Biomarker Discovery for Pre-Clinical Diagnosis of Tasmanian Devil Facial Tumour Disease

by

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Declarations

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Jessica Gathercole

22nd of October 2012

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Abbreviations

ACN	Acetonitrile
BGE	Back Ground Electrolyte
CE-MS	Capillary Electrophoresis-Mass Spectrometry
DPIPWE	Tasmanian Department of Primary Industries, Parks, Water and the Environment
ESI-MS	Electrosprayionisation-Mass spectrometry
GC-MS	Gas Chromatography-Mass Spectrometry
i.d.	Internal capillary diameter
MeOH	Methanol
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoroacetamide
PC	Principal component
PCA	Principal component analysis
PDDMAC	Poly(diallyldimethylammonium chloride)
PSSS	Poly(sodium 4-styrene-sulfonate)

Abstract

Currently, there are no pre-clinical diagnostic tests to test for devil facial tumour disease (DFTD). This disease is a contagious cancer that has spread through the population of Tasmanian devils over 60 % of the island of Tasmania. The disease appears to always prove fatal and has decreased the overall population of Tasmanian devils by 84 %.

This research investigated the potential of three mass spectrometry techniques to identify changes in the serum metabolome between DFTD and non-DFTD wild Tasmanian devils. Initially, a pilot study was conducted using a set of Tasmanian devil serum samples obtained from a total of 16 DFTD or non-DFTD devils to investigate the different mass spectrometry techniques. The serum was obtained from both males and females over the course of one year from locations in the Eastern half of Tasmania in known DFTD regions. One of these techniques examined the metabolites directly with mass spectrometry (MS) using electrospray ionisation - quadrupole – time of flight – mass spectrometry (ESI-MS). The other two techniques examined, capillary electrophoresis – mass spectrometry (CE-MS) and gas chromatography – mass spectrometry (GC-MS), involved the separation of metabolites prior to MS detection. The CE-MS method was unable to detect as many compounds as the other two methods so only the ESI-MS and GC-MS techniques were investigated further.

The ESI-MS and GC-MS techniques were used to examine the metabolites in three separate sets of samples obtained from wild Tasmanian devils that contained increasing amounts of sample variability using principal component analysis (PCA). Using the set with the least sample variability, both of the methods were successful at showing variability between all of the non-DFTD and all of the DFTD samples. Each of these two techniques was also able to classify devils with DFTD up to six months prior to visible

tumours and this ability to identify DFTD devils pre-clinically continued with the other sample sets.

The ESI-MS method was used to analyse a set of ~100 samples obtained from male devils in the Forrester Peninsula. This method provided a sensitivity of 66 % and a specificity of 80 %. The lower sensitivity was caused by uncertain classification of 6 of the DFTD samples and one DFTD sample incorrectly classified as non-DFTD. Unfortunately, because of the contagiousness of DFTD, all of the blinded samples in this sample set either had other diseases or developed DFTD within 12 months so the suitability of diagnosing healthy devils could not be determined.

The final sample set examined included samples from both male and female devils obtained from locations throughout Tasmania. The results were improved by separating the samples according to gender. When the female samples were analysed, a sensitivity of 95 % was obtained using both methods, with specificities of 80 and 72 % for the GC-MS and ESI-MS method respectively. The PCA results for the male samples were not as successful at showing variability between the DFTD and non-DFTD devils which may have been caused by the bias in the sampling locations of the male DFTD devils.

The results showed the presence of changes in the serum metabolome of DFTD Tasmanian devils that appear prior to clinical signs of the disease. With the correct validation, these methods could be used to diagnosis DFTD up to at least 6 months prior to clinical signs.

1 Introduction

Early diagnosis of contagious diseases has the potential to reduce the universal impact of the disease by quarantining infected individuals to provide treatment; and limiting the spread of the disease [1, 2]. Devil facial tumour disease (DFTD) is a fatal contagious cancer that has dramatically reduced the population of Tasmanian devils [3]. This unique creature is endemic to Tasmania and its presence at the top of Tasmania's food chain cements its ecological importance [4]. The low genetic diversity of Tasmanian devils [4] has allowed DFTD to develop into a contagious cancer that is spread by allograft transfer (cell-to-cell transfer between individuals of the same species) [5, 6]. A pre-clinical test for DFTD would allow for the isolation of DFTD-positive devils potentially before the tumour can spread to other individuals.

The majority of devils affected by this devastating disease are in the wild since devils in captivity are generally not exposed to infected individuals. The choice of sample obtained for diagnosis, therefore, needs to be relatively easily obtained from wild devils. Blood samples from trapped wild Tasmanian devils can be routinely obtained via an ear prick to obtain blood serum irrespective of whether they have observable tumours. This relatively easily obtained sample then has the potential to be used to screen for DFTD biomarkers. The successful development of a diagnostic blood test that examines DFTD specific biomarkers could be used in two ways to aid in the survival of this iconic creature. Firstly it could be used to eliminate DFTD devils from a wild population to reduce the spread of DFTD, and secondly, devils that have been obtained for insurance populations could be tested to ensure that they do not have the disease before allowing interaction with other non-DFTD devils.

1.1 Biomarkers and Diagnostic tests

Much research has been undertaken in biomarker discovery due to the potential for biomarkers to be used in the diagnosis of disease prior to other clinical signs [7-10]. Biomarkers are biological molecules that can show biological processes and pathological states of individual organisms. These biomarkers can then be used to determine if that individual is healthy, diseased [11] or pre-disposed [12] to a disease. In the case of disease diagnosis, biomarker profiles of body fluids may be used for diagnosis before the discernment of other clinical signs of the disease [13].

Biomarkers consist of chemicals from a range of chemical types and include DNA, mRNA, proteins and metabolites [11, 14] and these biomolecules can be observed in a range of biological fluids. Fluids are generally selected by their ease of accessibility and practicality and can be obtained from serum, plasma, whole blood, urine, saliva, sweat, ascites fluid, cerebrospinal fluid (CSF), hair or faeces [11, 15, 16]. There are two different approaches that can be used in biomarker discovery: targeted and non-targeted. Targeted approaches look at specifically known biomolecules; sub-classes of biomolecules such as esters or amines; or the metabolites involved in a certain metabolomic cycle. Non-targeted approaches examine as many molecules as possible at once so that the complete metabolome can be observed [16] and allow for biomarker discovery without prior knowledge of the biological pathway of the disease [17]. In the case of metabolites, searching for biomarkers using a non-targeted approach requires universal methods that have the ability to simultaneously separate and detect metabolites from multiple chemical classes [17]. This can be difficult because of the range of physical and chemical properties of the different metabolites. One technique is, therefore, not suitable to examine all types of metabolites simultaneously [18, 19].

1.1.1 Requirements of Diagnostic Tests

During the development of diagnostic tests there are many factors that need to be considered. It is important that the disease biomarker profile identified is specific to the disease; and has high specificity (ability to identify non-diseased individuals) and high sensitivity (ability to detect diseased individuals) to be useful [11]. The design of biomarker discovery studies must carefully consider other variations that can cause biomarker differences between individuals such as when the subject last consumed food, their diet, age, genetics, local environment, gender and reproductive cycle [16, 20, 21]. These factors can all affect the presence, absence and even the concentration of metabolites and other biomolecules [11, 22]. Appropriate validation of biomarkers, therefore, requires a large number of samples that come from both diseased and healthy individuals to ensure that differences observed between individuals with and without the condition are being identified and not symptoms of another affect [11]. For example, studies looking for disease metabolite biomarkers in saliva, conducted by Sugimoto and colleagues showed some situations where race or gender could affect the differences observed between healthy and diseased samples [23]. Metabolites are often chosen for biomarker discovery studies because they show biological changes more readily than other biomolecules for example, proteins [24]. Changes in metabolite profiles have already been observed in ovarian cancer [7], breast cancer [23] and prostate cancer [10].

1.1.2 Metabolomics

Metabolites are molecules of small molecular weight (generally less than 1500 Da) that are products from metabolic processes which occur as part of normal function, maintenance and growth of an organism [25, 26]. Metabolites include molecules from numerous chemical classes [24] and include sugars [27], carboxylic acids (including amino acids) [9], amines, esters [28], steroids [29], lipids and nucleotides [24]. They can vary greatly in

molecular weight, polarity and concentration [18], and often vary between 7-9 orders of magnitude [30]. Metabolomics is the study of the complete metabolome (the complete set of metabolites) [26]. Studies have been conducted on the metabolome of microbes [26], animals [26, 31], plants [26, 32] and humans [9] and they are similar across all species [21]. Unlike proteomics or genomics, metabolomics allows the study of changes in the phenotype of a subject [16] which provides the prospect of early cancer diagnostic tests because the metabolomic fingerprints for cancer cells are distinctive [16].

1.2 Analytical Techniques

In metabolomics, samples are most commonly analysed using nuclear magnetic resonance (NMR) or mass spectrometry (MS). These two methods can also be preceded with a separation technique such as liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE) [25]. The majority of techniques that involve separation utilise MS detection rather than NMR.

1.2.1 Nuclear Magnetic Resonance

NMR is a popular technique for metabolite analysis that has been used since the early 1970s and is commonly used to examine metabolic profiles of serum and urine samples [24, 33]. This technique is fast, non-destructive and requires minimal sample preparation. Its shortfall is that it has poor spectral resolution [34] and low sensitivity when compared with MS [15, 35] with a limit of detection for ^{1}H NMR metabolite analysis of around 100 μM in tissue and biofluids [24]. Resolution and sensitivity in NMR can be improved by increasing the magnetic field strengths thus the majority of metabolomic studies are conducted at 11.7 Tesla. Resolution is often compromised especially when looking at cells or tissues because of the presence of macromolecules, the binding of small molecules in the sample and differences in sample composition [34]. NMR is highly reproducible and

suitable to directly analyse liquid and solid samples [24, 36]. In one dimensional ^{1}H NMR, the technique is only suitable to distinguish between different functional groups that are present. For example, this technique is incapable of distinguishing between different sizes of lipids and is only able to determine the total amount of saturated lipids in a sample [24] but more specific information such as lipid sizes can be obtained when using two dimensional NMR [24]. NMR is useful to obtain qualitative data but quantitative data on the other hand can be difficult, especially in complex samples such as plasma because of overlapping signals from the different components in the sample. Quantitation can also be improved by using 2D heteronuclear NMR such as ^{1}H-^{13}C but the lack of natural abundance of ^{13}C and poor signal-to-noise ratios, decreases the sensitivity that can be obtained using this type of analysis. Sensitivity can be improved using long scan times of around10 hours since NMR sensitivity is proportional to the squared-root of the number of scans [37]. To improve sensitivity, Gowda *et al.,* developed a heteronuclear NMR method using ^{15}N-^{13}C that was able to examine 27 metabolites in plasma within 30 min [37].

In one study, NMR was used to determine that serum lactate may be a biomarker for weight gain in postmenopausal women with breast cancer undergoing chemotherapy. This study was conducted because of the higher risk of breast cancer re-occurring in patients that increase in weight [38]. A number of NMR studies have also discovered metabolites that are significantly altered in breast cancer tumours when compared to healthy tissue [16]. Proton NMR (^{1}H-NMR) has been used to identify disease metabolite biomarkers in brain tumours and one study has also compared histology results with magnetic resonance spectroscopy imaging (MRSI). This MRSI technique allows *in vivo* studies of metabolites without exposure to radiation by combining NMR and magnetic resonance imaging (MRI). The study suggested that this technique could be used to obtain more information to determine more suitable and precise positions for brain biopsy sites [16].

1.2.2 Mass Spectrometry

Mass spectrometry (MS) is a more sensitive technique than NMR and can thus provide quantitative information for a greater number of metabolites [37]. Mass spectrometry techniques have the ability to detect metabolites in the order of 100 nM whereas ^{1}HNMR only has a limit of detection of 100 μM [24].

Many different types of ion sources and MS analysers have been used to look at metabolites [26]. Some of the ion sources provide soft ionisation which produces only a small amount of fragmentation which decrease the complexity of data when compared to other types of ionisation because the m/z ratio of the ionised compounds are generally detected rather than the fragmented compounds. These types of soft ionisation include atmospheric pressure chemical ionisation (APCI), chemical ionisation (CI) and electrospray ionisation (ESI). Types of ionisation sources that do produce mass fragments include, atmospheric pressure photo ionisation (APPI) and electron impact (EI) [26]. APCI is suitable for polar or semi-polar metabolites. To ionise the metabolites the solution (such as the eluent from prior LC analysis) is passed through a tube that is heated ≥ 500 ˚C. The solvent is present in excess and these molecules along with the metabolite molecules are vapourised within the tube prior to detection with a mass analyser [26]. Chemical ionisation involves bombarding the metabolites with gaseous substances such as methane or ammonia to transfer charge or ions to ionise the metabolites. Electrospray ionisation (ESI) involves passing the solution through a charged capillary (charged between 2-5kV) [26] and during this process the solvent evaporates and the droplets formed shrink, resulting in charged analytes which are then analysed by a mass detector [39]. Electrospray ionisation is suitable for detecting polar molecules but non-polar molecules are better examined using APCI [16]. APPI uses photons from UV lamps to inject electrons from non-polar molecules to form ions at atmospheric pressure [26]. Electron impact uses

electrons to produce positively charged ions at vacuum pressure. The resulting fragments from each analyte are very reproducible which aid in identification. This method is usually coupled to GC [26]. Another type of ion source is matrix assisted laser desorption ionisation (MALDI) [40] which is popular in protein or peptide studies but it has also been used to look at metabolites with high molecular weights such as phospholipids in mammalian tissue [41]. MALDI involves spotting the sample onto a plate along with a matrix solution. When the solvent evaporates the analytes crystallise with the matrix molecules. Once the MALDI plate is placed in the mass spectrometer, a laser excites the matrix which allows the molecules to partially vaporise which leads to the vaporisation of the analytes. In the vapour stage protons are exchanged between the matrix and analyte to produce both negative and positive ions. The presence of buffers and salts are less problematic when using MALDI compared to ESI. MALDI cannot be analysed on-line after chromatographic or electrophoretic separations like ESI can; although automatic fraction collections can be conducted after separation onto the MALDI plate prior to insertion into the mass spectrometer [39].

There are also many different types of mass analysers including ion trap, time of flight (TOF), quadrupole (Q) and fourier transform ion cyclotron resonance (FTICR). Ion trap analysers have the ability to accumulate ions in the device by trapping the ions over time. This trapping ability was limited though until the development of linear ion trap instruments. Ion trap analysers have lower resolution capabilities than some other types of analysers but they do have the ability to conduct multiple-stage sequential MS-MS which allows for fragment ions to be further fragmented. This procedure is useful in determining post-translational modifications in proteins [42]. TOF analysers operate by measuring the time it takes for an ion to move through a tube of a specific length under vacuum. This time is then used to deduce the mass-to-charge ratio of the ion. TOF instruments can

provide mass accuracies of in the low parts per million range [42]. Another type of analyser, Quadropoles, cost less than the other analysers but they also have the lowest mass resolution. This instrument involves applying different potentials of direct current (DC) and radio frequency (RF) to four parallel rods. The ratio of DC and RF is always kept constant and the varying potential is used to select only the ions in the range of interest and the ions with a mass-to-charge ratio less or more than the range are not analysed by the MS [26]. FTICR has an accuracy of < 1 ppm and resolving power over 1,000,000 but it has a lower limit of detection than the hybrid Q-TOF analysers [26, 40]. FTICR measure the orbital frequency of ions orbiting under ultra-high vacuum and high magnetic field strength. The orbital frequency of the ions is dependent upon their mass-to-charge ratio. FTICR is the most accurate mass analyser currently available and consequently the most expensive. The high resolving power that can be obtained using FTICR allows for the ability to analyse more complex samples [26, 40].

Hybrid instrumentation have also been developed that merge two mass analysers together such as quadrupole-time of flight (Q-TOF), or triple quadrupole (QQQ) [26]. The merging of analysers improves the resolution and accuracy of MS analysers [42]. In Q-TOF analysers, the ions are guided with the quadropole and then analysed with the TOF in MS mode. In MS-MS mode the ions selected in one quadropole, are fragmented in a second quadropole by collision-induced dissociation and then analysed in the TOF [42]. Another commercially available instrument which consists of multiple analysers is the triple quadropole (QQQ). This instrument is suitable for targeted analysis of metabolites and other analytes because knowledge of the analytes is required at the beginning of the experiment to determine which ions to fragment and monitor [26].

1.2.2.1 Direct Injection Mass Spectrometry

Direct injection mass spectrometry (DIMS) allows for samples to be analysed in usually less than 5 min allowing for a large amount of samples to be analysed quickly [41, 43]. Direct injection mass spectrometry can also produce more reproducible results when compared to methods using prior separation such as LC-MS because degradation of columns can cause changes to retention times in subsequent runs [44]. However, one of the limitations of DIMS is that ionisation suppression can occur because of the production of droplets when using ESI for example. This phenomenon is caused when less volatile components in the sample cause evaporation difficulties which in turn reduce the efficiency of droplet formation. This drop in efficiency, reduces the amount of analytes that become charged which leads to reduced sensitivity because less ions reach the detector [45]. Part of the ionisation suppression can also be caused by components in the matrix which may ionise easier than the analytes. This effects and can be reduced using nano-electrospray [41] or certain sample preparation techniques [46]. Lecchi *et al.* states that simple sample preparation techniques used to remove proteins from blood samples with organic solvents can also reduce the amount of inorganic salts and thus reduce the matrix affects causing ionisation suppression [47]. Han *et al.* also showed that sample dilution can decrease ionisation suppression and found a 1:100 dilution of mouse serum to be the optimum in their FTICR-MS studies to allow quantitative comparison of non-targeted metabolite spectrums from mouse serum [43].

FTICR has also been used to detect over 10,000 components in a petrochemical sample which suggests that this technique is well equipped to simultaneously analyse all of the mammalian metabolome which is estimated to contain approximately 3000 components. The high accuracy allows for more accurate molecular identification because of the precise mass to charge ratio that can be obtained [40]. Han *et al.* used isotopically-labelled

internal standards to quantify metabolites from human plasma using non-tandem MS and formulas generated from Bruker Daltonics *Data Analysis*™ software using the *Generate Molecular Formula* tool. This group also conducted non-targeted metabolite analysis on serum obtained from 49 inbred mice involved in an alcohol study. They were able to analyse all of these samples in both positive and negative ion mode three times in approximately 24 hours. During this study they found 298 metabolites in positive ion mode and 133 metabolites in the negative ion mode. If prior sample separation was conducted before MS detection this number of samples would have required a number of days to analyse [43]. Ritchie *et al.* used ESI or APCI-FTICR-MS to conduct a non-targeted metabolomics search for markers for colorectal cancer using three independent sets of serum samples. These sets of samples were obtained from the USA and Japan. The researchers identified 44 different metabolites that were capable of distinguishing between serum samples from controls and colorectal cancer patients. This shows that metabolite disease biomarkers can be indentified when the samples are obtained from different geographical areas [48]. This result may not have been as promising if these researchers had looked at all three sets together instead of independently, using a subset of the complete sample to use as a "training set" for identifying the metabolite differences between the controls and cancer patients. Using the complete set of samples though may have helped to eliminate changes that occur from other variables such as sampling location and diet etc.

1.2.3 Advantages of Separation Techniques Prior to NMR or MS Detection

Detection of metabolites using NMR or MS can be improved when an additional technique is also employed that looks at different physiochemical properties. This can be done by initially utilising a separation technique such as liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE) prior to detection [49]. Further

separation using other physiochemical properties can be conducted by analysing with multidimensional separation techniques such as GCxGC-MS or LCxLC-MS. This is conducted by using separation columns with different types of stationary phases that contain different ratios of alkyl groups [26]. All of these techniques can provide complementary or further information on metabolites from a range of types of samples [15].

Improvements with ion suppression can also be achieved with utilisation of chromatography or electrophoresis techniques prior to MS analysis by separating the components of interest so that they are not eluted at the same time as the less volatile components in the sample [25, 45].

1.2.3.1 Gas Chromatography – Mass Spectrometry

Gas chromatography (GC) is a technique that separates volatile compounds according to their interaction with the stationary phase (column) and the mobile gas phase [26]. Gas-chromatography-mass spectrometry (GC-MS) is a very well developed technique which was published as early as the 1950s [26] and is now one of the most commonly used techniques for metabolite analysis. GC-MS provides good separation efficiency with the use of capillary columns and high sensitivity by using MS detectors [41].

GC columns can have non-polar or polar stationary phases and the differences in the stationary phases of the column determine how the analytes are separated. The analytes are primarily separated by the boiling point of the compound when a non-polar stationary phase is used but when a polar stationary phase is used the compounds are mainly separated by the polarity of the compound [41]. The analytes in the sample interact with the column (liquid stationary phase) and the gas mobile phase as the column is heated and allows the separation of species in the sample [26].

In metabolomic studies GC-MS can also be used in targeted and non-targeted approaches [50] and metabolites up to at least 350 Da can be observed [26]. However, a key requirement of metabolomics by GC-MS is that the samples need to undergo sample pre-treatment prior to injection into the GC. One approach is to use solid phase microextraction (SPME) which is an equilibrium processes to extract analytes from a sample but using SPME for multiple samples carries a high risk of sample carryover. To desorb the sample from the SPME syringe into the GC column cryofocusing is required because the analysis needs to start at low temperatures to give enough time for the sample to enter the column. To successfully use this technique with samples such as plasma, the majority of proteins need to be removed and the analytes of interest must be highly volatile. The presence of polymers, for example proteins, and non-volatile analytes not present at high concentrations are not suitable for SPME [51]. An alternative approach to remove proteins and matrix ions from a sample off-line is via protein precipitation with organic solvents [52, 53].

Many metabolites are not volatile and these can be analysed by GC with prior chemical derivatisation. This is generally performed using alkoxyamines and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA). Alkoxyamine stabilises sugars by converting the carbonyl groups to oximes in the open-chain conformation. The alkoxyamine is also used to prevent decarboxylation in α-ketoacids. The alkoxyamine reaction is conducted first so that the active hydrogens from acids, alcohols and amines are substituted with a trimethylsilyl group via a silylation agent such as MSTFA in a subsequent derivatisation reaction [41]. This type of two step derivatisation procedure allows for many types of metabolites to be analysed such as amino acids, sugars, sterols, carboxylic acids (such as fatty acids), lipids, phosphates and alcohols [26]. There are limitations to detect large polar molecules with GC-MS even with the chemical derivatisation because steric difficulties

make it difficult to modify all of the functional groups with the silylation agent [30]. The requirement for derivatisation prior to analysis increases analysis time and cost for this method [16] but when compared to SPME, derivatisation of the metabolites has several advantages. This method is not disadvantaged if some protein still remains in the sample and it allows for an increase in the number of analytes that can be analysed because many of the low volatile metabolites become volatile.

Nishiumi and colleagues examined serum metabolites with GC-MS to determine differences between 9 healthy volunteers and 21 patients with pancreatic cancer. The development of a diagnostic test for early stage pancreatic cancer could save many lives since 96 % of patients experience mortality within a year of diagnosis because the cancer is often diagnosed when it is in an incurable stage. A metabolite library obtained commercially from Shimadzu was then used to identify some of the metabolites seen in the chromatograms and the researchers were able to identify 60 metabolites. From these 60 identified metabolites they found 18 to be significantly different using Student's *t*-test between samples from pancreatic cancer patients and those from the healthy control group. Using partial least squares discriminant analysis (PLS-DA), Nishiumi *et al.* were not only able to successfully separate the patients from the healthy samples but they could also distinguish between the different stages of pancreatic cancer. They concluded that their research showed that metabolites could be used to develop an early stage diagnostic test for pancreatic cancer and thus increase the chance of curing the disease in many patents but further trials will need to be conducted with a larger sample group [9].

To increase the number of metabolites that can be identified, methods have been developed that use two-dimensional GC with a MS detector (GCxGC-MS) [49]. GCxGC is used to increase the peak capacity by using two columns with differing polarities to

separate compounds that co-elute when only one column is used which increases the number of metabolites that are identifiable [41, 49]. In a study conducted by Welthagen *et al.* 1200 components were seen when using GCxGC-MS but only 500 components when they used GC-MS. Increased detection sensitivity can also be obtained using GCxGC-MS which allows for smaller amounts of sample to be injected into the system [49].

It can be difficult to identify all metabolites detected in non-targeted metabolite studies because of the number of metabolites that are observed [30]. Part of this complication stems from difficulty in obtaining some metabolite standards [30]. Commercial metabolite identification libraries can be obtained for GC-MS which makes the technique more suitable than others such as CE-MS and LC-MS since libraries for these latter two techniques are not yet as widely available [26]. To identify the metabolites without commercial libraries, metabolite standards need to be obtained to compare the retention time and mass spectrum of the standard with that observed experimentally. When metabolite standards cannot be obtained, identification can only be determined by manually deciphering the mass spectrum of the analyte. Identification can be difficult if the peaks are not well resolved because of the presence of additional peaks in the mass spectrum from co-eluting analytes.

1.2.3.2 Liquid Chromatography – Mass Spectrometry

Liquid chromatography (LC) is a separation technique that separates analytes according to physiochemical properties which are determined by the type of column that is used. Size exclusion chromatography uses a column that separates the analytes according to size; and ion-exchange chromatography (IC) separates the analytes according to the charge of the molecule. Other LC columns such as those used in reverse phase liquid chromatography (RPLC) or hydrophilic interaction liquid chromatography (HILIC) separates the analytes

according to how hydrophobic or hydrophilic the analytes are respectively [54, 55]. The mode that will be discussed here is RPLC.

Techniques to examine metabolites using liquid chromatography – mass spectrometry (LC-MS) have only recently been developed [16]. Although LC-MS is slower and has limited commercial metabolite libraries when compared to GC-MS, it has the ability to look at analytes with a greater range of molecular weight and polarity than GC-MS [56] without prior chemical derivatisation [15]. However, it can be difficult to separate small hydrophilic peptides by LC [56].

LC when coupled to mass spectrometry (MS) is normally operated in the reverse phase (RP) mode because mobile phases used with (RPLC) are suitable at atmospheric pressure for ionisation in the MS [57]. Other practical issues to consider when looking at biomolecules with LC-MS are that the samples generally require preliminary sample preparation prior to analysis by the instrument. The amounts of organic modifier and salt also need to be kept low. Excess in organic modifier can cause the samples to be trapped on the column and excess salt can cause blockages in the column [56]. Metabolites from biological fluids can be difficult to separate by RPLC because many of these compounds are very polar and ionic. To counteract these issues HILIC columns have been developed which are more apt than RPLC at separating polar compounds [15, 55].

Yang and colleagues used a non-targeted metabolite approach to discover biomarkers for hepatitis B in humans by examining serum metabolites from 50 controls and 37 hepatitis B patients. After peak alignment and removal of noise, the peaks were analysed by partial least squares discriminant anlaysis (PLS-DA) and this plot was able to clearly separate the healthy samples from the diseased. Potential biomarkers were then determined by using a formula to determine the Variable Importance in the Projection (VIP) and then examining

which of the top 20 analytes determined with this formula were significantly different ($p < 0.05$) using Student's *t*-test. Examination of the mass spectra data led to the identification of 5 metabolites which are also involved in liver function. Further study was required to ensure the metabolites identified are biomarkers specific to hepatitis B [18].

1.2.3.3 Capillary Electrophoresis – Mass Spectrometry

Capillary electrophoresis (CE) separates components via the mass to charge ratio and is therefore suitable to separate polar and ionic compounds which includes many of the compounds found in biological fluids [15]. This technique is cheaper and more environmentally friendly than other techniques such as LC-MS. The reasons for this include limited or no use of organic solvents; using fused silica columns which are considerably cheaper than LC columns; and the requirement of very low samples volumes (nanolitres) [15]. Greater resolution is obtained in CE when compared to LC when electroosmotic flow (EOF) is present because EOF may help to focus the analyte bands [41]. The use of low sample volumes and capillaries with a narrow internal diameter provides very low pathlengths when detecting with UV which leads to poor detection sensitivity when compared to LC. Sensitivity in detection can be improved through the use of MS detection and/or using pre-concentration techniques inside the separation capillary [15]. There are many different modes of CE that are used to separate components in different ways such as capillary isoelectric focusing (cIEF), capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MEKC), or capillary gel electrophoresis (CGE). Many of these can be interfaced via ESI to MS but the presence of surfactants and polymers in MEKC and CGE are not suitable for connection to MS detectors [39]. CZE is the most popular method for analysing metabolites [15] and is the most popular method used with CE-MS [39]. The sharp peak profiles that are obtained with CE and low sample volume require high-scan-speed mass spectrometers to obtain

adequate detection sensitivity. This lack of sensitivity is also intensified further by the requirement of sheath flow in CE-ESI-MS which dilutes the analytes further prior to detection [19, 41].

CE is generally coupled to MS via ESI. The buffers that can be used in CE-ESI-MS need to be volatile and are therefore restricted to those comprising chemicals such as acetic acid, formic acid, ammonium acetate or ammonium formate. The ionic strength of the buffers also needs to be limited when analysing by ESI-MS to limit ionisation (ion) suppression [58, 59]. This limitation can cause CE-ESI-MS separations to lack the resolution that is seen in CE runs that are conducted with optical detectors [59] since the optimal buffers for separation cannot always be used.

CE-MS has been used to test various metabolites in a range of cases. Sugimoto *et al.* used CE-MS to determine saliva metabolites that could distinguish between oral cancer; pancreatic cancer; breast cancer; individuals with periodontal disease; and healthy individuals. This study looked at 69 patients with oral cancer, 30 with breast cancer 18 with prostate cancer, 11 with periodontal diseases and 87 healthy controls. They found 57 metabolites that were significantly different between the samples from the patients (cancer and periodontal disease) and the healthy individuals to the 5 % confidence level using the Steel-Dwass test. However, the metabolites that were found to be statistically different between breast cancer patients and healthy individuals were not found to be specific to breast cancer [23]. Baidoo *et al.* used pH-mediated stacking to improve sensitivity so that the higher-resolution and more accurate FT-ICR MS could be used to detect amino acids after CE separation. They tested their developed method by injecting a bacterial lysate from the species *Desulfovibrio vulgaris* and identified 27 metabolites from their mass-to-charge ratios and chemical standards [19]. A method has also been developed to screen for

amino acids and acylcarnitines in dried blood spots for inborn errors of metabolism (IEM). Online desalting and sample pre-concentration was conducted through electrophoresis since the strong electrolytes migrated thorough the capillary faster than the metabolites of interest. These researchers developed a method that allowed for a 1:1 methanol:water extraction of the proteins rather than a 100 % methanol extraction to isolate the metabolites in the supernatant. When using other techniques such as direct injection ESI-MS or LC-MS, internal isotope standards or chemical derivatisation are used which are not required when analysing by CE-ESI-MS. The absence of isotope standards and chemical derivatisation, along with the ability to remove unwanted salts reduces the ion suppression. This along with a reduction in solvent consumption reduces the cost of analysis of the dried blood spots using this method [60].

When compared with GC-MS or LC-MS, CE-MS is more appropriate for looking at phosphorylated or sulphated small molecules but less suitable for secondary metabolites [49]. The small sample volumes and high resolution of CE can decrease the sensitivity of MS detection especially when high-scan speed MS instrumentation are not used but low solvent volumes and separation of analytes in relatively cheap fused silica capillary makes this considerably cheaper than chromatographic methods.

1.2.3.4 Comparisons of Techniques

All of the techniques mentioned above are complimentary as they use different mechanisms to analyse the analytes in a sample. DIMS provides the quickest method to analyse metabolites. This method would be suitable for a quick screening test that could be later confirmed with one of the separation methods. Identification of the metabolites with DIMS is difficult because information such as retention time provided when separating the analytes prior to detection is not obtained. Migration or retention times can provide further

information on the structure and type of the chemical, cannot be used to aid in identification of metabolites that may have the same mass-to-charge ratio. Peaks of interest in DIMS could be further analysed with tandem mass spectrometry which would allow for manual identification of the metabolites.

The availability of commercial libraries and the ability to derivatise non-volatile metabolites with GC-MS advocates the advantages that this method has over the other methods. Although the relative migration times of analytes is quite reproducible with CE-MS actual migration times can vary. In non-targeted metabolites it is possible to observe 10-100's of peaks. To efficiently analyse the data it is beneficial to produce peak tables that allow for automatic integration of peaks. When migration times change, this can be difficult.

These three separation techniques separate analytes using different mechanisms for separation so the chemical classes that each are apt at examining differs. When choosing a method for metabolomics the method of choice depends on the goal of the study and whether or not a specific class of metabolites is being examined. Liquid methods such as LC and CE are more suitable when looking at large polar molecules where as GC is more suitable to look at the smaller metabolites such as amino acids, other organic acids and sugars. Each method will provide different information and in many cases utilising two methods will be more beneficial in obtaining the information required for the study being conducted.

A comparison of these three techniques, CE-MS, GC-MS and LC-MS, was conducted by Büscher *et al.* when looking at 75 different metabolites involved in central carbon and energy metabolism pathways. The majority of metabolites were polar and there were isomers of 16 of the metabolites in addition to the 75 in the sample. As can be observed in

Figure 1.1 approximately a third of the metabolites were observed in all methods and only three were not observed with any. The two liquid based methods (CE and LC) showed the most overlap in respect to the metabolites that were observed with these methods. This is likely to be because these methods are suitable to analyse non-volatile components. They were all found to have similar sensitivity and ability to examine isomers. The authors of this paper showed that LC and CE methods take between 20-60 min to analyse each sample but because of the need for chemical derivitisation of metabolites, GC takes between approximately 45-90 minutes. Overall the authors suggested that LC was the superior technique for the analytes they were studing and could be used complementary with GC-MS. This was suggested because CE was less robust and provided similar information to the LC methods [30].

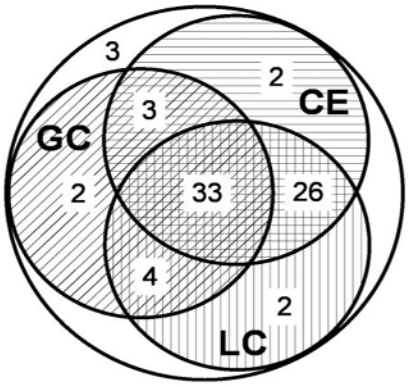

Figure 1.1: Distribution of the identification of 75 metabolties involved in central carbon and energy pathways using GC-TOF-MS, CE-TOF-MS and LC-TOF-MS. Figure taken from [30].

1.3 Sample Preparation for Metabolomic Studies

Prior to metabolite analysis there are numerous ways that samples can be collected, stored, and prepared for metabolite extraction. All of these procedures have the potential to modify the structure of the metabolites and can therefore affect the results and reproducibility of the analysis [16, 53].

Serum or plasma can be isolated from blood for metabolite analysis depending on how the blood is first stored immediately after collection. Plasma is obtained from blood samples that have been added to tubes containing anticoagulants or additives which allows the samples to be analysed quickly since there is no need to wait for the blood to clot. The types of chemicals used with blood to obtain plasma include EDTA, heparin, sodium fluoride/potassium oxalate or citrate. Serum is obtained from blood that has been allowed to clot naturally. Dettmer *et al.* showed in a study that compared serum verses EDTA-plasma that differences in the concentration of some analytes can occur between these two types of samples [52]. It is, therefore, important to ensure that all of the samples are either plasma or serum in metabolomic biomarker discovery projects.

Sample storage is known to influence the abundance of metabolites and in some instances metabolomic reactions can still occur if the samples are not immediately frozen at -80 ˚C or lower. The length of sample storage and number of freeze/thaw cycles has the potential to increase variability even more than what is observed with proteins or RNA. This can be more of an issue with target metabolomics than non-targeted metabolomics since the small amount of changes can generally be counteracted in non-targeted metabolomics if large sample sizes are utilised [53].

Potential interferants need to be removed prior to analysis by isolating the type of biomolecules that are being examined as potential biomarkers. In metabolomic studies, proteins are often extracted using solvent extraction [61]. The process of extraction can also affect how the metabolites are separated or detected during anlaysis [60]. Metabolites can be extracted from biological fluids using solid phase extraction, liquid-liquid extraction [40] or more commonly by precipitation (using either certain types of acids; salts or water-miscible organic solvents [40, 62]. When using the common method of using

organic solvents to precipitate and remove the proteins from the sample, the supernatant (which contain the metabolites) is collected and used for further analysis. If the correct ratios of solvent to sample are used the majority of metabolites of interest remain in the supernatant. There is a risk of losing important metabolites when using liquid-liquid extraction or solid phase extraction [40, 43].

In mass spectrometry analyses, the presence of involatile salts can cause ion suppression and the use of methanol extraction can help reduce the amount of involatile salts in a sample [60]. It is also sometimes beneficial to reduce the presence of phospholipids which are ionic species that can increase ion suppression during MS detection [62]. A study by Alzweiri *et al.*, examined the suitability of four organic solvents to extract metabolites from biological fluids. The dielectric constants and viscosity of solvents affects the efficiency of protein precipitation and thus the extent of protein removal from the sample. Alzweiri *et al.* suggest that acetone and acetonitrile are more apt at protein removal than methanol or ethanol [62]. Different extraction methods can be tailored to improve the efficiency of extracting certain chemical classes of metabolites. For example, amino acid extraction can be improved by using a 1:1 MeOH/H_2O extraction [60]. This increases the solubility of the amino acids since some of these acids are hydrophobic and some are hydrophilic [63].

Overall samples need to be obtained, stored and prepared in a consistent way to reduce changes in the metabolite composition between samples after collection. Different procedures to isolate the metabolites should be tested to obtain optimum results.

1.4 **Data Analysis**

The development of pre-clinical diagnostic tests using NMR or MS techniques can produce extensive amounts of data. To obtain meaningful information, chemometric

techniques are used to determine disease biomarkers. When using hyphenated techniques such as GC-MS, CE-MS or LC-MS, the data needs to be tabulated so that all of the peaks from each sample are correctly aligned with the other samples [64].

After the results are tabulated feature selection can be conducted to decrease the complexity of the data by removing redundant features so that further analysis can be conducted to determine biomarkers of the disease [65, 66]. Levner believes that this step is necessary to obtain the full potential of mass spectrometry techniques in discovering biomarkers for disease diagnosis [65]. Feature selection can be conducted using a range of statistical techniques including Student's *t*-test [61, 67], Kolmogorov-Smirnov test (KS-test) [65], ANOVA[61, 68] or Fisher test [69]. The feature selection step is then followed by multivariate analysis such as principal component analysis (PCA), partial least-squares (PLS) [64] hierarchical clustering [70] or linear discriminant analysis (LDA) [32]. Using techniques such as PCA and PLS allows the visualisation of complex data which can show differences in different groups of data according to patterns of analytes rather than a single analyte [71, 72]. This would be useful in biomarker studies because it can show if the presence and/or relative variance of different sample groups such as healthy and diseased according to a selection of biomarkers rather than a select few.

Feature selection and multivariate analysis can be conducted on a subset of the total data (training set or test set) and the resulting algorithm is then used to classify the samples that were not included in the training set. Stentiford *et al* found the use of Student's *t*-test as a feature selection step prior to hierarchical cluster analysis was required to show differences between fish with and without liver cancer. If the feature selection step was excluded the differences between the diseased and non-diseased fish were not observed [8].

The number of samples in the training set is important as work has shown that decreasing the number of the samples in the training set can decrease the accuracy of the algorithm to classify the samples [65]. Biomarker identification can be conducted at a later stage [66] or by using libraries that help to identify the biomarkers from mass fragments and retention times [9].

The identification of metabolites can involve some difficulties and generally require chemical standards. Identification can be obtained using a commercial library of the chemical standards along with retention indices [26]. The metabolites identified in this way must still be manually checked to ensure the library spectrum is a good match for the experimentally obtained spectrum. Non identified spectrums can also manually be identified. When this is done losses of water or sodium ions must be considered so that the assumed quassi-molecular ion is correct. In some circumstances, it may be difficult to determine the correct isomer for a selected metabolite especially if a standard is difficult to obtain and cannot be observed separately. Prior to identifying the metabolite as a potential disease biomarker, the biological function of the identified metabolite must also be examined to ensure that the metabolite identified is potentially associated with the disease being examined [18].

1.5 The Effect of Devil Facial Tumour Disease on Tasmanian Devils

1.5.1 Tasmanian Devils

Tasmanian devils (*Sarcophilus Harrisii*), once prominent throughout Australia, are now endemic to Australia's island state of Tasmania. The devil has been the world's largest living carnivorous marsupial [73] since the death of another iconic Tasmanian creature - the Tasmanian tiger (thylacine). The Tasmanian tiger was declared extinct in 1986 [74] though the last known Tasmanian tiger died at Hobart zoo in 1936 [75]. Tasmanian devils

are nocturnal [76], non-territorial creatures that generally reside in areas of greater than 10 km^2 although they have been known to travel 50 km in one day [73]. Tasmanian devils have an overall low genetic diversity but there is still a distinct genetic difference between devils from eastern Tasmania and those from north-western Tasmania [4, 77]. A study of the genetic diversity of Tasmanian devils conducted by Jones *et al.* examined the variability between devils of the same subpopulation and different subpopulations. Jones and colleagues observed very low genetic diversity within all sub-populations of these creatures. The greatest difference between populations was observed between the well-connected populations in the eastern half of Tasmania and those of north-western Tasmania. The terrain between these two locations in Tasmania is uninhabitable for Tasmanian devils so there is limited dispersal of devils between these two populations. This observation was collaborated with model-based clustering anlaysis. The north-western population of devils is smaller than the population found in eastern Tasmania [4]. Historically, there have also appeared to be declines in Tasmanian devil populations around 1909 and 1950 [76] which could have contributed to a decrease in the genetic diversity of this species.

Although devils are considered to be solitary animals, recent work published by Hamede *et al.* showed that a certain wild population of devils had broad contact networks. This study examined the interaction between 27 devils at Narawntapu National Park, Tasmania and showed that all devils had some contact with each other either directly or via another individual in the testing group. The study also found that interactions between different devils differed between mating and non-mating season. During mating season the most common interactions occurred between male and female devils. These interactions also appear to occur more frequently with the same individuals as a pair. In non-mating season the most common interactions occur between females. Interestingly, there were not many

male to male interactions in either season [2]. This knowledge could now shed light on how a contagious disease can spread though an entire devil population.

Tasmanian devils are scavengers and predators and consume all types of meat including fish, insects, birds and mammals (both marsupials and domesticated animals), generally as carrion which more than one devil often feeds upon. Their sharp teeth allow them to consume all of the carcass including the skull [76]. Devils are dimorphic in size, the females grow to 4.5-9 kg in size and the males grow to a size of 7.5-13 kg. They normally reach maturity at two years of age although since at least 1970 females have been known to breed from the age of one [73, 78]. Originally it was noted that less than 10 % of one year old female devils reproduced [73] but more female devils are now breeding earlier in regions where devil facial tumour disease (DFTD) is well established [78, 79]. Tasmanian devils only breed once a year which generally results in a total of four times during their lifetime if they survive until the age of 6. It is possible to breed captive devils but this can be difficult [80, 81]. Mating occurs in underground burrows [2] generally between March and April. The female does not ovulate until a few days after mating which is followed by a gestational period of between 14-22 days. Overall the mother gives birth 28-31 days after mating [2] to around 20 young at a time. The young then travel up to the pouch and the first to find one of the four teats are the only ones to survive. These three or four young devils (called imps) continuously stay hold of the teats until they leave the pouch to prevent them from falling out [74]. A female devil will lactate for 30 weeks with the imps living in the pouch for 15 of those weeks [76]. In the wild Tasmanian devils can live to five-six years of age [73]. Each female, therefore, can produce around 16-20 offspring in their lifetime but this is dramatically reduced if they die from disease and only reproduce once or twice in their lifetime.

1.5.2 Devil Facial Tumour Disease

Devil Facial Tumour Disease (DFTD) is a contagious cancer that forms in the facial area of Tasmanian devils but can metastasise throughout their body [3, 82]. As at December 2010, the disease had been observed in over 60 % of the island of Tasmania and had decreased the total population of devils to 20 % of what it was in 1996 when the DFTD was first observed. The disease still has not been observed on the west coast of Tasmania [83].

DFTD was first witnessed at Mt William National Park in Tasmanian's north east when a Tasmanian devil was noted to have tumours on their face. Even though there appears to be less male-male contact between devils and pre-dominantly female-female contact between devils in non-mating seasons [2], DFTD appears to have no gender bias [3, 84, 85] and does not appear to be present in juvenile devils. This disease had not been observed previously by the six biologists who had been trapping devils between 1964-1995 [73] although there is anecdotal evidence from people that saw devils with facial tumours as early as the 1950's [74]. In 1999, 3 years after the disease had been observed in Tasmania's far north-east, Jones, another Tasmanian biologist, observed tumours at Little Swanport on Tasmania's east coast, 250km south of Mt William National Park [74]. The disease was not publicly announced until September 2003. Even at this time very little was known about the disease. Work was conducted to determine if the cancer was spread by a retrovirus or if it had been initially caused by certain chemicals such as 1080 [74] but no chemicals were determined to cause the cancer. Biopsies from DFTD tumours were also examined for six viruses that are known to be involved in cancer and no evidence was gained to support the hypothesis that the etiology of this disease was caused by a virus [6]. Other research suggested that the cancer was spread by allograft [5] and a considerable amount of evidence has been obtained to support this theory [6] which is discussed more

completely below. Since then, further research has shown that DFTD is a cancer that easily metastasises by spreading hematogenously [86]. Tasmanian devils are prone to developing cancers but none of these are similar to DFTD [82]. DFTD cells have been shown to be of neuroendocrine in origin and also similar to Schwann cells which was supported from miRNA studies and the fact that DFTD cells also express Schwann cell markers [82]. DFTD microRNA (miRNA) profiles were shown by Murchison *et al.* using Pearson's correlation statistics, to be more similar to brain tissue than the nine other tissues that were tested (heart, testis, kidney, pancreas, spleen, bone marrow, lymph node, skin and liver) [82]. This cancer has also been shown to be neuroendocrine in origin by Loh *et al.*'s [77] using imunohistochemistry with cells that were histologically DFTD according to the diagnostic method published by Loh *et al.* [85] as well as positive and negative controls. As well as suggesting that the cancer was most probably neuroendocrine in origin their work also showed that devils from a range of geographical areas were affected with the same disease [77].

DFTD has been monitored in a range of geographical areas around Tasmania. Although DFTD is spreading west, the population of devils in western Tasmania is still majorly DFTD free [83] which could be partially because of genetic differences as well as the uninhabitable terrain between the northwest and eastern devils. Further research has also been conducted on the diversity on a class of proteins called major histocompatibility complex (MHC). MHC are antigens that are present on cell walls. The genetic diversity of MHC informs an individual's immune system if the cell is foreign or not. In humans only identical twins are likely to have the same MHC. Lack of diversity in the MHC between devils has been hypothesised to allow for allograft transmission of DFTD cells [87, 88] [89]. Siddle *et al.* observed genetic differences in MHC between the north-western and eastern Tasmanian devils. Tasmanian devils in eastern Tasmanian have low genetic

diversity in MHC although not all have the same MHC as DFTD cells. The low diversity in eastern devils and the differences between the north-western devils could contribute to the slower rate of transmission of DFTD in the north-western populations [88].

1.5.2.1 A Contagious Cancer

DFTD is a highly contagious disease; appears to always prove to be fatal after contact between devils [3, 76]; and is one of only a few known contagious cancers. Another example of a contagious cancer is canine transmissible venereal sarcoma (CTVS) which is sexually transmitted in dogs [90]. The transmission of DFTD appears to occur via allograft and the most plausible theory is that this occurs when the devils are bitting and fighting each other. The spread of this disease could also occur via cannibalism [86] and there is a possibility that healthy devils could be infected by DFTD cells that are left on carrion by DFTD positive devils [84]. There does not appear to be any vertical transmission between mothers and their young [86]. The allograft theory has been supported by results from chromosomal tests [5, 6], Amplified Fragment Length Polymorphism (AFLP) [6] and transmission trial [6] studies. Early chromosomal studies showed that all DFTD tumours from different animals had identical chromosomal abnormalities. This was shown with Pearse and Swift's work that looked at samples from 11 different DFTD-positive devils that had been obtained from different areas around eastern Tasmania. They saw that whereas normal devil cells contain 14 chromosomes (including the sex chromosomes), DFTD cells only contain 13 chromosomes. There were also abnormalities within the remaining chromosomes. Some of the chromosomes were decreased in size; four of the 13 chromosomes that are present in DFTD cells are not observed in normal Tasmanian devil cells; and neither of the sex chromosomes were observed in DFTD cells (see Figure 1.2). Although chromosomal changes occur in other types of tumours the fact that these abnormalities are identical in multiple devils suggested something more than conventional

cancer and the possibility that the cancer was contagious and spread by allograft [5]. This allograft theory was further supported in transmission trial studies [6], and research that was conducted by Siddle *et al.* that showed that Tasmanian devils have low major histocompatibility complex (MHC) diversity [87]. The low MHC diversity seen in devils allows for the allograft transmission of this disease because the MHC on the tumour cells does not differ from the host Tasmanian devil and are therefore not destroyed by the Tasmanian devil's immune system [87]. If DFTD cells have originated from Schwann cells this could also have contributed to this disease being transmissible since Schwann cells are involved in the immune response in the peripheral nervous system [82].

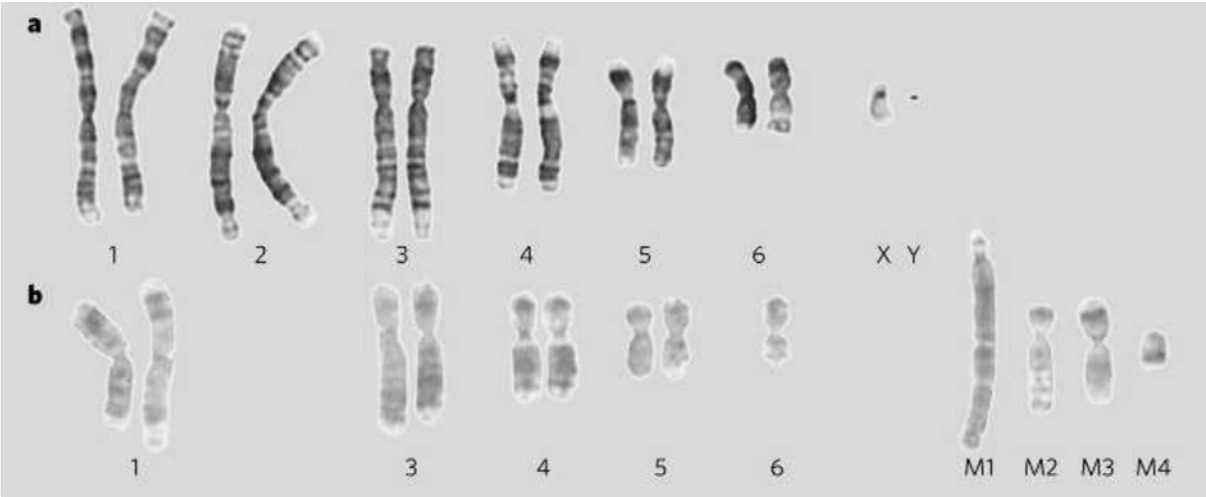

Figure 1.2: Comparison of a healthy male Tasmanian devil set of chromosomes (a) and the profile of chromosomes from DFTD cells (b) that were observed in 11 individual devils. The DFTD karotype shows an enlarged chromosome 1; the removal of both chromosome 2's, one of the chromosome 6's and both sex chromosomes; and the presence of four extra chromosomes that are not present in healthy cells (labelled M1-M4). This figure is reproduced from [5].

At present DFTD is tested for by taking a biopsy from a suspected tumour and undertaking a time consuming histological examination [73]. This does not allow for devils where no suspected tumours are seen to be tested for the disease, since no tumour biopsy can be taken. At present there are no methods to test for DFTD before the tumours are seen [91].

1.5.2.2 An Endangered Species

In the past, infectious diseases have not been considered to be a major driving force for species extinction [92]. Although a number of population declines in mammals have been caused by infectious diseases, one study has shown that only 8 % of endangered animal and plant species including approximately 1 % of endangered mammals are listed as endangered because of infectious diseases. DFTD gives us an example of how devastating a highly contagious disease can be to a species [92]. Low genetic diversity of wild species increases the chance that an infectious disease will put the species at risk of extinction [93]. This has likely been a major contributor to the devastation that DFTD has had on the Tasmanian devil population [87]. In DFTD areas most devils succumb to the disease by the age of two or three which is resulting in some females only breeding once [94]. This would also contribute to a reduction in the population of Tasmanian devils. In the early 1990s it was estimated that there was a population of 150,000 devils [95] but since the disease was first seen in 1996 there has been an 80 % decline in the total wild population of this species [83]. This decline in population led to the Tasmanian devil being listed as endangered under the Tasmanian Governments *Threatened Species Protection Act 1995* in May 2008 [96] as well as the Commonwealth *Environment Protection and Biodiversity Protection Act 1999* in May 2009 [97]. There is a high risk that the devil will be extinct in the wild from this disease in the next 20-30 years [98]. Local extinction in some areas is also possible within 5 years [78]. As is the case with all species, the extinction of the Tasmanian devil will alter the Tasmanian ecosystem which in turn will affect other species that reside in Tasmania. The loss of Tasmanian devils will reduce the competition at the top of Tasmania's food chain and it is feared that this could lead to increased establishment of foxes in Tasmania which could be detrimental to Tasmania's livestock industry.

1.5.2.3 Suppression Trials

Disease suppression in contagious wild animal diseases is often conducted by trapping and removing the diseased animals to limit the spread of the disease. A suppression trial for Tasmanian devils was set up at the Forestier and Tasman Peninsulas (see Figure 1.3) encompassing a total area of 360 km^2. To decrease the spread of DFTD all positive DFTD devils that were trapped were removed. This area was chosen since the only land contact between the peninsulas and mainland Tasmania is via the Dunalley Bridge at Dunalley. The initial suppression trial study occurred between June 2004 to June 2005 and then an intensive study commenced in January 2006. The suppression trial was conducted by removing and euthanizing all devils that either had DFTD or characteristic signs that were considered to show that they would develop DFTD. Devils were chosen this way since the only available diagnostic test is to histologically test visible tumours. The devils were removed during 10 day trapping trips that occurred four to five times per year in an area of the Forestier Peninsula that included an area known have DFTD devils as well as a buffer zone. The initial suppression trial showed good preliminary results with a decrease in the number of devils with late stage tumours (tumours larger than 4 cm in size) and no noticeable decrease in the population. The results from the suppression trial were compared to a similar site at the Freycinet Peninsula (see Figure 1.3). Whereas there was no noticeable decrease in devil population in the suppression trial area the population density of devils at the Freycinet Peninsula decreased from 0.9 to 0.6 devils per km^2 in a similar time frame to the 12 month initial suppression trial. In December 2009, the trial still had not succeeded in producing a DFTD free population but it had not been completely unsuccessful though since the population of devils in this area has not decreased since the start of the suppression trial and DFTD has also not spread to other places around the peninsulas. There are two possible reasons why DFTD had not been

eradicated. Firstly, it had been observed using hair samples that around 25 % of the population were not being trapped. This population could contain DFTD-positive devils that are spreading the disease to other devils. The second reason the disease may still be present is because DFTD-positive devils are not being removed soon enough to prevent them from spreading the cancer to other devils. The development of the preclinical test discussed in this work would aid in removing the devils before they can spread the disease [99]. Successful implantation of this suppression trial will allow for DFTD-free devils to be obtained from an eastern Tasmanian devil population which will allow for increased genetic diversity as part of insurance populations [86].

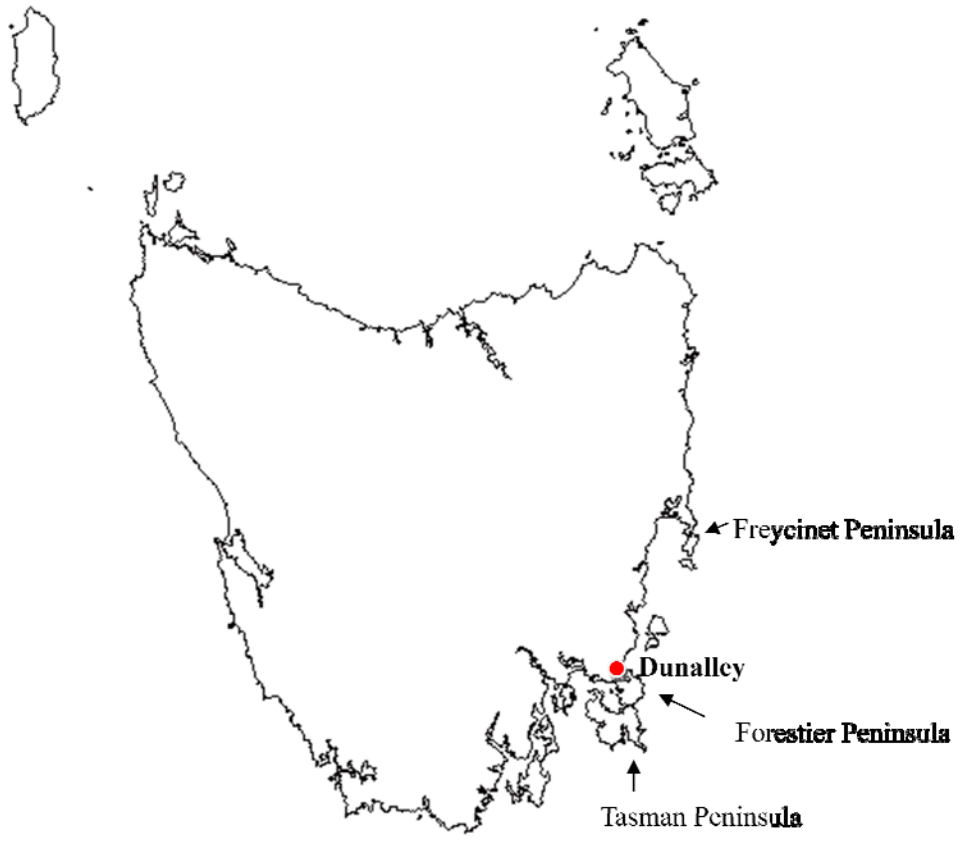

Figure 1.3: Map of Tasmania showing the Freycinet and Forestier Peninsulas where DFTD research is being conducted.

1.5.2.4 Insurance Populations

To try and prevent the extinction of this species the highest priority at present is the development of insurance populations in wildlife parks and zoos across Australia [100]. These insurance populations were developed to ensure that there was a DFTD-free population of devils that could be used to repopulate mainland Tasmania in the case that the species becomes extinct in the wild [98]. The Tasmanian devil population is known to have low genetic diversity so the purpose of the insurance population was also to maintain as much of this genetic diversity as possible. The best way to maintain genetic diversity in the insurance populations would be to conduct managed breeding. Managed breeding would require less captive devils but it could also cause problems of re-introducing the population into the wild because the living conditions of the captive devils in this situation would not be representative of the wild. To increase genetic diversity it is also possible to transplant devils from one population into other populations [98]. The initial *Insurance population strategy* [98] suggested that 150 DFTD-free devils that varied in as much genetic diversity as possible, would be bred to a population of 1500 devils (if breeding was managed) or 5000 devils (if breeding was not managed) or at a relative number if both these type of population groups exists. These numbers were considered as an "effective population" which is very close to the actual population when the gender numbers are identical and all animals are breeding at an equal rate.

At present, since there is no pre-clinical diagnostic test to determine if a devil has the disease before tumours are seen and the latent period of the tumour is also unknown [78], Tasmanian devils for the insurance population are maintained in quarantine for two years [86].

1.5.2.5 DFTD Diagnostic Tests

Currently DFTD is diagnosed histologically by examining biopsies of suspected tumours. The cells from the biopsies were considered to be DFTD if the neoplastic cells had been derived from dermis or subcutaneous tissue and were round to spindle-shaped [85]. DFTD cells can also be identified karotypically since the chromosomes from DFTD cells differ from those of the host in a distinct way as seen in Figure 1.2, page 32 [5].

This type of diagnosis may not be appropriate for biopsies obtained from non-facial DFTD tumours or metastasis of DFTD tumours. To aid in diagnosing these abnormal DFTD tumour biopsies, another diagnostic test was developed by Murchison *et al.* This diagnostic test involves staining suspected DFTD cells with an antibody for periaxin (PRX) a Schwann cell-specific myelin protein. This stain was able to correctly diagnose 20 DFTD tumours plus an additional 10 DFTD metastases from different types of organs. It could also correctly identify the nine non-DFTD biopsies that were tested [82].

All of these methods require a biopsy of the suspected tumour so at present there are no methods to test for DFTD before the tumours are seen [3, 91]. The availability of a pre-clinical diagnostic test will aid in the managing the spread of DFTD [2]. The development of this diagnostic test could be used to aid in two of the major projects that are currently being conducted to ensure that a healthy population of Tasmanian devils is maintained. Firstly this test could be used to ensure that only DFTD free devils enter into insurance populations. Having a pre-clinical test for DFTD would allow healthy Tasmanian devils to enter into the insurance population straight away allowing breeding to occur earlier. This is desirable since Tasmanian devils only have a lifespan of around six years [80, 81]. The insurance populations need to maintain as much genetic diversity as possible. To do this, devils will need to be included from eastern and western Tasmania since there are distinct

genetic diversities between these two groups of devils. Unfortunately almost all Tasmanian devils eventually develop DFTD in eastern Tasmania so to include these devils, an accurate pre-clinical test is required to ensure that these devils do not all ready have DFTD which could spread to the other individuals in the insurance population. The ability to determine DFTD pre-clinically will also allow a decrease in the time required for quarantine since the latent period for DFTD is uncertain and could be anywhere up to 12 months.

The availability of a diagnostic test would also aid in the suppression trial. Pre-clinically diagnosing DFTD would allow for the removal of DFTD devils potentially before the tumour cells could transfer and infect another devil.

For use in the suppression trial or to aid in ensuring a DFTD-free population in the insurance trial, a diagnostic test would be required to have the ability to test all Tasmanian devils with and without the presence of tumours in the wild. This means that a test that diagnoses the disease by a biopsy is not suitable. A biological sample is therefore needed that is easy to obtain from wild trapped devils. Blood is easier to collect than urine or other biological fluids when collection is taking place out in the field. A serum blood test is suitable since blood can be collected into clean tubes without additives in the field; allowed to clot; spun down at the end of the day in a portable centrifuge to remove the cells; and stored in a freezer prior to transportation back to the laboratory for testing.

1.6 **Project Aims**

The purpose of this work was to search for biomarkers for DFTD that could be used in the development of a pre-clinical diagnostic test to aid in the survival of Tasmanian devils. There have not been many if any diagnostic tests developed for diseases of wild animals where other factors affecting biomolecules are not controlled. Hines *et al.* though actually

noted less metabolite variability from mussels (*Mytilus galloprovincialis*) that had been sampled straight from the field compared to when mussels collected at the same location, were first stabilised in the laboratory for 60 hours prior to analysis [22]. This suggests that obtaining samples for biomarker determination from wild animals is not unfeasible. Wild fish have also been studied to try and determine biomarkers for liver cancer in flatfish (*Limanda limanda*) found in waters surrounding the United Kingdom. The researchers involved in this study looked at healthy and cancerous liver tissue which showed greater variability between individuals than between healthy and diseased tissue [8]. They also looked at peptides in wild flatfish plasma but their results showed greater difference between the location that the fish was caught and the age of the fish rather than the liver cancer status of the fish. Even removing these effects the researchers were still unable to completely distinguish between healthy and diseased fish. The results were only able to determine 7 out of the 10 fish that had liver tumours [101].

Although previous disease biomarker work on wild animals has not been as successful as hoped, the low genetic diversity of Tasmanian devils may aid in the discovery of DFTD specific metabolites for a diagnostic test. As a small blood sample obtained through an ear prick is the easiest sample to obtain from a wild devil, this work focused on the use of various types of mass spectrometry techniques with and without prior sample separation to examine serum metabolites obtained from non-DFTD and DFTD devils. The techniques, CE-ESI-MS, GC-MS and ESI-MS, were used in collaboration with multivariate analysis to discover differences in Tasmanian devil serum that are observed in DFTD positive individuals but not in non-DFTD devils to use as potential disease biomarkers for DFTD. Their applicability to be used for routine screening of samples to diagnose DFTD is also discussed.

2 Method Development

2.1 Introduction

As discussed in the introduction (see section 1.2), there are numerous well established instrumentation and methods that are suitable to study metabolomics based on NMR or MS detection [25, 102]. Mass spectrometry is a more sensitive technique which allows quantitative data to be obtained for a greater number of metabolites [37]. This work involved the investigation of three different mass spectrometry (MS) methods to distinguish differences in the metabolome between devil facial tumour disease (DFTD) and non-DFTD Tasmanian devils. One of the methods, electrospray ionisation – mass spectrometry (ESI-MS), had no separation step prior to MS detection. The other two methods, gas chromatography – mass spectrometry (GC-MS) and capillary electrophoresis – mass spectrometry (CE-MS) separated the metabolites prior to MS detection. GC-MS and CE-MS were both investigated because each technique a) provides a different process of separation; b) requires different types of sample preparation prior to analysis [15, 26, 41]; and c) different chemical classes of metabolites are better observed with different separation techniques [30]. These methods were examined to determine appropriate approaches to mine for specific metabolite changes that occur with DFTD.

2.2 Experimental

2.2.1 Samples

An initial pilot set of Tasmanian devil serum samples was obtained from 16 devils (including males and females) caught in the eastern half of Tasmania in 2004 (see Table 2.1) collected by the Department of Primary Industries, Parks, Water and the Environment

(DPIPWE), Mount Pleasant, Tasmania. All of the devils came from areas in which DFTD was known to be present. A pooled rat serum sample was obtained from female Sprague Dawley rats and a pooled mouse serum sample was prepared from serum obtained from non-obese diabetic-severe combined immunodeficient (NOD/SCID) mice.

Table 2.1: List of serum samples used for the pilot study with information on DFTD status, sampling location, gender and age. Further details can be observed in Appendix 1, Table A 1.

Sample #	Location	DFTD status	Sex	Age
04/0560	Bronte Park	DFTD 69 days later	M	3
04/0603	Bronte Park	Non-DFTD	F	3
04/0605	Bronte Park	DFTD	F	3
04/0735	Bronte Park	DFTD	M	3
04/0960	National Park	Non-DFTD	M	3
04/1021	National Park	Non-DFTD	F	4
04/2058	Mount William	DFTD	F	3
04/2062	Mount William	Non-DFTD	M	1
04/2064	Mount William	DFTD	M	4
04/2065	Mount William	Non-DFTD	F	3
04/3000	Fentonbury	DFTD	F	?
04/3003	Fentonbury	Non-DFTD	F	4
04/3008	Fentonbury	DFTD	M	4
04/3011	Fentonbury	Non-DFTD	M	4
04/3176	St Helens	Non-DFTD	F	3
04/3201	St Helens	Non-DFTD	M	3

2.2.2 Reagents

Water was purified with a Millipore Milli-Q water purification system (North Ryde, Australia). Methanol (> 99.7 %, isocratic HPLC grade (254nm)) was obtained from Scharlau Chemie S.A. Methylhydroxylamine hydrochloride and formic acid (98 %) were obtained from Fluka. Poly(sodium 4-styrene-sulfonate 70kDa was obtained from Aldrich, St Louis, USA; HPLC grade acetonitrile; very low molecular weight Poly(diallyldimethylammonium chloride), polybrene (≥ 95 %); N-Methyl-N-

(trimethylsilyl)trifluoroacetamide (MSTFA); pyridine (≥ 99.0 %) and sodium hydroxide (99.99 %) were obtained from Sigma Aldrich.

2.2.3 GC-MS

Proteins were removed from serum using a crude methanol extraction by precipitation. The serum (15 µL) was added to 500 µL of cold methanol. The mixtures were vortexed (Ratek VM1 vortex mixer) for 5-10 s to mix and then incubated at 4 ˚C for over 20 min. After incubation the samples were centrifuged at 14,000 rpm (Eppendorf bench top centrifuge 5424) for 5 min. A 200 µL aliquot of the supernatant was transferred into a glass sample vial which were then covered with parafilm and pierced. The samples were dried in a vacuum oven at 55 ˚C.

On the day of analysis the metabolites were derivatised using a method described elsewhere [103] using 20 µL of 40 mg/mL methylhydroxylamine hydrochloride in pyridine and 80 µL of N-Methyl-N-(trimethylsilyl)trifluoroacetamide. The solutions were then vortexed to mix and left at room temperature for 90 min.

After derivatisation, the samples were analysed using a Shimadzu QP2010-plus GC-MS fitted with a BPX-35 capillary column (30.0 m or 15.0 m in length, film thickness 0.25 µm and an internal diameter of 0.22 µm). The separation method for the 30 m column was as follows: 1 µL of sample was injected using splitless injection for 45 s at 230 ˚C. The temperature program was set to hold the column at 55.0 ˚C for 2 min and then increased at 15 ˚C/min until it reached 330 ^{o}C. The column was kept at this temperature for a further 6 min. The column pressure was kept constant at 88.6 kPa with an initial carrier gas (helium) flow rate of 0.88 mL/min. The temperature of the mass spectrometer ion source and interface was 200 ˚C and 250 ˚C respectively. The detector voltage was set to 0.88 kV.

Spectra were obtained from 6.5 min into each run between 85-500 m/z with a scan speed of 5000 amu/s.

The timing for the temperature program for the 15 m column was modified from the 30 m program to take into consideration the decrease in column length using Method Translation software (Agilent) as follows: 1 µL of sample was injected using splitless injection for 30 s at 230 ˚C. A temperature program consisted of holding the column at 55.0 ˚C for 0.7 min. The temperature was then increased at 42.4 ˚C/min until it reached 330 ^{o}C. The column was then kept at this temperature for a further 2.10 min. The column pressure was kept constant at 33.2 kPa with an initial carrier gas (helium) flow rate of 0.88 mL/min. The temperature of the mass spectrometer ion source and interface was 200 ˚C and 250 ˚C respectively. The detector voltage was set to 0.84 kV. Spectra were obtained from 2.2 min into each run between 85-501 m/z with a scan speed of 10,000 amu/s.

Data analysis was performed using the GCMS solutions software (Version 2.50SU1, Shimadzu 1999-2006). Integration was done with two different processes in *GCMS Postrun Analysis,GCMS Solution Version 2.50 SU1, © 1999-2006 Shimadzu Corporation.* For each metabolite peak, the base peak was determined and the total area for this ion was calculated to more accurately determine peak areas for overlapping peaks. The first process involved manual integration of the peaks. The second process used the *Quantitative Parameters Peak Integration* option in the program. For the second process, initially the main ion for each peak was determined and the list of m/z and retention times were inputted into the parameters table. The quantitative parameters were then adjusted to obtain the parameters that most efficiently integrated the peaks. Each peak was manually checked to ensure that the peak had been correctly identified. The peak table was then analysed using Microsoft Excel 2007 and XLSTAT (Addinsoft 1995-2010).

2.2.4 ESI-MS

Dilution Study: Two extraction methods were used to isolate the serum metabolites of interest from proteins and other large molecules. In the first instance 15 µL of Tasmanian

devil serum was added to 500 µL of cold method (sample M1) and the second method involved the addition of 30 µL of Tasmanian devil serum 500 µL cold methanol (sample M2). Both of the mixtures were vortexed to mix and then incubated at 4 ˚C for over 20 min. After incubation the samples were centrifuged at 14,000 rpm (Eppendorf bench top centrifuge 5424) for 5 min. Further dilutions of M1 were prepared before injection into the instrument with methanol to give final dilutions shown in Table 2.2. Sample M2 was not diluted further and had an overall dilution of the serum of 17.7 times (See Table 2.2). Each sample was acidified with addition of 10 % v/v aqueous formic acid to give an overall formic acid concentration of 0.1 % v/v.

Table 2.2: Description of serum sample dilutions for ESI-MS method development.

Sample #	Mixture	V(of prepared sample) (µL)	V(10 % Formic acid) (µL)	V(MeOH) (µL)	Total vol (µL)	Overall serum dilution
1	M1	198	2	0	200	1:34.7
2	M1	20	2	178	200	1:347
3	M1	2	2	196	200	1:3468
4	M1	100	2	98	200	1:68.7
5	M1	50	2	148	200	1:137.3
6	M2	198	2	0	200	1:17.8
7	-	-	2	198	200	N/A (blank)

The analysis of the samples was conducted on a Bruker micrOTOF-Q II instrument (Bruker Daltonics) as follows: The sample was infused into the instrument for 2-3 min via a syringe pump at a constant flow rate of 180 µL/hr (kd Scientific syringe pump with a 0.10 mL Gastight #1710 syringe, Hamilton Co, Reno, Nevada, USA). Spectra were collected between 50-1200 m/z in positive ion mode. The capillary voltage was set to -4500 V, nebulizer pressure was 0.3 bar, the flow rate and temperature of the dry gas was 4.0 L/min and 180 ˚C respectively. The energy of the Collision cell was 10.0 eV with a transfer time of 110.0 µsec. For further analysis the average spectrum was obtained between 1.0-2.0 min after the sample was injected into the instrument. Mass spectra were collected using MS software Compass 1.3 for micrOTOF-SR 1 micrOTOF control version 3.0 © 2002-2009 Bruker Daltonik Gmbh and analysed using MS software Compass 1.3 for

micrOTOF Sr 1 micrOTOF- SR 1 DataAnalysis version 4.0 SP 1 © 1992-2009 Bruker Daltonik Gmbh.

Quality control samples: A sample of rat serum was used as a quality control (QC) sample. The quality control samples were prepared by placing 15 µL of serum in 500 µL of cold methanol. The samples were vortexed to mix followed by a 4 ˚C incubation for at least 20 min. The mixtures were then centrifuged at 14,000 rpm for 5 min. Two 100 µL aliquots of the supernatant were then obtained and each aliquot was diluted by the addition of 98 µL of methanol and acidified by the addition of 2 µL of 10 % v/v aqueous formic acid. Generally each day two quality control samples were prepared to obtain a total of four aliquots of the quality control. Prior to the analysis of samples two reagent blanks were run followed by a quality control. Another reagent blank and quality control sample were run after every ten samples. The QC samples were run in alternating order to determine if outliers were caused by errors with the instrument or during sample preparation.

2.2.5 CE-MS

Instrumentation: Analysis was conducted with an Agilent CE-ESI-Iontrap MS system using an Agilent 3D capillary electrophoresis system connected to an Agilent 6320 Ion Trap LC/MS with the aid of an Agilent 1200 Series pump. The CE was controlled with Agilent ChemStation software and the MS was controlled with 6300 Ion Trap Control software (Waldbronn, Germany). Mass parameters were set as in Ramautar *et al.* [33] where the dry gas temperature was set to 180 ˚C, 4 L/min N_2, 7.3 psi nebulizer pressure and an ESI capillary voltage of - 4500 V. Spectra were collected between 70 – 400 m/z.

Separation and sheath buffers: 1 M formic acid was used for the background electrolyte (BGE). The sheath liquid consisted of 1 % v/v formic acid in 1:1 methanol:water and was used at a flow rate of 4 µL/min.

Polybrene-PSSS coated capillaries: Capillaries were coated using a Harvard apparatus PHD 2000 infuse/withdraw syringe pump and Hamilton gastight #1725 syringe with a flow rate of 300 μL/hr. The capillary was rinsed with Milli Q water for 15 min followed by 15 min with NaOH. Water was then passed through the capillary until the flow through was neutral. The capillary was then flushed with polybrene (10% w/v in water) for 30 min, water for 5 min, PSSS (5% w/v in water) for 30 min and finally water for a further 5 min.

PDDMAC-PSSS coated capillaries: Capillaries were coated using a Harvard apparatus PHD 2000 infuse/withdraw syringe pump and Hamilton gastight #1725 syringe with a flow rate of 300 μL/hr. The capillaries were rinsed for 5 min with Milli-Q water, 15 min with 1 M NaOH, more water until the flow through was neutral, 30 min with 0.1 % PDDMAC/0.5 M NaCl, 3 min with water, 30 min with 5 % w/v PSSS, 3 min water, 30 min with 0.1 % PDDMAC/0.5M NaCl, 3 min with water, 30 min with 5 % w/v PSSS, and finally 5 min water. At the beginning of each day the capillary was reconditioned by removing the outlet from the mass spectrometer and flushing for 5 min with 5 % PSSS, 5 min with water and 10 min with BGE to maintain capillary stability. This type of coating still allowed for EOF but eliminated the coating of analytes onto the capillary wall which reduces reproducibility by increasing migration times in subsequent runs.

Sample preparation: The organic solvent study was conducted by placing one volume of serum to two volumes of acetonitrile or methanol. The mixtures were vortexed to mix and incubated at 4 ˚C for greater than 20 min. After the incubation the samples were centrifuged at 14,000 rpm (Eppendorf bench top centrifuge 5424) for 5 min. The supernatant was injected into the instrument without further sample treatment. Prior to injection a 73.8 cm PDDMAC-PSSS coated capillary was conditioned for 5 min with 1 M formic acid as the BGE. The supernatant from the protein precipitation step was injected

into the capillary at 50 mbar for 120 s. Prior to sample separation, a sample plug of Milli Q water was injected into the capillary for 5 s at 50 mbar. 25 kV was applied to the column to separate the metabolites along with 30 mbar pressure to aid in moving the analytes through the sheath. The sheath fluid consisted of 1 % v/v formic acid in a solution of 1:1 methanol:water. Instrument and mass spectrometry parameters were used as stated above. To determine the ultimate ratio of solvent to serum, equivalent of one volume of Tasmanian devil serum was added to one, two and three volumes of acetonitrile. The samples were prepared and analysed by CE-MS in the same way as the organic solvent study.

Sample injection study: The sample injection study looked at rat serum samples. In both instances serum metabolites were collected by the addition of serum 1:1 with acetonitrile according to the method described under sample preparation. The samples were then injected 75, 90 and 120 s at 50 mbar followed by a 5 sec 50 mbar injection of water. A voltage of 20 kV and a pressure of 30 mbar was applied to the capillary during separation.

Sample separation: The optimum separation voltage was determined by the injection of rat serum metabolites using the same method as the sample injection study. Prior to injection the 80cm 50µm id PDDMAC-PSSS capillary was conditioned for 5 min with BGE. The rat serum metabolites were injected for 120 s at 50 mbar. A plug of water was then injected into the capillary for 5 s at 50 mbar. Analyte separation was conducted at a pressure of 30 mbar and separation voltages of either 15, 20 or 25 kV.

2.2.6 Analysis of quality controls

To ensure that the quality controls were reproducible the samples were analysed using the Grubbs test. Samples were considered to be an outlier if some of the variables (analytes) were outside of the range calculated by the formula: $f(x)_{\pm} = \bar{x} \pm (G_{n=x} \times \sigma)$ for each

analyte. Where $G_{n=x}$ was dependent upon the number of quality controls with a significance of 2.5 % [104].

2.3 Results and Discussion

2.3.1 GC-MS

Comparisons of the same sample between runs: Samples were analysed in duplicate and using two different column sizes (30 and 15 m). The shorter capillary size was investigated to determine if similar results could be obtained in a considerably shorter run time. Having a shorter run time could dramatically increase the sample output if the method was used to screen a large number of devils for DFTD.

Initially these samples were prepared and run in duplicate using the 30 m column. Overlaying the chromatograms showed good reproducibility which suggested that there was limited variability in the sample preparation and separation steps (see overlay of replicates in Figure 2.1). Because of the good reproducibility, preparing and running the samples in duplicate was not continued.

Originally these samples were analysed with the 30 m BPX35 column over 26.3 min. The same samples were later analysed on the same column cut to 15 m in length. The method times were modified according to Blumberg and Klee [105] with Method Translation software (Agilent) to take into consideration the shorter column, and the separation was completed 9.3 min after sample injection. The chromatograms of the serum metabolites obtained from a male DFTD positive devil (sample 04/0735) separated in the 30 m and the 15 m column can be viewed in Figure 2.2.

The shorter column was translated to provide a similar separation according to Blumberg and Klee [105] as would be expected, the reduction of plate numbers reduced the

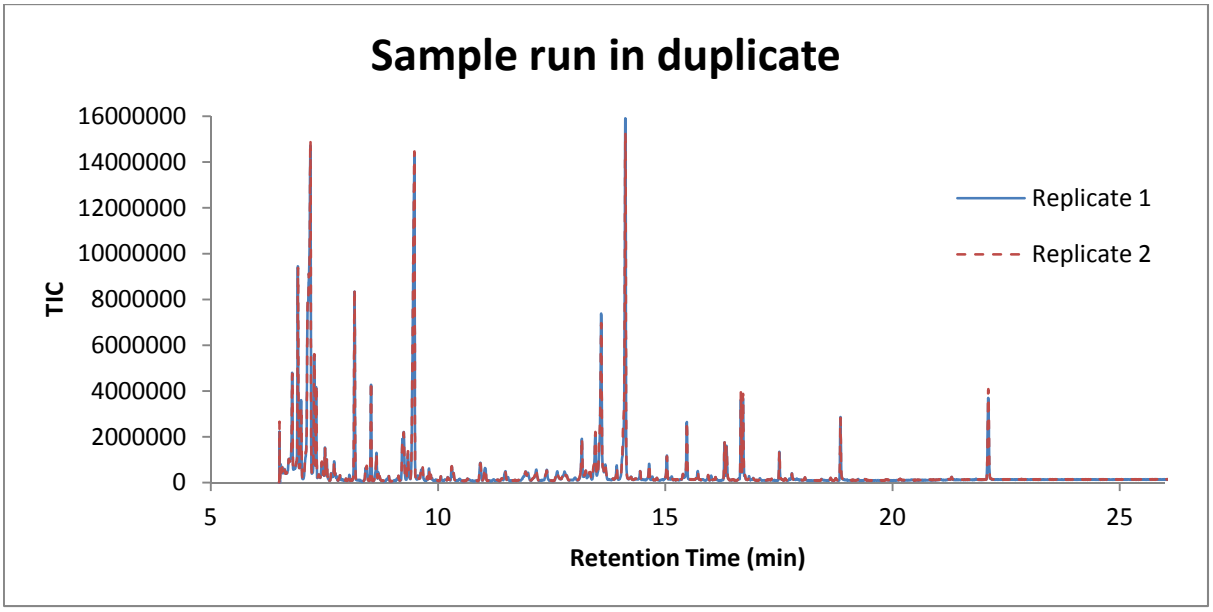

Figure 2.1: Reproducibility of samples: This figure shows the overlay of two chromatograms from the separation of serum metabolites from a DFTD male devil run in duplicate by GC-MS as follows: The samples were injected into the GC using a splitless injection for 0.75 min at 230 ˚C into a 30 m BPX-35 column. The temperature of the column was held at 55 ˚C for 2 min and then ramped to 330 ˚C at a rate of 15 ˚C/min. The column was held at 330 ˚C for 6 min. The acquisition of data was initiated 6.5 min into the run.

separation efficiency in the shorter column. The majority of peaks that were observed in the longer 30 m column could still be observed in the 15 m column. The relative intensity of the peaks though did differ between these two runs. An example of this can be observed by comparing the relative intensities of the cholesterol peak with the two dominant sugar peaks in the two chromatograms. In the 30 m column separation, the cholesterol peak is approximately half as intense as the small of the two large sugar peaks where as in the 15 m separation the intensity of the cholesterol peak is on par with the intensities of the two abundant sugar peaks. This change in relative intensity of the cholesterol peak was also observed with the other samples run. The change in peak height was mirrored when the peak areas were also examined (in the longer column the areas were 3 and 7 times higher than for cholesterol and in the shorter 15 m column the areas were approximately 4 and 5 times that of the cholesterol peak). Longer separation time increases diffusion of the analyte in the column so the peak of intensity for cholesterol is likely lower in the longer

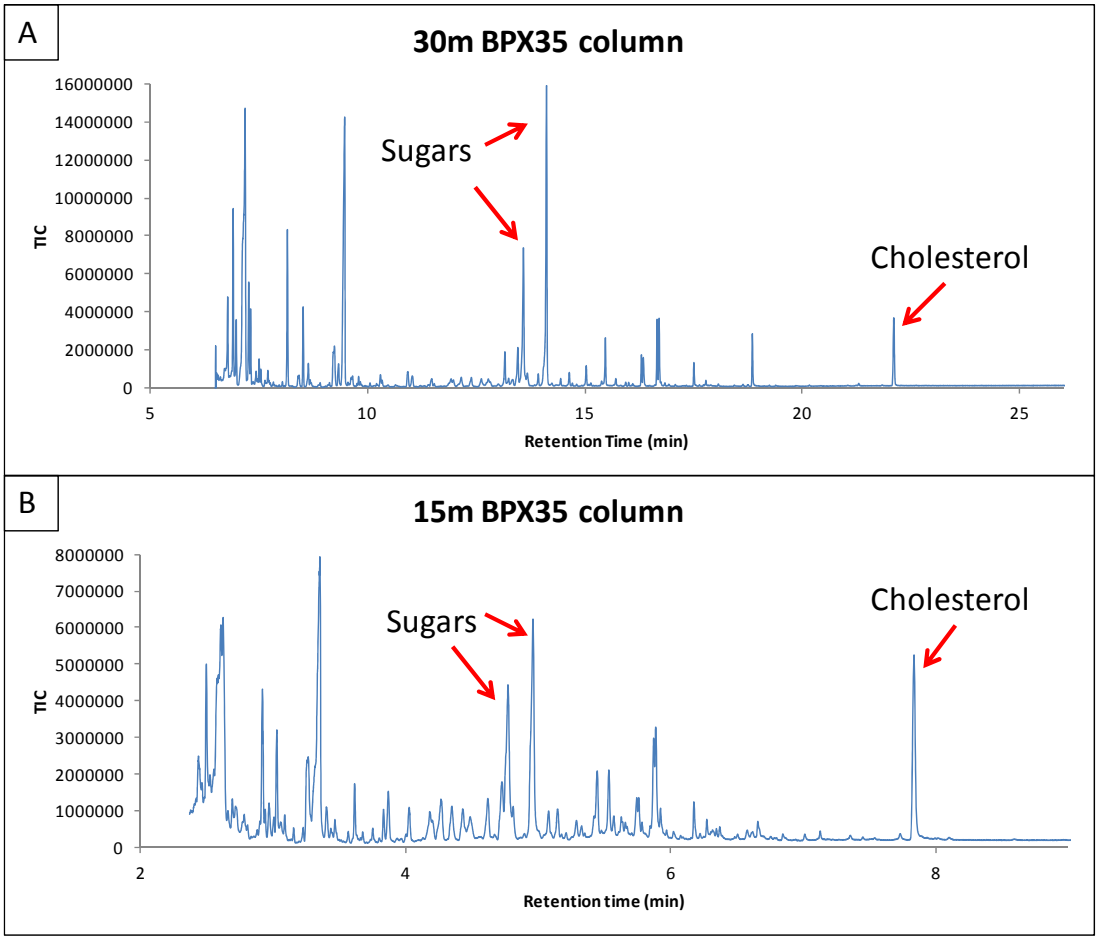

Figure 2.2: Comparison of the separation of serum metabolites from a male DFTD devil in a 30 m and 15 m column: In part A the sample was analysed by GC-MS as follows: The samples were injected into the GC using a splitless injection for 0.75 min at 230 ˚C. The temperature of the column was held at 55 ˚C for 2 min and then ramped to 330 ˚C at a rate of 15 ˚C/min. The column was held at 330 ˚C for 6 min. The acquisition of data was initiated 6.5 min into the run. In part B the sample was analysed by GC-MS as follows: The samples were injected into the GC using a splitless injection for 0.50 min at 230 ˚C. The temperature of the column was held at 55 ˚C for 0.70 min and then ramped to 330 ˚C at a rate of 42.40 ˚C/min. The column was held at 330 ˚C for 2.10 min. The acquisition of data was initiated 2.20 min into the run.

column because of peak broadening. The number of peaks observed in the shorter column was 105, around half the 215 peaks that were observed in the longer column for this sample. Although there were changes in peak intensity of the peaks compared to the 30 and 15 m columns, the shorter column allowed for at least twice as many samples to be analysed in the same time frame and was still able to provide enough information to discriminate between DFTD and non-DFTD samples to be observed by PCA (see chapter 3). The 15 m column was therefore used in future analysis.

2.3.2 ESI-MS

Electrospray ionisation (ESI) is known to be affected by ion suppression. Ion suppression is caused by the presence of less volatile components present in the solution. These ions can decrease the efficiency of droplet formation and evaporation which then affects the amount of analyte that becomes charged in the gas phase which reduces the amount of analyte that is then detected by the MS [45]. There are a number of processes that can be undertaken to decrease the effect of ion suppression in ESI-MS as discussed in section 1.2.2.1. The methods discussed here used the precipitation of proteins using organic solvents and sample dilution to reduce ion suppression. To do this, serum proteins were removed from a Tasmanian devil serum sample using methanol and various dilutions of the serum metabolites were prepared and injected into the ESI-MS (see Table 2.2). The resulting spectra were examined to determine the best dilution to use in further experiments (see Figure 2.3).

The spectra showed that lower dilutions (17.8 to 137.3 times dilutions) were more applicable when looking at the higher molecular weight ions (> 500 m/z) but higher dilutions (347 and 3468 times dilutions) gave better intensity for the lower molecular weight ions (< 500 m/z). Higher molecular weight ions have also previously been shown by Sterner *et al.* to decrease the intensity of lower molecular weight ions [106]. Taking into consideration the differences in intensity of the high and low molecular weight ions it was determined that the best dilution factor to use for further runs was 68.7 which showed good responses for both the low and high molecular weight ions. This was comparable to a study conducted by Han *et al.* which analysed mouse serum diluted 100 times by ESI-MS [43].

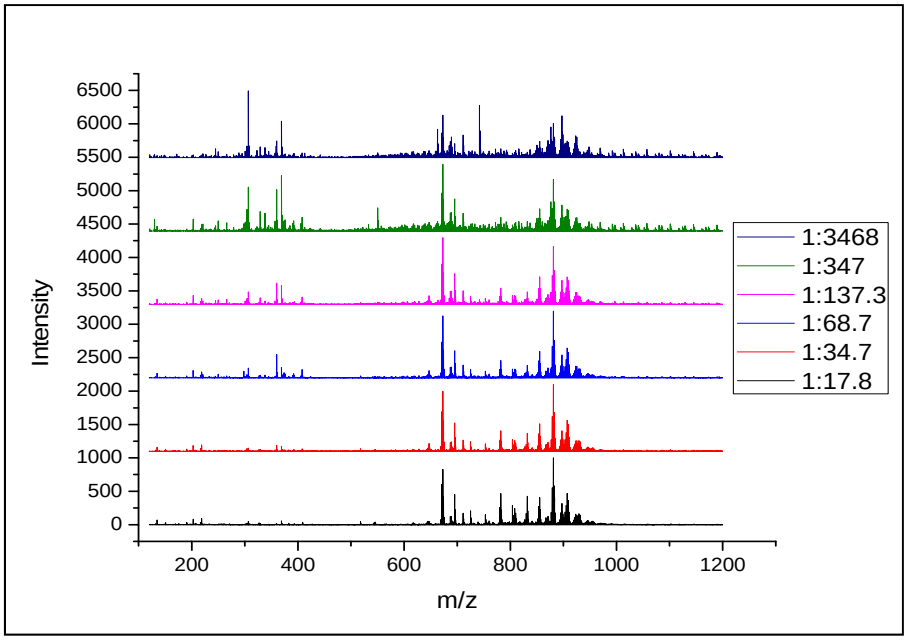

Figure 2.3. Mass spectra of a devil sample prepared with various amounts of dilutions (see right hand side legend) prior to injection into the ESI-MS.

Quality controls: The reproducibility of this method was shown by using a rat serum quality control which was used with a set of samples over three consecutive days and again with another set of data run one day approximately six weeks later (see Figure 2.4 for spectra). A total of 10 aliquots (see experimental) of this quality control were run with the initial set of samples and 4 were run in the second set. In the second set only 3 samples could be analysed because one of the samples had a corrupt file. The reproducibility of the initial 10 aliquots of the control sample were analysed using the Grubbs test as explained above in section 2.2.6. Quality controls on each day were within the outer ranges determined by the formula: $f(x)_{\pm} = \bar{x} \pm (G_{n=x} \times \sigma)$; where G was 2.29 (n=10); and 2.46 (n=13).

The three QC that could be analysed from the runs conducted six weeks later were analysed using the same formula (n=13) with all of the initial QC's. These three QCs were all within the range determined by the Grubbs test. This shows that this test is reproducible

over time. It would therefore be suitable for continued analysis of samples over a range of time such as what would be required to continually test Tasmanian devil serum for DFTD.

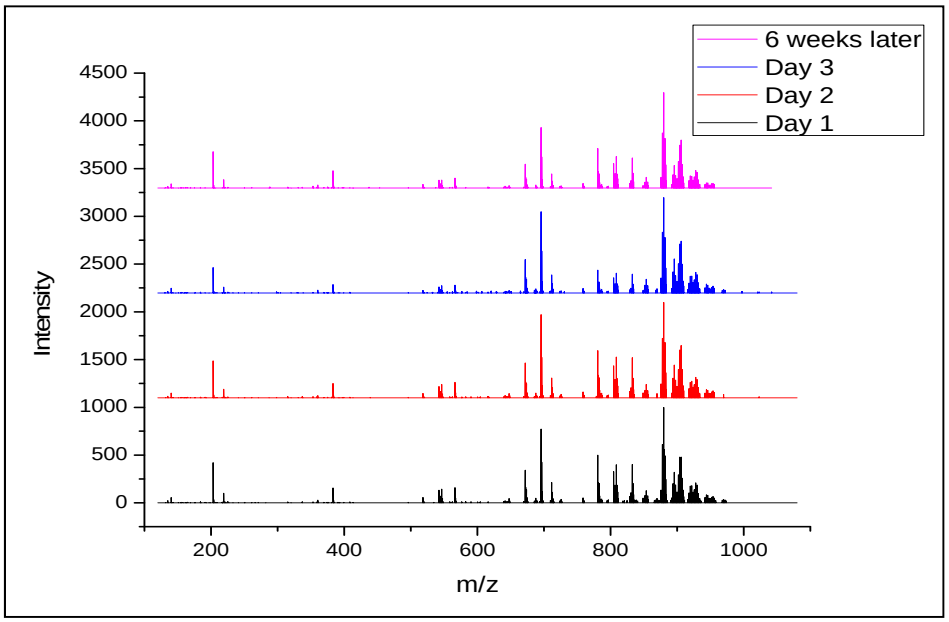

Figure 2.4: Showing the reproducibility of the rat quality control sample analysed of three consecutive days and again 6 weeks later using ESI-MS

2.3.3 CE-MS

Capillary coating: Fused silica columns are cheap and provide EOF which can aid in separation in metabolite studies, but separation and migration time can be affected by the binding of amino acids and peptides to the capillary wall which can also affect the separation in subsequent runs. These effects can be reduced or eliminated by coating the capillary. In the case of proteins, linear polyacrylamide capillaries are often used to prevent the proteins binding to the walls which also eliminates EOF [107]. In this work, a coating method based on Ramautar *et al.* was used to non-covalently coat the capillary with a bilayer of polymers consisting of an anionic and a cationic polymer [33] which would not eliminate EOF [108]. Ramautar *et al.* used a polybrene - polyvinylsulfonate coated capillary. Initially because of compound availability the polyvinylsulfonate polymer was replaced with another anionic polymer, poly (sodium 4-styrene-sulphonate)

(PSSS). Capillaries were also coated with another cationic polymer, poly (diallyldimethylammonium chloride) (PDDMAC) that was used instead of polybrene. The two different capillary coatings were compared for the separation of metabolites obtained with a 1:1 acetonitrile protein precipitation (see Figure 2.5). In both instances additional flushes of PSSS were required to maintain reproducibility between runs. The PB-PSSS capillary showed better separation between peaks than the PDDMAC-PSSS capillary but very few peaks were observed between 20 and 25 min. By observation, it appeared that the PDDMAC-PSSS capillary separated a similar number of metabolites in a third or the time of the PB-PSSS capillary without the observed peak free area, so PDDMAC-PSSS coated capillary was chosen for future work.

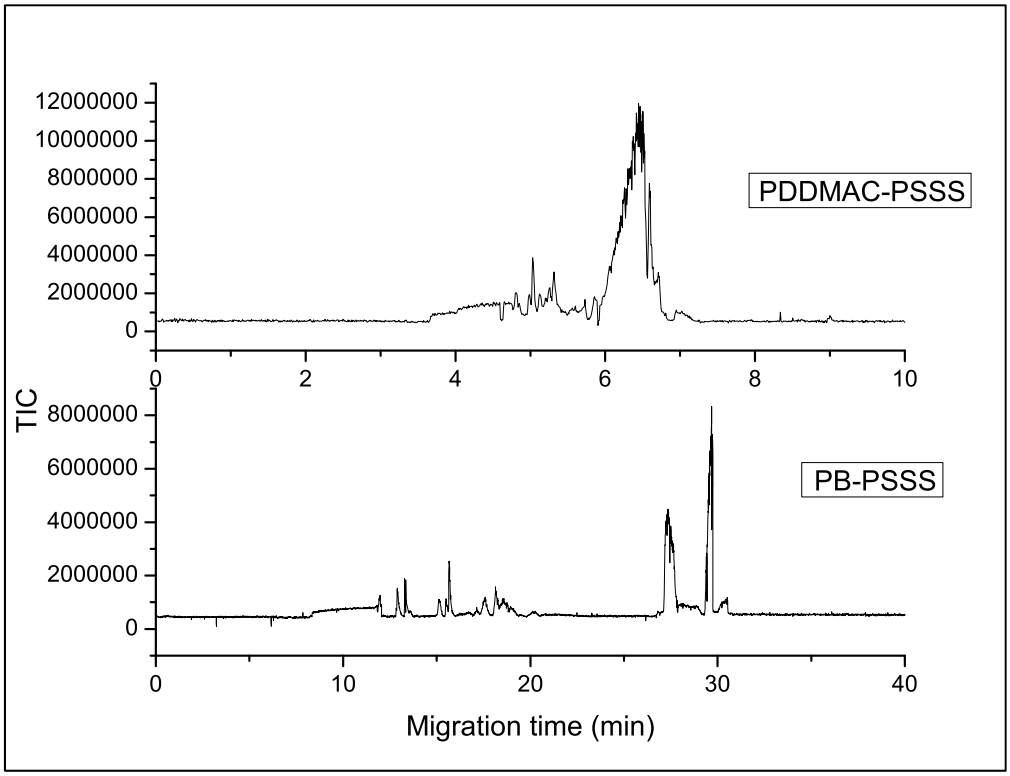

Figure 2.5: Comparison of two different capillary coating methods PDDMAC-PSSS and PB-PSSS. In both instances samples were prepared by obtaining the supernatant of a 1:1 mixture of pooled rat serum and acetonitrile. Prior to injection the capillaries were flushed for 3 min with BGE (1 M formic acid). Samples were injected for 120 s at 50 mbar followed by a 5 s 50 mbar water injection. The metabolites were then separated at 25 kV and 30 mbar in an 80 cm 50 μm i.d. capillary coated with either PDDMAC-PSSS or PB-PSSS.

Sample reproducibility: To test the reproducibility of CE-MS, samples of Tasmanian devil serum metabolites were run over the course of three days along with rat serum metabolites obtained from a pooled rat sample to use as a quality control. The rat quality controls in this sample set showed variation in migration time up to 5 min. Shifts in migration time changes and peak resolution were also observed between the devil samples. The capillary was re-coated with PSSS after approximately every 10 runs. When examining the quality controls, the migration time reproducibility was optimum when comparing runs that were conducted immediately after PSSS re-coating (see Figure 2.6). This was observed when comparing the electropherogram of the QC run immediately after PSSS re-coating and 13 runs after PSSS re-coating (see QC 5 and QC 7 respectively in Figure 2.7). As shown in Figure 2.7 the migration times increased with subsequent runs. This suggested that the capillary wall was being modified by either the metabolites coating the capillary or the PSSS polymer was being removed during runs. The removal of the PSSS polymer would expose the underling PDDMAC polymer and reverse the EOF because of the change in the charge of the capillary wall [108]. The modification in the capillary wall was affecting subsequent runs by changing the EOF or increasing analyte-capillary interaction during the run and thus increasing the migration times of subsequent runs. This result shown in Figure 2.7 suggests that the capillary should have been re-coated with PSSS more regularly to obtain greater reproducibility. In general capillaries can be re-coated in CE as part of the method which allows for continue analysis of subsequent samples when using an autosampler. Automated PSSS re-coating could not be conducted as part of the method in this case because the capillary outlet needed to be unconnected from the MS to prevent the large polymer from contaminating the MS. This method could be improved by coating before each run but this would increase analysis time and would prevent running samples overnight unless a device could be developed which allowed for the outlet to go to waste

during re-coating. It would be beneficial to determine a more robust capillary coating method that did not require frequent recoating.

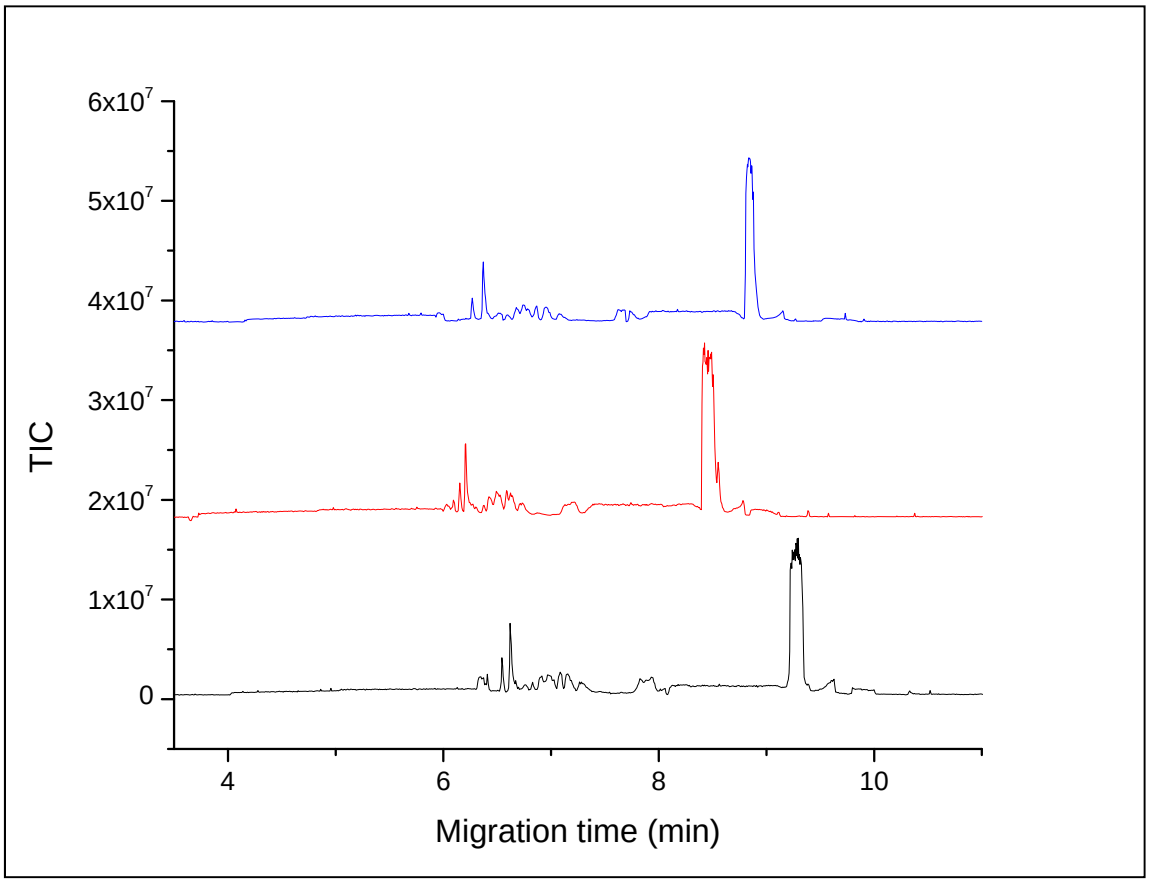

Figure 2.6: Showing the reproducibility of this CE-MS method. Samples were prepared by obtaining the supernatant of a 1:1 mixture of pooled rat serum and acetonitrile. Prior to injection the capillaries were flushed for 5 min with BGE (1 M formic acid). Samples were injected for 120 s at 50 mbar followed by a 5 s 50 mbar water injection. The metabolites were then separated at 20 kV and 30 mbar in an 80 cm 50 µm i.d. capillary coated with PDDMAC-PSSS.

Sample preparation: To determine the optimum conditions for metabolite isolation, two different organic solvents, methanol and acetonitrile (ACN) were investigated with different ratios of serum-to-solvent for protein precipitation. To determine the optimum organic solvent the proteins from a Tasmanian devil serum sample were precipitated by the addition of 1 volume of serum to two volumes of organic solvent (1:2). The electropherograms showed superior peak shapes and separation with the ACN protein precipitation (see Figure 2.8) thus different ratios of ACN to Tasmanian devil serum were

examined (see Figure 2.9). The electropherograms showing the separation of devil serum metabolites prepared with the 1:1 and 1:2 ACN ratios showed the most optimum and similar separations. The 1:1 ratio of ACN to serum was chosen since ACN provided better metabolite separation than the methanol preparation method and this ratio of solvent to sample, introduced less organic solvent during injection which could affect the ionic strength within the injection plug.

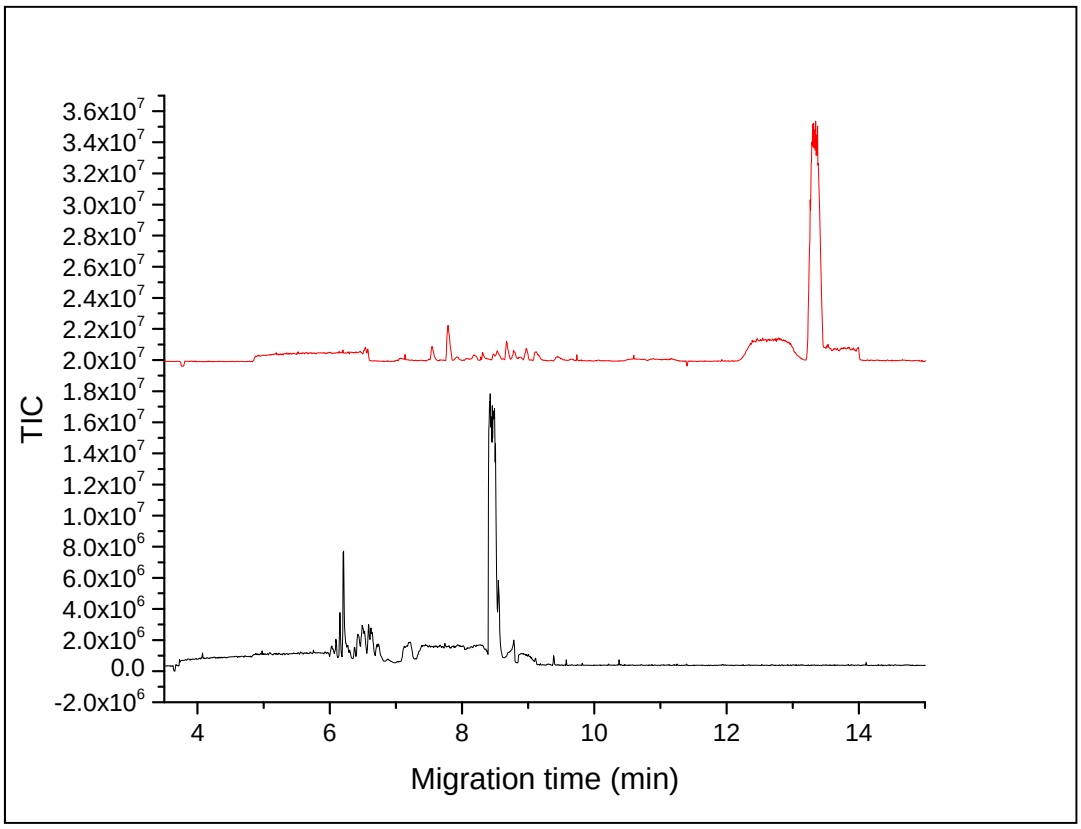

Figure 2.7: Showing the change in migration time that occurs between a run conducted immediately after a PSSS re-coating (bottom electropherogram) and 13 runs after a PSSS re-coating (top electropherogram. Samples were prepared by obtaining the supernatant of a 1:1 mixture of pooled rat serum and acetonitrile. Prior to injection the capillaries were flushed for 5 min with BGE (1 M formic acid). Samples were injected for 120 s at 50 mbar followed by a 5 s 50 mbar water injection. The metabolites were then separated at 20 kV and 30 mbar in an 80cm 50 µm i.d. capillary coated with PDDMAC-PSSS.

Sample injection study: To determine the optimum amount for sample injection, rat serum metabolites were injected into a PDDMAC-PSSS capillary for 75, 90 and 120 s and separated at 20 kV and 3 mbar. A longer injection of 120 s was considered to be optimum

since the electropherogram showed a higher number of separated peaks that were sharper and showed greater sensitivity (see Figure 2.10).

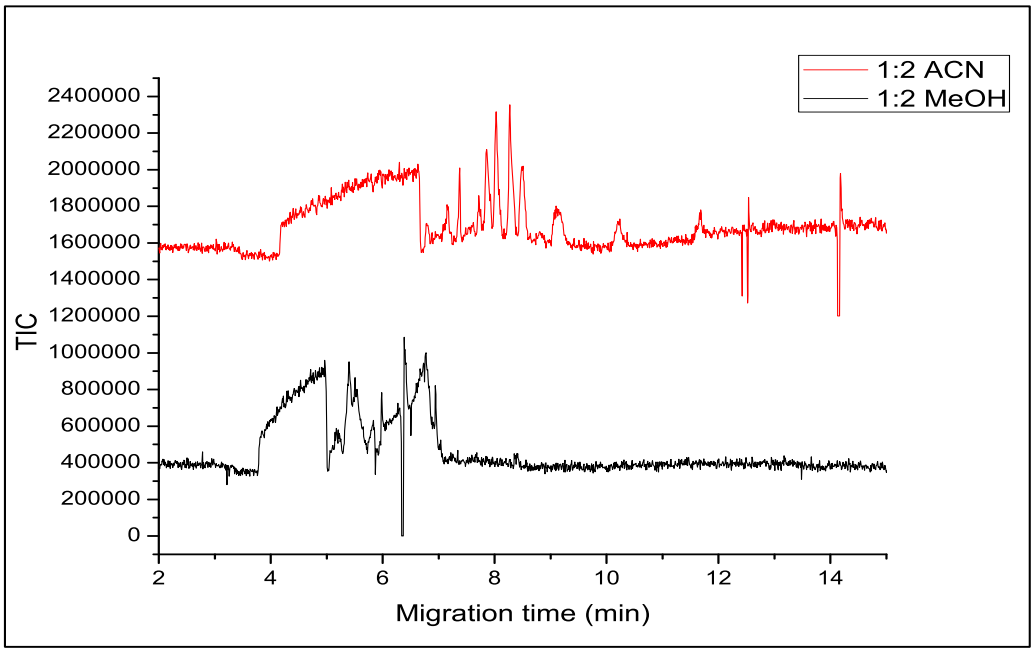

Figure 2.8: Comparison of acetonitrile and methanol protein precipitation sample preparation steps. In both instances samples were prepared by obtaining the supernatant of a 1:2 mixture of Tasmanian devil serum and acetonitrile (top) or methanol (bottom). Prior to injection the capillaries were flushed for 5 min with BGE (1 M formic acid). Samples were injected for 90 s at 50 mbar followed by a 5 s 50 mbar water injection. The metabolites were then separated at 20 kV and 30 mbar in a 73.8 cm 50µm i.d. capillary coated with PDDMAC-PSSS.

Separation voltage: Separation of rat serum metabolites (with a 120 s 50 mbar injection) were investigated with voltages of 15, 20 and 25 kV with a constant separation pressure of 30 mbar (see Figure 2.11). The 15 kV separation was not completed within 30 min which was around three times slower than the 20 kV and 25 kV separations. Although there was considerably greater resolution between peaks when separating at 15 kV there was also substantial peak broadening. On the other hand the 25 kV separation had very low resolution so 20 kV separations were conducted for further work.

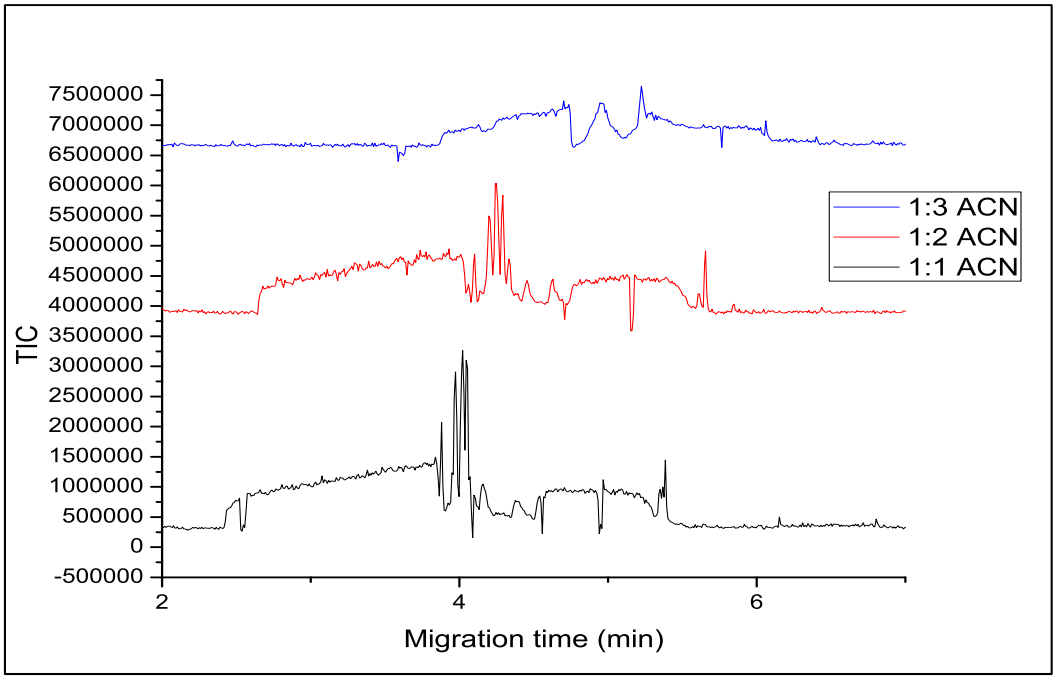

Figure 2.9: Comparisons of different ratios of acetonitrile to serum for protein precipitation. In all instances samples were prepared by obtaining the supernatant of a 1:1, 1:2 or 1:3 mixture of Tasmanian devil serum and acetonitrile. Prior to injection the capillaries were flushed for 5 min with BGE (1 M formic acid). Samples were injected for 120 s at 50 mbar followed by a 5 s 50 mbar water injection. The metabolites were then separated at 25 kV and 30 mbar in an 80 cm 50µm i.d. capillary coated with PDDMAC-PSSS.

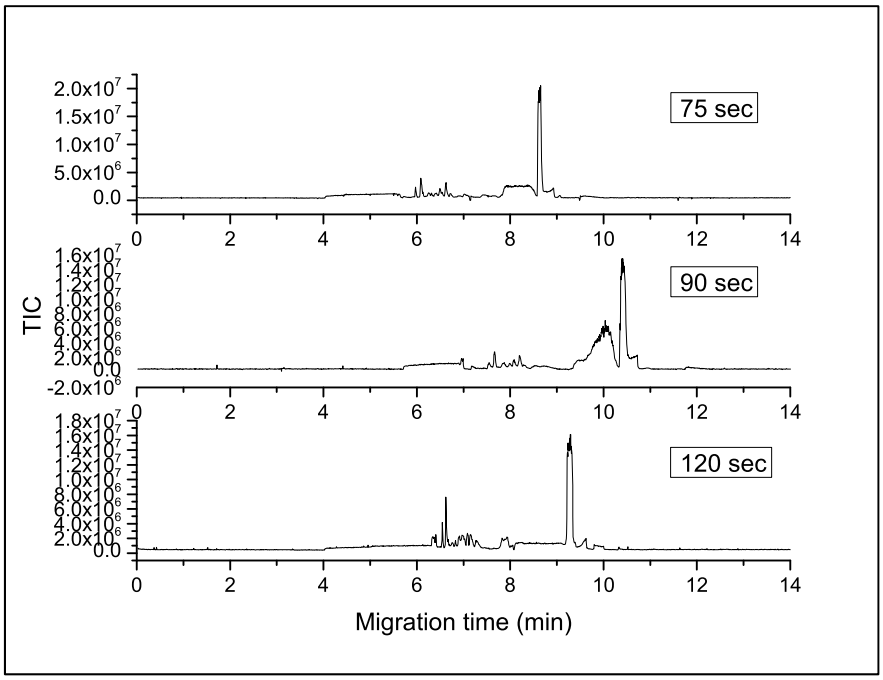

Figure 2.10: Comparison of different injection times. In all instances samples were prepared by obtaining the supernatant of a 1:1 mixture of rat serum and acetonitrile. Prior to injection the capillaries were flushed for 5 min with BGE (1 M formic acid). Samples were injected for 75, 90 or 120 s at 50 mbar followed by a 5 s 50 mbar water injection. The metabolites were then separated at 20 kV and 30 mbar in an 80 cm 50 µm i.d. capillary coated with PDDMAC-PSSS.

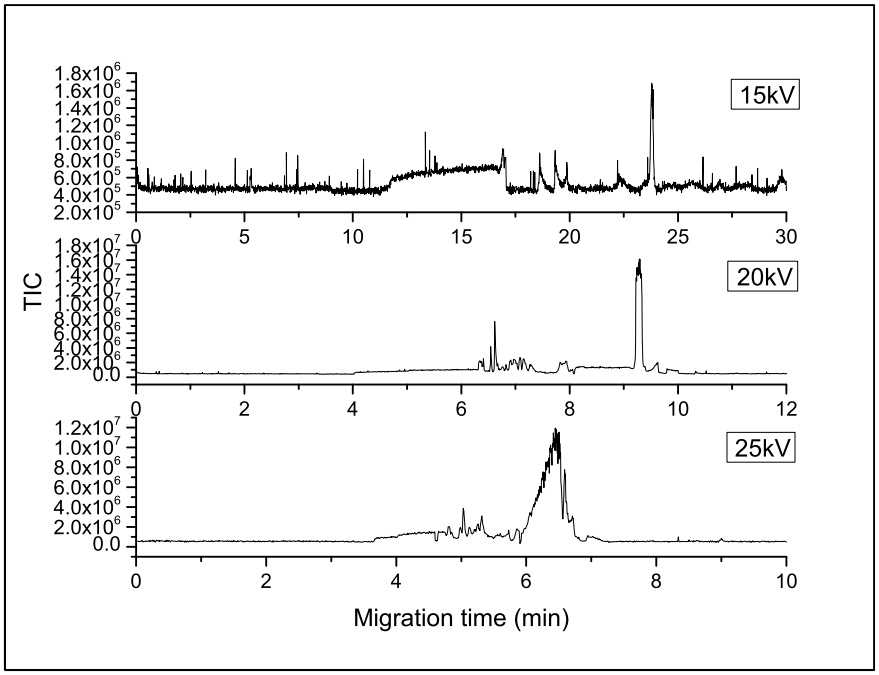

Figure 2.11: Examination of different separation voltages. In all instances samples were prepared by obtaining the supernatant of a 1:1 mixture of rat serum and acetonitrile. Prior to injection the capillaries were flushed for 5 min with BGE (1 M formic acid). Samples were injected for 120 s at 50 mbar followed by a 5 s 50 mbar water injection. The metabolites were then separated at 15, 20 and 25 kV (as stated) and 30 mbar in an 80 cm 50 µm i.d. capillary coated with PDDMAC-PSSS.

2.4 Data Processing

2.4.1 Gas Chromatography

Peak Normalisation and Feature Selection: The serum metabolites were analysed by GC-MS as stated in section 2.2.3. The data for each full chromatogram was tabulated according to retention time and the peak area of the base peak (most intense peak) ion for each metabolite. PCA was selected for the analysis of the data to allow for easy visualisation of the potential variance between DFTD and non-DFTD samples [71]. Initially PCA was conducted using all of the peaks without prior normalisation or feature selection (see Figure 2.12). Although the majority of DFTD and non-DFTD samples could be separated in this PCA, there were three non-DFTD samples (04/3011, 04/3003 and 04/0560) that clustered with the DFTD samples. Two of these non-DFTD devils had been obtained from Fentonbury and the remaining sample, 04/0560 was obtained at Bronte Park

which is approximately 100 km north of Fentonbury. Biases of gender and sampling location could be observed with samples 04/3000, 04/3003, 04/3008 and 04/3011. These four samples were obtained at Fentonbury from four different devils (see microchip numbers in Table A 1). It was observed that the two samples obtained from Fentonbury female devils, samples 04/3000 and 04/3003 clustered close together near the majority of the DFTD samples. The other two samples from Fentonbury, samples 04/3008 and 04/3011, also clustered close together near the majority of the non-DFTD samples. The second principal component showed a general gender bias in this plot which was shown by the majority of the male samples clustering in the bottom half of the plot. This showed that the variability of the samples was not only affected by the presence of DFTD but potentially gender and sampling location as well. Gender, disease and sampling location [8, 10, 20, 21, 109] have all been previously shown to affect the presence and abundance of metabolites in biological samples. Various procedures, such as normalisation were then conducted to observe further variability between DFTD and non-DFTD samples.

To remove any potential bias between samples a normalisation step is recommended when analysing biological samples such as urine, blood or tissue [110, 111]. There were a number of potential normalisation steps that could be investigated which included the addition of an internal standard [30, 112], normalising to a metabolite already present in the samples or by obtaining the ratio of each peak to the total area [32, 110]. Internal standards can also be added to the sample prior to sample injection and used to normalise the response for each metabolite. An internal standard would be required to not interact in any way; be absent in the sample and also elute away from the other components. One possibility is the use of labelled isotopes [30]. Since serum metabolite samples are already quite complex, an internal standard was not considered to be a viable option.

Initially all of the metabolites were normalised to the area of the cholesterol peak, a metabolite observed in all samples that elutes at the end of the chromatogram away from other metabolites. After the metabolites were normalised all the metabolite peaks were analysed using PCA and three clusters of samples were observed. One cluster contained

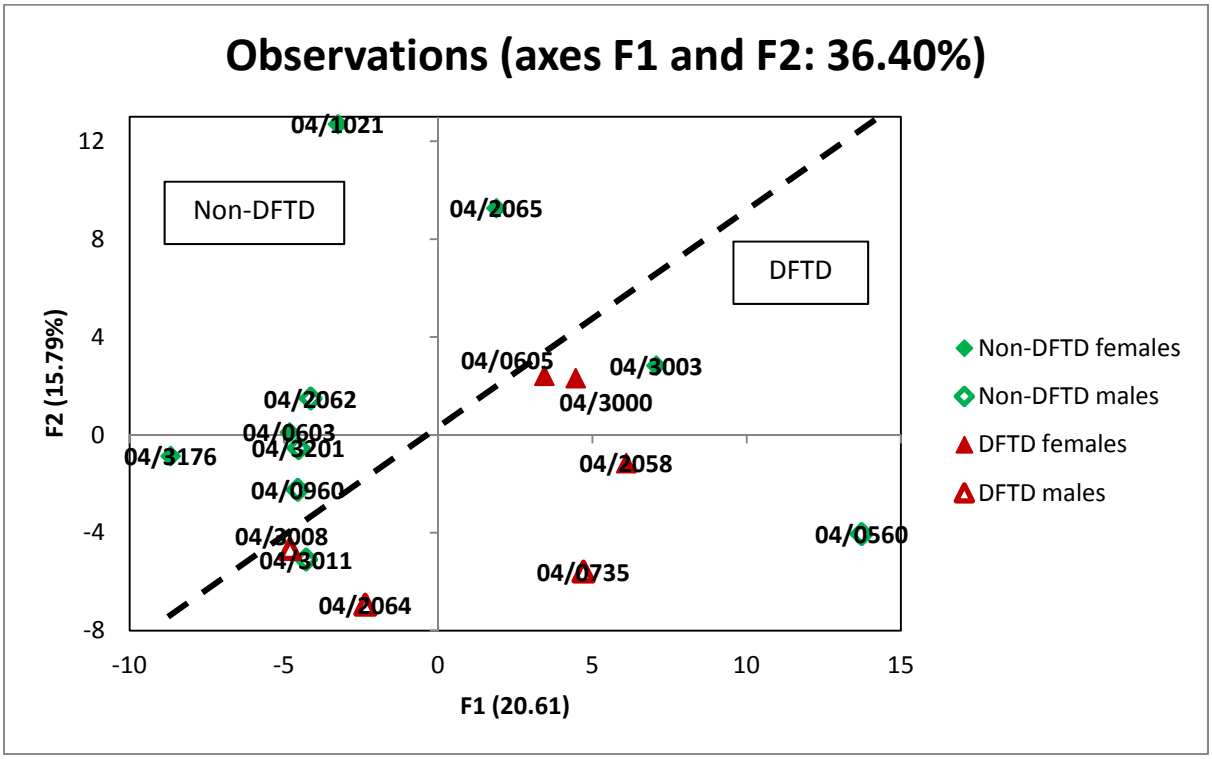

Figure 2.12: PCA of the first and second principal components showing the variability of devil serum metabolites without any peak normalisation or feature selection. The samples came from 16 male and female devils that were trapped in 2004 in known DFTD areas around the eastern half of Tasmania. The samples were analysed by GC-MS. The samples were injected into the GC using a splitless injection for 0.75 min at 230 ˚C into a 15 m BPX-35 column. The temperature of the column was held at 55 ˚C for 2 min and then ramped to 330 ˚C at a rate of 15 ˚C/min. The column was held at 330 ˚C for 6 min. The acquisition of data was initiated 6.5 min into the run.

just healthy samples, another contained a mixture of healthy and DFTD-positive samples and the third cluster contained two samples obtained from two different DFTD-positive male devils. This third cluster was positioned a considerable distance away from the other samples. The chromatograms for the two samples in the third cluster were examined further and it was found that the cholesterol peak was considerably smaller in these samples than in the other samples. The samples in the third cluster also had a decreased

abundance of glucose. Cholesterol levels in blood are increased by dietary intake of animal fat [113]. Animal fat would be digested by Tasmanian devils when they feed on carcases and other meat since they consume the whole carcass including the skeleton [76]. When DFTD becomes advanced, the position of the tumours could make it difficult for the devil to eat. If these samples came from devils with advanced disease this could explain the decrease in serum cholesterol and glucose. Other diseased or injured devils may also find it difficult to obtain food and would thus also show decreased serum cholesterol and glucose levels so this observation is not necessarily a good predictor for DFTD. The separation of samples in the PCA plot in Figure 2.13 did not appear to be separated according to other known parameters such as gender and/or sampling location. These results suggest that normalisation did aid in removing these biases but cholesterol was not an appropriate metabolite to normalise to. A feature selection step though can help to remove variables that are not dependent upon the disease or other criteria that is being explored.

Feature selection is often conducted to decrease the complexity of data that is further analysed with PCA [65, 66]. As the number of samples is increased in the sample set the amount of information also increases and it is best to try and decrease the information prior to multivariate analysis. A feature selection step pre-selects the variables (in this case metabolite peaks) that have the highest chance of showing a difference between different classes of samples such as non-DFTD and DFTD. Two ways to do this is by ANOVA and Student's *t*-test [68, 69]. Since only two groups were being investigated here (non-DFTD and DFTD) and ANOVA is used to analyse 3 or more groups, *t*-test was used in this instance [114]. Using the data normalised to cholesterol, a feature selection was conducted using Student's *t*-test to obtain a list of metabolite peaks that were significantly different between the DFTD and non-DFTD samples to the 95 % confidence level ($p \leq 0.05$). There were 15 peaks that were found to be significantly different and these peaks were further

analysed via PCA (see Figure 2.14). The PCA showed that there was variability between the DFTD and non-DFTD samples. In this analysis all of the non-DFTD samples were separated from the DFTD samples. This is logical since the variables that are not affected by the presence of DFTD are not involved in the analysis which removes variability of the samples that are caused by factors other than the presence of DFTD.

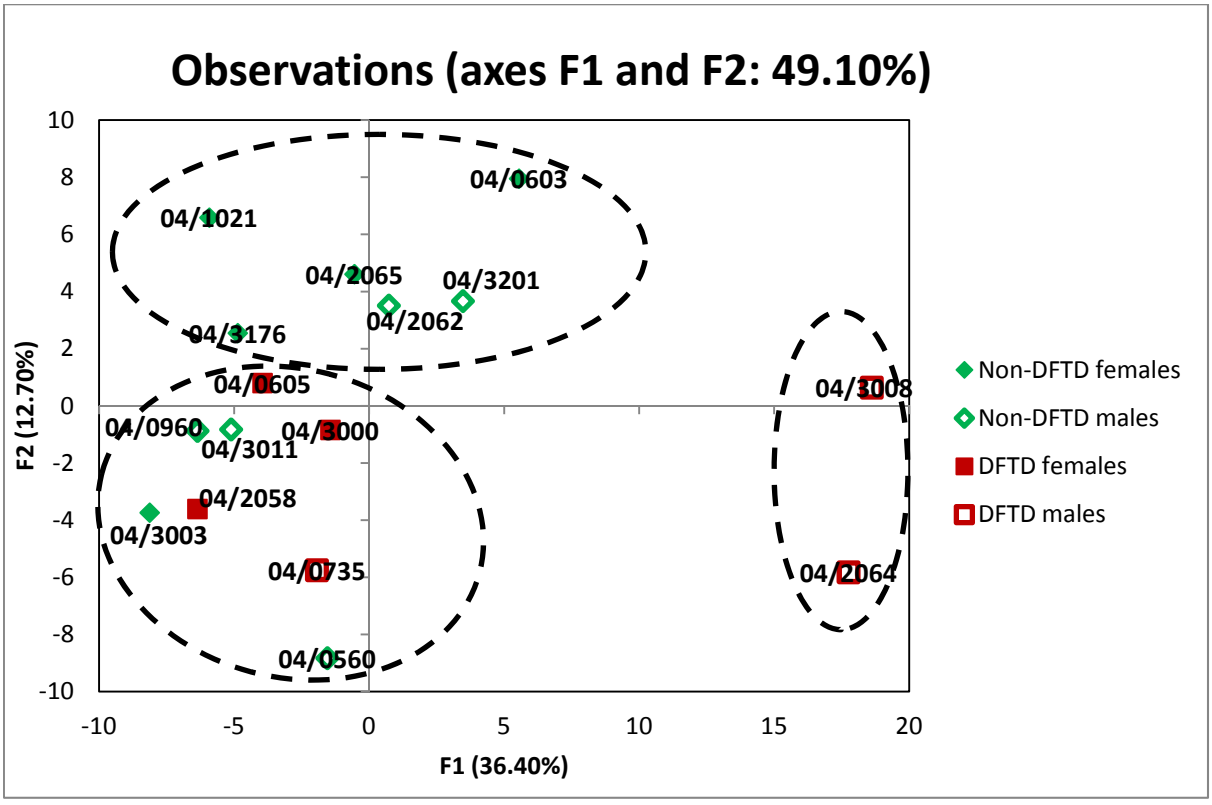

Figure 2.13: Identifying variability between samples normalised to cholesterol: This PCA plot shows the separation from the first and second principal components. The samples came from 16 male and female devils that were trapped in 2004 in known DFTD areas around the eastern half of Tasmania. The samples were run by GC-MS and injected into the GC using a splitless injection for 0.75 min at 230 ˚C into a 30 m BPX-35 column. The temperature of the column was held at 55 ˚C for 2 min and then ramped to 330 ˚C at a rate of 15 ˚C/min. The column was held at 330 ˚C for 6 min. The acquisition of data was initiated 6.5 min into the run. Each metabolite peak was normalised to cholesterol and all the peaks were analysed by PCA without prior feature selection.

Another pre-selection process that can be used prior to PCA analysis is the Fisher test [31] and this process was also investigated for use as a feature selection step. The metabolite peaks determined to have a Fisher value of greater than one were further analysed with PCA where, $F = \frac{[\sigma(all\ samples)]^2}{[\sigma\ (DFTD\ samples)]^2}$. There were 68 metabolites where F >1 and the second

and third principal components showed the best separation between the non-DFTD and DFTD samples (see Figure 2.15). Since the Student's *t*-test selected metabolites that were more apt at showing the variances between DFTD and non-DFTD this method of feature selection was used in future data analysis.

Although the addition of a feature selection step did allow for the separation of non-DFTD and DFTD samples, cholesterol was not viewed as suitable for peak normalisation because diet can affect the concentration of cholesterol levels in the blood and this may affect the prediction of other DFTD and non-DFTD samples that are analysed since DFTD is not the only reason that a devil may have a decrease of food intake. Further normalisation procedures were therefore investigated.

An alternate process used to normalise the data was to divide the response of each peak by the total sum of all peak areas [32, 110, 115]. Before calculating the sum of all peaks, peaks that were not observed in 80 % of the samples were removed. Previous work with normalising to cholesterol had shown improvement when a feature selection step was added to the analysing process so future analysis was conducted using a feature selection step. This method of analysis was able to show variability between non-DFTD and DFTD samples (see Figure 2.16). Two of the non-DFTD samples (04/0560 and 04/3003) were still positioned on the same side as the other non-DFTD samples but they clustered lower in the plot. These two samples also clustered with the DFTD samples in Figure 2.12. The other non-DFTD sample that had plotted with the DFTD samples in Figure 2.12 actually plotted with the non-DFTD samples well away from the DFTD samples. Overall though this plot generally separated the DFTD and non-DFTD samples.

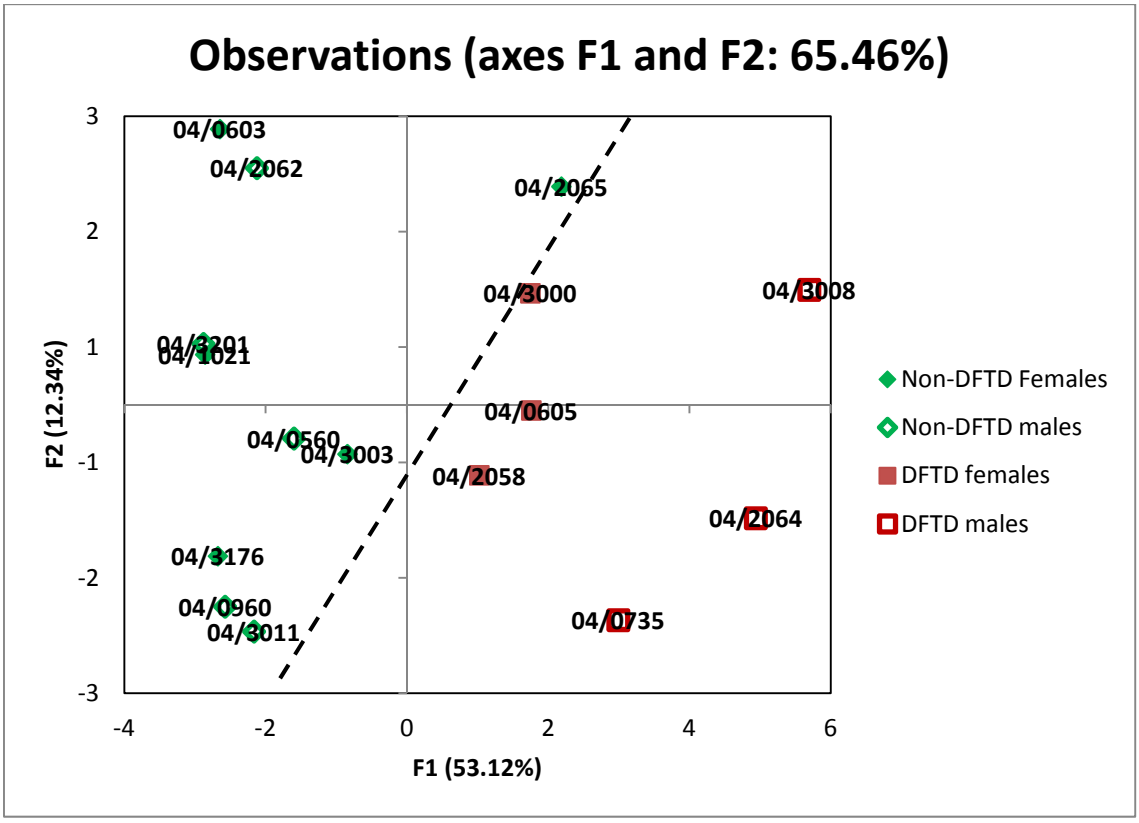

Figure 2.14: PCA plot of the first and second principal components of metabolites that were normalised to cholesterol and reduced via a feature selection step using Student's *t*-test. The samples came from 16 male and female devils that were trapped in 2004 in known DFTD areas around the eastern half of Tasmania. The samples were run by GC-MS. The samples were injected into the GC using a splitless injection for 0.75 min at 230 ˚C. The temperature of the column was held at 55 ˚C for 2 min and then ramped to 330 ˚C at a rate of 15 ˚C/min. The column was held at 330 ˚C for 6 min. The acquisition of data was initiated 6.5 min into the run. Each metabolite peak was normalised to cholesterol and a feature selection was conducted using Student's *t*-test. The 15 peaks with $p \leq 0.05$ were analysed by PCA.

To confirm that a feature selection step was still required when the data was normalised to total area, a PCA using all of the identified metabolite peaks was conducted. The variables with the greatest loadings were not identical to those identified using Student's *t*-test although many of the *t*-test peaks still had significant loadings in the loadings plot (see **Error! Reference source not found.** for the list of significant peaks determined using Student's *t*-test and Figure 2.18 for the loadings plot). The first four principal components were examined but none were suitable at clustering the non-DFTD samples away from the DFTD samples (see Figure 2.17) which showed that a feature selection step is required to efficiently show differences between DFTD and non-DFTD samples using PCA.

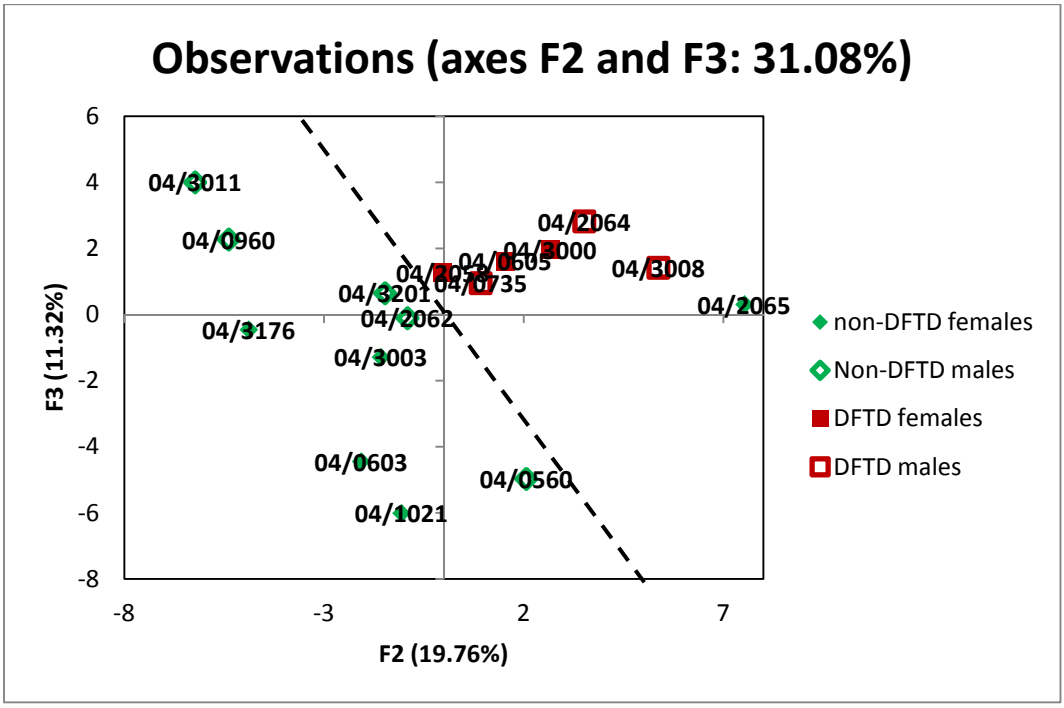

Figure 2.15: PCA plot of the second and third principal components of metabolites that were normalised to cholesterol and reduced via a feature selection step using the Fisher test. The samples came from 16 male and female devils that were trapped in 2004 in known DFTD areas around the eastern half of Tasmania. The samples were run by GC-MS. The samples were injected into the GC using a splitless injection for 0.75 min at 230 ˚C. The temperature of the column was held at 55 ˚C for 2 min and then ramped to 330 ˚C at a rate of 15 ˚C/min. The column was held at 330 ˚C for 6 min. The acquisition of data was initiated 6.5 min into the run. Each metabolite peak was normalised to cholesterol and a feature selection was conducted using the Fisher test. The 68 peaks with F > 1 were analysed by PCA.

Although some good results were obtained when normalising to cholesterol, analysis of all future samples was normalised to total area to eliminate possible differences caused by food intake. When the data was analysed after normalising to total area of all peaks, and conducting a feature selection using Student's *t*-test, the samples were efficiently separated between DFTD and non-DFTD samples using PCA analysis. Rather than continued exploration of the numerous types of chemometric analysis available, further analysis of data was therefore conducted using Student's *t*-test for feature selection following by PCA.

Table 2.3: The list of peaks identified to be significantly different between DFTD and non-DFTD samples analysed by GC-MS and normalised to total peak area according to Student's *t*-test.

Peak #	Retention time	m/z	p-value
9	7.44	142	0.0117
10	7.48	134	0.0273
14	7.72	131	0.0143
16	7.78	168	0.0440
26	8.27	209	0.0204
79	11.55	155	0.0043
87	12.15	156	0.0043
88	12.38	147	0.0475
90	12.73	217	0.0003
102	13.46	147	0.0428
105	13.68	156	0.0215
107	13.93	147	0.0114
110	14.44	147	0.0434
120	15.7	147	0.0229
127	16.14	149	0.0474
133	16.65	117	0.0020

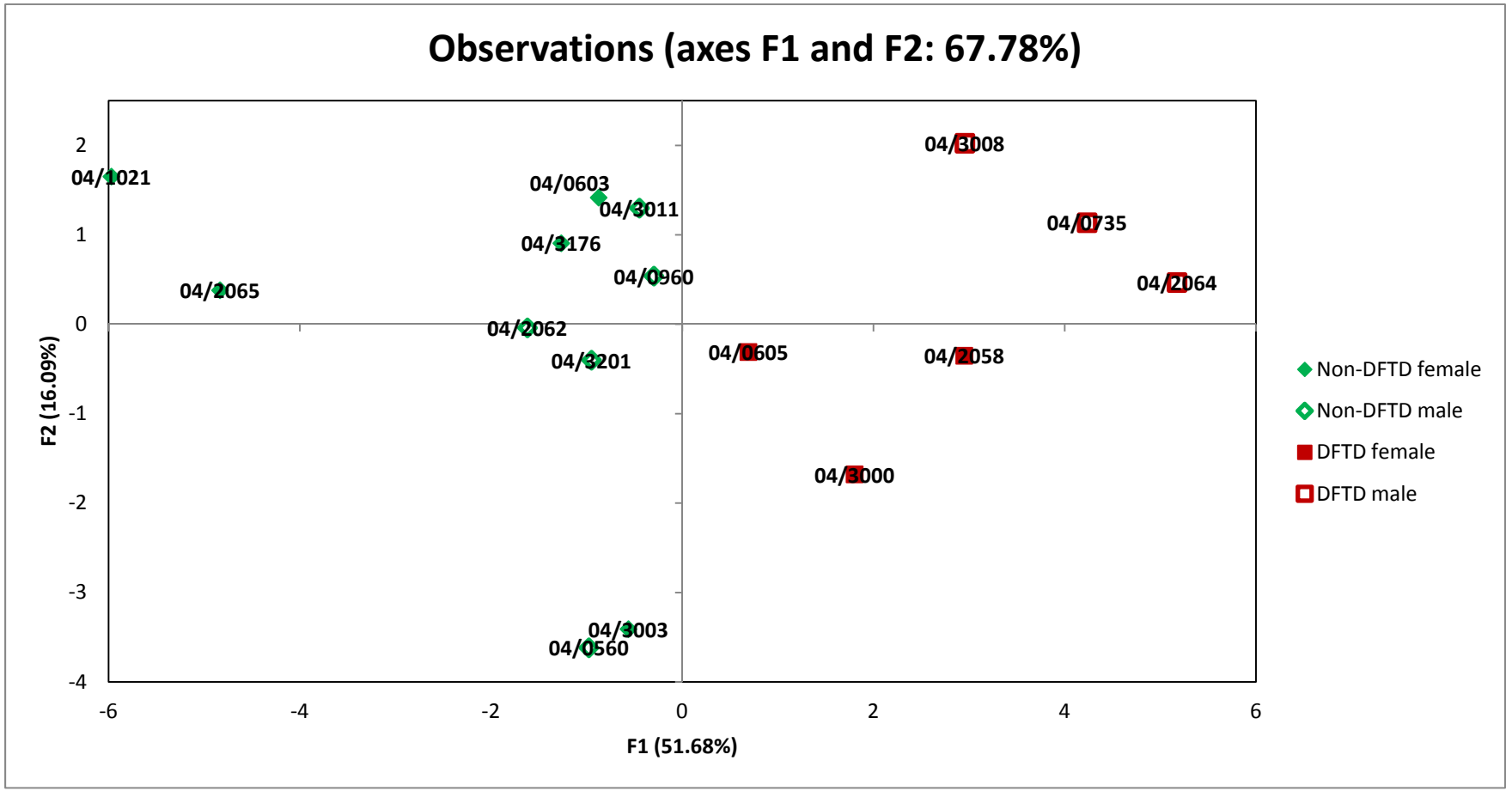

Figure 2.16: PCA plot of the first and second principal components after normalisation to the sum of the total area. The samples came from 16 male and female devils that were trapped in 2004 in known DFTD areas around the eastern half of Tasmania. The samples were run by GC-MS. The samples were injected into the GC using a splitless injection for 0.75 min at 230 ˚C. The temperature of the column was held at 55 ˚C for 2 min and then ramped to 330 ˚C at a rate of 15 ˚C/min. The column was held at 330 ˚C for 6 min. The acquisition of data was initiated 6.5 min into the run. Each metabolite peak was normalised to the sum of the total area of each individual sample and a feature selection was conducted using Student's *t*-test. The 16 peaks with $p \leq 0.05$ were analysed by PCA.

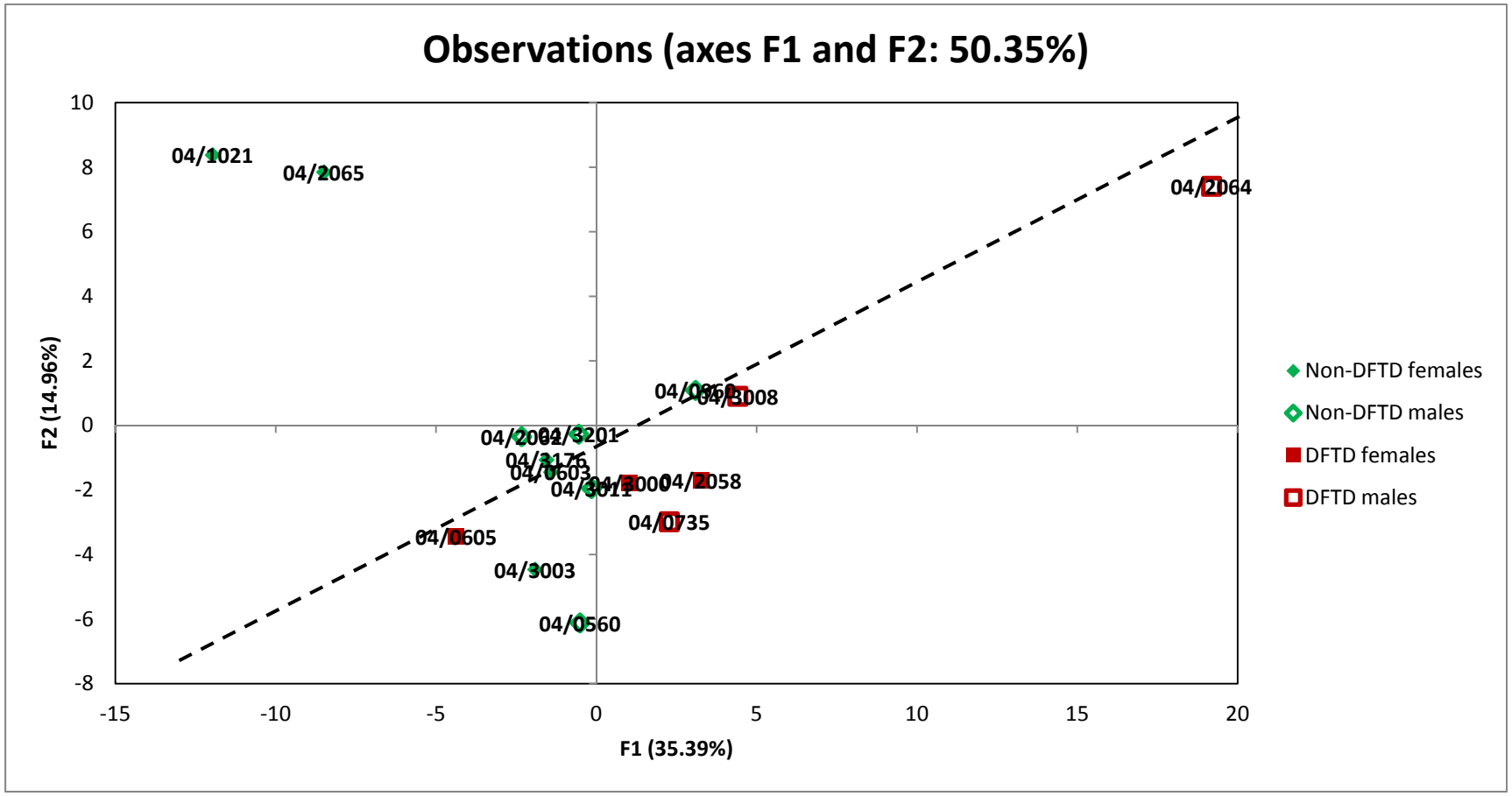

Figure 2.17: PCA of the first and second principal components showing the variability of devil serum metabolites normalisation to total peak area with no feature selection. The samples came from 16 male and female devils that were trapped in 2004 in known DFTD areas around the eastern half of Tasmania. The samples were analysed by GC-MS. The samples were injected into the GC using a splitless injection for 0.75 min at 230 ˚C into a 15 m BPX-35 column. The temperature of the column was held at 55 ˚C for 2 min and then ramped to 330 ˚C at a rate of 15 ˚C/min. The column was held at 330 ˚C for 6 min. The acquisition of data was initiated 6.5 min into the run.

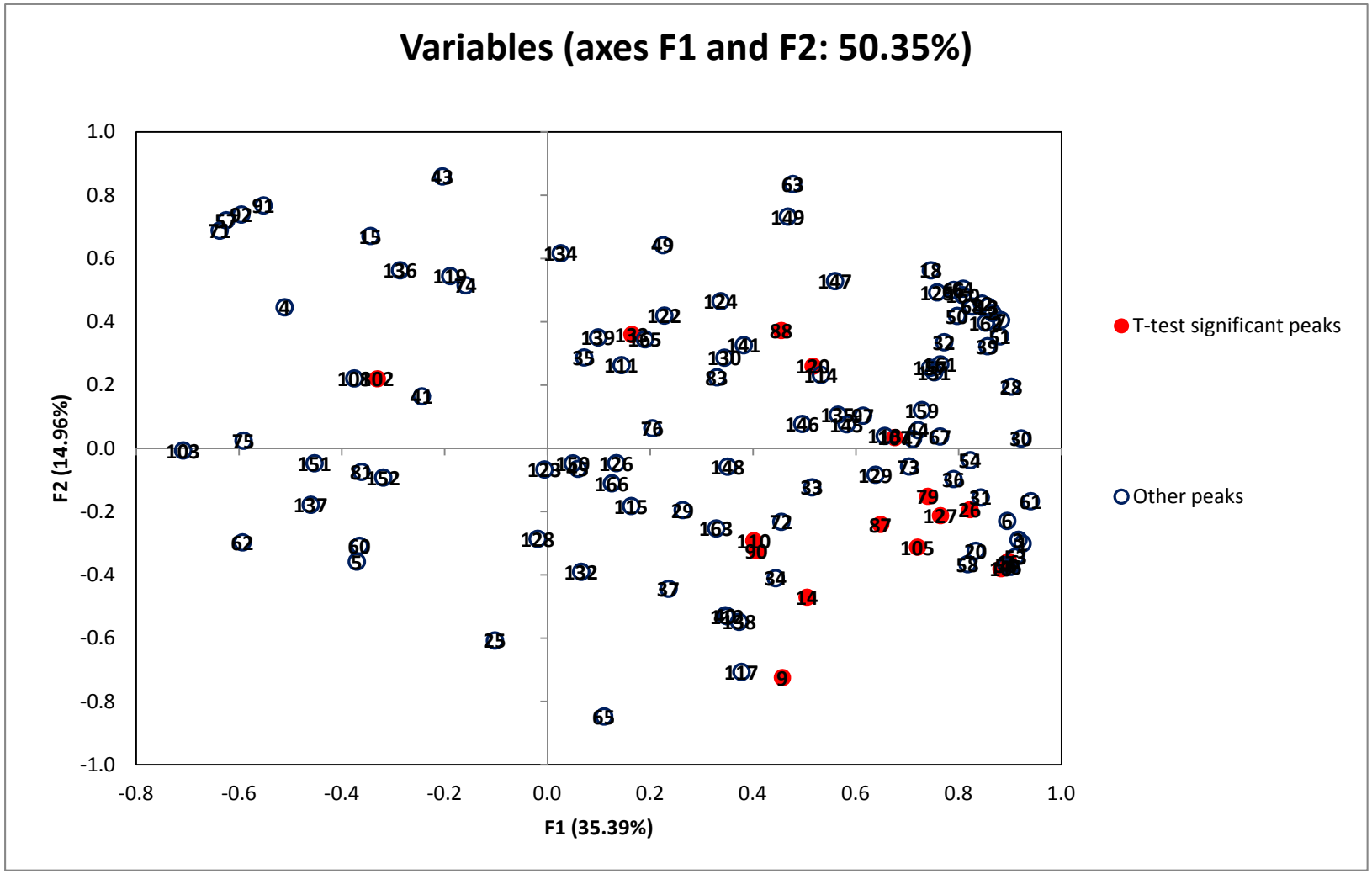

Figure 2.18: The plot of variables (metabolites) analysed for the PCA after normalisation to total area but without prior feature selection.

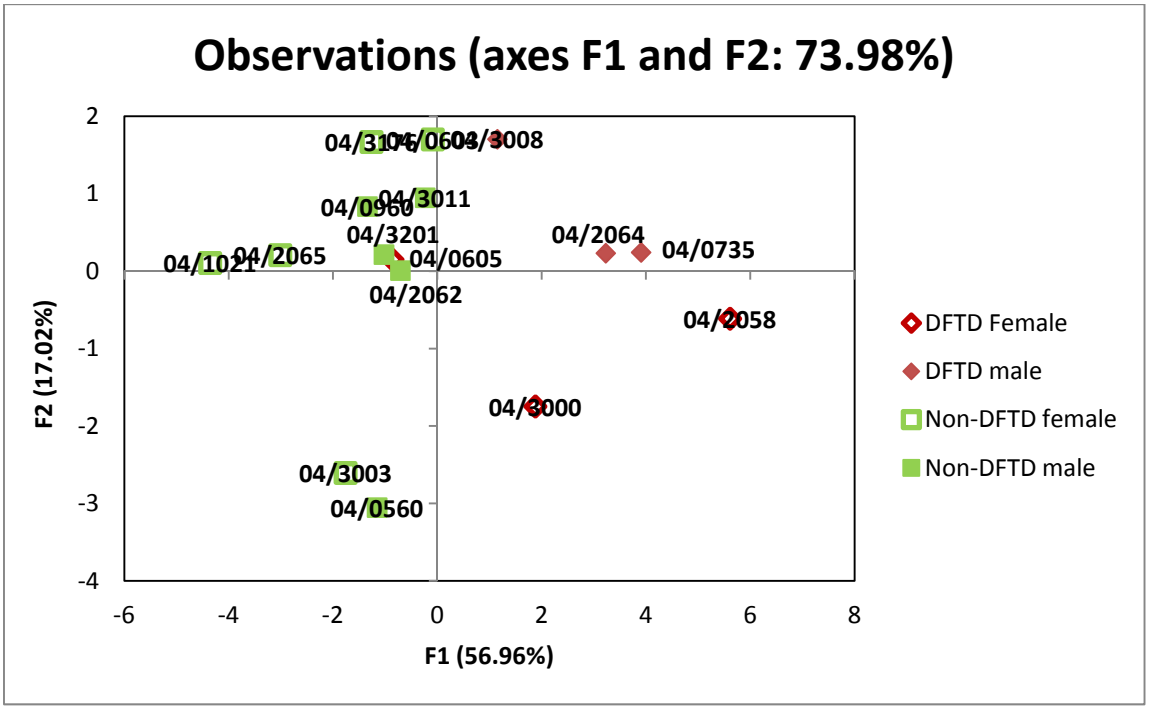

Figure 2.19: PCA analysis showing the variability of samples using the automated integration method. This PCA plot shows the variability determined using the first and second principal components. The samples came from 16 male and female devils that were trapped in 2004 in known DFTD areas around the eastern half of Tasmania. The samples were run by GC-MS. The samples were injected into the GC using a splitless injection for 0.75 min at 230 ˚C. The temperature of the column was held at 55 ˚C for 2 min and then ramped to 330 ˚C at a rate of 15 ˚C/min. The column was held at 330 ˚C for 6 min. The acquisition of data was initiated 6.5 min into the run. Each metabolite peak was automatically integrated as discussed above, normalised to the sum of the total area of each individual sample and a feature selection was conducted using Student's *t*-test. The 11 peaks with $p \leq 0.05$ were analysed by PCA.

Peak Integration: During the pilot study of this research the peaks present in the gas chromatogram were integrated by both manual and automatic integration methods as discussed in section 2.2.3. Both of these processes involved determining the area of the most abundant mass peak for each peak in the chromatogram. Automatic integration was conducted using the same chromatograms used in Figure 2.16. The metabolite peaks were then analysed the same way by normalising each peak to the total peak area for each sample; conducting a feature selection using Student's *t*-test to determine the peaks to the 95 % confidence level and analysing by PCA (see Figure 2.19). Although the PCA plot that was obtained when the metabolite peaks were integrated manually (see Figure 2.16) was more able to separate all of the DFTD samples from the non-DFTD samples the

automatic integration process (Figure 2.19) greatly reduced the amount of time required to process the data and was therefore preferential. The PCA for the automatic integrated peaks included more of the sample variability in the first two components than the manual peak integration method which is desirable. Only sample 04/0605 in Figure 2.19 was not clustered with the correct samples. This sample in Figure 2.16, which used data that was manually integrated, clustered close to the DFTD samples. The analysis of future sample sets used the automatic integration process because of the dramatic reduction in analysis time. Using this method still provided good results for samples obtained from devils with DFTD were efficiently separated from the samples obtained from non-DFTD devils.

2.4.2 ESI-MS

No normalisation was required for the ESI-MS data since the data exported from the DataAnalysis (Bruker Daltonics) software was already normalised. In a similar way to the GC-MS data, a feature selection step was employed as a data reduction step using Student's *t*-test. The variables (metabolite peaks) that were significantly different ($p \leq 0.1$ or $p \leq 0.05$) were analysed further with PCA. This method of analysis as with GC-MS was found to successfully show variability between the DFTD and non-DFTD samples analysed by ESI-MS (shown in chapters 3, 4 and 5).

2.5 Comparison of the different techniques

The three methods discussed here (CE-MS, GC-MS and ESI-MS) required different sample preparation steps; used different types of MS analysers and involved different separation mechanisms. The techniques thus required varying analysis times and provided different degrees of analyte sensitivity.

The CE-MS method could identify 36 distinct mass responses in the separation that were observed in the majority of devil samples compared to 107 and 142 peaks observed in 80

% of samples with the GC-MS and ESI-MS method respectively (Figure 2.20 shows the separation of serum metabolites from a DFTD and a non-DFTD Tasmanian devil using the CE-MS method). Capillary electrophoresis is known for the analysis of small sample volumes [15] which can reduce analyte sensitivity. The CE-MS method used an injection volume of just under 130 nL and taking into consideration the dilution factor of the samples prior to sample injection this injection was equivalent to 65 nL of serum. This was close to the equivalent amounts of serum that was analysed from the GC-MS and ESI-MS methods investigated which was 60 nL (from a 1 µL injection) with the GC-MS method and 45 nL with the ESI-MS method (taking an average spectrum between 1 to 2 min into

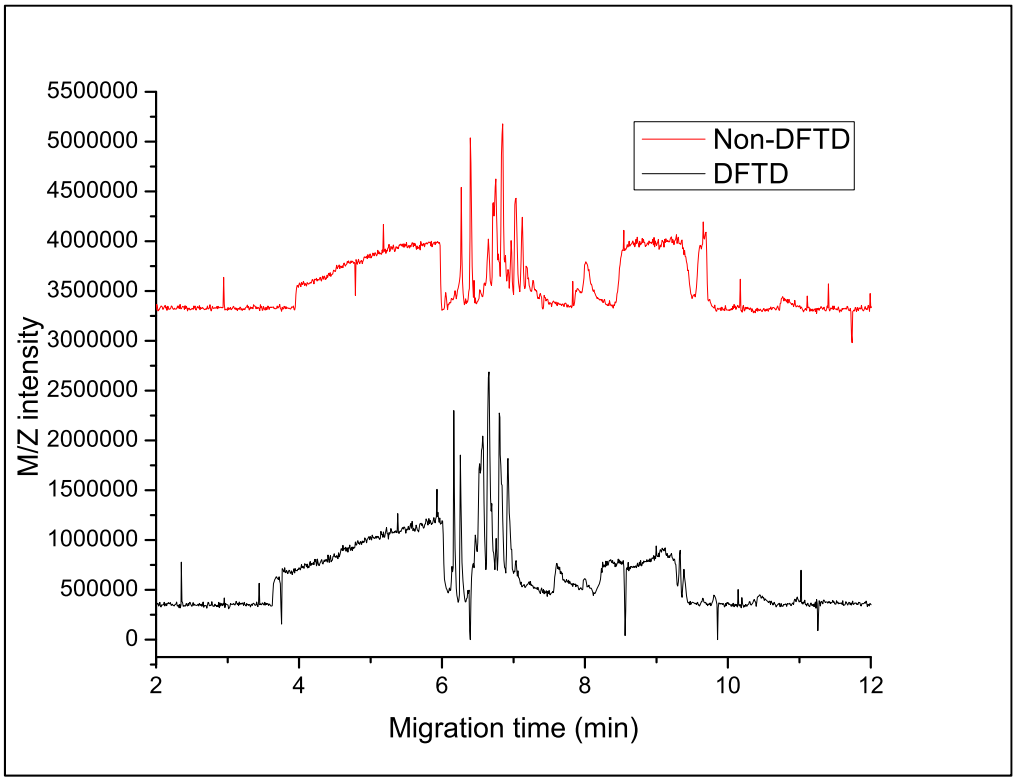

Figure 2.20: Typical electropherograms showing the separation of serum metabolites from a non-DFTD (top) and DFTD (below) Tasmanian devil. In all instances samples were prepared by obtaining the supernatant of a 1:1 mixture of non-DFTD or DFTD devil serum (as stated) and acetonitrile. Prior to injection the capillaries were flushed for 5 min with BGE (1 M formic acid). Samples were injected for 120 s at 50 mbar followed by a 5 s 50 mbar water injection. The metabolites were then separated at 20 kV and 30 mbar in an 80 cm 50µm i.d. capillary coated with PDDMAC-PSSS.

the injection which was equivalent to 3 µL of sample). This suggests that the low sample volume was not the cause for the decrease in the number of metabolites observed with CE-MS. The sensitivity of CE-MS is also reduced because of the need to dilute the sample further with the addition of sheath fluid [116]. Unlike the ESI-MS method, CE-MS requires additional sheath fluid because normal capillary flow does not provide efficient solute for electrospray ionisation [25, 39]. Extra fluid is not required with GC-MS since the analytes are all ready present in the gas phase and were ionised by an electron impact mass analyser.

Each of these methods used a different type of mass spectrometer. Whereas an iontrap analyser was used with the CE-MS approach, a QTOF was utilised in the ESI-MS approach and a quadrupole was employed after electron impact with the GC-MS method which all have different amounts of mass resolution which can affect the number of metabolites observed [26]. Different mass analysers also provide different degrees of mass accuracy which is important for accurate determination of the elemental composition of the analyte and metabolite identification [117]. Iontraps and quadrupoles both do not provide the resolution that can be observed with other mass analysers [26, 42]. The most accurate MS analyser used in this work was the QTOF using for the ESI-MS method [42].

The sensitivity of MS detection when electrospray ionisation is utilised can also be reduced by the presence of salts in the sample. The first broad peak observed in the CE-MS electropherogram (generally between 4-6 min in both the rat and devil samples) showed a pronounced peak consisting of sodium formate clusters. The large injection time required to show good analyte sensitivity had the fall back of also introducing a lot of sodium chloride into the capillary which can then react with the formic acid to form the sodium formate clusters. The presence of this cluster would contribute to ion suppression

which could be hiding the presence of metabolites that might co-elute with the sodium formate clusters.

Small sample injections in CE can lead to poor analyte sensitivity so stacking techniques are commonly used in CE to improve detection ability [116]. Shihabi used acetonitrile precipitation to remove the proteins from plasma and as a stacking technique to examine peptides and drugs. The acetonitrile also allowed for increased sample injections up to 50 % of the total capillary volume where as conventional CE uses injection volumes equal to 0.5-2 % of the total capillary volume. Shihabi's method though was only suitable to quantify drugs present in the body with concentrations in the range of mg/L [116]. This low sensitivity was also shown in the work discussed in this chapter (see section 2.3.3) where it appears that only the abundant metabolites were observed in the electropherogram when an injection equal to ~10 % of the capillary volume was used. Another process for sample stacking is to prepare the sample in buffer at a concentration 10 x less than the BGE. During this procedure a longer sample injection is used and the analytes move faster in the area of low ionic strength and then slow down as they enter regions of the full strength BGE. Injecting the sample electrokinetically can also improve analyte sensitivity by reducing the amount of solvent that is injected into the capillary [116]. This process of stacking could have been used by evaporating the solvent in the metabolite sample and resuspending the metabolites in dilute BGE.

When comparing the two methods that involved the separation of metabolites prior to MS detection the GC-MS method was superior to the CE-MS method. The CE-MS method, given time, could have been improved via the investigation of different types of stacking; using different types of MS analysers; or testing other capillary coatings. The GC-MS method without extensive method development provided better separation efficiency than

CE-MS and improved sensitivity. This could be explained by the fact that the analytes were all ready in the gas phase and did not need to be diluted with a sheath liquid to be analysed by the MS. The GC-MS and ESI-MS methods also provided consistently reproducible chromatograms / spectrums. The GC-ME and ESI-MS method were therefore used in further work as the CE-MS method required further method development to obtain appropriate sensitivity and reproducibility.

2.6 **Summary**

The pilot sample set examined with GC-MS showed variability between the DFTD and non-DFTD samples when the correct normalisation procedure was implemented. In the development of the appropriate data analysis procedures though variability had been observed to be correlated with gender and sampling location (see section 2.4.1 and Figure 2.12). The purpose of this study was to discover differences in the serum metabolome of DFTD devils prior to the manifestation of visible tumours. To aid in this discovery the sample variability was reduced to include samples obtained from male devils caught in the one location over the course of one year. These samples were analysed using both GC-MS and ESI-MS.

3 Comparison of ESI-MS and GC-MS Methods to Isolate Pre-Clinical Devil Facial Tumour Disease Biomarkers

3.1 Introduction

The work discussed in chapter 2 suggested that GC-MS and ESI-MS may be appropriate analytical techniques to study serum metabolites. This was shown when considering sample throughput and the sensitivity and selectivity of identifying non-DFTD and DFTD Tasmanian devils. In this chapter, these two mass spectrometry techniques are compared using non-targeted metabolite approaches to determine differences in the serum metabolome between non-DFTD and DFTD devils. The first method, GC-MS provided prior separation of metabolites before MS detection and the second method used MS detection without prior separation of metabolites. Low volumes of serum were obtained from DFTD and non-DFTD male wild devils trapped in eastern Tasmanian during 2004. This sample set was chosen to remove biases of sample storage times, gender, and genetics since devils from western Tasmania (which are yet to develop DFTD) are genetically different from eastern devils [4]. This chapter discusses the potential of identifying variability between DFTD and non-DFTD samples that is capable of diagnosing DFTD prior to clinical signs of the disease.

3.2 Methods

3.2.1 Samples

Tasmanian devil serum collected in 2004 was obtained in 2007 from the Tasmanian Department of Primary Industries, Parks, Water and the Environment (DPIPWE), Mount Pleasant, Tasmania; and stored at -80 ˚C (see Table 3.1).

Table 3.1: List of samples obtained from male devils at Bronte Park with the addition of the male samples part of the pilot study (see chapter 2). Further details on these samples can be observed in Table A 1 and Table A 2 in the appendix.

Accession number	Graph sample ID	DFTD status	Sampling Location
04/0276	1	Non-DFTD	Bronte Park
04/0284	2	DFTD	Bronte Park
04/0446	3	DFTD	Bronte Park
04/0448	4	Pre-clinical	Bronte Park
04/0451	5	DFTD	Bronte Park
04/0560	6	Pre-clinical	Bronte Park
04/0602	7	DFTD	Bronte Park
04/0720	8	DFTD	Bronte Park
04/0722	9	Non-DFTD	Bronte Park
04/0725	10	DFTD	Bronte Park
04/0728	11	Non-DFTD	Bronte Park
04/0731	12	Non-DFTD	Bronte Park
04/0735	13	DFTD	Bronte Park
04/1167	14	Non-DFTD	Bronte Park
04/1769	15	DFTD	Bronte Park
04/1771	16	Non-DFTD	Bronte Park
04/1849	17	Non-DFTD	Bronte Park
04/1852	18	Non-DFTD	Bronte Park
04/1853	19	DFTD	Bronte Park
Additional Samples from the Pilot Set			
04/2062	20	Non-DFTD	Mount William
04/2064	21	DFTD	Mount William
04/3008	22	DFTD	Fentonbury
04/3011	23	Non-DFTD	Fentonbury
04/3201	24	Non-DFTD	St Helens
04/0960	25	Non-DFTD	National Park

These 25 samples had been collected from wild male Tasmanian devils in the eastern half of Tasmania, Australia. DFTD status, sampling location and other information on the sample can be seen in Table 3.1. Pooled mouse serum was used as a quality control.

3.2.2 Reagents

Methanol (> 99.7 %, isocratic HPLC grade (254nm), was obtained from Scharlau Chemie S.A., European Union); Milli-Q water (Millipore);≥ 98 % o-methylhyrodxylamine hydrochloride and 98 % Formic acid were obtained from Fluka; N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and pyridine (≥ 99.0 %) were obtained from Sigma Aldrich.

3.2.3 Sample Preparation

Proteins were removed from the serum by precipitation with methanol by the addition of 15 µL of serum to 500 µL of cold methanol as stated in section 2.2.3. The supernatant was analysed using two complementary mass spectrometric techniques, gas chromatography-mass spectrometry (GC-MS) and electrospray ionisation-mass spectrometry (ESI-MS).

Gas chromatography - mass spectrometry: Samples were prepared by evaporating 200 µL of the supernatant to complete dryness under vacuum and derivatising the metabolites with methylhydroxylamine hydrochloride and MSTFA as described in section 2.2.3.

Electrospray ionisation – mass spectrometry: A 100 µL aliquot of supernatant for each sample and mouse quality control was placed into a tube with 2 µL of a 10 % v/v formic acid (in milli-Q water) and 98 µL of methanol to give a 0.1 % acidified solution. Reagent blanks were prepared by adding 2 µL of a 10 % v/v formic acid (in milli-Q water) to 198 µL of methanol.

3.2.4 Instrumentation

Gas chromatography - mass spectrometry: The samples were analysed using a Shimadzu QP2010-plus GC-MS fitted with a 15 m BPX-35 capillary column as described for the 15 m column in section 2.2.3.

Electrospray ionisation – mass spectrometry: These experiments were conducted by electrospray ionisation quadropole – time of flight- mass spectrometer (ESI-QTOF-MS) with a Bruker micrOTOF-Q II instrument. The prepared sample was infused into the instrument into the same way as stated in section 2.2.4 and analysed using the same parameters. Prior to every 10 samples a reagent blank and quality control sample was run. All analyses were performed on the same day. The average spectrum obtained between 1.0-2.0 min was then used for further analysis and collected using the same software according to section 2.2.4.

3.2.5 Data Analysis

Gas chromatography – mass spectrometry: Data interrogation initially involved obtaining the total area of the base peak for each chromatographic signal. The response for each peak was normalised to the total response of all the peaks. Automated data reduction was performed across the entire data set to locate sample class-differentiating metabolites by using two-tailed *t*-tests to determine differences between the DFTD and non-DFTD conditions. A p-value less than 0.05 or 0.10 (as stated) was considered statistically significant. The significant peaks were then analysed with PCA using XLSTAT (version 2010.2.01 Copyright Addinsoft 1995-2009).

Electrospray ionisation – mass spectrometry: Data interrogation was performed by chemometric analysis of the extracted spectra (50-1200 m/z). First a feature selection was performed using a series of Student's *t*-tests to extract ion responses that were significantly different ($p < 0.1$ or $p < 0.05$) between the DFTD and non-DFTD samples. The

significantly different ion responses were then analysed further using PCA (XLSTAT Version 2010.2.01 Copyright Addinsoft 1995-2009).

3.3 Results and Discussion

From the 19 samples obtained at Bronte Park, 9 samples were obtained from animals that exhibited visible DFTD tumours, 10 were apparently disease-free although 2 of these animals were later found to have showed signs of DFTD in a subsequent trapping trip. These two samples came from devils that were confirmed to have DFTD when they were captured 35 and 69 days afterwards (samples 4 and 6 respectively). The only variables that were controlled were sample storage time, gender and the requirement for the serum to have come from locations known to contain DFTD devils in the eastern half of Tasmania. These variables were controlled to remove bias between gender and the genetics of eastern and north-western devils.

3.3.1 GC-MS

Since promising results had been obtained from the pilot study (see chapter 2), these samples were analysed using GC-MS. Examples of chromatograms from a non-DFTD and DFTD sample can be observed in Figure 3.1. To determine if there was variability between the non-DFTD and the DFTD samples, the samples were analysed by PCA. To reduce the amount of data prior to PCA analysis a feature selection was conducted using Student's *t*-test. The eleven chromatographic signals with a p value < 0.1 were analysed further with PCA since only six signals had p values < 0.05. The PCA plot that shows the first and second principal components can be observed in Figure 3.2. The plot can be separated into an area of only non-DFTD samples but the DFTD area contained two samples that had come from apparent non-DFTD individuals. Continued trapping trips had been undertaken

at this location so information was sought to determine if any of the devils that had provided these samples later showed signs of DFTD.

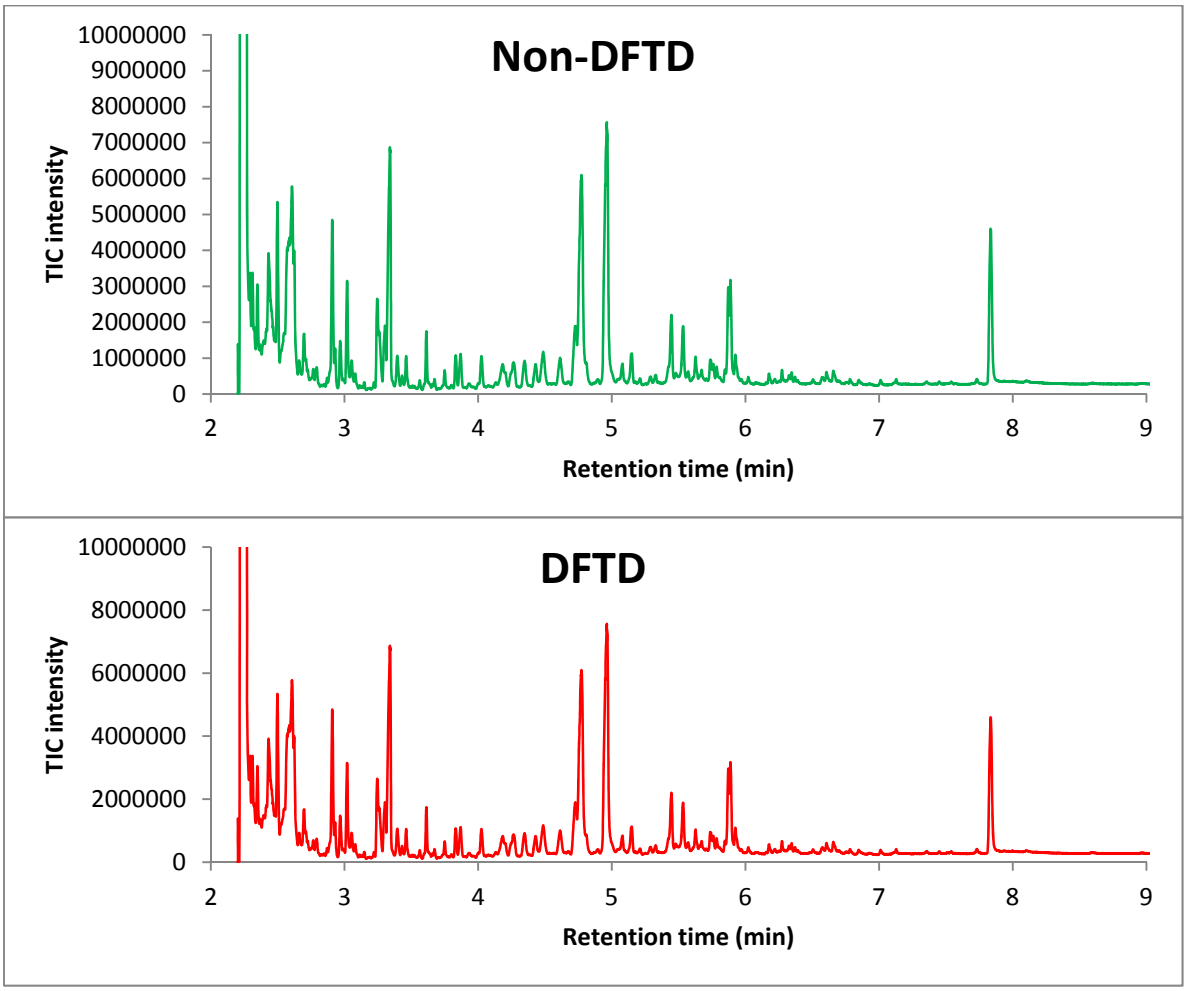

Figure 3.1: Comparision of GC-MS chromatograms of a non-DFTD and DFTD serum sample. The samples were injected into the GC using a splitless injection for 0.50 min at 230 ˚C. The temperature of the column was held at 55 ˚C for 0.70 min and then ramped to 330 ˚C at a rate of 42.40 ˚C/min. The column was held at 330 ˚C for 2.10 min. The acquisition of data was initiated 2.20 min into the run.

Unfortunately, the devil that provided sample 04/1771 (graph ID 16) was not caught again so further information on this devil could not be obtained. Interestingly though, two of the samples from this set were shown to later develop DFTD. Sample 04/0448 (graph ID 4) clustered with the non-DFTD samples in Figure 3.2 but this devil showed signs of DFTD when it was trapped again 35 days later. Sample 04/0560 (graph ID 6) which had also been part of the pilot study, was diagnosed with DFTD after it was trapped again 69 days later. It is interesting that the sample that showed signs 69 days later clustered with the DFTD

samples where as the sample from the devil with DFTD signs 35 days later clustered with the non-DFTD samples in the PCA plot in Figure 3.2. There are a couple of hypotheses that could explain this. Firstly, it is possible that sample 6 developed visible tumours prior to sample 4 but this could not be determined since the devils were only examined if they were trapped during trapping expeditions. Secondly, it is possible that the presence of these two pre-clinical DFTD samples (samples 4 and 6) in the feature selection step as non-DFTD samples may have also influenced the results.

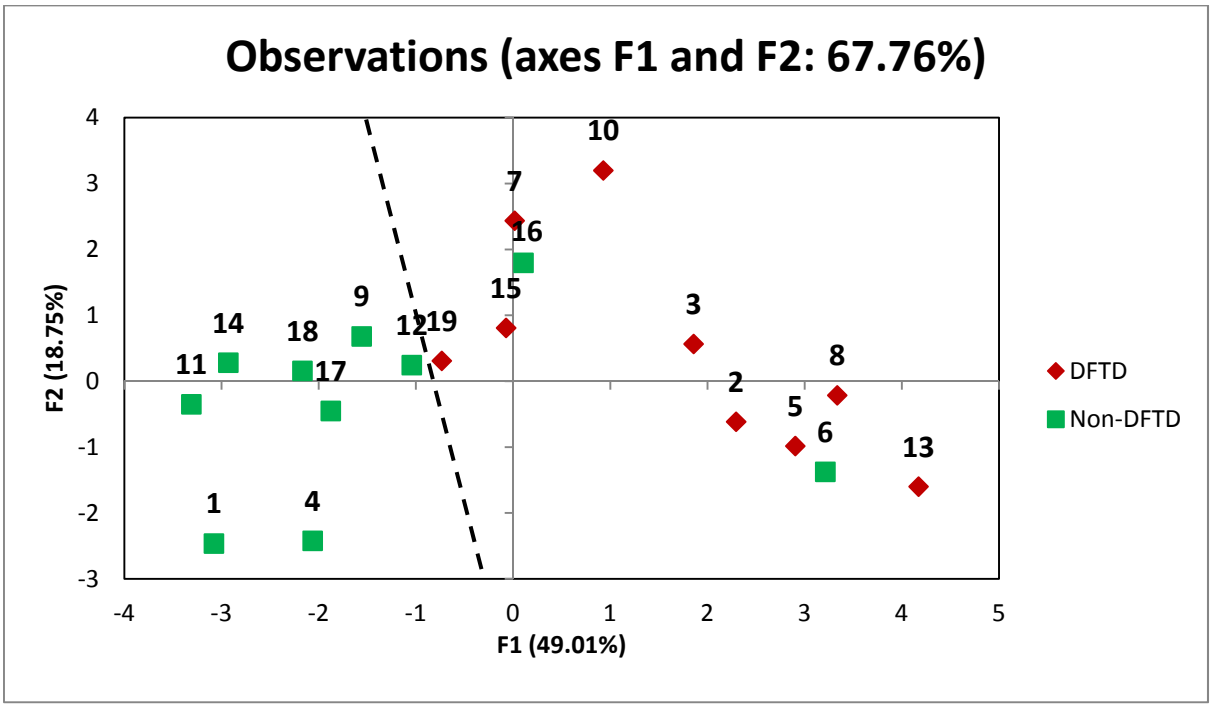

Figure 3.2: Principal component analysis (PCA) of the 11 signals determined to be significantly different (p-value < 0.1) between ten apparent non-DFTD serum samples and nine DFTD samples analysed by GC-MS.

To eliminate the influence of the two pre-clinical samples as well as sample 16 on the feature selection, these samples were removed and the remaining samples reanalysed. This time, feature selection with Student's *t*-test was used to determine signals that were significantly different from the remaining seven non-DFTD samples and the nine DFTD samples. The 19 signals which had p-values < 0.1 from all 19 samples were then analysed again with PCA (see Figure 3.3). With the three samples removed from the feature

selection, the two pre-clinical samples (samples 4 and 6) clustered with the DFTD samples. This exciting result showed that there are serum metabolite differences between DFTD and non-DFTD devils that can be observed before observable tumours. Both of the pre-clinical samples did not cluster with the DFTD samples in the first PCA (Figure 3.2) because including these samples in the feature selection would interfere in the selection of metabolites that are different between DFTD and non-DFTD samples. Interestingly, sample 16, which was also removed from the feature selection step, still clustered with the DFTD samples. It is possible that this devil did have DFTD with no observable tumours but this cannot be determined for certain because the individual was not trapped again.

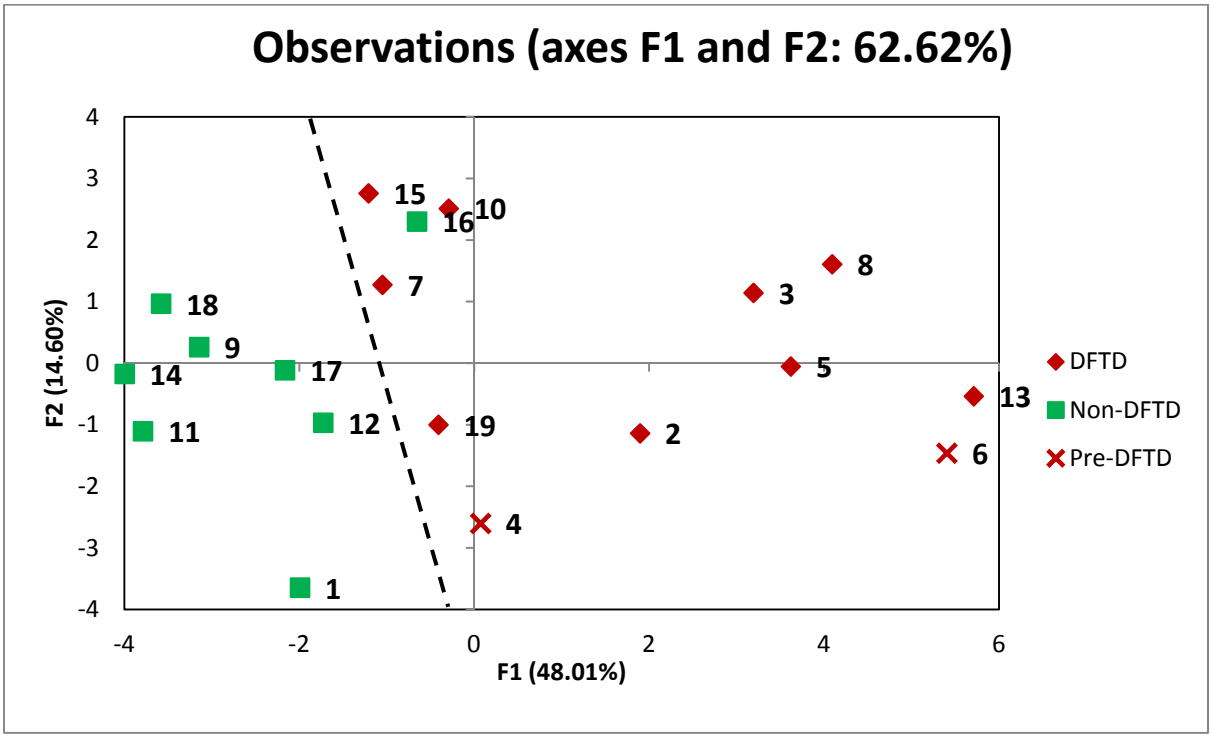

Figure 3.3: Showing the variability of samples without including the pre-clinical samples in the feature selection. PCA of the 19 signals determined to be significantly different (p-value < 0.1) between 7 non-DFTD serum samples and 9 DFTD samples analysed by GC-MS. The two pre-clinical DFTD samples along with sample 16 that had clustered with the DFTD samples in Figure 3.2 were not included in the feature selection.

Since good results were obtained with the Bronte Park samples the remaining male samples from the pilot study that had not already been included in the Bronte Park set were added to the analysis. This was to show if the addition of other devils from around eastern

Tasmania could still show metabolite variability between non-DFTD samples and samples obtained from devils with DFTD including individuals that developed DFTD in the future (pre-clinical). Another feature selection was conducted in the same way as above and looked for differences between the 11 DFTD samples and the 11 non-DFTD samples (this feature selection did not include the two pre-clinical samples or sample 16 that had clustered with the DFTD samples in both of the plots in Figure 3.2 and Figure 3.3). The 17 signals with p values < 0.05 were analysed further using principal component analysis (PCA). The first and third principal components were required in this PCA to adequately separate the DFTD and non-DFTD samples (see Figure 3.4). The pre-clinical DFTD samples as well as sample 16 still clustered with the DFTD samples. Interestingly, the sample that showed clinical signs of DFTD 35 days after the sample had been taken still clustered close to the non-DFTD samples as it had done in the two previous PCA plots. Even though the majority of samples were obtained from Bronte Park, the samples obtained from other areas in eastern Tasmania still separated according to DFTD status in the PCA plot. As in these two former plots, the pre-clinical sample that was observed to have clinical signs 69 days later (sample 6) was positioned on the opposite side of the non-DFTD cluster on the PCA plot. As was mentioned above, it is possible that the devil developed DFTD tumours earlier than the other pre-clinical sample (sample 4).

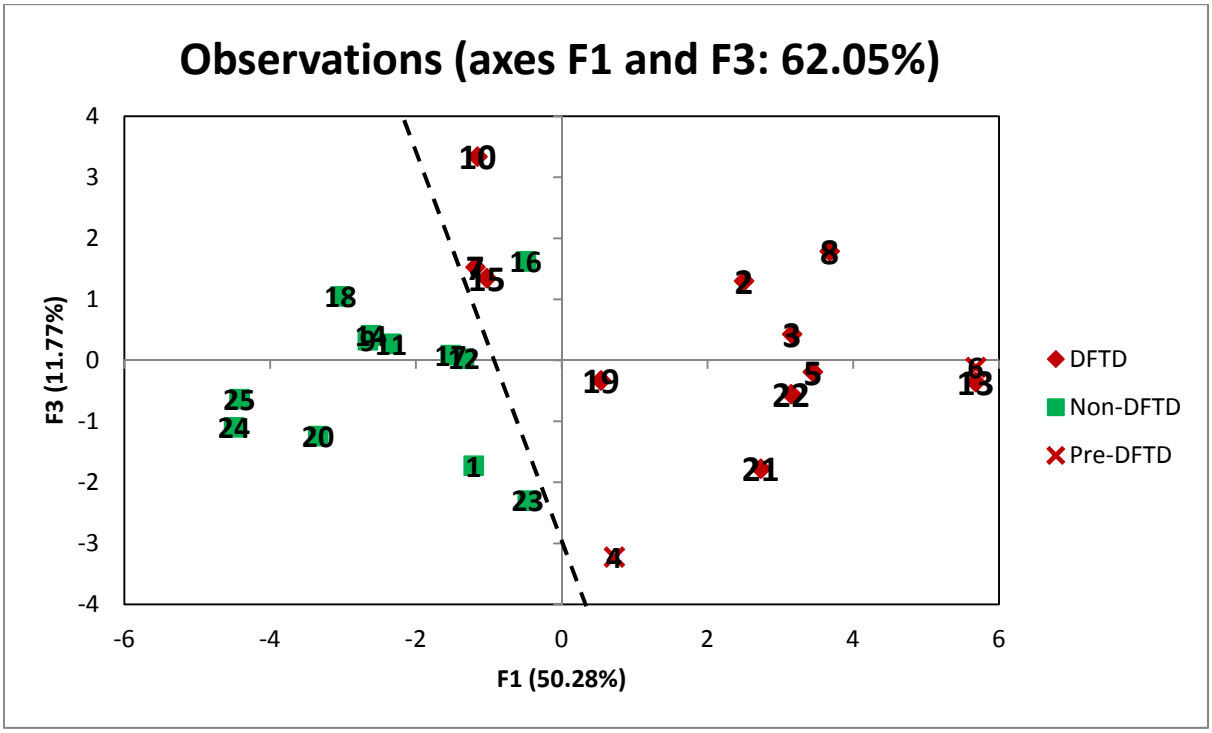

Figure 3.4: Variability between devil serum samples obtained from male devils in eastern Tasmania. This plot shows a principal component analysis using the first and third principal component, of DFTD, pre-clinical DFTD and non-DFTD serum samples from male Tasmanian devils analysed by GC-MS. The chromatographic data was analysed initially with Student's *t*-test to determine significantly different peaks between the DFTD and the non-DFTD (not including the potential pre-clinical samples and sample 16) samples. The 17 signals with a p value < 0.05 along with the 25 samples were then analysed via PCA.

3.3.2 Metabolite Identification

It is not necessary to identify the metabolites when the data is analysed with multivariate pattern analysis to determine the metabolite biomarkers [24]. The identification of these metabolites though, could be useful for understanding the disease or in the development of a more practical method to analyse samples for the disease. With prior separation, metabolites can be tentatively identified from the retention time and the mass spectrum using metabolomics libraries or standards [9]. Metabolites can be hard to identify though, because the lack of commercially available metabolites limits the number of metabolites that are included in metabolomics libraries [25] or as metabolite standards.

The GC-MS peaks that were determined to be significantly different between the DFTD and non-DFTD samples of all the males analysed in this chapter were preliminarily

identified using a private metabolite library obtained from a collaboration with the Max Planck Institute (Golm Database). The spectra of each of the peaks were compared to the spectra in the metabolite database. Some of the metabolites could not be identified with enough confidence from the library and these were identified as being unknown. Identification was aided using retention indices provided in the Golm Database. To ensure that the identified metabolites was appropriate, retention indices values were plotted against the retention time along with two known metabolites phosphate and cholesterol which eluted at the beginning and end of the chromatogram respectively (see Figure 3.6). The metabolite identifications and average abundance of each metabolite are listed in Table 3.2. Except for peak 38 the average abundance of each of the metabolites listed in Table 3.2 was higher in the DFTD samples than it was in the healthy serum samples. Compound 76, which was identified as being an unknown, was around twice as abundant in both the DFTD and the pre-clinical samples. Interestingly this peak was on average more abundant in the pre-clinical samples than it was in the samples obtained from devils with visible tumours. Previous research has also shown changes in the concentration of some of the suggested metabolites in Table 3.2 between healthy and cancerous samples from animals or humans.

Glycine has been shown to increase in cells as they become more tumourgenic [118]. This work also showed an increase in the concentration of glycine in serum samples from DFTD devils both before and after visible signs. Yang *et al.* found in their study of the central metabolism of human cancer cells that the concentration of *Myo*-inositol decreased in tumour tissues [118]. *Myo*-inositol though has been shown to be increased in a selection of cancers including colon adenocarcinoma, ovarian carcinomas and schwannomas [24]. We found that the concentration of this metabolite increased in serum from DFTD devils which appear to have developed from a Schwann cell or a precursor of Schwann cells [82].

Erythrose is a type of aldolase and in 1943, research was published that showed that rats with tumours had an abundance of aldolase. This has also been shown in human sera from cancer patients although the increase in aldolase was not very pronounced [119].

Table 3.2: Identification of metabolites that were significantly different between DFTD and non-DFTD serum samples including fold changes with respect to non-DFTD samples.

Pk #	Identification	Average fold change in pre-clinical compared to non-DFTD	Average fold change in DFTD compared to non-DFTD
30	Unknown	1.3	1.6
37	Glycine	1.3	1.3
38	Unknown	0.9	0.9
46	Erythrose	1.2	1.3
55	Aspartic acid	2.3	1.4
56	Unknown	1.5	1.3
59	Oxalacetic acid	1.3	1.3
60	Unknown	1.3	1.4
62	Oxoproline	1.7	1.7
64	Ribitol derivitive	1.5	1.5
65	Unknown	1.4	1.3
70	Unknown	1.4	1.4
71	Unknown	1.8	1.6
73	Inositol-β-glactoside	1.7	2.3
74	Myo-inositol	1.4	1.3
76	Unknown	2.3	1.8
78	Unknown	1.6	1.6

It was promising that some of the identified metabolites or metabolite classes had previously been associated with other cancers especially *myo*-inositol which has previously been associated with schwannomas. The identification of metabolites specific to DFTD could be used to study the biology of DFTD and the effects it causes to Tasmanian devils. These identifications should be confirmed using metabolite standards (if available) and then could be used to develop a more targeted metabolomic approach to diagnose DFTD in future research.

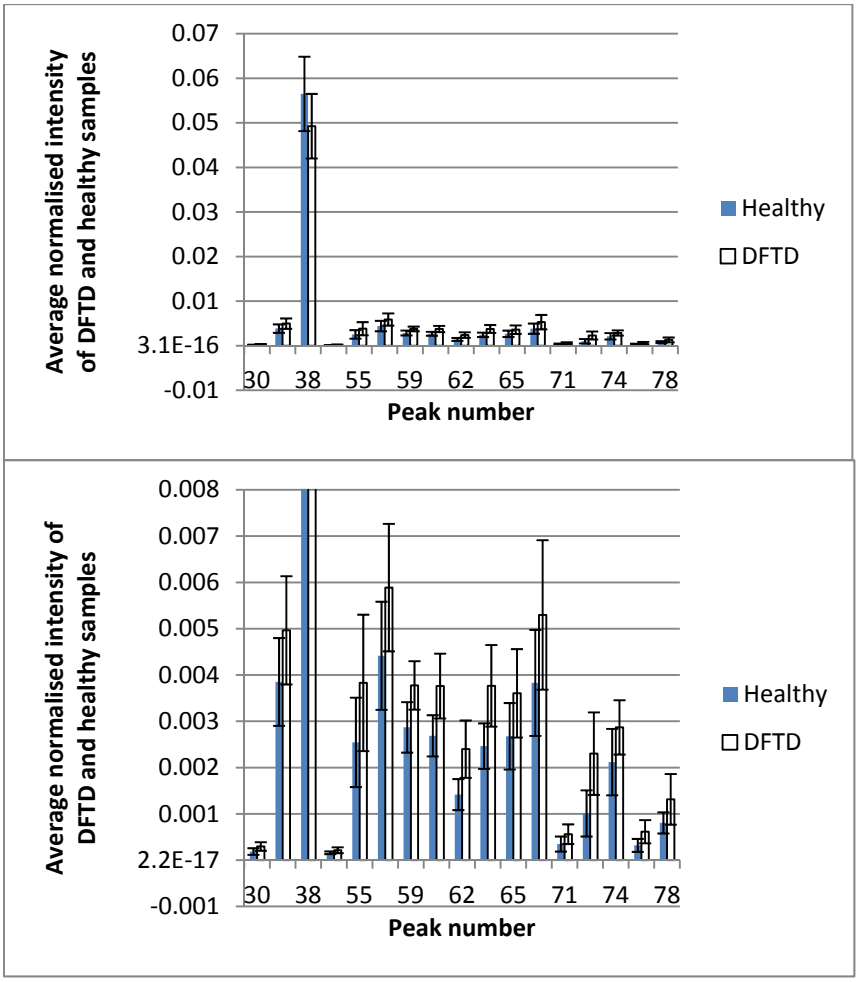

Figure 3.5: The average normalised intensity significantly different metabolites identified with GC-MS. This graph compares the normalised intensity between DFTD (clear box) and healthy (filled box) samples. The error bars are ± one standard deviation from the average. The bottom graph is zoomed in to show more detail.

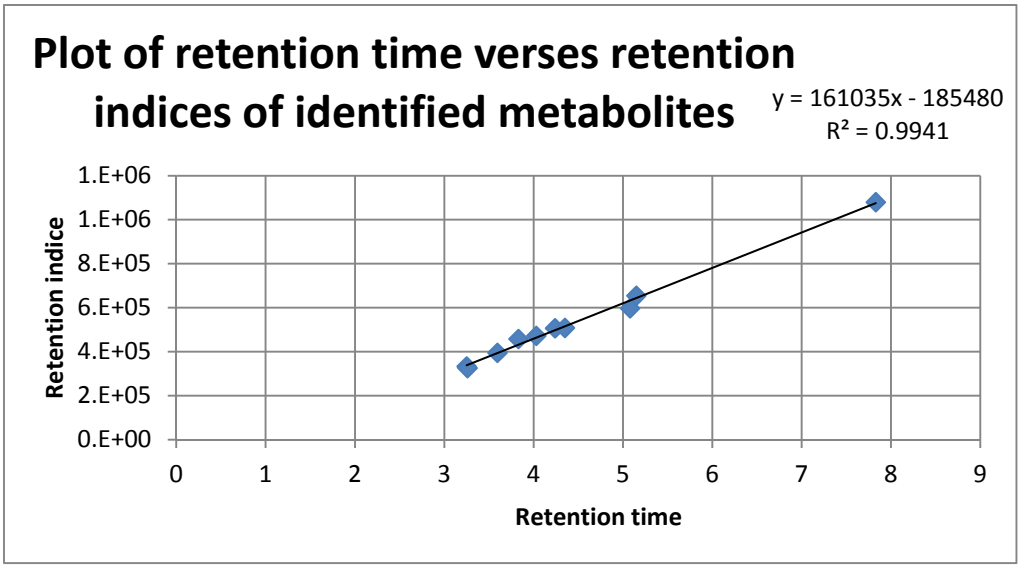

Figure 3.6: Plot of retention time verses retention indices of identified metabolites. The first and last data points are phosphate and cholesterol respectively.

3.3.3 ESI-MS

Although compound identification with mass spectrometry is more difficult without the information that can be gained though prior separation, analysis time is dramatically reduced with the removal of the separation step. These samples were analysed via electrospray ionisation - mass spectrometry (ESI-MS) to confirm the results obtained from the GC-MS data and determine if a faster technique could be used to diagnose DFTD. Examples of chromatograms from a non-DFTD and DFTD sample can be observed in Figure 3.7.

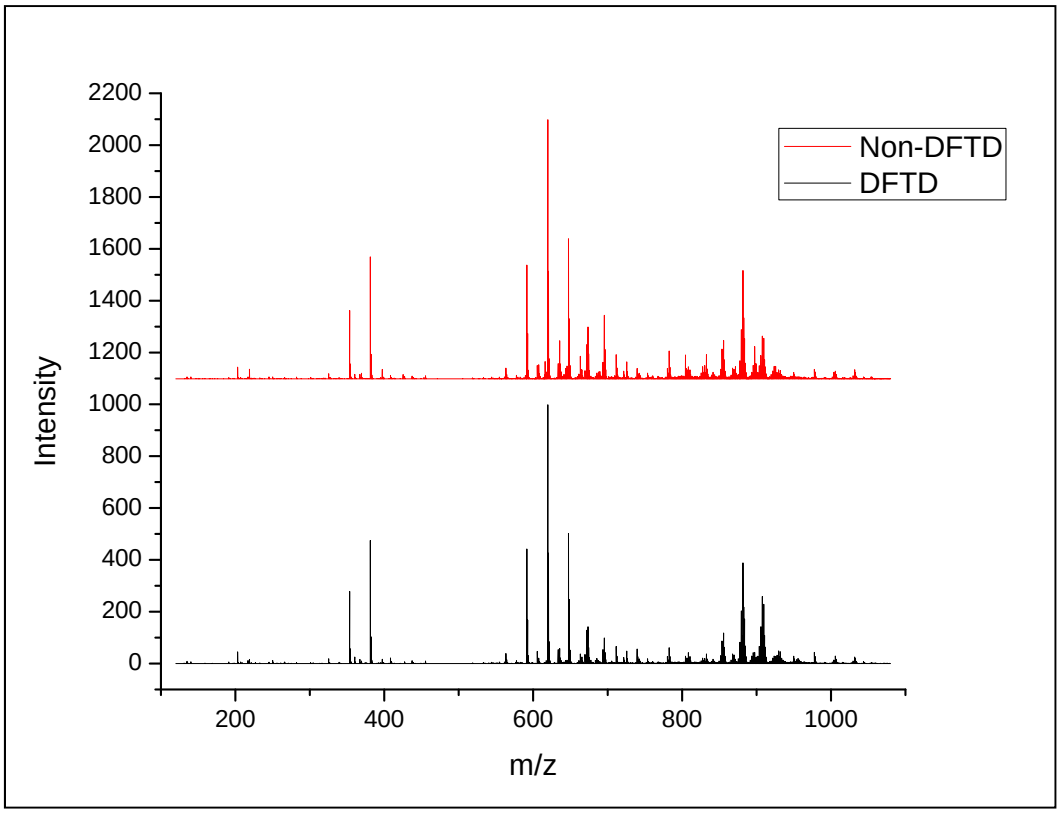

Figure 3.7: Comparison of mass spectra from a non-DFTD and a DFTD serum sample analysed in positive ion mode via ESI-QTOF-MS.

A feature selection using Students *t*-test was again conducted to reduce the amount of data prior to analysis by PCA with the XLSTAT software. Since the two pre-DFTD samples (sample 4 and 6) and sample 16 continually clustered with the DFTD samples when the samples had been analysed by GC-MS, the ESI-MS feature selection step did not include

these three samples. Initially only the Bronte Park males were analysed and the PCA plot can be observed in Figure 3.8. The Student's *t*-test identified 57 signals with p-values < 0.05 and these signals from each of the 19 samples were analysed with PCA. The variability between the non-DFTD and DFTD samples was more pronounced when the samples were analysed by ESI-MS than when they were analysed by GC-MS analysis (compare plots in Figure 3.3 and Figure 3.8). Encouragingly both of the pre-clinical samples (samples 4 and 6 marked with "x" on the plot) still clustered with the DFTD samples with this method. Sample 16 clustered with the non-DFTD samples instead of with the DFTD samples as it did after GC-MS analysis. There are two hypotheses that could explain this. The first possibility is that the GC-MS method is capable of identifying DFTD earlier than the ESI-MS method because of the prior separation of metabolites prior to MS detection. The second hypothesis is that the ESI-MS method is more accurate at diagnosing non-DFTD individuals than the GC-MS method which would suggest that the devil that provided sample 16 was in fact non-DFTD. These theories cannot be confirmed because the devil was not re-captured. Increasing the sample set could help to examine if ESI-MS can identify samples with DFTD earlier than the GC-MS method.

Since variability between the DFTD and non-DFTD samples was successfully shown with the Bronte Park samples analysed by ESI-MS, the remaining male samples from the pilot study were also included as they had been with the GC-MS approach. The feature selection identified 130 MS signals that had p-values < 0.05 with Student's *t*-test. The PCA successfully separated the non-DFTD samples from the DFTD samples using the first and second principal components (see Figure 3.9). The variability between the DFTD and the non-DFTD samples was not as pronounced in this PCA plot as it was in Figure 3.8 when only the Bronte Park samples were anlaysed. This could be explained from the increase in sample variability by the addition of multiple sampling locations. The pre-clinical samples

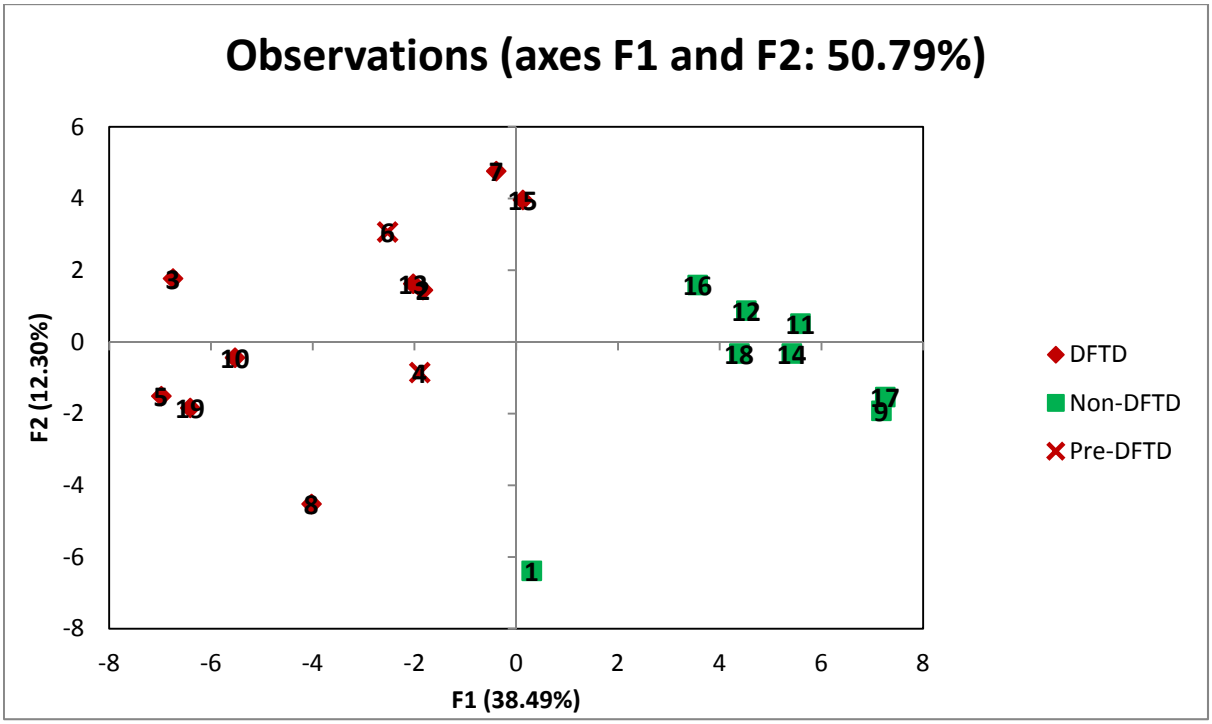

Figure 3.8: Variability between samples obtained from devils at Bronte Park. This plot shows the principal component analysis (PCA) of the 57 signals determined to be significantly different (p-value < 0.1) between 7 non-DFTD serum samples and 9 DFTD samples analysed by ESI-MS. The two pre-clinical DFTD samples along with sample 16 that had clustered with the DFTD samples in Figure 3.2 were not included in the feature selection.

were positioned in the DFTD cluster close to the non-DFTD samples whereas sample 16 remained clustered with the non-DFTD samples. There were other DFTD samples that were positioned on the plot close to the pre-clinical samples. Two of these samples came from the same devil (samples six and ten; devil A) and were sampled four months apart. Samples 9, 8 and 4 were sampled respectively over the course of 50 days from another devil (devil B). The samples that were taken later were positioned further to the left on the PCA plot. Sample 7 was sampled from the same devil as sample 24 (devil C), 35 days later. The positions of samples from devils A, B and C could suggest that the first principal component and thus the positions of samples on the PCA plot do coincide with the length of time that a devil has had DFTD but this is inconclusive.

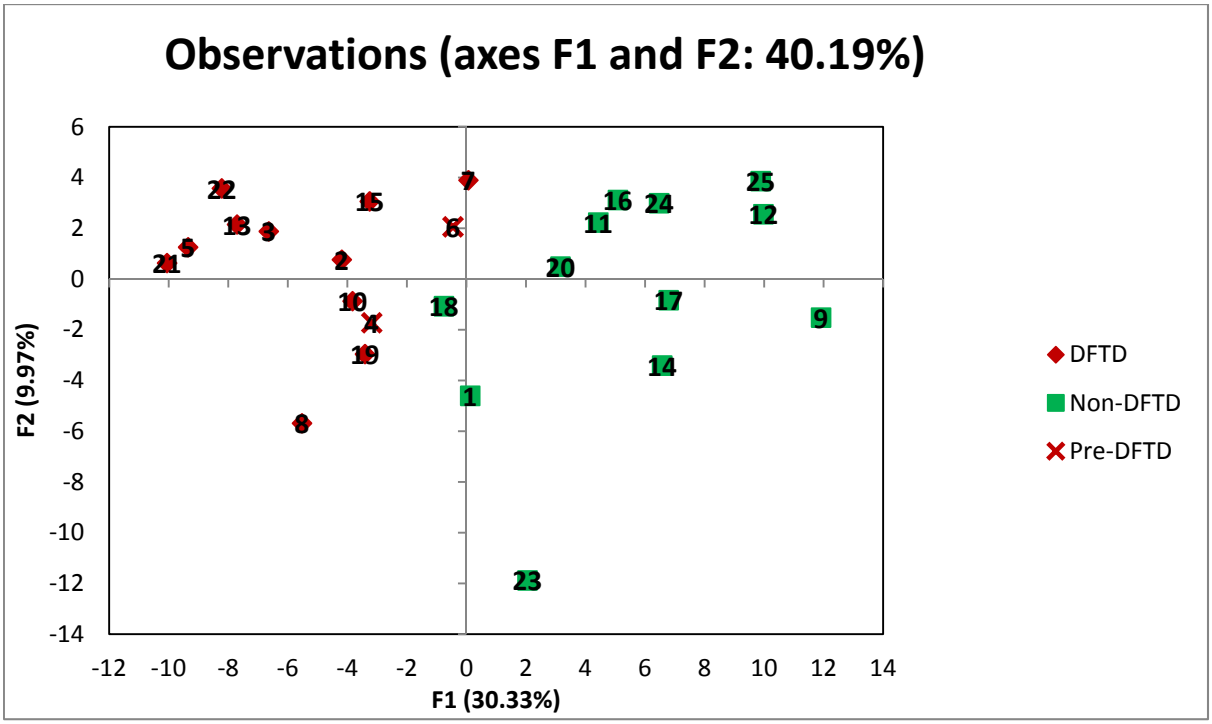

Figure 3.9: Variability between samples obtained from male devils throughout eastern Tasmania. This plot shows the PCA using the first and third principal component, of DFTD, pre-clinical DFTD and non-DFTD serum samples from male Tasmanian devils analysed by ESI-MS. The MS data was analysed initially with Student's *t*-test to determine significantly different peaks between the DFTD and the non-DFTD (not including sample 16) samples. The 130 signals with a p value < 0.05 along with the 25 samples were then analysed via PCA.

3.3.4 Method comparison

The results from the ESI-MS and the GC-MS sets of data showed that there are differences in serum metabolite profiles between non-DFTD and DFTD positive devils for a sample of wild male devils that were caught over the course of one calendar year from a range of geographical areas in the eastern half of Tasmania. There can be many issues when mining for potential disease biomarkers in wild animals because of the lack of control such as genetics or diet [20, 21] that can affect an individual's metabolome. This work was able to overcome the issues that can be seen with wild animals and successfully distinguish between samples taken from DFTD and non-DFTD devils including classifying pre-clinical samples as DFTD positive by limiting the variability of other factors that can affect the metabolome. At present pre-clinical diagnosis cannot be conducted on Tasmanian devils for DFTD. Both of these methods show evidence of the ability to diagnose DFTD prior to visible tumours by the analysis of serum metabolites. The GC-MS

method was capable of obtaining preliminary metabolite identifications. These identifications need to be confirmed using metabolite standards. Identifying these metabolites could increase the knowledge of components that help to characterise DFTD [6] and help to further understand the disease.

The development of two complementary techniques for DFTD diagnosis is advantageous for multiple reasons. If uncertain results are obtained with one of the methods the diagnosis could be evaluated using the alternate method. The GC-MS method is able to give additional information because of the prior separation of metabolites and production of mass fragments allows for identification of the metabolites. The results obtained in this study also showed the possibility that prior separation of metabolites before MS detection could help diagnose DFTD earlier since sample 16 was only identified as DFTD in the GC-MS method. The ESI-MS method could be utilised as a quick screening test for DFTD. The isolation of metabolites is identical for both methods so after the metabolites have been isolated they can be analysed by either GC-MS or ESI-MS. Since GC-MS could potentially show DFTD earlier than the ESI-MS method the GC-MS method could be used to just re-analyse the samples classified as non-DFTD to confirm the diagnosis which would decrease diagnosis time. The methods discussed here show the potential to test individuals for DFTD within a day using a small amount of serum. Currently DFTD diagnosis takes a number of days and can only be determined after the presentation of visible tumours so both of these methods show improvement on the current DFTD diagnostic test.

4 Blind Study to Investigate Differences in the Metabolome of DFTD and Non-DFTD Devils Involved in a DFTD Suppression Trial

4.1 Introduction

The major purpose of research into DFTD is to prevent the possible imminent extinction of the Tasmanian devil and this has resulted in two major initiatives being undertaken. The first project involves the establishment of a captive insurance population. This insurance population was obtained in part by capturing Tasmanian devils in DFTD-free areas in western Tasmania. This population has been established in numerous wildlife parks and zoos across Tasmania and the rest of Australia. The second project involved a suppression trial in the Forestier Peninsula in south-eastern Tasmania. This location was chosen because the only connection to the mainland of Tasmania is via a bridge over a canal. The suppression trial involved capturing and euthanising infectious animals to limit the spread of DFTD [86].

At present all current methods for the diagnosis of DFTD require biopsies of the suspected DFTD tumours [82, 85]. This is disadvantageous in the suppression trial because DFTD-devils can only be identified and removed once they are all ready contagious. Although there was no increase in the abundance of DFTD during the suppression trial, the disease was not eliminated from the Forestier Peninsula population [120]. As the spread of DFTD is by allograft, the visible presence of DFTD tumours required for diagnosis leaves the possibility in which the disease can still be spread. The availability of a pre-clinical test along with frequent trapping trips could improve the success of this trial by eliminating DFTD devils prior to the transmission of the disease to another individual.

This chapter builds upon the results of chapter 3 by increasing the number of devils in the sample set and using the metabolite profiling methods in a diagnostic manner with blind samples. Samples were obtained from male devils trapped in the Forestier Peninsula between 2008 - 2010. Concentrating on devils from this specific location removed many of the sample variables that would be present in a sample set representing devils from throughout the state. Although a pre-clinical test that is suitable for all Tasmanian devils is required to help with the establishment of captive insurance populations, the development of any pre-clinical diagnostic test for this specific population has direct and immediate benefits if implemented to assist in a suppression trial. In chapter 3, the ESI-MS method was shown to give comparable results to the GC-MS analysis in a considerable shorter time frame. The ESI-MS method was, therefore, used to examine serum metabolites from a set of 97 samples obtained from healthy devils; devils with DFTD; devils with other diseases; and devils that later developed DFTD. All of the samples were blind except for 18 samples that were analysed to train the chemometric algorithms.

4.2 Methods

4.2.1 Samples

A set of 97 Tasmanian devil serum samples was obtained from the Tasmanian Department of Primary Industries, Parks, Water and the Environment (DPIPWE) and stored at -80 ˚C. The samples were obtained from 59 wild male devils that had been trapped between 2008 and 2010 on the Forestier Peninsula, south eastern Tasmania, Australia and included 18 sample replicates. The sample set included 10 known DFTD samples and 8 known healthy samples from devils that had not shown any signs of DFTD that were used as a training set. The known DFTD samples were labelled as sample 10, 15, 20, 49, 50, 77, 80, 90, 95 and 100 and the healthy samples were samples 24, 28, 29, 40, 42, 62, 67, and 70. All the remaining samples were blind. Quality control samples were prepared from pooled rat serum.

4.2.2 Reagents

Methanol (> 99.7 %, isocratic HPLC grade (254 nm)), was obtained from Scharlau Chemie S.A., (European Union); a 10 % v/v solution of formic acid was prepared with formic acid (98 %, Fluka) and diluted with Milli-Q water (Millipore).

4.2.3 Sample Preparation

Each day up to 40 devil samples plus two aliquots of rat serum were prepared and run in the same day. The samples were randomised prior to sample preparation. Serum metabolites were extracted by mixing 15 µL of serum with 500 µL of cold methanol as stated in section 2.2.3 and prepared for ESI-MS analysis according to section 3.2.3. Two aliquots of supernatant from each of the prepared rat samples were prepared for quality controls.

4.2.4 Instrumentation

ESI-MS experiments were conducted using a QTOF Mass spectrometer (Bruker micrOTOF-Q II). The samples were randomised again prior to injection into the ESI-MS. Prior to every 10 samples a reagent blank and quality control sample was run. Each sample or reagent blank in turn was infused into the instrument the same way as stated in section 2.2.4 and analysed using the same parameters. The average spectrum obtained between 1.0-2.0 min was then used for further analysis and collected using the same software in the same way as stated in section 2.2.4. On each day of analysis the first Tasmanian devil sample was run 3 times over the course of the day. Two quality control samples were prepared each day and two aliquots were obtained from each of these prepared QC samples and run on the instrument in alternating order.

4.2.5 Data Analysis

The 18 training set samples undertook automated data reduction to locate sample class-differentiating metabolites by using two-tailed *t*-tests to determine differences between the

DFTD positive and DFTD negative devils. A p-value ≤ 0.05 was considered statistically significant and the significant peaks were then analysed using PCA (XLSTAT Version 2010.2.01 Copyright Addinsoft 1995-2010). Each peak in the quality control samples were examined to see if they were outside an acceptable range determined by the following formula: $f(x)_{\pm} = \bar{x} \pm (G_{n=x} \times \sigma)$, where $G_{n=12}$ was the Grubbs test value for 12 samples which is 2.412.

4.3 Results

4.3.1 Quality Controls

The Grubbs test analysis showed good reproducibility with the QC samples (example spectrums can be observed in Figure 2.4, see spectrums for day 1, 2 and 3).

4.3.2 Identification of Metabolome Differences between DFTD and Non-DFTD Serum Samples

The 18 known samples mentioned in section 4.2.1 formed a training set that was used to aid in establishing the DFTD footprint from each individuals metabolite fingerprint. This was conducted using Student's *t*-test as a feature selection step. The Student's *t*-test determined 16 peaks to be significantly different between the DFTD positive and DFTD negative samples to the 95 % confidence level. The masses are listed below in Table 4.1. This is lower than the number identified with the Bronte Park samples (see section 3.3.3) which may be because of the increase in sample variability since the samples were not all obtained during the one year or the increase in the number of samples which may have led to the identification of metabolites more specific to DFTD.

Table 4.1: List of mass-to-charge ratios determined to be significantly different (p < 0.05) according to Student's *t*-test.

134	191	192	200.9	207	519.3	547.4	584.4
622.4	709.5	818.6	829.7	841.5	885.8	936.7	953.7

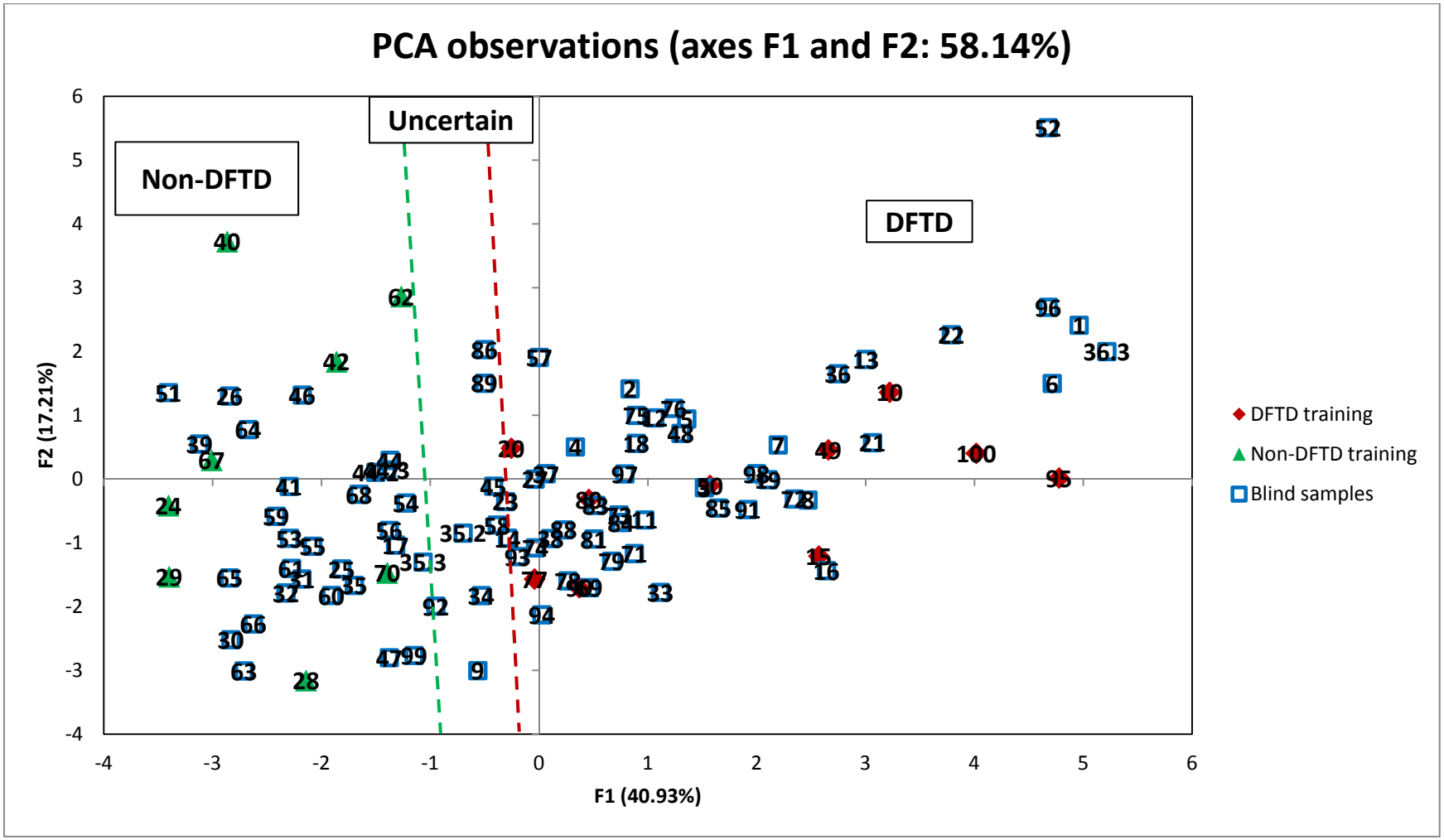

Figure 4.1: Principal component analysis using the first and second principal components sowing the variability between samples obtained from Forestier. A Students *t*-test was conducted and the 16 peaks that had p-value ≤ 0.05 between the 8 healthy and 10 DFTD samples were used to develop this PCA. The blind samples were plotted onto the PCA as supplementary observations. (Replicates were labelled X.1, X.2 and X.3 if they had been analysed on the same day.

Principal component analysis (PCA) was then conducted on the 18 training set samples with only the peaks that were determined to be significantly different. The analysis was then used to plot the remaining 79 samples (see Figure 4.1). The plot of variables shows which of these mass-to-charge ratios contributed most to the variability between the left and right side of the plot which corresponds to the non-DFTD and DFTD samples respectively (see Figure 4.2).

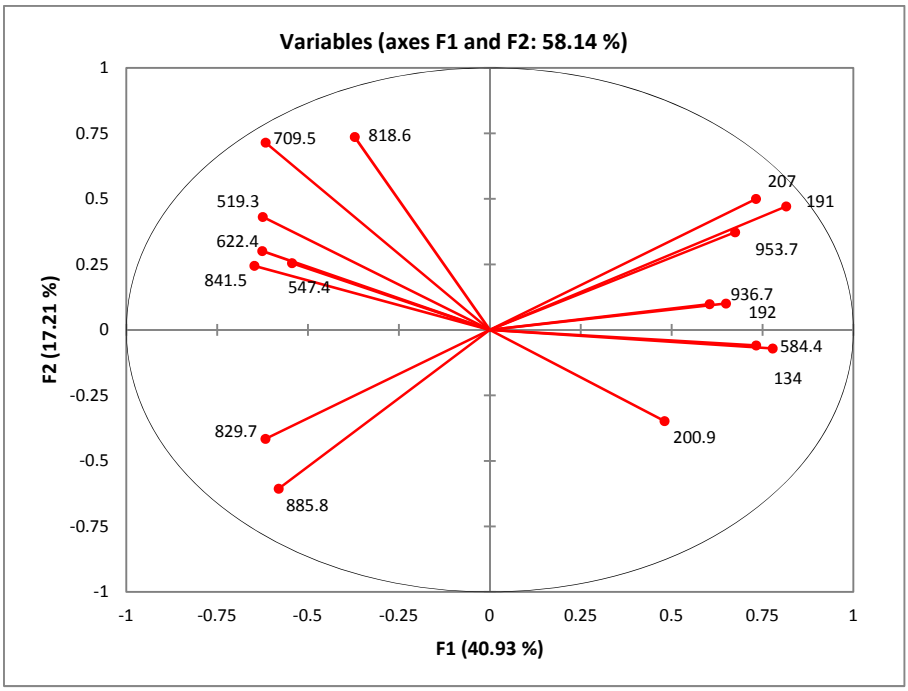

Figure 4.2: Plot showing the correlation between variables and principal components. It shows the importance each variable (m/z) had in the position of each sample on the PCA plot.

The PCA plot (see Figure 4.1) was separated into three sections, non-DFTD, DFTD and uncertain, according to the position of the samples in the training set. These three sections were used to predict the DFTD status of all of the blind samples. Once the disease diagnosis predictions had been completed the samples were unblinded to determine the accuracy of this method in predicting DFTD. The identification for each sample can be seen in Table 4.2 and are summarised in

Table 4.3.

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Table 4.2: DFTD classifications for all of the unknown Forestier samples obtained using 20 Forestier samples for the training set. Samples that were wrongly classified are in *italics*. Samples run in triplicate are labelled X.1, X.2 and X.3, where X is the sample number.

Sample ID	PCA classification	Prior Clinical Diagnosis		Sample ID	PCA classification	Prior Clinical Diagnosis
1	DFTD	DFTD		30	NON-DFTD	DFTD in 12 month
2	DFTD	DFTD		31	NON-DFTD	DFTD in 12 month
3	DFTD	DFTD		32	NON-DFTD	DFTD in 12 month
4	DFTD	DFTD		33	DFTD	DFTD
5	DFTD	DFTD		34	Uncertain	DFTD in 6 month
6	DFTD	DFTD		35	NON-DFTD	Other disease
7	DFTD	Other disease		35.2	Uncertain	Other disease
8	DFTD	DFTD		35.3	NON-DFTD	Other disease
9	Uncertain	DFTD		36	DFTD	DFTD
11	DFTD	DFTD		36.3	DFTD	DFTD
12	DFTD	DFTD		37	DFTD	DFTD in 12 month
13	DFTD	DFTD		38	DFTD	DFTD in 6 month
14	Uncertain	DFTD		39	NON-DFTD	DFTD in 6 month
16	DFTD	DFTD		41	NON-DFTD	DFTD in 12 month
17	*NON-DFTD*	*DFTD*		44	NON-DFTD	DFTD in 6 month
18	DFTD	DFTD in 6 month		44.2	NON-DFTD	DFTD in 6 month
19	DFTD	DFTD		44.3	NON-DFTD	DFTD in 6 month
21	DFTD	DFTD		45	Uncertain	DFTD in 6 month
22	DFTD	DFTD		46	NON-DFTD	DFTD in 6 month
23	Uncertain	DFTD		47	NON-DFTD	DFTD in 12 month

Sample ID	PCA classification	Prior Clinical Diagnosis		Sample ID	PCA classification	Prior Clinical Diagnosis
25	NON-DFTD	DFTD in 6 month		48	DFTD	DFTD
26	NON-DFTD	DFTD in 12 month		51	NON-DFTD	DFTD in 6 month
27	DFTD	DFTD in 6 month		52	DFTD	DFTD
53	NON-DFTD	DFTD in 12 month		75	DFTD	DFTD in 1 month
54	NON-DFTD	DFTD in 6 month		76	DFTD	DFTD
55	NON-DFTD	DFTD in 12 month		78	DFTD	DFTD
56	NON-DFTD	DFTD in 12 month		79	DFTD	DFTD in 6 month
57	DFTD	DFTD in 6 month		81	DFTD	DFTD in 1 month
58	Uncertain	DFTD		83	DFTD	DFTD
59	NON-DFTD	DFTD in 6 month		84	DFTD	DFTD
60	NON-DFTD	DFTD in 6 month		85	DFTD	DFTD in 6 month
61	NON-DFTD	DFTD in 6 month		86	Uncertain	DFTD
63	NON-DFTD	Other disease		88	DFTD	DFTD
64	NON-DFTD	DFTD in 6 month		89	Uncertain	DFTD in 12 month
65	NON-DFTD	DFTD in 6 month		91	DFTD	DFTD in 1 month
66	NON-DFTD	DFTD in 6 month		92	Uncertain	DFTD
68	NON-DFTD	DFTD in 12 month		93	DFTD	DFTD
69	DFTD	DFTD		94	DFTD	DFTD
71	DFTD	DFTD		96	DFTD	DFTD
72	DFTD	DFTD in 1 month		97	DFTD	DFTD
73	DFTD	DFTD		98	DFTD	DFTD
74	DFTD	DFTD		99	NON-DFTD	DFTD in 6 month

Table 4.3: Summary of DFTD classifications for the unknown Forestier samples using a Forester training set (these figures do not include replicates).

		DFTD status according to ESI-MS method		
		DFTD	**Non-DFTD**	**Uncertain**
Clinically diagnosed DFTD status	**DFTD**	32	1	6
	Other disease	1	2	0
	12 month pre-clinical	1	10	1
	6 month pre-clinical	6	13	2
	1 month pre-clinical	4	0	0

All of the blind samples came from devils that had DFTD, other diseases or developed DFTD within 12 months, so the accuracy of determining healthy devils could not be determined. The only samples obtained from devils that did not show signs of DFTD within 12 months had been provided as part of the training set. The latency period of DFTD could be anywhere up to 12 months so the samples that have been obtained from devils that developed DFTD within 12 months need to be considered as pre-clinical. The longest observed latency period for DFTD was observed in a devil that developed DFTD 10 months after it had been placed into a wildlife park (personal communication from Nolan Fox).

The sensitivity of the results was calculated to be 66 % (defined as $\frac{TP}{TP+FN}$, where TP is the number of true positives and FN is the number of false negatives. In other words sensitivity refers to how accurate the test is at classifying only the diseased samples as diseased). The specificity was calculated to be 80 % (defined as $\frac{TN}{TN+FP}$, where TN is the number of true negatives and FP is the number of false positives. This shows how accurate the test is at identifying the non-diseased samples as non-diseased [65]). From the samples analysed only 8 samples were classified as uncertain. Only one sample was classed

wrongly (sample 17 italicised in Table 4.2). This sample was classed as non-DFTD but was obtained from a DFTD-positive devil. One devil that had another disease was classed as having DFTD (sample 7). It is possible that this disease was another type of cancer with a similar metabolomic footprint to DFTD which could explain the wrong diagnosis. This could not be determined because the details of this devils disease were not given. Overall the results were promising.

The sample classed incorrectly, Sample 17, plotted close to the samples determined to be uncertain on the PCA plot which suggests that the line separating non-DFTD and uncertain should have been further left. This was not done though as it would have changed the classification of the non-DFTD samples from the training set to uncertain. It is also possible that this sample was an outlier in some other way compared to the DFTD and non-DFTD samples in the training set. To investigate the possible differences between sample 17 and DFTD samples, the average intensity for each of the significantly different mass-to-charge peaks (see Table 4.1) was obtained for the DFTD and the non-DFTD samples in the training set. These responses were plotted and compared with sample 17 (see Figure 4.3). The first five peaks (peaks 134, 191, 192, 200.9 and 207) were representative of the non-DFTD samples. All of these peaks except for peak 191 were also observed in the comparison of variables and principal components plot (see Figure 4.2) to effect the position of samples in the non-DFTD area. Sample 17 also gave a response for peaks 829.7 and 841.5 which were not observed at all in the average spectrum for DFTD samples. These peaks also were correlated with a negative value for the first principal component and representative of the non-DFTD samples. Sample 17 also had responses that were more representative of the average DFTD spectrum. For example, a strong response was observed with peak 936.7 which was absent in the average non-DFTD spectrum and no peak was observed for 622.4 which was also observed in the average

DFTD spectrum. The spectrum for 17 was not abnormal (see Figure 4.4) and the decrease in intensity in the small mass ions could also be observed in the full spectrums when compared to a representative DFTD sample. The increased intensities for the small ions were also observed in other DFTD samples. An investigation using tandem mass spectrometry to identify the compounds corresponding to these small mass ions as well as ion 622.4 and 936.7 could help shed more light on why the DFTD-positive sample 17 was classified as non-DFTD in this analysis.

The replicates of Tasmanian devil samples plotted very close together in the PCA plot showed limited variability. The sample with the greatest variability according to the PCA plot was sample 36. One of the three replicates of this sample had a corrupt file so only two were analysed. They have almost no variability according to the second principal component but some variability with the first principal component. They were both still significantly positioned correctly with the DFTD samples. The other two devil samples (samples 35 and 44) were clustered with limited variability in both the first two principal components. Two of the replicates for sample 35 were classified as uncertain and the remaining replicate was classified as non-DFTD but all three were still clustered close together on the PCA plot suggesting good reproducibility. Each of the replicates for sample 44 were positioned very close together and classified as non-DFTD.

All of the samples that came from devils that showed signs of DFTD one month later were classed as DFTD, and all bar one of the samples that came from devils who showed signs of DFTD 12 months later were classed as non-DFTD. The six month pre-clinical samples were classed as a mixture of DFTD, non-DFTD and uncertain. It is entirely feasible that the samples classed as non-DFTD were indeed not infected at the time of sampling and contracted the disease after the sampling point.

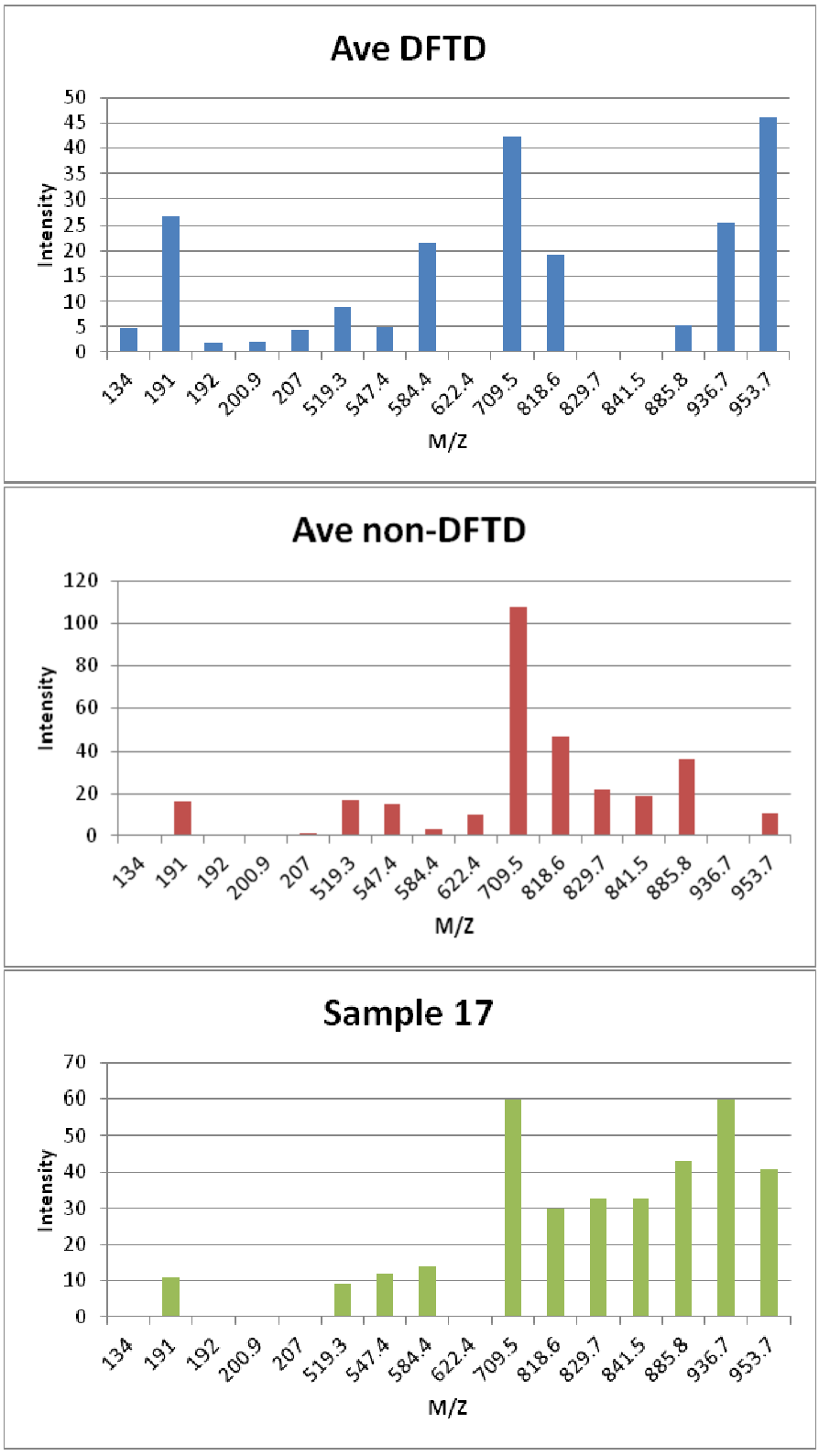

Figure 4.3: Comparing the average significantly different m/z responses (determined using Student's *t*-test) for the DFTD and non-DFTD training set samples with sample 17 that had been wrongly classified.

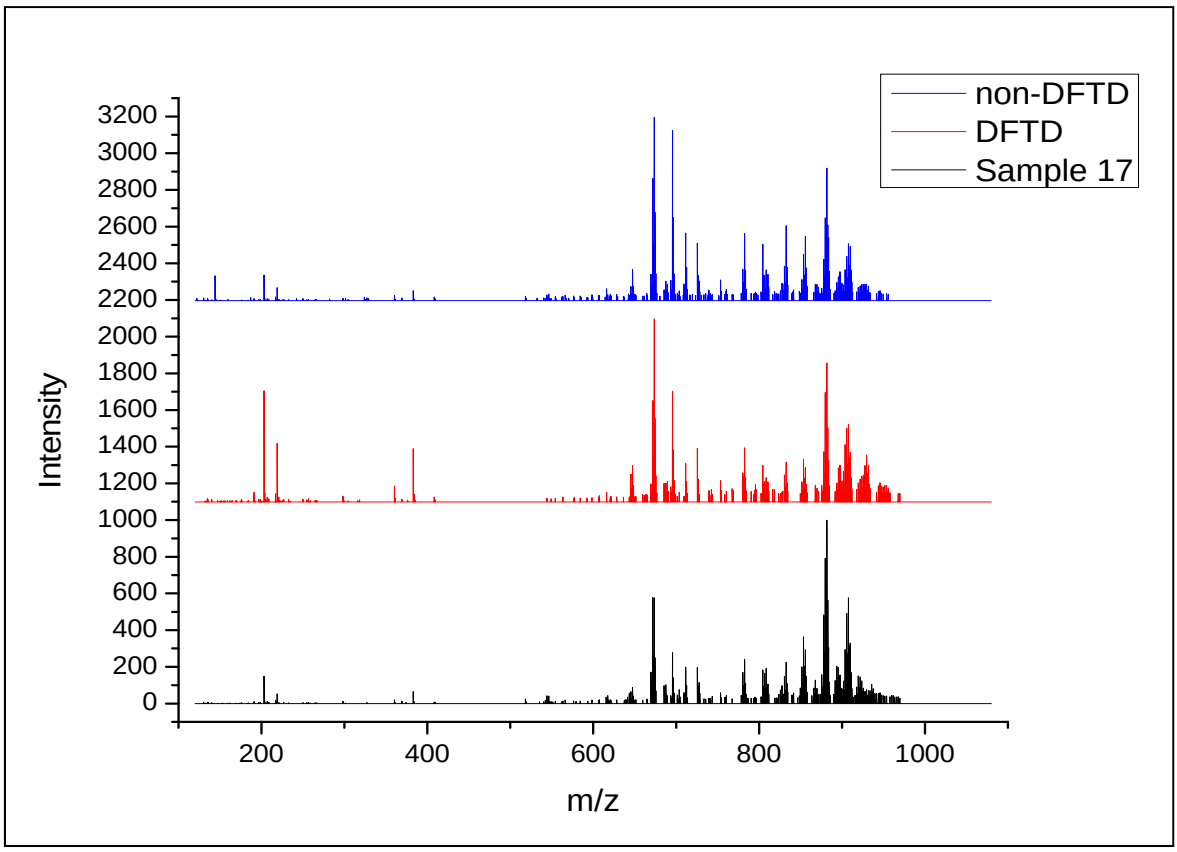

Figure 4.4: The mass spectrum of sample 17 compared to a representative spectrum from a DFTD and a non-DFTD sample.

These results are compelling because of their ability to diagnose DFTD up to six months before signs of a tumour are present. It is especially promising that all of the devils that showed signs of DFTD one month later were identified as having DFTD. Unfortunately, because of the contagiousness of this disease, the only samples obtained from healthy Tasmanian devils were part of the training set so it is impossible to determine how accurate these metabolites are at correctly classifying non-DFTD devils.

4.4 Summary

A suppression trial was conducted at Forestier Peninsular in south-eastern Tasmania between 2004-2010, which involved the removal of all DFTD devils that were trapped during trips occurring 4-5 times a year. The removal of DFTD devils at the Forestier Peninsula has recently been shown to not be preventing the spread of DFTD. Lachish *et al.*

suggested that more frequent trapping and the utilisation of a pre-clinical diagnostic test could allow DFTD devils to be removed prior to the individual transmitting the disease to another devil, would improve the results of this trial [120]. In 2010, field trips were increased to every month to decrease the chance of a DFTD-positive devil spreading the disease to another individual but the suppression trial was suspended because of the need for a pre-clinical diagnostic test.

These results showed the ability to identify DFTD up to six months prior to the presentation of tumours using ESI-MS. Only one DFTD sample was classified wrongly as non-DFTD. This technique allows samples to be prepared and analysed in less than hour. If the suppression trial was initiated again a sample of serum could be obtained, transferred to a laboratory facility and analysed within a day. The individuals diagnosed without DFTD could then be released after a limited time in captivity. The removal of pre-clinical DFTD devils would likely improve the outcomes of a suppression trial and potentially eliminate DFTD in the area.

5 Discovery of differences in metabolites between DFTD and non-DFTD devils with extensive sample variability

5.1 Introduction

The overall goal of this work is to develop a pre-clinical diagnostic test that can be used to determine DFTD biomarkers and which is not biased towards a particular subset of devils such as gender or territory which have been found to alter the metabolites in an individual [20, 21]. This chapter describes results from a blind study that contained samples representative of the conditions previously mentioned that can affect the metabolome. This study looked at a set of 130 serum samples collected between 2005-2009 from male and female Tasmanian devils. The devils were obtained throughout Tasmania and also included one sample from Badger Island in Bass Straight. The samples came from areas where DFTD was prevalent and from DFTD free regions; included devils with other known diseases; and also included devils that were known to have DFTD or another disease at a later date. Previous sample sets that were restricted to eastern Tasmania had shown the ability to determine devils that had DFTD up to six months prior to visible tumours (see chapters 2, 3 and 4).

It was proposed that by using feature selection and multivariate analysis, serum metabolites changes specific to DFTD could still be discovered even with the presence of other metabolite changing variables such as those mentioned above. The previous chapter (Chapter 4) showed the ability to identify DFTD devils using a relatively quick method with ESI-MS. The GC-MS method requires more analysis time but also provides a

separation dimension which could help to diminish the effects of having increased sample variables in this sample set. The discovery of DFTD specific metabolite changes in a sample set with this many variables would allow for the ability to diagnose DFTD in all Tasmanian devils rather than a subpopulation. Metabolites that show the presence of DFTD pre-clinically could aid firstly in obtaining disease free devils for insurance populations and secondly to remove DFTD devils before they can spread the disease to another individual. Both GC-MS and ESI-MS were used in this study to mine for differences between non-DFTD and DFTD samples to determine features that are significantly different between non-DFTD samples and, pre-clinical and clinical DFTD samples from devils throughout Tasmania.

5.2 Methods

5.2.1 Samples

130 Tasmanian devil serum samples obtained from male and female devils around Tasmania were provided by DPIPWE. This set of samples included a training set of 20-30 samples of which 20 were replicates of 20 of the other 110 samples (see Table 5.1 for a summary of the details of each sample and Table A 4 in the appendix for further information). A pooled mouse serum sample was used for a quality control.

5.2.2 Reagents

≥ 98 % o-methylhydroxylamine hydrochloride and 98 % Formic acid were obtained from Fluka, Switzerland; Pyridine, and N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were obtained from Sigma Aldrich; Methanol (> 99.7%, isocratic HPLC grade (254 nm) was obtained from Scharlau Chemie S.A., (made in the European Union); and Millipore Milli-Q water.

5.2.3 Sample Preparation

The samples were randomised prior to sample preparation. Metabolites were collected from the Tasmanian devil and pooled mouse serum by placing 15 µL of serum into 500 µL of cold methanol. The mixtures were vortexed (Ratek VM1 vortex mixer) to mix and then incubated at 4 ˚C for at least 20 min to ensure that as much protein as possible had precipitated out. The samples were then centrifuged at 14,000 rpm for 5 min (Eppendorf bench top centrifuge 5424) and an aliquot of supernatant was obtained and prepared for either GC-MS or ESI-MS.

Gas chromatography – mass spectrometry: Precipitation of proteins and solvent evaporation was conducted on one day to decrease sample variability. The metabolites were obtained by taking 200 µL of the supernatant from the protein precipitation step and evaporated to dryness in a vacuum oven between 50-55 ˚C. The samples were then stored at -20 ˚C. Prior to analysis, 10 µL of 40 mg/mL o-methylhydroxylamine hydrochloride in pyridine was added to the dried metabolite extracts. The samples were then vortexed to mix and then placed on a heating block at 30 ˚C for 60 min. The samples were then incubated at room temperature for at least 17 hours. After the incubation, 90 µL of MSTFA was added to each sample, vortexed to mix and incubated for a further 30 min at 37 ˚C on a heating block. This exact procedure was also applied to reagent blanks and quality controls (mouse sera extracts).

Electrospray ionisation – mass spectrometry: A 100 µL aliquot of supernatant for each sample and mouse quality control was placed into a tube with 2 µL of a 10 % v/v formic acid (in milli-Q water) and 98 µL of methanol to give a 0.1 % acidified solution. Reagent blanks were prepared by adding 2 µL of a 10 % v/v formic acid (in milli-Q water) to 198 µL of methanol.

Table 5.1: List of samples

Sample #	Sex	DFTD status	Other disease information	Sample #	Sex	DFTD status	Other disease information
D1	m	DFTD		6	f	NON-DFTD	
D2	m	DFTD		7	f	NON-DFTD	Pre-Non-DFTD disease
D3	m	DFTD		8	f	DFTD	
D4	f	DFTD		9	m	DFTD	
D5	m	DFTD		10	m	DFTD	
D6	f	DFTD		11	f	NON-DFTD	
D7	m	DFTD		12	f	NON-DFTD	Pre-Non-DFTD disease
D8	m	DFTD		13	f	NON-DFTD	
D9	f	DFTD		14	f	NON-DFTD	Pre-Non-DFTD disease
D10	m	DFTD		15	m	DFTD	
H11	m	NON-DFTD		16	f	NON-DFTD	
H12	m	NON-DFTD		17	m	DFTD	
H13	f	NON-DFTD		18	m	DFTD	
H14	m	NON-DFTD		19	f	NON-DFTD	
H15	f	NON-DFTD		20	m	NON-DFTD	Pre-Non-DFTD disease
H16	m	NON-DFTD		21	f	DFTD	
H17	f	NON-DFTD		22	f	NON-DFTD	
H18	f	NON-DFTD		23	f	NON-DFTD	
H19	m	NON-DFTD		24	f	NON-DFTD	
H20	f	NON-DFTD	Another disease	25	m	DFTD	
1	m	NON-DFTD		26	f	DFTD	
2	f	DFTD		27	f	DFTD	
3	m	NON-DFTD		28	m	NON-DFTD	
4	f	NON-DFTD		29	f	DFTD	
5	m	NON-DFTD		30	f	NON-DFTD	Neoplasia

Sample #	Sex	DFTD status	Other disease information	Sample #	Sex	DFTD status	Other disease information
31	f	NON-DFTD		56	m	NON-DFTD	Skin inflammation
32	f	DFTD		57	m	PRE-DFTD	
33	m	NON-DFTD		58	m	NON-DFTD	
34	f	NON-DFTD		59	m	DFTD	
35	f	NON-DFTD		60	m	NON-DFTD	Pre-Non-DFTD disease
36	m	DFTD		61	m	DFTD	
37	f	NON-DFTD		62	m	NON-DFTD	Non-DFTD growths
38	f	DFTD		63	m	DFTD	
39	m	NON-DFTD		64	m	DFTD	
40	f	PRE-DFTD		65	m	DFTD	
41	f	NON-DFTD		66	f	NON-DFTD	
42	f	NON-DFTD		67	f	NON-DFTD	Neoplasia
43	m	PRE-DFTD		68	m	DFTD	
44	f	NON-DFTD		69	m	NON-DFTD	Pre-Non-DFTD disease
45	m	DFTD		70	f	DFTD	
46	m	DFTD		71	m	DFTD	
47	f	NON-DFTD		72	f	DFTD	
48	m	NON-DFTD		73	f	DFTD	
49	m	NON-DFTD		74	m	DFTD	
50	m	DFTD		75	m	NON-DFTD	
51	m	DFTD		76	f	DFTD	
52	m	NON-DFTD		77	m	NON-DFTD	
53	m	DFTD		78	f	NON-DFTD	
54	f	DFTD		79	m	NON-DFTD	
55	m	NON-DFTD	Non-DFTD growths	80	f	NON-DFTD	

Sample #	Sex	DFTD status	Other disease information	Sample #	Sex	DFTD status	Other disease information
81	m	DFTD		96	f	NON-DFTD	Another disease
82	m	DFTD		97	f	PRE-DFTD	
83	f	DFTD		98	m	NON-DFTD	Urinary Infection
84	m	NON-DFTD		99	m	DFTD	
85	m	DFTD		100	f	NON-DFTD	Developed another disease
86	f	PRE-DFTD		101	m	NON-DFTD	Ulcers
87	f	NON-DFTD	Pre-Non-DFTD disease	102	f	DFTD	
88	m	NON-DFTD	Non-DFTD growths	103	f	NON-DFTD	Pre-non-DFTD growths
89	f	NON-DFTD	Alopecia (baldness)	104	m	PRE-DFTD	
90	m	PRE-DFTD		105	m	DFTD	
91	f	DFTD		106	m	NON-DFTD	Non-DFTD growths
92	f	NON-DFTD	Neoplasia	107	m	DFTD	
93	m	NON-DFTD	Kidney disease	108	f	NON-DFTD	Skin lesions
94	m	NON-DFTD	Malformation	109	m	NON-DFTD	
95	f	NON-DFTD	Another disease	110	m	NON-DFTD	

5.2.4 Instrumentation

Gas chromatography – mass spectrometry: The samples were randomised again before being analysed by the GC-MS and analysed with an Agilent 6850 GC system connected to an Agilent 5975C VLMSD mass spectrometer fitted with a SGE capillary column (part number 054702, Scientific Glass Engineering Analytical Sciences (SGE), Ringwood, Australia) (59.5 m in length, column thickness 0.25 μm and an internal diameter of 0.25 μm). 1 μL of sample was injected using splitless injection at 285 ˚C. A temperature program of 55.0 ˚C for 6 min and then ramped at a rate of 6.00 ˚/min to 340 ^{o}C with an isothermal period of 10 min at 340 ^{o}C was applied. Helium was used as the carrier gas at a flow rate of 1.5 mL/min for 50 min and then increased to 3.0 mL/min at a rate of 0.20 mL/min. MS detection was initiated 12.00 min into the run. Spectra were collected between 40-550 m/z. The spectra were analysed using the GCMS solutions software (Version 2.50SU1, Shimadzu 1999-2006). 10 Tasmanian devil serum metabolite extracts were run daily. A reagent blank was run in the first and ninth run of the day and a quality control was run in the fifth and final run of the day to ensure that variability over the course of the day was minimal.

Electrospray ionisation – mass spectrometry: The instrument was calibrated with sodium formate (10 mM sodium hydroxide, 0.1 % formic acid in 1:1 water-isopropanol). Samples were analysed using a Bruker micrOTOF-Q II instrument using the same procedure as chapter 3 using metabolites obtained from a pooled mouse sample for the quality control.

5.2.5 Data Analysis

Gas chromatography – mass spectrometry: The samples were randomised again before commencement of data analysis. The base peaks of each separated metabolite in the chromatograms of two healthy samples, two DFTD-positive samples and two blind

samples was determined and tabulated. The area of the base was then automatically tabulated using the GCMS Postrun software (Version 2.50SU1, Shimadzu 1999-2006). Obtaining the area of the base peak instead of the total ion chromatogram allowed for more accurate comparisons between runs with peaks that overlapped. The peaks from the GC-MS chromatograms were then normalised by obtaining the ratio of the peak area with the sum of all the base peak areas for each individual run. The known DFTD and healthy samples were used as a training set to classify the blind samples. The data was analysed by principal component analysis (PCA) (XLSTAT Version 2010.2.01 Copyright Addinsoft 1995-2009). Prior to PCA, a feature selection using Student's *t*-test was conducted with the training set. Signals that were not significantly different between the healthy and DFTD samples ($p \leq 0.05$ or ≤ 0.10 as stated) were removed and not considered further. Using these significantly different signals, PCA was performed using the known DFTD and healthy samples. The blind samples were not involved in the PCA but plotted on the PCA plot as supplementary observations. In the PCA, if the first two principal components were not successful in separating the non-DFTD samples from the DFTD samples, combinations of the first four principal components were evaluated to determine if any of those combinations could successfully separate the DFTD samples from the non-DFTD samples.

Electrospray ionisation – mass spectrometry: Data interrogation for ESI-MS experiments was performed by chemometric analysis of the extracted spectra (50-1200 m/z). A Student's *t*-tests to extract ion responses (from the spectra) that were significantly different ($p \leq 0.05$ or ≤ 0.10 as stated) between a selection of DFTD and non-DFTD samples that had been selected as the training set. The significantly different ion responses were then analysed further using principal component anlaysis (PCA).

5.3 Results and Discussion

5.3.1 GC-MS

The known DFTD (samples D1 to D10) and non-DFTD samples (samples H11 to H20) were used to conduct the feature selection to select the most significant peaks. The feature selection only identified 10 peaks with a p-value ≤ 0.1 so these were used to develop the PCA (see Figure 5.1).

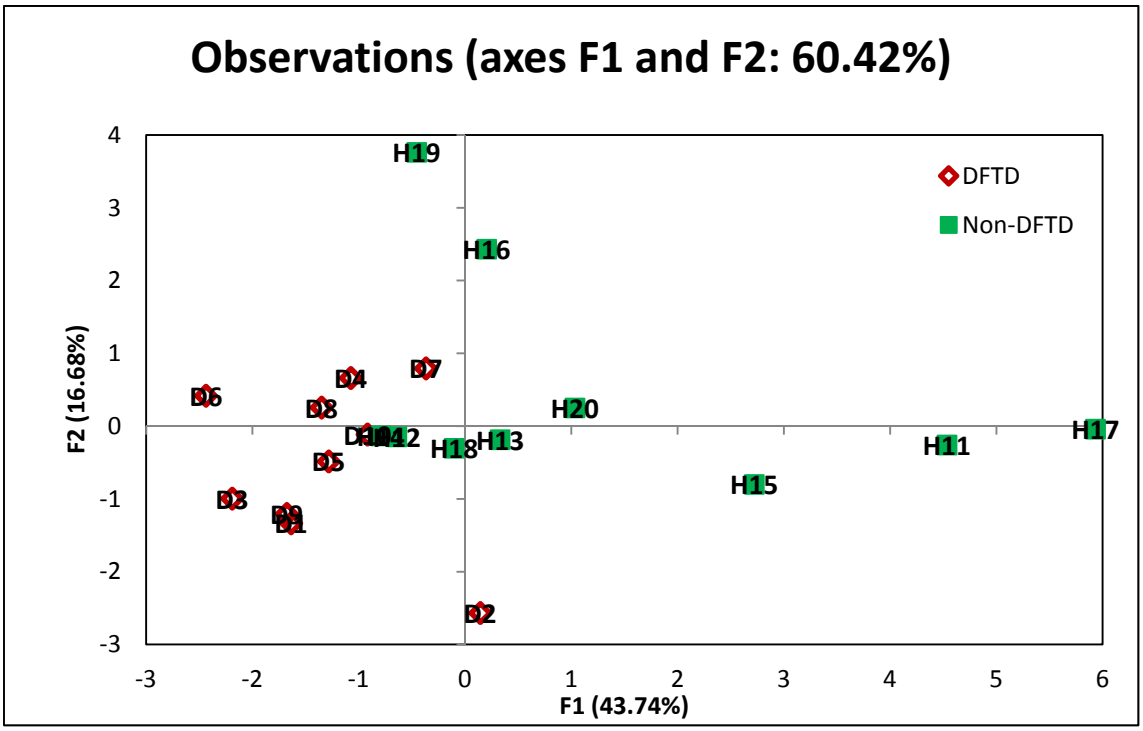

Figure 5.1: Principal component analysis of samples in the training set using the first two principal components. The chromatographic data of 10 DFTD and 10 non-DFTD samples was analysed with Student's *t*-test to determine the peaks that were significantly different between these two groups (p ≤ 0.10). The resulting peaks were then analysed by PCA.

The PCA showed more variability between the non-DFTD samples than the DFTD samples. Even with the loss of information that occurs with feature selections, this process of handling data allows for the pre-selection of DFTD specific signals. This pre-selection was efficient at selecting the signals that were DFTD specific which was shown by increased variability in the non-DFTD samples when compared to the DFTD samples. It was also supported by the evidence that the first principal component (labelled F1 on the

axes) contributed the greatest to separating the DFTD samples from the non-DFTD samples.

PCA plots that plotted the first principal component against the third and fourth principal components were also examined. These plots were not more efficient in separating the DFTD and non-DFTD samples (see Figure 5.2) than the plot that separated the samples using the first two principal components.

As previously mentioned, this set of samples contained a number of sample variables that were not present in the previously analysed sample sets (see chapters 3 and 4). The samples in this blind study were obtained from male and female devils located in eastern and north-western populations that have different genetics [4, 87] and come from different habitats [121]. To reduce the affect of these added variables an additional five DFTD and five non-DFTD samples were added to the training set chosen randomly from the blind samples. The DFTD samples that were un-blinded were samples 27, 71, 83, 99 and 107. The non-DFTD samples were samples 23, 41, 42, 55 and 92. The increase in the training set was done in an attempt to eliminate some of the variability that is observed in the PCA plot that could be attributed to some of the sample variables such as sample location and gender. A Student's *t*-test was conducted with these 30 samples. This test found 5 metabolite peaks with a value of $p \leq 0.05$ and 14 peaks with a value of $p \leq 0.1$. Since there were only 5 peaks with a p-value ≤ 0.05, the peaks that had a p-value ≤ 0.1 were further analysed by PCA. Two dimensional PCA plots using various combinations of the first four principal components (PCs) were examined to determine the plot that separated the DFTD samples from the non-DFTD samples most efficiently using two dimensional plots. The first and second PCs were observed to give the best separation between the DFTD and

non-DFTD samples (see Figure 5.3). The addition of more samples in the training set did not improve the separation of the DFTD and non-DFTD samples. Unlike in Figure 5.1,

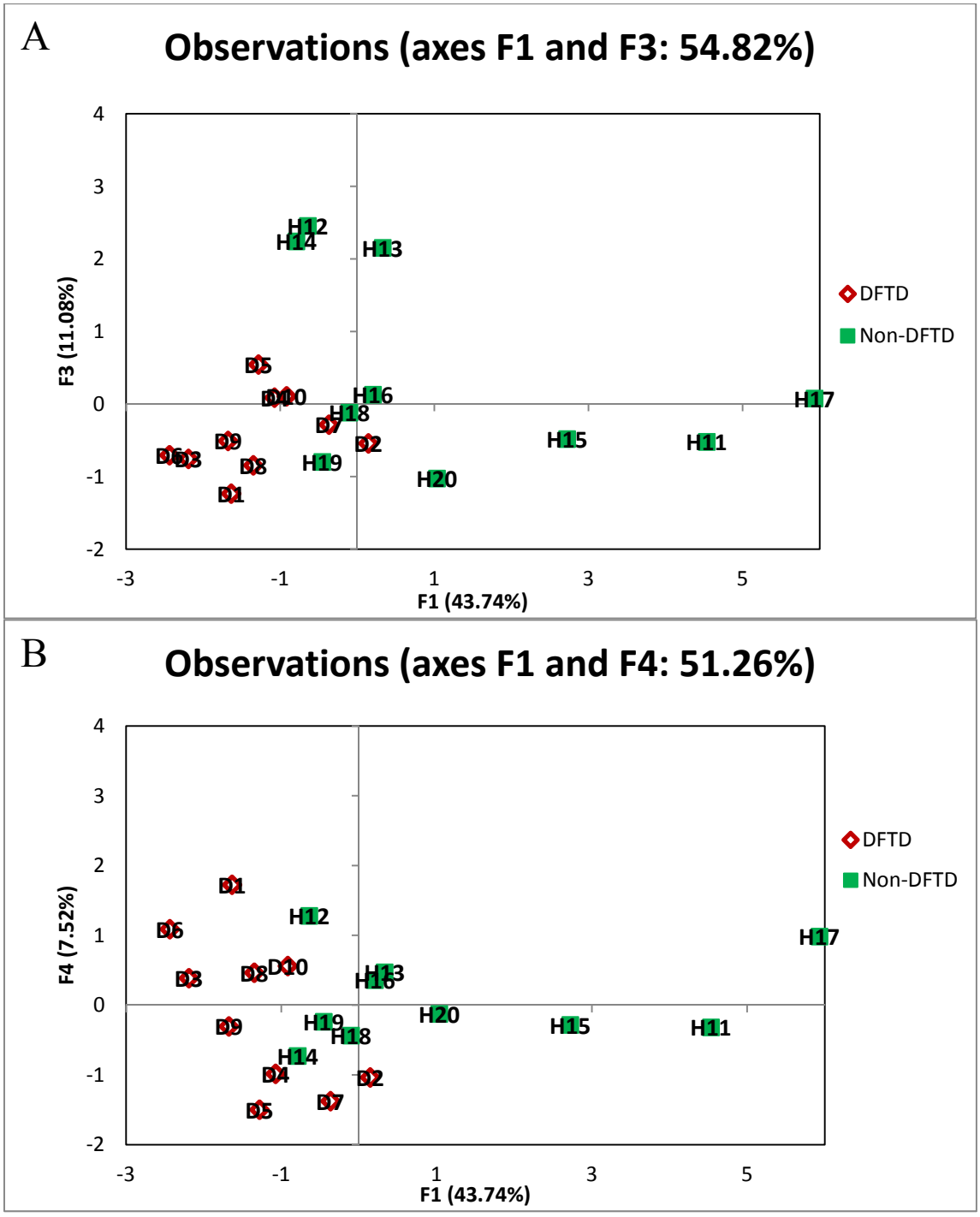

Figure 5.2: Principal component analysis of samples in the training set of the first and third principal component (A) and first and fourth principal component (B). The chromatographic data of 10 DFTD and 10 non-DFTD samples was analysed with Student's *t*-test to determine the peaks that were significantly different between these two groups ($p \leq 0.10$). The resulting peaks were then analysed by PCA.

where the first PC had the highest influence in separating the DFTD samples from the non-DFTD samples, both of the PCs are involved in separating the DFTD and non-DFTD samples in Figure 5.3. The two eclipses in Figure 5.3 show the distribution of the DFTD and non-DFTD samples. The eclipse showing the distribution of the non-DFTD samples overlaps with the eclipse surrounding the DFTD samples as there are four non-DFTD samples that clustered in the midst of the DFTD samples. When the sample set was un-blinded, information on the gender, age and location the animal was caught was obtained. These samples came from two females and two males of various ages ranging between one to six years old and from four different locations around mainland Tasmania. None of these sample variables explained why these four samples may have clustered with the DFTD samples. After the un-blinding it was found that three of the samples in the training set (samples 55, 92 and H20) had come from devils that had non-DFTD growths. The presence of samples from devils with other diseases would hopefully increase the specificity that this test could be specific to DFTD and not be indicative of any disease in general. These three samples from devils with non-DFTD growths did cluster close to or within the DFTD samples which suggests that the metabolites obtained in the feature selection show changes that occur with the presence of all abnormal growths not just those caused by DFTD. The other three non-DFTD samples (H11, H14 and H15) clustering with the DFTD samples in this plot (Figure 5.3) were considered to be healthy when they were trapped. Sample H11 was obtained from a three year old male that had been caught at West Pencil Pine. He was caught nine months later in the same location and still appeared to be DFTD free. The latency period of DFTD is still unknown, but it is believed to be less than 12 months so it is unlikely that sample H11 was a pre-clinical DFTD sample. No information on the devils that provided sample H14 and H15 was obtained from subsequent trapping trips so there is a possibility that these devils had contracted DFTD.

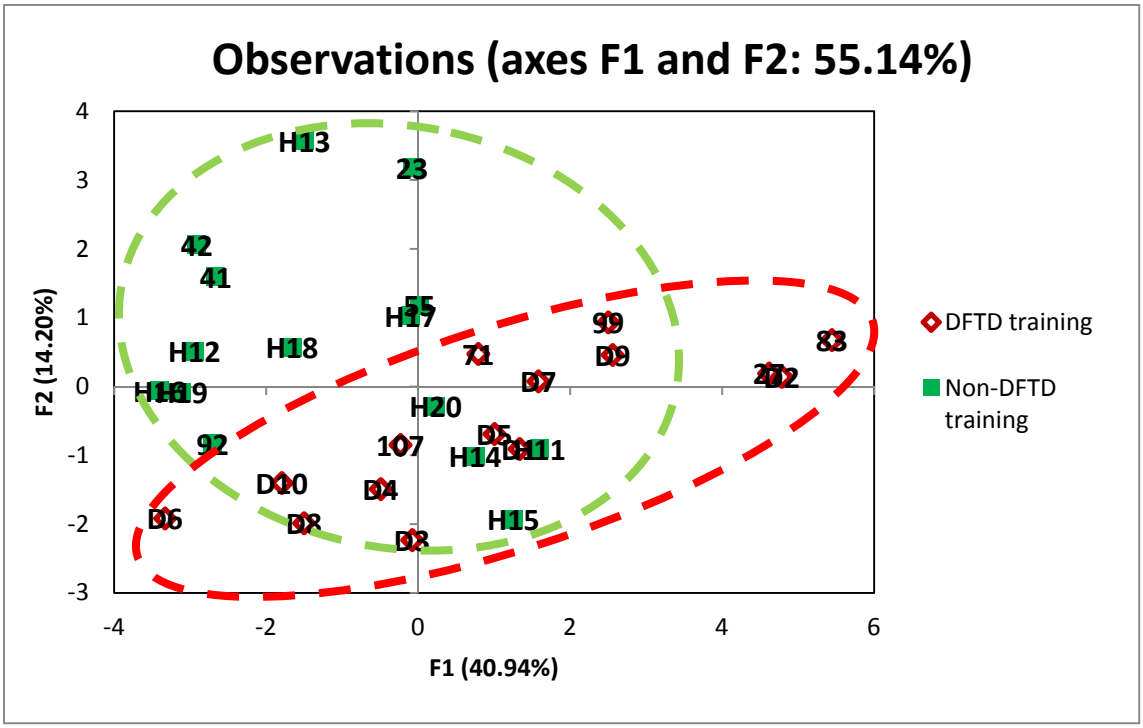

Figure 5.3: Principal component analysis of samples in the extended training set. The chromatographic data of 15 DFTD and 15 non-DFTD samples was analysed with Student's *t*-test to determine the peaks that were significantly different between these two groups ($p \leq 0.10$). The resulting peaks were then analysed by PCA.

A simple way to eliminate one of the sample variables that could affect the metabolome was to analyse each gender separately. Studies on gender differences in species have been observed previously. A study by Hines *et al.* showed increases in the presence of some metabolites in mussels (*Mytilus galloprovincialis*) and found that some metabolites were consistently higher in one gender or the other [22]. Another study by Plumb *et al.* investigated differences in the urine from mice between males and females. They analysed data from HPLC-ESI-TOF-MS using PCA and the PCA plots showed distinctive differences between male and female mice [122].

To analyse the males and females separately the training set of 30 that was used above was split into males and females and the gender of each sample was obtained. The female training set included nine non-DFTD samples (samples H13, H15, H17, H18, H20, 23, 41, 42 and 92) and five DFTD samples (samples D4, D6, D9, 27 and 83). The male training

set included six non-DFTD samples (samples H11, H12, H14, H16, H19 and 55) and ten DFTD samples (samples D1, D2, D3, D5, D7, D8, D10, 71, 99, and 107). These samples were analysed in the same way. A Student's *t*-test was conducted initially to determine which peaks had p-values ≤ 0.05 and 0.1. These sets of peaks were then analysed by PCA and combinations of the first four principal components were examined to find the combination that showed the greatest variability between the DFTD and non-DFTD samples.

The anlaysis of the female samples determined seven peaks that had a p-value ≤ 0.1 from Student's *t*-test that were analysed further by PCA. The first and third principal components showed the most variability between the DFTD and non-DFTD samples (see Figure 5.4). The DFTD samples along with sample H15 clustered together. H15 also clustered with the DFTD samples in Figure 5.3 where the males and females had been analysed together with the 30 sample training set. The rest of the female samples were also plotted onto this PCA as supplementary observations (samples that were not involved in the development of the PCA) but there was not good separation between the DFTD and non-DFTD samples (see Figure 5.5). As mentioned above, when the sample set became un-blinded, further information showed that two of the non-DFTD samples in the female training set (samples 92 and H20) were obtained from devils that had non-DFTD growths. This could explain why these samples were positioned close to the DFTD samples in the PCA plot. It is encouraging though that these samples did cluster with the non-DFTD observations even though the samples came from devils with non-DFTD disease.

In the anlaysis of the male samples the feature selection identified 7 peaks that had a p-value ≤ 0.05 and 11 peaks that had a p-value ≤ 0.10 from Student's *t*-test. The first and fourth principal components showed greatest variability between the DFTD and non-

DFTD samples in both instances but the $p \leq 0.05$ peaks showed the most separation between the DFTD and non-DFTD training samples which can be observed in Figure 5.6. Although sample H14 clustered close to the DFTD samples a line could be drawn that separates this sample away from the DFTD samples along with the rest of the non-DFTD training samples. The remaining male samples were added to the PCA plot as supplementary observations (see Figure 5.7). Only 5 out of the 22 DFTD samples that were not part of the training set clustered below the dotted line with the non-DFTD training samples but the majority of the non-DFTD samples also plotted above the dotted line with the DFTD training samples.

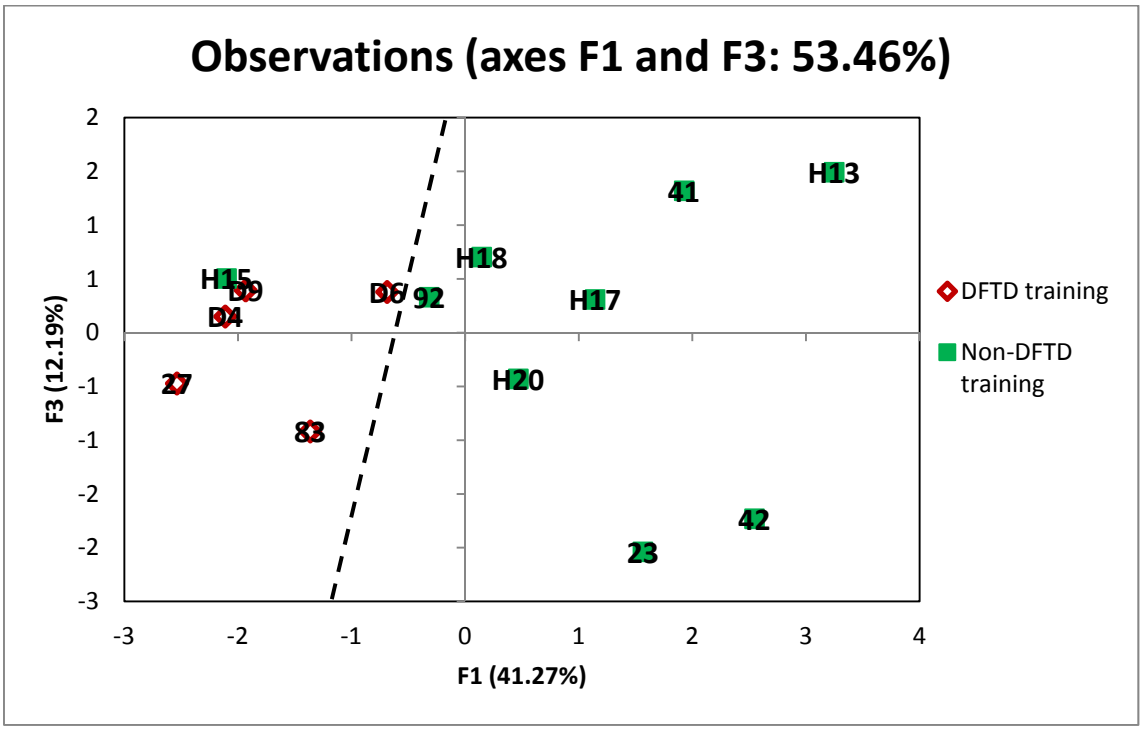

Figure 5.4: Principal component analysis of female training set samples analysed by GC-MS. 9 non-DFTD and 5 DFTD samples were used in the training set. A Students *t*-test was conducted and the seven peaks that had $p \leq 0.1$ were used to develop this PCA. The first and third principal components are used.

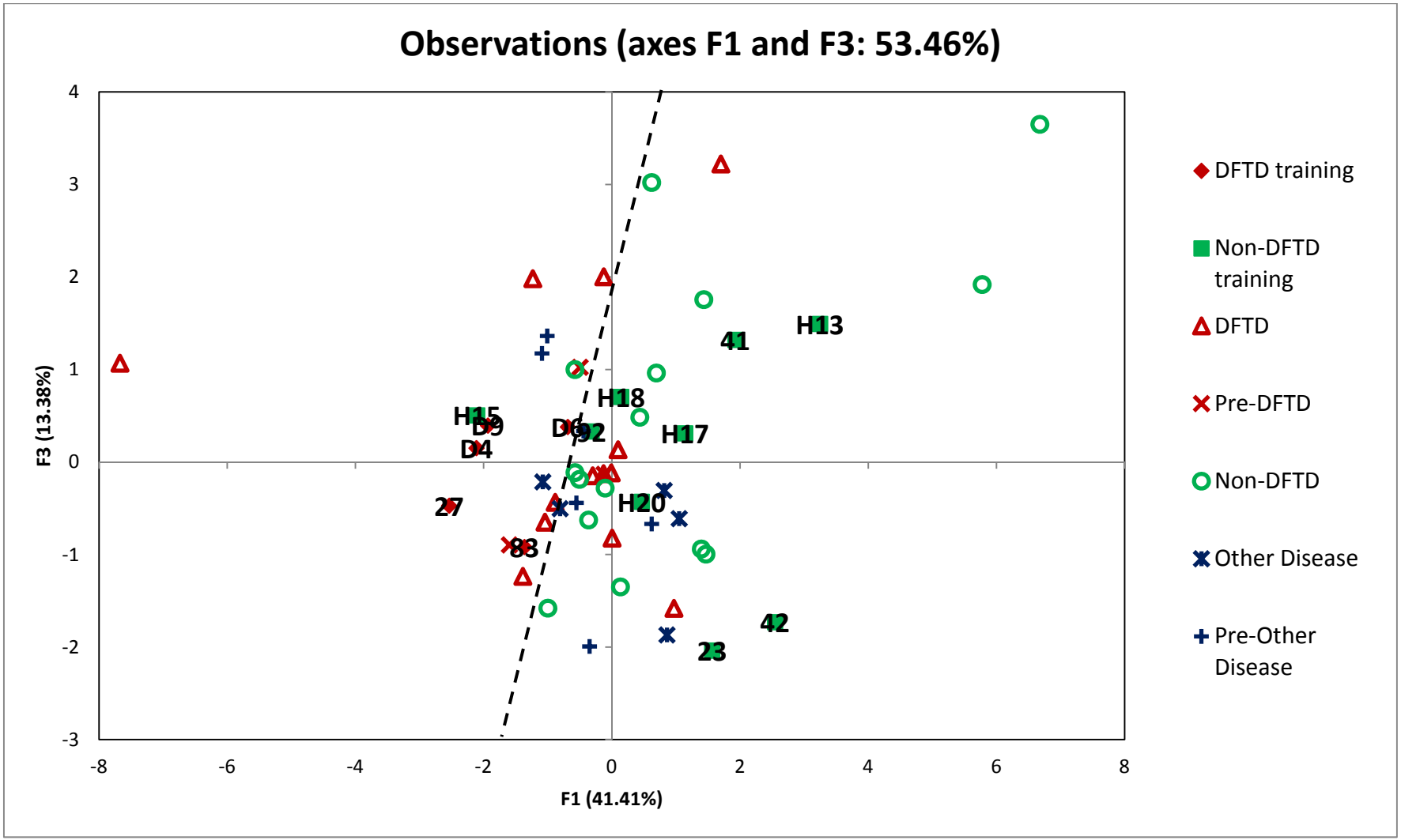

Figure 5.5: Principal component analysis of all female samples analysed by GC-MS. 9 non-DFTD and 5 DFTD samples were used in the training set. A Students *t*-test was conducted and the 7 peaks that had $p \leq 0.1$ were used to develop this PCA. The first and third principal components are used.

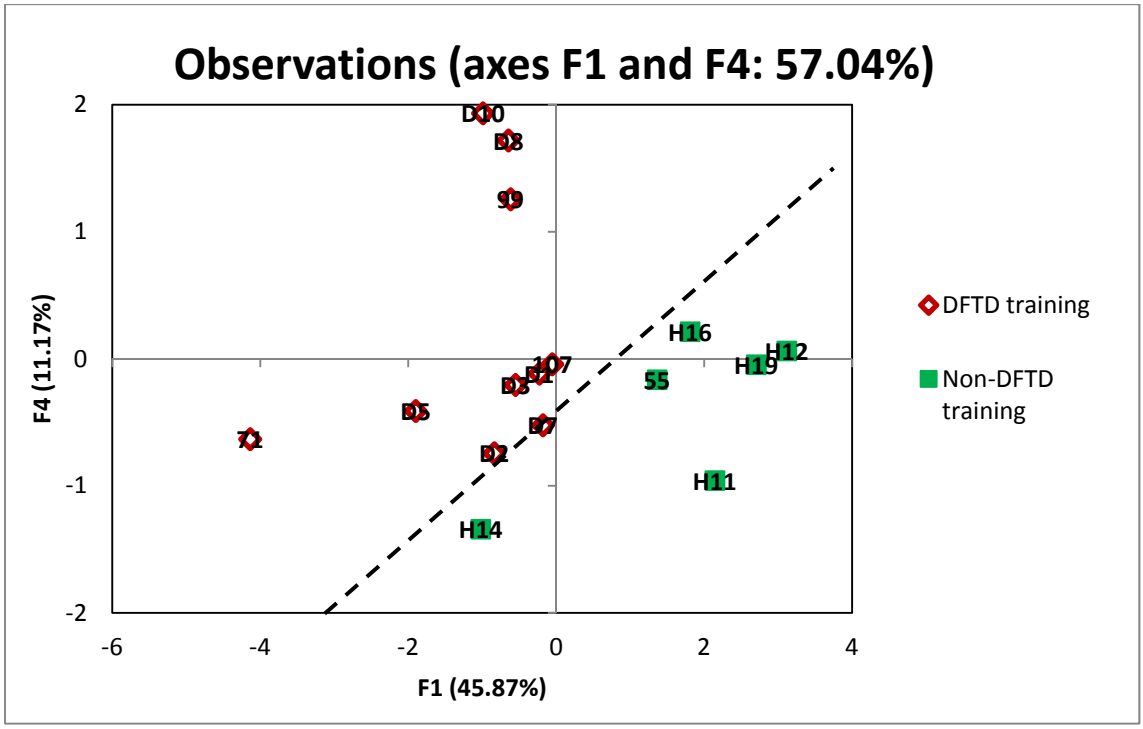

Figure 5.6: Principal component analysis of male training set samples analysed by GC-MS. 6 non-DFTD and 10 DFTD samples were used in the training set. A Students *t*-test was conducted and the 7 peaks that had $p \leq 0.05$ were used to develop this PCA. The first and fourth principal component are used.

The feature selection pre-selects the peaks that contribute the greatest in distinguishing between DFTD and non-DFTD samples. The PCAs developed with 14 samples for the female training set and 16 in the male training set was still unable to successfully distinguish between the DFTD and non-DFTD samples when all of the samples were added to the PCA plot. The PCA plots that just showed the training set did show enough variability between the DFTD and non-DFTD samples to separate these two sets more efficiently than when both genders were analysed together. The procedure was more successful at identifying differences between the DFTD and non-DFTD samples in the male devils. A higher proportion of DFTD samples were included in the male training set than in the female training set which may have contributed to the improved separation of DFTD and non-DFTD samples in the male PCA plots.

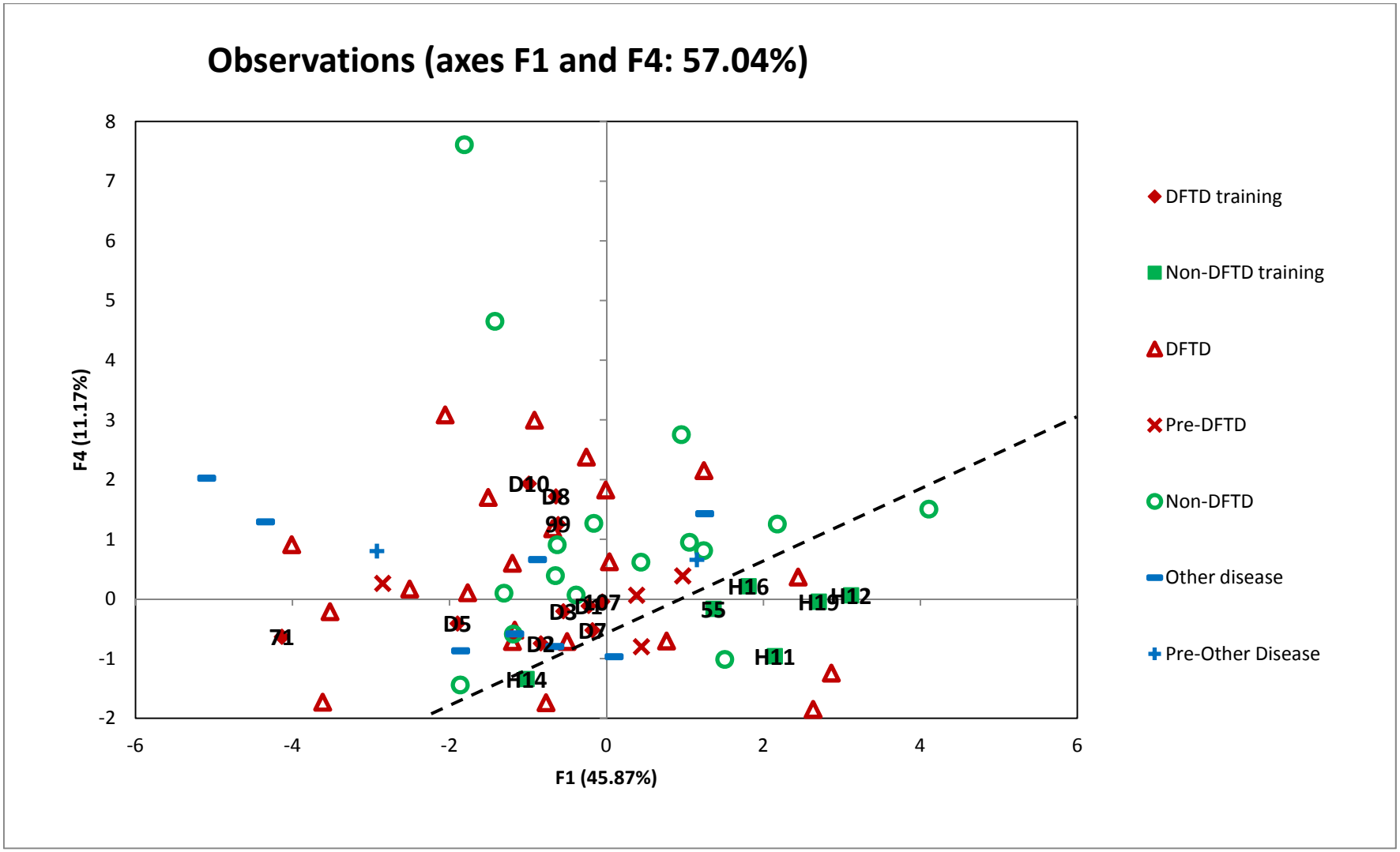

Figure 5.7: Principal component analysis plotting all of the male samples analysed by GC-MS. 6 non-DFTD and 10 DFTD samples were used in the training set. A Students *t*-test was conducted and the 7 peaks that had $p \leq 0.05$ were used to develop this PCA. The first and fourth principal components are used.

Separating the samples according to gender showed greater variability between DFTD and non-DFTD samples than the PCA plot including samples of both genders (see Figure 5.3, Figure 5.4 and Figure 5.6). The small number of samples in the training set is not efficient though to counteract the other variables such as diet; presence of other diseases; and sample location that can also affect the presence and abundance of metabolites. Once the set was un-blinded a larger training set was used to see if this could improve the results. If this was positive it would suggest that there were too many variables in this set for the number of samples that was being analysed.

It was believed that a larger sample set was still required to represent the number of sample variables other than gender that were represented in this sample group. The training set was increased to include a total of 70 % of the DFTD and healthy samples (samples without any disease or that were known to develop another disease later on) from the blind set. These were randomly selected to be used along with the original training set samples. The samples were also chosen so that equal numbers of DFTD and non-DFTD samples were used in the training set. Since looking at each gender separately had improved the results with the smaller training sets each gender was still examined separately. Overall there were 18 DFTD and 18 non-DFTD samples used in the male training set and 15 DFTD and 15 non-DFTD samples used in the female training set. As before, a PCA was developed using the peaks identified with p-values ≤ 0.05 and ≤ 0.1 using Student's *t*-test. Combinations of the first four principal components were examined to find the plot that showed the highest variability between the DFTD and non-DFTD samples.

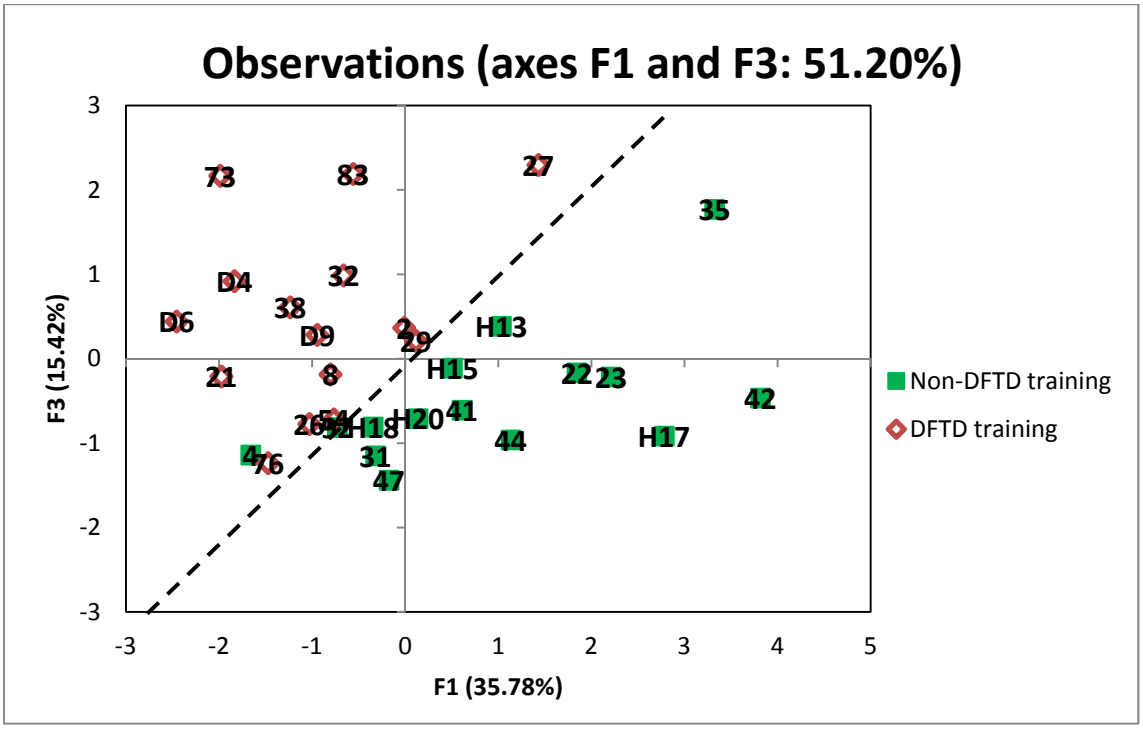

Figure 5.8: Principal component analysis of female training set samples analysed by GC-MS. 70 % of the non-DFTD and DFTD (15 non-DFTD samples and 15 DFTD samples) samples were used in the training set. A Students *t*-test was conducted and the 7 peaks that had $p \leq 0.05$ were used to develop this PCA.

The 70 % training set that was used with the female samples showed more promise than the 70 % male training set. The principal components that showed the greatest variability between the DFTD and non-DFTD samples were the first and third that was analysed the 7 peaks that had a value of $p \leq 0.05$ in the Student's *t*-test. Although some of the DFTD and non-DFTD samples clustered close together, it was possible to obtain a line of separation between the DFTD and non-DFTD samples (see Figure 5.8) except for one non-DFTD sample, sample 4. No further information was obtained from the devil that provided sample 4 so no supported theory on why this sample may have clustered with the DFTD samples could be determined except for the same explanation stated above for why four non-DFTD samples clustered with the males in the 70 % training set. Sample 92 also clustered closer to the DFTD samples than the other non-DFTD samples. This had also occurred when the smaller female training set was used (see Figure 5.4). As was

mentioned above, this sample had come from a devil that had a non-DFTD growth (neoplasia) which could explain the position of this point in the PCA plot. It is possible that the metabolites identified in the feature selection could be representative of changes that occurs in multiple types of tumours and abnormal growths not just DFTD which would explain why observations of samples from devils with non-DFTD growths cluster close or along with the DFTD observations. Since the PCA plot shown in Figure 5.8 showed promise in distinguishing the DFTD samples from the non-DFTD samples the remaining samples from the set were added to the PCA plot (see Figure 5.9). Once the sample set was un-blinded, it was very promising to see that the only DFTD sample to cluster with the non-DFTD sample was a pre-DFTD sample. The other two pre-DFTD samples clustered on the DFTD side of the plot. Another promising observation was that all of the samples that came from devils with other diseases clustered on the non-DFTD side of the plot except for one that came from a devil not with abnormal growths but with alopecia (baldness). There were samples that came from devils that later developed a non-DFTD disease that plotted with the DFTD devils. The "pre-other disease" sample that was positioned the furthest away from the non-DFTD section of the plot came from a devil that developed non-DFTD growths at a later stage which could explain why it was positioned with the DFTD samples. There were 3 samples that came from healthy devils that were not part of the training set that clustered amongst the DFTD samples (samples 11, 78 and 80). No further information was obtained on these devils so it is uncertain if these devils did later develop DFTD or another disease. The results are summarised in Table 5.2. The results showed this method to have a sensitivity of 95 % to correctly identify the DFTD and pre-DFTD samples and a specificity value of 80 % to correctly identify the healthy and devils with other diseases as DFTD-negative.

Table 5.2: Summary of the results obtained by analysing the female samples by GC-MS and PCA. 70 % of the samples were included in the training set. The PCA can be observed in Figure 5.9.

		DFTD status according to PCA (Figure 5.9)	
		DFTD	**Non-DFTD**
Clinically diagnosed DFTD status	**DFTD**	16	0
	Pre-DFTD	2	1
	Healthy	4	20
	Other Disease	1	4
	Pre-other disease	2	4

A PCA plot of the 70 % training set for the male devils was not as successful at separating the non-DFTD samples from the DFTD samples as the initial smaller male training set (see Figure 5.10). In the male plot the first two principal components from the PCA that used the signals with p-values ≤ 0.1 determined in the *t*-test showed the greatest variability between the DFTD and non-DFTD samples. There was a clear section in the plot that showed separation of non-DFTD samples from the DFTD samples in the training set but there were still four non-DFTD samples that clustered with the DFTD samples. Since there was a definite non-DFTD section on the plot the remaining samples were added to the plot as supplementary observations (see Figure 5.11). 2/4 of the non-training healthy (no disease) samples clustered within the DFTD section of the plot and 3/13 of the non-training DFTD samples clustered within the non-DFTD section of the plot. Promisingly all of the pre-DFTD samples clustered with the DFTD samples, even though there was not complete separation between the non-DFTD and DFTD samples in the training set. Once the sample set was un-blinded, no more information was available on the devils that

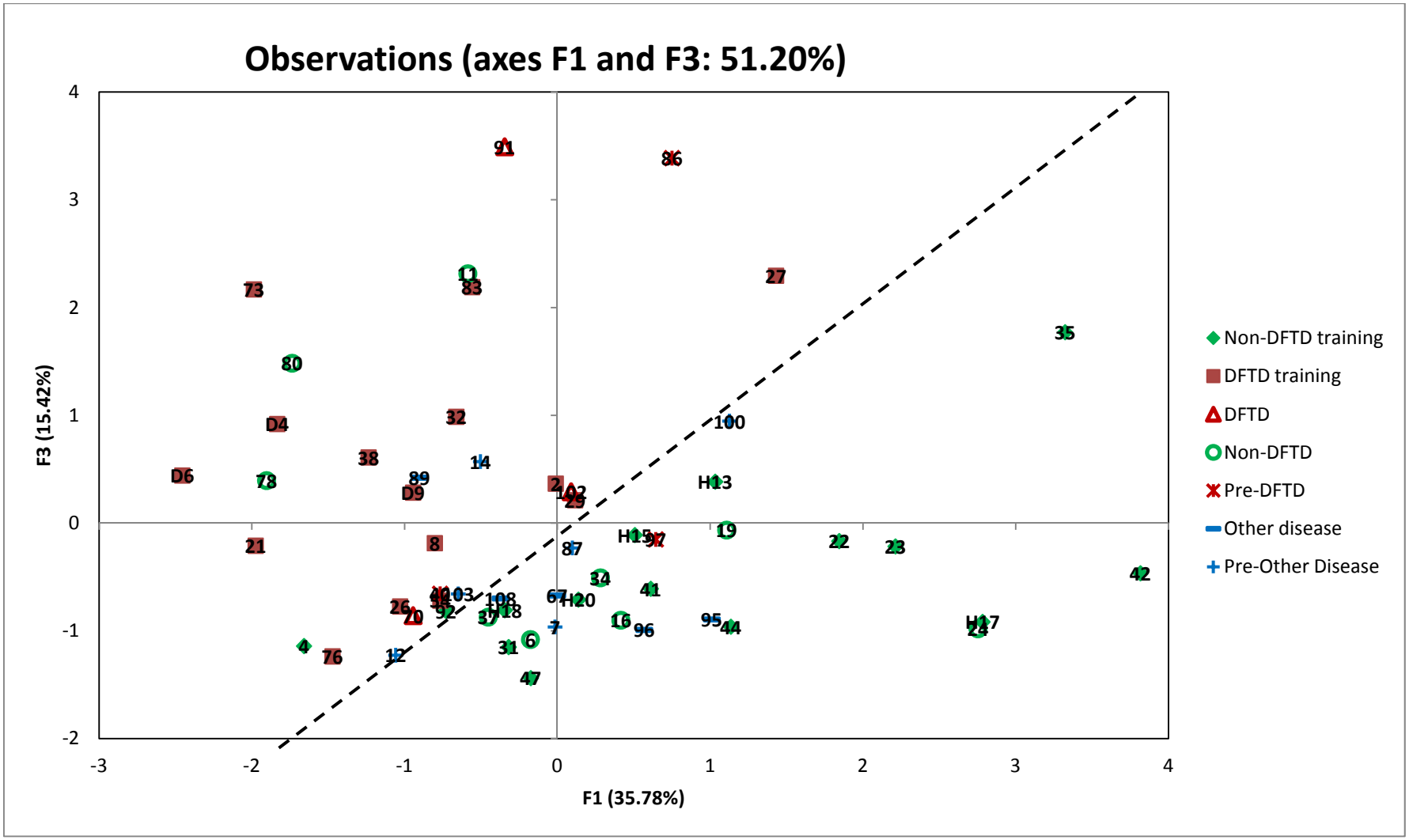

Figure 5.9: Principal component analysis of all female samples analysed by GC-MS. 70 % of the non-DFTD and DFTD (15 non-DFTD samples and 15 DFTD samples) samples were used in the training set. A Students *t*-test was conducted and the 7 peaks that had $p \leq 0.05$ were used to develop this PCA.

provided the four non-DFTD samples from the training set that clustered within the DFTD section of the plot and neither of the devils had appeared to be affected by other diseases at the time of sampling. The analysis of the male samples was inferior to the results obtained when analysing the female samples. In the female 70 % training set, the DFTD samples were obtained from ten different locations around Tasmania whereas in the male training 70 % training set the samples were obtained from six different locations and two thirds of these samples were obtained from only two separate locations, Forestier Peninsula and Bangor. This suggests that sample location does increase the variability of the samples and should be well represented in the training set as was the case with the females (see Figure 5.8 and Table 5.3). If the training set of the males represented samples from more locations these results could potentially be improved. A training set more representative of the different sampling locations could not be obtained from this set of samples to test this because 58 % of all the male DFTD samples in this blind study were obtained from either the Forestier Peninsula or Bangor (see Table 5.3) for distribution of samples according to sampling location and DFTD status). Samples of non-DFTD samples from these two locations were not provided from the Forestier Peninsula and only two were provided from Bangor which increased the bias these samples had with the DFTD training samples.

Only two of the metabolites (peaks 67 and 166) used in the PCA's to classify DFTD and non-DFTD samples were identified as being significant in both the female and male samples (see 7.2 Appendix 2: Fold Changes Observed for the Significantly Different Peaks for Both Blind Studies,

Table A 6) when using 70 % of the samples in the training set. Peak 166 showed < 0.5 fold change in pre-DFTD and DFTD females but only in the pre-DFTD males. There was a greater abundance of the metabolite peak 67 in the pre-DFTD males and females. The selection of different significant peaks between the genders correlates with other research that gender can affect the metabolome footprint of a disease [23].

Table 5.3: Distribution of sampling locations for the male and female samples also showing the distribution of samples obtained from non-DFTD and DFTD devils for each gender.

Sampling Location	All males	DFTD males	Non-DFTD males	All females	DFTD females	Non-DFTD females
Forestier	9	9	0	2	2	0
Reedy Marsh	2	2	0	2	2	0
Buckland	2	2	0	2	1	1
Bangor	12	10	2	6	4	2
West Pencil Pine	10	2	8	11	2	9
Fentonbury	4	2	2	5	2	3
Coles Bay	5	2	3	2	2	0
Granville	5	0	5	4	0	4
Bicheno	3	0	3	0	0	0
Trowunna	6	0	6	5	0	5
Mount Pleasant	5	2	3	8	1	7
Wisedale	1	0	1	3	1	2
Bronte Park	1	1	0	0	0	0
Taranna	3	0	3	0	0	0
Narawntapu	1	1	0	1	0	1
Marrawah	1	0	1	0	0	0
Hobart	0	0	0	2	0	2
Deloraine	0	0	0	1	1	0
Taroona	0	0	0	1	1	0
Westbury	0	0	0	1	0	1
Mole Creek	0	0	0	1	1	0
Temma	0	0	0	1	0	1
Alcomie	0	0	0	1	0	1
Sidmouth	0	0	0	1	0	1
Total samples	*70*	*33*	*37*	*60*	*20*	*40*

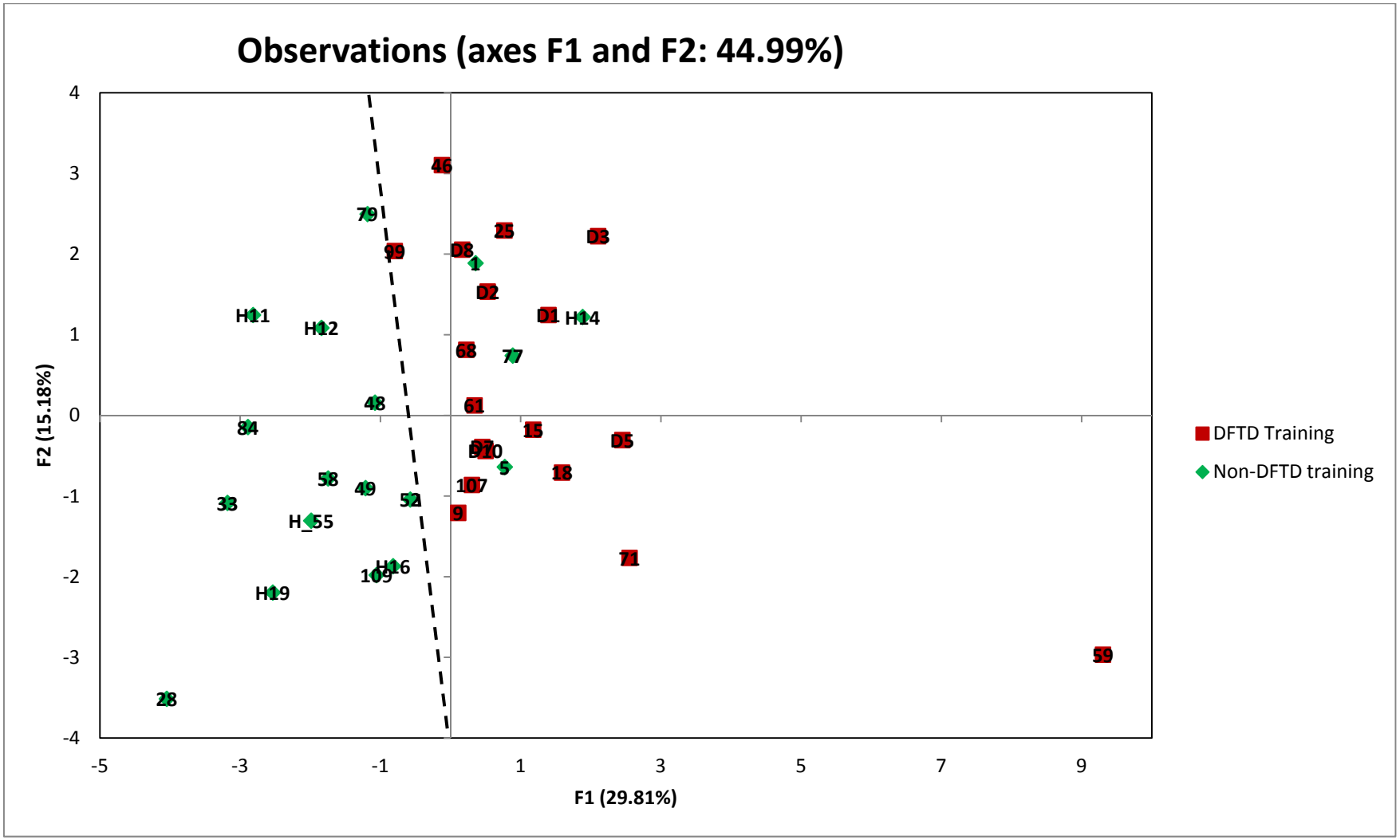

Figure 5.10: Principal component analysis of male training set samples analysed by GC-MS. 70 % of the non-DFTD and DFTD (18 non-DFTD samples and 18 DFTD samples) samples were used in the training set. A Students *t*-test was conducted and the 17 peaks that had $p \leq 0.1$ were analysed with PCA.

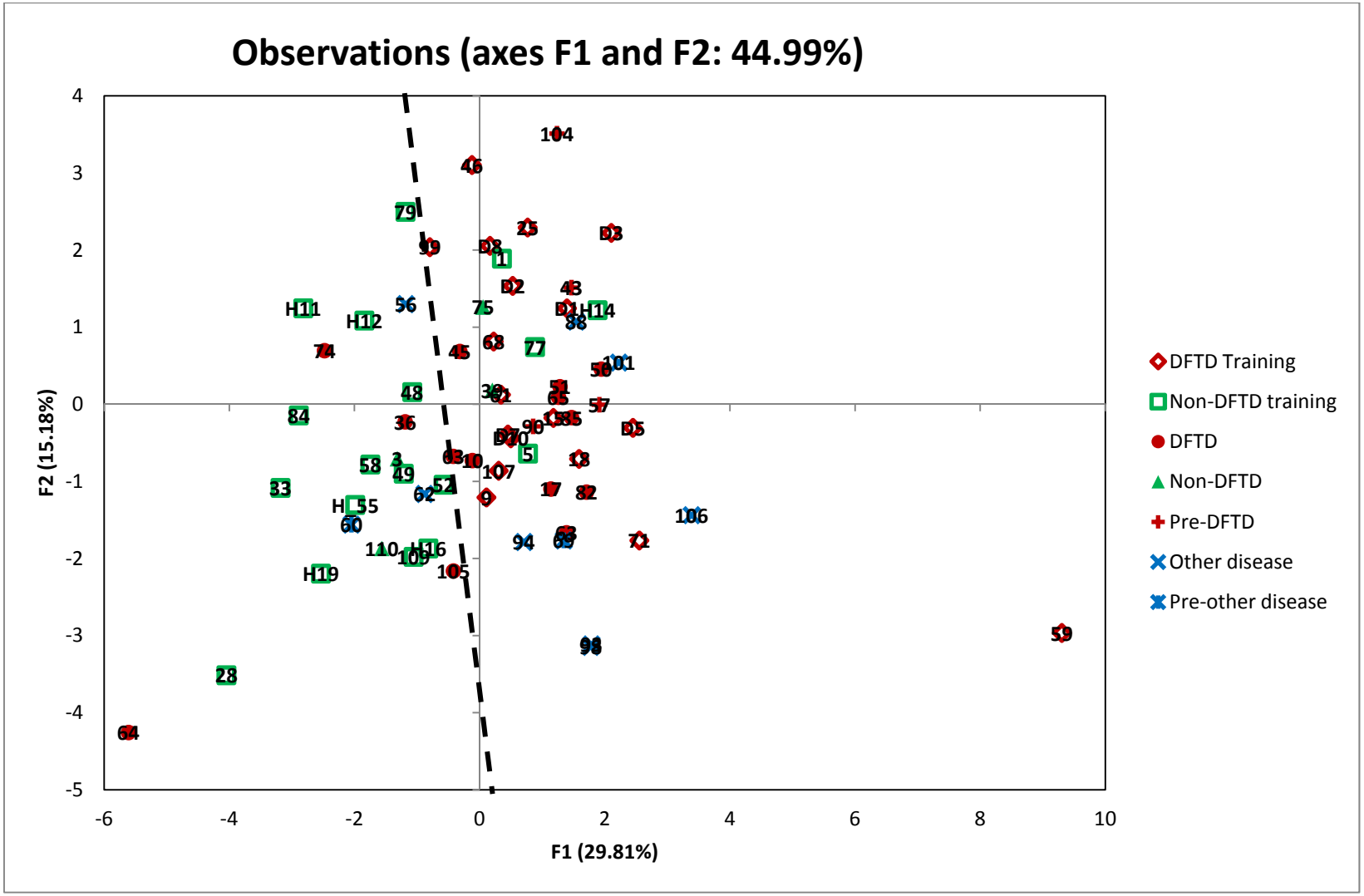

Figure 5.11: Principal component analysis of all male samples analysed by GC-MS. 70 % of the non-DFTD and DFTD (18 non-DFTD samples and 18 DFTD samples) samples were used in the training set. A Students *t*-test was conducted and the 17 peaks that had $p \leq 0.1$ were used to develop this PCA. Samples not included in the training set were added as supplementary observations.

5.3.2 ESI-MS

The data obtained using the ESI-MS method was analysed by PCA and looked at each gender individually since the previous work on the data obtained with GC-MS showed superior results when analysing the genders separately with PCA. The mass spectrums of these samples gave responses for over 2000 m/z values so Student's *t*-test was used to conduct a feature selection and decrease the number of peaks that would participate in further analysis. Feature selections were conducted with 70 % of DFTD and healthy samples. In the females there were less DFTD samples than healthy samples so 70 % of DFTD samples were included in the 70 % feature selection (13) with the same number of healthy samples. The male set included 15 non-DFTD (healthy) samples and the same number of DFTD samples. The samples included in these feature selections were chosen randomly using the "random function" in Microsoft Excel.

5.3.2.1 Females

The feature selection on the female samples analysed via ESI-MS, determined that 64 peaks were significantly different (p-value ≤ 0.05) between the DFTD and healthy samples in the training set. A PCA was then conducted on all of the samples using these peaks and the results are summarised in Table 5.4. This PCA was able to successfully separate the DFTD samples from the healthy samples as well as all but one "pre-other disease" samples. Information on what disease this "pre-other disease" (sample 87) was not given. The sensitivity of this test for the females was 95 % and the specificity was calculated to be 72 %. Promisingly the pre-DFTD samples also clustered with the DFTD samples except for sample 97. This PCA was not able to separate all of the "other disease" samples from the DFTD samples though. Interestingly some of the samples that came from devils with other types of cancers/growths (samples 92 and 95) clustered with healthy and DFTD samples so the significant peaks obtained from the feature selection were not specific to

diagnosing all types of cancer. There were other samples that also came from devils with non-DFTD growths (samples 67, 103) that did cluster with the DFTD samples. Samples that came from devils with other types of diseases (skin lesions, 108; baldness, 89) also clustered with the DFTD devils.

Table 5.4: Summary of the results obtained by analysing the female samples by ESI-MS and PCA using 70 % of the samples in the training set. The PCA can be observed in Figure 5.12.

		DFTD status according to PCA (Figure 5.12)	
		DFTD	**Non-DFTD**
Previously Determined DFTD status	**DFTD**	17	0
	Pre-DFTD	2	1
	Healthy	4	18
	Other Disease	5	3
	Pre-other disease	1	4

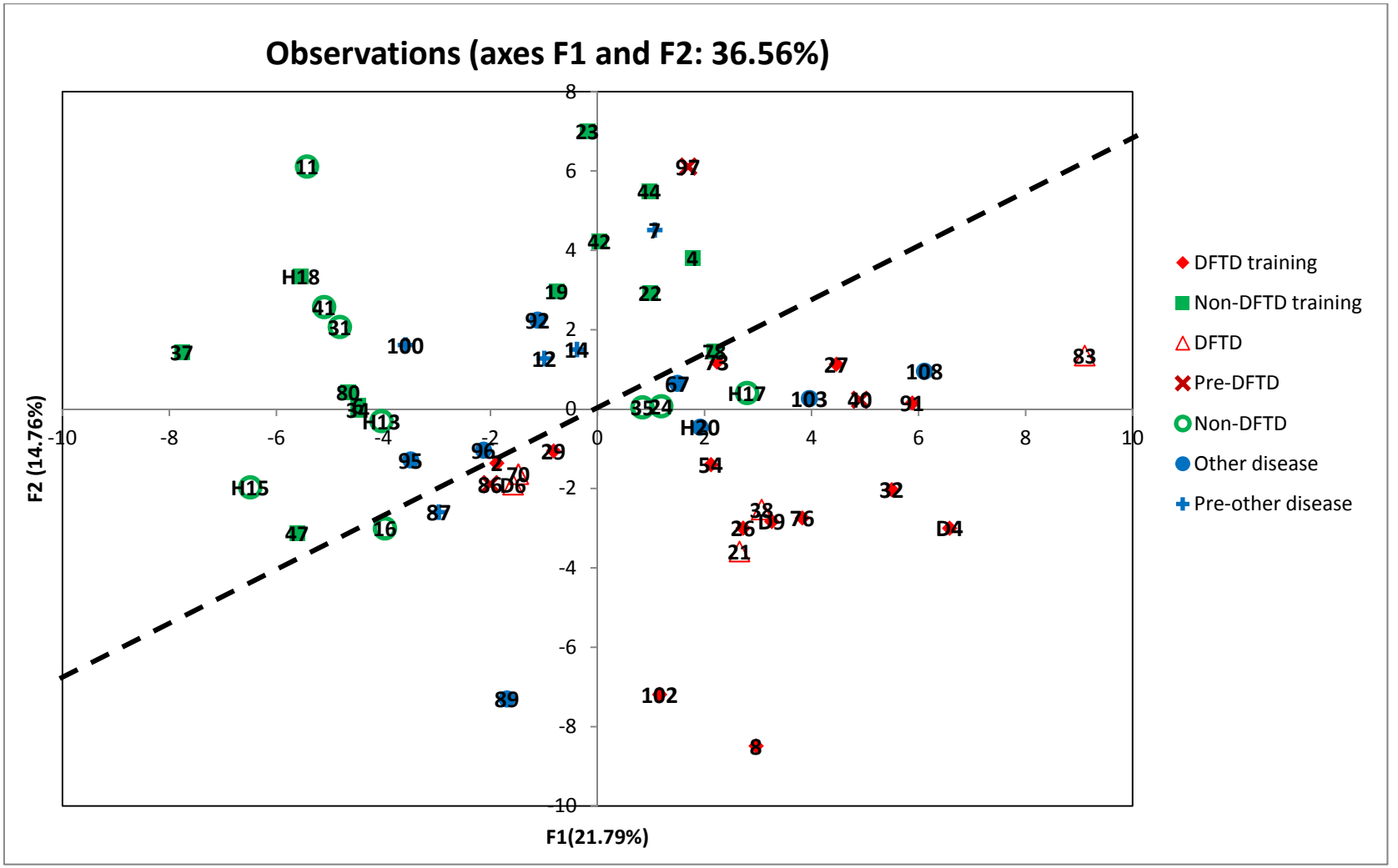

Figure 5.12: PCA plot of female samples analysed by ESI-MS. Initially a feature selection was conducted using Student's *t*-test and a PCA was conducted using the peaks that were found to be significantly different between 70 % of the DFTD and healthy samples to $p \leq 0.05$.

5.3.2.2 Males

A PCA analysis of the male samples was conducted using the 103 peaks that had a value of $p \leq 0.05$ that were determined with Student's *t*-test on the training set samples (see Figure 5.13). The PCA score plot did not completely separate the healthy samples from the DFTD samples when the first principal component was plotted with the second, third or fourth PC. The score plot that plotted the first and second principal component gave the best separation but this score plot sufficiently separated the majority of the healthy samples from the DFTD samples. Since one of the purposes of this test is to ensure that only non-DFTD devils are placed into insurance populations this is better than DFTD devils clustering with healthy samples. Only one of the pre-DFTD samples (sample 104) clustered with the DFTD samples. The remaining pre-DFTD samples clustered together in the lower right hand side of the plot along with sample 88 which came from a devil that had non-DFTD growths. This could be for a couple of reasons, firstly this test may not be sensitive enough to detect that these samples were diseased and secondly, it is possible that these devils had not developed DFTD at the time of analysis. Interestingly, all of the pre-other disease and other disease samples clustered with the DFTD samples except for sample 88. This suggests that the variability of the metabolites between these samples is more apt at showing the presence of disease generally than being specific to DFTD. The PCA of the male samples was not as sufficient as separating the DFTD and non-DFTD samples as the analysis on the female samples was which, as with the GC-MS results, is likely to be because of the bias in sample location for the male DFTD samples (see section 5.3.1).

From the metabolites considered to be significantly different using Student's *t*-test, only 3 were the same as those identified in the female samples (see 7.2 Appendix 2: Fold

Changes Observed for the Significantly Different Peaks for Both Blind Studies, Table A 5). This is a similar to the result obtained with analysis via GC-MS (see section 5.3.1). Interestingly the 560.3 mass ion was observed to have an average ~2 fold change in female pre-DFTD samples but ~ 0.5 fold change compared to non-DFTD samples in pre-DFTD males and both male and female DFTD samples. A similar response is observed with the 799.7 mass ion where the response for DFTD samples is very similar in both male and females but no fold change is observed in female pre-DFTD samples but this metabolite is down-regulated on average in samples obtained from male pre-DFTD devils. The third metabolite ion observed to be significant in both male and female samples from this sample set when analysed by ESI-MS was the 948.5 mass ion. Interestingly this metabolite was up-regulated in male pre-DFTD and non-DFTD samples but either not present or present in low amounts, in the female samples. From all of the metabolites considered to be significantly different only one was the same as those identified with in the blind study with low sample variability (discussed in chapter 4). These results show that differences in gender (as also observed with the GC-MS results) and sampling location can affect a disease metabolome as also observed by Sugimoto and Ward and their respective colleagues [23, 101].

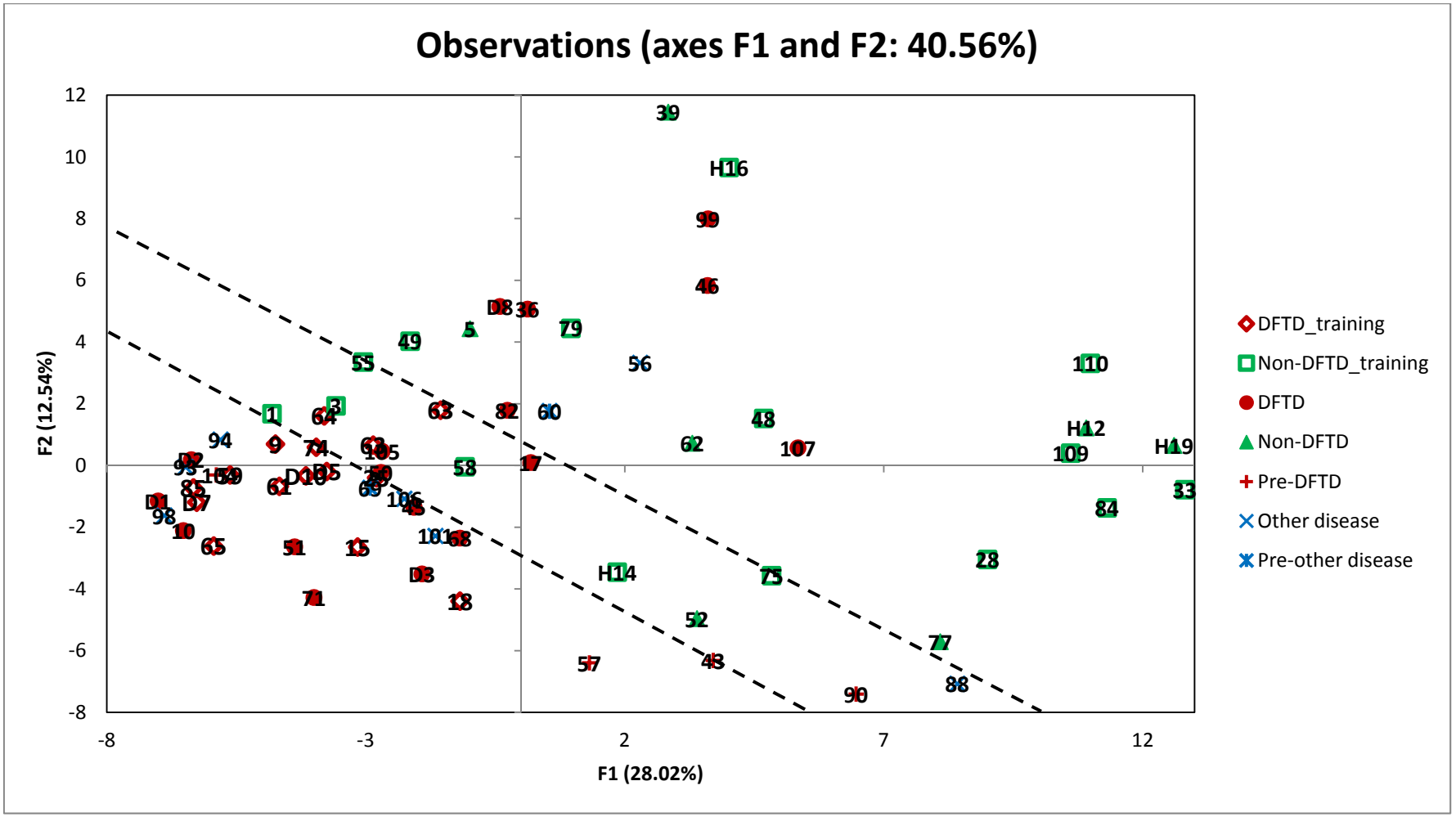

Figure 5.13: PCA plot of male samples analysed by ESI-MS. Initially a feature selection was conducted using Student's *t*-test with 70 % of the healthy samples (15 samples) and the same number of DFTD samples. A PCA was then conducted using the peaks that were found to be significantly different between all DFTD and healthy samples to $p \leq 0.05$. The samples in the bottom right hand corner were considered to be DFTD and the samples above the top line were considered to be non-DFTD. Samples positioned between the two lines were classed as uncertain.

5.3.3 Comparison of results between ESI-MS and GC-MS

To compare the methods the predicted DFTD status with each method, when 70 % of the samples were included in the training set, was compared for each sample (see Table 5.5 and Table 5.6). This comparison included the position of the training set samples since the training set was chosen randomly for both methods. The GC-MS method showed slightly better accuracy that the ESI-MS method which is likely because of the prior separation of the metabolites before detection with MS. For the female samples, 82 % were correctly identified using the GC-MS method; 75 % using the ESI-MS method; and 64 % of the samples were correctly identified with both methods. For the males, 60 % were identified using GC-MS; 58 % were correctly classified using the ESI-MS method; and 48 % of the samples were identified correctly with both methods. If a DFTD status had been uncertain in one of the methods or the methods gave contradicting results the diagnosis was determined to be uncertain. There were 17 uncertain results with the females and over all, only 3 (5 %) samples were classified wrongly using both methods. In the males, 28 samples had uncertain results and 7 samples were classified wrongly with both methods. As had been seen when looking at the males using the GC-MS and ESI-MS methods, the results for the males was inferior to the results obtained for the female samples probably because of the bias in sampling locations (see section 5.3.1 and Table 5.3).

One potential for the practical applications for using both of these methods to test for DFTD, would be to use the quicker ESI-MS method to screen for DFTD and then use the slower GC-MS method for confirmation or to diagnose the samples that were considered uncertain with the ESI-MS method. When looking at the female samples, 6 were considered uncertain with the ESI-MS method. All of these via one were correctly identified with the GC-MS method. This sample was classified as DFTD positive with the GC-MS method. If this method is used to ensure devils entering into insurance populations

are DFTD free it would be more important to ensure that there are no false negatives than ensuring that all healthy devils are classified as DFTD. The samples classified wrongly with the ESI-MS method (7 samples from the female set) were all classified as DFTD except for one pre-DFTD sample that was classified as non-DFTD. This sample (sample 97) was also classified as non-DFTD using the GC-MS method. It is possible that this devil had not contracted the disease at the stage of sampling. These results suggest that the ESI-MS method would be suitable to initially screen the samples and the GC-MS method is suitable to confirm any results considered as uncertain. Further validation though would be required before this could be confirmed.

Table 5.5 Comparison of DFTD diagnosis of female samples between GC-MS and ESI-MS methods.

Sample	Clinical diagnosis	GC-MS training	GC-MS prediction	ESI-MS training (Y/N)	ESI-MS prediction	Overall result	Correct?
D4	DFTD	Yes	DFTD	yes	DFTD	DFTD	yes
D6	DFTD	Yes	DFTD	no	DFTD	DFTD	yes
D9	DFTD	Yes	DFTD	yes	DFTD	DFTD	yes
H13	Non-DFTD	Yes	Non-DFTD	no	Non-DFTD	Non-DFTD	yes
H15	Non-DFTD	Yes	Non-DFTD	no	Non-DFTD	Non-DFTD	yes
H17	Non-DFTD	Yes	Non-DFTD	no	DFTD	Uncertain	no
H18	Non-DFTD	Yes	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
H20	Other Disease	Yes	Non-DFTD	no	Non-DFTD	Non-DFTD	yes
2	DFTD	Yes	DFTD	yes	Uncertain	Uncertain	no
4	Non-DFTD	Yes	DFTD	yes	Non-DFTD	Uncertain	no
6	Non-DFTD	No	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
7	Pre-Other Disease	No	Non-DFTD	no	Non-DFTD	Non-DFTD	yes
8	DFTD	Yes	DFTD	yes	DFTD	DFTD	yes
11	Non-DFTD	No	DFTD	no	Non-DFTD	Uncertain	no
12	Pre-Other Disease	No	Uncertain	no	Non-DFTD	Uncertain	no
14	Pre-Other Disease	No	DFTD	no	Non-DFTD	Uncertain	no
16	Non-DFTD	No	Non-DFTD	no	Uncertain	Uncertain	no
19	Non-DFTD	No	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
21	DFTD	Yes	DFTD	no	DFTD	DFTD	yes
22	Non-DFTD	Yes	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
23	Non-DFTD	Yes	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
24	Non-DFTD	No	Non-DFTD	no	DFTD	Uncertain	no
26	DFTD	Yes	DFTD	yes	DFTD	DFTD	yes

Sample	Clinical diagnosis	GC-MS training	GC-MS prediction	ESI-MS training (Y/N)	ESI-MS prediction	Overall result	Correct?
27	DFTD	Yes	DFTD	yes	DFTD	DFTD	yes
29	DFTD	Yes	DFTD	yes	DFTD	DFTD	yes
31	Non-DFTD	Yes	Non-DFTD	no	Non-DFTD	Non-DFTD	yes
32	DFTD	Yes	DFTD	yes	DFTD	DFTD	yes
34	Non-DFTD	No	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
35	Non-DFTD	yes	Non-DFTD	no	DFTD	Uncertain	no
37	Non-DFTD	no	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
38	DFTD	yes	DFTD	no	DFTD	DFTD	yes
40	Pre-DFTD	no	DFTD	no	DFTD	DFTD	yes
41	Non-DFTD	yes	Non-DFTD	no	Non-DFTD	Non-DFTD	yes
42	Non-DFTD	yes	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
44	Non-DFTD	yes	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
47	Non-DFTD	yes	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
54	DFTD	yes	DFTD	yes	DFTD	DFTD	yes
67	Other Disease	no	Non-DFTD	no	Uncertain	Uncertain	no
70	DFTD	no	DFTD	no	DFTD	DFTD	yes
73	DFTD	yes	DFTD	yes	Uncertain	Uncertain	no
76	DFTD	yes	DFTD	yes	DFTD	DFTD	yes
78	Non-DFTD	no	DFTD	yes	Uncertain	Uncertain	no
80	Non-DFTD	no	DFTD	yes	Non-DFTD	Uncertain	no
83	DFTD	yes	DFTD	no	DFTD	DFTD	yes
86	Pre-DFTD	no	DFTD	no	DFTD	DFTD	yes
87	Pre-Other Disease	no	Non-DFTD	no	DFTD	Uncertain	no
89	Other Disease	no	DFTD	no	DFTD	DFTD	no

Sample	Clinical diagnosis	GC-MS training	GC-MS prediction	ESI-MS training (Y/N)	ESI-MS prediction	Overall result	Correct?
91	DFTD	no	DFTD	yes	DFTD	DFTD	yes
92	Other Disease	yes	Uncertain	no	Non-DFTD	Uncertain	no
95	Other Disease	no	Non-DFTD	no	Non-DFTD	Non-DFTD	yes
96	Other Disease	no	Non-DFTD	no	Uncertain	Uncertain	no
97	Pre-DFTD	no	Non-DFTD	no	Non-DFTD	Non-DFTD	no
100	Pre-other Disease	no	Non-DFTD	no	Non-DFTD	Non-DFTD	yes
102	DFTD	no	DFTD	yes	DFTD	DFTD	yes
103	Pre-other Disease	no	DFTD	no	DFTD	DFTD	no
108	Other Disease	no	Non-DFTD	no	DFTD	Uncertain	no

Table 5.6: Comparison of DFTD diagnosis of male samples between GC-MS and ESI-MS methods.

Sample	Clinical diagnosis	GC-MS training	GC-MS prediction	ESI-MS training (Y/N)	ESI-MS prediction	Overall result	Correct?
D1	DFTD	yes	DFTD	no	DFTD	DFTD	yes
D2	DFTD	yes	DFTD	no	DFTD	DFTD	yes
D3	DFTD	yes	DFTD	no	DFTD	DFTD	yes
D5	DFTD	yes	DFTD	yes	DFTD	DFTD	yes
D7	DFTD	yes	DFTD	yes	DFTD	DFTD	yes
D8	DFTD	yes	DFTD	no	Non-DFTD	Uncertain	no
D10	DFTD	yes	DFTD	yes	DFTD	DFTD	yes
H11	Non-DFTD	yes	Non-DFTD	Not run	-	Non-DFTD	yes

Sample	Clinical diagnosis	GC-MS training	GC-MS prediction	ESI-MS training (Y/N)	ESI-MS prediction	Overall result	Correct?
H12	Non-DFTD	yes	Non-DFTD	no	Non-DFTD	Non-DFTD	yes
H14	Non-DFTD	yes	DFTD	yes	Uncertain	Uncertain	no
H16	Non-DFTD	yes	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
H19	Non-DFTD	yes	Non-DFTD	no	Non-DFTD	Non-DFTD	yes
1	Non-DFTD	yes	DFTD	yes	Uncertain	Uncertain	no
3	Non-DFTD	no	Non-DFTD	yes	Uncertain	Uncertain	no
5	Non-DFTD	yes	DFTD	no	Non-DFTD	Uncertain	no
9	DFTD	yes	DFTD	yes	DFTD	DFTD	yes
10	DFTD	no	Uncertain	no	DFTD	Uncertain	no
15	DFTD	yes	DFTD	yes	DFTD	DFTD	yes
17	DFTD	no	DFTD	no	Uncertain	Uncertain	no
18	DFTD	yes	DFTD	yes	DFTD	DFTD	yes
25	DFTD	yes	DFTD	yes	Uncertain	Uncertain	no
28	Non-DFTD	yes	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
33	Non-DFTD	yes	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
36	DFTD	no	Non-DFTD	no	Non-DFTD	Non-DFTD	no
39	Non-DFTD	no	DFTD	no	Non-DFTD	Uncertain	no
43	Pre-DFTD	no	DFTD	no	DFTD	DFTD	yes
45	DFTD	no	Uncertain	no	DFTD	Uncertain	no
46	DFTD	yes	DFTD	no	Non-DFTD	Uncertain	no
48	Non-DFTD	yes	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
49	Non-DFTD	yes	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
50	DFTD	no	DFTD	no	Uncertain	Uncertain	no
51	DFTD	no	DFTD	no	DFTD	DFTD	yes

Sample	Clinical diagnosis	GC-MS training	GC-MS prediction	ESI-MS training (Y/N)	ESI-MS prediction	Overall result	Correct?
52	Non-DFTD	yes	Non-DFTD	no	Uncertain	Uncertain	no
55	Non-DFTD	yes	Non-DFTD	yes	Uncertain	Uncertain	no
56	Other Disease	no	Non-DFTD	no	Non-DFTD	Non-DFTD	yes
57	Pre-DFTD	no	DFTD	no	DFTD	DFTD	yes
58	Non-DFTD	yes	Non-DFTD	yes	Uncertain	Uncertain	no
59	DFTD	yes	DFTD	yes	DFTD	DFTD	yes
60	Pre-Other Disease	no	Non-DFTD	no	Non-DFTD	Non-DFTD	yes
61	DFTD	yes	DFTD	yes	DFTD	DFTD	yes
62	Other Disease	no	Non-DFTD	no	Non-DFTD	Non-DFTD	yes
63	DFTD	no	DFTD	yes	Uncertain	Uncertain	no
64	DFTD	no	Non-DFTD	yes	Uncertain	Uncertain	no
65	DFTD	no	DFTD	yes	DFTD	DFTD	yes
68	DFTD	yes	DFTD	no	DFTD	DFTD	yes
69	Pre-Other Disease	no	DFTD	no	DFTD	DFTD	no
71	DFTD	yes	DFTD	no	DFTD	DFTD	yes
74	DFTD	no	Non-DFTD	yes	Uncertain	Uncertain	no
75	Non-DFTD	no	DFTD	yes	Uncertain	Uncertain	no
77	Non-DFTD	yes	DFTD	no	Non-DFTD	Uncertain	no
79	Non-DFTD	yes	Uncertain	yes	Non-DFTD	Uncertain	no
82	DFTD	no	DFTD	no	Non-DFTD	Uncertain	no
84	Non-DFTD	yes	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
85	DFTD	no	DFTD	yes	DFTD	DFTD	yes
88	Other Disease	no	DFTD	no	Uncertain	Uncertain	no
90	Pre-DFTD	no	DFTD	no	Uncertain	Uncertain	no

Sample	Clinical diagnosis	GC-MS training	GC-MS prediction	ESI-MS training (Y/N)	ESI-MS prediction	Overall result	Correct?
93	Other Disease	no	DFTD	no	DFTD	DFTD	no
94	Other Disease	no	DFTD	no	DFTD	DFTD	no
98	Other Disease	no	DFTD	no	DFTD	DFTD	no
99	DFTD	yes	Uncertain	no	Non-DFTD	Uncertain	no
101	Other Disease	no	DFTD	no	DFTD	DFTD	no
104	Pre-DFTD	no	DFTD	no	DFTD	DFTD	yes
105	DFTD	no	Uncertain	no	Uncertain	Uncertain	no
106	Other Disease	no	DFTD	no	Uncertain	Uncertain	no
107	DFTD	yes	DFTD	no	Non-DFTD	Uncertain	no
109	Non-DFTD	yes	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes
110	Non-DFTD	no	Non-DFTD	yes	Non-DFTD	Non-DFTD	yes

5.4 Summary

These results highlighted how different sample variables such as gender and sampling location (habitat), can cause interferences in predicting disease specific metabolite biomarkers. This was shown by the PCA plots that were developed with ≤ 30 samples in the training set that were not efficient at distinguishing between the DFTD samples the non-DFTD samples that were not part of the training set. The inferior results obtained with the male samples also highlighted problems with sample variability potentially because of the bias in sampling locations of the DFTD samples.

The removal of some of the variability between samples improved the results. This was shown by the dividing the samples according to gender. With the larger training set that contained 70 % of the healthy and DFTD samples the female PCA plots were quite proficient at separating the DFTD and non-DFTD samples. The females provided more promising results than the males with sensitivities of 95 % with both the GC-MS and the ESI-MS method. This may have been because of the lack of variability in the sampling locations of the samples obtained from DFTD-positive males in this sample set. The results highlighted the need to ensure that the many variables that can affect an individual's metabolome are well represented in the set of samples used as the training set in biomarker discovery. A broader set of male samples is needed to try and improve the results obtained from this gender. Especially since better sensitivity and specificity was obtained with the females using this sample set than that obtained from the samples examined in chapter 4 which had less variability because of the restricted sampling area.

Overall this set of samples was too small for the number of variables to be able to identify metabolomic differences between all of the DFTD and non-DFTD samples. The results also highlighted that the presence of other diseases can be wrongly diagnosed as having

DFTD when examining the metabolites identified in the Student's *t*-test. The results discussed in this chapter were comparable to results obtained by Stentiford and co-workers who found greater differences in age and sampling location than disease in liver fish. They were able to diagnose 7 out of 10 fish that had liver tumours when looking at peptides [8, 101]. Further work is needed to look at the identified metabolites more closely to determine if these metabolites are specific to DFTD or are just changed because the presence of cancer or other diseases.

The techniques discussed in this chapter showed good sensitivity and specificity at correctly classifying non-DFTD and DFTD female devils. Both methods provided a sensitivity of 95 % and specificities of 80 and 72 % for the GC-MS and ESI-MS methods respectively. As discussed in section 5.3.3 there is a viable potential in using the quicker ESI-MS method as a quick screening method and the GC-MS method to confirm uncertain diagnoses.

6 Summary and Conclusions

6.1 Discussion of Techniques

The aim of this research was to develop a screening test for DFTD that was firstly applicable for sampling wild Tasmanian devils and secondly did not require a biopsy of the suspected DFTD tumour. Three different mass spectrometry methods (capillary electrophoresis – mass spectrometry (CE-MS), gas chromatography – mass spectrometry (GC-MS) and electrospray ionisation – mass spectrometry (ESI-MS)) were investigated to mine for disease metabolite biomarkers using non-targeted approaches. All three methods involved the injection of roughly equivalent volumes of serum considering the different sample preparation steps and dilutions conducted prior to injection. CE-MS and GC-MS involve the separation of metabolites prior to detection with mass spectrometry. The CE-MS method was not as sensitive or reproducible as the other two methods and therefore not investigated further. Both the ESI-MS method and GC-MS method, when analysed with a feature selection step followed by principal component analysis (PCA), were able to distinguish differences in the serum metabolites from devils with DFTD up to six months prior to visible tumours. This positive result obtained from two complimentary techniques supported the hypothesis that differences in the serum metabolome can be observed between DFTD-positive and DFTD-negative devils prior to visible manifestation of tumours around the face and mouth of diseased Tasmanian devils.

In the case of the ESI-MS method, the removal of a separation step decreased the analysis time for the sample. The preparation of samples for the ESI-MS method could be completed in less than 30 min and analysed within five min including pre and post instrument washings. Prior to GC-MS analysis the metabolites needed to be derivatised in

two steps. The first reaction with o-methylhydroxylamine hydrochloride was conducted either for at least 17 hours or for 90 min prior to the addition of MSTFA which was incubated further prior to sample injection. Each sample run then took between 10 to 60 min depending upon the length of the column. Even though the preparation and analysis time for the GC-MS method was considerably longer, prior separation of metabolites allowed the collection of further information by separating the metabolites by chemical type.

Encouragingly some of the metabolites identified had previously been associated with cancer. The most interesting of these was the observed increase in *myo*-inositol which has been previously associated with schwannomas [24]. This result supports the evidence presented by Murchison *et al.* that suggests that DFTD may be Schwann cell in origin [82]. The preliminary identification of these metabolites could aid in understanding more about DFTD and the discovery of potential treatment strategies.

There were two reasons for the development of a pre-clinical diagnostic test. Firstly, to ensure that devils entering into insurance populations were DFTD-free to decrease quarantine time and secondly to remove DFTD-positive devils in the wild before the display of tumours to eliminate or at least reduce the spread of the disease to other devils. It is beneficial to lean on the side of caution in the first instance to ensure that no DFTD devils enter into the insurance populations. On the other hand the genetic diversity of Tasmanian devils is quite low so the removal of healthy devils from wild populations because of lack of specificity in the diagnosis is not advisable.

After validation it would be possible to use this test to diagnose DFTD in wild devils since serum samples can be obtained during routine trapping trips of wild populations. The sample preparation method to obtain metabolites from the serum samples are obtained

identically for both the ESI-MS and GC-MS method. This allows for simultaneous analysis of samples using both of the developed methods. A pre-clinical metabolite biomarker diagnostic test for DFTD could be used to confirm diagnosis of DFTD to eliminate the removal of healthy devils from a wild population. The shorter, ESI-MS method could be developed further to ensure that no DFTD devils are wrongly classified as having DFTD. The devils could be held for short periods of time and then released back in the wild after the analysis of the sample. In turn the GC-MS method could be further developed as a highly sensitive technique to ensure that only DFTD devils are diagnosed as DFTD-positive. This proposition is plausible since the size and geography of Tasmania would allow for samples to be obtained; delivered to a lab; and analysed within 24 hours.

Currently, there are also portable GC-MS instruments on the market. The GC-MS method could be modified for a portable instrument so that samples could be analysed at a base camp on the field. The devils are also micro-chipped on initial capture so it is possible to obtain a blood sample, test for the disease and then remove the devil on subsequent trips if a positive DFTD diagnosis is obtained. These latter two options are more plausible because of the cost and effort that would be required to temporarily hold animals during the time necessary to transport the samples back to the laboratory and analyse for DFTD.

6.2 **Discussion of Sampling**

Prior to routine testing of devils for DFTD, a potential diagnostic test will need appropriate validation. The methods developed here could be validated for use or the metabolites identified with the GC-MS method could be used to develop a more targeted approach for a diagnostic test. The validated diagnostic test will be required to be effective to test all Tasmanian devils including individuals from DFTD and non-DFTD areas and diagnose DFTD pre-clinically. To aid in this, samples for this study were obtained from wild devils

trapped throughout Tasmania. The spread of DFTD is very extensive in eastern Tasmania and almost non-existent in the majority of north-western and western Tasmania. Unfortunately, there are other variables that are also different between these two subpopulations of devils that can affect an individual's metabolome which include habitat, diet and genetics as well as the presence of other diseases.

There were challenges in selecting appropriate samples to be included in the sample sets because of the variables between north-western and eastern devils. To effectively determine metabolite biomarkers specific to DFTD, ideally the non-DFTD and DFTD samples needed to both include samples from western and eastern Tasmania. Unfortunately, there are very limited devils that will remain DFTD free for 12 months in eastern Tasmania and limited DFTD positive devils in the western half of Tasmania. The presence and rate of spread of DFTD appears to be dependent upon the variability of the MHC protein found on the surface of cells that inform an individual's immune system of invading cells from other individuals from the same or different species. The Tasmanian devils from north-western and western Tasmania appear to express a different combination of the MHC proteins than their compatriots in eastern Tasmania which is believed to explain the absence of DFTD in western Tasmania.

The results in chapter 5 illustrated the importance of appropriately handling sample variables. When the samples were separated by gender enough variability could be observed between the non-DFTD and DFTD female samples. When additional samples were added to the female training set both the GC-MS and the ESI-MS methods had sensitivities of 95 % and specificities > 70 %. In the case of the male samples, the distinction between non-DFTD and DFTD devils was not observed as it had been with the females because of the bias in sample location of the DFTD samples. This highlighted the

importance of ensuring that the training set is well representative of as many metabolite varying variables as possible in both the DFTD and non-DFTD set of samples. The spread of this disease makes this a difficult requirement to obtain. The training set for the males had 18 non-DFTD and 18 DFTD devils where as the training set for the females had 15 of each. The feature selection step does aid in pre-selecting only the metabolites that are DFTD specific. Gender was also observed to affect the metabolites that are significantly different between non-DFTD and DFTD samples. From the ESI-MS metabolites identified as significant, only 3 were identified in both the male and female samples from the blind state wide study sample set. The fold differences between non-DFTD and pre-DFTD and non-DFTD and DFTD samples were also not the same with both genders. The metabolites observed using the GC-MS method only identified two metabolites that were significantly different with both genders although the fold changes with this method were similar for both male and female samples. The results for the females in Chapter 5 suggests that with careful training set selection that was representative of as many sampling locations as possible a training set of around 40 could be used to mine for significant metabolites that differ between DFTD and non-DFTD devils if the genders are examined separately.

6.3 Applicability as a Pre-Clinical Diagnostic Test for DFTD

The success of many potential intervention projects to combat DFTD is dependent upon the development of a diagnostic test that has the ability to diagnose all wild devils with or without signs of DFTD.

The initial work involved in this project showed variability between devils without DFTD and those with DFTD including samples obtained from devils 69 days prior to clinical signs. This result showed the potential to use metabolomics to diagnoses diseases in wild populations which can be difficult because of uncontrolled variables such as diet or when

the individual last ate. The techniques discussed in this project showed the ability to detect differences between DFTD and non-DFTD devils up to six months prior to the presentation of visible facial tumours even with sample sets of increased variability (see chapters 4 and 5). With the correct validation these techniques could be used on any devil to ensure that devils entering into insurance populations are DFTD-free and to help form wild populations of DFTD-free devils in eastern Tasmania through the re-location of DFTD devils in areas such as the Forrester Peninsula. Further research should examine the accuracy of diagnosing DFTD between one to six months pre-clinical. If all devils that show signs of DFTD three or four months after the initial trapping, quarantine times for devils entering into insurance populations could be reduced.

In the case of suppression trials, trapping frequencies could be reduced from once a month to every three months. Removing DFTD devils prior to identifiable tumours should decrease the rate of spread for this contagious disease. The ability to reduce trapping frequencies will also dramatically reduce the cost of a suppression trial. Tasmanian devils have low genetic diversity which follows the island founder effect but there are differences in the genetics between the northwest and eastern devils [4]. Many of the devils that have been placed into the captive insurance populations come from north-western populations since these populations are predominantly DFTD-free but this limits the range of genetic diversity. The success of a suppression trial in eastern Tasmanian could establish a population of wild DFTD-free east-coast devils which will help to sustain the genetic diversity of this unique and valuable carnivorous marsupial.

The techniques developed here could also be used for biomarker discovery for conditions and diseases in humans and other types of wildlife. The identification of diseases in early stages, as in the case of this work, could help to eliminate the spread of contagious

diseases or aid in early diagnosis and thus better outcomes for diseased individuals by providing options for early treatment. Confirmation of metabolite identification could also help shed light on the biology DFTD to help develop treatment strategies for this disease.

7 Appendices

7.1 Appendix 1: Sample sets

7.1.1 Pilot Set

Table A 1: Set of samples obtained for a pilot study to determine the feasibility of discovering metabolite differences between DFTD and non-DFTD Tasmanian devils.

Sample #	Microchip #	Location	DFTD status	Sex	Age
04/0560	982009101334983	Bronte Park	DFTD 69 days later	M	3
04/0603	982009101328318	Bronte Park	Non-DFTD	F	3
04/0605	982009101682197	Bronte Park	DFTD	F	3
04/0735	982009101449680	Bronte Park	DFTD	M	3
04/0960	985120016080819	National Park	Non-DFTD	M	3
04/1021	Unknown	National Park	Non-DFTD	F	4
04/2058	985120016023130	Mount William	DFTD	F	3
04/2062	985120016063210	Mount William	Non-DFTD	M	1
04/2064	985120016051065	Mount William	DFTD	M	4
04/2065	985120016102779	Mount William	Non-DFTD	F	3
04/3000	985120016084089	Fentonbury	DFTD	F	?
04/3003	985120016105311	Fentonbury	Non-DFTD	F	4
04/3008	985120015622584	Fentonbury	DFTD	M	4
04/3011	985120016101849	Fentonbury	Non-DFTD	M	4
04/3176	985120015992482	St Helens	Non-DFTD	F	3
04/3201	985120016101940	St Helens	Non-DFTD	M	3

7.1.2 Set 1

Table A 2: Samples obtained from male devils at Bronte Park during 2004.

Sample #	Microchip #	Location	DFTD Status	Sex	Age
04/0446	982009101663132	Bronte Park	DFTD	Male	3
04/0448	982009101564196	Bronte Park	DFTD 35 days later	Male	?
04/0720	982009101357889	Bronte Park	Non-DFTD	Male	3
04/0728	982009101162213	Bronte Park	Non-DFTD	Male	?
04/0731	982009101382207	Bronte Park	Non-DFTD	Male	?
04/1167	985120016021605	Bronte Park	Non-DFTD	Male	2
04/1771	985120016684224	Bronte Park	Non-DFTD	Male	2
04/0451	982009101449680	Bronte Park	DFTD	Male	3
04/0602	982009101538392	Bronte Park	DFTD	Male	3
04/0725	982009101564196	Bronte Park	DFTD	Male	3
04/1769	982009101357889	Bronte Park	DFTD	Male	3
04/0284	982009101449680	Bronte Park	DFTD	Male	?
04/0722	Unknown	Bronte Park	Non-DFTD	Male	3
04/0735	982009101449680	Bronte Park	DFTD	Male	3
04/1849	985120015506893	Bronte Park	Non-DFTD	Male	1
04/1852	985120015994043	Bronte Park	Non-DFTD	Male	2
04/1853	982009101538392	Bronte Park	Non-DFTD	Male	3
04/0276	982009101382207	Bronte Park	Non-DFTD	Male	4
04/0560	982009101334983	Bronte Park	DFTD 69 days later	Male	3

7.1.3 Blind Study samples obtained from the Suppression Trial

Table A 3: Details of serum samples obtained from devils located at the site of the suppression trial at Forestier Peninsula including government accession numbers microchip numbers and DFTD status.

Sample ID	Accession #	Microchip #	DFTD Status	Sample ID	Accession #	Microchip #	DFTD Status
1	08/0134	982009104349396	DFTD	**22**	08/3904	982009104327264	DFTD
2	08/0177	982009104246774	DFTD	**23**	08/4046	982009104931509	DFTD
3	08/0178	982009104877244	DFTD	**24**	09/0235	985154000001083	Healthy
4	08/0741	982009104326821	DFTD	**25**	09/1418	982009104914402	6 month pre-clinical
5	08/1046	982009104272295	DFTD	**26**	09/1430	982009104925396	12 month pre-clinical
6	08/1047	982009104833287	DFTD	**27**	09/1438	982009104907340	6 month pre-clinical
7	08/1072	982009104246128	Other disease	**28**	09/1439	982009104720533	Healthy
8	08/1130	982009104254912	DFTD	**29**	09/1440	982009104884473	Healthy
9	08/2378	982009104809064	DFTD	**30**	09/1441	982009105175668	12 month pre-clinical
10	08/2379	982009104877259	DFTD	**31**	09/1443	982009104929919	12 month pre-clinical
11	08/2385	982009104836888	DFTD	**32**	09/1444	982009105195643	12 month pre-clinical
12	08/2386	982009104811311	DFTD	**33**	09/1448	982009104965330	DFTD
13	08/2380	982009104248590	DFTD	**34**	09/1449	982009104815996	6 month pre-clinical
14	08/2480	982009104834357	DFTD	**35**	09/1451	982009104925128	Other disease
15	08/3673	982009104810858	DFTD	**36**	09/1478	982009104857449	DFTD
16	08/3594	982009105181458	DFTD	**37**	09/1494	982009104876128	12 month pre-clinical
17	08/3595	982009102344207	DFTD	**38**	09/2535	982009104840088	6 month pre-clinical
18	08/3605	982009104328024	6 month pre-clinical	**39**	09/2536	982009104975946	6 month pre-clinical
19	08/3695	982009104815960	DFTD	**40**	09/2538	982009104907551	Healthy
20	08/4006	982009104931509	DFTD	**41**	09/2539	982009104725775	12 month pre-clinical
21	08/3727	982009100741969	DFTD	**42**	09/2540	982009104884473	Healthy
43	09/2543	982009104720533	Healthy	**69**	09/4408	982009104914402	DFTD

Sample ID	Accession #	Microchip #	DFTD Status	Sample ID	Accession #	Microchip #	DFTD Status
44	09/2532	982009104879401	6 month pre-clinical	**70**	09/4436	982009106092189	Healthy
45	09/2533	982009105195643	6 month pre-clinical	**71**	09/4440	982009104243151	DFTD
46	09/2531	982009104858662	6 month pre-clinical	**72**	10/0148	982009104975946	1 month pre-clinical
47	09/2545	985154000001106	12 month pre-clinical	**73**	10/0150	982009104858662	DFTD
48	09/2549	982009104940952	DFTD	**74**	10/0135	982009104789723	DFTD
49	09/2554	982009104863462	DFTD	**75**	10/0147	982009104879401	1 month pre-clinical
50	09/2561	982009104271638	DFTD	**76**	10/0129	982009105195643	DFTD
51	09/2573	982009104849679	6 month pre-clinical	**77**	10/0134	982009104840088	DFTD
52	09/2578	982009104995020	DFTD	**78**	10/0130	982009104849679	DFTD
53	09/2597	982009104929919	12 month pre-clinical	**79**	10/0156	982009106039877	6 month pre-clinical
54	09/2601	982009104914402	6 month pre-clinical	**80**	10/0157	982009104943898	DFTD
55	09/2602	982009104932691	12 month pre-clinical	**81**	10/0148	982009104975946	1 month pre-clinical
56	09/2605	982009104919715	12 month pre-clinical	**82**	09/4345	982009104884473	Healthy
57	09/2603	982009104906424	6 month pre-clinical	**83**	08/2480	982009104834357	DFTD
58	09/2615	982009104940952	DFTD	**84**	10/0129	982009105195643	DFTD
59	09/4338	982009104902572	6 month pre-clinical	**85**	10/0156	982009106039877	6 month pre-clinical
60	09/4339	982009104348760	7 month pre-clinical	**86**	09/2615	982009104940952	DFTD
61	09/4343	982009104840088	8 month pre-clinical	**87**	10/0157	982009104943898	DFTD
62	09/4345	982009104884473	Healthy	**88**	10/0150	982009104858662	DFTD
63	09/4346	982009106601685	Other disease	**89**	09/1494	982009104876128	12 month pre-clinical
64	09/4347	982009104789723	6 month pre-clinical	**90**	09/1478	982009104857449	DFTD
65	09/4348	982009104943898	6 month pre-clinical	**91**	10/0147	982009104879401	1 month pre-clinical
66	09/4349	982009104858662	6 month pre-clinical	**92**	10/0135	982009104789723	DFTD
67	09/4351	982009104720533	Healthy	**93**	10/0134	982009104840088	DFTD
68	09/4407	982009104925396	12 month pre-clinical	**94**	10/0130	982009104849679	DFTD
95	08/3695	982009104815960	DFTD	**98**	08/1130	982009104254912	DFTD

Sample ID	Accession #	Microchip #	DFTD Status	Sample ID	Accession #	Microchip #	DFTD Status
96	08/2379	982009104877259	DFTD	**99**	09/1449	982009104815996	6 month pre-clinical
97	08/0741	982009104326821	DFTD	**100**	08/3727	982009100741969	DFTD

7.1.4 Blind State Wide Study Sample Set

Table A 4: List of samples obtained from Tasmanian devils throughout Tasmania included in a blind study with no control of metabolite affecting sample variables. Details of government accession numbers, devil microchip numbers, sampling location, gender, age and disease status are listed.

Sample #	Accession #	Microchip #	Sampling Location	Sex	Age	DFTD status	Other Disease Information
D1	06/2022	982009100819644	Forestier	m	3	DFTD	
D2	06/2023	982009104356213	Forestier	m	3	DFTD	
D3	06/2024	982009104252965	Forestier	m	2	DFTD	
D4	06/2026	985120016101456	Forestier	f	2	DFTD	
D5	06/2027	985120016108883	Forestier	m	2	DFTD	
D6	07/1872	982009102626938	Reedy Marsh	f	N/A	DFTD	
D7	07/1875	982009102327363	Reedy Marsh	m	4	DFTD	
D8	06/2673	982009104842051	Buckland	m	1	DFTD	
D9	07/0189	982009100873904	Coles Bay	f	2	DFTD	
D10	07/1490	982009102731064	Bangor	m	4	DFTD	
H11	07/1531	982009104868089	West Pencil Pine	m	3	Non-DFTD	
H12	07/1237	982009105190043	Fentonbury	m	1	Non-DFTD	
H13	07/1279	985120016097550	Mount Pleasant	f	4	Non-DFTD	
H14	07/1323	982009104335482	Coles Bay	m	1	Non-DFTD	
H15	07/1366	982009102229973	Granville	f	4	Non-DFTD	
H16	07/1367	982009104358597	Granville	m	2	Non-DFTD	
H17	07/1368	982009102725368	Granville	f	4	Non-DFTD	
H18	06/2057	982009102432250	Bangor	f	3	Non-DFTD	
H19	08/0153	982009104859118	Bicheno	m	1	Non-DFTD	
H20	09/0402	982009100786171	Mount Pleasant	f	6	Other disease	Not Specified
1	06/3112	000682FBF5	Trowunna	m	2	Non-DFTD	
2	06/2177	982009102645810	MTP	f	2	DFTD	

Sample #	Accession #	Microchip #	Sampling Location	Sex	Age	DFTD status	Other Disease Information
3	06/3115	00065ED672	Trowunna	m	N/A	Non-DFTD	
4	06/2057	982009102432250	Bangor	f	3	Non-DFTD	
5	06/3145	00062D5B41	Trowunna	m	4	Non-DFTD	
6	06/3144	982009100795775	Trowunna	f	3	Non-DFTD	
7	05/1879	00065D83FA	Trowunna	f	NA	Pre-other disease	Pre-Non-DFTD disease
8	05/0051	N/A	Deloraine	f	N/A	DFTD	
9	06/2023	982009104356213	Forestier	m	3	DFTD	
10	06/2022	982009100819644	Forestier	m	3	DFTD	
11	06/3113	00068351D8	Trowunna	f	2	Non-DFTD	
12	06/3448	982009100786171	Mount Pleasant	f	N/A	Pre-other disease	Pre-Non-DFTD disease
13	06/3114	00065DC74E	Trowunna	f	2	Non-DFTD	
14	06/3451	982009100852046	Mount Pleasant	f	3	Pre-other disease	Pre-Non-DFTD disease
15	06/2109	985120015995993	Forestier	m	3	DFTD	
16	05/3164	985100010685532	Taroona	f	4	Non-DFTD	
17	06/2027	985120016108883	Forestier	m	2	DFTD	
18	06/2024	982009104252965	Forestier	m	2	DFTD	
19	07/0298	982009104863314	Wisedale	f	1	Non-DFTD	
20	06/1005	982009100819358	Granville	m	4	Pre-other disease	Pre-Non-DFTD disease
21	07/1315	982009100818058	Fentonbury	f	3	DFTD	
22	06/2672	982009104881249	Buckland	f	1	Non-DFTD	
23	07/1240	982009104809696	Fentonbury	f	1	Non-DFTD	
24	06/3447	985120016024404	Mount Pleasant	f	3	Non-DFTD	
25	06/2401	982009100371954	Mount Pleasant	m	3	DFTD	
26	06/2026	985120016101456	Forestier	f	2	DFTD	
27	06/2671	982009102220925	Buckland	f	3	DFTD	
28	07/1531	982009104868089	West Pencil Pine	m	3	Non-DFTD	
29	07/0187	982009102287979	Fentonbury	f	2	DFTD	

Sample #	Accession #	Microchip #	Sampling Location	Sex	Age	DFTD status	Other Disease Information
30	07/0374	00065D83FA	Trowunna	f	5	Other disease	Neoplasia
31	07/0295	982009104272592	Westbury	f	2	Non-DFTD	
32	07/0817	00065DC9C5	Mole Creek	f	6	DFTD	
33	07/0300	982009104817698	Wisedale	m	1	Non-DFTD	
34	07/0301	982009104246213	Wisedale	f	4	Non-DFTD	
35	07/0711	982009105197308	Temma	f	3	Non-DFTD	
36	07/0192	982009100873958	Coles Bay	m	2	DFTD	
37	07/1366	982009102229973	Granville	f	4	Non-DFTD	
38	07/0189	982009100873904	Coles Bay	f	2	DFTD	
39	07/1367	982009104358597	Granville	m	2	Non-DFTD	
40	07/1316	982009100831798	Fentonbury	f	3	Pre-DFTD	
41	07/1279	985120016097550	Mount Pleasant	f	4	Non-DFTD	
42	07/1241	982009104841310	Fentonbury	f	1	Non-DFTD	
43	07/1285	985120016101682	Mount Pleasant	m	N/A	Pre-DFTD	
44	07/1368	982009102725368	Granville	f	4	Non-DFTD	
45	06/2449	982009104234181	Bangor	m	3	DFTD	
46	06/2673	982009104842051	Buckland	m	1	DFTD	
47	07/0473	982009104967035	West Pencil Pine	f	2	Non-DFTD	
48	07/0479	982009104350998	West Pencil Pine	m	5	Non-DFTD	
49	07/1369	982009100816855	Granville	m	3	Non-DFTD	
50	07/1324	982009102247939	Coles Bay	m	2	DFTD	
51	06/1785	985120016101682	Mount Pleasant	m	4	DFTD	
52	07/1323	982009104335482	Coles Bay	m	1	Non-DFTD	
53	07/1875	982009102327363	Reedy Marsh	m	4	DFTD	
54	07/1253	985120016064617	Bangor	f	4	DFTD	
55	07/1372	982009100819358	Granville	m	5	Other disease	Non-DFTD growths

Sample #	Accession #	Microchip #	Sampling Location	Sex	Age	DFTD status	Other Disease Information
56	07/1534	982009104354989	West Pencil Pine	m	5	Other disease	Skin inflamation
57	07/1286	982009100371954	Mount Pleasant	m	N/A	Pre-DFTD	
58	07/0474	982009104862874	West Pencil Pine	m	5	Non-DFTD	
59	07/1856	982009100819338	Bangor	m	2	DFTD	
60	07/0481	982009104354989	West Pencil Pine	m	5	Pre-other disease	Pre-Non-DFTD disease
61	07/1855	982009100895084	Bangor	m	2	DFTD	
62	07/1540	982009104960787	West Pencil Pine	m	5	Other disease	Non-DFTD growths
63	07/1238	982009100917481	Fentonbury	m	2	DFTD	
64	07/1492	982009100747693	Bangor	m	N/A	DFTD	
65	07/2258	982009100754517	Bangor	m	2	DFTD	
66	07/0478	982009104976856	West Pencil Pine	f	4	Non-DFTD	
67	07/2124	982009101687230	Narawntapu	f	6	Other disease	Neoplasia
68	07/1490	982009102731064	Bangor	m	4	DFTD	
69	07/1914	982009105007128	Taranna	m	1	Pre-other disease	Pre-Non-DFTD disease
70	07/1212	982009102751068	Bangor	f	3	DFTD	
71	07/2259	982009100906846	Bangor	m	2	DFTD	
72	07/1213	982009100834894	Bangor	f	2	DFTD	
73	07/2257	982009100847763	Bangor	f	2	DFTD	
74	07/0500	982009100662150	Bronte Park	m	2	DFTD	
75	07/0477	982009104833285	West Pencil Pine	m	5	Non-DFTD	
76	07/1872	982009102626938	Reedy Marsh	f	N/A	DFTD	
77	07/1325	982009105363992	Coles Bay	m	1	Non-DFTD	
78	07/0476	982009104832117	West Pencil Pine	f	3	Non-DFTD	
79	07/1878	00065D7157	Taranna	m	5	Non-DFTD	
80	07/0480	982009104253340	West Pencil Pine	f	5	Non-DFTD	
81	07/1239	982009105165660	Fentonbury	m	2	DFTD	

Sample #	Accession #	Microchip #	Sampling Location	Sex	Age	DFTD status	Other Disease Information
82	07/1489	982009102757183	Bangor	m	N/A	DFTD	
83	07/1714	982009104267602	Wisedale	f	3	DFTD	
84	07/1237	982009105190043	Fentonbury	m	1	Non-DFTD	
85	07/3341	982009104859904	Narawntapu	m	3	DFTD	
86	07/0488	982009104269739	West Pencil Pine	f	3	Pre-DFTD	
87	07/0490	982009104357109	West Pencil Pine	f	2	Pre-other disease	Pre-Non-DFTD disease
88	08/0266	982009104880604	Marrawah	m	5	Other disease	Non-DFTD growths
89	08/4048	N/A	Alcomie	f	4	Other disease	Alopecia (baldness)
90	07/1478	000658C2A0	Trowunna	m	4	Pre-DFTD	
91	07/0486	982009104869208	West Pencil Pine	f	4	DFTD	
92	09/1583	982009104250147	West Pencil Pine	f	4	Other disease	Neoplasia
93	07/3448	982009105007128	Taranna	m	1	Other disease	Kidney disease
94	09/0193	985154000001207	Trowunna	m	1	Other disease	Malformation
95	09/0402	982009100786171	Mount Pleasant	f	6	Other disease	
96	09/0991	00068B7E56	Hobart	f	3	Other disease	
97	09/1612	982009104269739	West Pencil Pine	f	5	Pre-DFTD	
98	09/1069	982009104984790	Mount Pleasant	m	3	Other disease	Urinary Infection
99	08/1620	982009105145532	West Pencil Pine	m	2	DFTD	
100	08/3764	982009104250147	West Pencil Pine	f	4	Pre-other disease	
101	09/1446	982009104905221	Bangor	m	1	Other disease	Ulcers
102	07/0487	982009104968776	West Pencil Pine	f	5	DFTD	
103	09/1278	985154000001173	Sidmouth	f	4	Pre-other disease	Pre-non-DFTD growths
104	06/3122	000658C2A0	Trowunna	m	3	Pre-DFTD	
105	08/0134	982009104349396	Bangor	m	2	DFTD	
106	09/1451	982009104925128	Bangor	m	2	Other disease	Non-DFTD growths
107	07/1537	982009104815428	West Pencil Pine	m	5	DFTD	

Sample #	Accession #	Microchip #	Sampling Location	Sex	Age	DFTD status	Other Disease Information
108	09/0992	982009104357109	Hobart	f	4	Other disease	Skin lesions
109	08/0153	982009104859118	Bicheno	m	1	Non-DFTD	
110	08/0153	982009104859118	Bicheno	m	1	Non-DFTD	

7.2 Appendix 2: Fold Changes Observed for the Significantly Different Peaks for Both Blind Studies

Table A 5: Fold changes between DFTD and non-DFTD samples of significantly different peaks analysed by ESI-MS in both of the blind studies (see chapters 4 and 5). These peaks were determined using Student's t-test ($p \leq 0.05$) and then used to classify DFTD status using PCA.

ESI-Metabolite	male Forestier		male whole state		female whole state	
	pre-DFTD	DFTD	pre-DFTD	DFTD	pre-DFTD	DFTD
134	1.5	3.7	-	-	-	-
135	-	-	0.6	0.7	-	-
150	-	-	-	-	1.8	1.9
151	-	-	0.5	0.6	-	-
164.9	-	-	-	-	10.0	6.4
165	-	-	-	-	2.2	2.1
191	1.2	1.8	-	-	-	-
192	1.7	8.0	-	-	Only in DFTD (av 2.3)	
193	-	-	0.2	0.5	-	-
200.9	2.9	4.0	-	-	-	-
205.1	-	-	-	-	2.6	2.7
207	1.0	3.1	-	-	-	-
221.2	-	-	-	-	1.4	1.4
222.2	-	-	-	-	2.0	1.5
244.2	-	-	-	-	3.0	2.8
250.9	-	-	0.7	0.7	-	-
252.9	-	-	0.2	0.5	-	-
261	-	-	0.7	0.8	-	-
266.2	-	-	-	-	0.9	1.3
267.2	-	-	-	-	0.0	1.9
271	-	-	0.5	0.8	-	-
274.9	-	-	2.6	2.3	-	-
287	-	-	-	-	5.4	6.0
304.3	-	-	-	-	1.1	1.5
308.9	-	-	0.7	0.6	-	-
310.9	-	-	0.8	0.5	-	-
323.2	-	-	-	-	1.5	0.0
329.2	-	-	-	-	1.1	1.3
332.3	-	-	-	-	2.1	1.7
332.9	-	-	6.2	7.6	-	-
334.9	-	-	4.7	5.6	-	-
362.3	-	-	-	-	1.5	1.4
370.3	-	-	0.3	0.3	-	-
370.4	-	-	1.1	1.6	-	-

ESI-Metabolite	male Forestier		male whole state		female whole state	
	pre-DFTD	DFTD	pre-DFTD	DFTD	pre-DFTD	DFTD
371.2	-	-	-	-	1.2	1.3
384.1	-	-	1.2	1.4	-	-
387.2	-	-	-	-	1.1	1.9
392.8	-	-	1.4	1.5	-	-
400.8	-	-	1.8	1.9	-	-
408.3	-	-	-	-	1.4	1.3
433.3	-	-	0.0	0.0	-	-
441.3	-	-	-	-	0.0	3.0
490.3	-	-	0.3	0.2	-	-
512.3	-	-	1.0	0.1	-	-
516.3	-	-	0.9	0.4	-	-
519.3	1.1	0.8	-	-	-	-
522.4	-	-	0.3	0.7	-	-
524.4	-	-	0.2	0.7	-	-
532.9	-	-	-	-	1.0	1.2
534.3	-	-	1.0	0.7	-	-
540.4	-	-	-	-	1.9	2.0
540.9	-	-	-	-	1.3	1.8
542.3	-	-	0.7	0.8	-	-
544.3	-	-	0.6	0.5	-	-
547.4	0.6	0.5	-	-	-	-
556.8	-	-	0.8	0.1	-	-
560.3	-	-	0.6	0.4	1.9	0.5
563.9	-	-	1.4	0.3	-	-
564.3	-	-	0.0	0.5	-	-
566.3	-	-	-	-	1.0	0.7
569.4	-	-	0.6	0.4	-	-
570.4	-	-	1.1	0.8	-	-
572.4	-	-	1.2	0.3	-	-
582.7	-	-	-	-	0.0	5.8
584.4	1.7	2.1	-	-	-	-
585.9	-	-	1.3	0.4	-	-
586.3	-	-	-	-	0.0	0.2
586.9	-	-	1.2	0.2	-	-
589.5	-	-	0.7	0.8	-	-
590.3	-	-	-	-	0.0	0.2
591.5	-	-	0.3	0.7	-	-
592.5	-	-	0.0	0.7	-	-
592.9	-	-	1.1	0.6	-	-
593.9	-	-	1.8	0.1	-	-
600.9	-	-	1.4	0.2	-	-
602.3	-	-	0.4	0.5	-	-

ESI-Metabolite	male Forestier		male whole state		female whole state	
	pre-DFTD	DFTD	pre-DFTD	DFTD	pre-DFTD	DFTD
604.3	-	-	0.2	0.5	-	-
605.5	-	-	0.8	0.7	-	-
606.9	-	-	-	-	1.2	1.2
607.9	-	-	1.2	0.6	-	-
614.4	-	-	1.2	0.8	-	-
615.9	-	-	1.7	0.3	-	-
619.5	-	-	0.6	0.8	-	-
620.5	-	-	0.4	0.6	-	-
621.9	-	-	1.2	0.2	-	-
622.4	1.8	0.1	-	-	-	-
630.9	-	-	2.8	0.0	-	-
631.5	-	-	0.7	0.8	-	-
639.4	-	-	0.5	0.4	-	-
643.9	-	-	1.4	0.3	-	-
644.9	-	-	1.4	0.4	-	-
658.4	-	-	1.0	0.8	-	-
658.6	-	-	0.4	0.2	-	-
659.9	-	-	1.3	0.4	-	-
663.5	-	-	-	-	1.5	1.3
665.5	-	-	1.1	0.8	-	-
666.5	-	-	1.1	0.9	-	-
668.5	-	-	-	-	0.0	0.3
669.6	-	-	1.2	1.6	-	-
670.6	-	-	1.2	1.6	-	-
671.6	-	-	-	-	1.1	0.7
672.6	-	-	-	-	1.1	0.7
672.9	-	-	-	-	0.9	1.3
677.6	-	-	-	-	1.2	1.5
680.4	-	-	1.4	0.3	-	-
683.5	-	-	-	-	1.2	1.4
695.6	-	-	-	-	1.1	0.8
696.6	-	-	-	-	1.0	0.8
697.6	-	-	-	-	1.1	0.8
698.6	-	-	-	-	1.0	0.9
702.9	-	-	1.1	0.6	-	-
709.5	1.0	0.5	-	-	-	-
709.9	-	-	0.8	0.0	-	-
710.9	-	-	1.5	0.4	-	-
717.9	-	-	-	-	0.0	5.8
730.6	-	-	-	-	0.0	8.8
731	-	-	-	-	1.2	1.3
732.9	-	-	1.9	0.2	-	-

ESI-Metabolite	male Forestier		male whole state		female whole state	
	pre-DFTD	DFTD	pre-DFTD	DFTD	pre-DFTD	DFTD
739	-	-	-	-	1.2	1.2
740	-	-	1.7	0.4	-	-
747	-	-	1.2	0.7	-	-
748.6	-	-	0.5	0.5	-	-
756.6	-	-	-	-	1.3	0.9
761	-	-	-	-	1.3	1.3
762	-	-	2.0	0.5	-	-
763.6	-	-	-	-	1.1	0.9
765.5	-	-	1.6	1.1	-	-
765.6	-	-	0.9	0.8	-	-
791	-	-	1.5	0.6	-	-
799.7	-	-	0.3	0.8	1.0	0.7
804.6	-	-	-	-	1.2	0.9
805.6	-	-	-	-	1.2	0.9
806	-	-	1.4	0.4	-	-
806.6	-	-	-	-	1.0	0.8
807.6	-	-	-	-	1.0	0.8
809.6	-	-	-	-	1.1	0.8
818.6	0.5	0.8	-	-	-	-
827	-	-	-	-	0.0	3.2
827.7	-	-	0.4	0.8	-	-
829.7	0.7	0.2	0.4	0.8	-	-
834.6	-	-	-	-	1.1	0.8
841.5	0.7	0.1	-	-	-	-
843.7	-	-	0.4	1.0	-	-
844.7	-	-	0.1	0.8	-	-
850.6	-	-	1.1	0.4	-	-
857.7	-	-	0.5	0.6	-	-
859.6	-	-	7.0	2.3	-	-
860.6	-	-	1.3	1.3	-	-
862.6	-	-	2.1	4.2	-	-
864	-	-	3.6	0.0	-	-
869.7	-	-	0.5	0.7	-	-
871.7	-	-	0.5	0.8	-	-
872.7	-	-	0.5	0.9	-	-
885.8	0.5	0.3	-	-	-	-
886.6	-	-	7.8	2.8	-	-
890.6	-	-	-	-	1.4	0.8
892.6	-	-	0.0	0.2	-	-
904.8	-	-	3.0	1.5	-	-
936.7	2.6	3.3	-	-	-	-
948.5	-	-	1.2	1.7	0.0	0.0

ESI-Metabolite	male Forestier		male whole state		female whole state	
	pre-DFTD	DFTD	pre-DFTD	DFTD	pre-DFTD	DFTD
953.7	2.1	4.0	-	-	-	-
965.8	-	-	-	-	1.7	1.3
997.7	-	-	-	-	1.4	1.4
1003.7	-	-	1.3	0.3	-	-
1005.7	-	-	1.4	0.3	-	-
1011.9	-	-	-	-	1.2	1.7
1012.9	-	-	-	-	1.1	1.7
1013.7	-	-	-	-	1.3	1.3
1016.7	-	-	1.6	0.5	-	-
1030.7	-	-	1.4	0.7	-	-
1040.9	-	-	0.7	0.6	-	-
1045.9	-	-	0.9	0.3	-	-
1048.9	-	-	0.9	0.7	-	-
1057.7	-	-	-	-	1.3	1.3
1063.9	-	-	-	-	0.9	1.9
1074.7	-	-	1.1	0.7	-	-
1076.7	-	-	2.0	0.2	-	-
1077.7	-	-	2.2	0.1	-	-

Table A 6: Fold changes between DFTD and non-DFTD samples of significantly different peaks analysed by GC-MS in the blind study with extensive sample variability (see chapter 5). These peaks were determined using Student's t-test ($p \leq 0.05$ for the females and $p \leq 0.1$ for the males) and then used to classify DFTD status using PCA.

GC-MS metabolite			male whole state		female whole state	
Peak #	Migration time (min)	Base peak (m/z)	pre-DFTD	DFTD	pre-DFTD	DFTD
4	**13.2**	**175**	0.9	0.9	-	-
12	**14.1**	**104**	1.3	1.2	-	-
34	**16.1**	**103**	3.5	1.7	-	-
35	**16.3**	**147**	0.7	0.7	-	-
41	**17.05**	**147**	1.3	1.3	-	-
53	**18.6**	**145**	-	-	0.8	0.7
56	**19**	**100**	1.9	1.3	-	-
57	**19.1**	**147**	2.2	1.4	-	-
67	**20.4**	**147**	1.0	0.9	1.3	0.7
68	**20.6**	**147**	-	-	0.4	0.2
80	**22.1**	**85**	-	-	0.9	1.3
81	**22.35**	**147**	0.7	1.0	-	-
84	**23**	**204**	-	-	2.6	2.1
116	**28.1**	**115**	0.6	0.6	-	-
136	**32.1**	**319**	1.4	1.3	-	-
137	**32.4**	**147**	1.6	1.3	-	-
143	**33.7**	**204**	2.6	1.6	-	-
151	**35.1**	**299**	1.1	0.9	-	-
156	**37.8**	**327**	1.1	0.9	-	-
166	**43.4**	**361**	0.4	0.9	0.3	0.4
167	**43.8**	**361**	0.2	0.8	-	-
170	**45.2**	**91**	-	-	0.7	0.3

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