^{1}H Nuclear Magnetic Resonance-Linked Metabolomics Investigations of Broiler Poultry and Human Saliva

By Devki D. Parmar

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy at Leicester School of Pharmacy (De Montfort University)

Submitted August 2019

1st Supervisor

Dr Randolph Arroo

(Leicester School of Pharmacy, De Montfort University)

2nd Supervisor

Dr Meng Wang

(Leicester School of Pharmacy, De Montfort University)

This PhD research work was funded by De Montfort University (Leicester, UK) and WET Engineering.

Acknowledgments

I would first like to thank Jake Paul Morgan. You have been very important to me during this process; providing me with lots of help, support and philosophical ramblings throughout the time of this Doctoral Programme. I could not have made it to the end without you.

I would like to express my sincere gratitude to my two supervisors, Dr Randolph Arroo and Dr Meng Wang of the School of Pharmacy at De Montfort University for the guidance and the knowledge I needed in order to finish this research.

Thank you, Dr Mark Fowler, for being my academic support, giving me advice when I really needed it and generally making me laugh throughout most of my time teaching at De Montfort University. We are the dream team! Also, thank you to Hetal Rana for being an amazing technician and always being the best at your job!

I want to thank Jops Dann, for all the support you have given me throughout my PhD, and for letting me become a part of your family. To Eileen Smith for our Thursday coffee meet-ups and your wonderful support. Also, to Josephine Conn who knows that even though I have a PhD I can have my silly moments!

I want to thank my lab friends Bhishak Pankhania, Mohammed Alqarni and Fereshteh Jafari for when we needed each other's ears to chew off!

Acknowledgments.

I want to thank my parents, Asit ad Bina for their love and support through the most difficult periods of my time at De Montfort University. Also, to my dear brother Hari-Arjun Parmar for all of the fun times we had whilst I was writing up my thesis as home, including the many hours of watching Adventure Time from beginning to end. In the words of LSP ”You’re all lumping awesome!”

Also thank you to Hari again, for drawing one of my favourite places for this thesis.

Acknowledgments.

Declaration

This thesis has not been accepted in any previous application for a degree and contains original work of the author except where otherwise indicated.

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List of Abbreviations and Acronyms

δ – chemical shift
Δ – change in energy
AAS – Atomic absorption spectroscopy
ANOVA - analysis of variance
B_0 – applied magnetic field
CKD - chronic kidney disease
CPMG - Carr-Purcell-Meiboom-Gill
D_2O – Deuterium Oxide
DNA – deoxyribonucleic acid
EI - electron-impact
FID – Free Induction Decay
FC – Fold Change
GC-MS – gas chromatography-mass spectrometry
h – plank's constant (6.626×10^{-34} js)
HMDB -The Human Metabolome Database
HPLC – high performance liquid chromatography
HSQC – heteronuclear single quantum correlation
Hz – Hertz
I – Value of nuclear spin
J – coupling constant
L - Litres
LC-MS – liquid chromatography-mass spectrometry
LC-MS/MS
MS – mass spectrometry
MS/MS - tandem mass spectrometry
NMR – Nuclear Magnetic Resonance
NOEPR – nuclear overhauser effect pulse train
OPLS-DA – orthogonal partial least squares- discriminatory analysis
PCA – principle component analysis
pHu – ultimate pH
PLS-DA – Partial least squares-discriminatory analysis
ppm- parts per million
PRESAT – Presaturation
RD – recycle delay
RF – radio frequency

s^{-1} – seconds
SPSS – Statistical Package for Social Sciences
T - Tesla
T_1 – spin lattice relaxation
T_2 – spin- spin relaxation
TMS - tertramethylsilane
TSP - trimethylsilyl propionate
UPLC - Ultra-performance liquid chromatography
VIP - variable importance in projection
WHC - Water-holding capacity
y – gyrometric ratio

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Abstract

^{1}H NMR-linked metabolomics was employed to investigate blood plasma and breast meat muscle from broiler poultry for quality assessment. The purpose of this research is to further explore the metabolomics profile of poultry breast meat muscle and plasma and comprehensively identify their metabolic components. NMR spectroscopy was also used to further investigate as to whether different ultimate pH levels of water would influence the metabolic profile. A total of 10 meat muscle samples were processed and the resulting ^{1}H NMR spectra were elucidated, identifying a total of 18 metabolites. A total of 49 plasma samples were processed for ^{1}H NMR analysis, where 35 metabolites were identified and confirmed. These metabolites were cross-referenced with metabolic quality indicators from previous studies and it was found that the group of samples treated by WET Engineering showed higher levels of these metabolites and therefore deemed higher quality. The application of multivariate statistical analysis also showed a significant difference between treatment groups. Therefore, highlighting that changes in the pH of water given to broiler poultry can indeed have an influence on their metabolic profile

^{1}H NMR-linked metabolomics was also employed in order to quantify ionic magnesium and calcium salivary concentrations via NMR characterisation of their chelation complexes with EDTA. At total of 82 samples were processed for ^{1}H NMR analysis and metabolic compounds were identified. Concentrations of ionic levels

were measured for each sample and quantified via calibration curve, giving average ionic concentrations of 212 µMol/L for magnesium and 932 µMol/L for calcium. These results were comparable to those levels found in previous studies, using alternative methodologies such as AAS. The repeatability and reliability of the technique was also investigated by preparing and measuring each sample in triplicate and then re-measuring these triplicate samples after a period of 48 hours. It was found that there was a very low standard deviation of concentrations between triplicate measurements. Giving deviations on average of 22µMol/L for magnesium and 36µMol/L for calcium. However, after a period of 48 hours, when samples were re-measured, they showed on average a standard deviation that was twice as high for magnesium and four times higher for calcium.

The overall objective of this thesis was to demonstrate the range of research that can be done, when using ^{1}H NMR-linked metabolomics for the analysis of biological fluids. In this case, the research areas of agriculture and oral health were chosen.

Chapter 1 - Introduction

1.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR is a technique that can be used to measure the properties of atomic nuclei. This method was initially discovered by sending a stream of hydrogen atoms through a homogenous magnetic field, which was subjected to a radiofrequency field [1]. The experiment that was conducted by Rabi *et al.* in 1938, where energy from the radiofrequency field was being absorbed by the nuclei at a defined frequency, and this absorption caused a detectable deflection within the stream of hydrogen atoms [1]. This event was established as the first observation of NMR, and Rabi received the Nobel Prize in 1943 for his contribution to the development of this particular technique.

In 1952 the Nobel Prize was awarded to Felix Bloch and Edward M. Purcell for their contribution towards 'the development of new methods for nuclear magnetic precision measurements, and connected discoveries' [2], [3]. Purcell described, in his Nobel lecture in 1952, the basic quantities and processes controlling NMR spectra, including the influence of molecular motion, chemical shift, magnetic dipolar splitting in solids, indirect spin-spin coupling, and aspects of a general theory of nuclear magnetic relaxation [4].

NMR is a spectroscopic technique, which is based on the absorption of electromagnetic radiation. It can be used to study a wide range of nuclei including

^{15}N, ^{31}P and ^{13}C, however the most common nuclei observed using this technique is ^{1}H NMR, as this is the most abundant [5].

1.1.1 The Theory of NMR

The theory of NMR is based on the magnetic properties of particles. If a nucleus of an atom is placed into a strong magnetic field and then exposed to pulses of electromagnetic radiation, it will resonate at a specific frequency of that radiation. This is what allows researchers to record a spectrum and gain structural information about molecules.

Nuclei are charged particles and a charged particle in motion can generate their own magnetic field. You can image a nucleus being very similar to a bar magnet. This is known as a magnetic moment and can be represented as shown in [Figure 1].

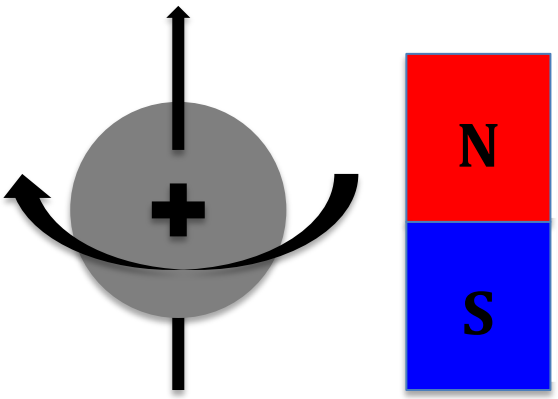


Figure 1 – Representation of a magnetic moment with regards to a bar magnet

Without an external magnetic field, the axis of the spins of nuclei are random, also known as degeneracy and can be in any direction, however when an external

magnetic field is applied the nuclei can either align with or against the magnetic field [Figure 2]. The way in which they align is known as spin states [6].

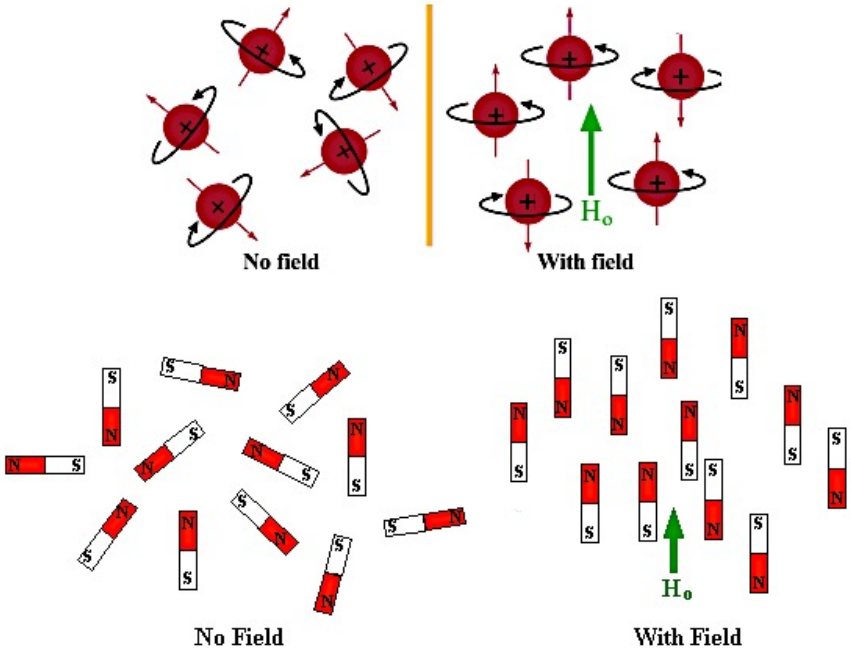


Figure 2 – Illustration of the behaviour of nuclei with and without an applied external magnetic field [7]

The total number of spin states that a nucleus can have is given by the equation; $2I + 1$, where (I) is the value of the spin. The vast majority of NMR uses ^{1}H or ^{13}C; which have a spin value of ½ would give 2 spin states [6].

The applied magnetic field is denoted as B_0 and is applied along the Z-axis [Figure 3]. For ^{1}H or ^{13}C these 2 spin states occur either with or against the magnetic field [Figure 2].

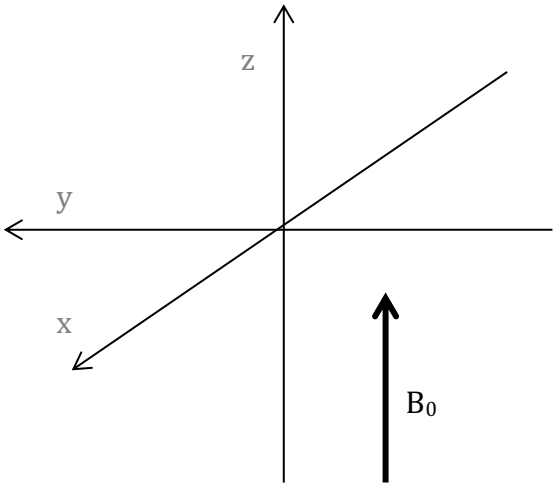


Figure 3 – Application of magnetic field to a nucleus

If the alignment is against the magnetic field, it will give a higher energy state as it is resisting the direction of the applied field. If the alignment is with the magnetic field then it will have a lower energy state, as there is no resistance with the applied field. Therefore, when there is no magnetic field being applied B_0 is equal to 0 and all of the spin states will be degenerate, and all have the same energy. As B_0 is applied the spin states appear [Figure 4].

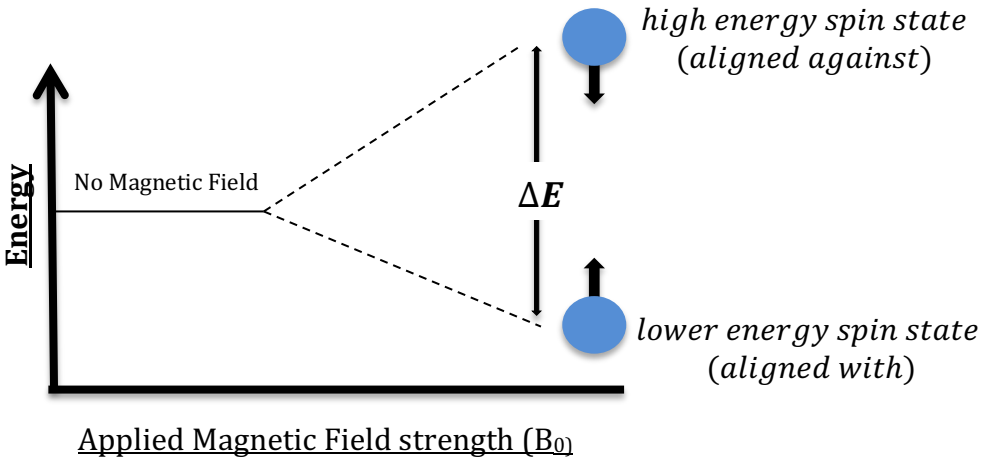


Figure 4 – illustration of transformation of energy states with the application of a magnetic field

The gap between the two states is known as ΔE or a change in energy. As the applied field strength increases so does ΔE.

The equation for ΔE is given by $\Delta E = \frac{h \cdot \gamma \cdot B_0}{2\pi}$, where h is Planck's constant (6.626×10^{-34} Js), γ is the gyrometric ratio, which is essentially a measure of how a particular type of nucleus responds to magnetic fields, so ^{1}H will have a gyrometric ratio of 267.512×10^6 rad T^{-1} s^{-1} and B_0 is the strength of the applied magnetic field by the spectrometer and is measured in Tesla (T).

For example if ΔE was calculated for a ^{1}H nucleus in a 9.39 T applied magnetic field we could denote this as the following [4];

$$\Delta E = \frac{(6.626x10^{-34}Js) \times 267.512x10^6 \times 9.39T}{2\pi}$$

This would give a ΔE value of 2.6144×10^{-24}. Which is a relatively small amount in change of energy.

An NMR spectrum is obtained by pulsing radio frequency (RF) radiation at a sample. The frequency of RF radiation, which is absorbed by the sample, causes the nuclei to resonate. This causes a peak to appear on an NMR spectrum at that frequency. As this corresponds to the energy of ΔE, multiple nuclei that have different ΔE within a sample will give rise to lots of signals at different frequencies, just like a typical NMR profile [4].

When RF radiation is applied to a nucleus, it can only be absorbed if it matches the ΔE. If the RF pulse is too high or too low in energy it will not be absorbed. As energy is equal Planck's constant and the frequency *(E=hv)* then there is only a specific energy of frequency radiation can be absorbed and in turn only a specific frequency that can be absorbed [Figure 5] [8].

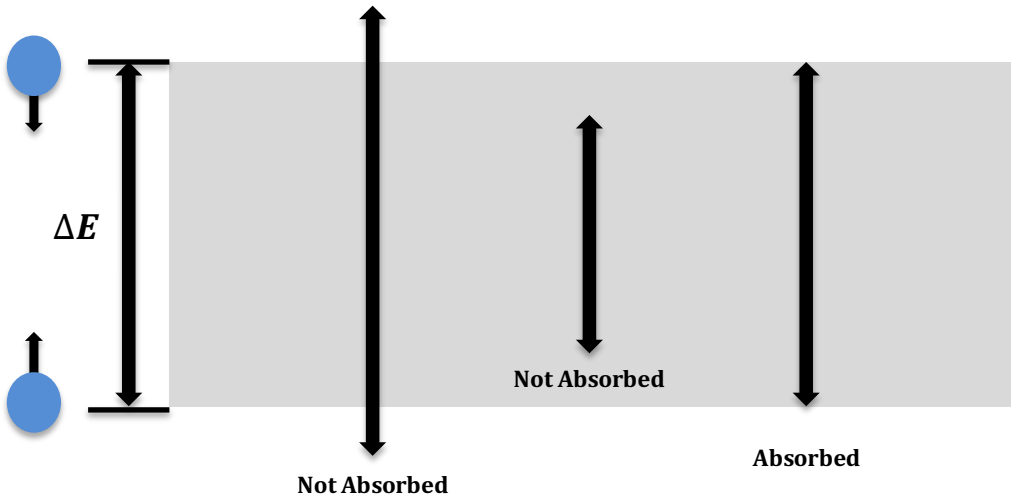


Figure 5 – Energy levels absorbed by nuclei

Another important factor of NMR theory is considering the populations of nuclei in both spin states. With ^{1}H NMR there will always be large populations of nuclei being observed with NMR spectroscopy. Typically, there will always be more nuclei in the lower energy state than in the higher energy state. This is known as the Boltzmann Distribution and can be seen in [Figure 6] [6]. This excess in population gives rise to something known as bulk magnetisation.

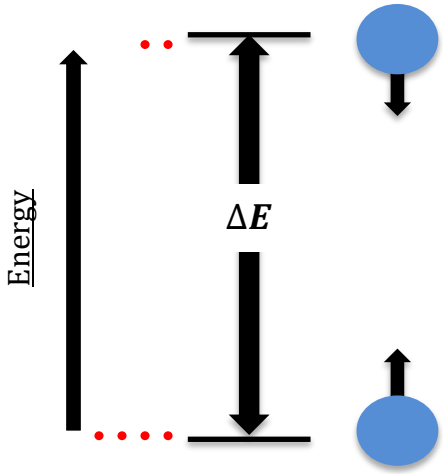


Figure 6 – Illustration of the Boltzmann distribution where populations of nuclei are shown as red dots.

When an NMR sample is subjected to multiple pulses of RF radiation, the energy from the RF radiation can be absorbed, this can promote one of the nuclei from a lower energy state spin state to the high-energy spin state. This will give a new population of nuclei at both energy levels. The more a sample is pulsed with RF radiation, the more nuclei will be promoted to a higher energy state as long as there is an excess of nuclei in the lower energy spin state [6].

When populations between spin states are equal and RF radiation is still being applied, there is an equal chance of a nucleus being promoted from a lower energy state to a high energy state by absorption, as there is that a nucleus will be demoted from a high energy state to the lower energy spin state through a process known as stimulated emission. Because each of these scenarios is equally as likely ultimately you reach a point where the population is equal between the two spin

states and this is known as saturation[6]. This kind of system can no longer provide valuable information in the form of an NMR spectrum; therefore, RF radiation needs to stop being applied allowing the system to relax.

By allowing the system to relax, this allows the nuclei to return to the Boltzmann distribution [Figure 6]. Meaning some of the higher energy nuclei will return to the lower energy state. This process is known as spin lattice relaxation and the time it takes for this process to occur is known as T_1. T_1 will depend on a number of different factors such as; the system you are using and the nuclei you are looking at [9].

The idea of pulsing and then allowing relaxation before another pulse occurs, is known as a pulse sequence. Examples of pulse sequences can be found in section 1.1.5. As samples are allowed to relax between multiple RF pulses, the resulting resonances can be combined, and the data can be averaged. The more acquisition data received the better the signal-to-noise ratio is and provides a much cleaner baseline in an NMR spectrum.

Another relaxation process involved in NMR is known as spin–spin relaxation. In addition to the incoming RF radiation having to match the resonant frequency, there is also a requirement for the nucleus to have an oscillating magnetic moment before it can absorb energy. This oscillating magnetic moment comes from the nucleus' spin. Normally nuclei do not spin perfectly straight on their axis and tend to 'wobble', which is known as precession [Figure 7] [9].

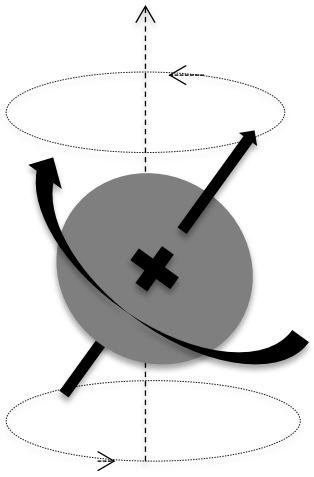


Figure 7 – Illustration of a nucleus in precession (Larmor Frequency)

As the nucleus is spinning on a 'tilt' the magnetic moment is moving around in the form of a circle [Figure 7]. This precession can be measured via NMR; however, the main issue is that all nuclei within a sample are at a different stage within the precession. This means that the magnetic moments of nuclei are not all pointing in the same direction at the same time. If the directions of all the magnetic moments were averaged, there would be no clear direction that could be measured [9].

As RF radiation hits the sample, it has the effect of knocking all these processions into alignment causing the bulk magnetisation to tip on its axis and the magnetic moment of each nuclei to be in the same phase of procession as each other. As the RF pulse if turned off this ordered procession becomes disordered again and we can measure this oscillating magnetic moment as an electric current. This process is known as spin-spin relaxation (T_2). This T_2 signal will decay over time and is what we detect and measure using an NMR spectrometer. This signal is a

measurement of frequency over time and is what we refer to as the free induction decay (FID) [9].

The instrumentation involved with NMR spectroscopy is also important to understand, as this is fundamentally how data for a sample is acquired. [Figure 8], shows a diagram of an NMR spectrometer, where the main components are highlighted [10]. The centre of the spectrometer contains a superconducting magnet, which is kept at 4 K, and therefore needs to be immersed in liquid helium, to enable it to stay at this temperature. An outer jacket of liquid nitrogen prevents the evaporation of any liquid helium from the inner chamber. A probe sits at the bottom of the magnet within the spectrometer. Samples are gently placed into the instrument via an air cushion provided by the airlift [11].

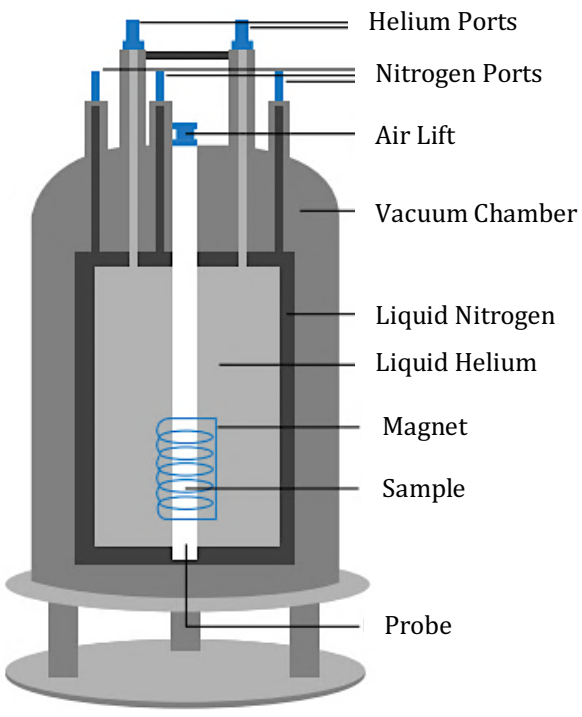


Figure 8 - NMR spectrometer [10]

1.1.2 Chemical Shift (δ) value

In NMR spectroscopy all nuclei have the same isotope, which experience the same applied magnetic field (B_0) and resonate at an identical frequency. You would expect this to not give you much information about the molecule, as all it would tell you is that your molecule has that particular nucleus, because all you would get is a single peak at the resonance frequency.

If we actually look at a nucleus in an applied magnetic field, we have to remember that electrons surround a nucleus. Electrons have their own magnetic fields, which oppose B_0. This has the effect of 'shielding' this nucleus from the applied field. This means the magnetic field the nucleus experiences, is actually weaker than the applied magnetic field (B_0) and this is dependent on the electron density around the nucleus [12].

If we have a nucleus that has a low electron density, the nucleus will experience more of the applied magnetic field (B_0) and vice versa with a high electron density nucleus. As a result the low electron density nucleus resonates at a higher frequency, known as deshielding and the high electron density nucleus resonates at a lower frequency, known as shielding [13]. The impact of this on an NMR spectrum is that a deshielded nucleus will appear at a higher chemical shift, whereas a more shielded nucleus will appear at a lower chemical shift [14].

A chemical shift is defined by the difference in parts per million (ppm) of the resonance frequencies between the nucleus of interest, such as ^{1}H and the reference nucleus, such as trimethylsilyl propionate (TSP) or tertramethylsilane (TMS). [6]. TSP and TMS are the most common reference compounds in NMR as they give a chemical shift of zero [5], [15].

1.1.3 Number of nuclei (resonance integrals)

The relative intensities of resonance signals can be attributed to the number of nuclei that generate those signals. Therefore, it is possible to quantify the relative proportions (numbers) of different, magnetically distinct ^{1}H nuclei within a molecule

1.1.4 Spin-spin coupling: number and placement of adjacent nuclei (multiplicity)

The interaction between the spins of neighbouring nuclei within a molecule may cause the splitting of an NMR signal peak. This splitting pattern is related to the number of equivalent hydrogen atoms at the nearby nuclei. This is known as spin-spin coupling, where one signal can be split into many peaks. Essentially the splitting pattern within a spectrum can tell you the number of different environments the nuclei are in. The separation between the different multiplet lines of the same resonance is known as the coupling constant, *J.* This can be measured as the distance in Hz between these resonance lines [5], [12].

1.1.5 Water suppression experiments

When using NMR spectroscopy, normally a pulse sequence involves the application of a 90˚ pulse, which can be reduced to decrease the time of the experiment and is followed by the acquisition of data where the free induction decay (FID) is recorded. However, in NMR-linked metabolomics research, the presence of water within the samples that are analysed can present a problem, since the great majority of samples examined, including tissue biopsies, blood serum and urine, all contain water.

Additionally, metabolite amounts within these samples are vastly lower than that of water. The water signal therefore overshadows the great majority of the observed ^{1}H signals within a spectrum. Rendering it difficult to assign those particular metabolites and assess any differences between the spectral profiles acquired.

To resolve this issue, water suppression experiments are employed. The particular pulse sequences applied in these experiments partially removes the water signal through various approaches. The two most commonly applied techniques used in the case of metabolomics are the Nuclear Overhauser Effect pulse train with Presaturation during relaxation and mixing time (NOEPR)[16] and Presaturation (PRESAT).

Presaturation (PRESAT)

PRESAT is the most commonly used water suppression technique since it allows the reliable quantification of signals that resonate close to the water signal [17]. The use of a solvent suppression technique is needed when the solvent used for a sample contains non-deuterated atoms. This would result in a solvent signal that would much more intense, when compared to other signals.

The method of presaturation is one that is most commonly used when it comes to solvent suppression, which involves small molecule samples[18]. The method is based on the selective saturation of the solvent resonances by a low powered radio frequency pulse, for example continuous wave radiation period of the solvent T_1, before the excitation pulse.[19] This selectively saturates the solvent at a specific frequency as it is irradiated during relaxation delay, with a non-specific pulse that excites the whole spectrum of resonances [20]. This pulse sequence allows the hydrogen atoms in –OH, -NH and -NH_2 functions that chemically exchange with water to become saturated, and therefore are rendered a less dominant signal. An example of this can be seen in [Figure 9], were (A) shows and NMR spectrum with no presaturation applied and (B) where presaturation has been applied and more of the smaller signals around 4-4.5ppm are visible [21].

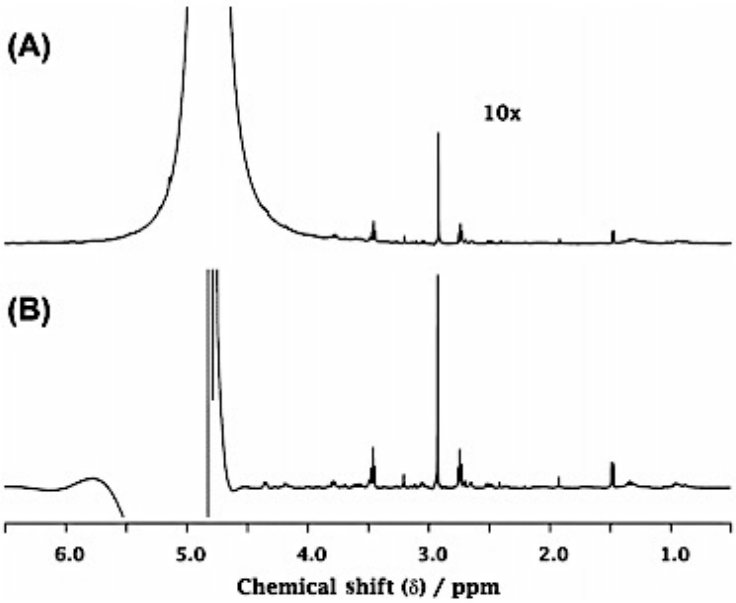


Figure 9 - An example of an NMR spectrum (A) before presaturation and (B) after presaturation [21]

The degree of saturation is determined by the rate of chemical exchange and the strength of the Nuclear Overhauser effect (NOE) [17].

1.1.6 Carr-Purcell-Meiboom-Gill (CPMG) Pulse Sequence

During the NMR-linked metabolomics analysis of plasma, there is a common issue that the spectrum produced will have a very noisy baseline, which is predominantly attributable to the resonances of proteins, lipids and other macromolecules, which are common and abundant.

As this is a common issue within metabolomics studies, two solutions have been proposed. It has been suggested that the proteins are removed prior to analysis,

via organic solvent precipitation or ultra-filtration [22]. However, more commonly, a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence is used with NMR data acquisition. Application of this pulse sequence allows the ^{1}H NMR spectrum to be acquired whist focusing mainly on signals that belong to low-molecular-mass metabolites and other small molecules [23].

CPMG pulse sequence involves the application of a 90° pulse and the collection of the initial few points resulting in the free induction decay [FID]. These initial few time points are averaged and stored as the first data point of the CPMG dataset. This pulse can be repeated anywhere from 500-2000 times, and will subsequently lead to the acquisition of a 500-2000 point CPMG data set [24]. This will lead to the production of an NMR spectrum that has a less noisy baseline that is normally caused by the presence of proteins and lipids.

1.2 Metabolomics

Over the past decade or so, most of the focus in 'omics' science has been sequencing the human DNA, the study of RNA or annotating the human proteome. Ever since the 1990's and predominantly since the determination of the human genome, there has been a real push forward with the techniques and approaches used in molecular biology and biochemistry [25]. The realisation for scientists that genetic differences might be able to account for disease processes and thus lead to new and innovative diagnostic approaches and hence better targeted therapies is key. This revolution in molecular biology progressed with the availability of micro-

array technology for detecting changes in gene expression. This type of study has been given the name transcriptomics. This subsequently expanded the ability to assay and then identify, using mass spectrometry-based methods [25]. This then led to carrying out studies that map the protein expression changes in cells and tissues and has been termed 'proteomics' [25].

There is, however, an equally important component of the human body that is starting to be studied more and more. This is the human metabolome. The human metabolome can be seen as the complete collection of metabolites that can be found in the human body. These small molecules include chemical entities that can be obtained through anabolism or catabolism [26].

Metabolites act as the 'bricks and mortar' of our cells [26]. The importance of metabolites is that they act as building materials for our macromolecules, which include proteins, DNA, carbohydrates, membranes and all other biopolymers that provide cellular structure and integrity. In combination with the genome, the human proteome and metabolome essentially define who and what we are [26].

However, in contrast to the genome and the proteome, the metabolome cannot be so easily defined. This is because it is not solely dictated by our genes, but also by our environment such as what we eat, drink and breathe, as well as our microflora such as bacteria residing in our intestinal tract, i.e. the human metabolome consists of a mix of both endogenous and exogenous compounds [26].

The beginning of the 17th century saw Santorio Sanctorius, considered the founding father of metabolic studies. In 1614, he published work that he had carried out on 'insensible perspiration'. He had determined that the total excrement, i.e. the total urine, faeces and sweat was less than the amount of fluid ingested into the body. This was due to metabolism in the body and water being lost through breathing. His work was one of the first to obtain physical data and provide a quantitative basis of pathology based on precise studies and instrumentations[27].

More recently in 1905, Otto Knut Olof Folin reported methods for the analysis of urine, particularly examining urea, ammonia, creatine and uric acid. In the 1960s and 1970s, the creation of the first quadrupole GC-MS allowed for investigations to be carried out on the quantitative analysis of metabolic profiles[27].

In 1971, Mamer and Horing performed the first mass-based metabolomics experiments. Consequently, the Horning group during this same time demonstrated how gas chromatography-mass spectrometry (GC-MS) could be employed to investigate a wide range of compounds that were detectable in human urine and tissue extracts[28]. Shortly thereafter, Arthur B Robinson and Linus Pauling investigated how biological variability might be explained by nutritional requirements. By studying early chromatographic separations of urine, Robinson and Pauling discovered that the chemical composition of the urine was a rich source of information for diseases, conditions, physiological age as well as metabolic processes [27].

One of the biggest databases dedicated to the human metabolome initially began as The Human Metabolome Project led by Dr David Wishart. This phase was completed in 2007, and the findings are accessible online via the Human Metabolome Database (HMDB) [29].

In 2009, Wishart et al stated that at the time at least 5 types of databases were used in metabolomics research. These included metabolic pathway databases; compound specific databases, spectral databases, disease/physiology databases and comprehensive organism-specified metabolomic databases [30]. The HMDB was fundamentally designed, as a primary resource, to facilitate with all metabolomics research [31]. It allows researchers to be able to have a reliable source in order to access qualitative information about human metabolites, as well as information about chemical structures, biological roles, metabolic pathways and transport mechanisms to name a few. It also references spectral data for each metabolite, acquired from; tandem mass spectrometry (MS/MS), gas chromatography-mass spectrometry (GC-MS) and NMR [31]. This allows researchers to utilise HMDB as a resource not only for general metabolite information but also as a reference tool for identifying metabolites through analytical chemical analysis [31].

Since 2007 the database has been significantly expanded and refined. Currently version 4.0 of HMDB has listed data on over 114,000 metabolites. This is partly due to the inclusion of both detected and expected metabolites [27], [33], [31]. As a

consequence, HMDB has helped to facilitate the research for many published studies in metabolomics, clinical biochemistry and systems biology [30]. An example can be found in a study of the human metabolic phenotype analysed by H-NMR of saliva samples, where HMDB was successfully used to assign and confirm a wide range of metabolites of interest [33]. Another study looking at disease biomarkers utilised HMDB to assign and confirm metabolites present in human urine [34].

When comparing the original 1.0 version of HMDB to the current 4.0 version (Table 1), it can be seen that the number of metabolites included in the database has greatly increased from 2180 to 114,000 [31]. Another increase is that of the number of experimental reference NMR ^{1}H and ^{13}C spectra, from only 385 initially to nearly 1500. Also tandem mass spectrometry spectral references have increased from only 390 to 2265, which is a huge increase [31]**.** One of the reasons for this is that the database is being used more now by researchers and has fast become much more comprehensive.

Table 1: Comparison of HMDB version 1.0 to current version 4.0 [31]**.**

Database Feature or Content Status	HMDB (Version 1.0)	HMDB (Version 2.0)	HMDB (Version 3.0)	HMDB (Version 4.0)
Number of Metabolites	2180	6408	40,153	114,100
Number of unique metabolite synonyms	27,700	43,882	199,668	1,231,398
Number of compounds with biofluid or tissue concentration data	883	4413	5027	7552
Number of compounds with experimental reference ^{1}H and or ^{13}C NMR spectra	385	792	1054	1494
Number of compounds with reference MS/MS spectra	390	799	1249	2265
Number of HMDB data field	91	102	114	130
Metabolite search/browse	Yes	Yes	Yes	Yes
Pathway search/browse	No	Yes	Yes	Yes
Disease search/browse	No	Yes	Yes	Yes

HMDB provides a simple and free user interface in order to match experimental chemical data from samples against the spectral database; which is why it is preferred over other databases, such as Chenomx, which requires researchers to buy licences and have training. It can also be argued that as HMDB is free, it allows more researchers to access the database and promotes collaborative work between research groups of all calibres, where budgetary constraints may have been an issue.

The growth of HMDB allows its continued use in various areas of published research. This shows how significant it has become in assigning metabolites and looking beyond that, into exploring metabolic pathways and roles of metabolites within the body.

Metabolomic analyses can usually be classified as either targeted or untargeted. Targeted metabolomics analysis primarily focuses on a specific set of intended metabolites. In most cases, these metabolites are usually identified and quantified. Using targeted analysis is crucial for assessing the behaviour of a specific group of compounds in a sample, under a set of defined conditions. Untargeted metabolomics, however, focuses more on the detection of as many groups of metabolites as possible in order to obtain patterns or 'fingerprints' without necessarily identifying or quantifying all, if any, specific compounds [35].

With metabolomics research, based on the specific objective of the research project and data manipulation, most studies carried out can be characterised into one of three categories;

- Discriminative,
- Informative
- Predictive

Discriminative analysis aims to seek differences between sample populations without creating basic statistical models or assessing possible pathways that may explain such differences. However, further significant discrimination of results can be achieved with the use of multivariate data analysis techniques. These techniques aim to maximise classification, and one of the most commonly used techniques is principal component analysis (PCA) (see section 1.3.3) [35]. PCA has been used in many studies, for many branches of investigations including agriculture to health and disease research. NMR was coupled with PCA in a study to investigate the pathophysiological responses of rabbits with coronary atherosclerosis. PCA plots within this study enabled the visualisation of differences between experimental groups [36]. Another experiment looking at the ^{1}H NMR metabolic profile of warty crab, blue crab and edible crab utilised PCA analysis to gain a general overview of the natural data grouping. Data obtained from the PCA analysis was able to show a higher level of alanine, glycine, and glutamate for the blue crab, a higher concentration of betaine and lactate in the warty crab, and a higher content of taurine in the edible crab [37].

Informative metabolomics analysis concentrates more on the identification and quantification of targeted or untargeted metabolites in order to obtain intrinsic information. It has been used in the 'development and continuous update of metabolite databases' such as the Human Metabolome Database [38]. Other areas of research can be carried out using informative analysis such as, possible pathways, the discovery of biomarkers, metabolite functionality studies as well as the creation of specialised metabolite databases [38].

Metabolomics research can also be predictive, in the sense that statistical models based on metabolic profiles and abundance can be created in order to predict a variable that could potentially be difficult to quantify by other means [35].

In human studies, metabolomics has regularly been used to define biomarkers that relate to prognosis or diagnosis of a disease or the efficiency/toxicity of a drug. Biomarkers are biological characteristics that are measured and evaluated as indicators of normal biological processes, pathological processes or pharmacological responses to therapeutic intervention [39]. Moreover, biomarkers are regularly used in clinical practice for diagnosis, severity assessment and response to therapy [39].

The importance of metabolomics studies is that it allows the simultaneous and absolute quantification of thousands of different metabolites within a sample [39].

1.2.1 Use of Bio-Analytical Chemistry for Metabolomics

As metabolomics research continues to grow, different analytical chemistry techniques have been employed as a means of analysis. Analytical chemistry applied to biological systems, allows for the identification of compounds and mixtures and determination of proportions of its constituents [40].

Most commonly, metabolomics research is supported by mass spectrometry (MS) and nuclear magnetic resonance (NMR) [41]. Hyphenated HPLC methods are

usually employed as a means of initial separation and ionisation before analysis via MS, again this is most commonly liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS)[41] [42].

LC-MS has become the major analytical technique in the field of metabolite profiling [43]. Liquid chromatography (LC) is said to be the most "versatile" of separation methods as it allows for a wider range of compounds with different polarity, with little effort in separation [41][43]. With the use of reverse-phase columns, semi-polar compounds can be separated. Conversely hydrophilic columns can be used to measure polar compounds such as sugars, amino acids and nucleotides. One of the main drawbacks to LC-MS is that there are many LC modes and MS analysers and analytical modes. This makes standardisation an issue as MS data obtained from different systems cannot be easily interpreted or compared [43]. The same can be said for other aspects of LC-MS as data obtained also becomes difficult to compare when; there is a difference in column type and material, differences in solvent mixtures are utilised between different studies, and also the age of the column [44]. Another issue with LC-MS is that is mostly uses soft ionisation sources such as atmospheric pressure or electrospray ionisation. The use of LC-MS for metabolite identification is conditioned by the capacity of the molecule to ionise whilst being part of a complex mixture as only ions can be measured by MS, therefore metabolites unable to ionise may not be detected [41]. These issue mean molecules that are separated and ionised don't always lead to a fragmentation pattern as you see mainly intact molecules, leading to the use of a

tandem mass spectrometry system such as LC-MS/MS, where you would see more of a fragmentation pattern [41].

Conversely, it has been argued that gas chromatography-mass spectrometry (GC-MS) is the most standardised method metabolomics research as it has nearly 50 years of established protocols for metabolite analysis, including; sugars, amino acids, sterols, hormones, fatty acids and many other intermediates of primary metabolism. There are also a vast accumulation of easily accessible libraries that reference mass spectra and chromatographic retention times collected under standardised conditions of 70 eV electron ionisation energy [45]. As GC-MS instrumentation uses hard-ionisation methods such as electron-impact (EI) ionisation, this results in higher sensitivity and resolution for metabolites and in turn provides rich and complex fragmentation patterns that gives a wealth of metabolite information [41]. Another advantage of GC-MS is that it can analyse more volatile compounds than compared to LC-MS, for example, the analysis of the breath for lung cancer or to quantify emissions of compounds from plants [46], [45]. As this may be an advantage to the technique, as gas phase or volatile substances can be easily and quickly analysed, there are risks of compounds that are liable to thermal degradation or decomposition to be lost [44]. Another issue can be that metabolites with higher boiling points for example pyruvate or lactate may need a derivatisation step in order to render them volatile enough for analysis [45].

Essentially, the main issue with LC-MS analysis is reproducibility of results, this reproducibility does not necessarily mean between samples but more between studies. In the case for GC-MS, retention of samples for alternative analysis is not possible and difficulty in achieving volatility for compounds can lead to a lengthier process. Conversely some compounds may not be thermally stable leading to a loss.

This is why it can be argued that NMR is becoming more central in metabolomics studies. NMR essentially produces a spectrum, which is a property of the material you are looking at, unlike the mass to charge ratio output in mass spectrometry. For example in NMR, ethanol will always have a triplet resonance at 1.17 ppm [29] regardless of the sample matrix, size of magnet, sample preparation.
Since the discovery and development of NMR spectroscopy, its initial use was primarily to identify small molecules and structure elucidation. It is now being applied to biological systems and linking this to the study of structure and function of macromolecules in order to investigate metabolism and metabolic processes. The use of ^{1}H NMR for the study of metabolomics has been described as early as 1977, when it was shown that ^{1}H signals could be observed from a variety of compounds within a suspension of red blood cells. The compounds detected included lactate, pyruvate, alanine and creatine [17].

Applications of NMR-based metabolomics have grown rapidly in recent years, and are now widely used across a number of disciplines [22]. They have particularly played a key role in the understanding of metabolism and metabolic processes

over the past 30 years [47]. NMR spectroscopy is a powerful tool for metabolite detection and identification since it has the ability to visualise metabolic profiles of intact biological fluids and biopsied tissue, and can both identify and quantify molecules in these complex mixtures [48].

The growing interest for the use of NMR as a diagnostic tool for biological fluids was initiated primarily for drug activity and toxicity research. Initially, NMR-linked metabolomics research was directed more towards identification for disease biomarkers for diseases such as tuberculosis, malaria, pneumonia, cancer and Parkinson's disease, amongst many others [49]. However, more recently the use of NMR has evolved to complement research within a variety of other fields from food quality [50],[51],[44] to oral health studies [52],[53].

NMR analysis has also advanced more towards the automation of sample processing. Samples can now be automatically processed by the use of NMR sampling belts. The power of the electromagnets used in instrumentation has also hugely increased in the past ten years, allowing for samples that would normally take 1-2 hours to process, now being completed in half the time, increasing the turnaround time for samples. Pre-programmed experiments, such as Bruker specific 'solvsupp' or 'HSQC', have also elevated turnaround time, as individual parameters do not have to be set up for each sample unless required.

There has always been a concern on the standardisation of sample preparation methods for NMR. Standard methodologies are very important to implement for

NMR especially in the case of biological fluids, as sample comparison can be a major part of analysis. Beckonert et al (2007), however, reported a set of protocols for the analysis of biofluids using NMR including, serum, plasma, urine and tissue [54]. These steps are crucial in order to provide a more standardised method of NMR analysis.

Since in NMR each organic molecule has a unique spectrum of signals, it should be possible to recognise the presence of them in a complex mixture. Thus, for metabolomics studies this requires a way in which to compare one sample to another and investigate this in a statistical manner in order for results to bear any significance.

1.3 Multivariate Statistical Analysis Techniques

When regarding statistical analysis of metabolomics data, many techniques from other 'omics' fields can be used as a tool for analysis. Traditionally a more classical approach can be utilised, aiming to assess group-wise differences in ether a univariate fashion such as a t-test or analysis of variance (ANOVA). An example of ANOVA analysis can be found in studies such as Lemanska et al looking at chemometric variance analysis of ^{1}H NMR metabolomics data on the effects of oral rinse on saliva[55]. Univariate techniques aim to reduce large numbers of measured analytes to those that only show the strongest response under investigatory conditions. However, univariate techniques can have drawbacks in

the sense that they fail to discriminate between groups if there are only minor differences, which can still be very important in metabolomics studies [56].

Conversely multivariate analysis can be applied, and this technique can help identify changes in singular metabolites between groups. The most commonly used techniques in the field are principal component analysis (PCA) and partial least squares regression (PLS)[56].

1.3.1 Multivariate statistical analysis software

There are a number of statistical analysis programmes available for data analysis such as SPSS, CAMO, R, XLSTAT and MetaboAnalyst. These software programmes allow for metabolomics data analysis and interpretation.

1.3.1.1 MetaboAnalyst

MetaboAnalyst is a comprehensive web-based tool. It is designed to carry out metabolomics data analysis, visualisation and functional interpretation. MetaboAnalyst was first introduced in 2009, which contained a single module for metabolomic data processing and statistical analysis. As more versions of MetaboAnalyst are introduced more modules are becoming available to use such as; metabolic pathways analysis, biomarker analysis and joint pathway analysis supporting both metabolites and genes [57]. More recently it has grown more popular as a tool in the field of metabolomics, processing around 1.8 million jobs from around 60,000 users. In 2017 MetaboAnalyst had been utilised in at least

one-fourth of all metabolomics publications in that year alone [57]. This included elucidating metabolic differences in studies looking at cancer, identifying highly predictive biomarkers for ketosis in dairy cows and many other studies looking biological processes and complex diseases. As MetaboAnalyst is continuously updated and features are enhanced, this enables the platform to work faster and created more robust results [57]. The recent update integrates a companion R package (MetaboAnalystR) and an accompanying R-command history panel. This method of data analysis is capable of exploring many areas of statistical analysis such as those seen in [Figure 10] [57].

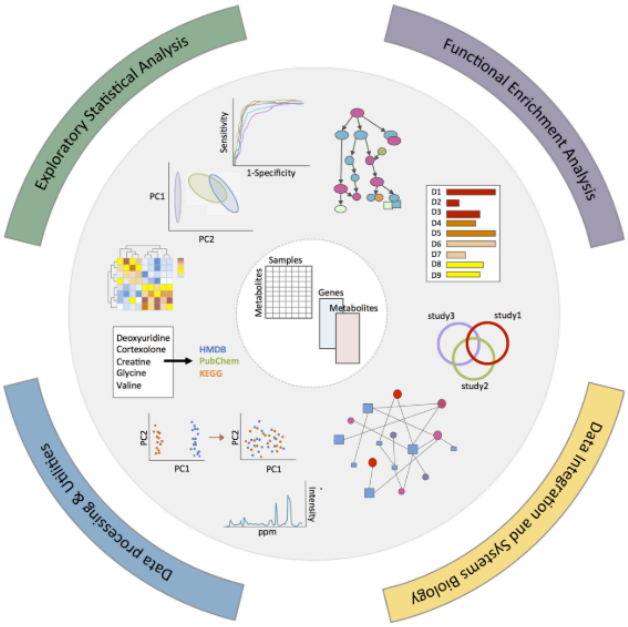


Figure 10 - Overview of MetaboAnalyst 4.0. Shows an overview of the current modules including; exploratory statistical analysis, functional analysis, data integration & systems biology [57].

The user-friendly interface allows you to perform pre-data analysis checks including the identification of missing values and normalisation. The process subsequently carries out multivariate analysis such as principal component analysis and partial lest squares discriminant analysis that can be reliably applied to metabolomics and chemometric data in order to obtain conclusive results. This easy and free method of data analysis is why MetaboAnalyst is fast becoming more popular for research groups to use when applying statistical analysis to their data sets as compared to other programmes, such as CAMO or SPSS that require training before the program can be used to its full potential. A lot of other alternative programmes also require a license to be purchased before use, which can be difficult for smaller research groups with restricted budgets.

1.3.2 Data Normalisation

1.3.2.1 Constant- Sum Normalisation

As with most metabolomics and chemometric data, before analysis, data is usually normalised. For biological fluids, such as urine or plasma, the concentrations of metabolites can be highly dependent on factors that are not necessarily completely controllable, such as the amount of water consumed before a sample is obtained. Normalisation of data allows for an effective method to reduce unwanted variables in spectral data. This is also, to account for variable dilution factors within samples that can arise, for example from variations in the number of cells, biofluid volume or tissue size [58].

Therefore, each observation may be normalised to ensure that all observations are directly comparable. Normalisation can be achieved through various means such as internally via computational means or with internal standards such as TSP in NMR analysis [59].

It can also be carried out externally via measurements of cell culture optical density or protein content. However, the most common form of normalisation is constant-sum normalisation. Constant-sum normalisation is achieved when each spectrum is normalised so that the total of all its integrals has a sum of 1[59].

1.3.2.2 Cube-Root Transformation and Pareto Scaling

Cube-root transformation is a method of data normalisation, which helps to form a more normal distribution of data.

Pareto scaling is currently a highly recommended prior treatment step. The process involves mean centring followed by the division of the square root of the X variable sample standard deviation [60]. The process if very similar to autoscaling, however instead of the standard deviation being used, the square root of the standard deviation is used as a scaling factor. It is best used when each variable varied in scale. It is preferred in NMR metabolomics as it's a good compromise between no scaling and autoscaling[61]. Pareto scaling in essence reduces the

relative importance of large concentrations of metabolites and partially preserves spectral line shapes and data structure.

Pareto scaling is preferred over other methods, as it stays closer to original measurements and is most commonly used to reduce the influence of intense peaks, whilst emphasising weaker peaks that may have more biological relevance [59]. This results in the corresponding loadings of the intense peaks to be reduced and loading from weaker peaks to be intensified[59].

1.3.2.3 Intelligent-Bucketing

The intelligent bucketing or 'binning' technique is a method that has become popular when dealing with NMR based metabolomics data. Bucketing can be used as a way to reduce a large data set. Instead of treating each chemical shift value as a separate integral, intelligent bucketing divides the spectrum up according to chemical shift resonances. This method is intelligent as resonances different in width and intensity are taken into account when integrating each peak [62]. The intensities within each bin are summed-up, so that the area under each spectral region is used instead of individual chemical shift intensities. This creates a new smaller set of variables [62].

1.3.3 Principal Component Analysis (PCA)

PCA is a widely used statistical techniques used in almost all scientific disciplines. It has also been described as one of the oldest multivariate techniques available [63].

PCA has been described as a multivariate statistical technique that analyses a data table. Within this data table, observations are described by a multitude of inter-correlated quantitative dependant variables. The main objective of PCA is to extract the important information from a table and represent it as a set of new variables, which are called principal components.[63]

PCA can be applied to a single set of variables, when a researcher is interested in discovering which variables in a set form coherent subsets that are relatively independent of another [60]. This is one of the main reasons why PCA is used for the analysis of multivariate metabolomics and chemometric datasets, since it is capable of dimensionality reduction, ranking, regression and clustering [64].

PCA is important to mention when it comes to multivariate statistics, as it is one of the primary forms of analysis. PCA however, is an unsupervised method meaning it assumes that the principal components are orthogonal and a linear combination of original features. If this is not the case for variables, PCA will not give you reliable results[65]. In essence with PCA you are projecting your data into a 2D space in order to observe sample clustering. For this reason, methods such as Partial Lest Squares-Discriminant Analysis (PLS-DA) are preferred. With

PLS-DA you are able to do a regression between your descriptors and the group of classes. Therefore you can define classes as a response to variables and this should provide more efficient separations[59], [65].

1.3.4 Partial Lest Squares Discriminant Analysis (PLS-DA)

PLS-DA is considered a frequently used classification method and is based on the PLS approach which was developed by Barker and Rayens (2003)[66]. PLS-DA analysis uses qualitative descriptor variables. However, in the case of PLS-regression analysis of a two-class dataset, the values of the dependent variables are coded numerically. In some cases, more than two classes can be defined.

Typically, using PLS-DA will provide a scores plot output of the classified samples. The plot is normally accompanied with a Q^2 and R^2 values. The Q^2 value estimates goodness of prediction where the R^2 value estimates goodness of fit [66][59]. Permutation tests are normally carried out alongside PLS-DA analysis, and these serve to evaluate whether the specific classification of individuals in the two or more groups is more significant than any random classification in the groups. These tests are used in order to explore the relationships between a set of variables and the hypothetical classification status. For example, the class labels of healthy controls and diseased patients are permuted and randomly assigned to different patients. Therefore, the incorrect class labels will be assigned, and the classification will again be computed. This should show that the incorrect class labels would be ineffective at class prediction since they have been generated

randomly. Normally, these permutations can have repetitions of up to 2,000 times, i.e. 200 times individually of each initially randomly assigned class label. Null distributions of classifications that are expected to be insignificant are formed [67].

Another feature of PLS-DA analysis is a variance important factor (VIP) score. A VIP score is a measure of a variable's importance in the PLS-DA model. It essentially summarises the contribution that a variable has made to the model. The score is calculated using the weighted sum of squared correlations between the PLS-DA components and the original values. The weights then correspond to the percentage variation explained by the PLS-DA component in the model [68]. In the case of metabolomics the model can be used as a tool to create a ranked list of the importance of an individual metabolite is for discrimination between experimental groups[69]. The higher the score is of a particular metabolite, the more significant it is in the PLS-DA model construction[70].

Outline/Objectives

The main objective of this thesis is to showcase the versatility of ^{1}H NMR spectroscopy within different fields of research. This will be done in this case by exploring the scope of ^{1}H NMR analysis in the fields of agriculture and the human oral health sector.

The contents of this work will explore the chemical profile of biological fluids using ^{1}H NMR for the analysis of;

- Broiler poultry plasma and meat muscle for the purpose of metabolic composition and evaluation of quality factors. The objective is to identify whether changes in drinking water will show a significant difference in the metabolomic profiles between study groups.

- Magnesium and calcium content in human saliva via the application of a novel technique using EDTA. The objective is to evaluate if the technique comparable to existing techniques as well as assessing the reliability and repeatability of this novel technique.

Chapter 2 - Materials and Methods

2.1 Chicken

The following information was outlined within the farm protocol pertaining to this study. Sample collection was limited during this study for health and safety reasons due to an outbreak of avian influenza.

This study observed a total of 10 poultry sheds. Each shed contained 30,000 birds; hence the total number of birds within all sheds amounted to 300,000. The specific breed of poultry was unknown.

The chickens arrived at the farm on the day they are born, and by protocol some sheds were dosed with antibiotics and methoxasol. Methoxasol is an agent used to treat bacterial infections associated with the middle ear, urinary tract, and respiratory and intestinal systems. It is important to note that broiler poultry in the sheds that pertain to the 'WET Engineering' sheds were not receiving any acids or antibiotics.

On site, there were 3 hatcheries and a number of parent flocks. In some cases, external hatcheries had been used to supply the chicks, whilst in others each crop was supplied by all three hatcheries, with flocks coming from parent flocks of different ages. This did have the ability to influence performance; however, the

farm limited this variability within the sheds by choosing similarly aged parent flocks that come from the same internal hatcheries.

Each crop cycle lasted 36-38 days with the additional time of 10-12 days of cleansing and sterilisation between each crop. Therefore, the total of one single crop cycle lasts 46-50 days.

Before each next crop began, the sheds all underwent a cleansing and sterilisation process. All old litter was incinerated on site for thermal energy production. All feeders underwent acid dousing with hydrogen peroxide/ peracetic acid (HPPA), a disinfectant containing 5% peracetic acid. All waste from this process went into a slurry tank and was taken away. The sheds were treated with a thermal fog that was used to eradicate *Campylobactor*, this was applied via an orchard spray. New chicken coop bedding is normally treated during the manufacturing process to reduce bacteria levels. Propionic acid was used to do this as it is used industry wide to inhibit growth of mould and some bacteria.

The environmental conditions the broiler poultry were kept under was highly regulated and controlled across all sheds. The humidity in all sheds was kept between 45-55%. The temperature was maintained at 33-34°C for the birds between days 1-25. This was then gradually reduced to 22°C from days 25-30. From day 30 until the end of the crop the temperature was further reduced to 20°C. Lighting was controlled by using artificial lights which were turned on at 2am when the birds were woken and turned off at 10pm to allow for sleep and

therefore allowed to drink for 20 hours per day. Ventilation was controlled by increases in temperature through inlets via pressure, which was set to around 40 Pascal's. Each farm manager was provided with ventilation rates, which they followed.

Vaccines may have also been administered during the course of the crop. Administration was carried out by way of an automatic in-line instrument placed in the entrance of each shed. The vaccine administered was been reported as 'GUMBO LZ228E'. When this administration occurred the SCOTMAS water system was turned off in order not to destroy the vaccine. The system was turned off for 36-48 hours prior to the vaccine being administered. This regime was kept the same throughout the trials. No information was received in terms of protocol during vaccination for sheds being supplied with the WET water system. Both the SCOTMAS and WET water treatment systems are outlined under the section of *Water Treatment Systems*.

The feeding programme was kept the same for all sheds and changes throughout the development over the 36-38 day cycle. Feed began with a high protein and low energy diet and moved onto a higher energy and lower protein diet, as the older the birds get the slower they grow. The regime consisted of four feed changes all together and these can be seen in Table 2 below.

Table 2: Feeding programme for all poultry (coccidiostat is a substance given to retard growth and reproduction of coccodian parasites)

Stage of cycle	Feed Regime
1-10 days	Started feed (common coccidiostat)
10-21 days	Grower feed (common coccidiostat)
21-28 days	Finisher (different coccidiostat with water intake depressing effect)
28 days - kill	Withdrawer (no coccidiostat)

The mineral content in the feed was kept the same throughout all sheds and was kept in line with normal industry standards. Minerals were fed to the birds via the food and therefore the mineral content of the water was kept low. Calcium was given to the chickens by the addition of limestone into their food and the calcium to phosphorus ratio was kept at 2:1. Calcium intake in the feed does decrease with bird age and the calcium to phosphorus ratio was still maintained at 2:1. Sodium levels in feed were higher at the start of the crop but these levels also decreased with bird age. Introducing magnesium and potassium ions into the diet could have caused diarrhoea; however, in order to prevent this, soya and wheat levels were carefully controlled.

At 35 days, 30-40% of the chickens were taken away for slaughter at an approximate weight of 1.88 kg; the remainder of the birds stayed until the end of the crop cycle and reached on average a weight of 2.2 kg. The birds that weighed 2.2 kg were the specimens from which samples were acquired.

2.2 Water Treatment System Specifications

Each shed used an average of 175,000 litres of water over the 36-38 day period, i.e. approximately 4861-4605 litres per day was used. The birds were awake for 4 hours a day and were free to drink when they needed to.

Two different water treatment systems were placed between each paired flock shed. These were the SCOTMAS system and the WET water treatment system. The SCOTMAS system primarily used chlorine dioxide, as a biocide, to treat the system. The system was installed within the pump room and the chlorine dioxide was diluted to a final concentration of 5 ppm before the birds could consume it. The purpose of this particular system was to treat municipal water, kill bacteria and reduce limescale. The resulted treated water was at pH6, which was achieved by adding anthium dioxide and anthium activator P10.

According to SCOTMAS Group, their pre-treatment of water can 'achieve the best flock performance and increased farm to plate production' [71]. The foundations for this company were laid in the early 1980s, where its directors first applied chlorine dioxide in order to rehabilitate the welfare of broiler flocks across Europe and Africa. Currently, their water treatment system consists of chlorine dioxide in combination with organic acids, which delivers improvements to feed conversion through their Floradox program [71]. The Floradox program involves improving stock health through a combination of water sanitation and enhanced gut performance. Water sanitation with the use of chlorine dioxide involves, the

destruction of biofilm at the source including lime scale and magnesium. Their blend of organic acids are then designed to stimulate activity within the gastrointestinal tract which reduces the need to use antibiotics, vitamins and supplements [72].

The WET water system was treated on an individual shed basis in order to achieve optimum pH.

Table 3: WET Engineering water treatment condition per shed

Shed Number	Water Conditions
1	pH 8-8.5
3	pH 9-8.5
5	pH 7.5 (WET pump house water)
7	pH 7.5 (WET pump house anti-bacterial)
9	pH 6.5

The pump house water tanks had a butyl or PVC liner and was topped with a hessian cover, so although they were covered, they were not airtight. Pipes that feed water into the sheds, both the 50mm and 32mm were made up of Mid Density Polyethylene (MDPE). The shed water metres measured water consumption in cubic metres, and this was recorded daily by the farm manager. The drinker lines across all 10 houses were the same but were arranged differently in sheds 6 and 8. The birds drank water from a stainless-steel nipple, which was delivered via a PVC

pipe, which sat inside a stainless-steel outer pipe. All wastewater was carried away through pipelines, which were connected to holes in the floor. This was carried away into a septic tank that was removed at the end of each crop.

2.3 ^{1}H NMR of Chicken Breast Meat

2.3.1 Sample Preparation

A total of 10 breast meat muscle samples were collected into sterile plastic bags and sealed at the farm site. The samples were transferred to the laboratory on ice and immediately processed. Samples were stored at –80°c.

Extraction of the breast meat muscle metabolites, was carried out using the methodology outlined by Beckonert et al, as published in nature protocols [54]. 80 mg of ground frozen breast meat muscle tissue was taken and placed into a sterile Eppendorf tube. A volume of 1.50 mL of ice-old extraction solvent was added (1:1 water and acetonitrile). This was then mechanically homogenised using an electric pestle rotor. The sample was then centrifuged at 10,000 x g for 10 minutes at 4˚C. A 1mL volume of the supernatant was then taken (containing hydrophilic metabolites) and freeze-dried in order to remove any remaining extraction solvent. The sample was reconstituted in 0.5 mL of D_2O containing 0.05 % (w/v) sodium 3-trimethylsilyl-(2,2,3,3-2 H4)-1-propionate (TSP, chemical shift reference and internal quantitative ^{1}H NMR standard). A volume of 50µL of pH 7.0 phosphate buffer and 50µL of a standard sodium azide (NaN_3) solution [final concentration

0.04% (w/v)] was added as a microbicide[54]. Samples were then rotamixed prior to transferring to 5-mm diameter NMR tubes.

2.3.2 Spectrometry Measurements

^{1}H NMR spectra from the 10 breast meat muscle samples were obtained at 300 K on a Bruker Avance 400 MHz spectrometer (Leicester School of Pharmacy, De Montfort University). The ^{1}H NMR spectra were acquired using the zg30 Bruker pulse sequence and each sample was analysed using an automated process and took on average a total of 12 minutes to complete. The specified parameters used are outlined in Appendix 1.

2.3.3 Spectral Post-Processing

Spectral data sets were analysed using NMR Spectrum Processor 2012 (ACD/Labs, Toronto, Canada). All spectra underwent manual correction for any phase or baseline distortion. Each spectrum also scaled and aligned to the internal reference of TSP (±0.05 ppm).

2.3.4 Spectral Assignments

Metabolites were assigned using a combination of chemical shift values, coupling patterns and coupling constants from literature and the online database HMDB [29].

2.4 ^{1}H NMR of Chicken Plasma

A total of 4-6 whole blood samples were collected via random sampling from each shed at the end of the crop cycle. In addition, one meat sample was provided from each shed.

2.4.1 Sample Preparation

A total of 49 whole blood samples were collected into clean 5mL tubes at the farm site. The samples were transferred to the laboratory on ice and immediately centrifuged and the clear plasma removed. Samples were stored at -80°C.

The preparation of plasma was carried out using the methodology outlined by Beckonert et al, as published in nature protocols [54]. A volume of 1 mL of whole blood was centrifuged at 10,000 x g for 10 minutes. Subsequently, 0.60 mL volumes of the plasma supernatant were treated with 100µL of D_2O, 50µL of pH 7.0 phosphate buffer and 50µL of a standard sodium azide (NaN3) solution [final concentration 0.04% (w/v)] was also be added as a microbicide. The sample was then vortexed prior to transferring into 5-mm NMR tubes using a sterile glass pipette [54].

2.4.2 Spectroscopy Measurements

^{1}H NMR spectra from 49 blood plasma were obtained at 300 K on a Bruker Avance 400 MHz spectrometer (Leicester School of Pharmacy, De Montfort University).

^{1}H NMR spectra were acquired using a spin-echo sequence [Carr-Purcell-Meiboom-Gill (CPMG)] with presaturation for water suppression (cpmgpr1d pulse sequence, Bruker). Each sample was analysed using an automated process and took on average a total of 14 minutes to complete. The specified parameters used are outline in Appendix 2.

2.4.3 Spectral Post-Processing

Spectral data were analysed using NMR Spectrum Processor 2012 (ACD/Labs, Toronto, Canada). All spectra underwent manual correction of any phase or baseline distortion. Each spectrum also scaled and aligned to the doublet signal of lactate (1.31 ppm).

2.4.4 Spectral Assignments

Metabolites were assigned using a combination of chemical shift values, coupling patterns and coupling constants from literature and the online database HMDB [29].

2.5 Statistical Analysis of Chicken Plasma Data

2.5.1 Multivariate Analysis Pre-Data Processing

All 49 plasma samples were included for data analysis. The spectra were analysed using NMR Spectrus Processor 2012 (Advanced Chemistry Development Inc., ACD/Labs, Toronto, Canada). Before analysis, all spectra were manually phased,

and baseline corrected for maximum removal of noise. Spectral integration was carried out between 0.80-8.50ppm; subsequently, the residual water signal between 4.60ppm and 5.05 ppm was removed using a tool within the Spectrus Processor programme, together with any baseline noise. All spectra were integrated using the intelligent bucketing process implemented through the above software.

The intelligent bucketing procedure divides all spectra into chemical shift buckets. Therefore, the position of a particular resonance peak within difference spectra is always incorporated into the same bucket, even if pH or ionic strength has shifted.

Advantages of using spectral binning include overcoming issues which arise from setting bucket widths which may cut a resonance peak in half and bias if it was to be carried out manually. Further to this all intelligent buckets were assessed, and buckets seen to contain no value was disregarded. This resulted in a total of 85 intelligently bucketed integrals.

For all 49 samples, a total of 85 resulting intelligently selected bucket integrals (data matrix) were imported into Microsoft Excel. Class labels for sample identification were then added before data analysis. This included identifying the different water systems used. This excel spread sheet was then exported as a CSV file.

The file was subsequently imported into MetaboAnalyst. The intensities for each sample were sum-normalised, cube root transformed, and Pareto scaled prior to analysis. The resulting normalised data was displayed including a visualisation of data distribution. PLS-DA was then employed in tandem with permutation analysis and Variable importance for prediction score analysis.

2.6 Human saliva

A total of 62 healthy, non-smoking, non-medically compromised participants were recruited locally. Written informed consent was acquired from all participants, ethics approved by the Faculty of Health and Life Sciences Research Ethics Committee, De Montfort University, Leicester UK. Unstimulated whole saliva was provided first thing in the morning in a plastic sterilised universal container after a period of 12 hours fasting. Participants were requested to refrain from oral activities during the period between awakening and sample provision (*ca*. 5 minutes).

Table 4 – Outline of Samples Collected Form Participants

Sex	Number of Participants	Average Age in Years (Range)
Female	37	24 (19-45)
Male	25	21 (17-27)
Both	62	23 (17-45)

2.7 ^{1}H NMR of Human Saliva

2.7.1 Sample Preparation

The preparation of saliva was carried out using the methodology outlined by Beckonert et al, as published in nature protocols [54]. Saliva specimens were transported to the laboratory on ice, and 2 ml volumes of whole saliva were centrifuged immediately for 5 min. at 3,500 rpm. Saliva samples were prepared in duplicate, since a sample without the presence of EDTA would serve as a control. 0.60 ml aliquots of salivary supernatants were removed, 0.10 ml of 50.00 mmol EDTA was added in excess, and the sample mixture then homogenised. 68µl of pH 7.00 phosphate buffer and 80µl D_2O containing 0.05% (w/v) sodium 3-trimethylsilyl-(2,2,3,3-2 H4)-1-propionate (TSP, chemical shift reference and internal quantitative ^{1}H NMR standard) was added to the sample and vortexed. Additionally, 50µL of a standard sodium azide (NaN_3) solution [final concentration 0.04% (w/v)] was also be added as a microbicide [54]. As noted above, an additional saliva sample was prepared in the same manner with 0.60ml HPLC-grade water instead of EDTA and this served as an essential control. The final solution was subsequently transferred into 5-mm diameter NMR tubes for ^{1}H NMR analysis

2.7.2. Spectroscopy Measurements

^{1}H NMR spectra from 62 saliva samples were obtained at 300K on a Bruker Avance 400 MHz spectrometer (Leicester School of Pharmacy, De Montfort University).

^{1}H NMR spectra were acquired using noesygppr1d (Bruker) pulse sequence for water suppression. Each sample was analysed using an NMR auto-sampler and each took on average a total of 12 minutes to complete. Parameters used to acquire ^{1}H NMR data for human saliva are located in Appendix 3.

2.7.3 Spectral Post-Processing

The spectra were analysed using NMR Spectrus Processor 2012 (Advanced Chemistry Development Inc., ACD/Labs, Toronto, Canada). All spectra underwent individual manual correction of any phase or baseline distortion. This assisted with eliminating any noise at the pre-data processing stage and also increasing reliability of metabolite identification. Each spectrum also scaled and aligned to the internal reference of TSP (-0.05 to 0.05 ppm). Spectral integration was carried out between 0.50-10.0ppm; subsequently, the residual water signal between was removed.

2.7.4 Spectral Assignments

Assignment of metabolites was carried out manually for all spectra. Metabolites were assigned using a combination of chemical shift values, coupling patterns and coupling constants from literature and the online database HMDB [29].

2.8 Calibration Curve of Magnesium and Calcium

2.8.1 Sample preparation

In order to determine concentrations of Ca^{2+} and Mg^{2+} for each saliva sample, a calibration curve was prepared by the preparation of serial dilutions of $MgCl_2$ and $CaCl_2$. Both powders were accurately weighed and dissolved in HPLC-grade water to produce the correct final concentration. 0.6mL of each solution was taken for each sample. An addition of 100µl of 50.00 mmol EDTA, 100µl of pH 7.0 phosphate buffer and 10% (v/v) of D_2O containing 0.05% (w/v) sodium 3-trimethylsilyl-(2,2,3,3-2 H4)-1-propionate (TSP, chemical shift reference and internal quantitative ^1H NMR standard) was then added, and the sample mixture was then thoroughly rotamixed. The final solution was subsequently transferred into 5-mm diameter NMR tubes for ^1H NMR analysis.

2.8.2 Spectroscopy Measurements

^1H NMR spectra for each calibration sample were obtained at 300K on a Bruker Avance 400 MHz spectrometer (Leicester School of Pharmacy, De Montfort University). ^1H NMR spectra were acquired using noesygppr1d (Bruker) pulse sequence for water suppression. Each sample was analysed using an NMR auto-sampler and each took on average a total of 12 minutes to complete.

2.8.3 Spectral Post-Processing

All spectra corresponding to Mg^{2+}-EDTA and Ca^{2+}-EDTA complex calibration curves and repeatability analysis were measured using NMR Spectrus Processor 2012 (Advanced Chemistry Development Inc., ACD/Labs, Toronto, Canada). All

spectra underwent individual manual correction of any phase or baseline distortion. This assisted with eliminating any noise at the pre-data processing stage and also increasing reliability of metabolite identification. Each spectrum also scaled and aligned to the internal reference of TSP (-0.05 to 0.05 ppm). Each ionic complex resonance was integrated relative to the internal standard of TSP set at 1.

2.8.4 Quantification of Mg^{2+}-EDTA and Ca^{2+}-EDTA complexes within saliva samples

For all 62 NMR spectra, each ionic complex resonance was integrated relative to the internal standard of TSP set at 1. Subsequently, the formula of the line from each calibration graph was utilised in order to calculate the concentration in mmol/L of each complex within the saliva sample. The results were compiled using Microsoft Excel.

2.9 ^{1}H NMR Repeatability and Reliability Analysis of Human Saliva

To assess reliability and repeatability between NMR measurements of samples, each participant's saliva was prepared in triplicate and measured using ^{1}H NMR. The triplicate samples were then kept for a period of 48 hours and re-measured using ^{1}H NMR.

2.9.1 Sample Preparation

A total of 21 samples from 7 participants were collected for this analysis.

Unstimulated whole saliva was provided first thing in the morning in a plastic sterilised universal container after a period of 12 hours fasting. Participants were requested to refrain from oral activities during the period between awakening and sample provision (*ca*. 5 minutes).

Saliva specimens were immediately transported to the laboratory on ice, and 2 ml volumes of whole saliva were centrifuged for 5 min. at 3,500 rpm. Each saliva sample was prepared in triplicate. 0.60 ml aliquots of salivary supernatant was removed, 0.10 ml of 50.00 mmol EDTA was added in excess, and the sample mixture then homogenised. 68µl of pH 7.00 phosphate buffer and 80µl D_2O containing 0.05% (w/v) sodium 3-trimethylsilyl-(2,2,3,3-2 H4)-1-propionate (TSP, chemical shift reference and internal quantitative ^{1}H NMR standard) was added to the sample and vortexed. Additionally, 50µL of a standard sodium azide (NaN3) solution [final concentration 0.04% (w/v)] was also be added as a microbicide [54].The final solution was subsequently transferred into 5-mm diameter NMR tubes for ^{1}H NMR analysis

2.9.2 Spectroscopy Measurements

All triplicate samples were measured on the day they were collected and again after a period of 48 Hours. ^{1}H NMR spectra from saliva samples were obtained at 300K on a Bruker Avance 400 MHz spectrometer (Leicester School of Pharmacy, De Montfort University). ^{1}H NMR spectra were acquired using noesygppr1d (Bruker) pulse sequence for water suppression. Each sample was analysed using

an NMR auto-sampler and each took on average a total of 12 minutes to complete. Parameters used to acquire ^{1}H NMR data for repeatability and reliability is located in Appendix 4.

2.9.3 Spectral Post-Processing

All corresponding spectra were analysed using NMR Spectrus Processor 2012 (Advanced Chemistry Development Inc., ACD/Labs, Toronto, Canada). All spectra underwent individual manual correction of any phase or baseline distortion. This assisted with eliminating any noise at the pre-data processing stage and also increasing reliability of metabolite identification. Each spectrum also scaled and aligned to the internal reference of TSP (-0.05 to 0.05 ppm). Spectral integration was carried out on each EDTA complex.

Chapter 3 - ^{1}H NMR-Linked Metabolomics Investigations of Chicken Breast Meat Muscle

3.1 Introduction

For hundreds of years, chickens have been widely used as a food source around the world. They have been farmed for both their eggs and meat. According to the food and farming industry from the Department for Environment, Food & Rural Affairs, the poultry meat statistics show that on average between September 2018 and September 2019 , 245.1 million broiler poultry birds were slaughtered in the UK [73].

Chicken products can be represented by the contents of their molecular and biomolecular agents. The combinations of these could provide each sample with individual indicators of their sources, rearing or farming husbandry. Therefore, methods available for the quality control and characterisation of chicken meat muscle samples, together with corresponding evaluations performed on their biological fluids, are of much importance and significance in a range of food research areas.

3.1.1 Farming Husbandry

Farming husbandry is the process of raising livestock, and consequently there are many animal welfare considerations needed when managing poultry livestock. In

the UK there are specific poultry farming guidelines, set up by the Department for Environment, Food & Rural Affairs, that help to manage and regulate the welfare of flocks in farms or poultry houses [74].

Within the guidelines, signs of good and bad health are specified, including a section on early signs of ill health. Other factors that directly relate to the health and quality of the chicken are extensively stipulated. The environment that the birds are to be kept in is included, as well as considering factors such as ventilation, temperature, light and litters [74].

Feed and water conditions for poultry are also mentioned within these guidelines. It is specified that birds should be fed a wholesome diet for their age and species in order to maintain good health, and that an adequate fresh supply of water should be provided. Water and feeding equipment should be designed and maintained to minimise competition between birds, avoid contamination, however contamination from what is not specified, and be able to function in all weather conditions. Feeding and watering equipment should also be regularly disinfected [75].

Reviewing these guidelines, there are no strict regulations governing feed and water, but simply that supplies should remain available and be kept clean. Governance of feed and water can ultimately have an influence on research that is conducted within this area since many farms treat water, using a variety of

different systems, before supplying it to birds, and levels of nutrients in feed can also differ depending on what is provided. It is important to recognise these guidelines, as they contribute to the quality of meat produced, which is one of the most important factors considered by both the industry and consumers.

The welfare of flocks can have a direct impact on the quality of meat produced. This ultimately has an impact on the industry and consumer. This is evident in many studies over the years, such as Campbell et al who carried out a review of the environmental enrichment for laying hens during rearing in relation to their behavioural and physiological development. Campbell concluded that there was a need to commercially validate any positive impacts of any cost-effective enrichments on the behaviour and physiology of poultry [76]. Campbell's study highlights a need within the agricultural research sector, to carry out further studies into the effects of the environment, on poultry and to assess why evaluating poultry on more of a physiological level is beneficial for this industry.

3.1.2 Applications of NMR-based Metabolomics in the Food Industry

The use of NMR in the analysis and quality control of meat has greatly progressed in recent years. The ability to simultaneously visualise all abundant molecules present in a complex system or raw materials makes NMR a favourable tool in food science[77]. Additionally, a wide range of molecules within a sample can give rise

to signals simultaneously, and therefore NMR spectroscopy is able to attain a molecular 'fingerprint' of the sample under study.

Using NMR to analyse product quality is becoming ever more prevalent in food research. This approach is being utilised to not only look at quality but also meat traceability, which are important factors for both consumers and manufacturers in the food industry [78]. The need to have a tractability system within the food industry, which can provide information on the origin, processing, retailing and final destination of foodstuffs, is fast becoming important, in order to enhance consumer confidence.

^{31}P NMR is also being introduced as a complementary tool of meat analysis. Currently as it offers only a limited amount of information, it is mainly applied to the post-mortem evaluation of animal muscle and assessing the water-holding capacity (WHC) of meat muscle. ^{31}P NMR spectroscopy, does also allow for the monitoring of added phosphorus compounds in brined muscle foods. This gives a better understanding of the role of these phosphorus compounds within WHC, and enable the control of these additives to the meat [79].

Previously, the use of NMR-based metabolomics has been combined with chemometric analysis for distinguishing the origins of various raw beef samples [80]. The study recognised the importance of growing consumer interest in the geographical origin of the meat they were purchasing, in view of an increased

number of 'mad cow' disease-affected animals and the implementation of the Free Trade agreement at the time.

^{1}H NMR spectroscopy was implemented in this case, coupled with a series of multivariate statistical analyses including; principal component analysis (PCA) and orthogonal projection to latent structure-discriminant analysis (OPLS-DA). The results acquired showed significant differences between extracts of beef originating from Australia, Korea, New Zealand and the United States. In particular, the OPLS-DA loading plots highlighted the major metabolites giving rise to the differences observed, including isoleucine, leucine, methionine, tyrosine and valine. It was noted that the considerable variability between these countries could be attributed significantly to breed, feeding regime, production system, environmental parameters and pre- and post-slaughter conditions [80]. Variability factors were not thoroughly investigated within the study, so it cannot be known whether certain variables such as breed or feeding regimes were the same or different between geographical locations.

It can be argued that the origin of any meat specimen is attributable to the conditions they have come from. However, attempting to define origin, with so many differing variables can become difficult to interpret. It is important to establish whether a single variation such as feeding regime or breed is attributable to significant differences between countries. This is because; it is unclear form the beef study to what degree each variable has contributed to the overall separation

between countries, as some factors may have had no effect on any significant difference. This therefore leaves room for research to be carried out, in order to assess whether significant differences can be detected between experimental groups, where only one independent variable is being measured and other extraneous variables are regulated so that results can be clearly comparable.

This study also did not conduct any research within the UK, and therefore there is potential for studies to be carried out not only to compare samples 'between countries', but also 'between regional farms' for various farmed animals. Considering this concept, extraneous variables such as the ones noted above would have to be heavily regulated in order to produce reliable, comparable results, which may not always be realistic.

Not only origin, but quality of meat is a growing area of interest within the food industry and is being assessed more regularly by the use of NMR spectroscopic-based methods. In 2013, a study was carried out assessing the relationship between age and quality of duck meat using ^{1}H NMR [81]. Ageing can cause changes in the metabolic profile, and therefore it was suggested that this could have a possible impact on the quality of meat. ^{1}H NMR spectroscopy allowed the metabolic composition of duck meat at four different time periods (27, 50, 170 and 500 days) to be elucidated. It was found that although lactate and anserine levels increased with age, fumarate, betaine, taurine, inosine and alkyl-substituted free amino acid concentrations decreased[81]. The results of this study create new

opportunities for analysis of other species of poultry, such as chicken meat, not only to assess metabolites that contribute to quality, but to be applied to research that assesses specific variables that can attribute to quality such as feed or water quality.

Authenticity of products can also be a major cause for concern within the food industry, as it is fast becoming apparent that the contents of a product may not be what it states on the packaging. Food fraud has grown considerably over the past few years[51], and adulterations of produce with additives such as preservatives or bulking agents may not always be apparent visually. However, further molecular analysis may show entirely different results. A study carried out into the authentication of roasted coffee, from a commercial producer, using ^{1}H NMR spectroscopy found four adulterants within their blends. These identified impurities could have been introduced during the manufacturing process.[51] The importance of studies such as these, show not only how the purity of a product can be identified, but also possibilities for manufacturing process development for tighter regulations. Being able to show a true representation of food products has the potential to create a biochemical fingerprint, unique enough to be traced directly to the individual producer themselves. The same can be said for meat authentication, as there are opportunities for more researchers to work with producers in order to build up more of a complete profile of the produce in question, so that authentication can become quicker, more accurate and easier to access.

A variety of metabolic quality factors have been identified as quality traits in meat, but little is known about which metabolic components are true reflections of high quality as this varies from study to study. A study into the determination of metabolites of porcine serum responsible for the meat quality by ^{1}H NMR, listed glucose and creatinine amongst their top biochemical properties for meat quality[82]. Another study investigating the quality traits of duck meat, lists creatine, betaine, anserine and carnosine as important indicative compounds[83]. Throughout the studies that have mentioned metabolic quality traits, creatine seems to be a quality indicator that is mentioned a handful of times, but by no means can this be assumed a universal indicator. Therefore, much more research has to be done in order to determine what metabolic quality factors pertain to certain types of meats, as only a handful of studies have been done all varying in the types of meat studied.

For produce such as chicken meat, techniques such as ^{1}H NMR spectroscopy could have the potential to distinguish between different producers, and also allow for 'on-site' manufacturing developments to enable product quality improvement.

Being able to utilise such a technique in this manner highlights how crucial NMR-based metabolomics analysis can be used for both the food industry and food science in general, and also the need for further research.

3.1.3 NMR-based metabolomics applications to chicken research

Previous research has been conducted into attempting to characterise the metabolic profile of chicken meat.

Xiao et al carried out a study, published in 2019, to assess the metabolic composition of chicken meat at various ages using a 600-MHz nuclear magnetic resonance spectrometer. It was noted by the study's author that 'to the best of our knowledge, few studies have focused on the analysis of the complete metabolome of chicken meat including primary and secondary metabolites.' [84] They mention that the only other study to attempt to comprehensively characterise chicken breast meat muscle was a study carried out by Le Roy et al [85]. The study by Xiao et al was able to ultimately identify 57 metabolic components within chicken meat muscle [84]. As this study was carried out in China there is a more of a need for data to be derived from other countries in order to have a fuller picture of the metabolic profile of chicken meat muscle.

Currently, there has been limited metabolomics research conducted directly into the evaluation, traceability and quality control of chicken breast meat muscle using ^{1}H NMR analysis.

The importance of exploring this is to understand the biological and biochemical (metabolic) mechanisms associated with the control of poultry quality and tractability. This could potentially allow a set of biomarkers to be defined, which

could provide an indication of source and rearing, and also aid the development of farming husbandry. Thus, one of the advantages of creating a predictive model would be to enable the optimisation of poultry meat quality.

Poultry in the form of turkeys, ducks, geese, quail, guinea fowl, and particularly chicken, is viewed as an acceptable dietary component in many countries today. However, in 1990, chicken was the only meat to increase in sales in Great Britain, accounting for 33% of the carcass market [86].

Extensive breeding and nutritional programmes have ensured that a shorter time is needed when growing birds to a marketable weight. This is particularly evident in the case of broiler chickens, where it has been reported that live weights of 2kg can be achieved by up to six weeks, compared to the 8 weeks required in previous years[86].

Since this ever more prevalent issue of quality assurance, research has been carried out into the characterisation of chicken tissues and biological fluids in order to cater for future research on poultry metabolomics. Le Roy et al. conducted preliminary research into the NMR-based metabolic characterisation of chicken biofluids and tissues in order to create a model for avian research. The purpose of this research was to use biofluids and tissues in order to provide metabolomics data for poultry meat. Research carried out involved the analysis of biological matrices, including the liver, kidney, spleen, plasma, egg yolk and white,

colon, caecum, faecal water, ileum, pectoral muscle and brain of n = 6 chickens. Approximately 80 metabolites were identified in total, highlighting the advantage of using NMR-linked metabolomics analysis as a technique for this purpose. However, this research should be viewed as preliminary, since there are a number of drawbacks that seem to compromise the reliability of this study. Only six 'NovoGen Brown' commercial laying hens in total were analysed. This limits the reliability of the metabolites identified, as there is no discussion to whether these 80 metabolites are breed- or geographically specific, or whether they contribute to any quality factors. It is mentioned that the chickens were purchased in Surrey, however there is not further information regarding this variable[85]. Chickens were also fed a diet of 'Chicken Layer Pellets' and water *ad libitum*. Feed can vary between different poultry farmers, which, in turn, can contribute to variability, and this factor was not highlighted in the discussion. The conditions of the system that supplied the water is also not stipulated, since this could exert an influence on the metabolic profiles of the chickens since there are risks associated with water consumption in poultry health. According to Amaral (2004) these include waterborne diseases such as; bacterial, viral and protozoan [87]. Therefore, system and basic information such as water pH is important to report. The research conducted, highlights major developments in metabolite identification within the area; however, it was reported that results produced high variability, which could be a reflection of the small samples size. This leaves room for further research to be conducted using a larger sample size, and also exploring the ideas of variability through factors such as diet, water consumption or geographical origin.

Consequently, metabolomics research has been carried out by the Institute of Food Research, into determining the origin of chickens using proton NMR as an analytical tool. This has provided an insight into how chickens can be statistically significantly distinguished by geographical location [50]. Defatted dry material from chicken breast was analysed by ^{1}H NMR analysis in order to determine whether discrimination by country of origin could be achieved through multivariate and univariate statistical methods. NMR was used, and combined 1D and 2D strategies in order to identify possible biomarkers for discrimination of origin. Chickens were obtained from four different, locations shown in Table 5 [50];

Table 5: Geographical origin, country and number of samples acquired for NMR analysis [50].

Geographical Origin	Country/Region	No. of NMR samples
Europe	**France**	19
	German	14
	Denmark	19
	UK	9
	Netherlands	2
	Poland	5
	Hungary	2
South America	**Brazil**	89
	Chile	43
	Argentina	6
Thailand		34
China	**Batch A**	58
	Batch B	83
	TOTAL	**383**

The samples provided, were obtained in the form of defatted dry material from chicken breast. Results from NMR analysis showed both Chinese batches A and B being clearly distinguishable from all other origins using ^{1}H NMR analysis. Samples

originating to Europe could be characterised by higher levels of alanine, glycine, branched chain amino acids (valine, leucine, isoleucine), betaine and dimethylglycine and lower levels of carnosine and anserine compared with the other regions [50].

There was very poor sample representation from areas in Europe such as the UK and the Netherlands and Thailand, as countries such as China and South America have almost double the number of samples when compared to all others. The study also compares countries like Thailand to whole continents like South America, which does not seem representative.

Additionally, since samples collected from China were the highest quantity amongst all other regions, this could have led to a more accurate representation. The feeding and drinking conditions between each sample from China were also more likely to be consistent with one another. Chinese samples that were divided in batches A and B were supplied at different times from two different regions; however, the regions involved were not specified.

With reference to Table 5, the number of samples obtained from various locations in Europe may not have been representative of the continent as a whole, since for some countries within Europe only 2 samples were provided. However, for countries covered in South America, a larger number were obtained from Brazil and Chile in order to provide more representative results. This perhaps could

suggest why there was no significant distinguishability between Europe and the rest of the samples

In addition, no other biofluid was used in analysis such as blood plasma. The breed of chicken was also not specified, so it is not clear if the same breed was observed throughout the different locations. Therefore, any variance found between locations could be simply ascribable to a difference in breed.

Variables such as breed, diet, post mortem conditions, were not seriously considered in this study, as it was mentioned that 'The metabolite profiles of organic compounds measured by these methods presumably reflect a number of factors including breed, diet, living conditions etc.' [50] Meaning there are just too many variables within sampling to give a meaningful conclusion, as it is not clear to what extent each variable contributes to any significant difference between groups. Factors such as feeding regimens and water consumption can also vary depending on location, budget, governmental laws and guidelines and individual preferences of the farmer. Thus, leaving room for research to be carried out into how much each individual factor, such as drinking conditions or feed, can impact the metabolic profile of chicken meat muscle. Conclusively, representative samples from continents were poorly met, such as two samples from Hungary surely cannot represent the metabolic profile for all the chickens within that country as a whole. Additionally, the conditions chickens were kept in within the European

groups cannot be assumed to all be consistent, since even UK welfare guidelines for some aspects are not tightly regulated [75].

Metabolomics work such as this, casts doubts on research that has been carried out identifying the origin of specific meats, as each individual variable has not been assessed for how much impact it has on the overall variation between groups. Although these studies have shown some significant differences amongst different sources of meat, is this really a true representation, if variables such as food, breed, light, temperature, storage or water supply could potentially have a varying effect to the significant variation between the groups that are observed?

Therefore, this leaves a requirement for studies to be conducted into observing whether singular variations, such as feed or treatment system of water can have an influence on the metabolic profiles of poultry, and whether these results could highlight any underlying issues when studying the quality and geographical origin of meat sources using metabolomics.

In the few studies that have used ^{1}H NMR to evaluate chicken-meat quality a number of certain metabolites have been identified that can be used as predictors.

According to a study carried out by Beauclecq et al the use of orthogonal projection to latent structure-discriminant analysis (OPLS-DA) multiblock analysis was used to the predict the quality of chicken meat [88]. Chicken muscle has previously been

classified as either pale, soft, exudative (PSE), where the value of the pH is below 5.7 at 15 minutes post-mortem; or dark, firm and dry (DFD), where pH value is above 6.4 [88]. This characteristic has been investigated, examining quality through variations in ultimate pH (pHu), and resulting changes in metabolism were investigated via ^{1}H NMR analysis [88]. This investigation argues that the variations in muscle glycogen storage are highly correlated with variations in pHu, and thus poultry meat quality. This is because the consumption of ATP produced from the stored glycogen in the muscle by anaerobic glycolysis during the transformation of muscle into meat releases lactic acid and protons, leading to a progressive fall in pH to the ultimate value [88]. Two divergent broiler lines were selected, where positive and negative pHu groups were characterised by a 17% difference in muscle glycogen storage [88].

Resulting muscle metabolites were analysed using ^{1}H NMR spectroscopy. A total of 17 discriminating metabolites between the two lines were identified using OPLS-DA chicken muscle[88]. The metabolites identified are included in Table 6 below [88]. These also include their variable importance in projection (VIP) in the OPLS-DA and fold-change ratio (FC).

Table 6 - Muscle Metabolites (n=25) Identified by OPS-DA Discrimination the pHu- and pHu+ lines [88]

metabolites	VIP[a]	FC[b]	*P*-value[c]
muscle polar extract ^{1}H NMR			
glucose	1.38	1.77	≤ 0.0005
glycine	1.36	0.60	≤ 0.0005
NAD^+	1.19	0.82	≤ 0.0005
betaine	1.16	0.67	≤ 0.0005
mannose	1.15	1.59	0.001
alanine	1.14	0.77	0.001
dimethylglycine	1.08	0.77	0.001
proline	1.06	0.84	0.001
phenylalanine	0.91	0.86	0.003
taurine	0.90	0.69	0.008
phosphocreatine, creatine	0.83	1.08	0.024
arginine, lysine	0.61	1.12	0.150
anserine	0.36	0.96	0.197
β alanine	0.32	1.05	0.473
niacinamide	0.06	0.97	0.839
muscle polar extract ^{31}P NMR			
glucose 6-phosphate	1.48	2.15	≤ 0.0005
fructose 1,6-bisphosphate	1.21	1.86	0.001
glycerophosphocholine	1.19	0.63	0.001
inorganic phosphate	1.13	0.84	0.001
AMP	0.95	0.60	0.008
mannose 6-phosphate	0.86	1.27	0.019
phosphocreatine	0.75	1.36	0.041
NAD(H)	0.74	1.14	0.051
ATP	0.53	1.08	0.127
phosphocholine	0.40	1.14	0.296

This study lists metabolites identified along with how important each factor seems to contribute to quality, which has seldom been previously done in other studies. Amongst the identifications, it can be seen that metabolites such as glucose, betaine and phenylalanine all rank within the top 10 VIP features observed.

This study indicates that there is a need to investigate poultry quality in more detail and to observe whether metabolic identifications and important quality features are shared amongst other studies. Also, to further research whether

additional factors such as water pH can show a statistical significance between broiler lines.

From previous studies reviewed, it is clear that there is room for more research to be carried out within the UK to comprehensively characterise the metabolic profile of broiler poultry. This is not just to further the research within this field but also to look ahead to potentially applying this to sectors such as food fraud.

The overall aim of this section is to characterise the metabolic profile of broiler poultry meat from the UK. ^{1}H NMR-linked metabolomics analysis will be employed to investigate the metabolic status of broiler poultry meat muscle to comprehensively identify and characterise metabolites therein.

3.2 Results

3.2.1 ^{1}H NMR Spectral Assignments

All ten NMR spectra of meat muscle were reviewed, and a representative example spectrum was chosen. This can be seen in [Figure 11] and is a representation of the typical metabolites identified from chicken breast meat muscle.

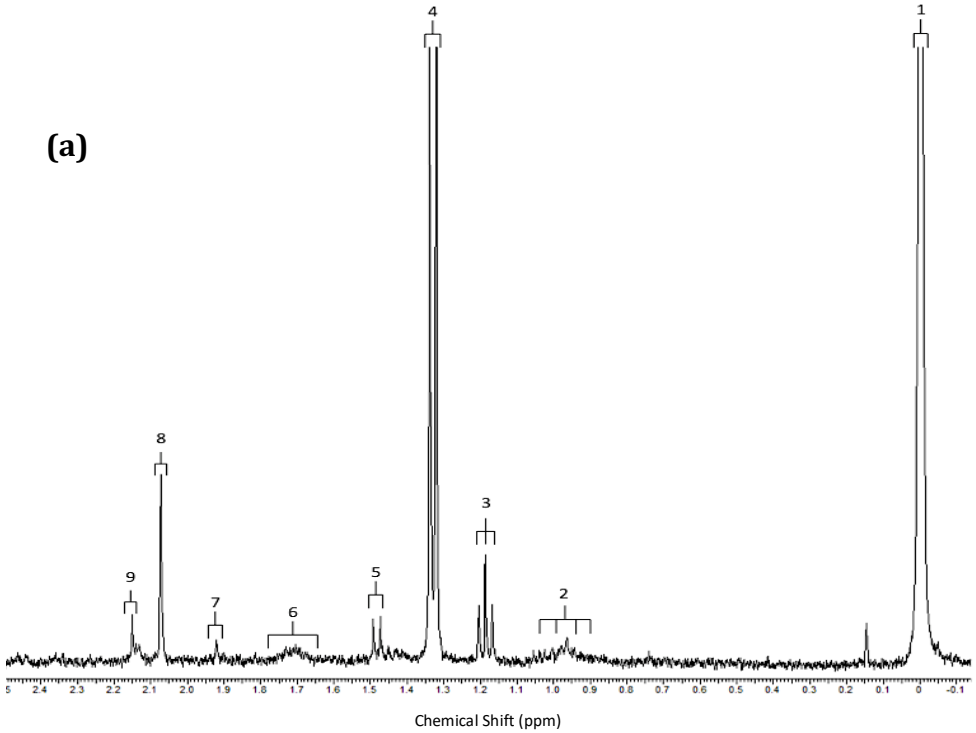
Initially each resonance was identified and the chemical shift was noted. For example, lactate which is denoted as a doublet in [Figure 11] as signal number four (4). Within metobolomics, there are a number of comon resonances you can assume to be present within certain biological samples, however, it is important to

always confirm this. This can be done by utilising the Human Metbolome Database (HMDB). Within this database there are various options that can be used in order to confirm the presence of a particular metabolite.

One method of confirmation is to enter the name of the metabolite, you wish to find a reference spectrum for, into the search bar. This will lead to a 'metabocard' of the metabolite, where information about the molecule can be found. This information includes; the common name, description, chemical structure, physiological effect as well as reference spectra from various analytical methods including GC-MS, LC-MS/MS, ^{13}C NMR and ^{1}H NMR. In this case, the reference lactate ^{1}H NMR spectrum can therefore be obtianed and compared to the chemical shift and coupling pattrerns of the resonance in question.

This, however can not be carried out for each resonance as it may not always be apparent which metabolite a signal belongs to. Therefore another method can be applied. The chemical shift of a resonance can be entered into the database. This will typically return a number of possibile metabolites that can cause this resonance. Each reference ^{1}H NMR spectrum must then be reviewed to find which chemichal shift matches that of the resonance most accuratly. In the case of signal 4 from [Figure 11] the chemical shift values of the doublet at 1.31ppm entered into the database. The chemical shift values that are entered are also given a peak tolerance of ± 0.02ppm due to minor variations in natural baseline drift [47]. As the results from the web-based database are produced, the first result given is of

Alpha-hydroxyisobutyric acid. This metabolite is discounted as the ^{1}H NMR reference resonance is only a singlet and does not match the precise chemical shift or coupling patterns of the resonance under investigation. The second result is that of D-lactic acid. The ^{1}H NMR reference spectrum matches the chemical shift patterns of the doublet resonance, at 1.31 ppm. The reference also gave indicaton of a quartet signal at 4.10 ppm, which is also observable on the spectrum belonging to the meat sample. The resonances were therefore confirmed as belonging to the metabolite lactate.



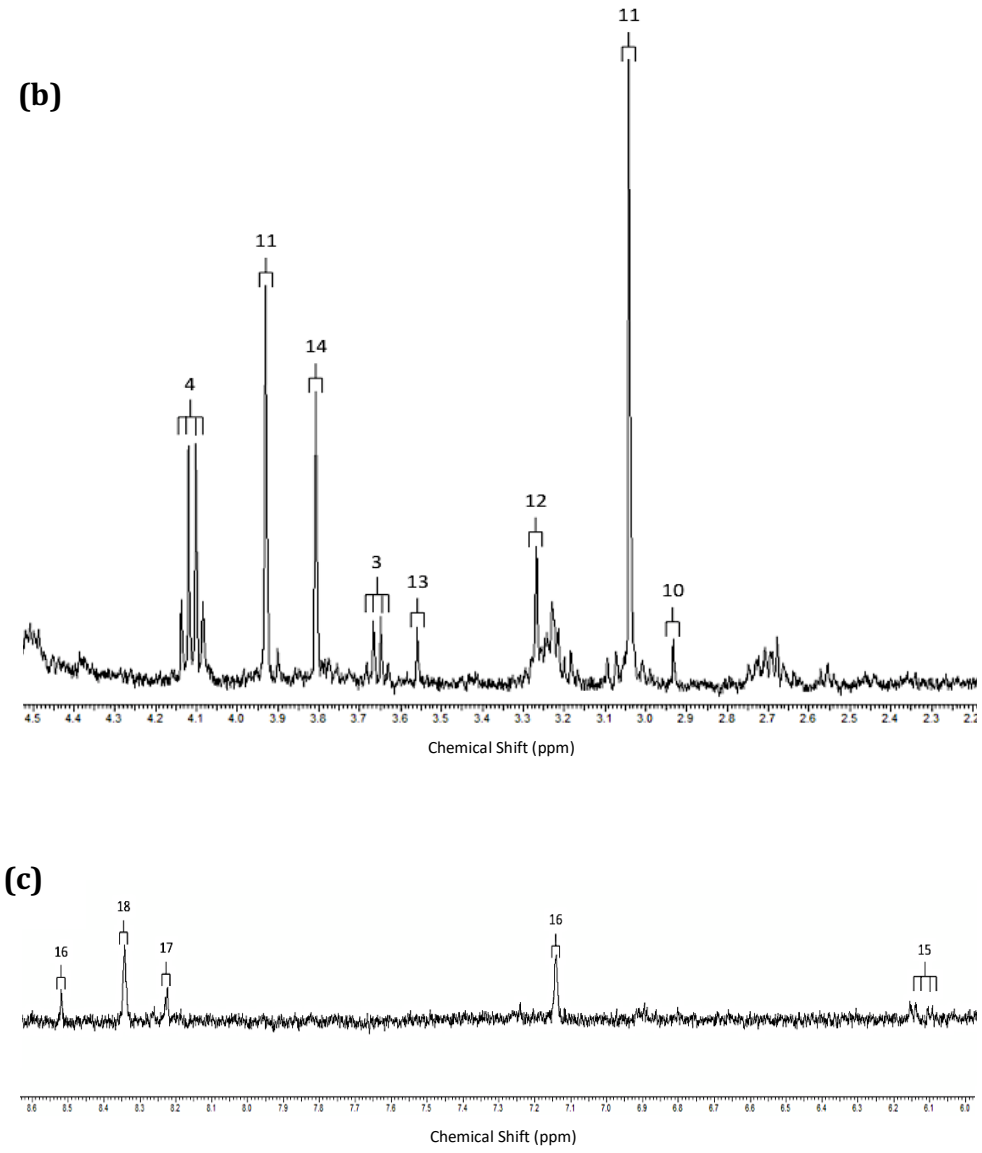


Figure 11: (a), (b) and (c), 0 – 2.4, 2.2-4.5 and 6.0-8.6ppm regions respectively of the 400 MHz meat muscle tissue ^{1}H NMR profile of poultry (a typical spectrum is shown):

<u>Abbreviations</u>: 1, TSP; 2, Not Distinguishable; 3, Ethanol; 4, Lactate-CH_3; 5, Alanine-CH_3; 6 Not Distinguishable; 7, Acetate; 8, N-Acetylglutamate; 9, Methionine; 10, Dimethylglycine-CH_3's; 11, Phosphocreatine-CH_3/Creatine; 12, Trimethylamine N-Oxide; 13, Glycine-CH_2; 14, Anserine; 15, NAD+, NADP+; 16, Histidine; 17, Hypoxanthine; 18, Formate.

Table 7 below, shows the list of metabolites identified and confirmed via HMDB in poultry breast meat muscle.

Table 7: Table of chemical shift values, coupling patterns and assignments of resonances present from Figure 11. Spectral assignment labels correspond to those visible in Figure 11 where; s – singlet; d- doublet; t-triplet q – quartet; m- multiplet

Spectral Assignment Label (11)	^{1}H NMR Resonance Chemical Shift Value (δ) / PPM	Coupling Pattern	Assignment
1	0	s	Trimethylsilylpropanoic Acid
2	0.9- 1.05	m	Not Distinguishable
3	1.16-1.18/3.63-3.67	t/q	Ethanol
4	1.31-1.32	d	Lactate-CH_3
5	1.45-1.47	d	Alanine-CH_3
6	1.17	m	Not Distinguishable
7	1.91	s	Acetate
8	2.07	s	N-Acetyleglutamate
9	2.15	s	Methionine
10	2.94	s	Dimethylglycine-CH_3's
11	3.02/3.92	s/s	Phosphocreatine-CH_3/Creatine
12	3.25	s	Trimethylamine N-Oxide
13	3.54	s	Glycine-CH_2
14	3.8	s	Anserine
15	5.98-5.99/6.09-6.10	d, d	NAD+, NADP+
16	7.10/7.90	s, s	Histidine
17	8.20	s	Hypoxanthine
18	8.54	s	Formate

Many of the spectral assignments within Table 7, were simple to identify, as the signals were visually clear, however broader signals such as that of signals; 2 and 6

were more difficult to distinguish from baseline noise and therefore classed as not distinguishable.

3.3 Discussion

From the research carried out on the ^{1}H NMR analysis of chicken breast meat muscle, a total of 16 metabolites were identified and confirmed with the use of HMDB.

Using HMDB for the assignment of peaks is common, as it has previously been used within studies such as Beauclercq et al, which looked specifically at identifying metabolic components of chicken meat muscle [88]. The use of this database has not been restricted to metabolic studies within the agricultural industry but also in areas such as the identification of disease biomarkers using NMR [49]. As the HMDB provides a simple free, user interface in order to match experimental chemical shifts from biofluid data against the spectral database, it was preferred over other databases, such as Chenomx, which tend to need licences and training. There is, however, opportunity for this data to be analysed using alternative programmes, like Chenomx in order to compare accuracy of the HMDB compared to others. However, as researchers universally use HMDB, it was deemed an appropriate method for this study.

All the metabolites identified in this study, correlate to metabolites previously found within other studies that have utilised NMR analysis for chicken meat, these studies include Beauclercq et al and Xiao et al [84], [88]. Between studies by other research groups and this study, a total of 18 metabolites identified were common throughout all studies. Whilst not all aspects of these studies are the same as the methodology used within this study, such as sample preparation, the key point to make about NMR is that, differences such as these do not majorly influence the chemical shifts of metabolites and therefore they are still comparable between studies, which perhaps cannot be said for methodologies such as GC-MS.

As, the studies by Beauclercq et al and Xiao et al have been carried out within the countries of France and China respectively, this study adds to data that can represent UK chicken metabolic profiles. This study has however, only been carried out specifically within the Midlands region of the UK. Therefore, this leaves room for additional research to be carried out within more northern and southern parts, so that a fuller picture can be created of the metabolic profile of chicken meat in the UK.

One particular issue however, that influences the comparability between this study and the studies carried out by Beauclercq et al and Xiao et al are sensitivity of the instrument. This study utilised at 400MHz NMR in order to obtain results whereas in studies by Beauclercq et al and Xiao et al, a 500MHz and 600MHz NMR was used to analyse samples. This does not largely affect chemical shift values, but it does

have an effect on how sharply the peaks can be visualised. This can explain why some resonances within this study, such as that of spectral assignment 2 and 6 in Table 7, appear broader when compared to other metabolic profiles of chicken meat. Knowing this it can be concluded that when dealing with biological samples pertaining to chicken meat muscle should not be analysed by an NMR with a strength of magnet that this below 400MHz, as anything below this would only hinder the ability of the research to be able to distinguish between components. It is also important to note that biological mixtures such as chicken breast meat muscle are complex and if the study was to be repeated it would have been beneficial to carry out analysis on an NMR with higher magnet strength in order to obtain the most accurately defined results as possible.

It is apparent that the results found within this study, do show a reflection of common metabolites that have been previously found within other studies that have investigated the metabolic profile of chicken meat. Creatine, for example, is a common metabolic compound found in many studies that investigate meat muscle, from Beauclercq et al who looked at chicken meat muscle quality to Zanardi et al who looked at metabolic profiling of ground beef from various geographical origins[77], [88]. Creatine is naturally synthesised in the liver, kidney and pancreas of vertebrates from the amino acids; arginine, methionine and glycine. In vivo, creatine is the product of the arginine biosynthesis pathway and metabolises to creatinine. The majority of creatine in the body is stored in skeletal muscles [89]. Creatine can be identified within a ^{1}H NMR profile from two distinct singlets

that appear at 3.02ppm and 3.92ppm [29]. For animal meat muscle, studies such as Kim et al and Lee et al have described that higher levels of creatine stored within meat muscles are associated with better quality [82], [83]. Beauclercq et al also found that within the top features that contribute to higher chicken meat quality, creatine was amongst one of the metabolites identified. Again, here there are gaps created within this field of food research to carry out further investigations that quantify levels of creatine within animal meat, but particularly chicken meat muscle, as this is yet to be performed within the UK.

Another compound identified within this study was acetlygluamate. Whist studies such as Beauclercq et al and Xiao et al, do not identify this compound within the NMR analysis they do, this compound has been identified in the form of glutamic acid and pyroglutamic acid from a study by Kelly, who identified it with the use of LC-MS/MS[50]. There have also been studies related to animal meat, in this case a study by Kim et al, which identified glutamic acid in porcine meat with the use of ^{1}H NMR at the chemical shift of 2.07ppm [82]. In 1952, Grisolia and Cohen found that a derivative of L-glutamic acid is required for the biosynthesis of L-citrulline[90]. They also found that that N- acetyl, N-chloroacetyl, N-carbamyl, N-propionyl and N-formyl derivatives of glutamate were active in producing citrulline, and that acetyl- and carbamyl-glutamate activates the biosynthesis of carbamyl phosphate [90][91]. This all takes place within the mitochondria and it is known that muscle contains the highest levels of mitochondria within the body. It may be possible that due to high concentrations of mitochondria within the

muscle, this has resulted in the ability to detect acetylglutamate within chicken meat muscle when subject to ^{1}H NMR.

The additional use of ^{31}P was considered but was not able to be utilised for this study. The consideration of ^{31}P NMR is important within the field of food research as it plays a major role in metabolic reactions[92]. Future studies should attempt to incorporate this within analysis of meat muscle as it can offer an additional level of separation between chicken meat or conversely show additional commonalities between meat samples, when dealing differences in geographical locations.

It is recognised that this study was only able to carry out on a total of 10 samples, which can be considered a small sample, as studies such as Beauclercq et al used a total of 20 chicken meat samples. Additionally, Xiao et al used 50 samples to characterise the metabolic profiles of Chinese Wuding chicken meat. The reason a small sample of 10 was used for this study was due to issues such as avian flu, which shows that, for research of this nature there are natural obstacles that can present themselves. When carrying out future investigations, it would be beneficial to analyse a much larger sample size in order to have much more of an accurate and reliable samples profile.

The metabolites identified from this study, add to the overall reliability of the metabolic assignments and help to 'fill in the gaps' in terms of exploring the total sum of metabolic components form various countries found in chicken breast meat

muscle via ^{1}H NMR. However, as this study focused on a particular farm within a particular areas and breed of poultry, this creates new opportunities to further comprehensively study poultry breast meat muscle metabolites from different geographical areas within the UK as well as a variety of breeds. The results from further study can aid in building up a range of profiles for samples from different parts of the country.

Using NMR technology shows many advantageous over techniques previously used such as IR, UV-Vis, X-Rays, electromagnetic waves including low and high frequency. As sample preparation and analysis is not labour-intensive or destructive to the samples. NMR analysis also allows for many parameters to be applied to the same sample; such as changing number of scans or applying ^{13}C NMR and ^{31}P NMR are still possible for the same sample.

Analysis of poultry meat muscle has moved on from simply using NMR to assess water distribution or water protein interactions, to studies such as this that help to pinpoint what particular metabolites contribute to the make-up of chicken meat. This is something that this study has assisted to develop. This research also opens up potential to begin quantitative as well as qualitative analysis as this will further individualise poultry meat muscle to all other available meat muscle available within the food industry. This has led to more of a platform for industries trying to minimise the occurrence of food fraud and additives to meat such as preservatives

and bulking agents that are not always visually apparent, which in turn help to further ensure higher quality.

3.4 Conclusion

From the developed methodology a total of 16 metabolites within poultry breast meat muscle have been identified including some, which have seldom been identified previously, identified using ^{1}H NMR spectroscopy. This helps to develop the overall research that has been done using ^{1}H NMR spectroscopy for poultry meat muscle as it helps to develop a more reliable fingerprint. This will eventually aid in both the food and farming industry and areas of meat fraud too enable higher quality of produce. It is still however, recommended that future research will help to develop the reliability, including use of ^{31}P NMR, so that there is a more standardised approach to analysis.

If this study were to be carried out again, there would be more of an emphasis on obtaining a higher quantity of samples, as this would further the reliability of the metabolites identified and potentially increase the number of metabolites identified. There is also opportunity for the use of ^{31}P NMR as the facilities were not available so this can allow for further identification of phosphorus-based metabolites.

Chapter 4 - ^{1}H NMR-Linked Metabolomics Investigations of Chicken Blood Plasma

4.1 Introduction

Over the past many years, the analysis of plasma has been commonplace in the realms of metabolomics research. In 2003, Graaf and Behar conducted a study to quantitatively measure the metabolites found in blood plasma using ^{1}H NMR [93]. This study is important to recognise as it shows that only in the last decade has NMR spectroscopy really taken off in terms of being applied to methods that would have been previously completed with LC-MS/MS or GC-MS [50]. Or perhaps the use of a radioimmunoassay, like in the study described by Leenstra et al in 1991, which looked at quantifying the levels of hormones, glucose, triglycerides and free fatty acids in the plasma of broiler poultry [94].

It was recognised that NMR analysis did not require any significant sample preparation, which led to the saving of a considerable amount of time of several hours, as preparation only took minutes. It was also discussed that minimal samples preparation also reduced the likelihood of sample contamination and therefore inaccurate and unreliable results [93]. In addition to this it was also noted that, the use of intact blood plasma was very important as it allowed the measurements of many important 'physicochemical interactions' that 'allows the direct study of serum macromolecules and lipoproteins' [93].

However as previously discussed, NMR analysis is expanding and being applied to more and more fields of research every day. In 2007 procedures for how plasma samples should be prepared for NMR analysis were standardised [54]. This study highlighted the need for standardisation of sample preparation for a technique that is becoming ever more popular to use in all areas of research.

The use of ^{1}H NMR to study plasma is now expanding to being applied to animal plasma and not just humans especially in cases of disease and health studies [95]. Kim et al, used plasma belonging to rats in order to model chronic kidney disease (CKD). Within this study rat plasma was prepared and analysed using NMR, the significance of the results was evaluated with the use of statistical analysis in the form of OPLS-DA [96]. The metabolic profiling and multivariate pattern recognition allowed for the simultaneous observation of a wide range of metabolites where the concentrations could be measured under the progression of CKD[96].

Of course, there are many studies that utilise both human and animal plasma for many areas of research. However, in the case of food quality research, it seems that meat is the biological sample of choice for many researchers as highlighted within the introduction of Chapter 3, and plasma can sometimes become neglected.

Taking live plasma samples for evaluation of food quality is seldom used. This is rather perplexing as plasma offers many advantages over the use of tissue analysis

in order to conduct food-based research as it can be taken before and after slaughter, whilst still keeping meat muscle intact. This means that a biological profile of the animal can be kept whilst the animal is still alive allowing for adjustments to be made if necessary, to improve quality. This also allows for less overall waste of potential produce for sale, therefore researchers as well as producers should be looking into alternatives such as the analysis of plasma to assess the metabolic profile of animals produce.

Metabolic profiles of chicken plasma have been studied very rarely, only a handful of researches have actually used plasma within their studies. Le Roy et al utilised NMR spectroscopy in order to analyse chicken plasma for metabolic characterization [85]. This study has been previously discussed within the introduction of Chapter 3, where it was concluded that the research conducted, highlights major developments in metabolite identification within the area of food research; however, it was reported that results produced high variability, which could be a reflection of the small samples size. This leaves room for further research to be conducted using a larger sample size, and also exploring the ideas of variability through factors such as diet, water consumption or geographical origin. However, only 6 samples were used in this study, leaving room for larger studies to be carried out to assess the reliability of the metabolites identified within this study. There was also no discussion to whether the metabolites identified are breed or geographically specific, which again leaves room for research to be carried out in order to assess whether changes in certain environmental factors can have an influence on metabolic profiles between chickens. These samples were

collected from Surrey, therefore leaving room for samples from other areas within the UK to be analysed. This will allow for a fuller profile of chicken plasma to be formed for future use whether that is for quality purposes or food fraud.

Beauclercq et al also used plasma as part of their study into the identification of key factors relating to chicken meat quality [88]. This study, discussed in the introduction of Chapter 3, used orthogonal projection to latent structure-discriminant analysis (OPLS-DA) multiblock analysis to the predict the quality of chicken meat [88]. A total of 39 samples were analysed and resulting serum metabolites were analysed using ^{1}H NMR spectroscopy. A total of 20 discriminating metabolites between the two lines were identified using OPLS-DA in serum respectively [88]. The metabolites identified are included in Table 8 below, which include their variable importance in projection (VIP) in the OPLS-DA and fold-change ratio (FC), with methylhistidine, hypoxanthine and phenylalanine being amongst the top factors that contribute to high quality within chicken plasma [88].

Table 8 - Serum Metabolites (n=26) Identified by OPS-DA Discrimination the pHu- and pHu+ lines [88]

metabolites	VIP[a]	FC[b]	*P*-value[c]
	serum ^{1}H NMR		
3-methylhistidine	2.44	**0.67**	≤0.0005
hypoxanthine	2.36	**0.68**	0.001
xanthine	2.22	**0.85**	0.002
1-methylhistidine	1.55	**0.81**	0.054
adenosine	1.54	**0.71**	0.034
uridine	1.41	**0.85**	0.067
fumarate	1.29	**0.86**	0.105
phenylalanine	1.12	**0.94**	0.142
glucose	1.03	1.06	0.058
formate	0.93	**0.88**	0.123
arginine	0.92	1.06	0.218
acetylglutamine	0.86	1.04	0.356
3-hydroxybutyrate	0.80	**0.93**	0.266
isoleucine	0.68	1.04	0.361
glutamine	0.65	**0.97**	0.426
tyrosine	0.65	**0.96**	0.523
maltose	0.34	1.06	0.523
citrate	0.31	1.02	0.621
lysine	0.20	1.01	0.864
phosphocreatine	0.15	1.02	0.720

Studies such as Beauclercq, show how analysis of chicken plasma is possible with the use of NMR, however, more has done in the way of metabolomics analysis in order to create more of a complete metabolic profile of chicken plasma, especially from various other countries, not just France where Beauclercq et al conducted their study.

Beauclercq et al, was not the only study to combine NMR analysis of plasma with multivariate analysis [88]. According to Cho et al, high-resolution NMR spectroscopy in combination with multivariate statistical methods has been widely used to investigate metabolic fluctuations in biological systems [97]. The study presented various methods for identifying the metabolic features that contribute

to the distinction of spectral samples among varying conditions in human plasma, which included; Principal Component Analysis (PCA) and Variable Importance in Projection (VIP) via Partial Least Square Discriminant Analysis (PLS-DA) [97]. The overall result of the study showed better classification was achieved using VIP values from a PLS-DA model than other unsupervised approaches such as PCA. It was concluded that the use of unsupervised methods such as PCA lead to potential problems in terms of accuracy and reliability of statistical conclusions [97].

Many studies have combined the analysis of human plasma using NMR spectroscopy with multivariate statistics. A study by Gebregiworgis and Powers applied PCA and OPLS-DA to human plasma metabolic profiles generated from NMR spectroscopy [49]. Elucidation of PCA and OPLS-DA score plots were done by visually inspecting the relative separation of class clusters. It was also noted that assigning a statistical significance to any clustering patterns is fundamental to inferring an accurate biological relevance [49].

It is not however only human plasma that statistical analysis has been applied to. As well as Beauclercq et al applying orthogonal projection to latent structure-discriminant analysis (OPLS-DA) in order to assess the significance of results from chicken plasma, Le Roy et al also utilised PCA in order to assess any impact of diet and infections on chicken metabolism.

The overall aim of this thesis is to comprehensively characterise the profile of broiler poultry plasma. It is also to further analyse broiler chicken plasma for

identifying potential quality traits and determine if the metabolic profile of blood plasma can be influenced by a difference water treatment system. Results will assist in evaluating the reliability of ^{1}H NMR-based metabolomics research that has been previously conducted into the broiler poultry.

^{1}H NMR-linked metabolomics analysis will be employed to investigate the metabolic status of broiler poultry plasma. Broiler poultry will be supplied with drinking water via two independent pre-treatment processes. The plasma from these birds will then be analysed and the data subjected to statistical analysis in order to determine whether there are any significant differences between broiler poultry.

4.2 Results and Discussion

4.2.1 ^{1}H NMR Spectral Analysis

All 49 NMR spectra of plasma were reviewed, and an example spectrum was chosen. This can be seen in [Figure 12] and is a representation of the typical metabolites identified from chicken blood plasma.

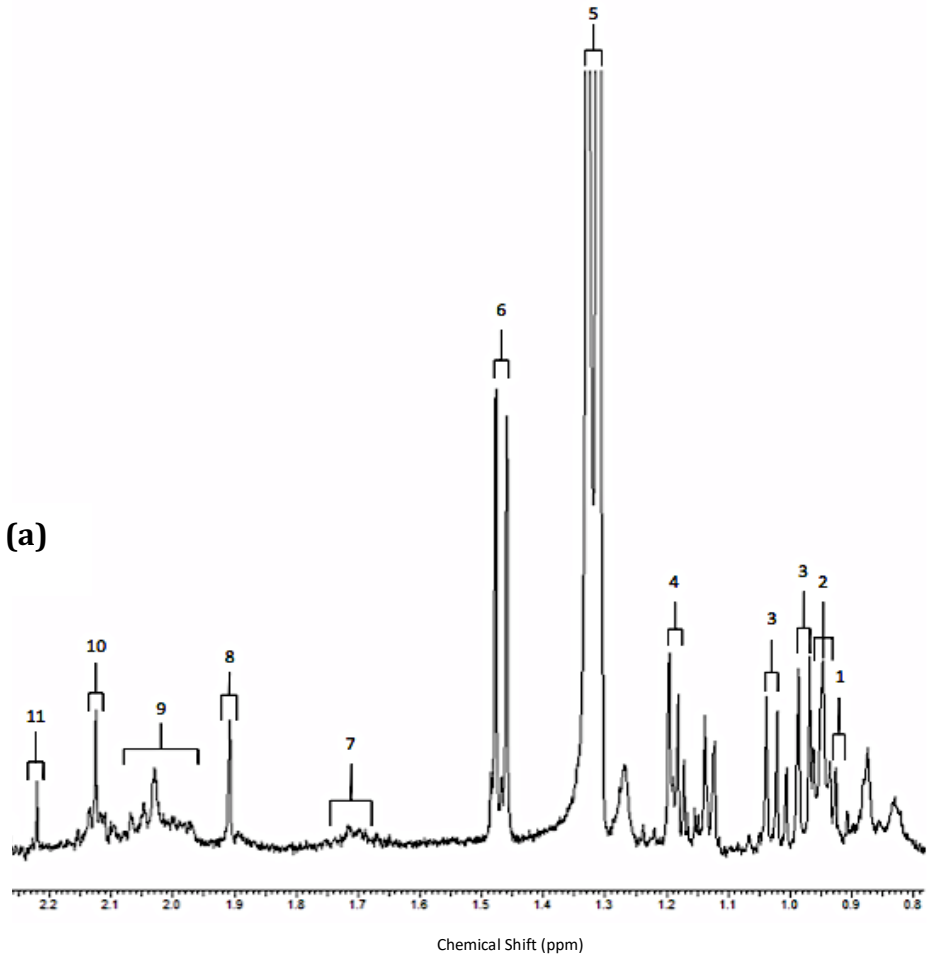
Initially each resonance was identified and the chemical shift was noted. For example, lactate which is denoted as a doublet in [Figure 12] as signal number five (5). Within metobolomics, there are a number of common resonances you can assume to be present within certain biological samples such as blood plasma,

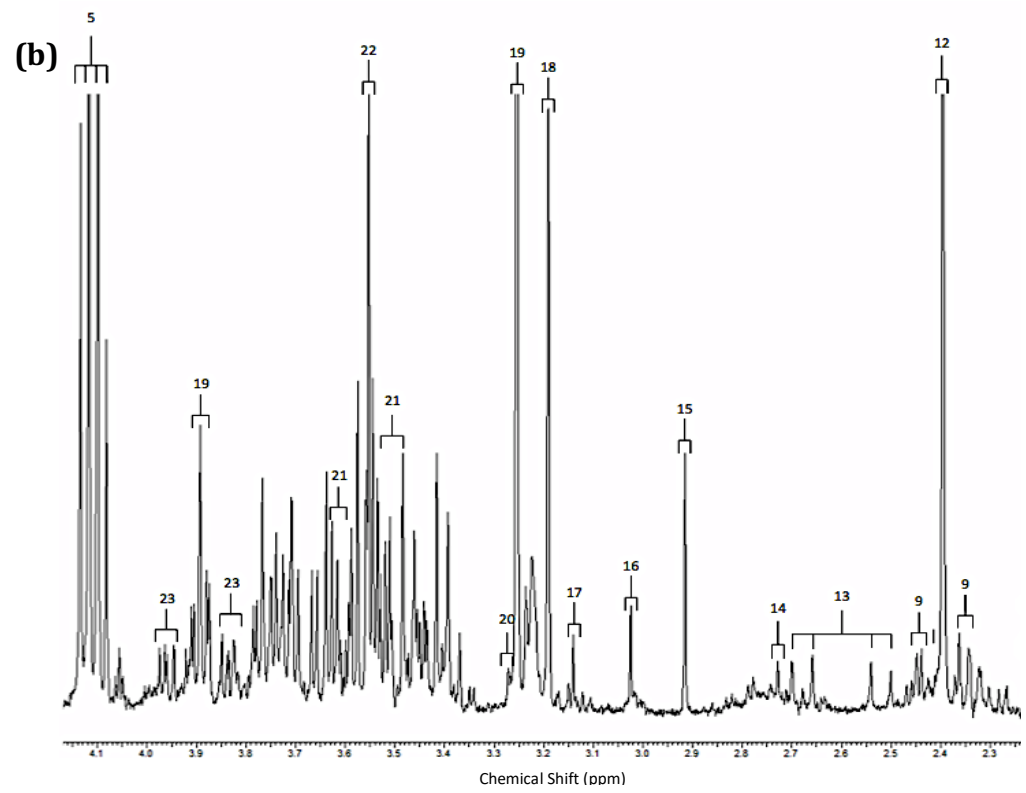
however, it is important to always confirm this. This can be done by utilising the Human Metbolome Database (HMDB). The database can be used to confirm the presence of a particular metabolite.

One method of confirmation is to enter the name of the metabolite, you wish to find a reference spectrum for, into the search bar. This will lead to a 'metabocard' of the metabolite, where information about the molecule can be found. This information includes; the commn name, decription, chemical structure, physiological effect as well as reference spectra from various analytical methods including GC-MS, LC-MS/MS, ^{13}C NMR and ^{1}H NMR. In this case, the reference lactate ^{1}H NMR spectrum can be obtianed and compared to the chemical shift and coupling pattrerns of the resonance in question.

This, however can not be done for all resonances, as it may not always be apparent which metabolite a signal belongs to due the cast mix of matabolites. Therefore another method can be applied. The chemical shift of a resonance can be entered into the database. This will typically return a number of possibile metabolites that can result at this ppm. Each reference ^{1}H NMR spectrum must then be reviewed to find which, if any, match that of the resonance most accuratly. In the case of signal 5 from [Figure 12], the chemical shift values of the doublet at 1.30ppm and 1.32ppm were entered into the database. The chemical shift values that are entered are also given a peak tolerance of ± 0.02ppm due to minor variations in natural baseline drift [47]. As the results from the web-based database are

produced, the first result given is of α-hydroxyisobutyric acid. This metabolite is discounted as the ^{1}H NMR reference resonance is only a singlet and does not match the precise chemical shift or coupling patterns of the resonance under investigation. The second result is that of D-lactic acid. The ^{1}H NMR reference spectrum matches the chemical shift patterns of the doublet resonance, at 1.30ppm and 1.32ppm. The resonances were therefore confirmed as belonging to the metabolite lactate.




(b)
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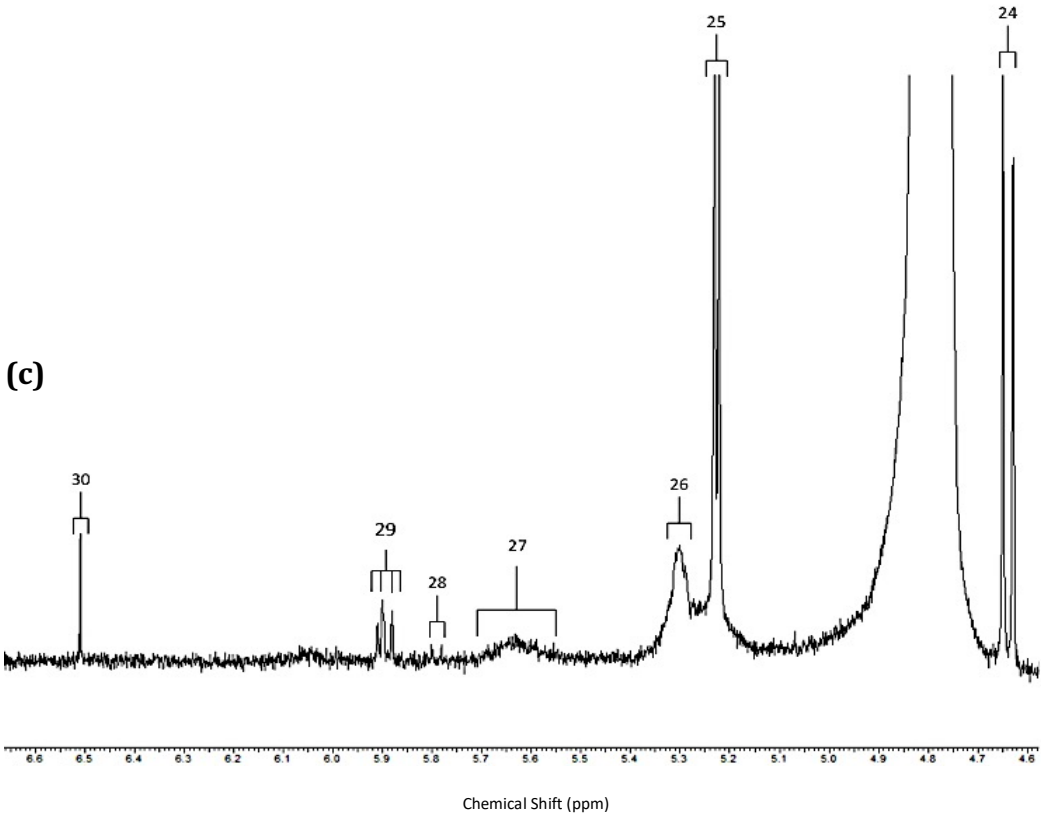
Chemical Shift (ppm)


(c)
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Chemical Shift (ppm)

(d)

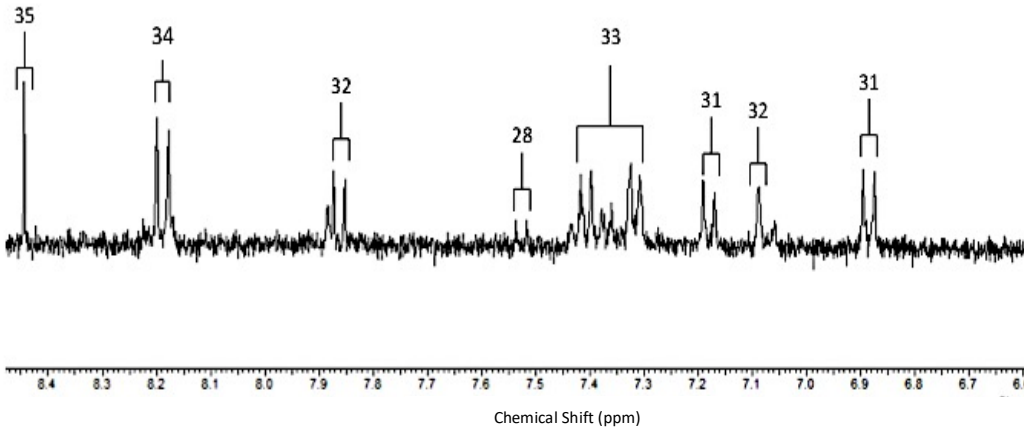


Figure 12: (a), (b), (c) and (d), 0.8-2.25, 2.25-4.15, 4.6-6.6 and 6.65-8.45 regions respectively of the 400 MHz plasma ^{1}H NMR profile of poultry (a typical spectrum is shown): Abbreviations: 1, Isoleucine-CH_3; 2, Leucine; 3, Valine-CH_3's; 4, 3-Hydroxybutyrate; 5, Lactate-CH_3; 6, Alanine-CH_3; 7, Not Distinguishable; 8, Acetate-CH_3; 9, Glutamine; 10, Trimethylamine; 11, Methionine; 12, Succinate-CH_2's; 13, Citrate; 14, Sarcosine-CH_3's; 15, Dimethylglycine-CH_3's; 16, Creatinine/Creatine; 17, Dimethylsulfone; 18, Trimethylamine Oxide; 19, Betaine; 20 Malonic Acid; 21, Taurine; 22, Glycine-CH_2; 23, Serine; 24, β-Glucose Anomer; 25, α-Glucose Anomer; 26, Vinylic Proton; 27, Not Distinguishable; 28, Uracil; 29,Uridine; 30, Fumarate; 31, Tyrosine; 32, Histidine; 33, Phenylalanine; 34, Hypoxanthine; 35, Formate.

Table 9 shows the list of metabolites identified in poultry plasma.

Table 9: Table of chemical shift values, coupling patterns and assignments of resonances present from Figure 12. Spectral assignment labels correspond to those visible in Figure 12 where; s – singlet; d- doublet; t-triplet q – quartet; m- multiplet.

Spectral Assignment Label (Figure 12)	^{1}H NMR Resonance Chemical Shift Value (δ)/ppm	Coupling Pattern	Assignment
1	0.92	d	Isoleucine-CH_3
2	0.94	t	Leucine
3	0.97/1.02	d/d	Valine-CH_3's
4	1.19	d	3-Hydroxybutyrate
5	1.31, 1.32	d	Lactate-CH_3's
6	1.45, 1.47	d	Alanine-CH_3
7	1.68	m	Not Distinguishable
8	1.91	s	Acetate-CH_3
9	2.05/2.34-2.44	m/m	Glutamine
10	2.12	s	Trimethylamine
11	2.15	s	Methionine
12	2.39	s	Succinate-CH_2's
13	2.51,2.54,2.64,2.67	q	Citrate
14	2.73	s	Sarcosine-CH_3's
15	2.94	s	Dimethylglycine -CH_3's
16	3.02	s	Creatinine/Creatine
17	3.13	s	Dimethyl Sulfone
18	3.18	s	Trimethylamine Oxide
19	3.25/3.89	s/s	Betaine
20	3.26	s	Malonic Acid
21	3.46-3.53	m	Glucose
22	3.54	s	Glycine-CH_2
23	3.38/3.95	t/t	Serine
24	4.62,4.66	d	B-Glucose
25	5.23	d	A-Glucose
26	5.32	m	Vinylic Proton
27	5.64	m	Not Distinguishable
28	5.79	d	Uracil
29	5.88-5.90	t	Uridine
30	6.51	s	Fumarate
31	6.87/7.17	d/d	Tyrosine
32	7.09/7.90	d/d	Histidine
33	7.36-7.42	m	Phenylalanine
34	8.17/8.20	s/s	Hypoxanthine
35	8.54	s	Formate

Most metabolites identified in this study, correlate with metabolites previously found in other studies that have utilised NMR analysis for chicken plasma, these studies include; Beauclercq et al and Le Roy et al [88] [85]. Some metabolites identified in this thesis, have not previously been attributed to broiler chicken plasma including sarcosine. According to HMDB, sarcosine is the n-methyl derivative of glycine. It is common to find this metabolic compound within chicken plasma, as it is normally present in many meats such as ham and turkey and even in egg yolks [29].

The majority of the metabolites identified in this study have indeed been recognised as constituents of chicken plasma from studies conducted by both Beauclercq et al and Le Roy et al [88] [85]. It is still important however to recognise its geographical location. Studies such as this conducted in other countries will allow researchers to investigate whether these typical metabolites found in broiler poultry plasma are indeed universal, as this cannot just be assumed. This will allow for further contribution to the metabolic profile of chicken plasma. It also highlights the benefit of utilising plasma rather than muscle. This is because the meat muscle can remain undestroyed, if only a plasma sample is taken and therefore, less meat is subjected to testing and ultimately being wasted. This study showcases that it is indeed possible to study broiler quality through the analysis of plasma rather than meat.

4.2.2 Multivariate Data Analysis

Herein well-known chemometric tools for data analysis were applied. In order to determine whether results from this data set were statistically significant, multivariate data analysis was employed. The PLS-DA method of multivariate analysis was used including permutation tests of data in order to explore and identify multivariate independence of any classification groups detected [59], [60].

Multivariate Data Analysis of Scotmas Water System versus the WET Engineering System

Before analysis, normalisation of data was performed. An example of the resulting distribution can be seen in [Figure 13].

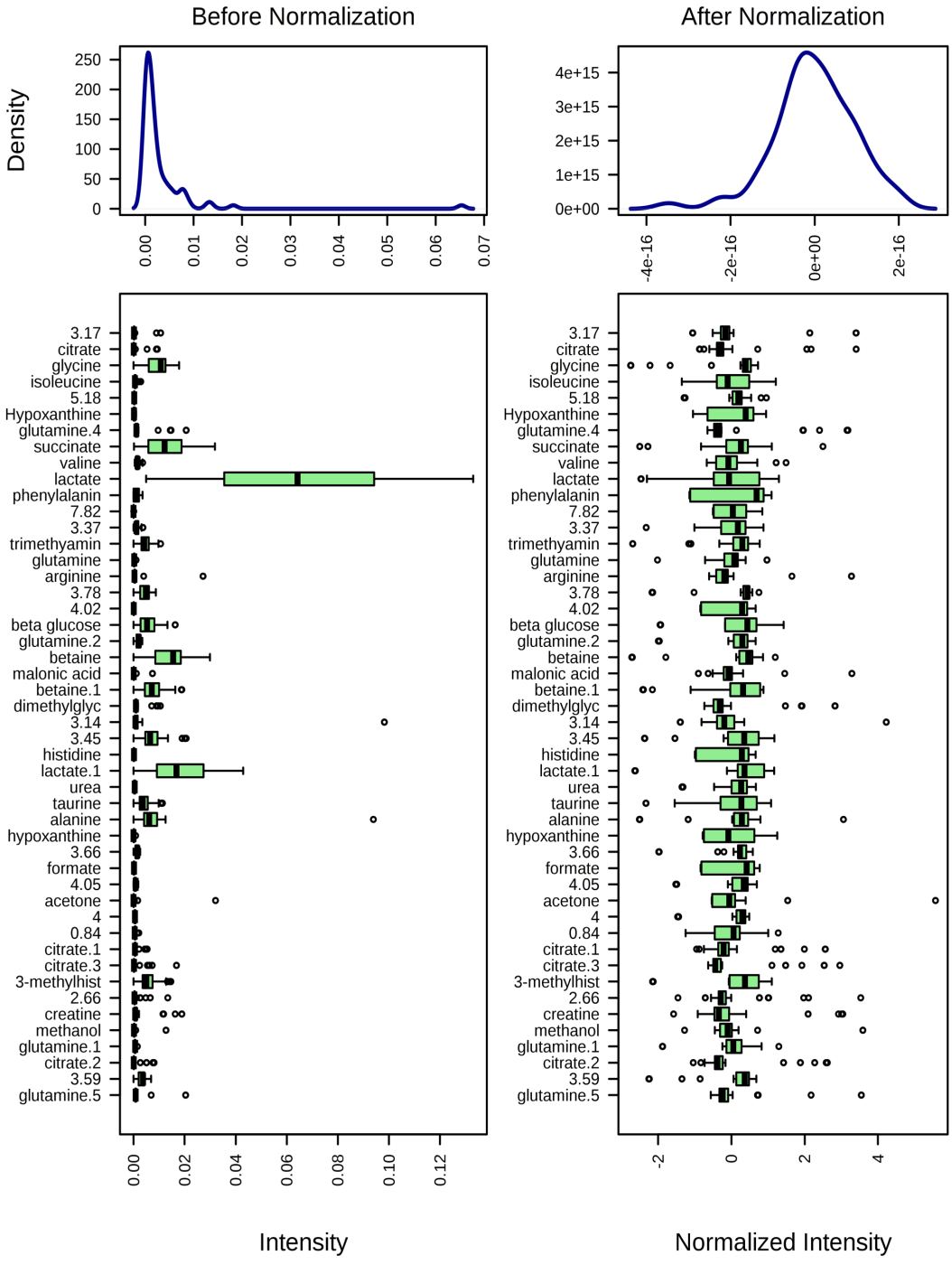


Figure 13: Normalisation of ^{1}H NMR poultry plasma samples comparing Scotmas Water System Vs. WET Engineering Water System

Following normalisation, PLS-DA analysis was used in order to reveal any potential classification clusters between the Scotmas water and WET engineering systems. The two-dimensional plot analysis of the results can be seen in [Figure 14].

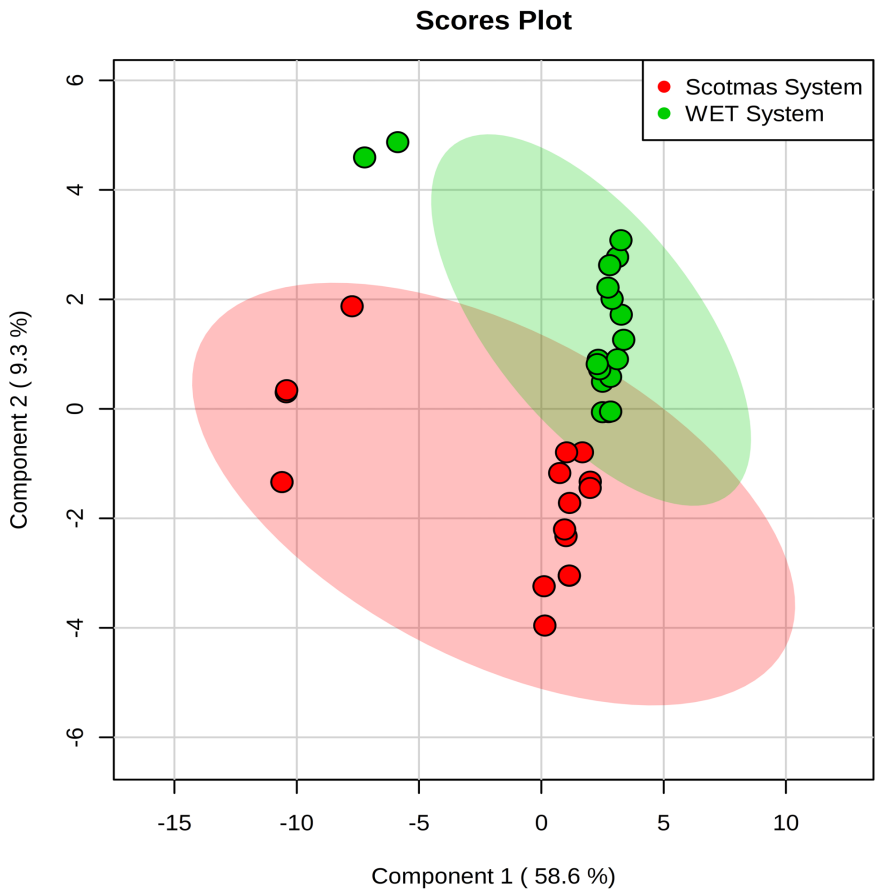


Figure 14 - PLS-DA Two-Dimensional Scores Plot of ^{1}H NMR poultry plasma samples comparing Scotmas Water System Vs. WET Engineering Water System

Samples pertaining to each water treatment system appear to cluster somewhat separately. This gives a primary indication that there may be a significant difference between metabolic levels between sample groups. However, there is a wide spread of sample data belonging to both data sets.

Accompanying cross-validation analysis was performed giving values of R^2 = 0.744 and Q^2= 0.57. The R^2 value highlights a good fit of the data and the Q^2 value highlights an adequate classification model. Therefore, it is also important to further investigate differences between groups by further assignment of classes to the WET engineering water system.

In order to determine if the two water systems showed statistically significant results the data set was then permutated. An explanation of this process can be found in section 1.3.4. A total of 1000 and 2000 permutations were employed in order to assess statistical significance. The significance level was set at 5%.

Permutation testing of the data set demonstrated that the differences between each classification were indeed statistically significantly different at (*p*= 5e-04) for 2000 permutations and (p= 0.001) for 1000 permutations.

As results showed significant differences between the metabolic profiles of broiler poultry plasma belonging to each water treatment system, further VIP (Variable Importance in Projection) score analysis was performed [Figure 15]. This assists in discriminating metabolic features that contribute to the significant difference between class groups [97].

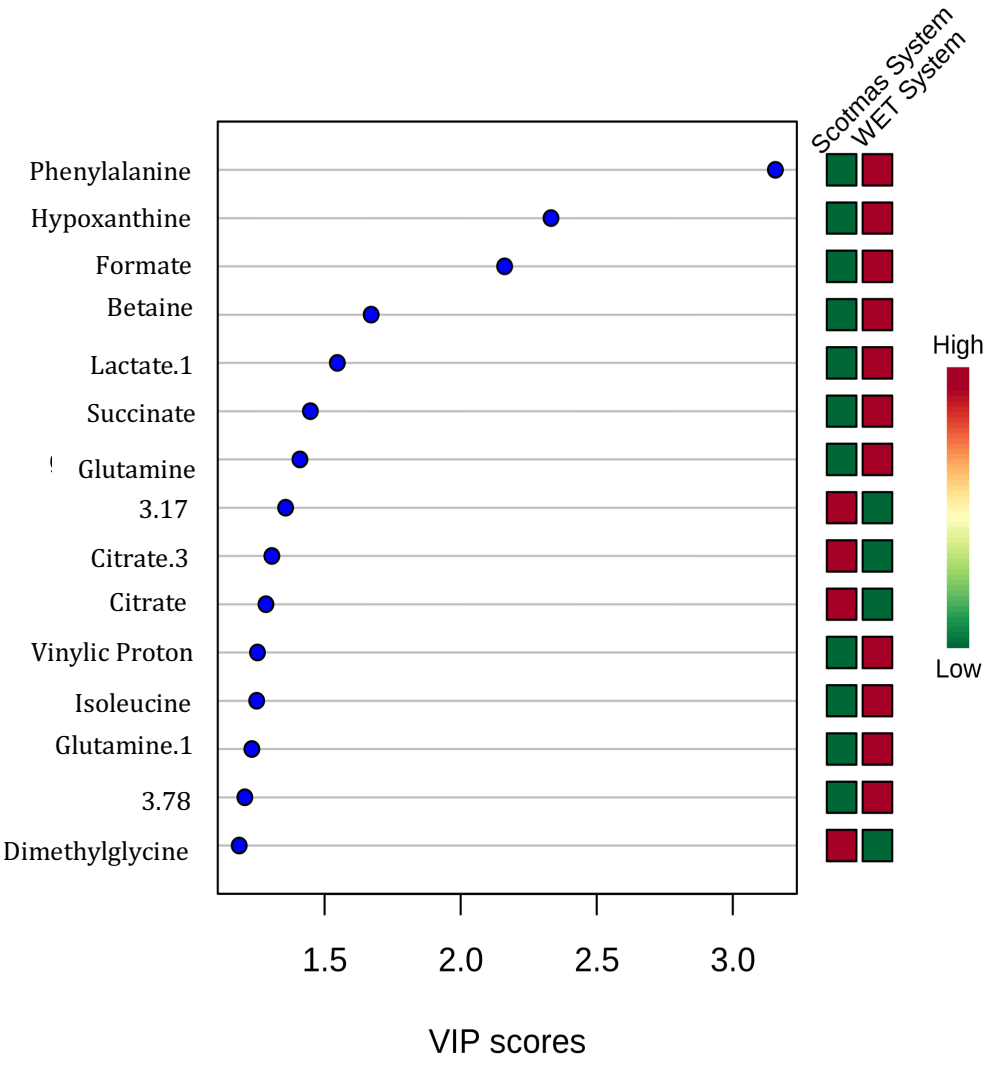


Figure 15 - PLS-DA VIP score analysis of ^{1}H NMR poultry plasma samples comparing Scotmas Water System Vs. WET Engineering Water System

VIP score analysis identified the top 15 features that contributed to significant differences, for metabolites that were unable to be identified; their chemical shift value is stated. Each VIP score is calculated using the weighted sum of squared correlations between the PLS-DA components and the original values [68]. Therefore, the higher the score the more that metabolite has contributed to any

significant difference. The heat plot located to the right, shows whether concentrations of the significantly contributing metabolites where higher or lower within the experimental groups.

For multivariate analysis, initially PLS-DA analysis was applied to the two water treatment systems. This was important to carry out in order to achieve a visualisation of data, which results in 2 broad clusters pertaining to each water treatment system. From [Figure 14] the two potential groups could be seen separating, with accompanying R^2 and Q^2 showing a good classification model and fit. To further explore and conclude whether there was a statistically significant difference in results, at this class level, permutation analysis was applied with a significance level set at 5%. The resulting permutations gave a p value of 0.001. This meant there is a significant difference in metabolic profiles between the Scotmas water treatment system and the WET engineering water treatment system.

Subsequently, the VIP score plot shown in [Figure 15] was explored in order to further understand which metabolites contribute to the significant difference. VIP score analysis highlighted that metabolites such as hypoxanthine, which is involved in the formation of nucleic acids, phenylalanine, which is an essential amino acid, and betaine which is important in metabolism and also widely regarded as an anti-oxidant, all according to the HMDB, were important factors in determining significant differences.

The only significant studies that have been identified, that look at metabolites found in poultry plasma are by Beauclercq et al and Le Roy et al described in the introduction of this chapter [88] [85]. The study by Le Roy et al only characterised components of poultry plasma, whereas Beauclercq et al studied and identified quality traits in plasma profiles under different group conditions.

Comparatively both this study and the study conducted by Beauclercq et al identified hypoxanthine, phenylalanine, formate, citrate and glutamine amongst the top significant contributing factors for the discrimination between both treatment groups.

The heat plot identified; hypoxanthine, phenylalanine, and betaine to appear lower in concentrations in Scotmas samples and higher concentrations in WET engineering samples. This could suggest the samples pertaining to the WET engineering treatment to perhaps be healthier and therefore of a better quality.

Results from Chapter 3 highlighted the studies that assigned particular metabolic compounds that correlate with quality. However, these were all done by conducting analysis on the meat [81][82][83]. These studies that assessed food quality only look at meat rather than plasma. Even though it is likely, it cannot be assumed that these quality indicators found in meat are mirrored in plasma.

This study looking at the comparison of plasma profiles resulting from two different water treatment systems and identifying any potential quality traits attributable to this difference is one of the first of its kind.

There is much room for more research to be done in order investigate what influence other factors have on broiler poultry, such as feeding regimes or even sleeping patterns. As this is all highly valuable information for not just the agricultural industry, but also governments that stipulate regulatory guidelines and laws especially when it comes to food fraud and the assurance of high-quality meat.

Initially comparing the two water treatment systems via PLS-DA, showed promising results. It was then decided that further investigation needed to be done in order to further assign classification labels. This would allow for further identification of separations within the WET water treatment system, as even though all shed water is largely processed identically, there was a difference in the ultimate pH throughout sheds. It is was also investigated as to whether these VIP scores will remain the same, when further classes are introduced within the WET water system samples.

Multivariate Data Analysis of Scotmas Water System versus individual WET engineering water Systems

The purpose of further investigation was to identify if significant differences arise when the Scotmas water treatment system was compared to the WET engineering water treatment system in the sheds.

As before data underwent normalisation. This was carried out again by applying constant-sum normalisation, cube root transformation and Pareto scaling, which resulted in a normal distribution of data.

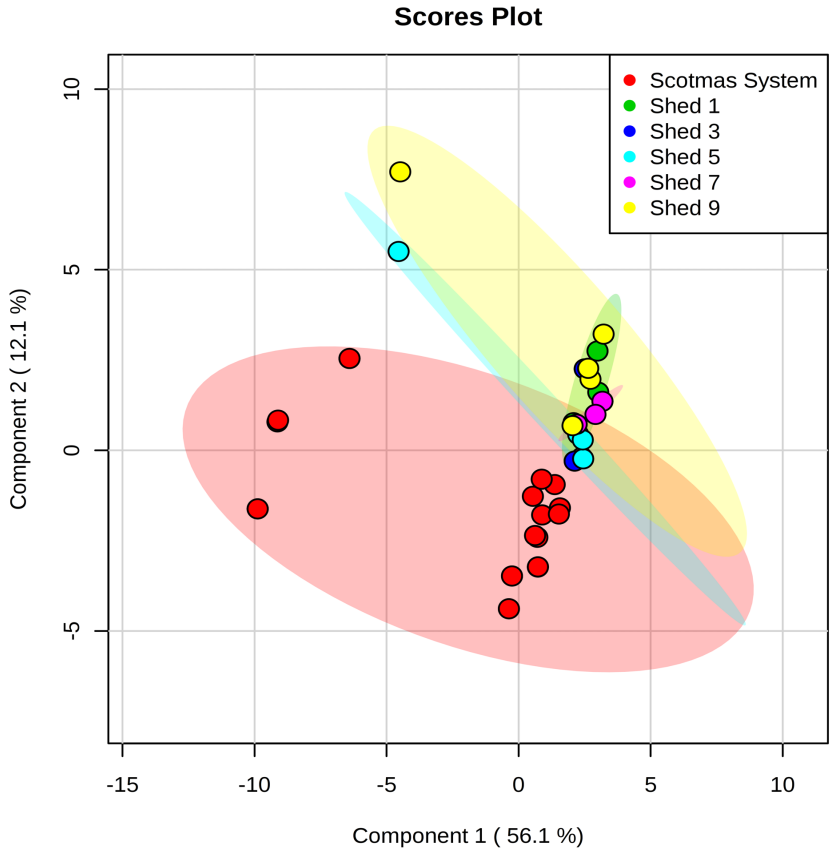


Figure 16 - PLS-DA Two-Dimensional Scores Plot of ^{1}H NMR poultry plasma samples comparing Scotmas Water System Vs. Individual WET Engineering Water Systems

Following normalisation, PLS-DA analysis was applied again in order to reveal any further classification clusters between the Scotmas water and individual WET engineering water systems. Two-dimensional plot analysis was initially analysed, the results of this can be seen in [Figure 16]. It is important to note that sheds, 1,3,5,7 and 9 belong to the WET engineering water treatment system.

Samples pertaining to each of the two water treatment systems still appear to cluster separately. It can be seen that there are potential further individual clusters within the data relating to the WET engineering system samples. Therefore, even further investigation will conclude whether there are any significant differences between just sheds 1, 3, 5, 7 and 9 which all belong to the WET engineering water treatment system.

In order to determine if results from [Figure 16] showed statistically significant results, the data set was permutated. A total of 1000 and 2000 permutations were employed in order to assess statistical significance.

Permutation testing of the data set demonstrated that the differences between each classification were indeed statistically significantly different at (p= 0.5 x 10^{-3}) for 2000 permutations and (p= 10^{-3}) for 1000 permutations. Results from permutation tests show there is still a significant difference between the metabolic profiles of broiler poultry with additional classifications, still showing significant variation between sheds.

As a result VIP (Variable Importance in Projection) score analysis was again performed in order to assist in identifying discriminating metabolic features that have contributed to the significant difference between the more detailed class groups [97].

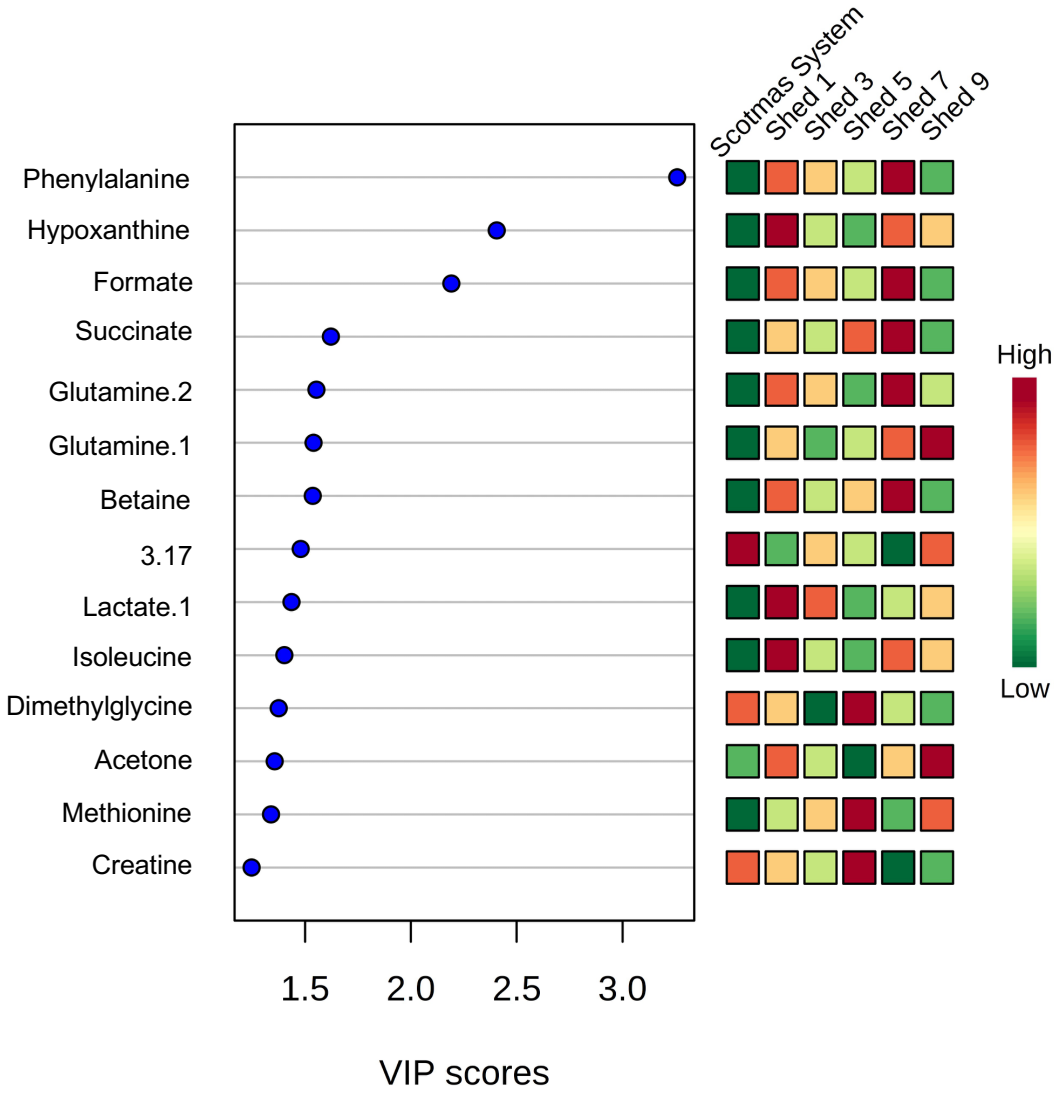


Figure 17 - PLS-DA VIP score analysis of ^{1}H NMR poultry plasma samples comparing Scotmas Water System Vs. Individual WET Engineering Water Systems

The VIP score analysis identified the top 14 features that contributed to significant differences. These results again showed metabolites such as phenylalanine, hypoxanthine and betaine to be lowest in samples pertaining to the Scotmas system, whilst showing higher levels in certain sheds belonging to the WET system.

The goal of this research study was to further identify the metabolic profile of poultry plasma and identify potential quality factors. It was also to assess if ^{1}H NMR spectroscopic analysis could be utilised in order to differentiate poultry treated with two different water treatment systems. This was done to determine whether this kind of change in variables could lead to significantly different metabolic profiles within poultry plasma.

The main difference between the two water systems was that the Scotmas system primarily used chlorine dioxide at a final concentration of 5ppm with the addition of Anthium Dioxcide® achieving a water pH of 6. The Scotmas system also incorporated the addition of organic acids through their Floradox program including antibiotics, vitamins and supplements, whilst the Wet Engineering water treatment system consisted of variety in levels of pH ranging from 6.5-9.

Whilst acquiring the NMR profiles of the chicken plasma, samples were analysed via a cpmg1d pulse programme. This allowed for the reduction of the water signal to between 4.7 and 5.0ppm, which normally overlaps a large number of resonances around 4.0 and 5.0ppm.

The resulting PLS-DA score plot shown in [Figure 16] still showed separation of samples pertaining to the Scotmas system but also possible separations between WET system sheds. However, to assess whether further individual classification assignments still gave a significant difference between all class groups permutation analysis was employed. Permutation analysis gave a resulting p value of 0.0001. This indicated that with the addition of more detailed class assignments there was still a significant difference between all class metabolic profiles.

With this, VIP score analysis was performed, shown in [Figure 17], in order to assess if the same or different metabolic features contributed to this significant difference as in [Figure 17]. Analysis from this VIP score plot still showed similar metabolic features, such as phenylalanine, hypoxanthine and betaine. It was seen that these metabolite levels were relatively low for samples pertaining to the Scotmas system and sheds 3, 5 and 9 and higher in sheds 1 and 7. This suggests that poultry quality was higher within sheds of an ultimate pH between 7.5 and 8.5. The reason for shed 1 to show more positive results was perhaps due to the high alkalinity of the water allowing for the irradiation of certain bacteria that may not have been able to survive at that pH, thus promoting higher metabolic health. This theory is further strengthened by shed 7 also showing some of the most positive results as this water system as fitted with the addition of anti-bacterial. It does pose a question to why shed 9 was not as successful at showing positive results as this shed was set to pH 8.5-9. However, this could be due to the higher

pH having more of a negative effect on gut bacteria and ultimately metabolic health.

However, from [Figure 17], creatine was additionally identified as a feature of significance. Creatine has also been mentioned in many previous studies such as Kim et al that highlight it as a metabolite that signifies high quality, as it increases muscle power, performance and mass, leading to a stronger healthier bird [82]. It was Shed 5 pertaining to the WET treatment system that yielded the highest levels of this metabolite. Indicating that the poultry quality could be highest within this sheds that used an ultimate pH between 7.5. [Figure 17] does show a high level of creatine within the samples belonging to the Scotmas treatment system. This could suggest a high level of quality; however, creatine is not a sole indicator of quality as highlighted by Beauclercq et al [88]. Samples belonging to the Scotmas treatment system scored lowest in the concentrations of all other metabolites.

This shows that significant differences have been found between water treatment systems, with particular attention to Shed 5 belonging to the WET treatment system.

4.3 Conclusion

In conclusion, this study showed that NMR could indeed be used as a robust methodology for the analysis of poultry plasma. It was also identified that there were indeed significant differences between poultry plasma samples when treated

with two different water treatment systems. A total of 33 metabolite identifications were made from a total of 49 samples. This assists in further comprehensively contributing to the complete identification of poultry plasma biofluids.

Metabolites that denote better health and thus higher quality include; betaine, hypoxanthine, phenylalanine and creatine, which were identified as main contributors to the significant differences between water systems. These same factors were also identified as significant quality contributors in the study by Beauclercq et al [88].

It can be concluded that changes in pH indeed do have an influence on the metabolic profile of poultry plasma and the metabolites that were contributed most significantly are involved in important metabolic processes. Thus, those samples with higher concentrations of these metabolites were denoted as being of a higher quality.

This study also opens up so many new platforms for further research such as potential metabolic fingerprinting of different poultry breeds and reliable method for identifying metabolites that constitute as quality factors. It additionally has the potential to aid with geographical origin information as farming methods like water treatments systems and feeding systems so vary amongst farms. Countries may also have different regulatory information on factors such as these, so it is important to investigate what influences these have.

The use of ^{1}H NMR in this study showed potential that may be integrated into industry. Due to the simple sample preparation and high throughput of samples it can surely be very beneficial in aiding with contributing to the construction of a poultry database. This has the potential cut down on food fraud; to create perhaps metabolic fingerprints for different poultry breeds. It can even possibly aid with geographical origin information as farming methods like different water treatments systems and feeding systems so vary amongst farms.

This study has also created additional research opportunities into further investigating what has been hypothesised for this study. As only 1 crop was used for this study there is scope for experimental parameters to be duplicated for a more longitudinal study, which can incorporate perhaps different breeds and higher overall sample numbers. There is also further potential for other species such as duck to be investigated, as that is also commonly eaten meat worldwide.

A new platform for origin research can be additionally investigated as a similar study could be conducted incorporating samples from around the world. This can be carried out in order to ascertain whether poultry origin is something that can be identified via ^{1}H NMR spectroscopy whilst controlling potential extraneous variables such as feed and the conditions the birds are housed in or at least recognising geographical variable difficulties.

Chapter 5 - Quantification of Mg^{2+} and Ca^{2+} EDTA Complexes in Saliva using ^{1}H NMR Spectroscopic Analysis

5.1 Introduction

5.1.1 Saliva

A healthy adult will produce approximately 0.50-1.50 L of saliva per day at a rate of 0.5 mL/min[98]. However, several physiological and pathological conditions can modify saliva production, such as smell or taste stimulation, chewing, hormonal states, drugs, age, hereditary influences, oral hygiene and physical exercise. Saliva is a biological fluid that originates from mainly three pairs of major salivary glands; parotid, sublingual and submandibular, and a large number of minor ones [98]. Salivary glands are composed of acini, and saliva is stored in secretion granules at this site [Figure 19]. These granules are filled with water, in which proteins, low-molecular-mass biomolecules and electrolytes are dissolved [99].

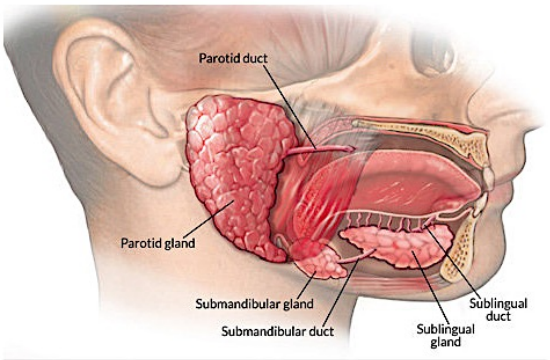


Figure 18: Salivary glands [100]

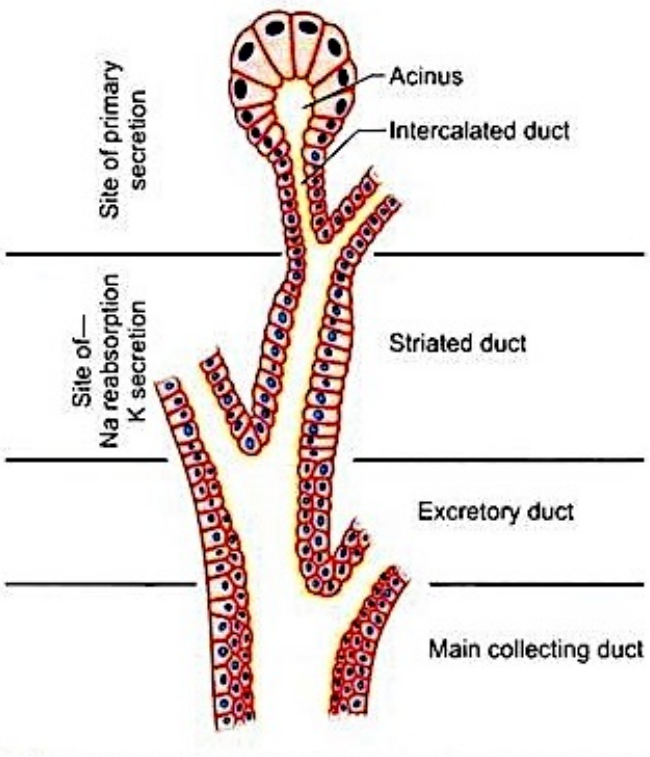


Figure 19: Histology of salivary gland showing the primary and secondary areas of secretion [101]

Each type of salivary gland will secrete a characteristic class of saliva. Differences in the concentration of components such as ions, proteins and metabolites amongst glands are observable. Salivary proteins are also expressed differentially amongst individual glands such as the sublingual and submandibular ones [98].

The primary secretion from salivary glands is plasma ultrafiltrate, but within salivary ducts there is an energy-dependant reabsorption of Na^+ and Cl^- ions, resulting in hypotonic fluid secretion with a lower ionic concentration than that of blood plasma[98]. Numerous factors may play key roles in the modification of salivary ionic concentrations such as differences in stimulated and unstimulated

saliva. Low-molecular-mass biomolecules detectable in saliva include urate, glucose, a range of amino acids, lipids such as cholesterol, and mono-diacylglycerols[98].

Although saliva ''*lacks the drama of blood, the sincerity of sweat and the emotional appeal of tears*' (quoting Mandel in 1990[99]), it remains as a biofluid of much importance to investigators, especially with regard to its chemical/biochemical composition. Investigations pertaining to saliva have expanded into areas from identifying its composition[102] to using it as a method of detection for diseases like oral cancer [53]. The potential for saliva to be used ever more readily for research purposes offers a high level of scope.

The properties and function of saliva have been investigated comprehensively for over 60 years. A major effort has been made to further understand its composition as a whole, and also to investigate variations and imbalances of biomolecules and relevant metal ions. Gaining a better comprehension of saliva and its biomarkers, could potentially allow it to be used as a relatively non-invasive diagnostic tool. Studies using proteomic and transcriptomic approaches could, in principle, permit the early detection of many oral and systemic chronic diseases [103].

For humans, calcium is the most abundant mineral in the body and is accountable for approximately 40% of all body minerals and in total 2% of total body weight [104]. Many enzymes require calcium ions, since they act as mediators for

hormonal responses. They play another vital role in smooth muscle contractions, since this process involves the release of calcium ions (Ca^{2+}) from intracellular stores. It is also essential for blood coagulation [105].

Ca^{2+} promotes the production of vitamin D, which is essential for the body. Vitamin D increases intestinal Ca^{2+} absorption. Low levels of Ca^{2+} within the body can cause the parathyroid gland to release hormones, which release more Ca^{2+} from the bones into the bloodstream, ultimately leading to osteoporosis. Low levels of Ca^{2+} can also lead to cardiovascular disease, amongst other health problems. Conversely, high levels of Ca^{2+} can lead to parathyroid disease and kidney stones.

The release of Ca^{2+} from teeth arise from eating and drinking, which causes the demineralisation of human tooth enamel and subsequently increases the susceptibility of teeth to decay in the oral cavity [106]. Hence, the quantification of this metal ion in human saliva may serve as a valuable oral health index and potentially offers valuable information regarding evaluations of damage to tooth enamel.

Magnesium is a mineral element that is found abundantly in nature. Magnesium also plays an important role in the human body, and its significance has previously been described as 'overlooked' [107]. It is ingested primarily through plant sources since it is present in the pigment chlorophyll [104].

Within the human body, magnesium ions (Mg^{2+}) are used as a cofactor for many enzymes using adenosine triose phosphate (ATP). Approximately 40-60% of the Mg^{2+} found in the human body is stored in bone, and the remainder stored in soft tissue and muscle. [104]

Mg^{2+} has been reported to be important for around 300 enzyme systems, and most aspects of biochemistry are dependent on this ion [107]. It is known to act directly on the myoneural junction, affecting neuromuscular irritability and contractions[104].

Furthermore, Mg^{2+} aids in regulation of blood pressure, assists with RNA synthesis, activates many of the B-complex vitamins, works as an excitable membrane stabilizer, and also assists the process of vasodilation. It is also used complimentarily to Ca^{2+} since it increases the bioactivity of this ion.

Therefore, it is extremely useful to be able to quantify both Mg^{2+} and Ca^{2+} ions in biofluids effectively [104]

5.1.2 Saliva as a Biological Fluid for Clinical and Clinically Related Investigations

Chiappin et al addressed the importance of saliva as a new tool for diagnostic and basic investigations [98]. This review highlighting the use of saliva has provided

information regarding disease processes and disorders. From studies reviewed, the use of salivary mineral content, such as magnesium, calcium, sodium and potassium, appears to be of much importance in terms of diagnostics. Analysis of saliva can also provide information regarding the functioning of various organs within the human body[98].

Dziewulska et al investigated salivary mineral composition involving patients with oral cancer [53]. The rationale for this study was to identify possible markers that could aid in the diagnosis of this critical condition. The group participating in this study consisted of n = 34 patients who had recently been diagnosed with oral cancer (aged between 35-72 years, including 7 women and 27 men). The control group, consisted of 30 healthy dental patients (aged between 37-70 years, including 5 women and 25 men). Un-stimulated whole saliva was collected into plastic containers for analysis. Potassium and sodium ions were analysed using ion-selective electrodes. The concentrations of calcium, iron, phosphorus and magnesium were determined using colorimetric methods. The concentration of calcium was assessed using a specific Calcium test by Konelab, at a wavelength of 600 nm. Magnesium levels were analysed using an Mg-Kit (bioMerieux,), with Camalgite, at 520 nm. Camalgite, is known to be used in conjunction with EDTA as it is a stronger binder to metal ions. Iron was identified using the Ferentest with Feren S (bioMerieux) at 593 nm, and phosphorus identified with phosphore UV with ammonium [53].

Results showed that there was a statistically significant decrease in levels of sodium within the oral cancer group. Dziewulska also found that salivary Mg^{2+} and Ca^{2+} levels were higher in cancer patients than in healthy patients [53]. This shows that comparing healthy and disease measurements of magnesium and calcium levels in saliva can be an important aspect in oral cancer studies.

Table 10 shows in detail the ionic levels identified with particular attention to the range of control calcium and magnesium levels at 0.18-2.43mmol/L and 0.08-2.20mmol/L respectively. It is also important to note that these levels were compared to values stated in previous literature. In a study conducted by Błoniarz et al a control average of 1.03mmol/L for calcium and 0.21mmol/L for magnesium was identified. Similarly in a study by Sphitzer et al a control average of 0.02mmol/L for calcium and 0.29mmol/L for magnesium was identified [53].

Table 10 - Mineral composition of saliva from study and control groups in present study compared to levels found in literature [53]**.**

Present study							
Mineral	**Group**	**Mean**	**SD**	**Median**	**Q3**	**Q1**	**Range (min.-max.)**
Sodium [mmol/L]	Study	27.29	13.61	20.5	17	35	13.00-61.00
	Control	20	8.09	18	15	21	12.00-49.00
Potassium [mmol/L]	Study	26.96	8.14	25	22.7	34.04	6.23-44.52
	Control	25.93	9.27	25.63	21.56	31.13	5.37-44.95
Calcium [mmol/L]	Study	0.99	0.66	0.77	0.46	1.55	0.02-2.72
	Control	0.82	0.55	0.75	0.35	1.04	0.18-2.43
Magnesium [mmol/L]	Study	0.44	0.26	0.38	0.22	0.66	0.08-0.96
	Control	0.42	0.39	0.36	0.2	0.5	0.08-2.20
Iron [mmol/L]	Study	0.74	1.26	0.36	0.18	0.72	0,00-7.2
	Control	0.57	0.92	0.36	0.18	0.36	0.00-4.14
Phosphorus [mmol/L]	Study	10.3	11.2	4.97	3.46	8.53	1.90-35.22
	Control	9.7	9.31	6.61	4.77	8.13	0.77-34.93
Data from literature							
		Błoniarz *et al.*, 2003			Sphitzer *et al.*, 2007		
Sodium [mmol/L]	Study	47.55			22.5		
	Control	8.35			19.8		
Potassium [mmol/L]	Study	23.42			20.7		
	Control	18.51			24.3		
Calcium [mmol/L]	Study	2.96			1.47		
	Control	1.03			0.92		
Magnesium [mmol/L]	Study	0.79			0.37		
	Control	0.21			0.29		
Iron [mmol/L]	Study	22.9			no data		
	Control	4.47			no data		
Phosphorus [mmol/L]	Study	no data			7.52		
	Control	no data			5.43		

The comparison between healthy patients in Dziewulska's study to that of Błoniarz and Sphitzer is interesting as it highlights the range in just control ionic levels found between studies, which could explain why they found no significant mineral markers. This kind of variation between patient levels could be normal as no two individuals would have the same ionic levels, however, there has been no study to investigate to what range these levels can vary. It can also casts doubts on the accuracy and precision of methodologies used for analysis, if there was no repeat measurement of the same sample. This creates an opportunity for alternative methods of analysis to be developed, to allow the same sample to be re-measured in order to increase precision and accuracy.

Even though this study found differences between ionic levels in the control and study group, they did not find any salivary mineral markers that could be useful for the diagnosis of oral cancer, since there were no statistically significant differences in age, sex, smoking status and alcohol consumption between the study and control groups [53]. This may be ascribable to sample size, since 34 patients perhaps may have not been sufficient to attain significance. There was also an imbalance of male to female participants. In addition, the method of how saliva was collected was not stipulated, and this may have exerted an influence on the results acquired, since during the day participants will normally go through a series of oral activities such as, brushing, smoking, eating and drinking, etc.

This investigation did however; highlight the ease of salivary sample collection and its use for such studies. Studies such as this provide a baseline for research to be conducted into alternate methods of saliva analysis that is supplementary to selective electrode and colorimetric techniques. It also shows that more research needs to be done, in order for saliva to prove as a useful diagnostic tool for cancer.

Another similar study investigated salivary levels of bivalent cations in patients with parotid gland tumours [108]. Parotid malignant tumours of stages II-III were studied in a total of 31 patients before surgical therapy and compared to a group of 27 control volunteers. Atomic absorption spectrometry (AAS) was employed in order to determine magnesium and calcium concentrations in saliva. Results revealed that from the control group salivary calcium levels were measured at 1.21

± 0.009 mmol/L and salivary magnesium levels at 0.14 ± 0.03 mmol/L. Mg^{2+} concentrations were found to be significantly higher in patients with parotid malignant tumours over those of the control group at 0.25 ± 0.04 mmol/L)[108]. This study highlights the significance for the quantification of salivary mineral content as an aid for the supplementary diagnosis of parotid gland tumours. It also demonstrates the potential for ionic levels measurements to be used as preliminary disease indicators. A drawback of this study, however, is the use of a destructive technique for analysis, as this would not allow the sample to be preserved and permit additional analyses.

As well as oral cancers, many additional oral diseases have been investigated primarily using saliva as the biofluid of interest. Chronic periodontitis is a disease caused by plaque, although disease progression is dependent on individual susceptibilities. A previous study, by Manea and Nechifor, established if chronic periodontitis can significantly modify the salivary concentrations of bivalent cations such as calcium, magnesium, zinc and copper [109]. For this investigation, experimental groups consisted of 30 adult patients with clinically-onset chronic periodontitis, and 30 healthy controls. It was also ensured that both groups were free from acute oral pathology and general illness, and each one was also sub-divided according to participants' smoking habits. Saliva samples were collected first thing in the morning and then immediately processed. Similar to previous studies, AAS was utilised in order to determine ion concentrations of copper, calcium and zinc. Also, ion chromatography was used for Mg^{2+} quantification.

Results showed control levels of calcium to be 2.10 ± 0.02 mmol/L and magnesium to be 0.02 ± 0.001 mmol/L. Comparison of salivary cations showed that there was a significant connection between salivary calcium, magnesium and zinc concentrations and chronic periodontitis. It was also concluded that salivary magnesium and calcium levels are influenced by smoking [109]. In comparison to the study by Dziewulska, the control concentration of calcium found by Manea and Nechifor does indeed lie within the control range Dziewulska found. However, the concentration Manea and Nechifor report for magnesium is outside the range found by Dziewulska. This again highlights a need for further research to be done in assessing variation in patients and developing a precise and accurate methodology for determining ionic concentration in saliva.

A study by Varghese et al. showed that when comparing smokers and non-smokers with chronic periodontitis, there was a significant increase in salivary calcium levels in smokers with periodontitis [52]. This highlights the decrease in calcium absorption efficacy of smokers. This research is important since it not only shows that diseases such as cancers can indeed be diagnosed and potentially monitored through the analysis of saliva, but also that these ionic levels such as calcium can be influenced by smoking, which is therefore a crucially important consideration for researchers when carrying out future investigations.

In 2009, a research group in China conducted a study into the increase and decrease of inorganic and organic anions such as phosphate, lactate and acetate

and inorganic cations such as calcium and magnesium in patients with sialolithiasis [110]. The objective of this study was to identify and compare any electrolyte alterations in submandibular/sublingual saliva in patients with sialolithiasis compared to those observed in healthy controls. Saliva samples were collected from n = 10 sialolithiasis patients and n = 10 healthy controls, and these were both age and sex matched. Saliva was collected at a fixed time point in the morning, 30 minutes after participants had brushed their teeth with deionised water, after a night of fasting; The saliva was then centrifuged at 14,000 rpm for 20 min. at 4°C to remove food debris, bacteria or epithelial cells. Ion chromatography was then utilised in order to quantify components and results are shown in Table 11.

Table 11 - Ion Chromatographic Results for Organic and Inorganic Anions and Inorganic Cations [110]

Inorganic cations	Sialolithiasis patients	Healthy controls	P value
Sodium (mmol/l)	4.335 (3.130–13.625)	3.540 (1.585–15.725)	0.880
Potassium (mmol/l)	15.750 (9.520–19.125)	16.150 (11.018–21.400)	0.791
Magnesium (mmol/l)	0.135 (0.099–0.300)	0.345 (0.185–0.558)	0.044
Calcium (mmol/l)	1.035 (0.758–1.558)	0.630 (0.588–0.753)	0.003
Data are expressed as median and quartile range (Q25–Q75).			
Chloride (mmol/l)	9.095 (4.923–13.175)	9.460 (5.905–18.150)	0.473
Nitrate (mmol/l)	2.075 (0.045–6.300)	1.645 (0.423–10.553)	0.940
Phosphate (mmol/l)	3.210 (1.363–4.722)	3.235 (2.085–4.058)	0.821
Sulphate (mmol/l)	0.135 (0.100–0.223)	0.175 (0.073–0.215)	0.850
Organic anions	Sialolithiasis patients	Healthy controls	P value
Thiocyanate (mmol/l)	0.220 (0.173–0.598)	0.340 (0.170–1.145)	0.520
Citrate (mmol/l)	0.007 (0.000–0.016)	0.015 (0.010–0.024)	0.018
Lactate (mmol/l)	0.031 (0.005–0.157)	0.170 (0.017–0.243)	0.212
Acetate (mmol/l)	0.149 (0.005–0.945)	0.059 (0.008–0.498)	0.791
Data are expressed as median and quartile range (Q25–Q75).			

Results showed that on average calcium concentrations were significantly higher in sialolithiasis patients than those of controls at 1.035 mmol/ and 0.630 mmol/L respectively. Magnesium concentrations, however, were significantly, on average, lower in sialolithiasis patients when compared to healthy controls at 0.135 mmol/L and 0.345 mmol/L respectively [110].

This study did manage to analyse a large number of inorganic and organic ions however this required a multitude of different guard columns to be used. If this study could be adapted to be used via another methodology such as ^{1}H NMR spectroscopy, as these components could potentially be detected simultaneously from just one sample, using an internal standard in order to quantify. The sample size was also very small. This is common and can depend on how much saliva a participant is able to produce; however, a larger size would be needed in order to improve the reliability and reproducibility of this methodology. This research was also carried out in China, so results would not be able to be generalised amongst other countries in view of differences in lifestyle and diet.

Smoking is a prevalent issue when carrying out oral studies since it has been shown to cause differences in salivary composition. A study by Poles et al specifically explored the elemental composition of saliva between smokers and non-smokers. Results showed that smoking produced more significant changes in the saliva of women than men as the concentrations of certain elements increased in the saliva of women much more then in male smokers[111]. According to the

World Health Organisation, it has been estimated that more than 1.2 billion people throughout the world use tobacco, and smoking-related diseases are responsible for approximately 6 million deaths worldwide [111]. Therefore, it is important to conduct research and educate the public on the dangers of smoking.

This leaves room to investigate, evaluate and compare the chemical composition of saliva pertaining to both smokers and non-smokers. His study also has a significant impact for the role that saliva has in studies considering the use of smoking participants, since it could act as an extraneous variable on results. For this study, 32 individuals were selected, half of which had smoked cigarettes on a daily basis, and the remaining half had never smoked. Saliva was collected with a sterile Pasteur pipette and sent to be analysed via X-ray fluorescence. Results showed there were significant differences in elemental composition, including those in the levels of sulphur, phosphorus, chloride, potassium, calcium, manganese, iron, copper, titanium, vanadium and nickel [111]. This study highlights how important it is to consider participant information such as smoking status, since this could influence the results of such investigations. One weakness of this study is that it could have applied metabolomics analysis in order to gain a fuller picture of the oral effects of smoking. Therefore, this leaves room for studies that assess salivary composition between smokers and non-smokers at a metabolic level.

Various studies have also been carried out to characterise the composition of saliva. The configuration of human saliva has always been of interest to

researchers, since this serves as a foundation to base future disease and related diagnostic studies. In studies reviewed to date, inorganic ions have primarily been of interest, and organic biomolecules have commonly been overlooked. Zhoufan Chen *et al.* (2014) carried out research in China in order to quantify levels of both inorganic ions and organic molecules in saliva. Unstimulated whole saliva was collected into a disposable tube from 11 undergraduate students (6 males, 5 females) after a period of overnight fasting. It should also be noted that volunteers received prophylaxis, which is a treatment given to prevent a disease from occurring, and also rinsed with deionised water before providing a saliva specimen [112].

71 samples were collected in total from the 11 subjects over an 18 month period [112]. Analysis was carried out via ion chromatography, and samples were processed under gradient ion-exchange mode for organic ion biomolecules, which had been optimised with respect to retention time and peak retention. Calibration was then performed, and linearity was checked in order to quantify these agents. Results are shown in Table 12.

Table 12: Compounds identified in saliva [112]

Anion	Concentration ± SD (mmol/L)	Coefficient of variation (%)
Chloride	11.80±4.92	41.75
Nitrite	0.14±0.40	282.19
Nitrate	0.76±0.99	129.46
Phosphate	2.62±1.07	40.72
Sulphate	0.12±0.10	76.88
Thiocyanate	0.33±0.24	72.15
Lactate	0.01±0.04	422.31
Acetate	0.24±0.38	153.79
Propionate	0.03±0.07	240.74
Formate	0.02±0.03	188.60

This study shows that low levels of organic anions could be detected throughout the 18-month period. The sample size was particularly small, however, but since the study was performed over 18 months, reliability could have been improved. This also highlights agents in saliva that should also be considered when performing such disease- or diagnosis-relevant research work. Therefore, there is scope for a technique to be developed that explores organic biomolecules and inorganic species simultaneously, such as NMR spectroscopy.

Many of the studies explored have used saliva for the purpose of oral disease identification. Many studies into the investigation of salivary mineral composition

primarily use ion chromatography as method of choice. Studies have used this method in order to observe a wide range of organic and inorganic components, however this has been achieved through the use of guard columns, separator columns and suppressors [110]. This does, however, create a platform to investigate whether alternative methods of analysis, such as NMR, can give similar results. This could create an alternative method for analysis and further characterise the metabolic profile of saliva as well as speed up sample turnaround time and explore the potential for minimum sample preparation and handling.

5.1.3 ^{1}H NMR Analysis of Saliva

In 2002, a study was performed in order to investigate the potential of using high-resolution NMR spectroscopy for the analysis of saliva [102]. The study highlighted the advantages of using this technique for this purpose, since it is virtually non-invasive, it can permit the simultaneous identification and quantification of metabolites, it requires minimal sample preparation, it has a fast turnaround time for analysis, and has a high degree of sensitivity[102]. 20 participants attending dental school aged between 21 and 49 years were recruited, and no participants had any periodontal disease or active dental caries. Whole saliva was collected into plastic universal containers immediately after waking in the morning, and this was repeated for a total of three days[102]. Before each sample was collected, participants were asked to refrain from any oral activity before waking and providing a sample. The samples were placed on ice and transported to the

laboratory where they were immediately centrifuged to remove any debris or cells, and the resulting supernatants then prepared for NMR analysis [102].

Further analysis involving random collections of saliva form the same participants during their normal daily activities was also performed. These samples were treated in the same manner noted above. The study showed that an excess of 60 metabolites could be identified via NMR analysis at an operating frequency of 600 MHz. Highly significant "between-subject" differences' in metabolite concentrations were found for 9 out of 11 components monitored [102]. The limitations of this study could potentially be the small sample size, of just 20, since the metabolites that were identified may not be representative of the total population; therefore, a larger sample size needs to be explored. However, this technique clearly has the potential to be developed in order to identify organic and inorganic components in saliva.

5.1.4 NMR Analysis of Magnesium and Calcium Ions using of Ethylenedinitrilotetraacetic Acid (EDTA)

The use of EDTA within NMR based studies in order to quantify metal ions is ever more prevalent, being using not only within the health sector but also food. Monakhva et al utilised NMR spectroscopy in order to quantify cations in mineral water. EDTA was used in order to form complexes with magnesium and calcium.

Reference standards were prepared and a resulting equation (Bharti & Roy, 2012) was used in order to quantify results [113].

[Figure 20] shows the results obtained via ^{1}H NMR analysis, showing clear complex formation from calcium and magnesium, whilst EDTA is being used in excess. Using EDTA is excess is important to allow all magnesium and calcium present to form a complex, thus providing more accurate results [113].

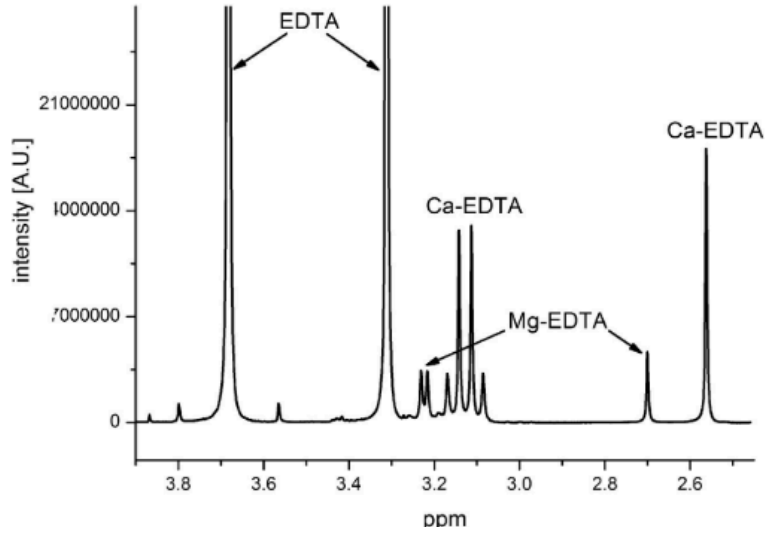


Figure 20: ^{1}H NMR spectrum of mineral water with 507 mg/L Ca2+ and 85.5 mg/L Mg2+ after adding excess of EDTA

The complexion that occurs to form both the calcium-EDTA complex and the magnesium-EDTA complex are illustrated in [Figure 21] below. These resulting complexes that are formed, cause the specific resonances shown in [Figure 20].

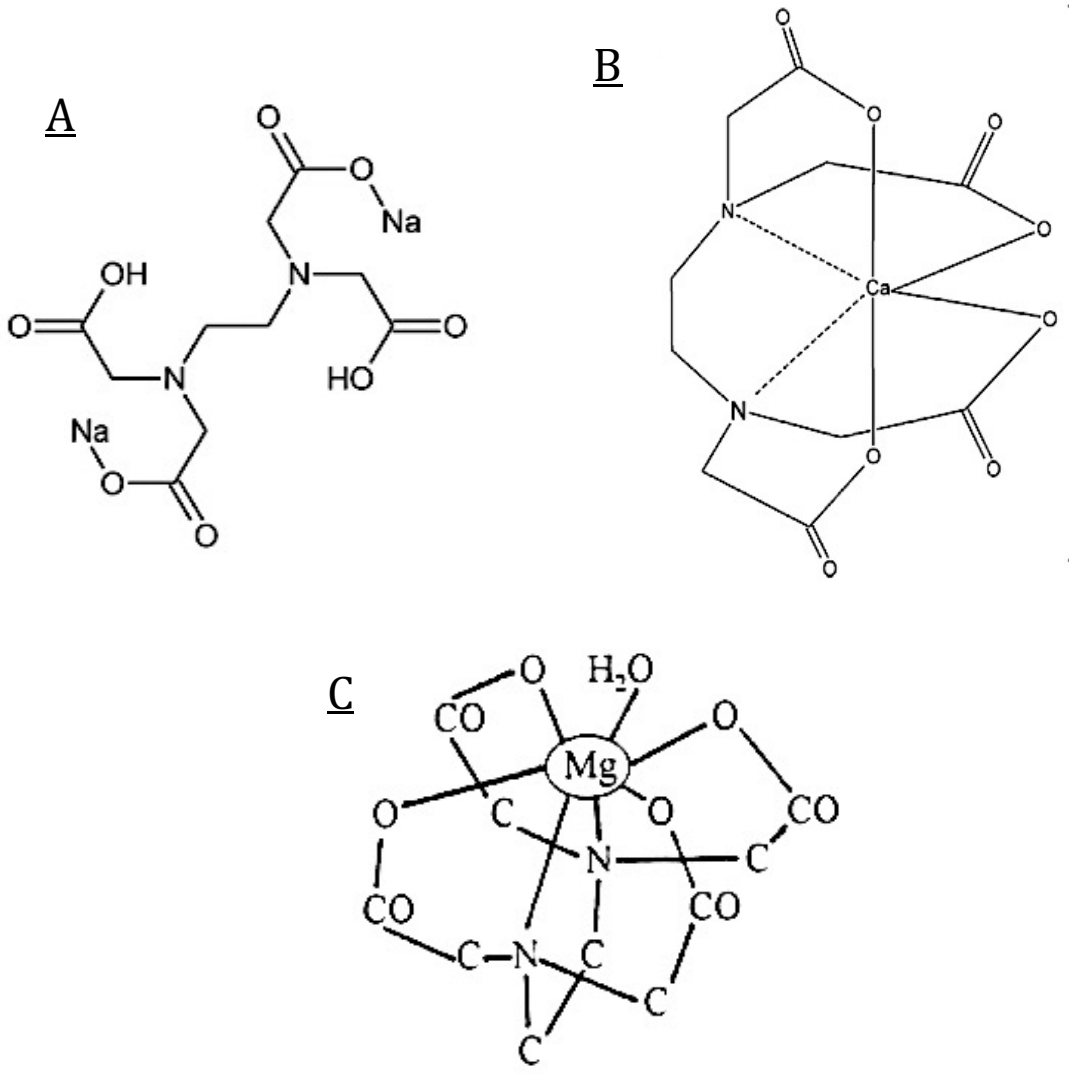


Figure 21 – Illustrating the complexes formed by EDTA when complexing calcium and magnesium ions. A) Structure of EDTA; B) calcium-EDTA complex; C) magnesium-EDTA complex [114], [115]

The method of analysis used by Monakhova et al was reported to have been applied successfully for the analysis of 31-water mineral samples and that the

method of the addition of EDTA to quantify mineral ions can be easily adapted to other matrices [113]. This study does indeed provide valuable information such as the identification of the chemical shifts for both calcium and magnesium complexes as well as the chemical shift of EDTA itself within the sample.

This method has also been applied to biological samples such as serum. Probert et al utilised EDTA to form complexes in human serum in order to identify differences in the NMR plasma metabolic profiles of Niemann Pick C1 [116]. Additionally, a study carried out by Somashekar et al used ^{1}H NMR to simultaneously determine Ca2+ and Mg2+, via their ETDA complexes. Samples were quantified using test solutions containing increasing concentrations of Ca2+ and Mg2+ from a stock solution. The spectrum of each sample was then recorded and concentrations were calculated from the signal integral for the corresponding EDTA complex [117].

Very little has been done to apply this methodology of chelation via EDTA to saliva. Grootveld et al, utilised ^{1}H NMR in order to monitor salivary calcium concentrations. This was done partly by using ethylenediamine tetra-acetate addition, as atomic absorption spectrometry was also used. The study carried out investigations on a total of 18 saliva samples. It was concluded that, NMR spectroscopy was a useful analytical tool for evaluating concentration of calcium ionic level in saliva. This study does, however, concentrate more on the effects on saliva following the application of a dentifrice, also, it only investigates a total of 18

samples and the focus on magnesium ionic level was very little as no conclusion was drawn for the use of EDTA for its identification. As little research on this subject had been conducted, it leaves room to further investigate this technique and to assess how reliable and repeatable using EDTA to quantify magnesium and calcium ions in saliva really is.

The investigation for this thesis will primarily explore the potential of ^{1}H NMR spectroscopic techniques to further comprehensively identify the metabolic profile and determine the concentrations of both Ca^{2+} and Mg^{2+} ions in healthy human saliva in the UK. It will also aid to explore the reliability and repeatability of the methodology.

Previous studies have highlighted the importance of being able to quantify levels of Mg^{2+} and Ca^{2+} in human saliva, in view of its importance for disease diagnosis such as periodontitis and sialolithiasis. There is, however, still potential for a technique to be developed that can measure Mg^{2+} and Ca^{2+} levels in saliva in tandem with identifying organic components whilst not destroying the sample [109] [52].

Within this investigation, initially the metabolic ^{1}H NMR profile of saliva will be elucidated. Additionally, levels of Ca^{2+} and Mg^{2+} in healthy human saliva will be quantified using ^{1}H NMR spectroscopy. The method for detecting salivary calcium and magnesium ion concentrations will be determined by measuring the intensity of the resonances of their complexes with ethylenediamine tetra-acetate (EDTA)

visible in the ^{1}H NMR profiles acquired. These ionic levels measured will then be compared to a set of prepared standards in order to quantify results.

Concentrations of Ca^{2+} and Mg^{2+} within samples will also be compared between same day and next day sample analysis in order to investigate the reliability and repeatability sample preparation as well as reliability of the of the technique and assess sample integrity.

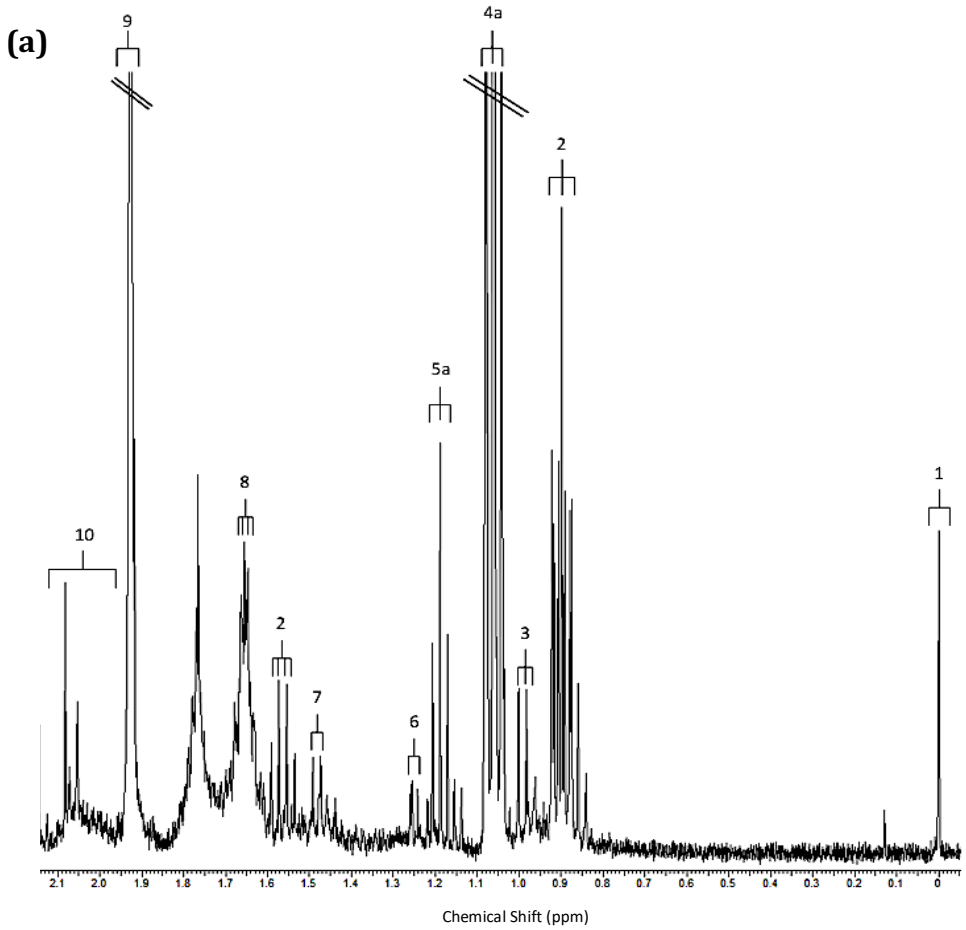
5.2 Results and Discussion

5.2.1 ^{1}H NMR of Human Saliva

All NMR spectra of human saliva, both control and experimental, were reviewed and an example spectrum of each was chosen. This can be seen in [Figures 22 and 23] and show a typical metabolic profile of human saliva, highlighting a total of 21 metabolic compounds.

[Figure 22] identifies a sample analysed without the addition of EDTA.
[Figure 23] shows samples analysed with the addition on EDTA, with the formation of complexes.

An outline of how spectral assignments were done, can be found at the beginning of section 4.2.1


(a)
9
4a
2
5a
8
1
10
2
3
7
6
Chemical Shift (ppm)
(b)
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Chemical Shift (ppm)

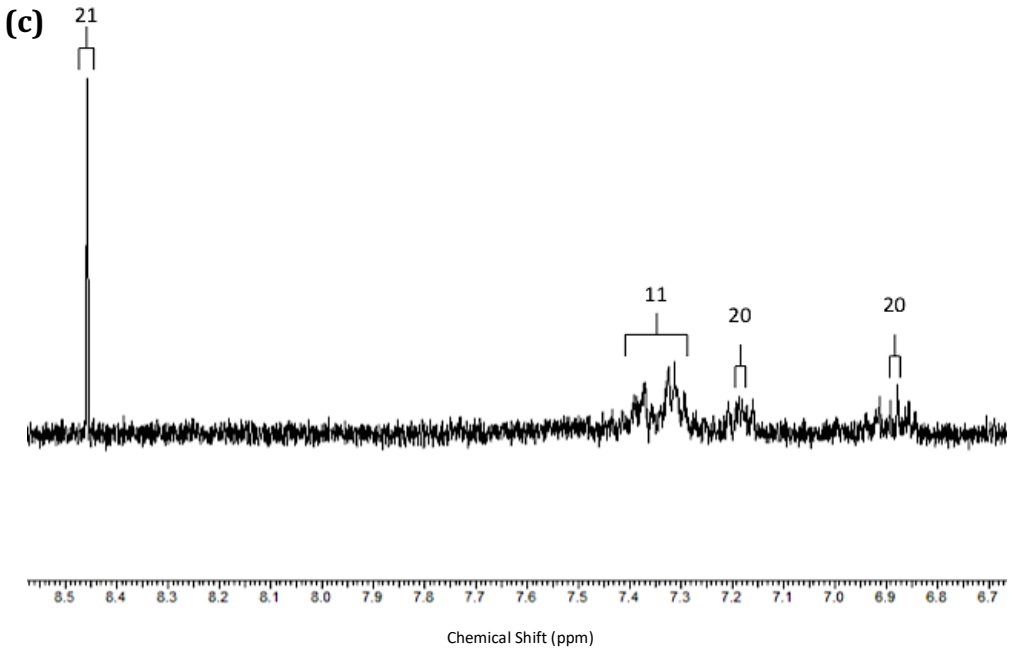
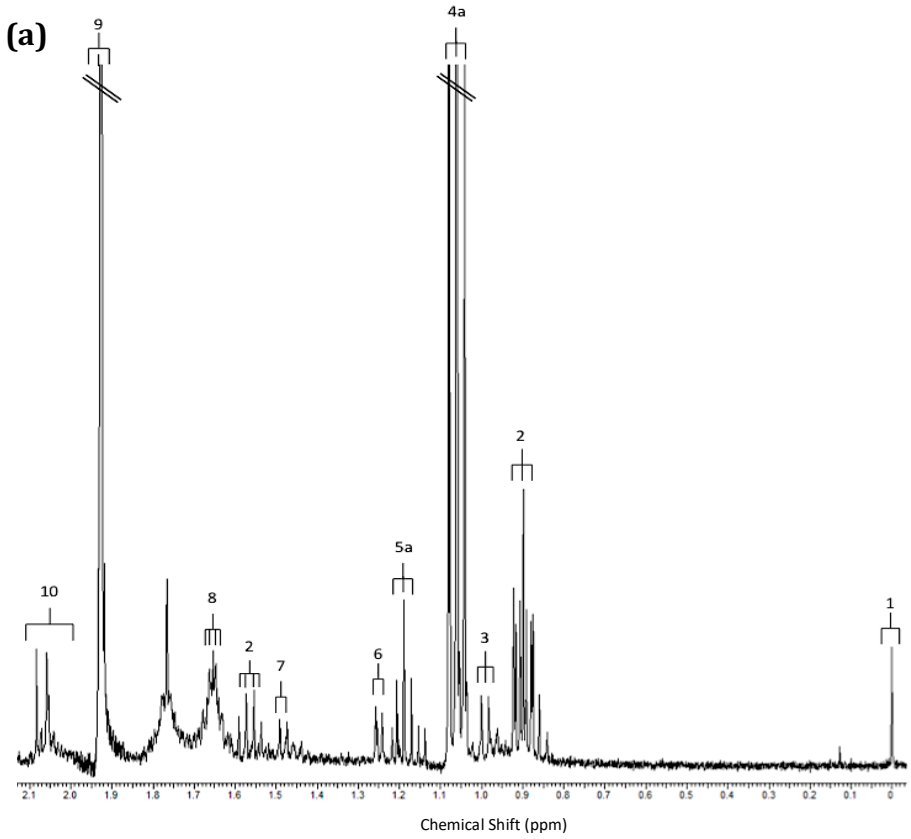


Figure 22: (a), (b) and (c), 0-2.10, 2.15-4.20 and 6.7 to 8.55 regions respectively, of the 400 MHz ^{1}H NMR profile of saliva control. Abbreviations: 1 TSP; 2, Butyrate; 3, Valerate–CH3; 4a, Propionate-CH3; 4b, Propionate-CH2; 5a, Ethanol-CH3; 5b, Ethanol-CH2; 6, 3-Hydroxybutyrate; 7, Alanine; 8, 5- Aminovalerate; 9, Acetate; 10, N-Acetyl Sugars; 11, Gamma-Aminobutyrate; 12, Pyruvate; 13, Methylamine; 14, Dimethylamine; 15, Trimethylamine; 16, Phenylalanine; 17, Choline; 18, Taurine; 19, Glycine; 20, Not Distiguishable; 21, Formate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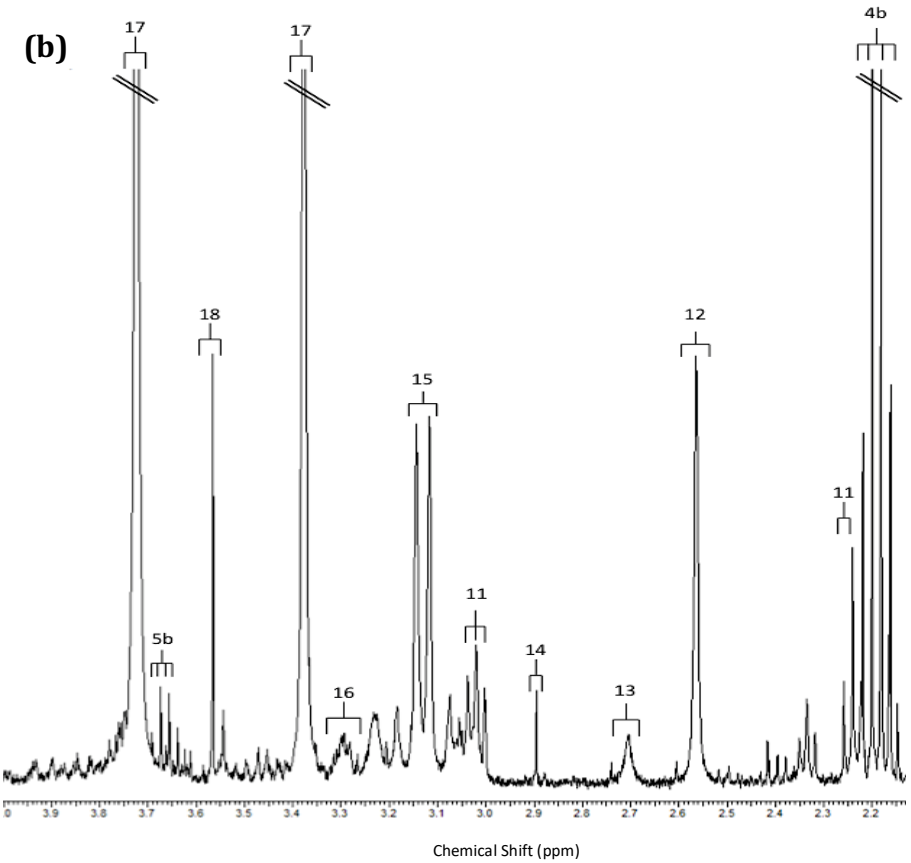
Chemical Shift (ppm)


(b)
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11
5b
14
16
13
Chemical Shift (ppm)

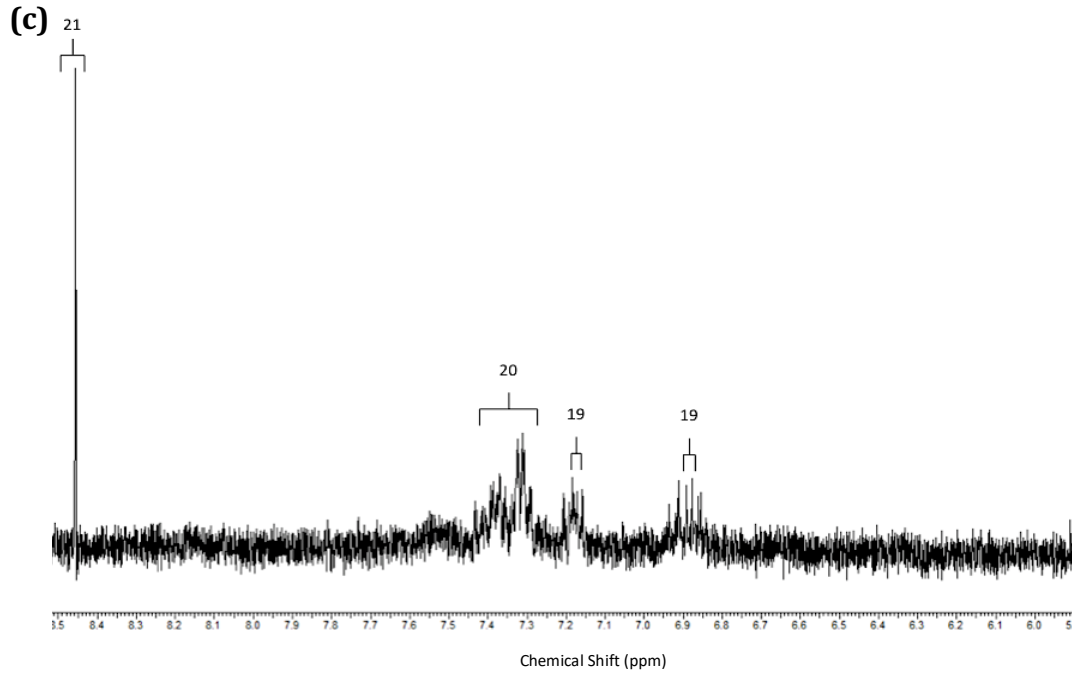

Figure 23: (a), (b) and (c), 0-2.1, 2.15-3.95 and 6.0-8.5 regions respectively, of the 400 MHz ^{1}H NMR profile of saliva with EDTA. Abbreviations: 1 TSP; 2, Butyrate CH3; 3, Valerate–CH3; 4a, Propionate-CH3; 4b, Propionate-CH2; 5a, Ethanol-CH3; 5b, Ethanol-CH2; 6, 3-Hydroxybutyrate; 7, Alanine; 8, 5- Aminovalerate; 9, Acetate; 10, N-Acetyl Sugars; 11, Gamma-Aminobutyrate; 12, Ca-EDTA Complex; 13, Mg-EDTA Complex; 14, Trimethylamine; 15, Ca-EDTA Complex; 16, Taurine; 17, EDTA; 18, Glycine; 19,Tyrosine; 20, Phenylalanine-aromatic ring protons; 21, Formate.

Table 13 and 14 below, shows the list of metabolites identified in human saliva

Table 13: Table of chemical shift values, coupling patterns and assignments of resonances present from Figure 22. Spectral assignment labels correspond to those visible in Figure 22 where; s – singlet; d- doublet; t-triplet q – quartet; m- multiplet.

Spectral Assignment Label (Figure 22)	^{1}H NMR Resonance Chemical Shift Value (δ)/PPM	Coupling Pattern	Assignment
1	0	s	Trimethylsilylpropanoic Acid
2	0.87-0.90	t	Butyrate-CH_3
3	0.91	t	Valerate-CH_3
4a	1.04	t	Propionate-CH_3
4b	2.17	q	Propionate-CH_2
5a	1.16-1.18	t	Ethanol-CH_3
5b	3.63-3.67	q	Ethanol-CH_2
6	1.24,1.25	d	3-Hydroxybutyrate
7	1.46,1,47	d	Alanine
8	1.64-1.68	m	Aminovalerate
9	1.91	s	Acetate
10	1.95-2.13	m	N-Acetyl Sugars
11	2.28/3.04	t/t	Gamma-Aminobutyrate
12	2.46	s	Pyruvate
13	2.59	s	Methylamine
14	2.49	s	Dimethylamine
15	2.89	s	Trimethylamine
16	3.13/7.25-7.44	m/m	Phenylalanine
17	3.19	s	Choline
18	3.30/3.42	t/t	Taurine
19	3.56	s	Glycine-CH_2
20	6.87/7.17	m/m	Not Distinguishable
21	8.54	s	Formate

Table 14: Table of chemical shift values, coupling patterns and assignments of resonances present from Figure 23. Spectral assignment labels correspond to those visible in Figure 23 where; s – singlet; d- doublet; t-triplet q – quartet; m- multiplet.

Spectral Assignment Label (Figure 23)	^{1}H NMR Resonance Chemical Shift Value (δ)/PPM	Coupling Pattern	Assignment
1	0	s	Trimethylsilylpropanoic Acid
2	0.87-0.90	t	Butyrate-CH_3
3	0.91	t	Valerate-CH_3
4a	1.04	t	Propionate-CH_3
4b	2.17	q	Propionate-CH_2
5a	1.16-1.18	t	Ethanol-CH_3
5b	3.63-3.67	q	Ethanol-CH_2
6	1.24,1.25	d	3-Hydroxybutyrate
7	1.46,1,47	d	Alanine
8	1.64-1.68	m	Aminovalerate
9	1.91	s	Acetate
10	1.95-2.13	m	N-Acetyl Sugars
11	2.28/3.04	t/t	Gamma-Aminobutyrate
12	2.56	s	Ca-EDTA Complex
13	2.70	s	Mg-EDTA Complex
14	2.89	s	Trimethylamine
15	3.12, 3.14	d	Ca-EDTA Complex
16	3.30/3.42	t/t	Taurine
17	3.37, 3.74	s/s	EDTA
18	3.57	s	Glycine-CH_2
19	6.87/7.17	m/m	Tyrosine
20	7.25-7.44	m	Phenylalanine-Aromatic Ring Protons
21	8.54	s	Formate

A total of 20 and 21 components were identified for saliva with no EDTA and saliva with EDTA respectively. The chemical shifts identified for both complexes were indeed, found to be comparable to that of Monakhova et al. This also aided with the identification and confirmation of the EDTA-complex signals.

5.2.2 Calibration Curve of Magnesium and Calcium

Before concentrations of Ca^{2+} and Mg^{2+} for each saliva sample can be determined, a calibration curve of each ionic compound would first have to be constructed. This was done by the preparation of serial dilutions outlined in section 2.8. The resulting integration of the EDTA complex was plotted against the concentration of each solution.

A calibration curve for both the Mg^{2+}-EDTA and Ca^{2+}-EDTA complexes were subsequently created using the integrals of the observed signals *versus* the molar concentration of ion in each solution. These values are shown in Tables 15 and 16. The calibration curves are shown below in [Figures 24 and 25].

Each ionic complex signal was integrated relative to the internal standard signal of TSP, set at 1. Consequently, the equation of the line was calculated for both EDTA complexes. Thus, allowing Mg^{2+} and Ca^{2} EDTA complexes measured from saliva samples to be quantified.

Table 15: Serial concentrations of $CaCl_2$ and the resulting integral values

$CaCl_2$ (mmol/L)	Integral value
4.85	5.71
4.36	5.13
3.59	4.23
2.85	3.35
0.93	1.10
0.50	0.58
0.29	0.34
0.05	0.011
0	0.06

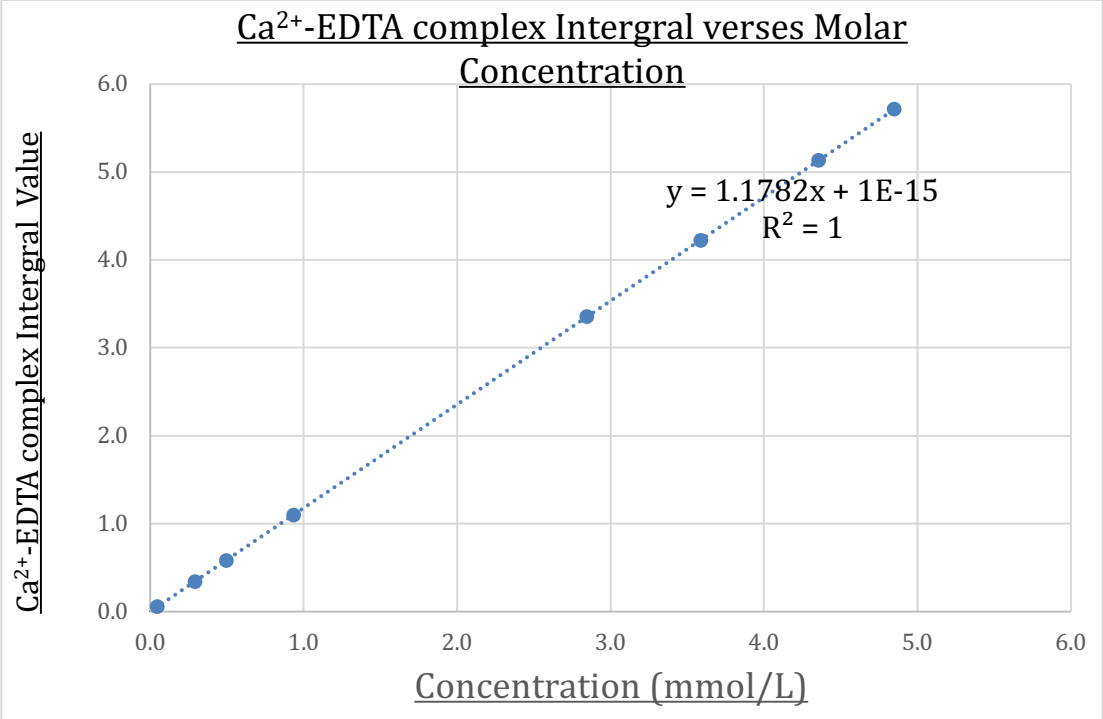

Figure 24: A graph to show the ionic concentrations of calcium versus Ca^{2+}-EDTA complex integral values

Table 16: Serial Concentrations of $MgCl_2$ and the resulting integral values

$MgCl_2$ (mmol/L)	Integral value
1.6134	1.850
0.9681	1.110
0.8067	0.930
0.4840	0.056
0.0533	0.060
0.0351	0.040
0.0122	0.014
0.0104	0.012
0.0095	0.011
0	0.00

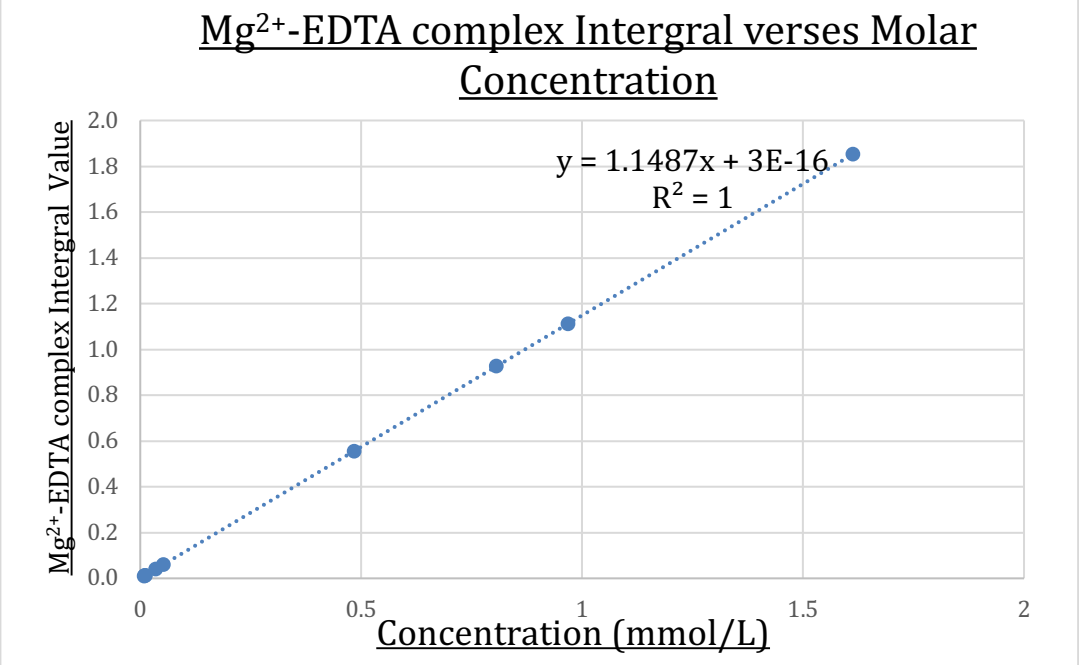

Figure 25: A graph to show magnesium ionic concentrations versus the Mg^{2+}-EDTA complex integral

The regression of these calibration curves was very important, as this would ultimately have an influence on accuracy and precision. A good linear regression was found resulting in an R^2 value of 1.

5.2.3 Quantification of Mg^{2+}-EDTA and Ca^{2+}-EDTA complexes within saliva samples

With the use of the calibration graphs, it was possible to quantify ionic levels of calcium and magnesium within the saliva. This was done using the equation of the line to find the concentration in mmol/L. The results of the Mg^{2+}-EDTA and Ca^{2+}-EDTA complex levels are shown in Table 17. These levels were also compared to levels found in previous studies.

Table 17: Mineral levels from saliva in present study and from literature [53], [110], [118]

Present Study						
Complex	**Group**	**Mean**	**Median**	**Standard Deviation**	**Standard Error**	**Range (Min.-Max.)**
Mg^{2+}-EDTA [mmol/L]	All	0.212	0.207	0.019	0.002	0.009-0.858
	Male	0.241	0.239	0.129	0.016	0.083-0.657
	Female	0.192	0.189	0.156	0.0198	0.009 - 0.858
Ca^{2+}-EDTA [mmol/L]	All	0.932	0.888	0.035	0.004	0.477-1.985
	Male	0.973	0.923	0.243	0.031	0.600-1.715
	Female	0.905	0.864	0.300	0.038	0.477-1.985

Data from Literature					
Mineral [mmol/L]	Dziewulska et al (2013)	Błoniarz et al (2003)	Sphitzer et al (2007)	Gradinaru et al (2007)	Su et al (2010)
Magnesium	0.42	0.21	0.29	0.14±0.03	0.345
Calcium	0.82	1.03	0.92	1.21±0.09	0.630

The purpose of this study was to explore and develop the novel application of ^{1}H NMR in order to quantify Mg^{2+} and Ca^{2+}, within human saliva. Subsequently analysis via ^{1}H NMR also allowed for the further characterisation of metabolites present within salivary biofluids.

Results from this study confirm that Mg^{2+} and Ca^{2+} can be measured via a complex formation from the addition of EDTA. These Mg^{2+} and Ca^{2+}EDTA complexes gave on average salivary mineral levels of 0.212 mmol/L for Mg^{2+} and 0.932 mmol/L for Ca^{2+}. Results also showed that on average male participants were seen to have higher levels of calcium and magnesium present within saliva than compared to female participants. Many studies try to provide a balance of male and female participants; however, it is always important to consider this when forming conclusions.

Many studies before this have preferred the use of spectral analytical methods such as ion chromatography or AAS. This technique, however, has demonstrated

that it is capable of quantifying magnesium and calcium mineral levels in saliva but also allowing for a metabolic profile of saliva to also be characterised. It provides many advantages over other techniques, as NMR is able to provide both ionic and metabolic information from a single sample. Sample preparation is minimal which reduces the risks of contamination. The sample is also preserved, so that multiple readings can be taken of the same sample to ensure instrumental reliability. This is in contrast to destructive techniques, where the sample is destroyed, and a new sample would have to be taken in order to repeat the experiment. Thus, not really being able to assess method reliability.

Due to the formation of complexes some data from a typical salivary NMR spectrum is lost such as taurine and phenylalanine. Also, only young, healthy participants were used for this study. This makes it difficult to apply findings to older patients, as a larger age range was not covered. This does however; create a new platform research to be carried out, particularly for disease investigations, as it opens up the opportunity for NMR to be utilised as a diagnostic tool.

Table 17 shows levels identified within this study compared to levels found within five additional studies. It is clear to see that even comparing the studies to themselves there is a great deal of variation between measurements. The average mineral levels found in this study agree with levels found by studies such as Sphitzer et al (2007) and Błoniarz et al (2003). It has to be mentioned that even though these measurements agree other studies, salivary mineral levels will

always vary from study to study. This can be due to factors such as country of study, sample preparation methodology, age and number of participants and the reliability of method of analysis as this is evident in some studies that did not require participants to fast before a saliva sample was taken or saliva samples were not taken at the beginning of the day. Therefore, variables such as these are always important factors to consider and precaution should always be taken before a comparison is made.

5.2.4 ^{1}H NMR Repeatability and Reliability Analysis of Human Saliva

5.2.4.1 ^{1}H NMR Sample Repeatability Analysis

Each triplicate measurement of both Mg^{2+}-EDTA and Ca^{2+}-EDTA complex were calculated using the calibration curves previously prepared and shown in section 5.2.2. The standard deviation of each triplicate was calculated as well as the standard error. Subsequently the average of all standard deviations and standard error measurements were calculated. This can be seen in Table 18 below.

Table 18: Standard Deviation and Standard Error Values for Repeat Sample Measurements

Complex	Standard Deviation Mean [mmol/L]	Standard Deviation Range [mmol/L] (Min.-Max.)	Standard Error Mean [mmol/L]	Standard Error Range [mmol/L]
Mg^{2+}-EDTA	0.022	0.0002- 0.194	0.012	0.0001- 0.112
Ca^{2+}-EDTA	0.036	0.0005 - 0.308	0.021	0.0003- 0.178

5.2.4.2 ^{1}H NMR Sample Reliability Analysis

Each sample measurement of both Mg^{2+}-EDTA and Ca^{2+}-EDTA complex were calculated using the calibration curves previously prepared. The standard deviation of each sample prepared on different days was calculated as well as the standard errors. Subsequently the average of all standard deviation and standard error measurements were calculated. This can be seen in Table 19 below.

Table 19: Standard Deviation and Standard Error Values for Measurements Taken Over Multiple Days

Complex	Standard Deviation Mean [mmol/L]	Standard Deviation Range [mmol/L] (Min.-Max.)	Standard Error Mean [mmol/L]	Standard Error Range [mmol/L]
Mg^{2+}-EDTA	0.054	0.001- 0.199	0.033	0.001 - 0.140
Ca^{2+}-EDTA	0.120	0.032 - 0.230	0.072	0.023- 0.163

Reliability and repeatability of this technique was important to investigate for this study. This was something not many previous techniques have been able to explore as sample analysis normally means the destruction of the sample. Also, as this is the application of a more novel technique, reliability and repeatability are important to assess.

Results showed that on average the standard deviation for magnesium and calcium were 22µMol/L and 36µMo/L respectively from triplicate samples prepared from

the same whole saliva. Standard error was also measured for calcium and magnesium minerals and was shown to be 21μMol/L and 12μMol/L respectively.

This shows that when using ^{1}H NMR spectroscopy, sample measurement values group very closely together and also that sample means are unlikely to greatly vary when compared to its true measure. Similarly, it was also important to assess sample reliability, as this would give an idea of how reliable and consistent samples of the same composition were. It was found that triplicate samples after a period of 48 hours showed an average standard deviation of 54μMol/L for magnesium and 120μMol/L for calcium mineral measurements. Standard error was also measured for calcium and magnesium minerals and was shown to be 72μMol/L and 33μMol/L respectively. These figures are higher and show that over time, measurements deviate more. When considering the variation between samples it is also essential to consider human errors such as variations in pipetting or loss of sample due to the transfer from one container to another.

It could be argued that if further research is carried out and the method is further standardised, it could provide a superior alternative when compared to other techniques, as you not only gain information on ionic levels but metabolic information as well. It also provides a primary indicator into the repeatability and reliability of this technique. These figures show that the reaction providing complex formation remains relatively stable but can change over time.

This study has also created new opportunities for additional research as it has showed to provide so much more than ionic information and allows for the sample to be kept intact in case of additional analysis techniques. This could provide very beneficial for the future of oral disease research and has created the opportunity to utilise ^{1}H NMR spectroscopy alongside ion chromatographic techniques to further enrich data. There are also further prospects of additional ionic complexes such as Zinc to be investigated and developed. This study has also created opportunities for additional research to be carried out on a wider age group. This study primarily focused on salivary mineral levels for the average age of 25. Therefore, more can be done to assess salivary mineral levels of older generation participants for comparison as some aspects of human physiology changes the older we become, including oral health.

5.3 Conclusion

Conclusively, this study shows a viable alternative to other techniques such as AAS when quantifying calcium and magnesium ions within saliva. With a total of 82 saliva samples ^{1}H NMR has shown it cannot only be used successfully, but also reliably for quantification. The technique has demonstrated accuracy as there was a low deviation of ionic levels between for multiple samples prepared from the same sample, on the same day. However, it also shows that over time ionic levels can deviate more after a period of 48 hours. The nature of using NMR also means the sample is preserved allowing for additional analysis such as ^{13}C.

This study also opens up many new platforms for further research such as; developing a more standardised process for this technique and even further investigation of the differences in ionic levels between male and female donors and how this should be considered very carefully within this field.

Chapter 6 - Final Discussion

The aim of this thesis was to use ^{1}H NMR and demonstrate how versatile it can be as an analytical technique in various fields of research. The chosen areas of research were agriculture and oral health.

For the field of agriculture, it was used as a tool to analyse the metabolomic profiles of both broiler poultry plasma and fresh meat muscle. The poultry were divided into two groups, where each group was given water of a specific pH to drink. The purpose of analysing their metabolic profiles was to assess if there were any significant changes between groups. The reason for doing this was an attempt to identify if there was any improvement in quality. This was done by looking at factors previously identified by other studies and comparing these to what was found by this study and to see if these quality traits could indeed be influenced by changes in the pH of water birds are given.

In contrast NMR was also chosen to be applied to the field of oral health. It was chosen to analyse human saliva with the application of a novel technique, to determine the concentrations of calcium and magnesium ions within this biofluid. The reliability and repeatability of this technique was also investigated by analysing the same sample multiple times and also preparing saliva samples in triplicate to assess precision and accuracy. The purpose of this was to indeed aid

with the standardisation of the methodology and look at how comparable results really were.

For the area of agriculture, the study of fresh poultry meat proved interesting. Fresh poultry meat has seldom been processed via NMR and the standard procedures for the extraction of polar metabolites was applied and proved successful in producing results. A total of 10 spectra were produced and elucidated and a total of 18 metabolites could be identified.

Including only one previous study, that looked at fresh poultry meat muscle, there has not been much research into the characterisation of poultry meat metabolomic profiles via NMR. Looking at studies of chicken meat in general, whether its aged or fresh meat, there is a lack of geographically representative data, as only a few countries have been investigated such as France and China. It is important to have this data, as it has been shown that geography does have an influence on metabolic profiles. The research from this thesis has helped represent data for the metabolomic profiles of poultry meat from the UK.

Poultry plasma was also looked at in this thesis and a total of 49 samples were processed with a total of 33 metabolites being identified. Multivariate analysis showed significant differences between metabolic profiles of the groups when treated with different water treatment systems. Metabolites from previous research, that identified good health and therefore good quality were identified to be significant contributors to the significant difference. Samples belonging to the

group termed WET Engineering, were indeed the group that showed higher levels of quality than compared to the Scotmas Water system.

It was concluded that changes in pH of water fed to broiler poultry can have a significant effect on the metabolic profiles and in turn quality.

Practical implications of this are important to consider as it allows for the potential of NMR to be integrated into industry as a method for identifying and comparing farming practices that could contribute to higher quality factor in broiler poultry. The continued data collection of poultry plasma and indeed meat muscle allows for a more detailed database, which has the potential to cut down on food fraud as it has the means to create metabolic fingerprints for breeds of poultry.

If a similar study were to be done, there would be more of an emphasis for more meat samples to be collected as this would allow for a more accurate representation of metabolite levels, which can then be quantified and compared this has seldom been done before. Phosphorus NMR could also be used as a method of analysis, allowing for the additional identification of phosphorus-based compounds, such as calcium phosphate to assess bone health [92]. Also, this study only looked at one crop cycle. For both plasma and meat muscle one crop cycle can be expanded to perhaps three of four crops within a year to see if results are consistent between generations of broiler poultry.

This study has shown that there are alternatives to existing techniques for measuring calcium and magnesium levels within saliva, which can be done accurately and precisely. However, there does need to be more of a standardisation with this practice in order for studies to be more comparable.

For the area of oral health, a total of 82 saliva samples were analysed and 21 metabolic compounds were identified. Calcium and magnesium ions were detected in saliva with the use of EDTA. The addition of EDTA acts as a chelating agent and binds to calcium and magnesium ions forming a complex that can be analysed and quantified using NMR spectrometry. Both these complexed compounds were amongst the 21 identified compounds. These complexes were quantified by producing calibration curves of both calcium and magnesium EDTA complexes.

These identified levels of calcium and magnesium in samples were compared to literature values. These literature values comprised of values obtained from studies that quantified these ionic levels via other methodologies such as AAS. It was clear to see that even comparing ionic levels from the studies themselves there is a great deal of variation between measurements. The average mineral levels found in this study agreed with levels found by 2 other studies. It can be concluded that even though these measurements are comparable, salivary mineral levels will always vary from study to study, due to participant variation.

It was also important to assess the repeatability and reliability of the technique as it has never been applied previously to saliva, so it was key to investigate accuracy

and precision. This was tested by preparing each sample in triplicate, these triplicate samples were then left for a period of 48 hours and then re-measured. The results showed that triplicate measurements had an average standard deviation of 22μMol/L and 36μMol/L for magnesium and calcium respectively. After a 48-hour period these values were measured at 54μMol/L and 120μMol/L for magnesium and calcium respectively. This is approximately twice as high for magnesium and four times as high calcium. This could suggest an unstable reaction over a long period of time, however as a study like this has never been done before a repeat of the experiment would provide us with information as to whether this is a common issue. Samples, however, realistically are not kept for so long after data collection and low deviation between triplicate samples that are freshly prepared show promising results for this technique.

What this study has done is create an opportunity for NMR to be utilised within the oral health sector for evaluation ionic levels which can be associated with oral diseases. Also, as NMR is able to provide a full metabolic profile of saliva, it opens up the opportunity for it to be used in conjunction with other techniques that utilise NMR for oral health analysis. There are also further opportunities to expand this to look at other potential complexes that could be formed with the addition of a chelating agent such as zinc. This study also looked at saliva provided by participant that were on average 25 years of age and future work could focus on more of a diverse age range in order to investigate how oral health can change over time.

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Appendices

Parameter	
Pulse Program	zg30
Spectrometer Frequency	399.9399

Time Domain (TD) (Size of FID)	131072
Dummy Scans (DS)	2
Number of Scans (NS)	128
Loop Count for 'td0'	1
Spectral Width [ppm]	20.6983
Acquisition Time [sec]	7.9167490
FID Resolution	0.126314
Filter Width	90000
Receiver gain	143.7
Dwell Time (DW) [µsec]	60.4
Pre-scan Delay (DE) [µsec]	6.5
Transmitter Frequency offset (O1P) [ppm]	6.175

Appendix 1: Parameters used to acquire ^{1}H NMR data for poultry meat muscle

Parameter	
Pulse Program	cpmgpr1d
Spectrometer Frequency	399.9399
Time Domain (TD) (Size of FID)	32768
Dummy Scans (DS)	2

Number of Scans (NS)	256
Loop Count for 'td0'	1
Spectral Width [ppm]	12.1142
Acquisition Time [sec]	3.3816576
FID Resolution	0.295713
Filter Width	90000
Receiver gain	362
Dwell Time (DW) [µsec]	103.2
Pre-scan Delay (DE) [µsec]	6.5
Transmitter Frequency offset (O1P) [ppm]	4.712

Appendix 2: Parameters used to acquire ^{1}H NMR data for poultry plasma

Parameter	
Pulse Program	noesygppr1d
Spectrometer Frequency	399.9399
Time Domain (TD) (Size of FID)	32768
Dummy Scans (DS)	2

Number of Scans (NS)	128
Loop Count for 'td0'	1
Spectral Width [ppm]	12.1142
Acquisition Time [sec]	3.3816576
FID Resolution	0.295713
Filter Width	90000
Receiver gain	128
Dwell Time (DW) [µsec]	103.2
Pre-scan Delay (DE) [µsec]	6.5
Transmitter Frequency offset (O1P) [ppm]	4.704

Appendix 3: Parameters Used to Acquire ^{1}H NMR Data for Human Saliva

Parameter	
Pulse Program	noesygppr1d
Spectrometer Frequency	399.9399
Time Domain (TD) (Size of FID)	32768
Dummy Scans (DS)	2

Number of Scans (NS)	128
Loop Count for 'td0'	1
Spectral Width [ppm]	12.1142
Acquisition Time [sec]	3.3816576
FID Resolution	0.295713
Filter Width	90000
Receiver gain	128
Dwell Time (DW) [µsec]	103.2
Pre-scan Delay (DE) [µsec]	6.5
Transmitter Frequency offset (O1P) [ppm]	4.704

Appendix 4: Parameters Used to Acquire ^{1}H NMR Data for method repeatability and reliability