

# **PRE-PROCESSING AND ANALYSIS OF HIGH-DIMENSIONAL PLANT METABOLOMICS DATA**

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## Abstract

Metabolomics technologies produce an overwhelming amount of complex data. Extracting the relevant information from such data is a challenging process, requiring a series of appropriate numerical treatments to transform the raw measurements into parsimonious outputs with clear biological meaning. In this thesis, a complete data analysis ‘pipeline’ for handling multivariate (high-dimensional) plant metabolomics data is presented. This pipeline is intended for data acquired by chromatographic techniques coupled to mass spectrometry, and includes four discrete steps: pre-processing, pre-treatment, statistical modelling and metabolite annotation.

All software elements in the pipeline are flexible and open source. Two programming platforms were employed for various different steps. The pre-processing step is conducted using XCMS software in the freely available ‘R’ environment. Pre-treatment and statistical analyses are conducted using ‘R’, and the commercial language, Matlab (The Mathworks, Inc). Comparisons were made between alternative statistical methods, as well as across different implementations of nominally the same method, at the level of coding of the algorithms. Thus, the open source nature of both languages was fully exploited.

The statistical modelling step involves a choice of multivariate/univariate and supervised/unsupervised methods, with an emphasis on appropriate model validation. Particular attention was given to a commonly encountered chemometric method, Partial Least Squares Discriminant Analysis (PLS-DA). Consideration is given to different variants of the PLS algorithm, and it will be shown these can impact quite substantially on the outcome of analyses.

Specific components of the pipeline are demonstrated by examining two experimental datasets, acquired from *Arabidopsis* wild type and mutant plants. The first of these comprises amino acid profiles of a set of lipid mutants, obtained by liquid chromatography mass spectrometry (LC-MS). Multivariate classification models were developed which could discriminate between the mutants and wild type, and also make predictions about mutants of unknown functionalities.

The second dataset concerns untargeted metabolite profiling, and is used for a thorough exploration of all steps in the pipeline. The data were obtained by gas chromatography mass spectrometry (GC-MS) from mutants deficient in starch synthesis or degradation. Supervised statistical modelling was able to discriminate between the mutants, even in the presence of strong batch effects, whilst in contrast, unsupervised modelling performed poorly. Although methodological and even algorithm differences can produce numerically quite different results, the final outcomes of the alternative supervised modelling techniques in terms of biological interpretation were very similar.

**CHAPTER 1:**  
**INTRODUCTION**

# **1 INTRODUCTION**

## **1.1 Introduction to metabolomics**

Metabolomics, the comprehensive analysis of all metabolites in a biological sample, has emerged in recent years as an important functional genomics tool that can significantly contribute to the understanding of complex metabolic processes (Oliver et al., 1998; Rochfort, 2005; Tweeddale et al., 1998; Weckwerth, 2003). Metabolomics can be used to describe the responses of biological systems to environmental or genetic modifications and is considered the key link between genes and phenotypes (Fiehn, 2002). The plant metabolome may include hundreds or thousands of different metabolic components that can vary in their abundance by up to 6 orders of magnitude (Weckwerth and Morgenthal, 2005). Any valid metabolomic approach must be able to provide unbiased and comprehensive high-throughput analysis of this enormous diversity of chemical compounds (Bino et al., 2004). The impressive progress in the development of high-throughput methods for metabolomics in the last decade is a result of both the rapid improvements in mass spectrometry (MS)-based methods (Shah et al., 2000), and in computer hardware and software that is capable of handling large datasets (Katajamaa and Oresic, 2007).

## **1.2 Analytical approaches**

A wide range of mass spectrometric techniques are used in plant metabolomics, each of them providing particular advantages regarding precision, comprehensiveness and sample throughput. At the end of the 1990's, GC-MS (gas-chromatography mass spectrometry) was the technology of choice for attempts at the simultaneous analysis of a very large number of metabolites in a range of plant species (Fiehn et al., 2000; Roessner et al., 2000). This work contributed to the development of spectral libraries for the identification of unknown metabolites (The Golm Metabolome Database by Max Planck Institute of Molecular Plant Physiology in Golm, Germany). Today, GC-MS remains one of the most popular technologies for identifying multiple metabolites in plant systems.

LC-MS (liquid-chromatography mass spectrometry) is another commonly used technology, well adapted to non-volatile and thermo-unstable analytes. Other popular mass spectrometric techniques include CE-MS (capillary electrophoresis),

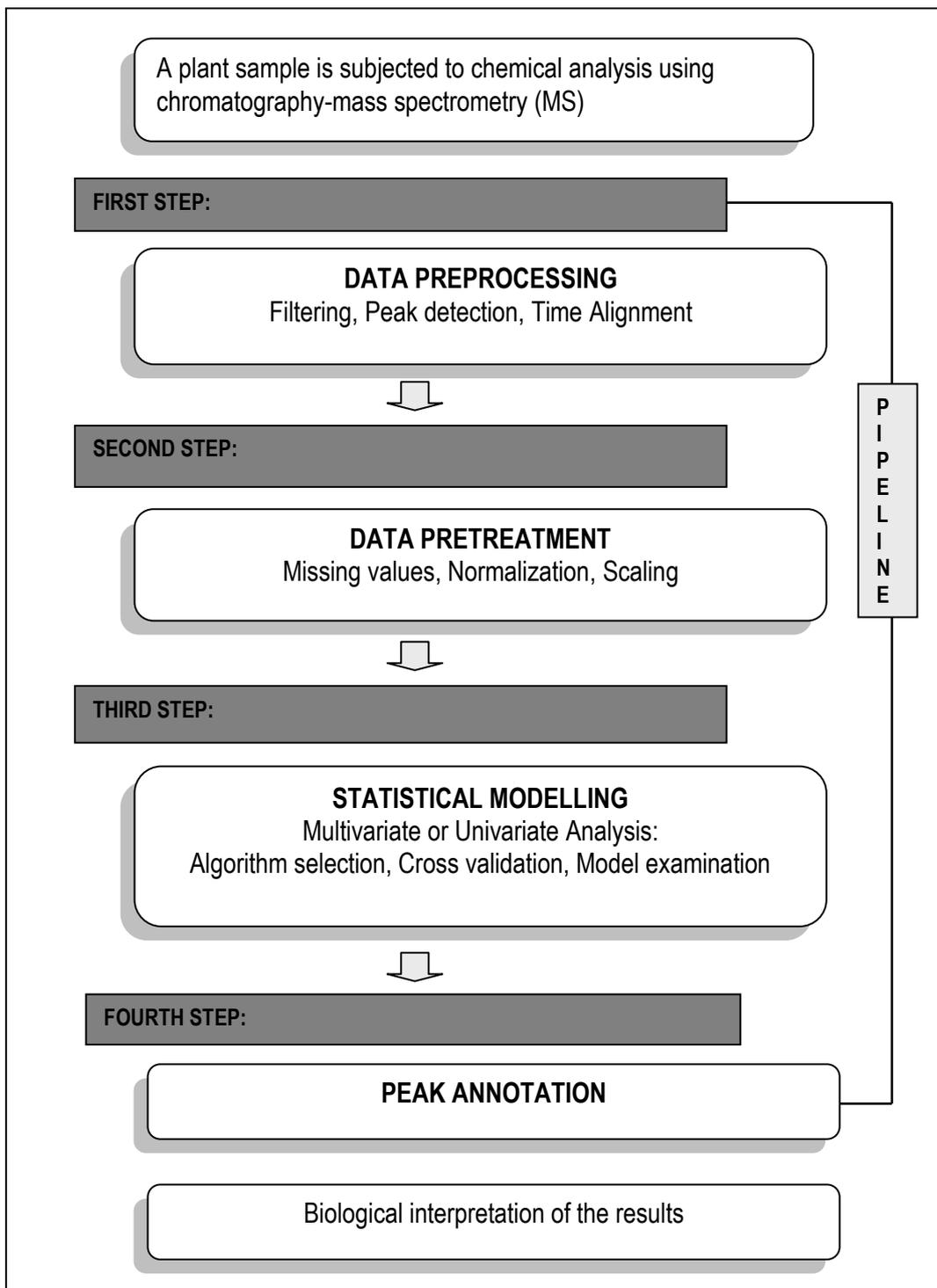
EI-MS (electrospray ionization liquid chromatography), and several combinations of technologies such GCxGC-MS, or tandem MS. Besides mass spectrometry, NMR is widely used in other areas of metabolomics and is becoming increasingly popular in plant systems (Krishnan et al., 2005).

While the capabilities of metabolomic technologies are constantly progressing, a global metabolite analysis is still constrained by the considerable challenges of covering the wide chemical diversity and range of concentration of all metabolites present in an organism. In fact, a combination of different technologies may always be necessary for a thorough metabolomic analysis (Bino et al., 2004; Moco et al., 2007b). Whichever technologies are used, a necessary requirement is the establishment of a robust **data handling pipeline**, in order to interpret the very large number of chromatographic peaks and mass spectra produced, and to make meaningful comparisons of data obtained from different instruments.

### 1.3 Data handling

Handling the large and complex datasets produced by metabolomic experiments is one of the prime challenges in the metabolomics research field (Boccard et al., 2010; Jonsson et al., 2005; Van Den Berg et al., 2006). Data handling can be considered as a pipeline of successive steps: data pre-processing, data pre-treatment, data analysis (usually statistical modelling), and annotation. Some of the main considerations for the choice of the appropriate data handling procedure are the analytical platform used to generate the data, the biological question to be answered and the inherent properties of the data. **In this work I present a pre-processing, pre-treatment, analysis and annotation pipeline for GC-MS and LC-MS metabolomic data (Figure 1.1).** This includes:

- pre-processing (condensing and extracting features from the raw data);
- pre-treatment (scaling and or normalization, to address specific properties of the data)
- statistical modelling (for example, dimensionality reduction and discriminant analysis steps)
- metabolite annotation (using appropriate databases)



*Figure 1.1. Metabolomic analysis pipeline*

Once a robust metabolomic analysis pipeline has been established, it can be used in various applications; from answering simple biological questions (for example, what are the differences between two cultivars?), to investigating complex metabolic networks. The steps in the pipeline will now be considered individually.

### **1.3.1 Data pre-processing**

In metabolomic analyses, a raw dataset may contain tens or hundreds of spectra, each of them containing many hundreds or thousands of intensity measurements. Low level pre-processing is often necessary in order to make sense of this large volume of data. Data pre-processing constitutes the initial step in data handling (Goodacre et al., 2007), and its main goal is to extract all the relevant information from the raw data and summarize them in a single table (data matrix). This procedure includes steps such as noise filtering, data binning, automatic peak detection and chromatographic alignment.

Pre-processing mass spectrometric data is one of the most challenging areas in the metabolomics field with regard to software development. Most of the technology manufacturers provide automated software intended to accomplish these tasks for instance AMDIS or SIEVE (Blackburn et al.; Styczynski et al., 2007), however, instrument dependent software packages have substantial limitations and are usually inefficient. Several free (open source) packages are increasingly being used in the field, such as XCMS (Smith et al., 2006), MZMine (Katajamaa et al., 2006), MetAlign (Lommen, 2009), and several others (Blackburn et al.). The pre-processing step is discussed fully in Chapter 3.

### **1.3.2 Data pre-treatment**

Certain properties of a dataset, such as unwanted technical variation can limit the interpretability of metabolomics data (Van Den Berg et al., 2006; Werf et al., 2005). Data pre-treatment methods are used to correct or at least reduce some of these aspects (Idborg et al., 2005). Initially, data may be **normalized** prior to analysis to remove certain types of systematic variations between the samples. Normalization aims to remove this unwanted variation whilst preserving biological information. There are several statistical methods for data normalization; one of the most common is area normalization (Craig et al., 2006). When internal standards are added, their

peaks may be used as scaling factors for more efficient normalization (Sysi-Aho et al., 2007).

Depending on the choice of statistical analysis method, the data may be further pre-treated prior to model fitting. Mean centering and variance scaling are common pre-treatment steps (Keun et al., 2003; Van Den Berg et al., 2006) that can optimize the fit of the model to the data. Data pre-treatment is often overlooked, but in fact it can have a great impact on the outcome of the statistical analysis. In this work it is emphasized that pre-treatment is an important step of the analysis pipeline, and that the assumptions and limitations of the pre-treatment method should always be taken into account.

### **1.3.3 Data analysis**

A common characteristic of all metabolomic techniques is that they produce **high-dimensional** data: performing an analysis of a single sample will result in a large number of discrete data values, or equivalently, a vector with a large number of elements (Goodacre et al., 2004). From a statistical point of view, it is a great challenge how to deal with these high-dimensional spaces, where hundreds of (possibly highly correlated) variables define the data matrix. Univariate methods such as the Student's t-test, one-way analysis of variance (ANOVA), or their non-parametric equivalents are useful for explanatory analysis purposes by providing an overview of the pre-processed data, albeit one variable at a time; their use can be rather limited when dealing with thousands of variables. A collection of statistical techniques, known as chemometrics (Trygg et al., 2006; van der Greef and Smilde, 2005), has become established as a valuable tool for handling multivariate metabolomic data. Of the various chemometric methods principal component analysis (PCA) and partial least squares (PLS) are the most popular.

PCA is a dimension reduction method that is widely used for data exploration and visualization. PCA was first proposed in 1901 by Pearson (Pearson, 1901), but as with all the multivariate methods, it was not widely used until the arrival of modern computing technology over the past three decades. The target of PCA is to reveal underlying patterns by compressing the data while retaining as much as possible of the original information. PLS is a technique similar to PCA, derived from the

concept of iterative fitting (Wold et al., 1983). In its basic regression form, PLS models the relationship between two datasets, using a series of local least square fits. This is the crucial difference between PLS and PCA: PLS is a supervised technique that makes use of additional information to produce a statistical model, whereas PCA is unsupervised not requiring a second data input.

An area that has attracted attention in the field is the use of metabolomic data for mutant classification problems, discussed further below. Both PCA and PLS can perform this kind of analysis when used as dimension reduction before discriminant analysis, forming the methods PCA-DA and PLS-DA respectively. These hyphenated methods are both highly effective supervised classification methods for application to multivariate data. However, as with all supervised techniques particular emphasis should always be given to model validation, as an important step of the model building.

Regarding the statistical software for multivariate analysis, MATLAB is considered a standard for the development and publication of chemometrics algorithms, while the open source statistically-oriented language R is rapidly becoming a popular alternative. These are the two development environments that have been used in the present work. There are many other commercial and open source statistical packages that offer options for multivariate analysis, including many with well-developed graphical user interfaces (GUIs), e.g. SIMCA (Eriksson et al.; Wold and Sjostrom, 1977). However, where algorithm development or indeed transparency is a priority, then a language-based package is the more flexible, preferred option.

#### **1.3.4. Metabolite annotation**

A big effort in the metabolomics field is directed towards the establishment of good databases for the identification of plant metabolites (Bais et al., 2010). Although substantial improvements have been made in the last years, the uniform annotation of metabolite signals in publicly available databases remains a challenge (Saito and Matsuda, 2010). The construction of metabolite databases in the plant field is particularly difficult because plants produce a huge diversity of metabolites – larger than that of animals and microorganisms. In fact, a single accession of *Arabidopsis thaliana* is expected to produce ~5000 metabolites or more. AraCyc (Mueller et al.,

2003) is one of the most extensive databases that contains 2,632 compound entries to date. Other databases that include plant metabolite data are KEGG (Okuda et al., 2008), PlantCyc and KNApSAcK (Yonekura-Sakakibara and Saito, 2009).

In metabolomic studies, metabolite signals are identified by comparing their chromatograms and mass spectra with those of standard compounds available in libraries. However, the pool of identified compounds for some of the technologies e.g. LC-MS, especially for secondary metabolites, is very much limited. Tandem MS may be employed for structural elucidation in these cases. The most thorough spectral libraries concern GC-MS technology. In the present work, the Golm Metabolome Database (Hummel et al., 2007) was used for the annotation of GC-MS data.

## **1.4 Applications of metabolomics in plant research**

### **1.4.1 Functional genomics**

Metabolomics as functional genomics tool aims to replace or complement the somewhat laborious and low-throughput classical forward genetic approaches. The key role of metabolomics in decoding the functions of genes has been reported extensively in the recent years (Bino et al., 2004; Fiehn, 2002; Hagel and Facchini, 2008; Hall, 2006; Oksman-Caldentey and Saito, 2005). In plant systems, metabolomics can be a valuable tool for the identification of responsible genes and their products, or plant adaptations to different abiotic stresses. The detailed characterization of metabolic adaptations to low and high temperature in *Arabidopsis thaliana* has already demonstrated the power of this approach (Kaplan et al., 2004). Metabolomics approaches have been successfully used to assess the natural variance in metabolite content between individual plants, an approach with great potential for the improvement of the compositional quality of crops (Fernie and Schauer, 2009; Schauer and Fernie, 2006). The determination of the role of both metabolites and genes can provide new ideas for genetic engineering and breeding.

### **1.4.2 Mutant analysis**

The analysis of phenotypic mutants can greatly contribute to our understanding of the structure and regulation of biosynthetic pathways in plants (Keurentjes, 2009). Metabolomics, due to its unbiased approach, has become a major tool in the analysis

of direct transgenesis/mutation effects, as well as for the investigation of indirect and potentially unknown alterations of plant metabolism. Metabolomics approaches have been successfully used to phenotype genetically and environmentally diverse plant systems, i.e. to determine the influence of transgenic and environmental manipulations on a number of transgenic potato tubers altered in their starch biosynthesis pathway, and wild type tubers incubated in different sugars using GC-MS (Roessner et al., 2001). Many approaches for phenotypic analysis have been described, ranging from changes in the whole plant phenotypes, or novel assays for detecting specific compounds. The ultimate aim is to switch from specific classes of molecule to more global metabolomics approaches.

The advancements in MS have allowed multiple compounds to be analysed simultaneously, for example, LC-MS/MS analysis was efficiently used for the screening of 10,000 Arabidopsis random mutant families for changes in levels of free amino acids in seeds (Jander et al., 2004). The combination of mutants screening and genetic mapping based identification can enhance the efficient discovery of genes that influence enzymes in multiple pathways, of relationships between different metabolites, and between metabolites and other traits.

The distinctiveness of mutant phenotypes was explored in a comparative analysis that employed different fingerprinting technologies (NMR, GC-MS, LC-MS, FTIR) and machine learning techniques (Scott et al., 2010). (The present thesis employs a subset of the same data (the “HiMet” project, Chapter 4)). The use of metabolite fingerprinting for the rapid classification of phenotypic Arabidopsis mutants has also been reported (Messerli et al., 2007). Both of these studies demonstrated that metabolomic analysis can successfully be used for the prediction of uncharacterized mutants, in this way assisting in the process of gene discovery.

### **1.5 Further challenges in plant metabolomics**

Considering the role of metabolites in biological systems, metabolomics can be a very important tool in efforts to decipher plant metabolism. However, the biochemical richness and complexity of plant systems will always remain one of the fundamental challenges. Future directions in the field are set to involve the improvement of the technological capabilities, the construction of public available

databases for plant metabolite annotation and finally the ultimate effort for systems biology approaches that integrate analyses from metabolomics, transcriptomics and proteomics experiments. Examples from studies in microorganisms show that this is a promising research field, and such data sets are beginning to become available for plant systems (Last et al., 2007; Redestig et al., 2011). In relation to the establishment of a thorough data analysis pipeline, the ultimate goal of metabolomics is to realize the full potential of technology and data handling methods, and leave biological interpretation as the only real bottleneck remaining.

**CHAPTER 2:**  
**METABOLOMIC TECHNOLOGIES**

## 2 METABOLOMIC TECHNOLOGIES

The development of high-throughput methods for measuring large numbers of compounds has been facilitated in recent decades by rapid improvements in analytical technologies. In order to enhance the information available from the enormous amount of recorded (raw) data by the different analytical instruments, a good understanding of the technologies used for the data acquisition is essential.

In this Chapter, I will present the technologies used to acquire the data in the present work, along with a number of issues common to all the high-throughput analytical techniques. In general terms, the capabilities of the different technologies to analyse small molecules differ in the amount and type of compounds analysed per run, in the quality of structural information they obtain, and in their sensitivity (Weckwerth, 2007). With regard to analysing the wide range of metabolites within a cell, each technology provides particular advantages and disadvantages. There is no instrument able to measure all compound classes involved in an ‘omic’ scale analysis (Dunn and Ellis, 2005), therefore a combination of different technologies is often necessary to gain a broad view of the metabolome of a tissue (Hollywood et al., 2006). The most commonly used metabolomics techniques are chromatographic techniques coupled to mass spectrometry (MS), and nuclear magnetic resonance (NMR). In this work, the data were acquired by either Liquid Chromatography mass spectrometry (LC-MS) or Gas Chromatography mass spectrometry (GC-MS), and they are discussed in depth below.

### 2.1 The extraction method

Metabolomics presents a significant challenge for the extraction methodology, due to the required comprehensiveness of the extract, which should represent as large number of cellular metabolites as possible. Moreover, in order to have reproducible measurements, the conditions and provenance of the biological material should be as homogenous as possible in terms of environment (e.g. light, temperature, time of sampling), and the enzymatic activity should be halted for the duration of the extraction process to prevent possible degradation or inter-conversion of the metabolites (Canelas et al., 2009; Lin et al., 2007; Moco et al., 2009).

The extraction method should also be adapted toward the analytical technique used and the required metabolite range. No single extraction method is ideal for all the metabolites within a cell or tissue. For metabolomics, with its implication of a hypothesis-free design, a fast, reproducible and unselective extraction method is preferred for detecting a wide range of metabolites. Wherever feasible, internal standards can be added to the extraction solutions for quality control and for subsequent quantification of the samples (Fiehn et al., 2008; Major and Plumb, 2006). Good analytical practice is also to conduct measurements on reference or “quality control” (QC) samples at regular intervals during a study. The aim is to be able to monitor and potentially correct for variations in the data due to changing instrument response, an inevitability in virtually all analytical technologies.

## **2.2 Mass Spectrometry**

The main requirement for metabolomic analysis is the ability of an instrument to detect chemicals of complex mixtures with high accuracy. MS is ideal for this kind of analysis because it can detect and resolve a broad range of metabolites with speed, sensitivity and accuracy (Dettmer et al., 2007). It produces mass spectra with very sharp peaks which to a great extent are independent of each other and reflect different metabolites. The key components of a mass spectrometer are shown in Figure 2.1.

The data produced by mass spectrometric systems can be used in metabolomic approaches without any knowledge of what chemicals are involved. However, mass spectrometers can also be useful tools for subsequent structural identification of unknown compounds. MS can be used to analyse biological extracts either directly via direct-injection MS, or following chromatographic or electrophoretic separation. (van Zijtveld et al., 2003). Direct injection mass spectrometry (DIMS) is a very rapid technique to analyse large number of metabolites, but it has drawbacks mostly because of a phenomenon known as ion suppression, where the signal of many analytes can be lost at the mass spectrometer interface. For example, if one chemical prevents ionisation of another, it may erroneously be concluded that the second is absent. Moreover, without tandem MS (that involves multiple steps of fragmentation), DIMS cannot distinguish isomers.

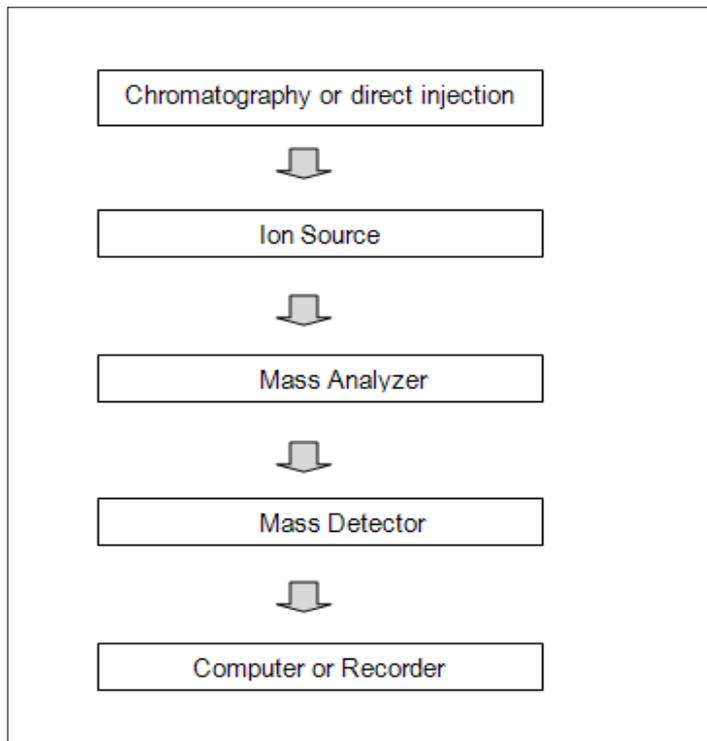


Figure 2.1. Basic diagram for a mass spectrometer

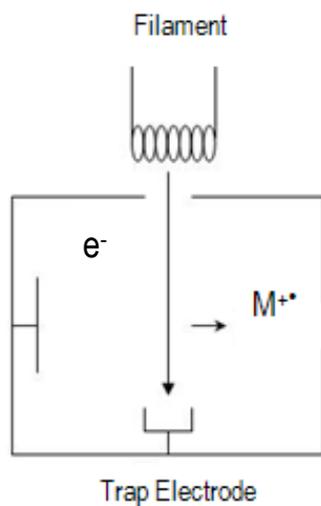


Figure 2.2. The ion source consists of a heated filament giving off electrons. The electrons are accelerated towards an anode and collide with the gaseous molecules of the analyzed sample injected into the source.

The first step for most of the techniques in mass spectrometry is the ionization of the neutral molecules and the following decomposition of the molecular ions that are produced. All these ions are separated according to their mass-to-charge ratio and are detected in proportion to their abundance. Ultimately, the fragmentation products provide information regarding the nature and the structure of the precursor molecule.

### **2.2.1 Ion Sources**

An ion source (Figure 2.2) converts the gas or the liquid phase sample molecules into ions. There are several techniques for the ionization of the samples prior to the analysis in mass spectrometers. Some of them are very energetic and cause extensive fragmentation, while others are softer and only produce molecular species. The physicochemical properties of the analyte are very important at this stage, as it is usually the ionization step that determines what types of samples can be analyzed by mass spectrometry, i.e. some techniques are limited only to volatile and thermally stable compounds.

Electron ionization and electrospray ionization (Figure 2.3) are very commonly used in GC-MS and LC-MS analysis respectively (Cole, 1997). These two methods are described in some more detail below. Others include: Field Desorption (FD), Plasma desorption, laser desorption and Matrix Assisted Laser Desorption Ionization (MALDI), fast atom bombardment (FAB), thermospray, atmospheric pressure chemical ionization (APCI), thermal ionization (TIMS), and gas-phase ion molecular reactions (De Hoffmann and Stroobant, 2007).

#### **2.2.1.1 Electron ionization**

Electron ionization is the most common form of ionization. It is suitable only for gas-phase ionization, which requires that the compounds are sufficiently volatile. Gases and samples with high vapour pressure are introduced directly into the source, while liquids are heated in order to increase their vapour pressure. This technique induces extensive fragmentation; the electron energy applied to the system is typically 70 eV (electron Volts), with the result that molecular ions are not always observed. Because of the extensive fragmentation, it works well for structural identification of the compounds (Figure 2.4).

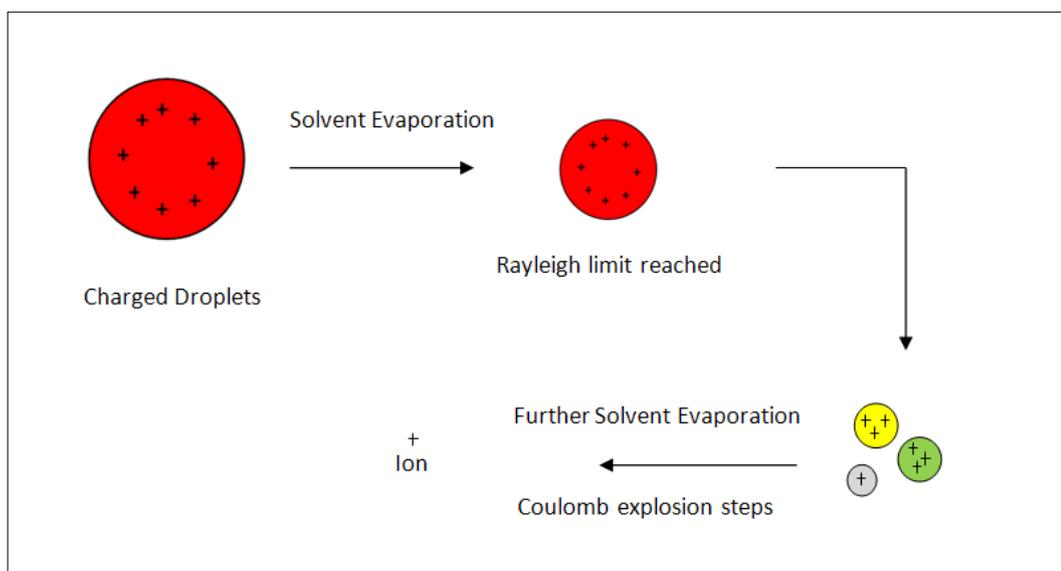


Figure 2.3. Electro spray ionization

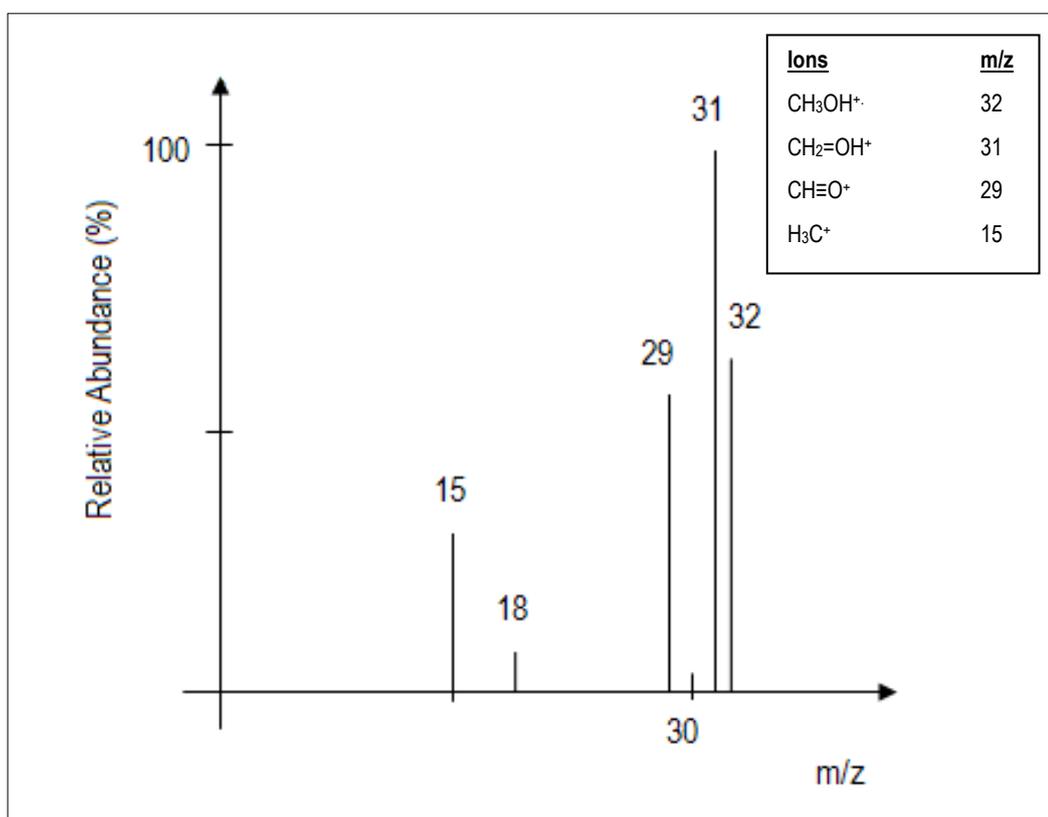


Figure 2.4. Mass spectrum of methanol by electron ionization. The y-axis is the relative abundance of each ion, which is related to the number of time an ion of that m/z occurs. All ions are shown as a percentage of the most abundant ion (CH<sub>3</sub>OH<sup>+</sup> in this spectrum).

Chemical ionization is a complementary method to EI, that produces ions with little excess energy, thus less fragmentation, and molecular species can easily be recognised.

#### **2.2.1.2 Electrospray ionization (ESI)**

Electrospray is a soft ionization technique and belongs to the liquid phase ion sources, where the analyte is in a solution. This solution is introduced by nebulisation (as droplets) into the mass spectrometer through some vacuum pumping stages. An electrospray is produced by applying a strong electric field, under atmospheric pressure, to the liquid through a capillary tube. The effect of the electric field as the solution emerges from the tip is to generate a spray of highly charged droplets that pass down a potential (and pressure) gradient towards the analyser. An important feature of ESI is its sensitivity to concentration and not to the total quantity of sample injected, as is the case in most other sources. The development of electrospray ionisation (ESI) has had a major impact on the mass spectrometric analyses of a broad range of analytes, and in particular for the analysis of macromolecules.

#### **2.2.2 Mass analyzers**

Once the ions have been produced, they need to be separated according to their masses. The ions are transported to a mass analyzer, which sorts them by their mass-to-charge ratio ( $m/z$ ) by the application of appropriate electromagnetic fields. The main characteristics of a mass analyzer are the upper mass limit, the transmission and the resolution. The upper limit determines the highest value of the  $m/z$  that can be measured. The transmission is the number of ions reaching the detector compared to the number of ions produced by the source. Finally, the resolving power is the ability to yield distinct signals from two ions with a small mass difference.

There are many types of mass analyzers (De Hoffmann and Stroobant, 2007), using either static or dynamic fields, and magnetic or electric fields (Table 2.1). Many mass spectrometers use two or more mass analyzers for tandem mass spectrometry (MS/MS). Some of the common types are the quadrupole mass filter, the quadrupole ion trap, the Time-of-flight (TOF), the Fourier transform ion cyclotron resonance,

**Table 2.1.A comparison of mass resolution, mass accuracy and linear dynamic range for different MS configurations**

| Different MS configurations |          |   |
|-----------------------------|----------|---|
| Quadrupole instruments      | Q-MS     | <i>Resolution about 2,500<br/>Good mass accuracy<br/>Limited dynamic range</i>  |
| Time-of-flight MS           | TOF-MS   | <i>Resolution about 10,000<br/>High mass accuracy<br/>Limited dynamic range</i> |
| Hybrid TOF-MS               | Q-TOF-MS | <i>Resolution about 10,000<br/>High mass accuracy<br/>Limited dynamic range</i> |
| Fourier Transform-MS        | FT-MS    | <i>Resolution about 100,000<br/>High mass accuracy<br/>Wide dynamic range</i>   |

and the Orbitrap. The first two are low-resolution, and the latter three high-resolution analysers. Of the most common analysers, which were used to acquire the data in the present work, are the quadrupole and the ion trap.

#### **2.2.2.1 The quadrupole**

Quadrupole is a mass filter that produces an oscillating field created between four parallel rods. A quadrupole mass analyzer acts as a mass-selective filter and only ions with a given  $m/z$  range can pass through the system. Quadrupoles are low resolution instruments. Usually, the quadrupoles are operated at unit resolution, i.e. resolution that is sufficient to separate two peaks that are one mass unit apart.

#### **2.2.2.2 Ion trap**

The ion trap analyzer is a type of mass analyzer in which ions are confined in space by means of a three-dimensional, rotationally symmetric quadrupolar electric field capable of storing ions at selected  $m/z$  ratios. The ions are trapped in a quadrupole field, in a space defined by the electrodes, and are sequentially ejected. It is also possible to build a linear ion trap using quadrupoles, which is the case in the LTQ (“linear trap quadrupole”) Orbitrap, for example.

#### **2.2.3 Detectors**

The final element of the mass spectrometer is the detector. The detector records either the charge induced or the current produced when an ion passes by or hits a surface. In a scanning instrument, it measures the value of an indicator of quantity (the signal produced in the detector during the course of the scan versus where the instrument is in the scan and produces a mass spectrum, representing the abundances of each ion present as a function of  $m/Q$ ).

#### **2.2.4 Important MS Parameters**

There are several instrumental parameters that describe the performance of a mass spectrometer, which are used to determine whether the instrument suits the intended analysis. The most important are the mass spectrometer’s resolving power and mass accuracy. Mass resolution is the ability of the detector to distinguish two peaks of slightly different  $m/z$  and it is described as the difference in mass-to-charge between the two adjacent mass signals. Mass accuracy is used to indicate the deviation of the

instrument's response from a known mass and it is described by the ratio of the mass error and the expected mass:

$$\Delta m = \frac{m(\text{measured}) - m(\text{real})}{m(\text{measured})}$$

where  $\Delta m$  is usually represented as parts per million, ppm. The quality and the quantity of mass signals can be significantly improved by the using high-resolution and ultra-high resolution accurate mass spectrometers.

The mass detector's sensitivity and the linear dynamic range are also very important. Mass sensitivity is the ability of an instrument to separate the intensity of a real analyte from the noise. Sensitivity is given by the ratio between the intensity level of the mass signal and the intensity level of the noise:

$$SNR = \frac{\textit{intensity of mass}}{\textit{noise}}$$

Linear dynamic range is the range over which the ion signal is linear with the analyte concentration. In general, the development of new analytical techniques is largely focused on increasing the resolution and the comprehensiveness of the metabolites that are measured and on increasing the speed and throughput of the analytical assays.

### **2.3 MS- chromatography coupling**

The coupling of MS to chromatographic techniques enables the separation of the mixture components before samples enter the mass spectrometer. By adding a separation technique, the number of ions being measured at a given time is reduced, which improves the analytical properties of the method by reducing ion suppression. Moreover, chromatography can separate isomers, providing a way to measure compounds with exactly the same mass. The separation properties usually reflect the type of molecule being measured, i.e. polar versus hydrophobic or positively charged versus negatively charged.

In the case of mass spectrometry-chromatography coupling, the instrument's resolving power in the time direction, i.e. a reasonably constant retention time scale, is a very important prerequisite for obtaining consistent data that can be properly combined across different sample acquisitions.

### **2.3.1 Gas Chromatography**

GC-MS technology is highly suitable for rapid metabolite profiling, because it is a very versatile technique which offers comprehensiveness for different compound classes. Many applications have been developed for the most common plant metabolites (Last et al., 2007). GC-MS is well established for chemical identification and there is a large knowledge-base of literature and spectral libraries for all the main metabolites (Schauer et al., 2005), the largest of which is the 2005 NIST/EPA/NIH Mass Spectral Library (<http://www.nist.gov/srd/nist1.htm>).

However, GC-MS has several limitations (Kopka, 2006). First of all, samples have to be sufficiently volatile. Such compounds are introduced directly, but for non-volatile components, chemical derivatization is required. Most metabolites analyzed by GC-MS can be partitioned into polar and non-polar fractions, and after specific derivatization, each fraction made volatile. There are a number of strategies for derivatising compounds prior to GC/MS analysis, e.g. silylation, alkylation, acylation and alkoxyamination, the standard procedure in plant metabolomics is to first derivatise them using methoxyamine ( $\text{CH}_3\text{-O-NH}_2$ ) in pyridine to stabilize carbonyl moieties in the metabolites. Chemical derivatization provides significant improvement in the compounds' separation but has the drawback that it adds an extra step into the analytical procedure, and it can introduce artefacts in the process, for instance multiple derivatives of some compounds (e.g. amino acids) or derivatives of reducing sugars.

GC-MS is most suited to small molecules. Large complicated molecules tend not to be particularly volatile, and their derivatization is not easy. Measurements of higher phosphates, co-factors and nucleotides have to be carried out using other techniques. Moreover the analysis of secondary plant metabolites, and metabolites with relative molecular masses exceeding  $m/z$  600-800 is not feasible using GC-MS techniques. Finally, samples are destroyed by the GC-MS sampling procedure.

### **2.3.2 Liquid Chromatography**

Similar to gas chromatography MS (GC-MS), liquid chromatography mass spectrometry (LC-MS) separates compounds chromatographically before they are introduced into the mass spectrometer. It differs from GC-MS in that the mobile phase is liquid, usually a mixture of water and organic solvents, instead of gas. LC-MS most commonly uses soft ionization sources.

LC-MS is being increasingly used in metabolomics applications due to its high sensitivity and the large range in analyte polarity and molecular mass it detects, which is wider than GC-MS. LC-MS has a strong advantage over GC-MS (Díaz Cruz et al., 2003), in that there is no need for chemical derivatization of metabolites (required for the analysis of non-volatile compounds by GC-MS). A substantial drawback for the LC-MS as a non-targeted profiling tool is the lack of transferable mass spectral libraries. On the other hand, LC-MS can be a very good tool for structural elucidation of unknown compounds, especially when it uses tandem MS.

### **2.4 Other technologies**

Capillary electrophoresis (CE-MS) is an alternative MS technology used in metabolomics, which has a very high resolving power and can profile simultaneously many different metabolite classes (Terabe et al., 2001).

Along with MS, NMR is one of the most important technologies in plant metabolomics (Krishnan et al., 2005; Ratcliffe and Shachar-Hill, 2005). It can detect a wide range of metabolites and provides both structural and quantitative results. It has the great advantage that is a non-sample-destructive method. The main drawback is that it provides lower sensitivity compared to other techniques regarding the analysis of low abundance metabolites, thus it is not efficient for very complex mixtures. For improved identification results the combination of NMR with MS can be a very powerful strategy (Exarchou et al., 2003; Moco et al., 2007a).

Other alternatives include thin layer chromatography, FT-IR (Johnson et al., 2004) and HPLC with ultraviolet (UV) but these give virtually no structural information.

## **2.5 Summary**

The various metabolomics technologies provide different standards in analytical precision, comprehensiveness and sample throughput. Each technique has particular advantages in the identification and quantification of the metabolites in a biological sample. LC-electrospray and NMR are considered as very important technologies in the metabolomic race; LC-ESI for its coverage and sensitivity, NMR for its coverage, resolution and structural aspects, especially where sensitivity is not the main concern (e.g. concentrated medical samples versus dilute plants). However, the comprehensiveness for different compound classes make GC-MS technology a superior technique for plant metabolomics. Moreover GC-MS is quick, cheap, has reasonable coverage, with good structural libraries, and was the technique of choice for the major study reported in this thesis (Chapter 5), on starch metabolism in *Arabidopsis*.

**CHAPTER 3:  
COMPUTATION**

### 3 COMPUTATION

#### 3.1 Pre-Processing – pipeline step 1

The first step in the data analysis pipeline is data pre-processing, which involves aligning and peak extraction/integration processes that prepares the multiple samples of raw data for the statistical modelling step. It is very important to perform this first step diligently, since the accuracy and reproducibility of results from analysing LC-MS and GC-MS data sets depend in part on careful data pre-processing.

Untargeted metabolite profiling yields a vast amount of complex data that can be difficult to handle. Figure 3.1 shows an example of a three-dimensional surface of LC-MS data that indicates the many components and the complexity of the nature of the chromatographic data. Data pre-processing includes a variety of different procedures for editing and analyzing mass spectrometric chromatographic data, such as signal detection, spectral calibration, de-noising, baseline correction and normalization (Bijlsma et al., 2006). The aim is to optimize the resulting matrix of identified peaks and transform the data into a format that makes the subsequent statistical analysis easier and more robust.

There are a number of tools for pre-processing MS-data, proposing different analysis methods and algorithms; in this work I extensively used the XCMS software ([metlin.scripps.edu/xcms/](http://metlin.scripps.edu/xcms/)). XCMS (Smith et al., 2006) has advanced capabilities for feature selection, and is emerging as a very important resource in the metabolomics field, not least because of its use of open source software (Corrado, 2005; Gentleman et al., 2004). The XCMS software suite was developed initially for pre-processing LC-MS data, and to our knowledge, it is used predominantly for this purpose. However, with appropriate modification, it should also be highly useful for treating GC-MS data. This approach is explored in the present work, in which I will disclose the application of XCMS to GC-MS data, identifying the most important parameters and the manner in which they need to be adjusted in order to optimize the pre-processing step for this different class of data.



### 3.1.1 XCMS – an overview

XCMS is a package developed in R ([www.r-project.org](http://www.r-project.org)) and made available by the Bioconductor Project (<http://www.bioconductor.org/>), for the treatment of (hyphenated) MS data. It is a sophisticated data analysis tool that includes many options for data handling and visualization. It includes novel algorithms for data analysis (Smith et al., 2006), taking advantage of the many statistical processing routines available in R, whilst allowing the user to control its features in order to optimise the analysis. However, because the software interface is a command line programming environment, it can be a challenge for users without programming experience.

In general terms, the XCMS software package transforms large, full-scan raw MS data into a much smaller matrix of pre-processed data. XCMS has some prerequisites regarding the input file formats. All data must be input in one of the following raw file types: aia/andi, NetCDF, mzXML and mzData. In these file formats, the data are stored as separate lists of mass/intensity pairs with each list representing one scan. NetCDF (Rew and Davis, 1990), which has been used in the present work, is a very common format and most MS instruments incorporate software for conversion to this file type. XCMS outputs the final matrix of processed data into a tab separated value (.tsv) file. This includes the intensity values for all masses ( $m/z$  values) detected, for each one of the samples. The number of values can range from a few hundred to a few thousand.

The pre-processed data may be subjected to further feature selection and subsequent multivariate statistical analysis. XCMS offers some statistical processing, but this is restricted to univariate ANOVA-type analyses on grouped data only (single grouping variable). Furthermore, to utilise the XCMS statistical analysis features, data files should be organised in subdirectories based on the sample grouping characteristics e.g. cell type or mutation. More commonly, the final matrix of pre-processed is output from XCMS and transferred to a dedicated package for statistical analysis (as implemented in the present work).

The most important advantages of XCMS is that it works quickly, and crucially, unlike the most common alternatives, it does not require the use of internal standards

for the retention time alignment (Elizabeth et al., 2006). The ability of its algorithms to work without internal standards is very important. It is sometimes desirable to avoid the addition of chemicals during sample preparation that may interfere with the experimentally relevant metabolites. The isotopic and the adduct peaks are treated as separate metabolite features, thus contributing to the total number of the identified metabolites.

### 3.1.2 The XCMS environment

XCMS is implemented as an object-oriented framework within the R programming environment. XCMS provides two main classes for data storage and processing, respectively represented by the `xcmsRaw` and `xcmsSet` objects. Each class includes several fixed algorithms and arguments that can be altered for the data analysis. The properties of the `xcmsRaw` and `xcmsSet` objects are compared in Table 3.1., where it can be noticed a considerable reduction in storage requirements that results from the pre-processing inherent to the `xcmsSet` object (in the example given, 6.34Mb from an entire experimental data set versus 38.5Mb from each individual sample). This also represents a substantial reduction in complexity, in terms of evaluating the experimental data, which is the principal reason for the use of a pre-processing package.

### 3.1.3 XCMS pre-processing steps

Pre-processing in XCMS is conducted in three main steps, applying a series of algorithms to achieve the following (see also flowchart in Figure 3.2):

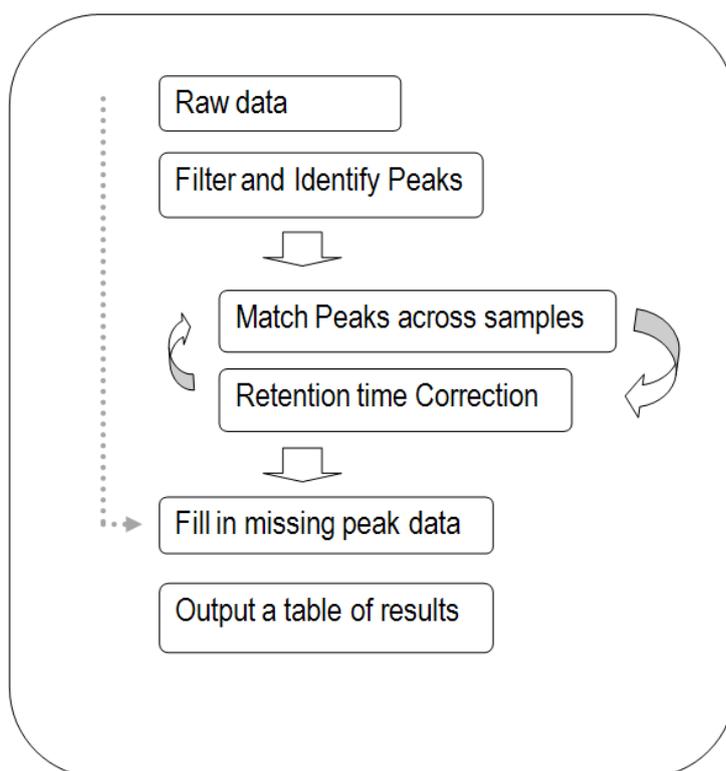
- (1) **Peak detection:** identify peaks in each of the samples;
- (2) **Retention time alignment:** match peaks with similar retention times across multiple samples, and use the groups of matched peaks for time alignment;
- (3) **Fill in any missing peaks** that peak identification initially failed to recognise, or fill in appropriate data for peaks that are genuinely missing from a sample, by integrating raw data at an appropriate retention time.

Each of these steps will now be described in detail.

#### 3.1.3.1 Peak detection – peak width considerations

The complexity of this initial step is related to a certain degree to the presence of noise, which can mask the important components of the chromatographic data.

| <i>Table 3.1. A comparison of the xcmsSet and xcmsRaw objects.</i> |   |  |
|--|---|--|
| <b>Object</b>  | <b>xcmsSet</b>  | <b>xcmsRaw</b>   |
| <b>Mode</b>  | “Batch mode”  | “Single run”   |
| <b>Purpose</b>   | Transformation of a set of peaks from <b>multiple samples</b> into a matrix of processed data | Processing and visualization of the raw data from a <b>single run</b>            |
| <b>Typical memory usage</b>  | An xcmsSet object with 42 samples with about 632 <b>peaks</b> per sample:<br><b>6.34 Mb</b>   | An xcmsRaw object with 1 sample and 5773 <b>mass spectra</b> :<br><b>38.5 Mb</b> |



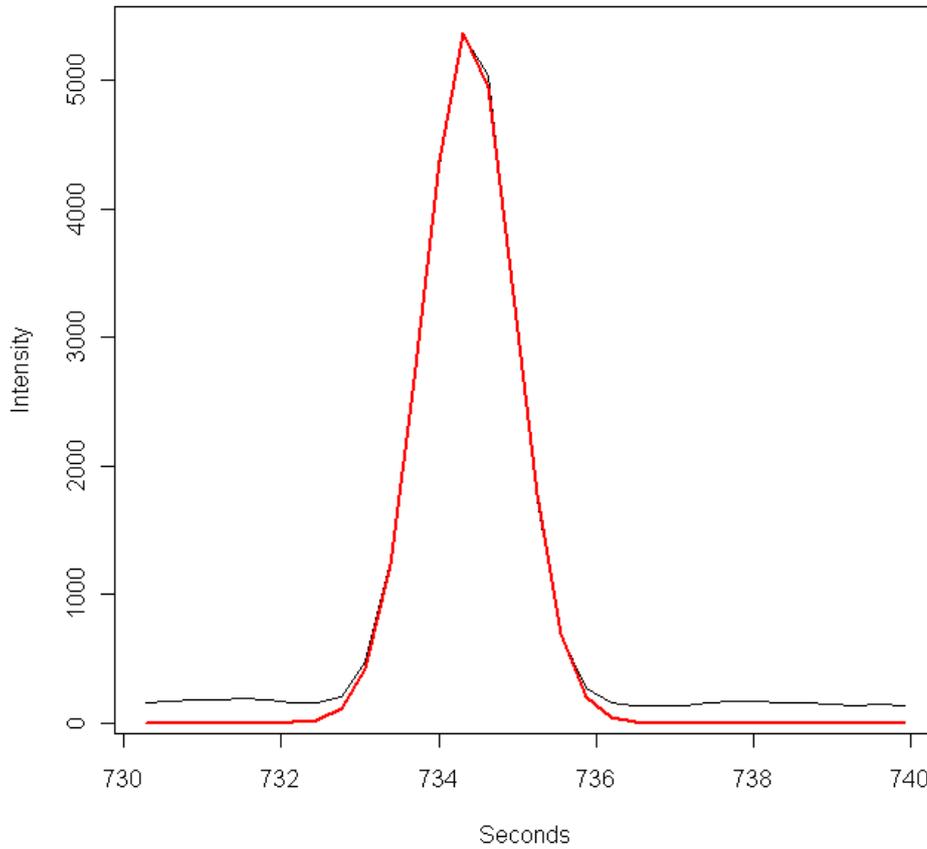
*Figure 3.2. Flowchart showing the pre-processing steps incorporated in XCMS*

A good peak detection method should be able to reduce the noise and read complex data in a comprehensive manner with the minimum loss of information. The XCMS peak detection step provides a robust and reproducible method able to filter out random noise and detect peaks with low signal-to-noise ratio.

The peak detection algorithm cuts the data into slices one tenth of a mass unit (0.1  $m/z$ ) wide, and then operates on the individual slices in the chromatographic domain. Each of these slices is represented as an extracted ion chromatogram (EIC, see Figure 3.1). Before peak detection, each slice is filtered with a “matched filter” that uses a second derivative Gaussian shape to generate a new, smoothed chromatographic profile. Match filtration is based on the application of a filter whose coefficients are equal to the expected shape of the signal, to be discussed below (Danielsson et al., 2002). After filtration, the peaks are detected using the mean of the unfiltered data as a signal-to-noise cut-off. Finally, the peaks are determined by integrating the unfiltered chromatogram between the zero-crossing points of the filtered chromatogram. The most important parameters that need to be chosen at this step are: the *peak width* of the filter, the boundaries of the *mass tolerance window*, and the *binning algorithm*, which are each described below.

- **Peak width.** The shape of a chromatographic peak can be very different depending on the type of chromatography and the type of instrument. For example, LC-MS peaks are much wider than those obtained by GC-MS and TOF-MS. For the best use of the matched filter, the characteristics of the model peak should fit the characteristics of the sample peak. The default XCMS value for the peak full-width at half-maximum (*fwhm*) is 30 (seconds). Note that this is appropriate for LC-MS, but not necessarily for the other techniques. In our work, I established an optimal *fwhm* value of 3 to be used in processing the starch GC-MS data set. The results are discussed in full Section 5.3.2, Figure 5.3; an example of the filter applied to a representative GC-MS sample peak from our data is shown in Figure 3.3.
- **Mass tolerance window (bin width).** Another important consideration is the relationship between the width of the mass peaks and the mass bin width, which

### Averaged Ion Chromatogram: 50-500 m/z



*Figure 3.3. Application of a matched filter to a typical GC-MS chromatographic peak of the starch dataset (Chapter 5). The black trace represents the original peak indicating a very small fwhm value of approximately 1.5 seconds; the red trace shows the fitted peak.*

- in turn is related to the resolution and scan-to-scan accuracy of the instrument. A peak can shift or become distorted for two reasons. First, in high resolution instruments or centroid mass spectral data, where the peak width can be significantly smaller than the slice width, the signal from an analyte may sit almost exactly on the boundary of two bins and oscillate between adjacent slices over chromatographic time, making an otherwise smooth peak shape appear to have a sharply uneven surface. In this case, the maximum signal intensity from adjacent slices is combined into overlapping Extracted Ion Base Peak Chromatograms (EIBPCs). Second, in low resolution instruments, where the peak width can be larger than the default 0.1  $m/z$  slice width, the signal from a single peak may split across multiple slices and the middle of the very broad peak (which is where the centroid line will be placed) will move around quite widely. In this case, instead of eliminating the extra peaks during detection, the algorithm incorporates a post-processing step where the full peak list is sorted and examined by intensity, eliminating any low intensity peaks surrounding the higher intensity peaks in a specific area. By altering the bin width, the XCMS peak detection algorithm can handle, in theory, different peak shapes in a flexible and robust manner.
- Binning algorithm.** The binning algorithm transforms the data from being separate lists of mass and intensity pairs into a matrix with a row representing equally spaced masses and a column for each sample. The software package provides four alternative algorithms, which mainly differ in the way the intensity in the mass bins is calculated, and the method used to interpolate areas with missing data. In this work I used the default parameters for this step.

### 3.1.3.2 Retention time alignment – across samples peak grouping

Time alignment starts with the matching of peaks that represent the same analyte across different samples. The matched peaks are subsequently used for the calculation of retention times and alignment. The important parameter here is the *band width of peak groups (bw)*. The grouping algorithm starts with binning all the samples in the mass domain. After grouping the peaks in bins, the algorithm resolves groups of peaks with different retention times in each bin and starts to operate in the

chromatographic domain. To avoid certain complications, it uses a kernel density estimator to calculate the overall distributions of peaks in chromatographic time (Figure 3.4), and from these distributions identifies groups of peaks with similar retention times. The algorithm employs several criteria for the optimum identification of the groups, i.e. it selects only groups that contain more than half of the samples. The effect of the grouping bandwidth can be seen in Figure 3.4.

The grouping information from the peak matching step is used to identify groups of peaks with a high probability of being well-matched, and these groups are used as temporary standards. For every one of the so-called “well-behaved” groups, the algorithm calculates the median retention time and the deviation from the median for every sample in the group (Figure 3.5). For parts of the chromatogram in which no well-behaved groups are identified, the algorithm uses a local regression fitting model, “loess”, to approximate differences between deviations, and interpolates sections where no peak groups are present. For increased precision, the alignment step can be repeated recursively.

### **3.1.3.3 Filling missing peak data**

XCMS includes a final step in which an algorithm identifies missing samples from the groups, re-reads the raw data and integrates the regions of the missing peaks. Missing samples from the groups can be a result of missed peaks during peak identification, or because a peak is genuinely absent from a sample. This step is very important because difficulties of handling missing values (or large numbers of zeros) may arise in later statistical analysis.

### **3.1.4 Competing software**

There are alternatives to XCMS for pre-processing MS data (Mueller et al., 2008). Amongst the most popular of these are Sieve, MZmine, and MetAlign. Sieve is a commercial software supplied by Thermofisher. It aligns chromatographic data, extracts ion chromatograms (EICs) for every aligned ion and outputs them in a table. Before the introduction of XCMS, Sieve was the only software used by the Metabolite Services (JIC) for metabolomics analysis. Sieve (with a license to Spotfire®) provides a very good user-friendly environment that allows interactive

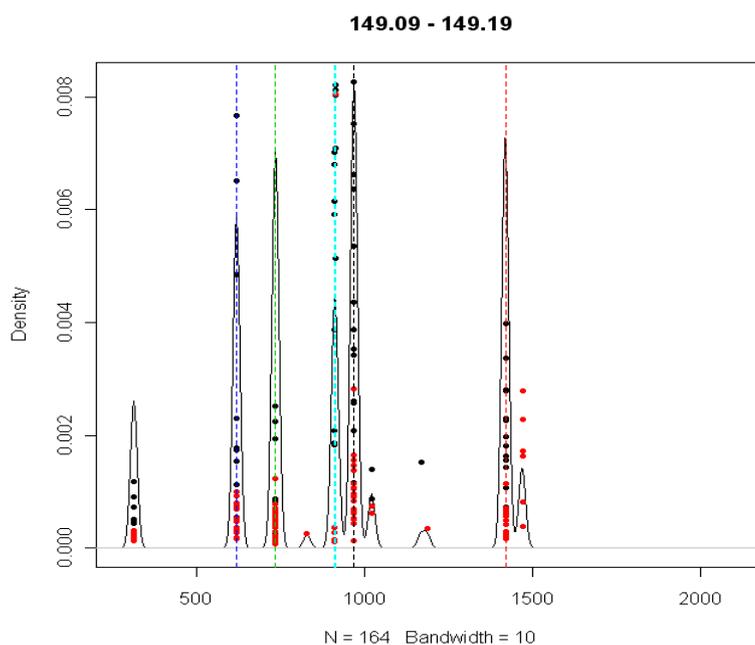
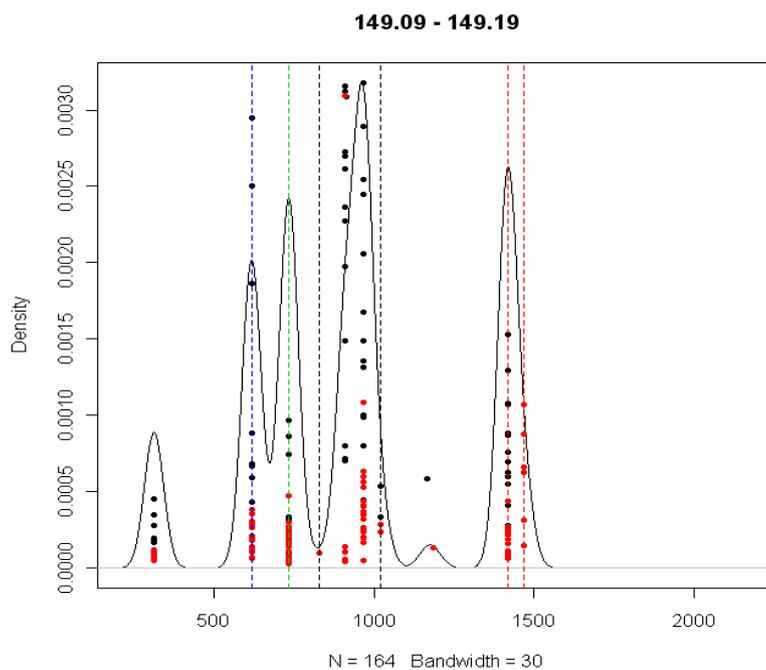


Figure 3.4. An example of cross-sample peak matching from the starch dataset (Chapter 5), using two different band widths. Individual peaks are shown as dots with y-position indicating relative intensity (density). A smoothed peak density profile, which was drawn using a kernel density estimator, is shown as a black continuous line. Coloured dashed lines indicate identified groups. Note that the lower Bandwidth (bw) value decreases the inclusiveness of the grouping only to the peaks with very similar retention times. The impact is more obvious when comparing the two graphs in the area 700-1000s.

### Retention Time Deviation vs. Retention Time

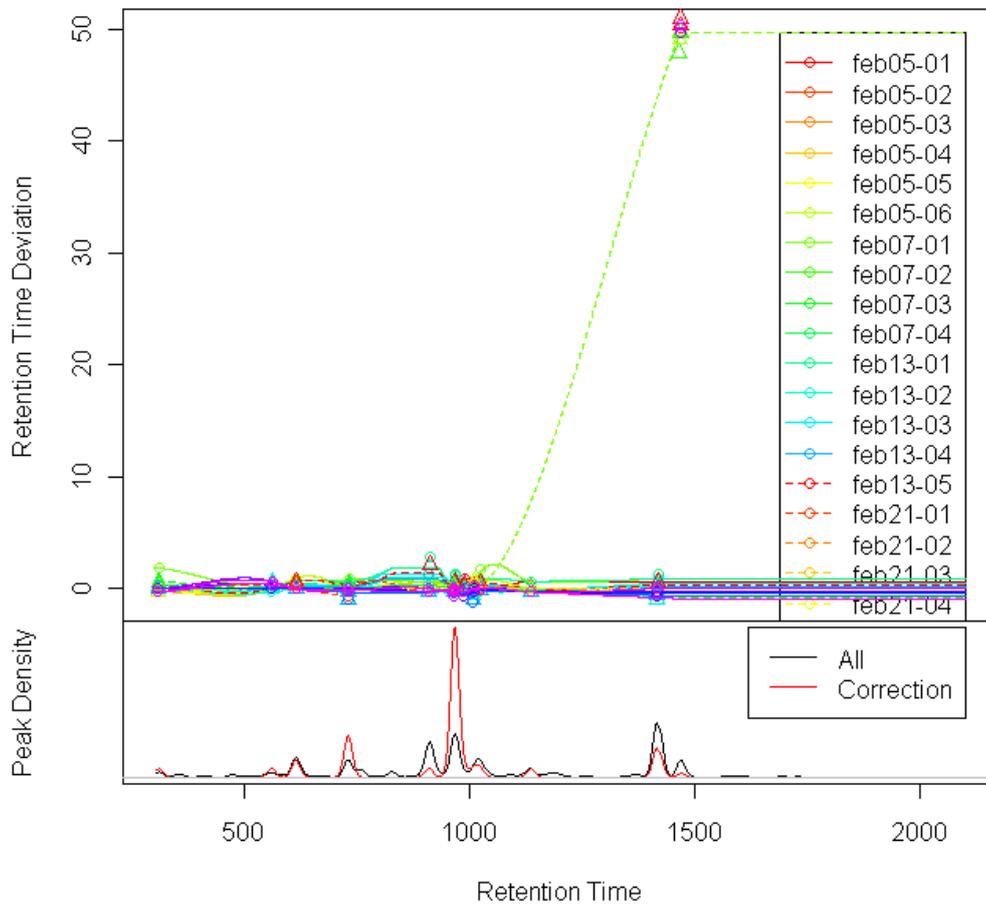


Figure 3.5. Deviation profiles that are used for aligning the samples in starch dataset (Chapter 5). In this example prominent retention time deviations were not observed; deviations in GC-MS data are generally expected to be small. Retention time deviations can be either positive or negative, with negative values indicating that a sample was eluting before most of the others and vice versa. On the bottom segment of the figure, a kernel density estimator is used to show the distribution of all peaks (black trace) and those peaks used as standards for retention time correction (red trace).

visual inspection of the EICs, but it has some crucial flaws. First of all, it is instrument dependent, compatible only with Thermofisher instruments. Moreover, it does not allow access to its proprietary algorithms, thus it is difficult for the user to fully understand how it works. The peak detection algorithms appear unrefined, identifying peaks using unsophisticated thresh-holding processes which are often inadequate.

MZmine (Katajamaa et al., 2006) is an open source package for the analysis of metabolic MS data. It has good functionality, and allows the user to perform a large amount of data pre-processing using EICs (Extracted Ion Chromatograms), and some basic multivariate analysis. It has several visualization algorithms for both the raw and processed data. The most important feature is the alignment tool, which can be used to process data for export to allow analysis in other statistical software packages. MetAlign (Lommen, 2009) is another very popular software programme for the pre-processing and comparison of accurate mass and nominal mass GC-MS and LC-MS data. Its algorithms incorporate several pre-processing steps i.e. data smoothing, local noise calculation, baseline correction, between-chromatogram alignment. It is capable of automatic format conversion and handling of up to 1000 data sets. Finally, many instrument manufacturers provide their own software packages for metabolomic analysis. However, as noted for the Sieve package, such proprietary software is in general instrument-specific and closed-source, so that the numerical methods by which the data are pre-processed are not transparent.

### **3.2 Pre-treatment – pipeline step 2**

This step mainly concerns data scaling processes (mean centring, variance scaling, normalization) and missing values treatment. Here, “scaling” is used to refer to treatments which are applied column-wise (to each variable, or metabolite intensity): for each variable, mean-centring simply consists of subtracting the dataset mean from each intensity, and variance-scaling of dividing each intensity by the dataset’s standard deviation. “Normalization” refers to treatments which are applied row-wise (to each observation or sample), and principally this involves applying a correcting factor so that the sum of all intensities equals unity, making overall intensity scales comparable across samples. The choice of scaling requires a very careful consideration, since scaling alters the relative distances between the observations

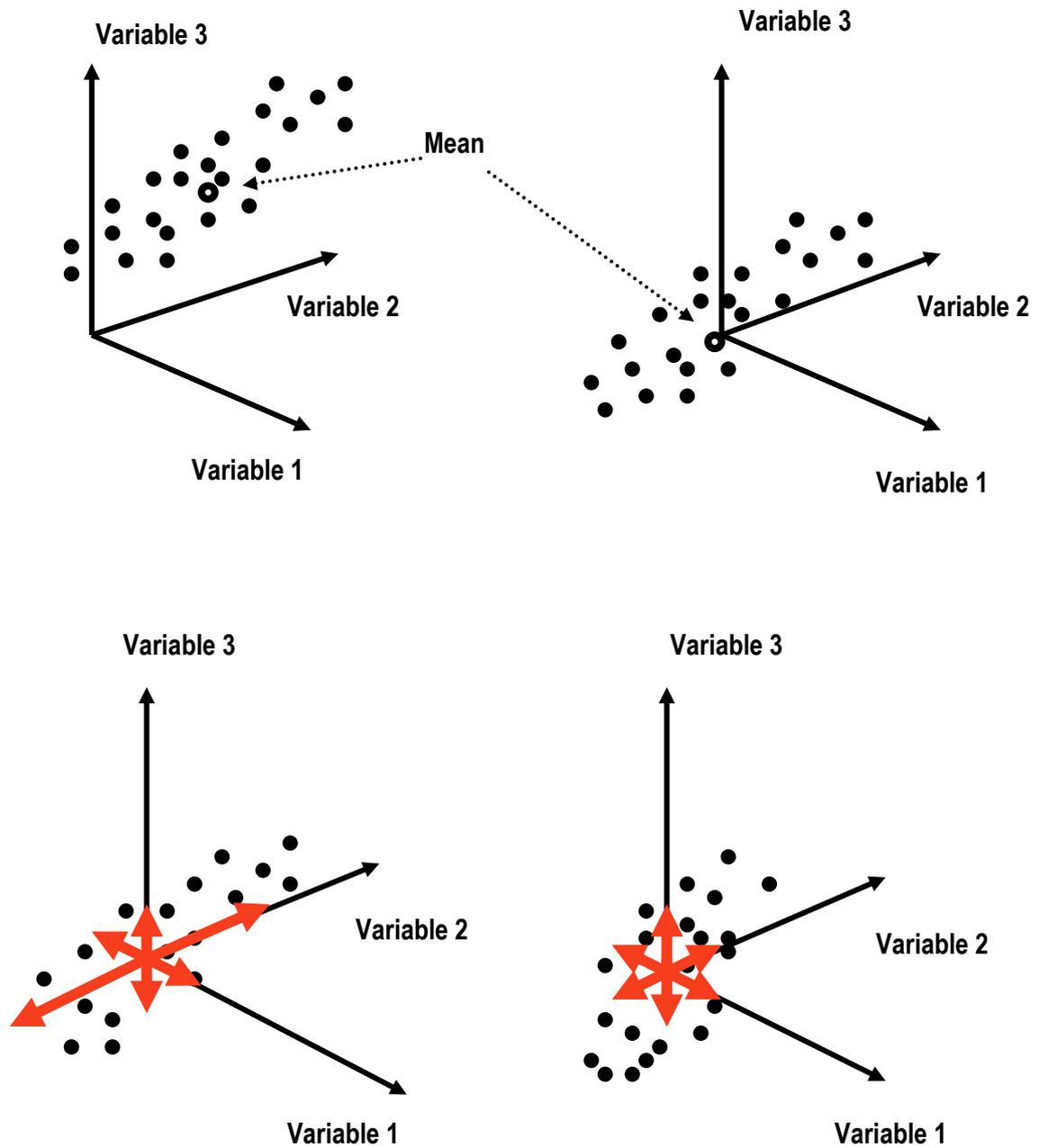


Figure 3.6. Graphical representation of mean centring and variance scaling for a three dimensional system (top left graph: original data cloud; top right graph: mean centred data; bottom left graph: mean centred data; bottom right graph: variance scaled data)

(Figure 3.6), and this can have a dramatic effect on the output of analyses. Similarly how one treats missing variables may have a significant effect on the position of individual samples in clustering diagrams. The effect of these pre-treatments are explored in detail in Chapter 4.

### **3.3 Statistical modelling – pipeline step 3**

The objective of this third step of the analysis pipeline is to find patterns or other sources of systematic variation within the data which can be translated into useful biological information. Because of the size of the data matrix produced by the pre-processing step, and because for metabolomic data, the biological differences between samples sometimes arise from comparatively small concentration differences across many metabolites, recognizing the patterns and interpreting them is not always straightforward.

The statistical methods used in this work can be placed in two main categories – univariate and multivariate approaches. There are very many competing software packages for carrying out statistical analyses, all of which will offer a variety of alternative approaches in both these categories. The packages can be broadly subdivided into two types: those that are front-ended with a GUI (graphical user interface), which must generally be regarded as “black boxes” (commercial examples of GUI-based packages are SPSS and Excel); and those that are based around a statistical programming language, with open access to all the algorithms offered. Matlab (The Mathworks, Inc, Cambridge) is a commercial example of a matrix programming language, and a worldwide standard for multivariate data handling. R is a free, open source language, originally arising from the statistics community. Both Matlab and R have been used throughout this work, including in direct comparisons of algorithm outputs, which as I will show in later chapters, have been found to differ at the level of method implementation. Therefore, it is essential to present the theory behind the statistical methods used in this work, for a thorough understanding of the statistical modelling processes, and representations of the models obtained.

### 3.3.1 Multivariate analysis

PCA (principal component analysis) and PLS (partial least squares) are the most commonly used techniques in the chemometrics field for analysing multivariate (high-dimensional) data (Kemsley, 1996). Both of the methods compress the original data matrix so that underlying patterns may be revealed. PCA is a very useful tool for data visualization and exploration; PLS makes use of a second matrix of data (in our case, categorical) to compress the data in a “supervised” manner. In this thesis, PLS and PCA are used as dimension reduction methods in predictive models, prior to linear discriminant analysis (PLS-DA, PCA-DA). The predictive capability of the hyphenated models is evaluated using cross-validation. In Section 5.4.2.2 a direct comparison between PCA-DA and PLS-DA is shown.

#### 3.3.1.1 Principal Component Analysis (PCA)

PCA can be viewed as a linear transformation of matrix  $\mathbf{X}$  to its principal component scores:

$$\mathbf{Z} = \mathbf{X} \mathbf{P}$$

where  $\mathbf{X}$  is the data matrix,  $\mathbf{Z}$  is the scores matrix and  $\mathbf{P}$  is the principal component (eigenvectors) matrix. The columns of  $\mathbf{P}$  (rows of  $\mathbf{P}^T$ ) are known as loadings, and the columns of  $\mathbf{Z}$  are known as scores (Figure 3.7). Graphically, the matrix  $\mathbf{X}$  can be thought of as occupying a multidimensional coordinate system, and the linear transformation corresponds to rotating the original variable axes onto a new coordinate system (Figure 3.9).

In PCA,  $\mathbf{P}$  is chosen as to satisfy the equation

$$\frac{\mathbf{X}^T \mathbf{X}}{(n-1)} \mathbf{P} = \mathbf{P} \mathbf{L}$$

where  $\mathbf{L}$  is a diagonal matrix whose elements are eigenvalues of the covariance matrix:

$$\frac{\mathbf{X}^T \mathbf{X}}{(n-1)}$$

and the columns of  $\mathbf{P}$  its corresponding eigenvectors. The eigenvalues also represent the variance of the columns of  $\mathbf{Z}$ . For many analysis methods the data matrix  $\mathbf{X}$  is mean-centred (column means subtracted from all entries).

There is also a formulation of PCA in which the  $\mathbf{X}$  matrix is variance-scaled (the mean-centred entries are divided by the respective column standard deviation), in which case the loadings are eigenvectors of the data correlation matrix. Variance scaling alters the relative distances between observations, thus the loadings and scores will differ between the correlation and covariance matrix methods. In the covariance matrix methods, the loadings retain the same units as the original data, which can sometimes allow the analyst to attribute physical meaning to individual PCs. However, in the correlation matrix method, small but potentially useful spectral features can influence the linear transformation as much as large spectral peaks.

### 3.3.1.2 Partial Least Square (PLS)

Partial Least Square analysis is a supervised multivariate data analysis method that particularly confronts the situation of many possibly correlated predictor variables, and relatively few samples. PLS bears a close relation to PCA. The main difference is that PLS, in addition to the  $\mathbf{X}$  matrix, uses also a second input vector  $\mathbf{y}$  of dependent variates. The linear transformation of the  $\mathbf{X}$  and  $\mathbf{y}$  vector (or  $\mathbf{Y}$  matrix, see below) can be thought of as a rigid rotation of the original coordinate system, chosen such that the scores along the transformed axes account for successively maximized covariance between  $\mathbf{X}$  and  $\mathbf{y}$ . The first PLS component maximizes the covariance between  $\mathbf{X}$  and  $\mathbf{y}$ , and is given by:

$$\mathbf{v}_i = \frac{\mathbf{X}_i^T \mathbf{y}_i}{(\mathbf{y}_i^T \mathbf{X}_i \mathbf{X}_i^T \mathbf{y}_i)^{0.5}}$$

where  $\mathbf{v}_i^T \mathbf{v}_i = 1$ . The scores vectors  $\mathbf{z}_i$  are calculated by projecting the data  $\mathbf{X}_i$  onto the loadings  $\mathbf{v}_i$ ,

$$\mathbf{z}_i = \mathbf{X}_i \mathbf{v}_i$$

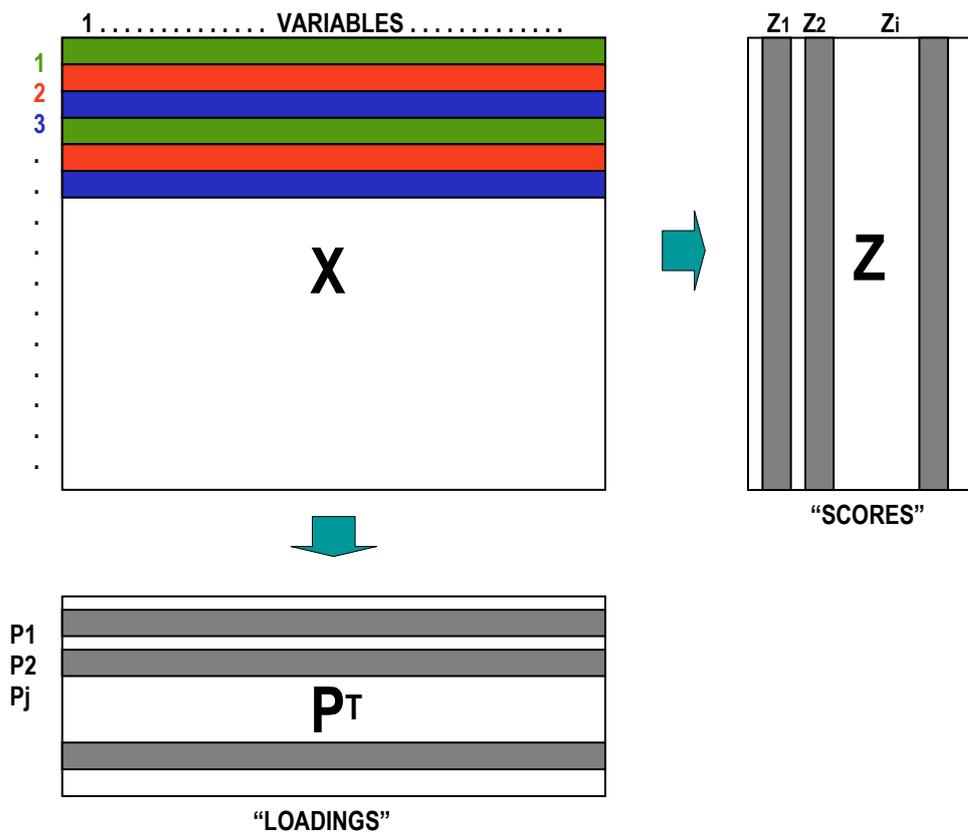


Figure 3.7.A schematic description of the decomposition of the  $X$  matrix to the scores ( $Z$ ) and loadings ( $P$ ) matrices

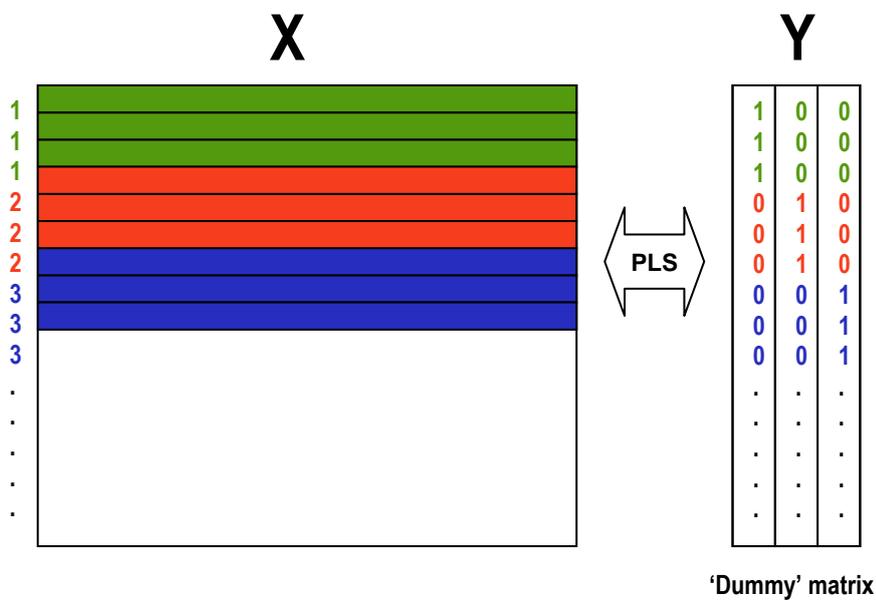
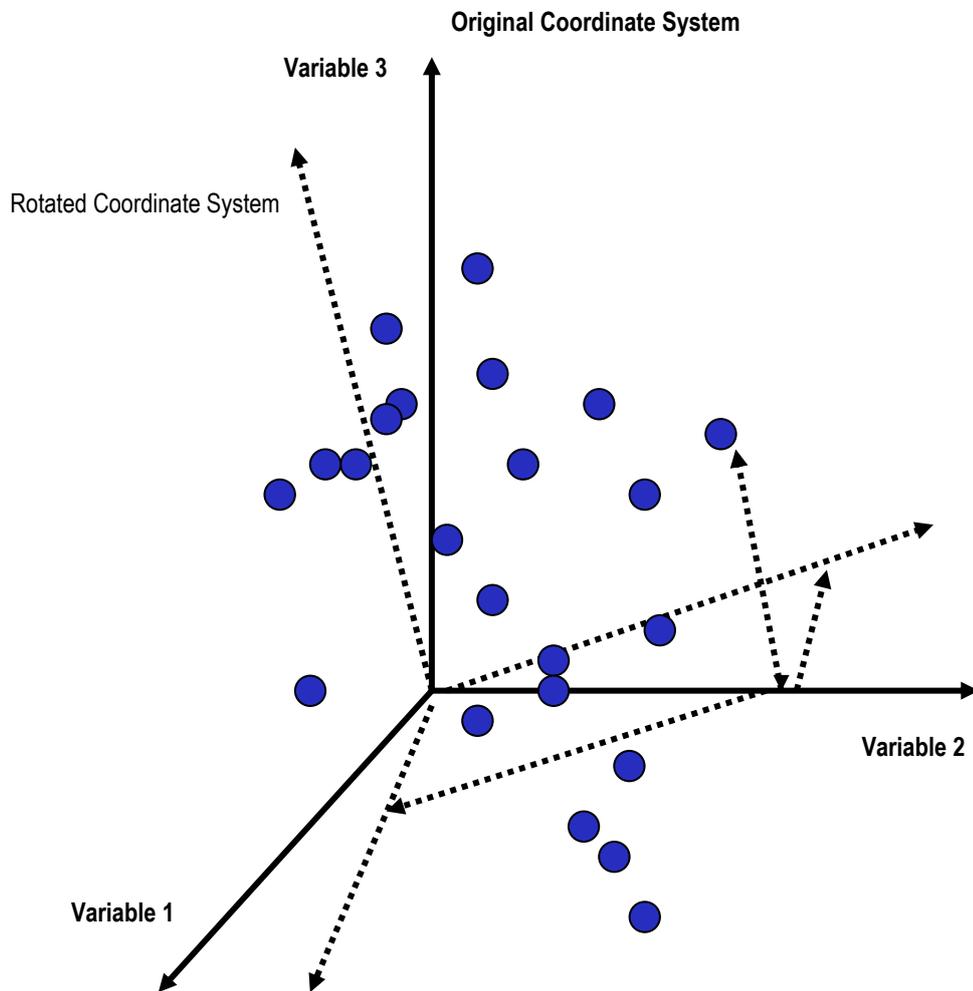


Figure 3.8. In case of supervised methods, in addition to  $X$  matrix a further set of input data (with the original grouping information) is required. For PLS2, this is a matrix  $Y$  of dependent variable as shown above.



*Figure 3.9. An illustration of the rigid data rotation, as occurs in principal component analysis and partial least squares. Each axis of the rotated coordinate system defines a loading, while the projection of each point onto the loadings produces the scores*

The subsequent components are orthogonal (uncorrelated) to the previous components. They are determined iteratively by calculating a residual- $\mathbf{X}$  and  $-\mathbf{y}$  (where the projected part of the data is subtracted from the complete dataset), maximizing each time the covariance between the  $\mathbf{X}$ -residual and  $\mathbf{y}$ -residual. In our work, a dummy matrix  $\mathbf{Y}$  (rather than vector) is required to represent the different groups or categories of data. In this case, the method is sometimes known as PLS2.

The original PLS method was first proposed in the 1980s, when computers were not as advanced as today, and the algorithm had to make compromises at the coding level in order for it to be practical to carry out the calculation of scores and loadings in a reasonable time. Since then, various algorithms have been developed that provide different definitions of PLS, some of which have the specific aim of improving the speed of calculation (Andersson, 2009; de Jong, 1993; Geladi, 1988).

Both R and Matlab provide routines for carrying out PLS regression; additionally for this work, in-house scripts have also been used for conducting the NIPALS PLS method (Miyashita et al., 1990; Rosipal and Krämer, 2006). Some examples of preliminary comparisons carried out in the different development environments are given in Appendix A1, which presents Matlab and R scripts for conducting cross-validated NIPALS PLS2. A Matlab in-house script for NIPALS PLS was applied to a model dataset (infrared spectra of olive oils, publicly available at [www.ifr.ac.uk/Bioinformatics/BSDataSets.html](http://www.ifr.ac.uk/Bioinformatics/BSDataSets.html)) and the script outputs compared with the original R version of PLS. This comparison resulted in a revision to the R script, also shown, to correct for the absence of cross-validated scores amongst the output arguments. Furthermore, in Chapter 5, I present the comparison of the output from two different variants of PLS2 as applied to one of the metabolomics datasets of interest. The methods in question are the NIPALS algorithm as described by Martens (Martens, 2001) and the SIMPLS algorithm (de Jong, 1993) provided as the PLS routine in Matlab).

### **3.3.1.3 Linear Discriminant Analysis (LDA)**

Discriminant analysis is used to find the linear combination of features which best separate two or more groups of observations. The LDA algorithms use the mean

observations of each group, calculate the distance of each observation from each group mean, and re-assign each observation to the nearest group mean. A common distance measure is the Mahalanobis  $D^2$  metric, which has been used in this thesis. The Mahalanobis distance between the  $j$ th observation and the  $k$ th group mean:

$$D^2 = (\mathbf{z}_{(j)} - \mathbf{z}_{(k)}) \mathbf{S}_p^{-1} (\mathbf{z}_{(j)} - \mathbf{z}_{(k)})^T$$

where  $\mathbf{S}_p$  is an average of the covariance matrices calculated separately for each group by:

$$\mathbf{S}_p = \frac{\sum_{i=1}^g (n_i - 1) \mathbf{S}_i}{n - g}$$

where  $n$  is the number of observations,  $n_i$  is the number of observations in group  $i$ , and  $g$  the number of group means. The result of the discriminant analysis is usually given as a list of the group indices to which the observations are re-assigned, and often summarized by the percentage of correct re-assignments.

A consequence of the use of the Mahalanobis distance is that LDA in this form cannot be applied directly to multivariate data sets which contain more variates than observations. Since this generally applies to almost all data matrices arising from modern analytical techniques, it is common practice to use PCA or PLS as a precursor to LDA, forming the hyphenated methods PLS-DA and PCA-DA. It is then the scores from PCA or PLS which are passed as variates into the LDA step.

### 3.3.2 Validation methods

Cross-validation, sometimes also called rotation estimation, is a technique that is used for assessing the goodness of fit of a statistical model, as well as the ability of the model to generalize to an independent data set. It is a vital stage of the modeling process, as it provides an estimate of the “true” (rather than overfit) performance of the predictive model. One round of cross-validation involves the split of the data into complementary subsets, the training and the test sets (Figure 3.10(a)). The analysis is performed on the training set, and the test set is used for model validation. All the

model parameters, i.e. the optimum number of components and the selected variables, are optimized on the training set. To reduce variability, multiple rounds of cross-validation are performed using different partitions, and the validation results are averaged over the rounds.

The differences between the various cross-validation types are based on differences of the sub-set partitioning process. In this work, leave-one-out and leave-segment-out cross validation was used. Both of the methods differ from repeated random partitioning in that all observations are used as both training and tests sets and each observation is used as a test set exactly once. In leave-segment-out (K-fold) cross-validation (Figure 3.10(b)), the original dataset is randomly partitioned into K subsets. A single subset is used as a test set and the remaining are used as training set. The cross-validation process is repeated K-times and each of the subsets is used exactly once as validation set. In leave-one-out cross-validation (LOOCV) a single observation from the original data set is used as test set, and the remaining observations as training set. This is repeated such that each observation in the sample is used once as test item. The various cross-validation methods are advantageous in situations where the number of independent observations in the dataset is relatively small (i.e., tens rather than hundreds), but they need to be used appropriately and with an awareness of their limitations. The choice of an inappropriate validation method can lead to overfitting, a phenomenon which is examined in Section 4.4.1.

### **3.3.3 Univariate analysis**

Univariate statistical analysis encompasses the wide range of traditional statistical methods in which only one predictor variable is considered at a time. In the context of metabolomics data analysis, univariate analysis is often used in the first stages of research for descriptive purposes, where individual metabolites are viewed (and sometimes modeled) singly, or also as a confirmatory tool following multivariate analysis. Generally though, it is supplemented by more advanced multivariate statistical methods. The focus of the present work is the application of multivariate techniques for the analysis of high-dimensional data. However, some selected univariate analysis methods (specifically, multi-way ANOVA (in Chapter 5) and non-parametric equivalents of t-tests (Chapter 4)) are used for comparative purposes, and these will be described briefly first.

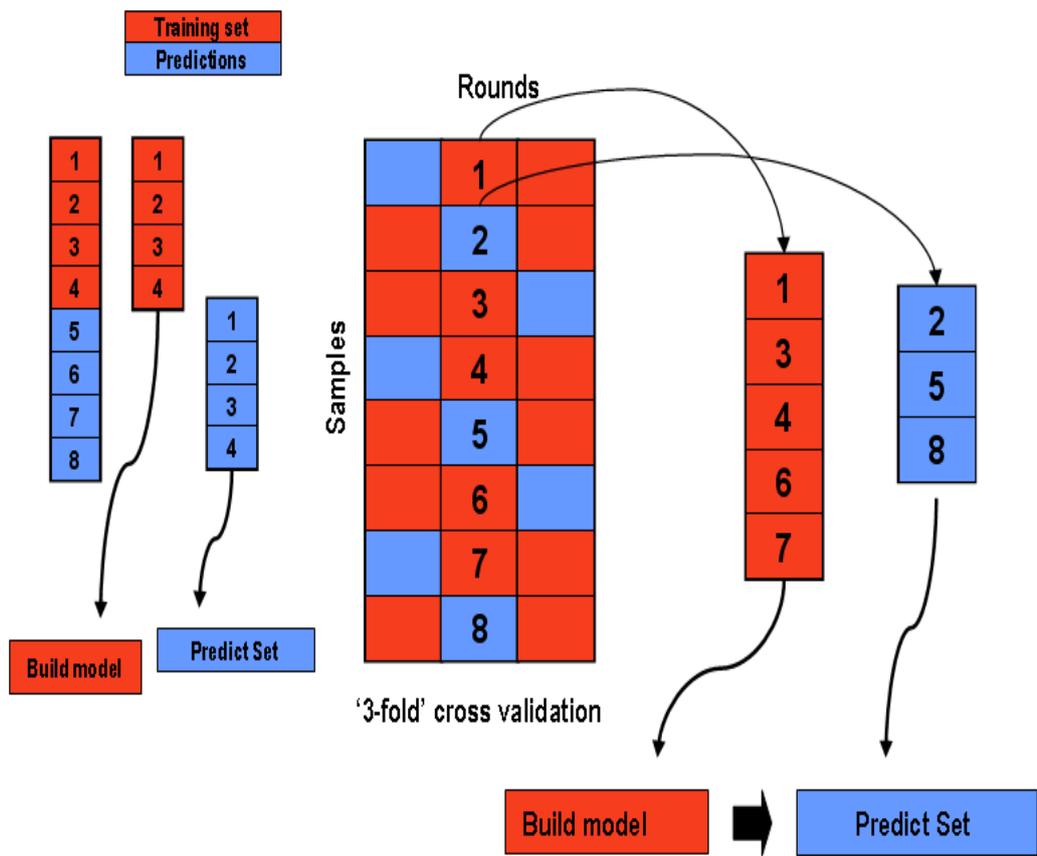
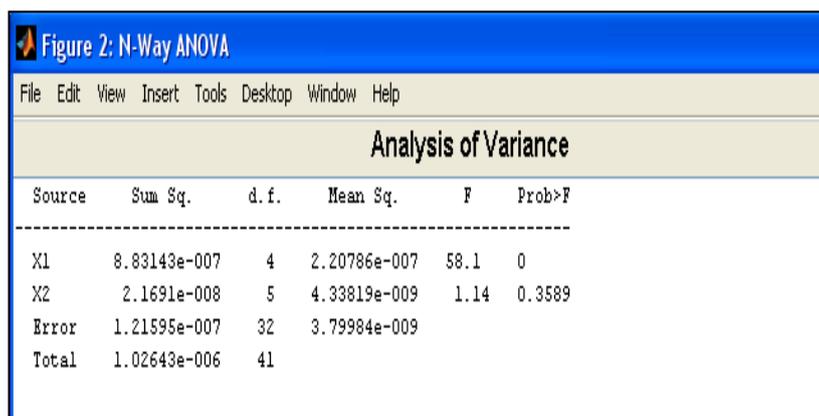


Figure 3.10. An illustration of the re-sampling mechanism in Cross Validation

### 3.3.3.1 Analysis of variance (ANOVA)

ANOVA is a technique that aims to discover whether data from nominally different groups are statistically different, that is, to determine whether the groups differ significantly with respect to the measured characteristic. It does this by testing the null hypothesis that the groups share a common mean.

The standard ANOVA output is a table containing elements as follows: sums of squares (SS), degrees of freedom (df), mean squares (SS/df),  $F$  statistic, and  $p$ -value (Figure 3.11). The  $F$  statistic is used in the hypothesis test, and the  $p$ -value returned informs on the significance. A small  $p$ -value is evidence for rejecting the null hypothesis, and suggests that the group means are significantly different. The  $p$ -value depends on assumptions about the random disturbances in the model equation. For the  $p$ -value to be valid, these disturbances need to be independent, normally distributed, and have uniform variance.



| Figure 2: N-Way ANOVA                           |              |       |              |      |        |
|---|--------------|-------|--------------|------|--------|
| File Edit View Insert Tools Desktop Window Help |              |       |              |      |        |
| Analysis of Variance                            |              |       |              |      |        |
| Source  | Sum Sq.      | d. f. | Mean Sq.     | F    | Prob>F |
| X1  | 8.83143e-007 | 4     | 2.20786e-007 | 58.1 | 0      |
| X2  | 2.1691e-008  | 5     | 4.33819e-009 | 1.14 | 0.3589 |
| Error   | 1.21595e-007 | 32    | 3.79984e-009 |      |        |
| Total   | 1.02643e-006 | 41    |              |      |        |

*Figure 3.11. Multi-way ANOVA table for two factors X1(day of analysis) and X2(genotype). The  $p$ -value (Prob>F) for X2 indicates whether or not the type of genotype is a significant factor for separating the groups of samples after compensating for the effect of day. This is an example from section 5.4.1., showing that one of the identified spectral features (a peak with  $m/z$  130 and retention time 11.35min) is not a discriminatory factor for the five genotypes involved in the experiment.*

**Multi-way** (N-way) ANOVA is used to determine whether the means in a set of data differ when grouped by multiple factors, and indicate which factors or combinations of factors are associated with the difference. For example in Chapter 5, these factors are the different genotypes as well as the day that the analysis was performed.

A graphical reassurance that the means of groups are different for each examined variable can be gained by looking at the boxplots (this is the way I will examine the variables in Section 5.4.1.2). However, it should be noted that the notches are (by default in this software package) used for a comparison of medians, not a comparison of means.

### **3.3.3.2 Multi-comparison tests**

Post-hoc multi-comparison tests are performed to determine not just whether there are any differences among the means, but specifically to assess which pairs of means are significantly different. The several available tests differ on the assumptions about characteristics of the statistical population. In this thesis I used two types of tests: the parametric **Student's t-test** and the non-parametric **Wilcoxon signed-rank test**. Student's t-test examines if two independent samples that come from normal distributions with unknown but equal (or, optionally, unequal) variances have the same mean, against the alternative that the means are unequal. In a t test, a t statistic is computed and compared to a critical value. The critical value is chosen so that when the means are the same (any difference is attributed to random chance), the probability that the t statistic will exceed the critical value is small (i.e. less than 5%, a commonly used threshold for the p-value). In Section 5.4.2.2, the critical values from the t distribution are calculated using a Bonferroni adjustment to compensate for multiple comparisons. The Bonferroni adjustment at the 5% level is calculated as:

$$\text{Bonferroni critical value} = 0.05 / (\text{number of variables})$$

A Wilcoxon rank sum test examines if two independent samples that come from identical continuous distributions have equal medians, against the alternative that they do not have equal medians. It is the non-parametric equivalent of the t-test. The

key difference is that the data are not used in their raw form, but are instead transformed into a ranked list before calculation of the test statistics. In this way, the distribution of the original data is irrelevant as far as the test is concerned (hence the term “non-parametric”).

#### **3.4 Peak annotation – pipeline step 4**

The final step in the metabolomics data pipeline is annotation of the individual peaks. Comprehensive identification of all detected metabolites is a challenging task; however, if the output from the statistical analysis includes, for instance, a subset or a ranked list of important peaks, then the task of annotation can be made less daunting by reducing the scale of the task.

In the present work, for the annotation of peaks I used AMDIS software, where metabolites are identified by comparing retention indices and mass spectra with the Golm Metabolome Database and NIST libraries. The data are imported in NetCDF format; an automated calibration process is run that converts the retention times to retention index values; and subsequently, within a few seconds, the software package outputs a list of identified compounds.

**CHAPTER 4:**  
**CONSIDERATIONS FOR METABOLOMICS DATA**  
**ANALYSIS: A CASE STUDY – THE HiMet PROJECT**

## 4 CONSIDERATIONS FOR METABOLOMIS DATA ANALYSIS: A CASE STUDY – THE HiMet PROJECT

### 4.1 An introduction to HiMet project

HiMet (from *Hierarchical plant Metabolomics*) is the acronym that was given to a cross-institute BBSRC project (Table 4.1) which ran for the period 2004-2007. Hierarchical plant metabolomics for gene function analysis refers to the use of metabolomic technologies for the assessment of the metabolic role of particular genes, and their contribution to the overall functioning of plant cells and organs (Jenkins et al., 2004). In this Chapter, I use a self-contained data subset from the HiMet project (from the “HiMet9” experiment, which has also been used in a paper that was developed during the life of this project by Ian Scott (Scott et al., 2010)) to explore the use of our data pipeline in hierarchical plant metabolomics.

The dataset was produced by the York collaborator, using LC-MS technology, and the peaks were integrated, de-convoluted and identified manually. Thus, in this Chapter, only the second and third step of the data analysis pipeline are addressed (see Figure 1.1; pre-treatment and statistical analysis). The pre-processing step is not explored, as I did not have access to the raw LC-MS data. PLS-DA modelling is initially used for the discrimination of a collection of samples of known Arabidopsis genotypes. Subsequently, the model is used for the classification of a selection of mutant samples with unknown gene functions (the “SM lines”, discussed below) into the groups of known genotypes.

The complete HiMet project involved the development of machine learning technologies (Scott et al., 2010) in combination with high-throughput metabolite analysis of mutant Arabidopsis plants, as well as the development of metabolome fingerprint databases reflecting perturbations in specific metabolic pathways and enzymes. The project examined a large collection of well established Arabidopsis mutants, and a selection of dSpm transposon-insertion mutants (SM lines). The SMLines selected were candidate metabolism mutants from within the ATIDB transposon-insertion database ([www.atidb.cshl.org](http://www.atidb.cshl.org)). These have been categorised according to biological function using the Gene Ontology (GO) consortium annotation.

**Table 4.1. Partners involved in HiMet Project and the metabolomic technologies used by each collaborator**

| Institute   | Expertise   |
|---|---|
| University of Wales, Aberystwyth (UWA)<br>(Coordinator) | <ul style="list-style-type: none"> <li>• Metabolic fingerprinting using FT-IR and ES-MS</li> <li>• ArMet development</li> <li>• Data analysis and explanatory machine learning</li> </ul> |
| John Innes Centre                                       | <ul style="list-style-type: none"> <li>• Plant cultivation, harvesting and preparation</li> <li>• Targeted metabolite profiling using LC-MS</li> </ul>                                    |
| Rothamsted Research                                     | <ul style="list-style-type: none"> <li>• Targeted metabolite profiling by GC-MS</li> <li>• Metabolite fingerprinting by NMR</li> </ul>  |
| University of York                                      | <ul style="list-style-type: none"> <li>• Targeted metabolite profiling by GC-MS, LC-MS, GC and LC-fluorescence</li> </ul>   |
| UMIST   | <ul style="list-style-type: none"> <li>• Data analysis and explanatory machine learning</li> <li>• Metabolic fingerprinting using FT-IR</li> </ul>  |

**Table 4.2. Arabidopsis mutants included in HiMet9 dataset and their metabolic role**

| Mutants       | Area of metabolism | Annotation   |
|---------------|--------------------|--|
| <i>act1</i>   | Lipids/fatty acids | The <i>act1</i> mutant is deficient in the plastidic acyl-ACP:glycerol-3-phosphate acyltransferase |
| <i>fad2-1</i> | Lipids/fatty acids | The <i>fad2-1</i> mutant is deficient in polysaturated fatty acid synthesis                        |
| <i>fae1</i>   | Lipids/fatty acids | The <i>fae1</i> mutant is deficient in the acyl-CoA elongation (fatty acid elongase)               |
| <i>WT-Col</i> |                    | Wild type (Col-0)  |

**Table 4.3. List of Amino acids measured in the examined dataset by LC-MS**

| <i>Letter codes</i> | <i>Amino acids</i>      |
|---------------------|-------------------------|
| A                   | Alanine                 |
| AAA                 | Lysine                  |
| C                   | Cysteine                |
| CIT                 | Citrulline              |
| D                   | Aspartic acid           |
| E                   | Glutamic acid           |
| F                   | Phenylalanine           |
| G                   | Glycine                 |
| GABA                | Gamma aminobutyric acid |
| H                   | Histidine               |
| I                   | Isoleucine              |
| J                   | Leucine or Isoleucine   |
| K                   | Lysine                  |
| L                   | Leucine                 |
| N                   | Asparagine              |
| ORN                 | Ornithine               |
| P                   | Proline                 |
| Q                   | Glutamine               |
| R                   | Arginine                |
| S                   | Serine                  |
| T                   | Threonine               |
| V                   | Valine                  |
| W                   | Tryptophan              |
| Y                   | Tyrosine                |

## 4.2 Materials and methods

### 4.2.1 Samples

The dataset's known-genotype samples (Table 4.2) include the wild-type (WT-Col), and three Arabidopsis lipid/fatty acid mutants: *act1* mutant - deficient in the plastid acyltransferase that catalyzes lysophosphatidic acid biosynthesis; *fad2-1* mutant – deficient in polyunsaturated fatty acid synthesis; and *fae1* mutant – related to fatty acid elongation. Additionally, a collection of eleven SM single-copy transposon-insertion lines (SM 15225, SM 15771, SM 17367, SM 18958, SM 19779, SM 19801, SM 19881, SM 20192, SM 21150, SM 270, SM 35810) was used to investigate previously unknown gene functions. These SM lines were selected via a TIGR.5 genome annotation of the ATIDB database, and the ATIDB entries were matched to MAPMAN for Gene Ontology Consortium categorization.

### 4.2.2 Plant growth and harvest

Plants were grown by the JIC partner in nine random blocks in an environment of 23°C/18°C, 16/8 h day/night photoperiods of 250 to 270 mmol m<sup>-2</sup> s<sup>-1</sup> light, and 70% relative humidity. Aerial tissues from stage 6.00 plants (Boyes et al., 2001) were harvested into liquid N<sub>2</sub> in mid light period, freeze dried, and powdered. Replicate plants from each block were allocated to each analytical method. Shipment and laboratory processing entailed a few days at ambient temperature.

### 4.2.3 Sample analysis

Twenty-four amino acids were measured by the York partner, with norleucine used as an internal standard, on a Thermo LCQ Classic LC-MS device (Thermo Scientific). Samples (2 mg) extracted in 70 µL of 80:20 ethanol:water (4°C, 30 min) were analyzed as isobutyl chloroformate derivatives (Husek, 1998) on a 100 mm porous graphitic column (5 mmHypercarb; Thermo Scientific) at 0.4 mL min<sup>-1</sup> with a 15min gradient of 100% solvent A (10 mM ammonium trifluoroacetate, 10 mM trifluoroacetic acid in 50:50 ethanol:water) to 100% B (10 mM trifluoroacetic acid in tetrahydrofuran). Amino acids were measured by positive-ion atmospheric pressure chemical ionization-tandem MS, with capillary at 4V and 150°C, vaporizer at 550°C, and discharge current of 6 mA (Scott et al., 2010).

### **4.3 Multivariate data exploration and pre-treatment**

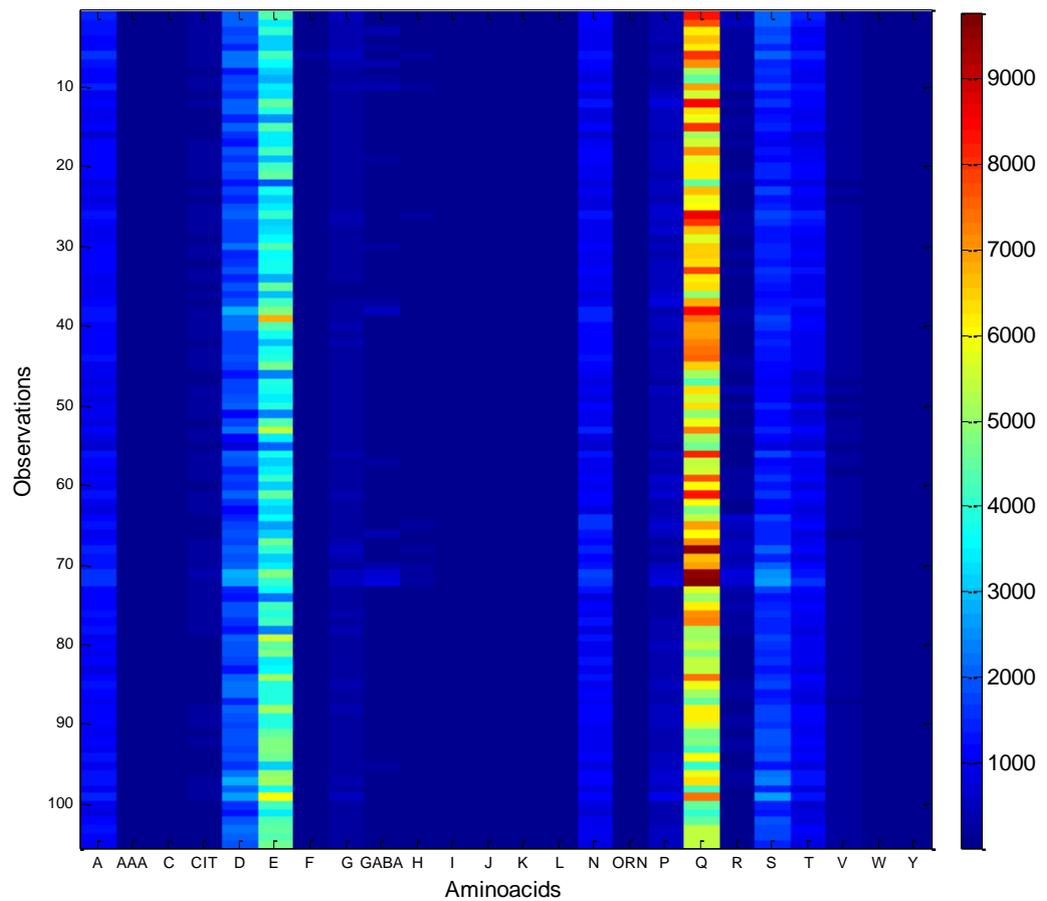
#### **4.3.1 The raw data**

All pre-processing (peak identification and integration) was carried out by the collaborators at York. The raw data matrix of the specific dataset incorporates the intensities of 24 amino acids (Table 4.3) for 105 samples. These 105 observations include the four genotypes (*act1*, *fad2-1* and *fae1* mutants, and the wild type), and consist of 9 independent biological replicates for each of the wild-type, *act1* and *fad2-1* mutants, 8 independent biological replicates of *fae1* mutant, and three technical replicates for each biological replicate (identified by unique sample codes). (see Appendix A2, Table 4.4).

It is good practice to use an appropriate way of examining the raw data in its entirety, as an initial means of quality control. Many different types of graphs can be used for this purpose, but amongst the most useful are the “heatmap” representations which make use of colour to represent intensities in the dataset. The complete table of raw data is shown as a heatmap in Figure 4.1. In this type of plot, each data value (metabolite intensity) is indicated by a patch of colour whose RGB (red-green-blue) value has been determined by mapping the intensity value onto the desired colour scale. Heatmaps of the intensity matrices provide an immediate impression of the general patterns in the data. For instance, it is apparent that the HiMet 9 data matrices are dominated by two amino acids, E (Glutamate) and Q (Glutamine).

#### **4.3.2. Missing values**

Missing values (i.e. empty cells where the respective metabolite has not been assigned to any numerical value) are very common phenomena in metabolomic measurements. The handling of missing values is an important step in the preparation of the data, as most of the multivariate methods require a fully defined matrix, or become computationally ineffective for incomplete data. How best to deal with sets containing missing values depends in part on the actual number of missing values and also, if there is an indication, on the mechanism which gave rise to them. For instance, missing values sometimes imply that the level of the respective



*Figure 4.1. Heatmap of HiMet 9 raw data matrix of all known genotypes (WT-Col, act1, fad2-1, fae1). In this representation each element of the raw data matrix [105(samples)x24(variables/aminoacids)] corresponds to a rectangular area and the values of the elements (intensity values) determine the colour of each patch; brighter colours correspond to higher intensity values as indicated by the colour bar(see Matlab “imagesc” function). The graph reveals a pattern of high intensity values for the amino acids E (Glutamate) and Q (Glutamine).*

metabolite was below the detection limit, in which case a sensible approach might be to replace them with a value that is smaller than this limit. However, if there are many missing values and the data are absent completely at random, then the most straightforward solution is to discard the entire column of observations, although discarding data has the potential cost of losing any valuable information that might exist in the remaining entries.

A number of methods for handling missing data have been proposed that usually involve estimating the missing values from the values of those variables which are available. The simplest approach is the replacement of the missing value by the mean (or the median) of the metabolite level across the remaining samples. A more sophisticated approach is to replace the missing value by the mean (or median) of its nearest neighbours; in the case of grouped data, these could be regarded as the remaining individuals from the same group. The replacement of missing values is mainly a computational issue, since many routines will either not work if there are large numbers of zeros present (as in certain circumstances this may lead to attempts to divide by zero), or will produce results that are dominated by the large apparent (but misleading) variance that will result from the presence of a significant number of zeros amongst an otherwise well-behaved normally-distributed collection of data values. The aim of dealing with missing values is to prevent errors or artefacts occurring, rather than to make an active contribution to the classification results. In the HiMet9 dataset, I elected to discard a number of columns (metabolite intensities), as they contained a high proportion of missing values. These columns corresponded to the amino acids B (aspartic acid or asparagine) and M (methionine), which are not included in Table 4.3.

### **4.3.3 Data scaling**

Another initial consideration is whether the data should be scaled and/or normalized before any modelling. Here “scaling” is used to refer to column-wise treatments of the variates in the data matrix, and “normalization” to refer to row-wise treatments of the observations. Scaling in particular can greatly affect the metabolites that are identified as important, thus selecting the appropriate data pre-treatment is a crucial step of the analysis. This choice depends on several factors. These include the biological question to be answered: do I have prior knowledge of which metabolites

might be important, or is this a hypothesis-free design; do I want to give all metabolites the opportunity to influence the modelling equally, or weight our results to primary compounds? The properties of the measurements also need to be considered, for example whether there are unwanted systematic variances (offsets, shifts) that could be eliminated or mitigated by particular scaling or normalizations.

Variance scaling, in which each variable is divided by its standard deviation, is a way to relatively reduce the influence of larger peaks (major compounds present in large concentrations) and increase the impact of the smaller spectral features (possibly interesting but potentially also noise-corrupted or suffering from missing values, as discussed above). This approach is useful when the impact of the low abundance metabolites needs to be considered, but it should be emphasized that the inflation of small values creates an increased danger of altering the biological meaning of the results. The influence of the measurement error, that is usually relatively large for small values, is increased as well. It is important to note that the effectiveness or otherwise of scaling cannot always be predicted in advance, particularly in hypothesis-free designs, and in general should be considered on a case-by-case basis. The effect of variance scaling as a pre-treatment before PLS-DA is discussed in the next section.

#### **4.4 Multivariate data analysis (PLS-DA)**

A cross-validated PLS-DA model (see example of the script in Appendix A1) was used to first discriminate the Arabidopsis wild type and known mutants (*act1*, *fad2-1*, *fae1*) and then predict the classification of mutants with unknown functionality (SMlines). The aim was to discover first, whether any separation of the predominantly fatty-acid mutants could be obtained from a dataset comprising intensities of amino acids; and second, whether any classification model obtained could be used to make meaningful statements or generate hypotheses about gene function in the SM lines.

Figure 4.2 shows the classification success rate of PLS-DA predictive models as a function of the number of PLS scores used, with and without variance scaling of the HiMet9 dataset. In these cases the classification success rate is derived from the observations that are correctly classified as members of their group.

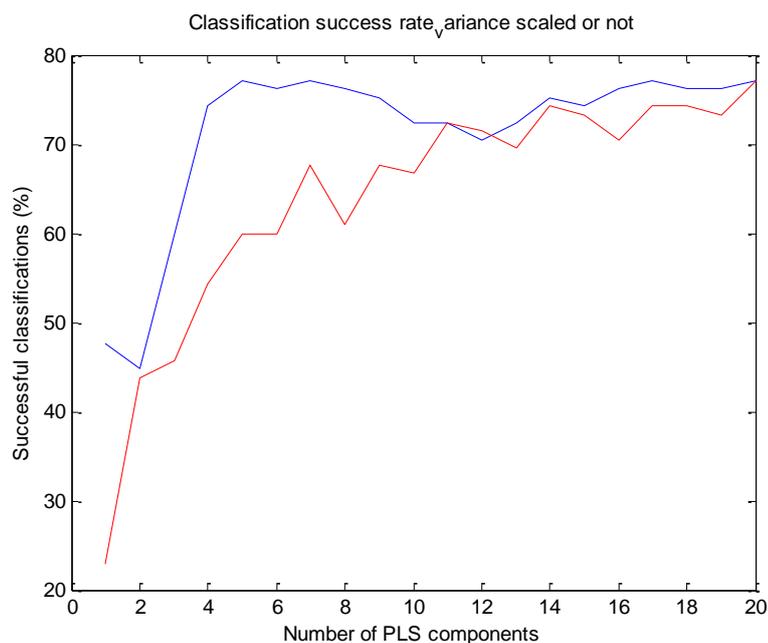


Figure 4.2. A comparison of the classification success rates between variance scaled (red trace) and non-scaled (blue trace) data.

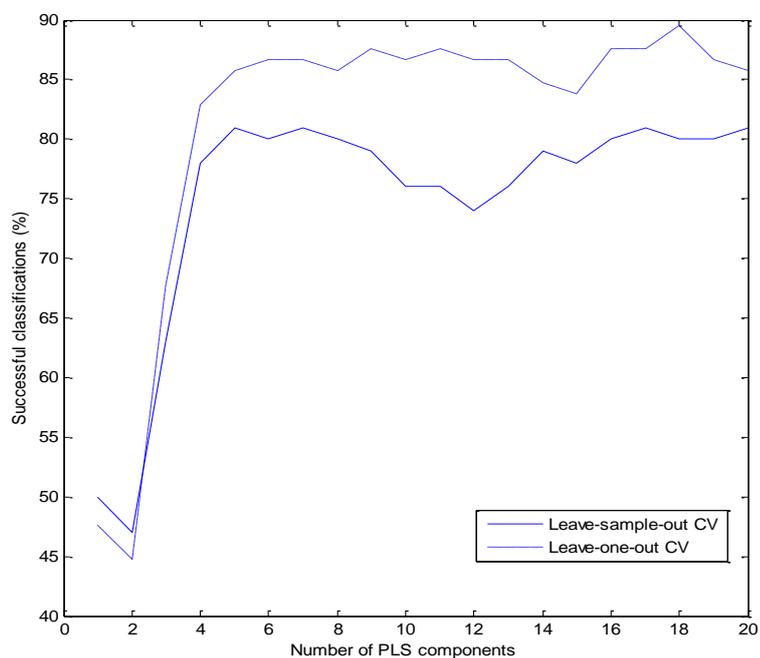


Figure 4.3. A comparison of the classification success rates between different validation procedures; Leave-one-out Cross Validation and Leave-sample-out Cross Validation.

The classification of the models themselves will be discussed below, but with regards to pre-treatment, in this particular instance, variance-scaling compromises the parsimony of the PLS-DA modelling. Amplifying the signals from the low abundance metabolites (and their inherent noise) has made the task of obtaining an effective model harder. Therefore, I henceforth employ mean-centering only as the pre-treatment of choice in the present chapter.

#### 4.4.1 Cross-validation design

Validation is a very important step in estimating the fit of a model to an independent data set. In this thesis, PLS-DA models are evaluated using a cross-validation design. The appropriate training/validation segmentation can be a crucial factor for constructing robust predictive models (Broadhurst and Kell, 2006). In this section I demonstrate an important parameter of the validation design related to the presence of replicate measurements, and a potential pitfall, known as overfitting. In an overfit model, the classification ability may superficially appear satisfactory, but in fact is not statistically significant and the model is destined to perform less well when validated on entirely new samples.

It is easy to highlight the numerical causes of gross overfitting by passing similarly dimensioned sets of random numbers through the same modelling procedure (Defernez and Kemsley, 1997). However, various more subtle forms of overfitting can be manifested with real experimental data, often related to the idea of what exactly constitutes an “independent” measurement. In the results shown on Figure 4.3, the dataset includes replicate measurements of each sample. In this case, leave-**one**-out cross validation provides an ‘overoptimistic’ (overfit) result. The reason for this behaviour is due to technical replicates tending to be classified together. Thus, as the model dimensions increase as the training segment is fitted, the single validation items follow suit and classify in the same way as their matching replicates in the training segment, rather than providing an independent test of each dimensionality. The cross-validation success rate is therefore unfairly augmented. In circumstances such as this, only leave-**sample**-out cross-validation can reflect the true predictive performance of the model. This demonstrates that the existence of technical

replicates is an issue that should be carefully considered in statistical modelling, and that replicates should by no means be treated as independent observations.

#### 4.4.2 Classification of the known genotypes

The classification results of a leave-sample-out PLS-DA model (including the wild type (WT-Col) and the three mutants) are shown in Appendix A2, Table 4.5. The model was obtained from mean-centered data, using leave-sample-out cross-validation. From the first four PLS components it yielded a classification success rate of 71.4%. (Figures 4.2-4.3) and describes 97.38% of the variation in matrix X (of the metabolite mass fragment intensities). This means that this PLS-DA model successfully predicted most of the observations (79 out of 105). In fact, only one biological replicate for each of the mutants *act1* and *fad2-1* is mis-classified (identified by the sample codes sampl 1110 and sampl 1097 respectively). The number of mis-classifications for the *fae* mutant and the wild type is slightly larger, and it appears that technical replicates may occasionally classify into different groups (e.g. technical replicates identified by the sample code sampl 111). It is observed that the mis-classification for *fae* and wild type involve largely only these two groups, indicating a close match between the amino acid profiles for these two genotypes.

In Figure 4.4, the classification results are visualized in score plots for the first four components. It is obvious from these graphs that wild type and *fae1* are hardly discriminated in any of the PLS dimensions. However, a very good discrimination of *act1* from the rest of the genotypes in the first and the second dimensions is observed, and a clear discrimination of *fad2-1* and *fae1* in the rest of the dimensions (i.e. the third dimension separates *fad2-1* and *fae1*, the fourth dimension separates *fae2-1* from the wild type). It is clear that separating *fae1* and wild type is the greatest difficulty in this experiment.

Loadings plots can be used to identify which of the amino acids are responsible for the observed classifications (Figure 4.5). As it was anticipated with regard to the fact that the data used in the model are not variance-scaled, amino acids with higher concentration have the largest impact on the classification result.

#### **4.4.3 Predictions of the unknowns (SMLines)**

The four-component PLS-DA model was used to classify the SMLines included in HiMet9 experiment. The classification result is shown in Appendix A2, Table 4.6. From a collection of 292 samples of SMLines, 231 samples were classified as wild type, 45 samples were classified as *fae1*, only 15 samples were classified as *act1* and three samples as *fad2-1*. As can be seen, some of the biological and technical replicates are assigned into two different groups. The reasons for such separation could be the variability there is between biological replicates, as well as other technical aspects (batch effects) that were not available in the metadata.

In order to enhance the interpretation of the classification result it is often useful to draw graphs that show how the SMLines are scattered across the groups of known mutants. In the Figures 4.6 to 4.9 the SMLines from each one of the groups are superimposed on the classification result of the known mutants for the first two PLS dimensions. Figure 4.6 shows the large amount of SMLines that were classified as wild type, which seem to be largely superposed across the original *fae1* group. This is a reflection of the close similarity between these two types in the original classification model. On Figure 4.7, it is very clear that the SMLines classified as *act1* are closer to the cluster of the *act1* mutant, however, some of them are also very close to the remaining groups. This is a reminder to exercise caution, and not to interpret an *act1* classification as entirely definitive.

Overall, the SMLines seem to be closely related to the wild type or the *fae1* mutant, however, considering that these two groups were the least well discriminated from one another in the original model, it is very difficult to assign the SMLines with absolute confidence to either of these groups and to come to firm conclusions about gene functions.

#### **4.5 Discussion on the limitations of the Himet9 work**

In this work only one dataset from the HiMet9 experiment I presented, which was kindly provided by Nigel Hardy of the University of Aberystwyth. However, during the course of this study, the full Himet data was published in a paper focusing on the application of machine learning (ML) to metabolomics data (Scott et al., 2010).

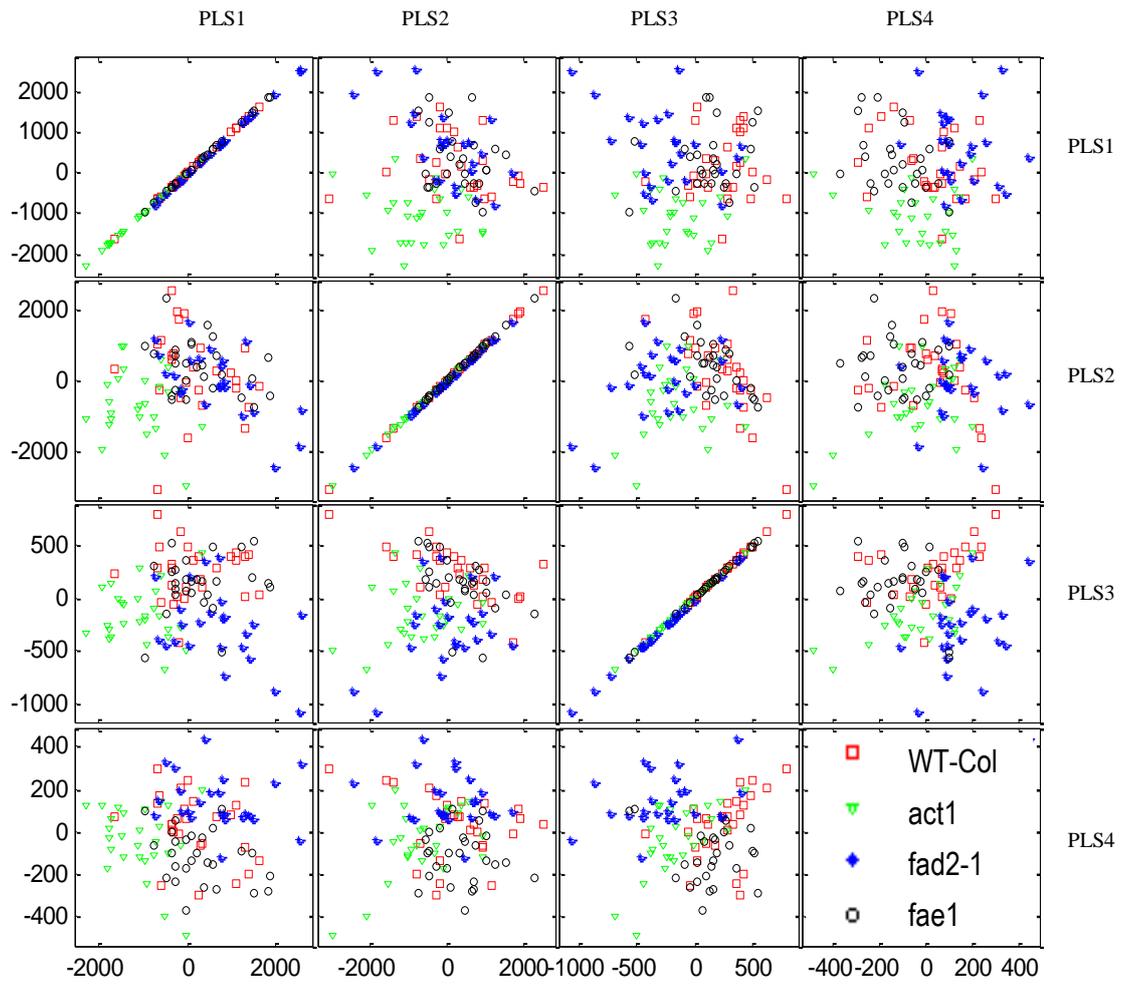


Figure 4.4. Score plots of the HiMet9 dataset on the first four components, using a leave-sample-out PLS-DA model (without variance scaling). The first and the second components (PLS1 vs PLS2) clearly separate *act1* mutant for the rest of the genotypes. The third and the fourth components separate *fad2-1* from *fae1* and wild type. Mutant *fae1* and wild type are hardly separated in any of the components.

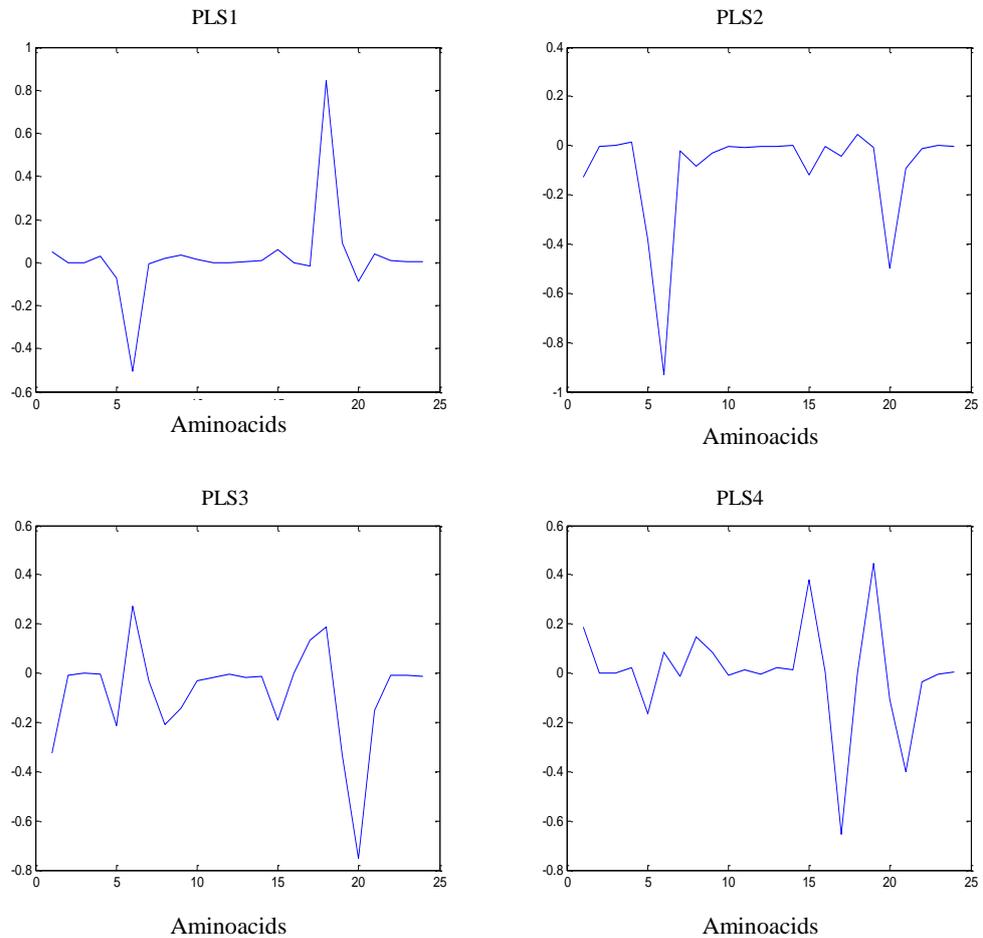


Figure 4.5. Loading plots for the first four components (PLS1, PLS2, PLS3, PLS4). On these graphs the x-axis corresponds to the measured amino acids (Table 4.3) and the sharp peaks point amino acids responsible for the discrimination of the groups. The first component reveal the amino acids *E* and *Q* (glutamate and glutamine) (variable 6 and 18 respectively); the second component additionally reveals aminoacid *S* (variable 20); the fourth component indicates amino acids *N*, *P* and *T* (variables 15, 17 and 21 respectively). The loadings are dominated by the most abundant amino acids, as the data used in the model are not variance scaled.

It was therefore considered that a more in depth analysis of Himet datasets was not justified, and the use of this dataset was limited to demonstrating the application of PLS-DA to classify known mutants and predict the classification of unknowns. However, it is interesting that I have been able to distinguish mutants of lipid metabolism (*act1*, *fad2-1*, *fae1*) solely on the basis of their amino acid profiles. This conclusion is confirmed by Scott et al. (2010), who showed that all (apart from *fad4* and *tag1*) mutants that were involved in HiMet experiments could be discriminated by amino acid profiles. One might assume that they would more readily be separated on the basis of carbohydrate or fatty acid profiles, but there is a close coupling of carbon and nitrogen metabolism in plants (Stitt and Fernie, 2003). Metabolomics, for example in potato tubers (Roessner *et al*, 2001) has been used previously to show such a link and there are complex regulatory mechanisms known to ensure a balance between carbon and nitrogen utilisation (Nunes-Nesi *et al*, 2010).

The incompleteness of the classification concerning the SMlines is partially due to the small number of mutants included in this experiment. Any attempt to come to conclusions regarding gene functions analysis would require the examination of hundreds of mutants from different metabolic pathways. Moreover, a more comprehensive collection of metadata could have helped the interpretation of the mis-classifications of the SM-lines.

In summary, in this Chapter, I examined an LC-MS dataset of known metabolites (24 amino acids; targeted metabolite analysis), where the peak detection and deconvolution was performed manually, consequently the first (and last) steps of the pipeline were not required. In the next Chapter, I will examine a complete GC-MS untargeted metabolomics experiment, including hundreds of ions within a mass range of 50-500 m/z representing known or structurally novel metabolites, which are produced by pre-processing the raw data files using the XCMS software, and use statistical modelling to make statements about the dataset that are of direct biological interest.

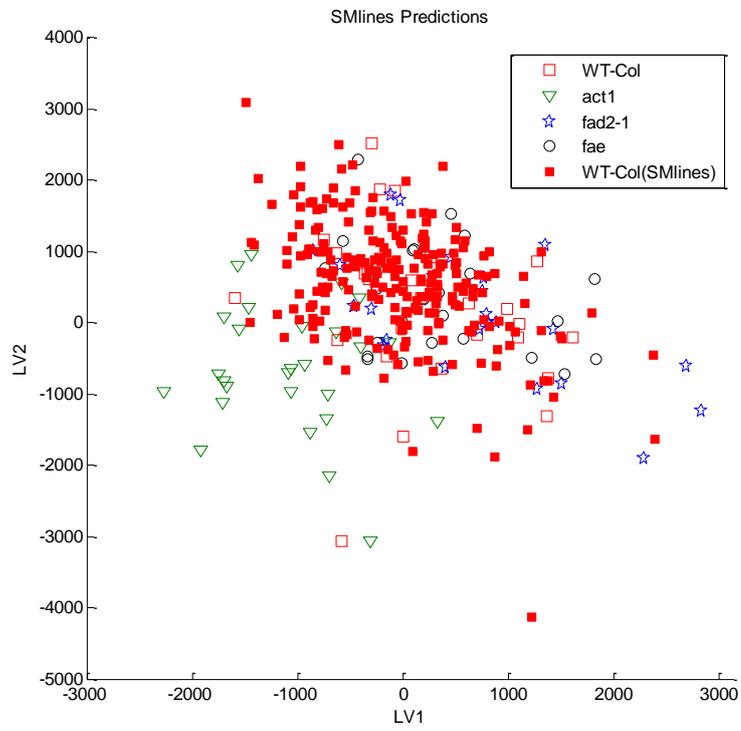


Figure 4.6. SM-lines assigned to the wild type (WT-Col) group (closed square symbols) superimposed on the clusters of WT-Col, act1, fad2-1 and fae1 (open symbols)

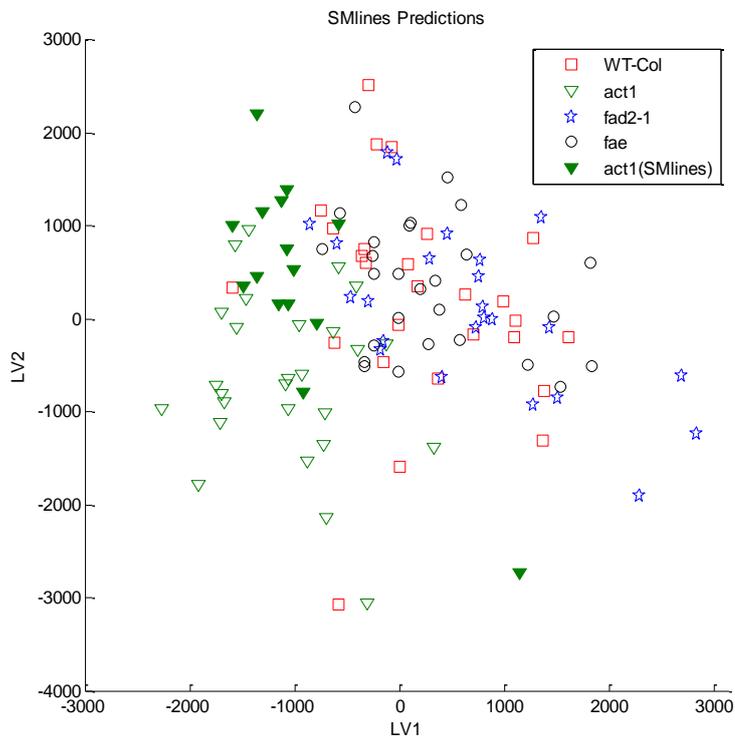


Figure 4.7. SM-lines assigned act1 group (closed triangle symbols) superimposed on the clusters of WT-Col, act1, fad2-1 and fae1 (open symbols)

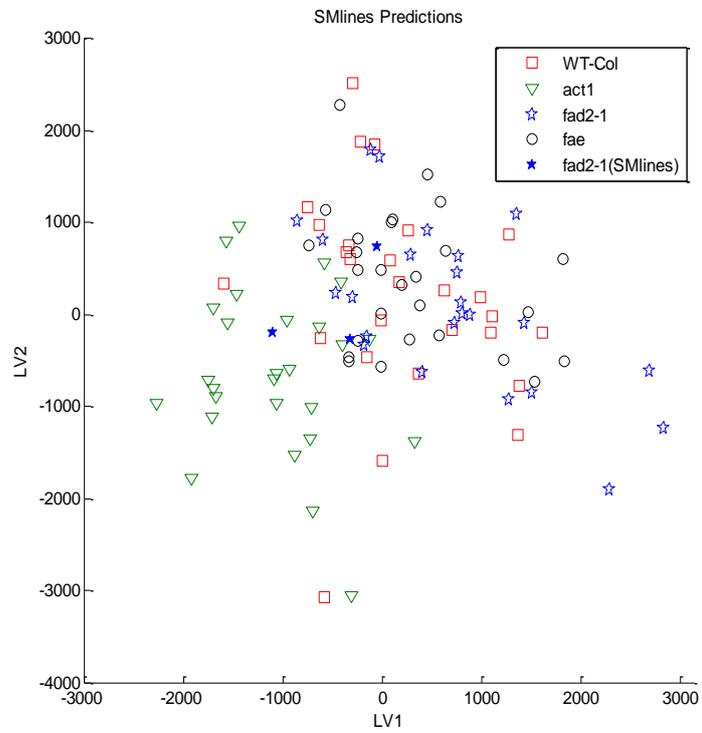


Figure 4.8. SM-lines assigned *fad2-1* group (closed star symbols) superimposed on the clusters of WT-Col, *act1*, *fad2-1* and *fae1* (open symbols)

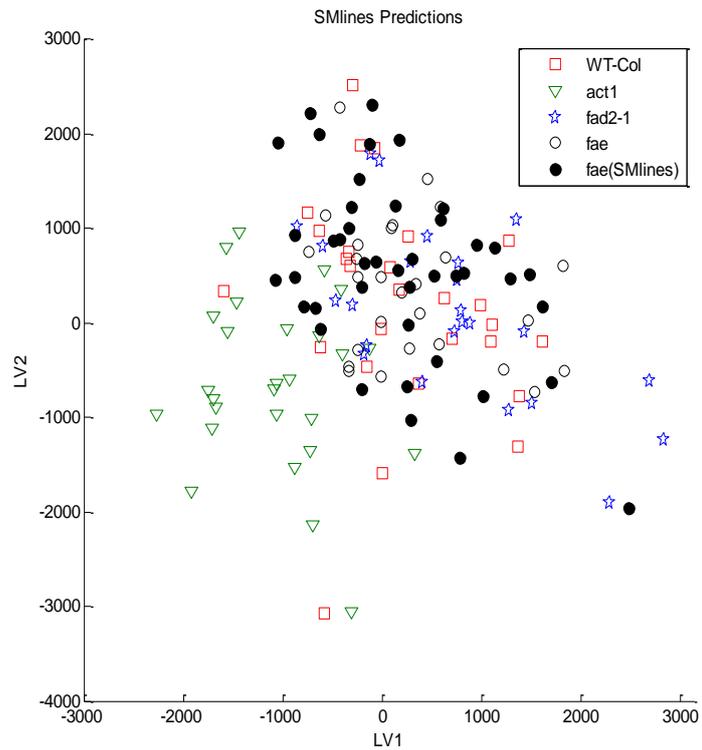


Figure 4.9. SM-lines assigned *fae1* group (closed circle symbols) superimposed on the groups of WT-Col, *act1*, *fad2-1* and *fae1* (open symbols)

**CHAPTER 5:**  
**A METABOLOMICS INVESTIGATION**  
**OF STARCH METABOLISM**

## 5 A METABOLOMICS INVESTIGATION OF STARCH METABOLISM

Starch is a principal storage carbohydrate in higher plants (Beck and Ziegler, 1989). It is found both in photosynthetic and non-photosynthetic parts of the plant. In many species, it accumulates in chloroplasts during the day and it is degraded to provide sugars for metabolism and growth at night. Starch metabolism is very important for the normal life cycle of the plant, thus deficiencies in starch biosynthesis and degradation may result in retarded plant growth (Zeeman et al., 2007). There are differences in starch structure, synthesis and degradation between species as well as between leaves, roots and seeds (Zeeman et al., 2002).

**The focus of the work presented in this chapter is starch metabolism in *Arabidopsis* leaves at night.** I will begin by describing, in the following sections, important components of the process of starch metabolism in *Arabidopsis* leaves at night. I will then present the findings of this part of the work, a study of a selection of mutants defective in starch biosynthesis and degradation. Briefly, gas chromatography-mass spectrometry was used for high-throughput profiling of their metabolite content. XCMS was used to pre-process the raw data. This incorporated an optimization step to determine the appropriate non-default bandwidth parameter to use for the GC-MS data. A variety of statistical methods were then explored to analyse the data, with the aim of producing robust and biologically meaningful results. These included supervised statistical multivariate techniques as methods for the classification of these mutants based on the metabolite levels. Comparisons were made across different algorithm implementations (PLS-DA), and across different statistical approaches (multivariate and univariate, and additionally an unsupervised technique, hierarchical cluster analysis).

### 5.1 Starch metabolism

#### 5.1.1 Starch biosynthesis

In *Arabidopsis* leaves, starch is synthesized along with sucrose as products of photosynthetic carbon assimilation. Sucrose is exported to the non-photosynthetic parts of the plant, whereas starch is retained in the chloroplasts and is degraded the subsequent night (Smith et al., 1997).

Starch is an insoluble glucan composed of two polymers of glucose: amylopectin and amylose (Buléon et al., 1998). Starch synthesis is catalysed by starch synthases (SS) which are encoded by five gene classes: GBSS (granule-bound starch synthase), SSI, SSII, SSIII and SSIV. Each of the SS isoforms has different properties and a distinct role in the synthesis of the starch polymers. The role and action of these enzymes in the pathway of starch synthesis in leaves is only partially understood. Most of the knowledge concerns how the glucose polymers are elongated and branched, but very little is known about how the starch polymers and the starch granules themselves are initiated.

Recent advancements suggest that the SSIV synthase may be necessary for the initiation of the starch granule (Szydlowski et al., 2009). It has been observed that in its absence the number of starch granules in the leaf is very low, i.e. *Arabidopsis ss4* mutants have just one large granule in the chloroplast, whereas the wild type leaf chloroplasts contain about five granules. A possible explanation for this behaviour is that unlike the other SS isoforms, SSIV proteins possess an N-terminal extension which enables the interaction with other proteins and contributes to the granule initiation. In the absence of SSIV, SSIII seems to be responsible for the initiation of the single granule per chloroplast and plants lacking both SSIV and SSIII lack starch in their leaves (Zeeman et al., 2010). The precise role of SSIV and SSIII in granule initiation is under investigation.

### **5.1.2 Starch degradation**

The pathway of starch degradation in leaves was not well understood until recently (Smith et al., 2005). Starch degradation has been extensively studied in germinating cereal endosperm, but there is evidence that this seed pathway is likely to be very different from that which takes place in leaves and other plant organs. In leaves, starch is degraded primarily by hydrolysis of the constituent glucans to maltose and glucose, both of which can be exported from the chloroplast and metabolized in the cytosol (Figure 5.1).

The pathway in leaves has two particular features that do not occur in the pathway in germinating cereal seeds (Smith et al., 2005): (1) first, the phosphorylation and dephosphorylation of the surface of the starch granule that is required for starch

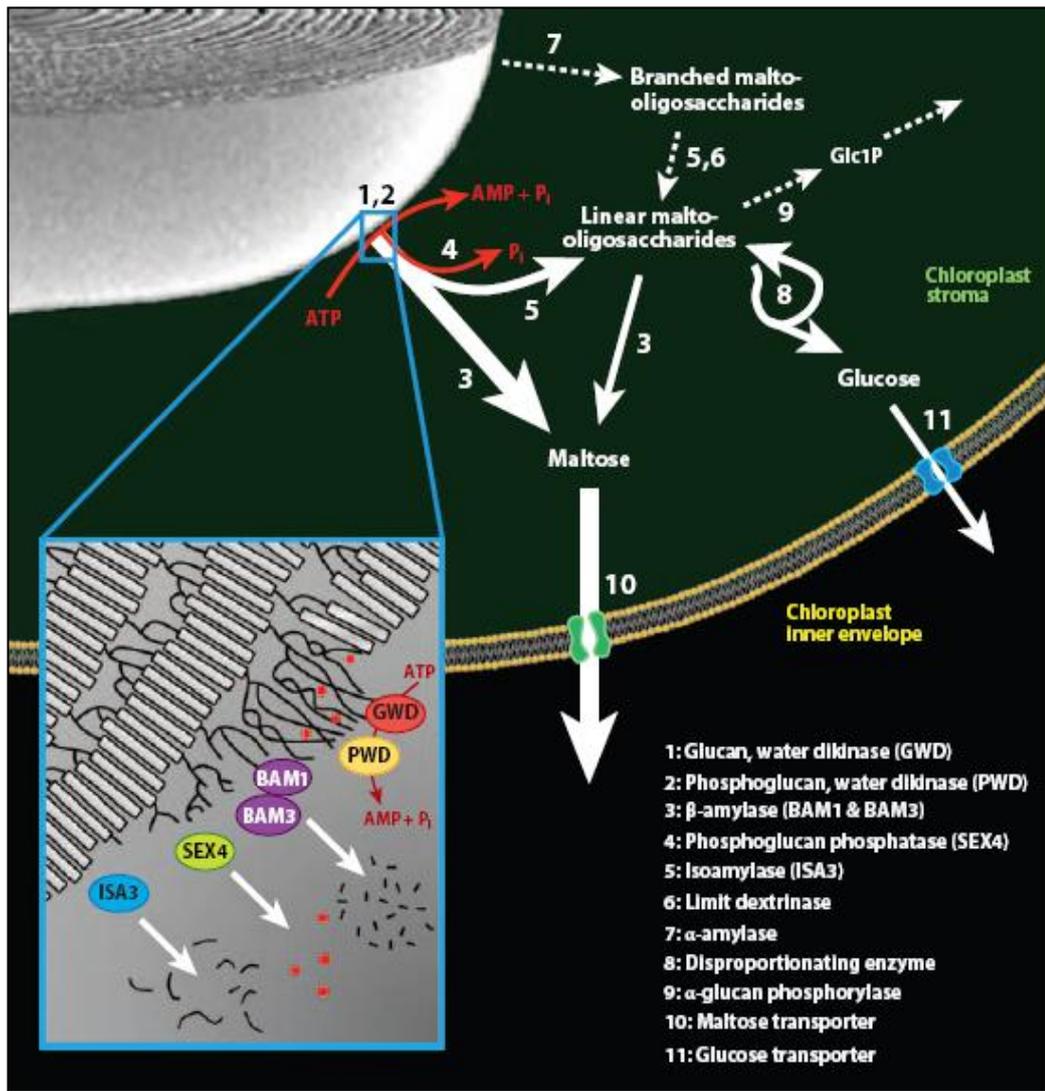


Figure 5.1. The starch degradation pathway includes a series of processes of converting granular starch in the chloroplast into hexose phosphate in the cytosol (Zeeman et al., 2010).

degradation (Yu et al., 2001), which involves the enzymes SEX1 (also known as GWD1) and SEX4, (2) second, the export of maltose from the chloroplast and its subsequent metabolism in the cytosol, which involves the maltose transporter protein MEX1 and the transglucosidase DPE2 (Niittylä et al., 2004). Neither of these features is yet fully understood, but it has been observed that mutations affecting the above key proteins decrease starch breakdown, resulting in the accumulation of starch over repeated diurnal cycles (Zeeman et al., 2010).

### **5.1.3 Phosphorylation and de-phosphorylation of the starch granule**

The phosphorylation of the starch glucans is a process which serves to solubilise the granule surface and allows the hydrolases access to the glucan chain. This process requires a class of enzymes called glucan water dikinases (GWD). GWD has a high affinity for crystalline malto-oligosaccharides, and phosphorylation results in extensive solubilisation of the constituent oligosaccharide chains. Mutations that eliminate the GWD protein or affect the dikinase domain of the enzyme dramatically reduce the rate of starch degradation. Loss of GWD in *Arabidopsis* leaves (*sex1* mutants) leads to a very severe starch-excess (*sex*) phenotype, accumulating amounts of starch up to seven times greater than those in wild-type leaves (Yu et al., 2005). A second enzyme, the phosphoglucan water kinase (PWD), is also required for normal starch breakdown but the *pwd* mutants have a mild *sex* phenotype.

### **5.1.4 Fate of maltose**

Maltose is produced by  $\beta$ -amylolysis inside the chloroplast, however none of the enzymes that are capable of hydrolyzing maltose to produce glucose is plastidial. There is strong evidence that maltose produced during starch degradation at night is exported to the cytosol via a specific protein, *MEX1*, which is located in the inner membrane of the chloroplast envelope. Mutations at the *MEX1* locus cause accumulation of both starch and maltose in *Arabidopsis* leaves. Maltose levels are at least 40 times those of wild-type leaves (Chia et al., 2004).

In the cytosol, maltose is metabolized via a transglucosylation reaction. Extracts of leaves lacking a predicted transglucosidase, DPE2, have a phenotype similar to that of *mex1* that lacks the maltose transporter. Maltose levels are many times higher than

those of normal plants and starch degradation is inhibited. The free glucose released from the maltose is likely to be converted to hexose phosphate.

### 5.1.5 Pathway elucidation

The roles and importance of key enzymes in starch metabolism have been mainly explored using two approaches: first, using forward genetics by selecting plants unable to degrade starch from a mutant population, then finding which gene has been mutated; second, using reverse genetics by obtaining mutants lacking expression of genes from the genome that are predicted to encode enzymes that might be important in starch degradation. In general, forward genetic *Arabidopsis* mutant studies have been among the most successful approaches to revealing roles of genes and their products, and elucidating biochemical, developmental and signalling pathways. Forward genetics approaches have the advantage over reverse genetics that there is no need for prior knowledge of the genes involved in the process; nevertheless it is sometimes technically challenging to discover the gene responsible for a phenotype by map-based cloning or discovery of the insertion element, as the selected phenotypes may arise by secondary changes which may or may not be related to the subject of study. Reverse genetics is also an excellent way to associate genes with phenotypes, though a large number of mutants with a wide range of phenotypic assays is required for producing detectable phenotypes. Ultimately, both of these methods are time consuming, thus restricting the rate of discovery of gene function.

A more rapid way to gain information about the functions of important enzymes is to use **metabolomics as a functional genomics tool** in order to explore what happens to the metabolism when they are lost, and to compare the effects of loss of different components of the pathway. Metabolite profiling is potentially a valuable method for these comparisons, because it enables a large number of metabolites to be analysed simultaneously and gives a broad view of metabolism. However, in order to gain meaningful information from very complex metabolite profiles, which would allow the effective preliminary characterization of classical genetic mutants, it is necessary to compare large metabolomic datasets, and to use robust statistical methods to make these comparisons.

## **5.2 Materials and methods**

### **5.2.1 Mutants selection**

Five mutants deficient in starch metabolism were used to determine the effect of loss of different proteins on the metabolite profile of *Arabidopsis* leaves. The collection of mutants includes one mutant deficient in starch biosynthesis, *ss4*, and four mutants deficient in starch degradation: *sex1* and *sex4* (involved with the phosphorylation and dephosphorylation of the starch granule) and *mex1* and *dpe2* (involved with the maltose metabolism). All mutants used were of Columbia (Col-0) ecotype background. Wild type plants (Col-0) were used for comparisons. In Table 5.1 I list the replicates (seven for each genotype) and the day on which the GC-MS analysis was performed. The latter will be discussed as an important consideration during the data analysis.

### **5.2.2 Plant growth**

*Arabidopsis* (*Arabidopsis thaliana*) ecotypes Col-0 and their mutants were grown in a climate-controlled chamber set to growth conditions comprising cycles of 12 hours light at 20°C followed by 12 hours dark at 16°C. Relative humidity was kept between 60 and 75%. Plants of each genotype were randomized with respect to position within the growth chamber shelving. Mature rosette stage, pre-flowering specimens were harvested 1 hour before the end of the dark period (Boyes et al., 2001) and immediately frozen in liquid nitrogen.

### **5.2.3 Extraction and GC-MS analyses of *Arabidopsis* leaf metabolites**

The extraction and the analysis were performed by Baldeep Kular (JIC) and Lionel Hill (JIC), respectively. The extraction method was based on that described by Roessner *et al.* (2000 & 2001). Leaves were ground to a powder while frozen in liquid nitrogen using a mortar and pestle and then freeze-dried and stored at -80°C until needed. For soluble metabolite profiling by GC-MS analysis, leaf material (40–60 mg) was extracted in 2 ml of 100% methanol together with an internal standard (0.050 mg/ml phenyl- $\alpha$ -D-glucopyranoside, Sigma P6626; Sigma-Aldrich Corporation, St. Louis, Missouri, USA). The mixture was heated and sonicated in a screw-capped glass tube at 80°C for 15 minutes. Insoluble material was removed by centrifugation. The samples were then evaporated to dryness under vacuum at 40°C. Samples were dissolved in 100  $\mu$ l of 2% methoxyamine hydrochloride (Aldrich,

| <b>Table 5.1 Dates of analysis of specimens of each genotype.</b> |                        |
|---|------------------------|
| <b>Genotype</b>   | <b>Day of Analysis</b> |
| WT-COL  | 05/02/2007             |
| WT-COL  | 05/02/2007             |
| WT-COL  | 05/02/2007             |
| Sex 4-3   | 05/02/2007             |
| Sex 4-3   | 05/02/2007             |
| Sex 4-3   | 05/02/2007             |
| SS4   | 07/02/2007             |
| dpe 2-5   | 07/02/2007             |
| Sex 1-3   | 13/02/2007             |
| Mex 1-1   | 13/02/2007             |
| WT-COL  | 21/02/2007             |
| Mex 1-1   | 21/02/2007             |
| Mex 1-1   | 21/02/2007             |
| Mex 1-1   | 21/02/2007             |
| Sex 4-3   | 21/02/2007             |
| SS4   | 22/02/2007             |
| SS4   | 22/02/2007             |
| SS4   | 22/02/2007             |
| Sex 1-3   | 22/02/2007             |
| Sex 1-3   | 22/02/2007             |
| Sex 1-3   | 22/02/2007             |
| Sex 4-3   | 22/02/2007             |
| Sex 4-3   | 22/02/2007             |
| Sex 4-3   | 22/02/2007             |
| dpe 2-5   | 22/02/2007             |
| dpe 2-5   | 22/02/2007             |
| dpe 2-5   | 22/02/2007             |

22,690-4; Sigma-Aldrich Corporation, St. Louis, Missouri, USA) in pyridine for 90 minutes at 30°C with constant stirring to protect the carbonyl moieties. The samples were then silylated with the addition of 100 µl of MSTFA (N-methyl-N-[trimethylsilyl] trifluoroacetamide, Pierce Biotechnology, now Thermo Scientific, Rockford, Illinois, USA) for 30 minutes at 37°C with constant stirring. The samples were transferred to glass GC vials and left for 2 hours before analysis.

The analyses was performed using (Agilent Technologies, Wilmington, Delaware, USA) GC 6890N coupled to a Mass Selective Detector 5973*inert*. Automated splitless injections (1 µl) were made using an Agilent 7683 automatic sampler. Conditions of chromatography were: inlet temperature 250°C; the carrier gas was helium at a constant flow rate of 0.9ml/min; nominal inlet pressure of 7.86 psi. The oven temperature program was: 80°C for 2 minutes, 10°C/min to 340°C then held for 7 minutes, giving a total run time of 35 minutes. The column was a ZB-5HT Inferno (Zebron: 7HG-G015-02, Phenomenex, Macclesfield, UK.) 30m x 0.25mm x 0.25 µm with a 5 meter guard column incorporated on the front end. The mass spectrometer parameters were: using electron ionisation in positive mode (70eV), with a source temperature of 230°C and a quad temperature of 150°C, according to the manufacturer's defaults. Total ion scans were made from 50 to 500 amu and all data was processed via the Agilent GC Chemstation software (D.01.00) in conjunction with the NIST Mass Spectral Library, V2.0 (National Institute of Standards and Technology, Gaithersburg, Maryland, USA) and the Gölml Metabolome database ([http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd\\_msri.html](http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_msri.html)) hosted at the Max Planck Institute of Molecular Plant Physiology, Potsdam, Germany.

## **5.3 Results and discussion**

### **5.3.1 Visual examination of metabolite profiles**

The composition of individuals of each genotype can be broadly visualized by displaying their metabolite profiles as annotated chromatograms. Figure 5.2 shows an illustrative comparison between all metabolite profiles of the wild type and mutant plants. Variations in metabolite levels in comparison to the wild type can reveal distinct patterns of change affecting central metabolism in each genotype. Nevertheless, it should be taken into consideration that firstly, the samples are a “snapshot” taken at a single time point, and secondly, a visual point-by-point

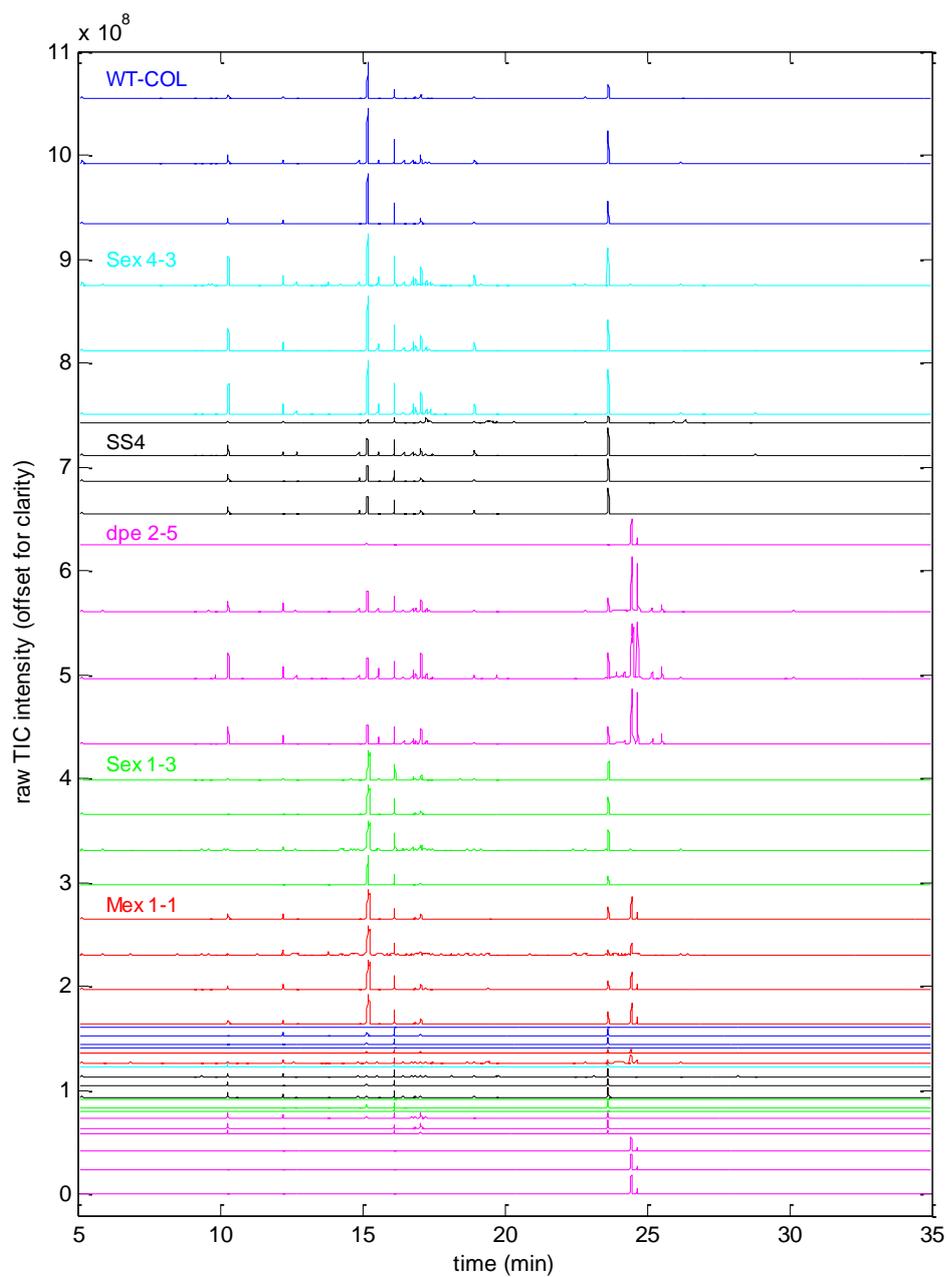


Figure 5.2. Chromatographic profiles for all observations (in the order shown in Table 5.1). The graph is produced by reading the raw netCDF files in Matlab using the Bioinformatics Toolbox.

analysis is not practical on the metabolomic scale. Furthermore, there is a substantial within-genotype variance, which is a clear indication of the need for data normalization, discussed further below.

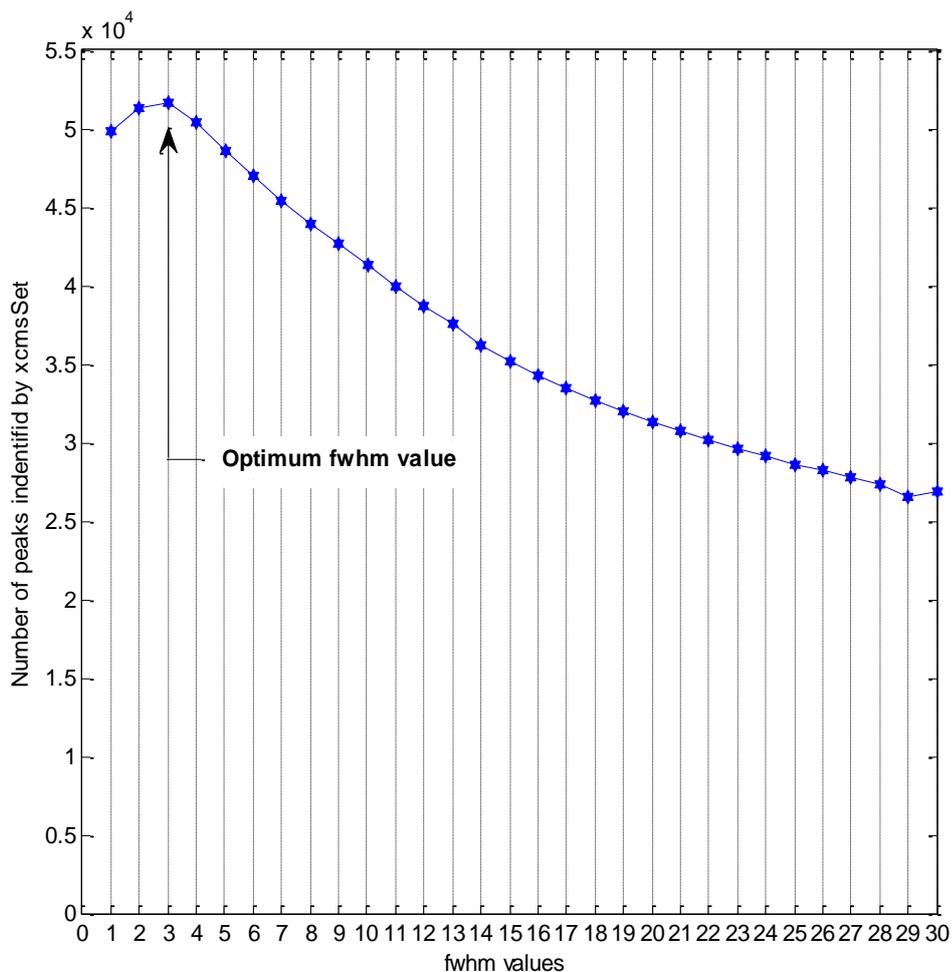
The peaks in the chromatogram were identified by using the Golm Metabolome Database ([http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd\\_msri.html](http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_msri.html)) through the NIST software. Over 100 metabolites were detected in each sample. These included a range of chemical classes, mainly represented by sugars, sugar alcohols, amino acids and organic acids.

### 5.3.2 Data pre-processing

XCMS software (version 2.10.1) was used to deconvolute and align mass ions from the 42 data files (samples) into a single data set. The input files were in NetCDF format, created by format conversion of the raw GC-MS datafiles.

As discussed in Chapter 3, the default XCMS parameters are by default intended for pre-processing LC-MS data. Historically, XCMS has largely been used for processing this type of data. However, modifications are required for handling GC-MS data, arising from a substantive difference in the width of the chromatographic peaks, which are much wider for LC-MS data. Specifically, the XCMS parameters (see section 3.1.3) which require new values are: *fwhm* (full-width half-maximum; `xcmsSet` function) and *bw* (band width; `group` function). Failure to adjust these parameters appropriately will lead to XCMS overlooking a large proportion of the peaks/compounds present in the data.

In Figure 5.3 it is shown how the *fwhm* parameter was optimized for the specific GC-MS dataset. On the basis of this, it was determined that the value for this parameter for handling GC-MS data should be set to 3 (a substantial change, compared with the LC-MS default of 30). The bandwidth parameter *bw* was set to 10. (Full details of the various XCMS parameters and pre-processing steps were discussed in Chapter 3, where a typical chromatographic peak of this dataset and the band width as a grouping variable are shown in Figures 3.3 and 3.4, respectively.)



*Figure 5.3. Number of peaks identified by xcmsSet for different fwhm (full width half maximum) values. This graph suggests that the default xcmsSet value (fwhm=30) which is suitable for LC-MS data has to be substituted by a much lower fwhm value. (The decrease in the number of peaks for larger fwhm values implies that narrow neighbouring peaks might be counted as one, while the increase observed in the beginning of the graph indicates missed out peaks when fwhm is too small.)*

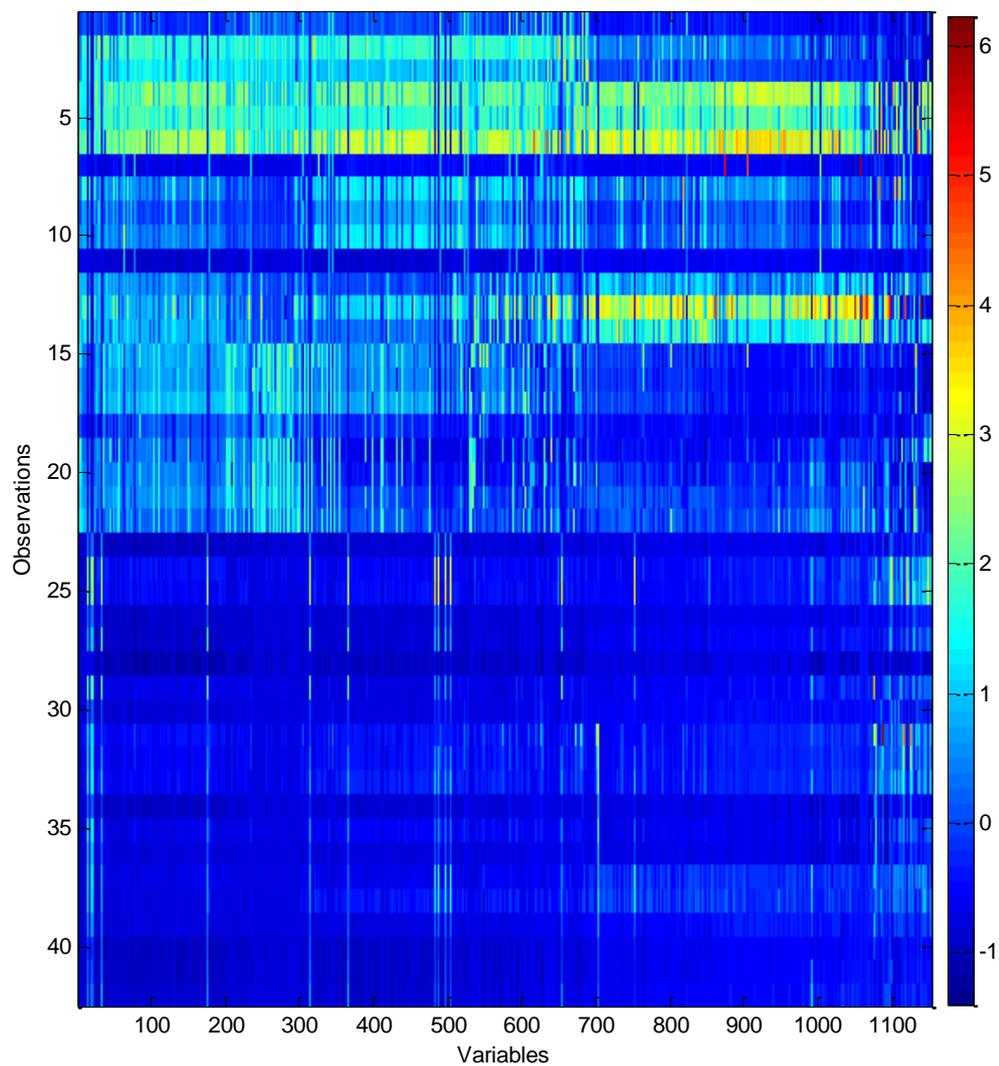
The data set of aligned mass ions was exported from XCMS as “tsv” format, which could be viewed using Microsoft Excel, or exported into Matlab for further analysis. XCMS identified 1153 variables (indexed as m/z – RT pairs), forming an intensities matrix of size [42x1153] data values.

### **5.3.3. Data normalization**

In chromatographic techniques, it often happens that non-biological experimental variances or “batch effects” are observed across the runs and/or from sample to sample, which makes the task of comparing data directly difficult. In order to increase the reliability of detecting biological phenomena, any non-biological biases should ideally be avoided, or if this is not possible, then removed (or at least mitigated) at the data analysis stage using numerical techniques. The corrections that are required often employ the use of normalization techniques.

In this specific data set, “batch effect” variability was observed, related to the day on which the measurements were made. This variability is likely to be due to any or all of the following: non-constant instrument calibration; instrumental drift that maps onto the sample run order; irreproducible or imperfect sample preparation. Figure 5.4 shows a heat-map representation of the intensities of the entire data set for variance scaled data. This is a useful alternative to the profile plots for representing the data, as it allows an entire matrix of numbers [42x1153] to be viewed simultaneously, whilst also offering the ability to visually identify certain properties of the data, such as trends or grouping effects. It is clear that the intensity values are higher for the early measurements.

One way to adjust for this type of batch effects is to use known internal standards. However, in this specific study no internal standards were used with the rationale to perform a purely untargeted analysis and avoid the use of external compounds that could interfere with the genuine metabolites. Instead, numerical normalization was carried out, which consisted of scaling each row of the raw data matrix so that the sum of intensities for every row (representing a sample) is equal to unity. This approach is intended to transform data acquired by different methods, or exhibiting a strong machine effect, onto a common intensity scale. The effect of normalization is



*Figure 5.4. Representation of the intensities of the entire data set for variance scaled data. Each one of the rows in the matrix represents a single run and each column an  $m/z$  and retention time pair. The runs (observations) are shown in chronological order and the intensities are represented as described in the colour bar. The different genotypes are randomly distributed across the days.*

pronounced for many of the variables (metabolites). Figure 5.5 illustrates the effect of normalization for one randomly selected variable.

Normalization reduces the unwanted systematic drift, but it cannot eliminate it entirely, and its effects are less successful for some variables than others. For example, it is interesting to note that within the data a few variables with zero (or near-zero) values for the latest days of analysis were observed; these are likely to be metabolites present in quantities near the detection limit. An example is shown in Figure 5.6. Normalization does not improve the distributional properties in this circumstance.

### 5.3.4 Correlation analysis

A useful means of gaining an overview of the relationships between all possible pairs of samples is to compute their correlation. The correlation in this case is the usual Pearson correlation. Below are shown visual representations of these relationships, presented as heatmaps of full [42 x 42] correlation matrices. In each case, the diagonal of the matrix (from the upper left corner to the lower right) represents the correlation of a sample with itself, and is thus equal to unity. This diagonal separates the matrix into two triangles that are mirror images of each other (since the correlation of a sample A with a sample B is always equal to the correlation of sample B with sample A). Considering that the data are affected by two main factors, the genotype and the day of analysis, two kinds of representations were used in this work: matrices where the data were ordered by day (Figure 5.7) and matrices where the data were ordered by genotype (Figure 5.8). All the matrices shown are computed from normalized data.

In order to find the level of correlation for any pair of samples, I examine the value in the heatmap on Figure 5.7 for the row and column intersection for those two samples. For instance, by a closer inspection of inter-sample covariance for day 5 (outlined by a black square for clarity), I find that four different genotypes (*ss4*, *sex1*, *sex4*, and *dpe2*, respectively) were analysed on this day, and this is reflected by four [3 x 3] squares of high correlation along the diagonal. This pattern of correlation suggests several findings: first, that within one analysis batch, the within-genotype variance is generally less than the between-genotype variance. Second, the

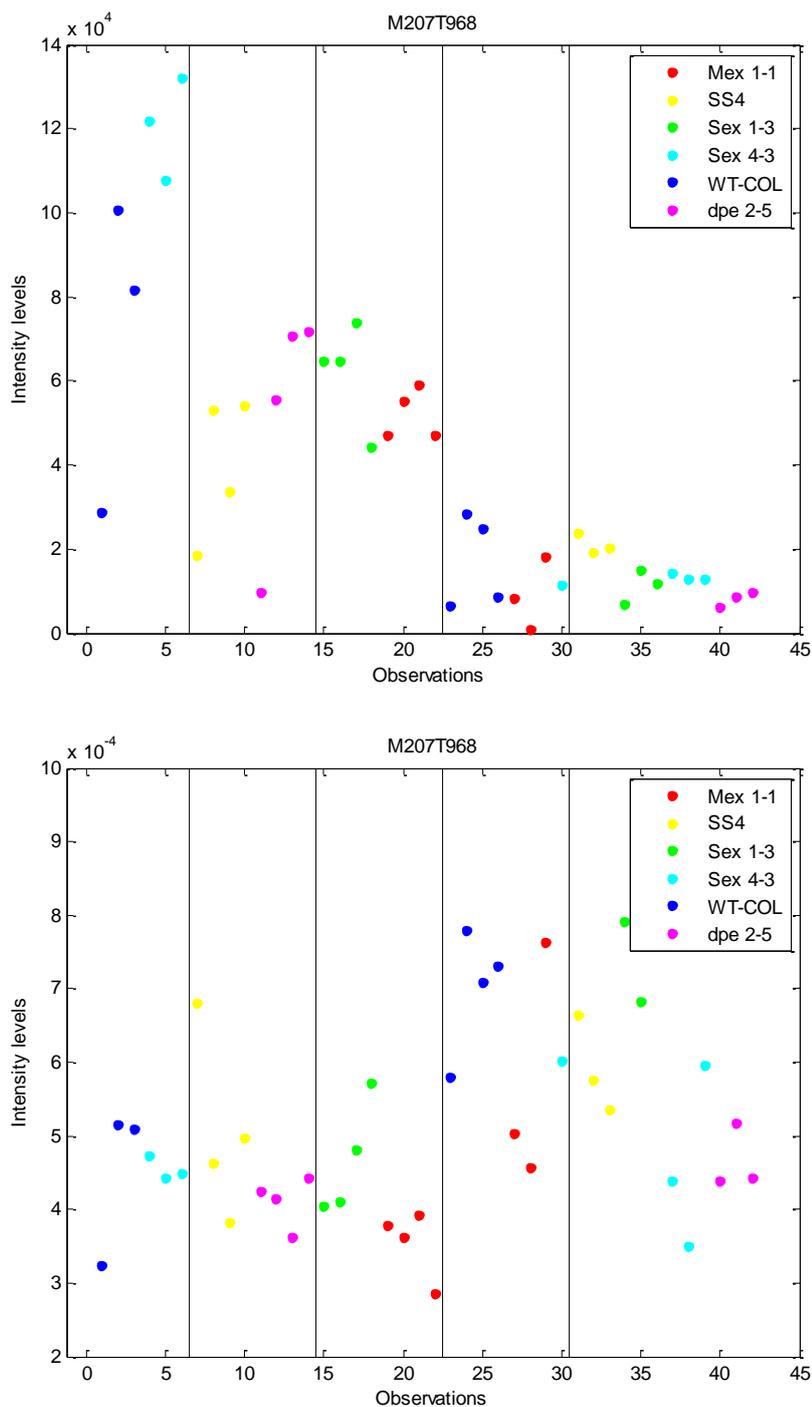


Figure 5.5. A randomly selected variable (metabolite) with  $m/z$  207 and RT 16.13min (968sec) before (top) and after (bottom) normalization. The horizontal lines separate the data from different days of analysis. Normalization mitigates the pronounced systematic shift over time observed in the raw data.

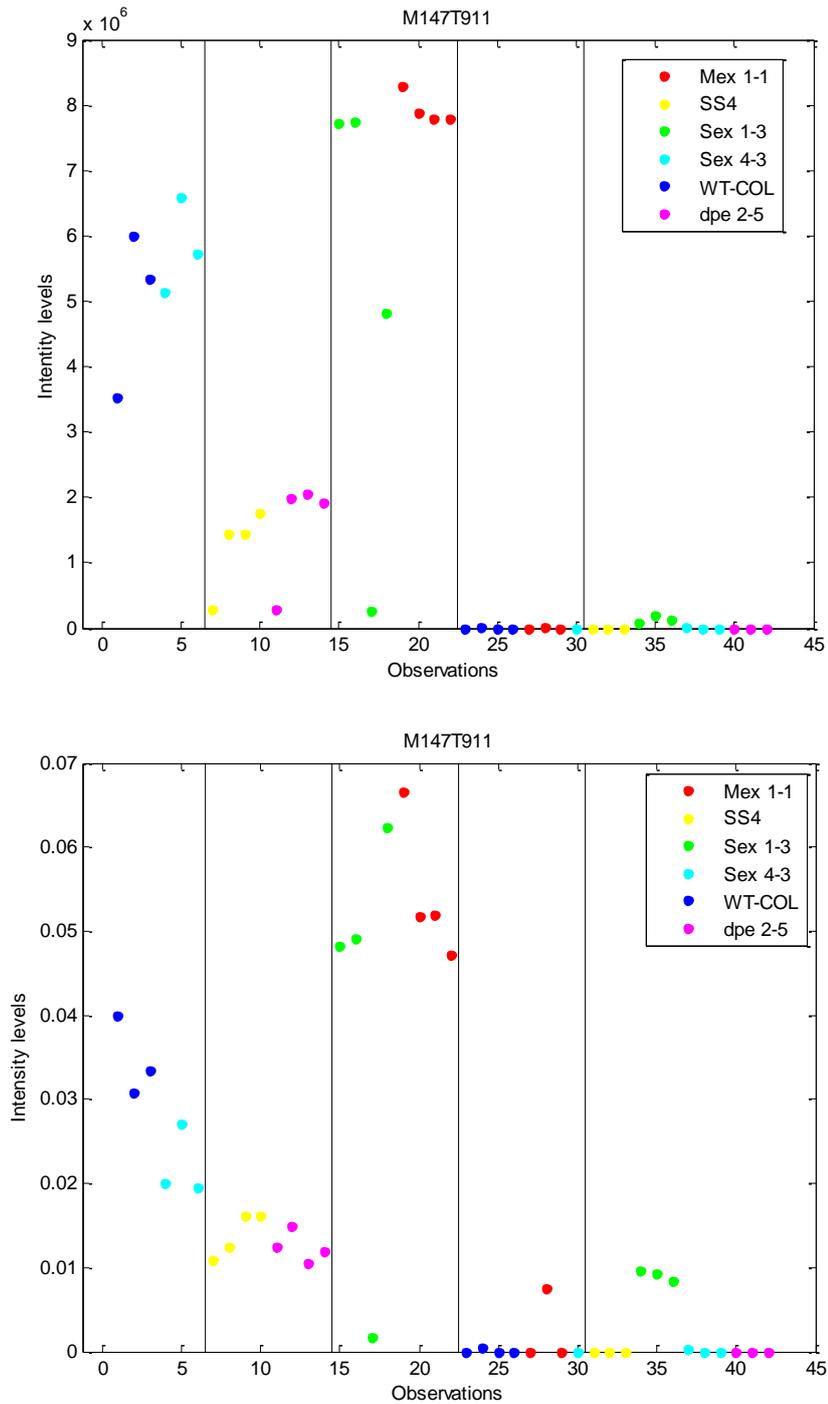
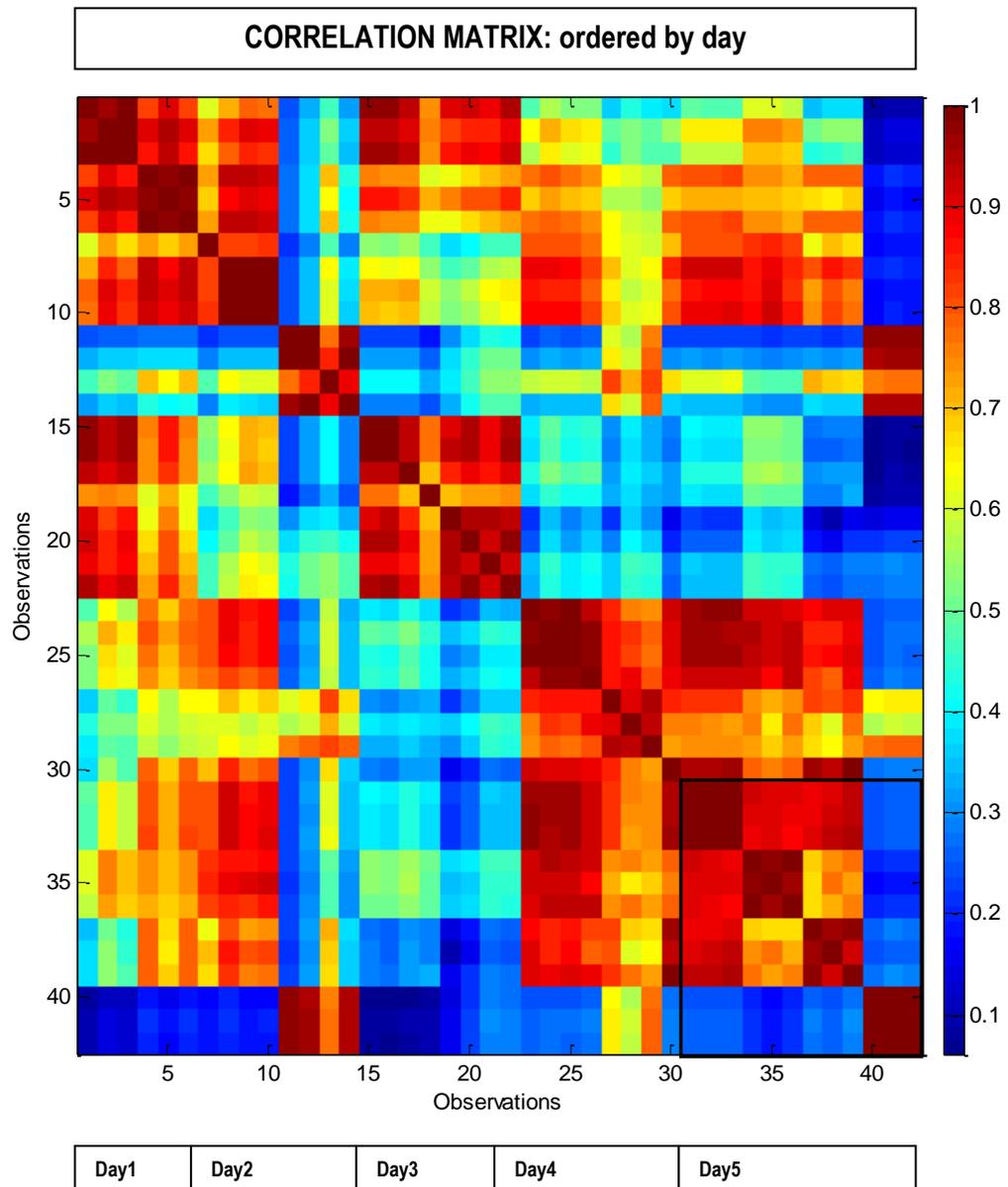
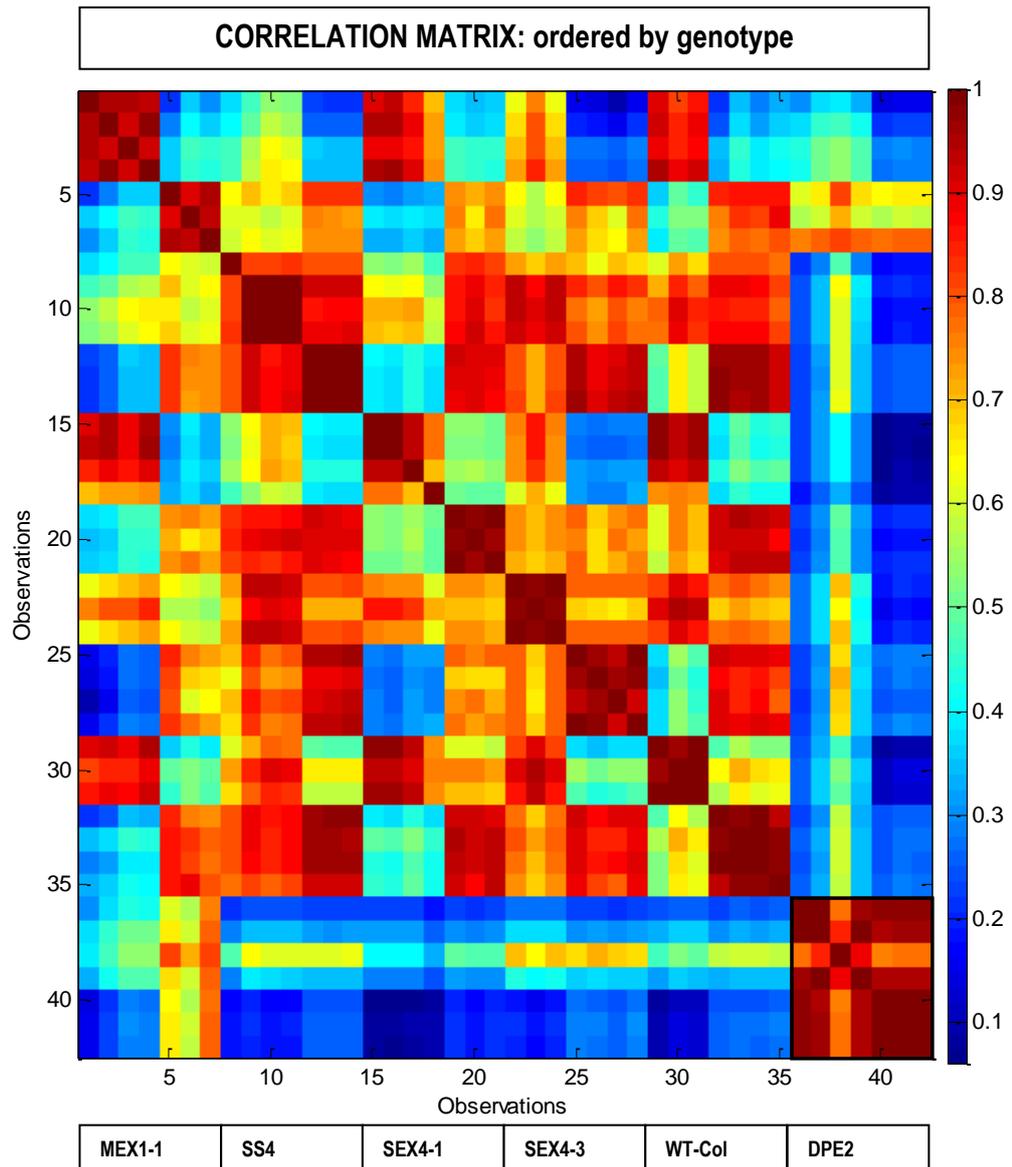


Figure 5.6. An example of the appearance of near zero values for the last days of analysis for a variable with  $m/z$  207 and RT 16.13min (968sec) before (top graph) and after (B) normalization. The horizontal lines separate the data in different days of analysis.



*Figure 5.7. Correlation matrix of normalized starch data (ordered by day of analysis). The above graph suggests that the within-genotype variance is generally less than the between-genotype variance, i.e. the highlighted black square in Day5 reveals the presence of four groups which correspond to mutants: *ss4*, *sex1*, *sex4*, and *dpe2* respectively.*



*Figure 5.8. Correlation matrix of normalized starch data (ordered by genotype). Squares of high correlation along the diagonal reveal the only partial success of the normalization. The black square (bottom right) highlights the separation of *dpe2* mutant from all the rest.*

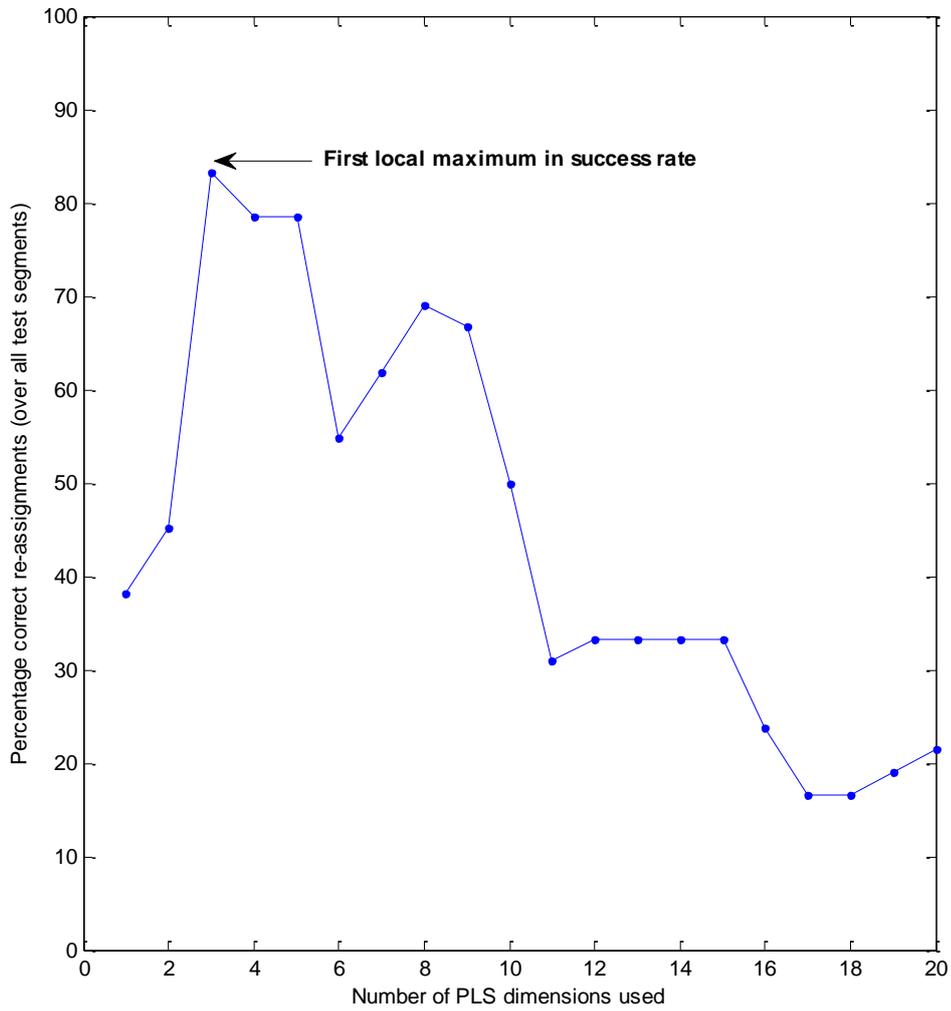
*sex4*, *sex1*, *ss4* (starch granule) mutants are relatively more co-varying than the *dpe2* mutants. By looking at the correlation matrix ordered by genotypes in Figure 5.8, it is seen that this distinction is observed for all the samples of the *dpe2* genotype. From Figure 5.8 further insight gained into the only partial success of the normalization: it is clear that some day effect is unavoidable.

#### **5.4 Multivariate modelling**

This data set, in common with many metabolomics studies, is characterized by a very large number of variables (specifically, 1153 different peaks) identified in each profile, and a relatively small number (42) of independent biological samples. In such circumstances, the family of data compression methods provide suitable statistical approaches for analysing the data. I have elected to use a supervised multivariate classification method, Partial Least Square Discriminant Analysis (PLS-DA), as an appropriate approach, discussed below. In the subsequent sections, this method will be compared with alternative approaches.

##### **5.4.1 Partial Least Square Discriminant Analysis (PLS-DA)**

PLS-DA analyses were carried out to determine whether the different genotypes could be systematically distinguished. The method (NIPALS algorithm routine; see Appendix A1) was implemented using leave-one-out cross-validation, as unlike in the HiMet9 dataset in Chapter 4, there are no technical replicates. All data were normalized, as discussed above. In addition, various data scalings were investigated (including variance-scaling and auto-scaling). In common with the initial studies on the HiMet data reported above, it was found that optimal results were obtained using mean-centering (“covariance method PLS”, see section ) only. This model resulted in very high classification success rate of 83.3% from the first three PLS components, as shown on Figure 5.9. I conclude that three PLS scores only are sufficient to provide a good discriminatory model for distinguishing genotypes. Scatter plots of the cross-validated scores for the first three PLS components are shown in Figures 5.10-5.12, with the points colour-coded by genotype. In most of the genotypes, the biological replicates clustered together. However, it should be noted that the “day effect” is still present, in some cases splitting the same genotypes in two groups.



*Figure 5.9. Number of classification successes vs the number of PLS factors used in the PLS-LDA method (using the NIPALS algorithm). The first local maximum on this graph indicates an optimum classification success rate of 83.3% accomplished for the first three components (35 out of 42 samples correctly classified).*

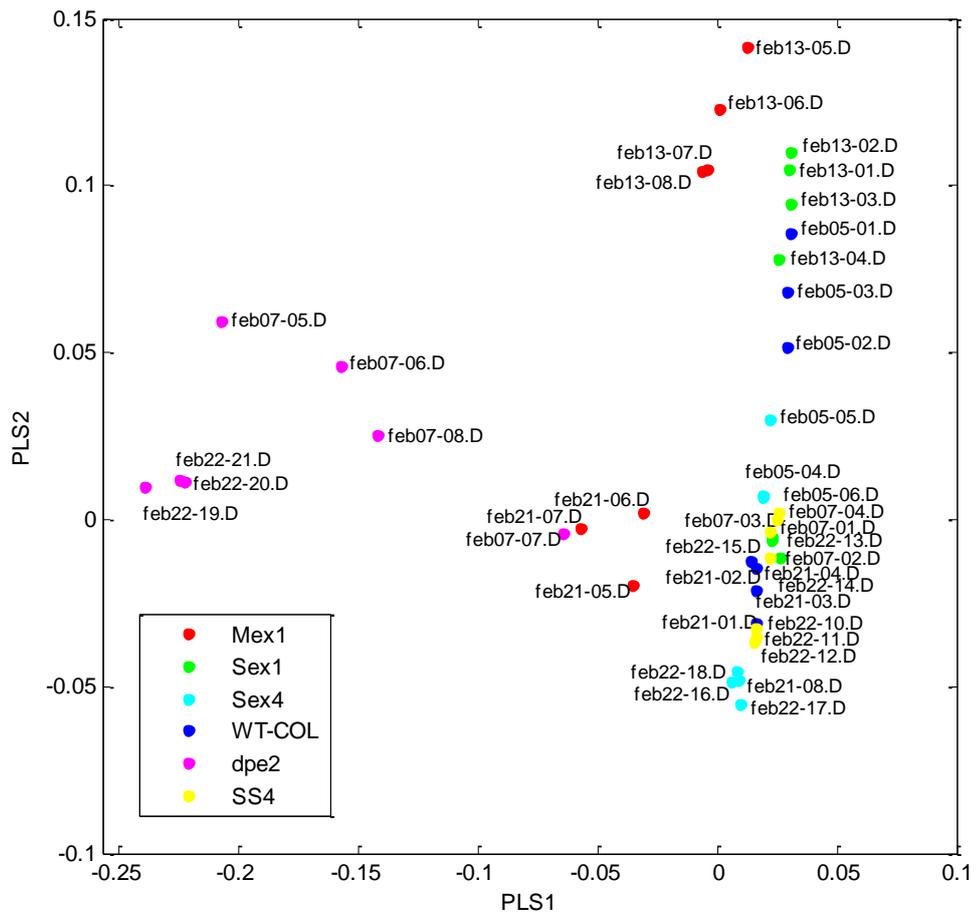


Figure 5.10. Scores plot of the first versus the second PLS components (PLS1 vs PLS2).

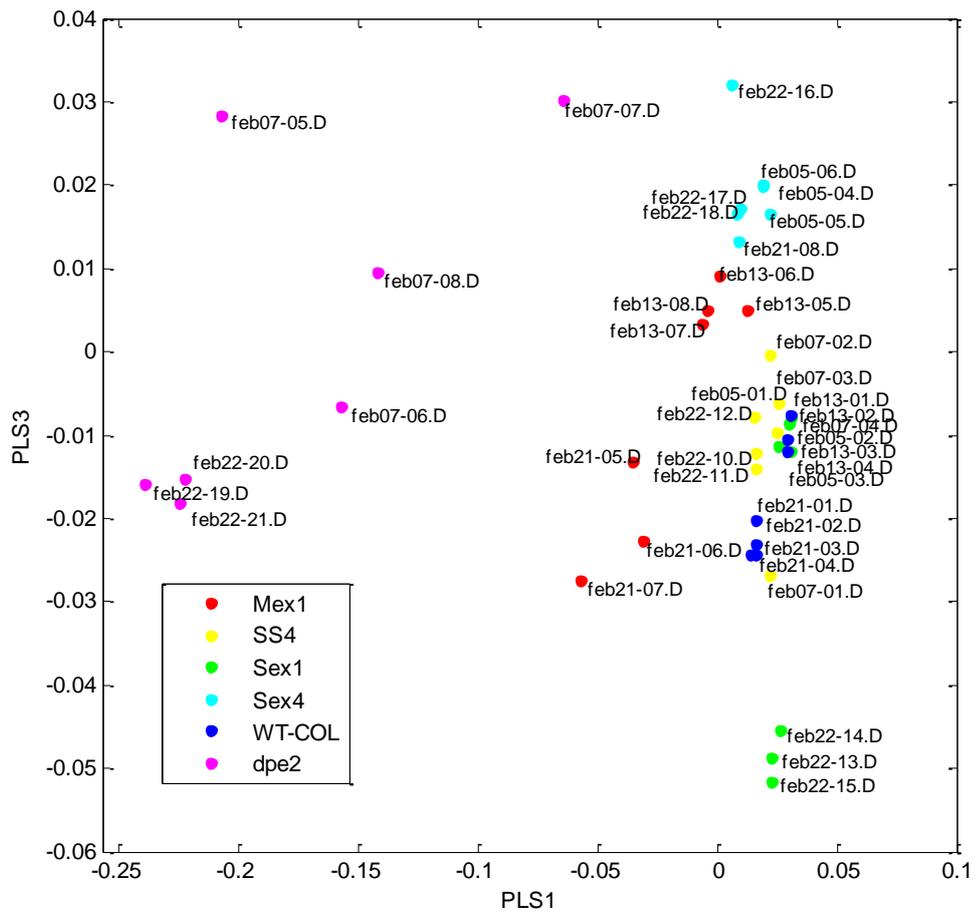


Figure 5.11. Scores plot of the first versus the third PLS components (PLS1 vs PLS3).

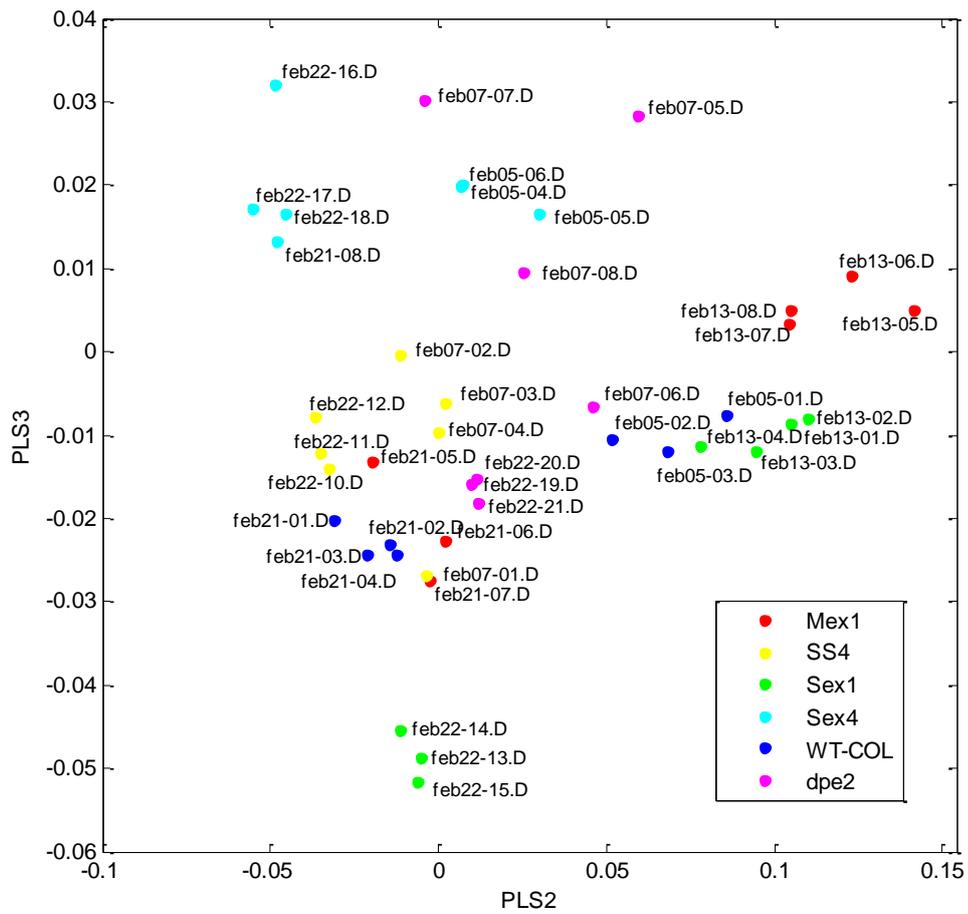
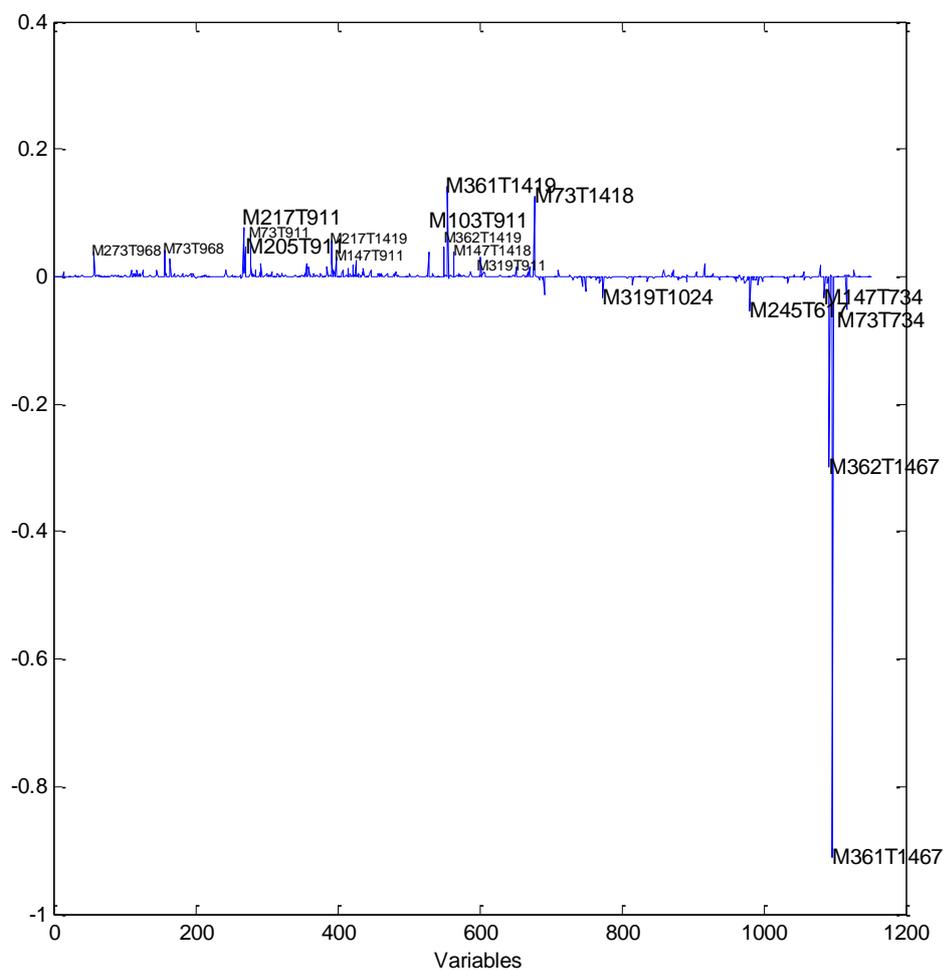


Figure 5.12. Scores plot of the second versus the third PLS components (PLS2 vs PLS3).

It is extremely clear that the *dpe2* mutant is separated from the rest of the genotypes (the magenta points in all three scores plots). The mutant *mex1* (red points) is well separated also, and in both cases, the first PLS dimension is sufficient to distinguish the groups. The second and third PLS dimensions are required to achieve almost (but not quite) complete discrimination between the remaining genotypes. The relationships between the mutants are clearer when the data are observed in the dimensions of the first and the third PLS components.

The separation of *dpe2* and *mex1* from the rest of genotypes was anticipated given that these mutants affect consecutive steps in the same pathway. This finding is confirmed by Masserli et.al. (2007), who found that *mex1* is classified to *dpe2* and none of the other genotypes seems like these two. Masserli et.al. (2007) used unsupervised methods (PCA, HCA) and in-house-developed supervised algorithms to investigate mutants affected in starch metabolism (including *dpe2*, *mex1*, *sex1* and *sex4*). Although the two metabolic profiles (*dpe2* and *mex1*) are not identical, both studies suggest that these two mutants are clustered together due to the very large individual effect of maltose.

The aim of PLS-DA is not only to establish if the metabolite profiles of the different genotypes can be systematically distinguished, but also to identify the variables (potential metabolites) that contribute to this distinction. The loadings plots can offer this information, and are particularly useful in low-dimensional models such as the present case. When PLS-DA is implemented with no data scaling (covariance PLS), the loadings reflect relative intensities in the original data (or more precisely, large variances; the tendency is for large features in the original data set also to dominate in the loading space). The first three loadings are shown in Figures 5.13-5.15. Each is marked with several m/z-retention-time values corresponding to the major loading weights. Many of these features can also be identified as present in the raw data. The next step is to identify these peaks, or at least, a subset of the most dominant ones, as these are clearly important metabolites for distinguishing the genotypes. However, both the identification and the subsequent biochemical interpretation pose many challenges, to be discussed below. Many of the metabolites had significant weightings in the PLS vectors, indicating that separation of the genotypes is due to changes in many metabolites. This implies that different genotypes are associated



*Figure 5.13. Loadings plot of the first PLS component (PLS1). The loadings peaks with absolute weights >10% of the maximum absolute weight value are labeled with their m/z-retention-time identifier.*

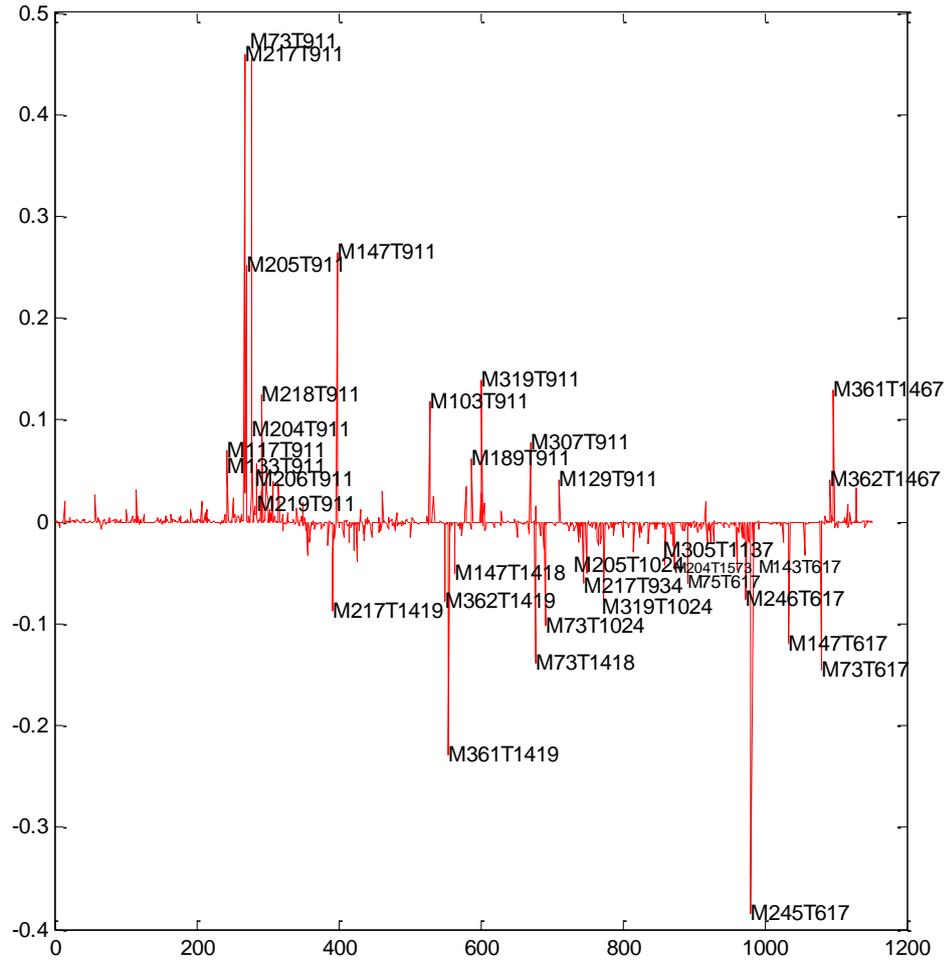


Figure 5.14. Loadings plot of the second PLS component (PLS2). The loadings peaks with absolute weights > 10% of the maximum absolute weight value are labeled with their m/z-retention-time identifier.

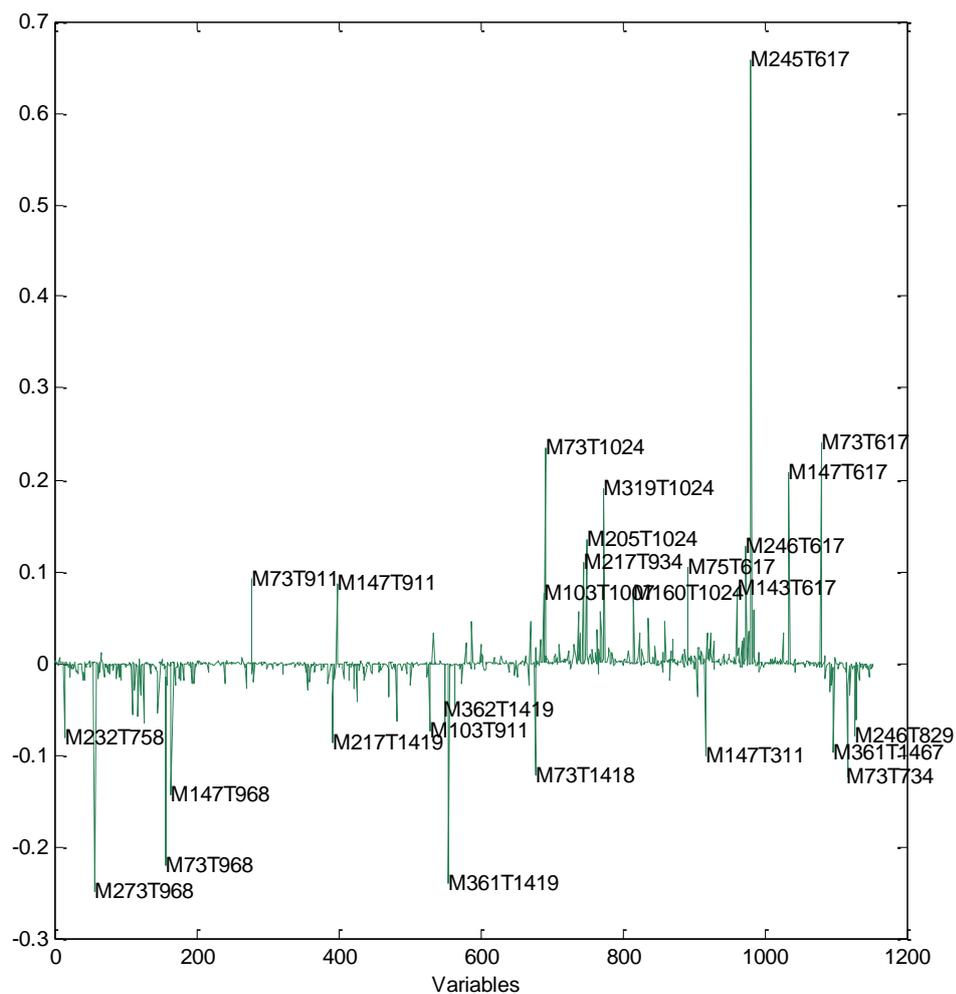


Figure 5.15. Loadings plot of the third PLS component (PLS3). The loadings peaks with absolute weights > 10% of the maximum absolute weight value are labeled with their  $m/z$ -retention-time identifier.

with individual “fingerprints” - distinct patterns of relative metabolite levels – rather than a change in only one individual compound for each genotype.

#### **5.4.1.1 Identification of significant metabolites**

In order to identify compounds corresponding to variables suggested as being important for discrimination between mutants by the PLS-DA analyses, the Golm Metabolome database was used. This currently has to be carried out using the raw data in the AMDIS software package, rather than be possible directly from within XCMS or indeed using the intensities integrated by XCMS. The important variables, however, are identified by XCMS in terms of m/z and retention time pairs. Therefore it is necessary to match these two pieces of information. Thus the mass spectra were extracted with XCMS and additionally with AMDIS, and the consistency of reads was cross-checked between the two software tools. This is done by comparing and cross-referencing major features of the MS spectrum at the retention time of the compound of interest, as is exemplified for the case of glucose (with retention time 1024sec) in Figure 5.16-5.17.

This identification process represents step 4 in our metabolomics data pipeline. Table 5.2 & 5.3 summarise the input and outcomes, respectively, of this process: Table 5.2 presents a list of the m/z and retention-time pairs of the variables identified on the basis of the loadings for PLS axes 1 to 3. The outcomes of the matching process and subsequent identification using the Golm Metabolome database are shown in Table 5.3, as a list of the discriminatory compounds including several sugars, amino acids and organic acids. In the next section, I will investigate the role of these compounds in starch metabolism.

#### **5.4.1.2 Role of the identified metabolites in starch metabolism**

Boxplots were constructed to display the median and range of the intensities for one fragment for each unique retention time of the compounds that were identified as discriminatory by PLS-DA. From these plots, it is possible to see how the multivariate modelling has identified and made use of the differences in levels in these components, in order to separate the groups in the PLS-DA.

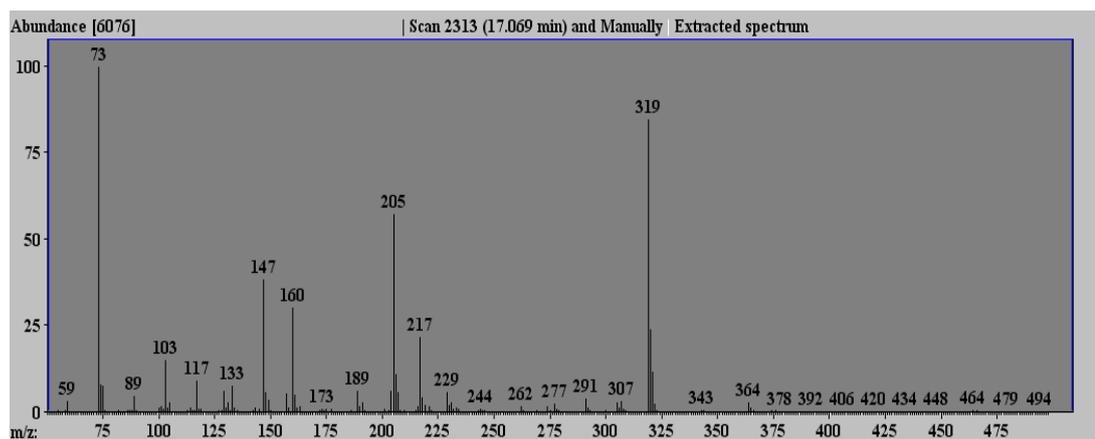


Figure 5.16. Manually extracted spectrum by AMDIS for retention time 17.069 min

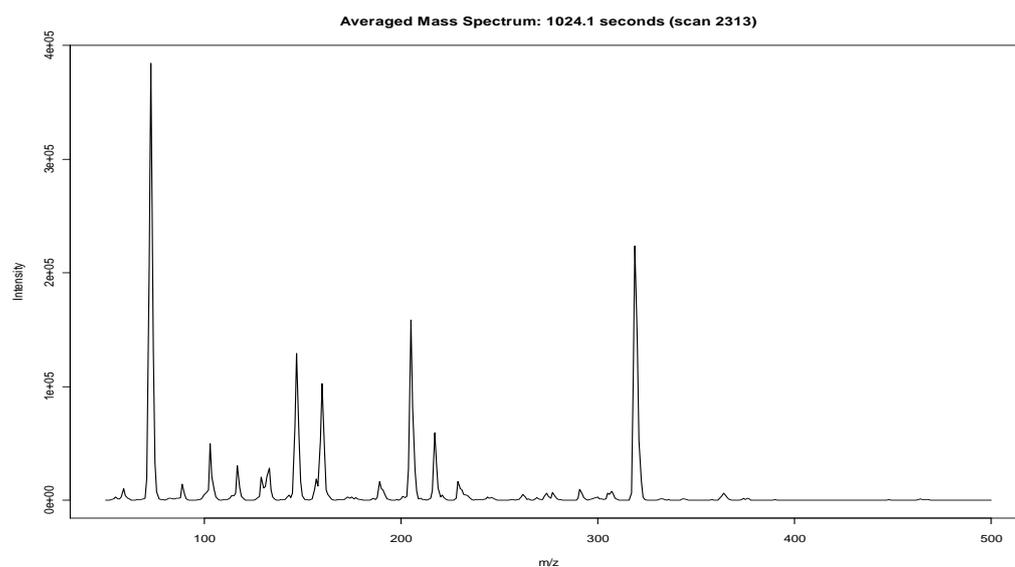


Figure 5.17. Average spectrum chromatogram extracted by xcms, 17.066 min

**Table 2.** Loadings peaks with absolute weights >10% of the maximum absolute weight values, identified as m/z (M) – retention time (T), when the fragments of maltose are removed.

| First PLS dimension (PLS1) | Second PLS dimension (PLS2) | Third PLS dimension (PLS3) |
|----------------------------|-----------------------------|----------------------------|
| 'M117T911'                 | 'M273T968'                  | 'M232T758'                 |
| 'M133T911'                 | 'M73T968'                   | 'M273T968'                 |
| 'M217T911'                 | 'M147T968'                  | 'M363T968'                 |
| 'M205T911'                 | 'M73T911'                   | 'M73T968'                  |
| 'M73T911'                  | 'M103T1418'                 | 'M147T968'                 |
| 'M204T911'                 | 'M218T1419'                 | 'M217T1419'                |
| 'M206T911'                 | 'M129T1418'                 | 'M218T634'                 |
| 'M218T911'                 | 'M217T1419'                 | 'M204T634'                 |
| 'M219T911'                 | 'M363T1419'                 | 'M362T1419'                |
| 'M277T911'                 | 'M147T911'                  | 'M361T1419'                |
| 'M217T1419'                | 'M169T1418'                 | 'M73T1418'                 |
| 'M147T911'                 | 'M271T1419'                 | 'M73T1024'                 |
| 'M103T911'                 | 'M437T1419'                 | 'M217T934'                 |
| 'M362T1419'                | 'M451T1419'                 | 'M319T1024'                |
| 'M361T1419'                | 'M103T911'                  | 'M305T1137'                |
| 'M189T911'                 | 'M362T1419'                 | 'M204T1573'                |
| 'M319T911'                 | 'M361T1419'                 | 'M75T617'                  |
| 'M307T911'                 | 'M147T1418'                 | 'M147T311'                 |
| 'M73T1418'                 | 'M319T1419'                 | 'M246T617'                 |
| 'M73T1024'                 | 'M73T1418'                  | 'M245T617'                 |
| 'M129T911'                 | 'M103T1007'                 | 'M233T734'                 |
| 'M217T934'                 | 'M73T1024'                  | 'M147T617'                 |
| 'M205T1024'                | 'M217T934'                  | 'M73T617'                  |
| 'M319T1024'                | 'M205T1024'                 | 'M147T734'                 |
| 'M204T1573'                | 'M320T1024'                 | 'M73T734'                  |
| 'M75T617'                  | 'M319T1024'                 | 'M246T829'                 |
| 'M143T617'                 | 'M160T1024'                 | 'M116T412'                 |
| 'M246T617'                 | 'M217T1024'                 |                            |
| 'M245T617'                 | 'M204T1573'                 |                            |
| 'M147T617'                 | 'M75T617'                   |                            |
| 'M73T617'                  | 'M147T311'                  |                            |
|                            | 'M143T617'                  |                            |
|                            | 'M246T617'                  |                            |
|                            | 'M245T617'                  |                            |
|                            | 'M247T617'                  |                            |
|                            | 'M233T734'                  |                            |
|                            | 'M147T617'                  |                            |
|                            | 'M117T1043'                 |                            |
|                            | 'M73T617'                   |                            |
|                            | 'M147T734'                  |                            |
|                            | 'M73T734'                   |                            |

**Table 5.3. Discriminatory metabolites as identified by the Golm library for plant metabolites using AMDIS**

| <b>Retention time<br/>(sec)</b> | <b>Retention time<br/>(min)</b> | <b>Related compounds or compound classes</b> |
|---------------------------------|---------------------------------|--|
| 311                             | 5.1833                          | Oxalic acid                                  |
| 617                             | 10.2833                         | Fumaric, succinic acid or maleic acid        |
| 734                             | 12.2333                         | Malic acid                                   |
| 829                             | 13.8167                         | Glutamic acid                                |
| 911                             | 15.1833                         | Ribitol                                      |
| 934                             | 15.5667                         | Glutamine                                    |
| 968                             | 16.1333                         | Citric acid                                  |
| 1007                            | 16.7833                         | Fructose methoxamine                         |
| 1024                            | 17.0667                         | Glucose                                      |
| 1137                            | 18.9500                         | Myo inositol                                 |
| 1418                            | 23.6333                         | Sucrose                                      |
| 1419                            | 23.6500                         | Raffinose                                    |
| 1467                            | 24.4500                         | Maltose                                      |
| 1573                            | 26.2167                         | Galactinol                                   |

The boxplots are representations of the data in the raw matrix X with each box corresponding to one of the mutants. The central (red) mark in the box is the median of all peak intensities for each mutant and the edges of the box are the 25th and 75th percentiles of the peaks distribution. The whiskers extend to the most extreme data points that are not considered outliers, while outliers are plotted individually. The below boxplots were constructed using Matlab (function `boxplot` with the default parameter), where points are drawn as outliers if they are larger than  $q_3 + w(q_3 - q_1)$  or smaller than  $q_1 - w(q_3 - q_1)$ , where  $w$  is the maximum whisker length (the default value equals 1.5), and  $q_1$  and  $q_3$  are the 25th and 75th percentiles, respectively. The default of 1.5 corresponds to approximately  $\pm 2.7\sigma$  and 99.3 coverage if the data are normally distributed.

As anticipated, data analysis revealed that a large amount of change in the metabolite content of the particular starch mutants is related to changes in the levels of sugars. (Figures 5.18-5.29).

Maltose is part of the pathway of the starch degradation in leaves. As referred in section 5.1.4, the mutants *mex1* and *dpe2* are deficient in the export of maltose from the chloroplast and its subsequent metabolism in the cytosol, respectively. Given that these are consecutive steps in the same pathway, it would be expected that their metabolite profiles are similar. The loadings for the first PLS vector (Figure 5.13) are dominated by two large peaks with retention time 24.45 min (identifiers respectively as 'M362T1467' and 'M361T1467' in Table 5.2). It is likely that these originate from the fraction corresponding to maltose. Since the weights in the loading are negative, and the scores of the *dpe2* and *mex1* mutants are also negative with respect to this PLS dimension, this would indicate relatively higher maltose contents in these two genotypes. This is confirmed by the boxplot on Figure 5.18 which shows that maltose is present only in these two genotypes, however the levels of maltose in *dpe2* are much higher. It additionally indicates that this is the main factor distinguishing these two genotypes from each other, and from the remaining genotypes.

Sucrose is the major transport sugar in plants, and typically in the dark (which is when these plants were harvested) a block in starch degradation is expected to result

in low sucrose levels. I observed a pattern of low intensity values for the variable at retention time 23.6 min, likely to be sucrose, for *mex1* and *dpe2* mutants, higher intensity values for *WT-Col*, *sex1*, *sex4* and considerably higher values for *ss4* mutant (Figure 5.19).

Another important metabolite in plant metabolism is myo-inositol. Figure 5.20 shows a pattern of low intensity values for *mex1*, *sex1* and *dpe2* and higher values for *ss4* and *sex4*. The next graphs (Figures 5.21-5.23) show the levels of glucose, methoxyamine and galactinol, respectively.

Organic acids and amino acids are major metabolites in primary metabolism, thus it is interesting to observe the alterations in their intensities in the different mutants.

However, the physiological explanation of these differences is not as straightforward as in the sugars, suggesting more wide-ranging effects. The organic acids are part of primary metabolism, and there are big carbon fluxes through them (citric acid etc. in the TCA cycle). On Figures 5.24- 5.28 are shown the levels of oxalic acid, fumaric acid, malic acid, glutamic acid, glutamine and a dicarboxylic acid. A mass fragment, M147, with retention time 12.33 min (734sec), which was identified by AMDIS as oxalic acid, appears in the third PLS loading vector. This compound seems to discriminate mutants which might have been expected to have similar profiles, i.e. *sex1* from *sex4*.

Six mass fragments, M73, M75, M143, M147, M245 and M246 were detected at retention time 10.28 min (617sec), identified by AMDIS as fumaric, succinic and/or maleic acid. These fragments appear mainly in the second and third PLS loading vector, indicating that one of the above associated dicarboxylic acids could be responsible for the classifications shown on Figures 5.10 and 5.12. The most prominent relationship as revealed on the boxplot is a strong discrimination between the mutants *sex4* and *sex1*.

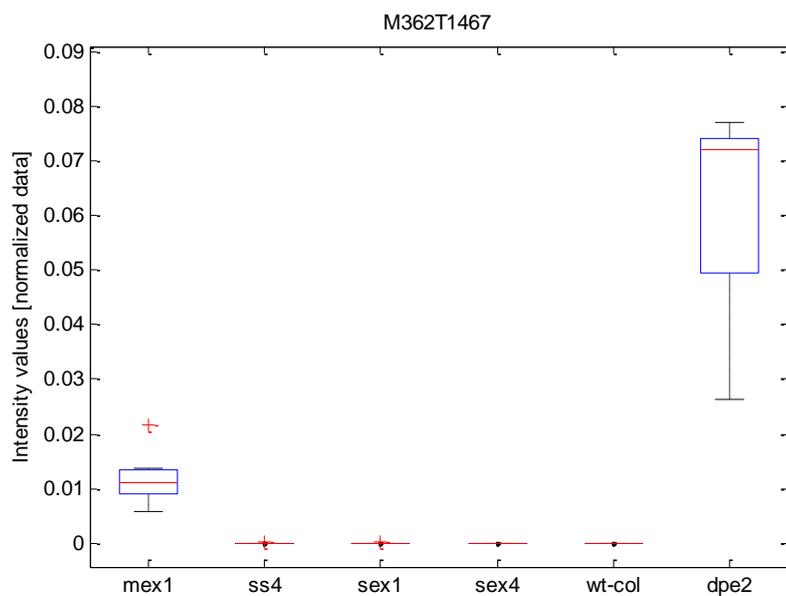


Figure 5.18. Boxplot of a variate with  $m/z$  362 and retention time 24.45 min (1467sec), which was identified as a fragment of maltose.

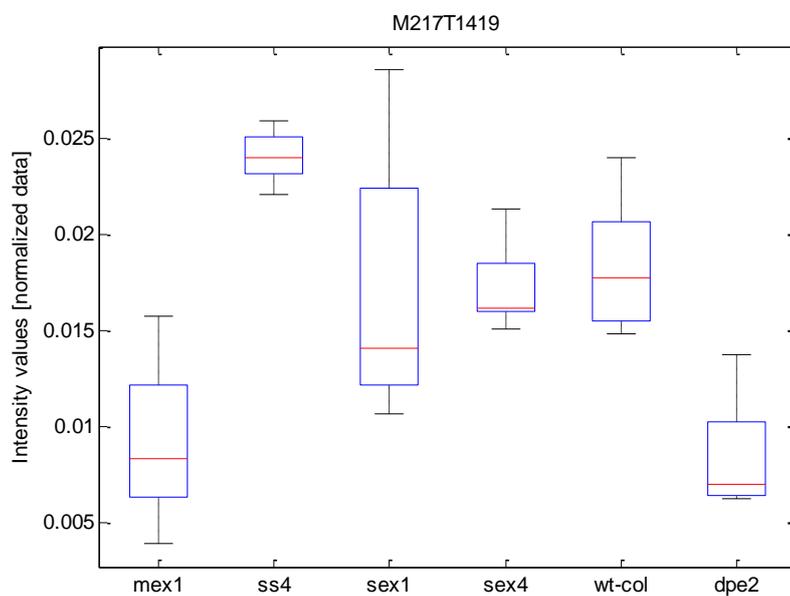


Figure 5.19. Boxplot of a variate with  $m/z$  217 and retention time 23.65 min (1419sec), which was identified as a fragment of either sucrose or raffinose.

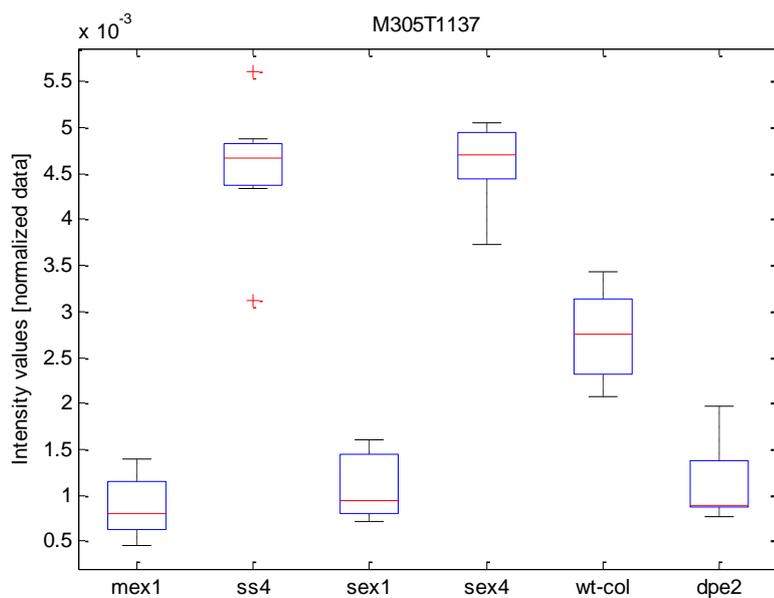


Figure 5.20. Boxplot of a variate with  $m/z$  305 and retention time 18.95 min (1137sec), which was identified as a fragment of myo-inositol.

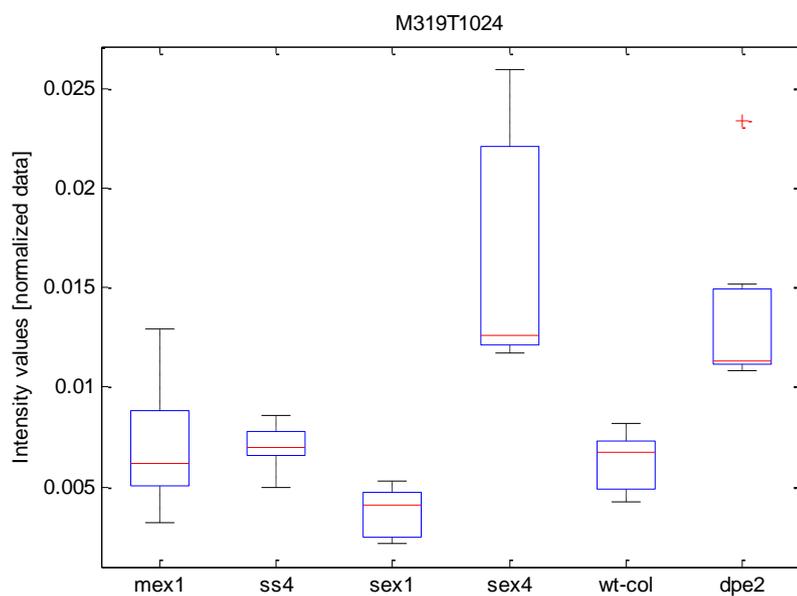


Figure 5.21. Boxplot of a variate with  $m/z$  319 and retention time 17.07 min (1024sec), which was identified as a fragment of glucose.

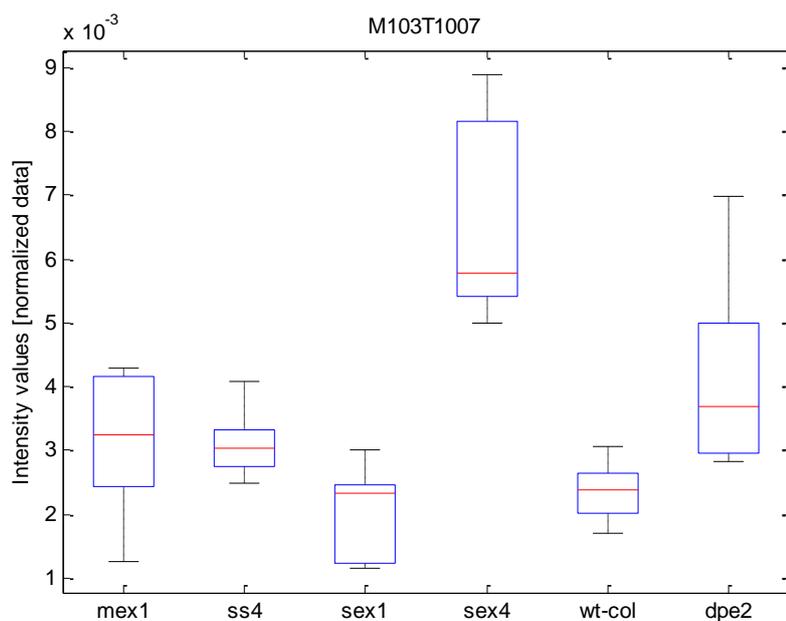


Figure 5.22. Boxplot of a variate with  $m/z$  103 and retention time 16.78 min (1007sec), which was identified as a fragment of fructose methoxyamine.

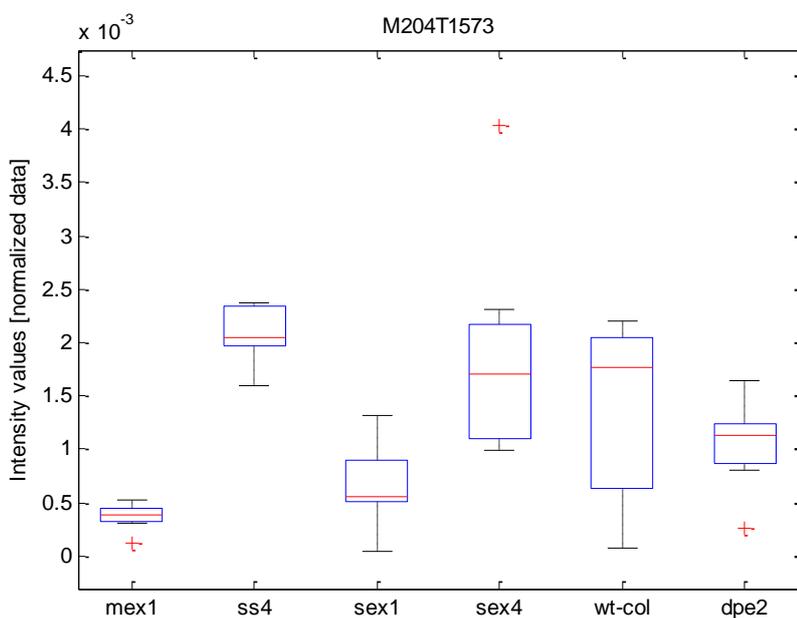


Figure 5.23. Boxplot of a variate with  $m/z$  204 and retention time 26.22 min (1573sec), which was identified as a fragment of galactinol.

Two unique mass fragments, M73 and M147, were detected at retention time 12.33 min (734sec), both of them identified as malic acid. These fragments appear in the first and third PLS loading vectors, indicating that malic acid could contribute to the discrimination of *dpe2* and *mex1* from the rest of the genotypes (Figure 5.10), or to any of the relationships on the third PLS component (Figure 5.12), among which the discrimination of *sex1* and *sex4* is most noticeable on the boxplot below (Figure 5.26). A mass fragment, M246, with retention time 13.82 min (829sec) identified by AMDIS as L-glutamic acid, appears in the third PLS loading vector. However, the boxplot (Figure 5.27) shows that there is large variance within each genotype, and incomplete discrimination.

Finally, glutamate and glutamine are involved in carbon/nitrogen balance in plants. It is found that glutamine is a clear discriminator between various genotypes (Figure 5.28). Glutamate did not reveal any strong relationship among the different genotypes.

#### **5.4.1.3 Summary of the mutant relationships**

Maltose appears in significant concentrations only in the *dpe2* and *mex1* mutants, with the highest levels in *dpe2*. Maltose has a very strong effect on the clustering obtained by the supervised modelling. The effects of differences in the other metabolite levels are somewhat more subtle. Raffinose and sucrose have relatively high concentrations in the *ss4* mutant, low concentrations in *mex1* and *dpe2*, and more intermediate concentrations for *sex1*, *sex4*, and wild type. The variable most likely identified as fumaric acid has higher values for *sex4* and very low for *sex1*, indicating that it is a separator of the *sex* mutants. Myo-inositol exhibits high levels for *ss4* and *sex4*, lower levels for wild type, and very low for *mex1*, *sex1* and *dpe2*. Again this provides a differentiator between the *sex1* and *sex4* mutants.

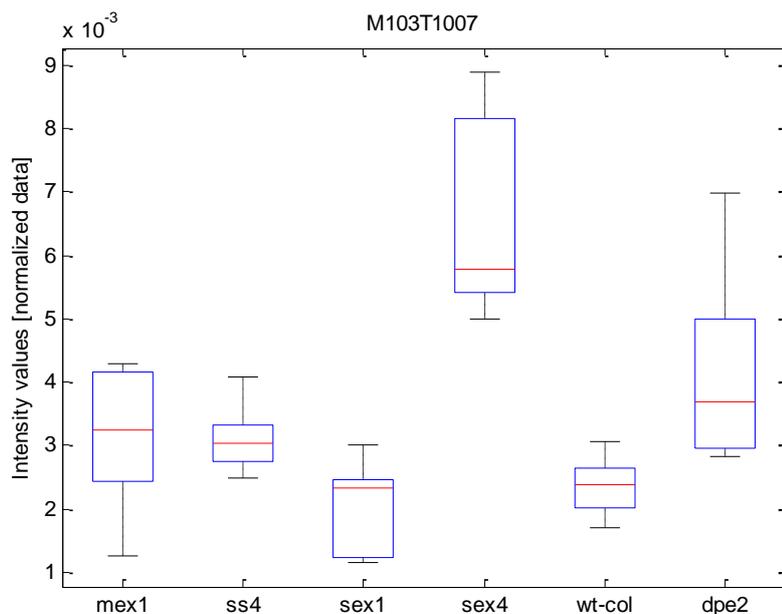


Figure 5.24. Boxplot of a variate with  $m/z$  147 and retention time 5.18min (311sec), which was identified as a fragment of oxalic acid.

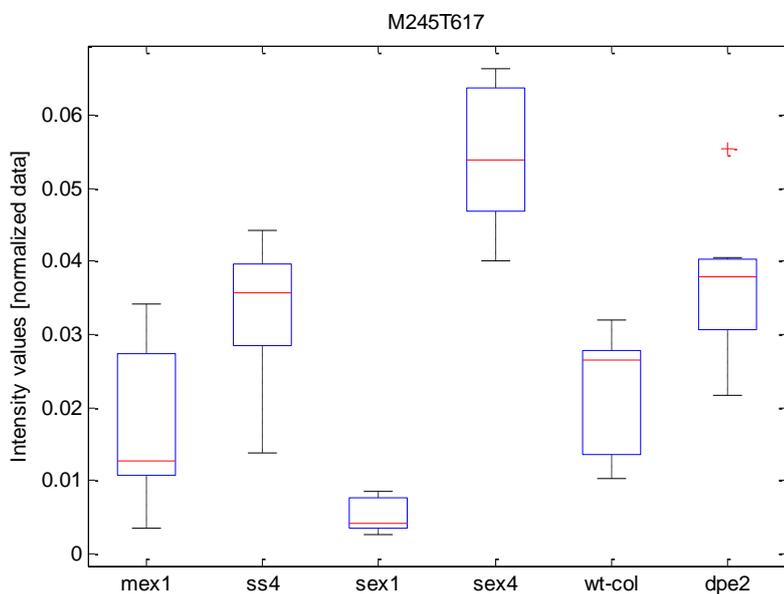


Figure 5.25. Boxplot of a variate with  $m/z$  245 and retention time 10.28 min (617sec), which was identified as a fragment of a dicarboxylic acid.

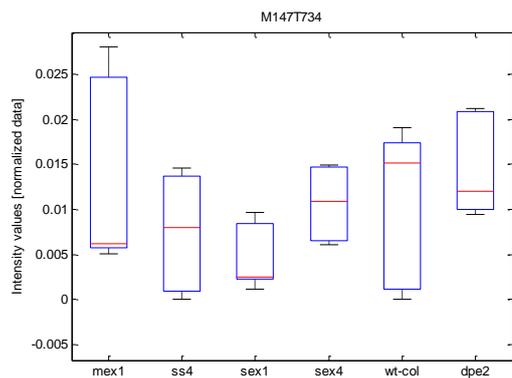


Figure 5.26. Boxplot of a variate with  $m/z$  147 and retention time 12.23 min (734sec), which was identified as malic acid.

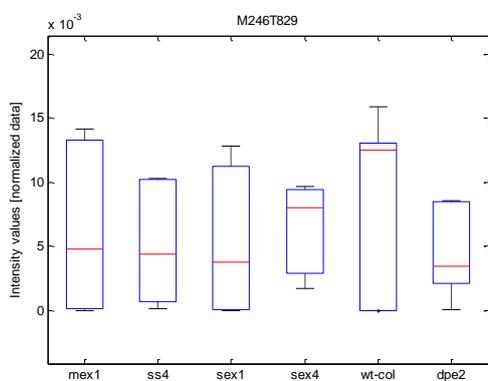


Figure 5.27. Boxplot of a variate with  $m/z$  246 and retention time 13.82 min (829sec), which was identified as glutamic acid.

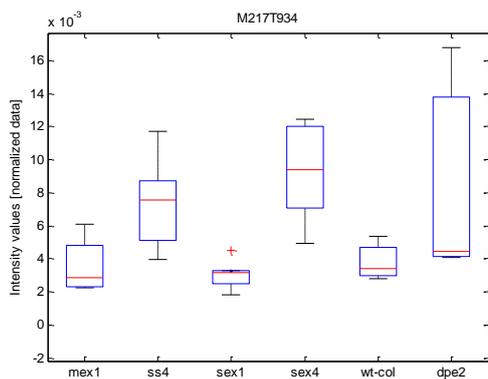


Figure 5.28. Boxplot of a variate with  $m/z$  217 and retention time 15.57 min (934sec), which was identified as a fragment of glutamine.

## 5.4.2 Comparison with alternative statistical methods

In order to evaluate the results obtained by PLS-DA, I examined the data first using alternative PLS-DA algorithms, and second using different methods of analysis. To begin, I compare two different PLS2 routines: the NIPALS algorithm which is the original, standard algorithm for PLS, and the SIMPLS algorithm which is the method provided in the *plsregress* function in Matlab. For comparison purposes, I additionally performed PCA-DA and an unsupervised method, hierarchical cluster analysis. Finally, I present the use of a univariate method as a means of identifying the most significant variables and hence as a variable selection method prior to multivariate modelling.

### 5.4.2.1 An alternative algorithm to perform PLS-DA

There are many alternative algorithms for performing Partial Least Square regression (Lindgren and Rännar, 1998), both for PLS1 (single vector as grouping Y variable (Andersson, 2009; Manne, 1987)), or for the method that I use throughout this thesis, PLS2, that uses a matrix as a grouping Y variable (Alsberg and Kvalheim, 1994; Manne, 1987).

I present here the compare between two algorithms: the NIPALS algorithm (Martens, 2001), which is considered the original, standard algorithm for PLS; and the SIMPLS algorithm (de Jong, 1993), a more recent development. The latter is provided as the default algorithm within the *plsregress* function in Matlab. The NIPALS algorithm(s) were written in house as Matlab scripts (Appendix A1). Note that these were cross-checked in their function against the default NIPALS algorithms in R (PLS regression through the generic functions *plsrr*), and were found to produce precisely the same results.

In Figure 5.29, it can be seen that the results of the scores obtained by the NIPALS and SIMPLS routines are very similar for the first few components, but they start to substantially differ after around the fifth component. This difference is partially related to a tolerance factor (see Appendix A1), but also to a substantive difference between the two algorithms. According to de Jong (1993), SIMPLS truly maximises the covariance criterion, whereas the standard PLS2 algorithms (i.e. NIPALS) lie closer to ordinary least-squares regression where a precise fit is sought. The SIMPLS PLS2 routine is expected to lie closer to PCA than the standard PLS2 algorithm; this is confirmed by the results of our PCA-DA analyses, discussed below.

The consequences of the differences between the NIPALS, the SIMPLS and the PCA approaches can be compared by looking at the success rate in discriminant analysis in each of the cases (Figures 5.30, 5.32 and 5.9). In all cases, there is general agreement for low-dimensional models, but as scores with smaller variances are included, the disagreement between the outcomes increases. A potential reason for the differences between the two PLS algorithms for a multivariate  $\mathbf{Y}$  in our specific case could be that the computations are affected by the size of the  $\mathbf{X}$  matrix, which consists of a relatively small number of observations/samples and a large number of variables, some of which have a large range of intensities.

Conclusively, it seems that the choice of algorithm for ostensibly the same method, PLS2, makes a clear and sometimes large difference to the scores with smaller variances. However, the overall impact on the classification results from, crucially, the optimal, low-dimensional models (and the discriminatory peaks identified) is not very great. Hence, provided care is taken to identify only parsimonious models, the choice of algorithm may not be a major concern.

#### **5.4.2.2 Principal Component Discriminant Analysis PCA-DA**

PCA-DA analysis was carried out as a comparison with PLS-DA. The method was also implemented using leave-one-out cross-validation. The model resulted in a very high classification success rate of 83.33% correct classifications for the first three components (Figure 5.32). The resulting scores (Figures 5.31 and 5.33) and loadings for this analysis were very similar to the results obtain by PLS-DA method for the optimal low-dimensioned model.

#### **5.4.2.3 Hierarchical Cluster Analysis**

In terms of multivariate analyses, I also analysed the data set using Hierarchical Cluster Analysis (HCA). In contrast to the hyphenated –DA techniques, this is an unsupervised method for examining groupings in the data. The results of the HCA analysis are shown in dendrograms (Appendix A3) that list all the samples, and indicate similarities among them. I examined several combinations of methods to calculate the pairwise distances between the metabolite profiles and different linkage methods to generate the clusters. **However, due to the strong batch effect that dominates the data, this method failed to reveal the relationships between the**

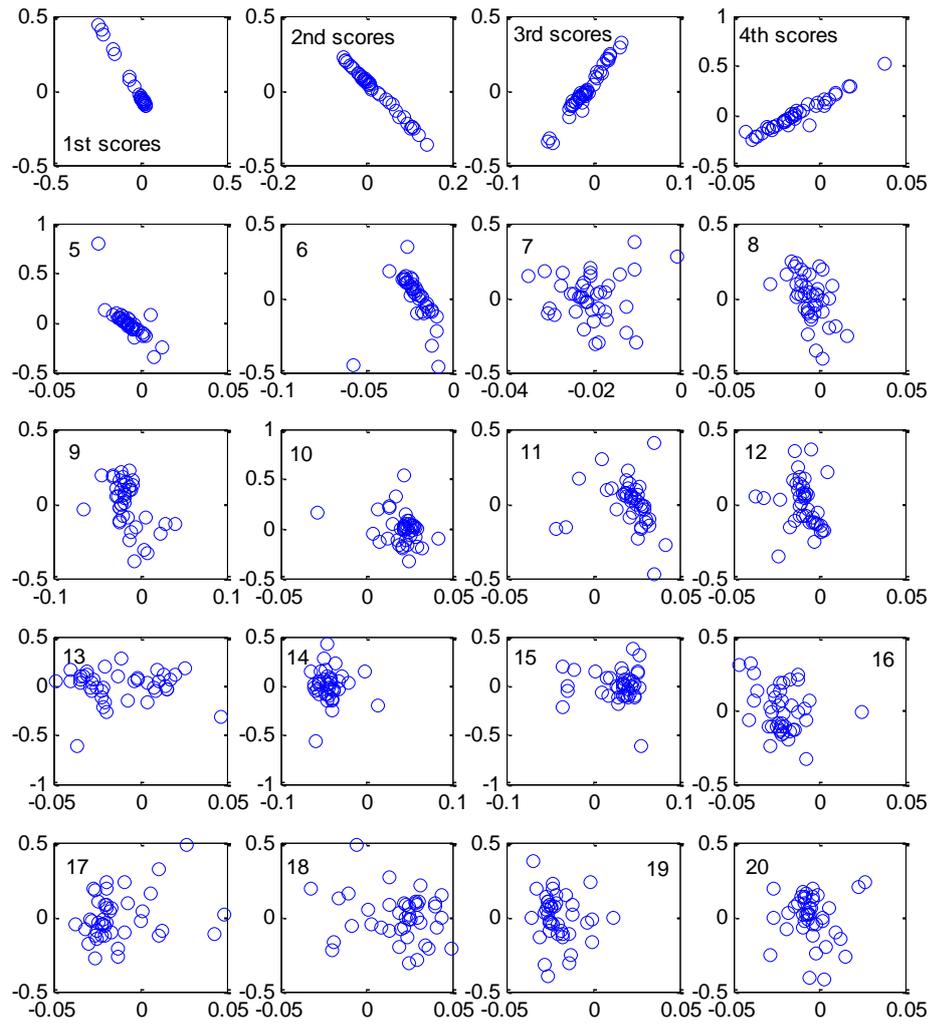
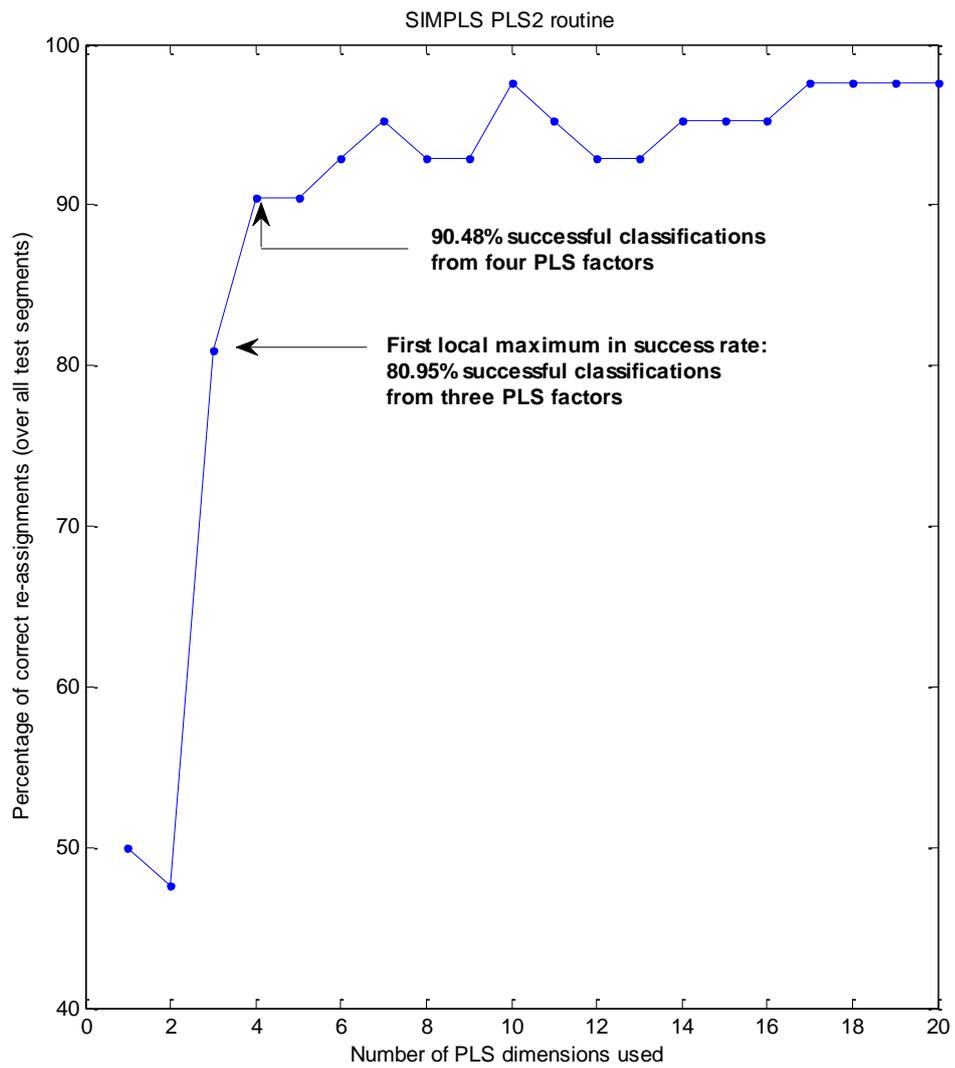
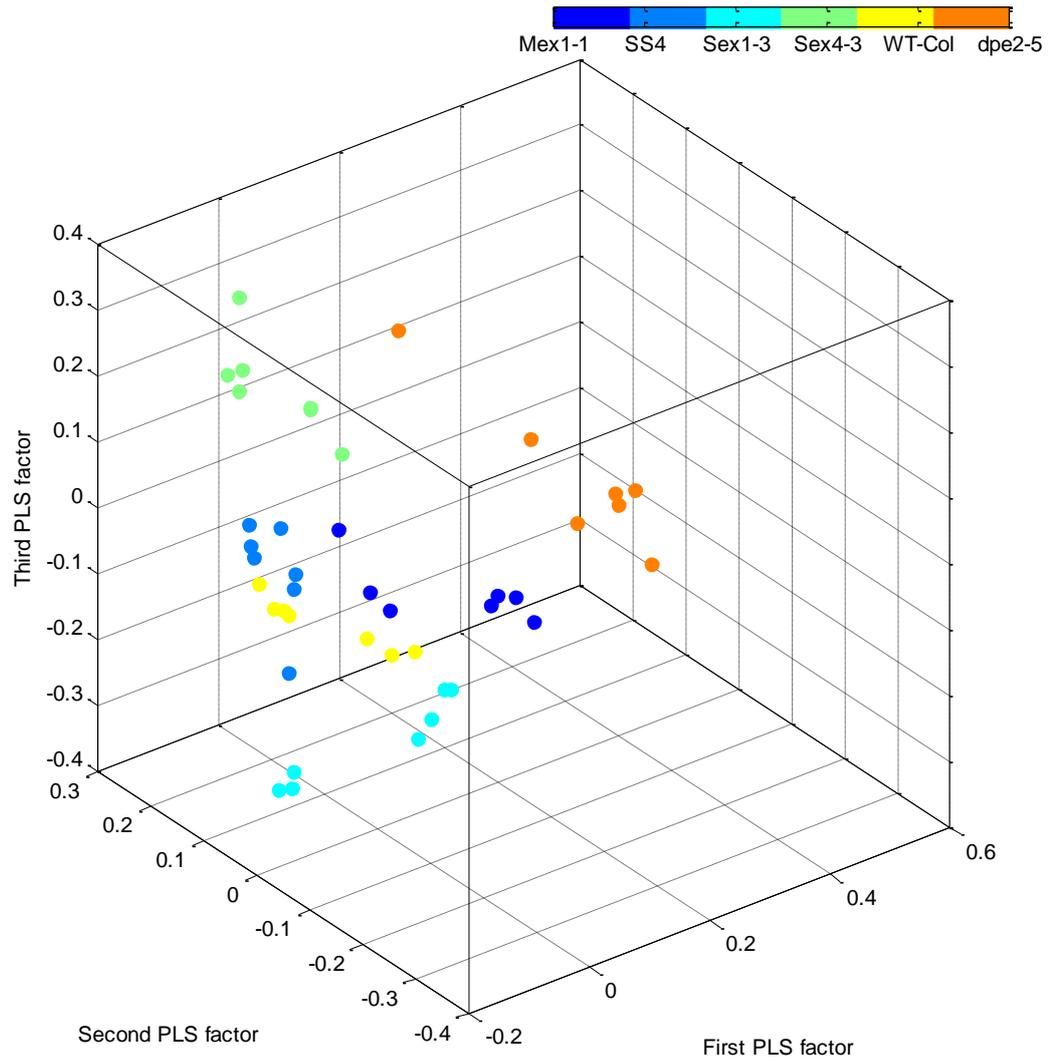


Figure 5.29. Comparison between PLS scores obtained by the standard NIPALS (horizontal axes) and the SIMPLS (vertical axes) algorithm. (SIMPLS as implemented in the Matlab `plsregress` function, NIPALS following the method of Martens and Nae with Tolerance  $10^{-7}$ )



*Figure 5.30. Number of classification successes vs the number of PLS factors used in the PLS-LDA method (using the SIMPLS algorithm).*



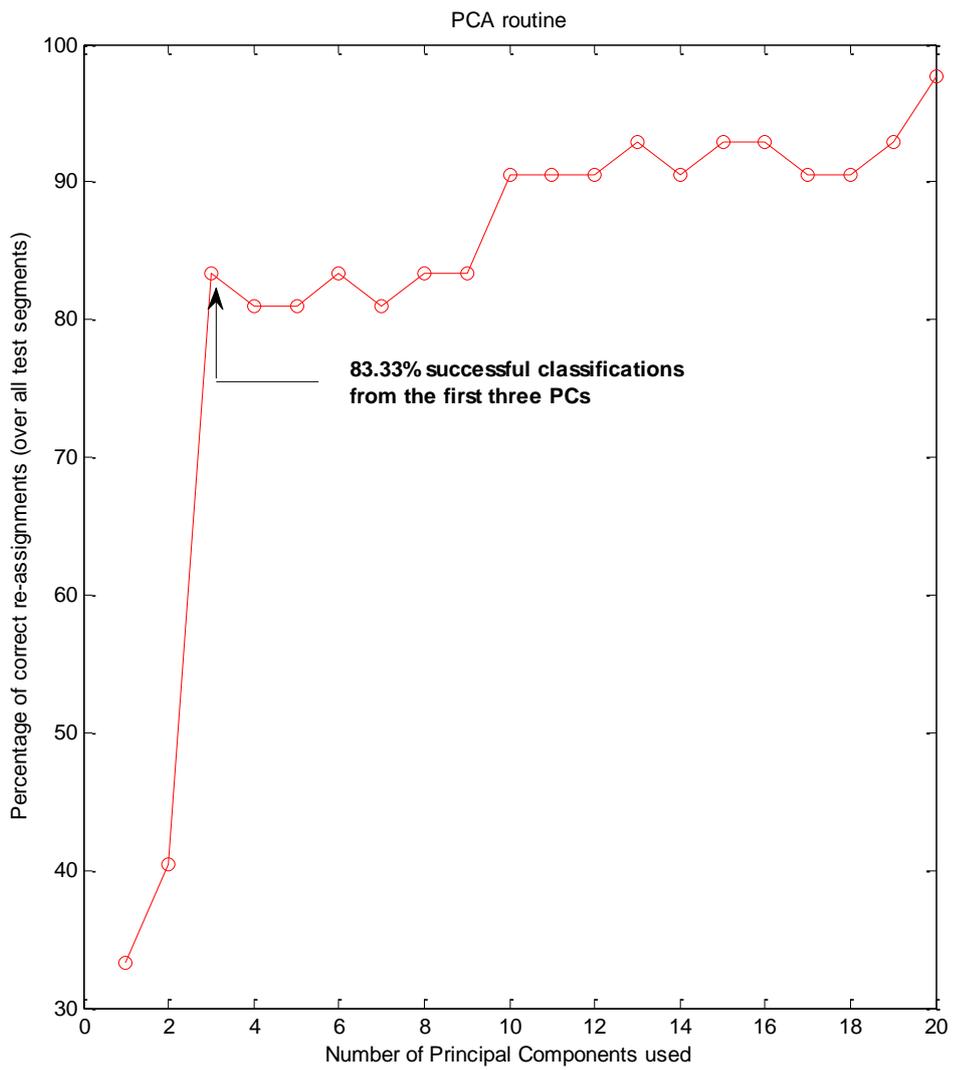
*Figure 5.31. Scores plots for the first three components (PLS1 vs PLS2 vs PLS3) using PLS-DA method (SIMPLS algorithm).*

**mutants as a result of the genetic variation.** The separation of *dpe2* from the rest of the mutants was the only clear observation of biological interest, which is consistent with the results of the other statistical modelling analysis.

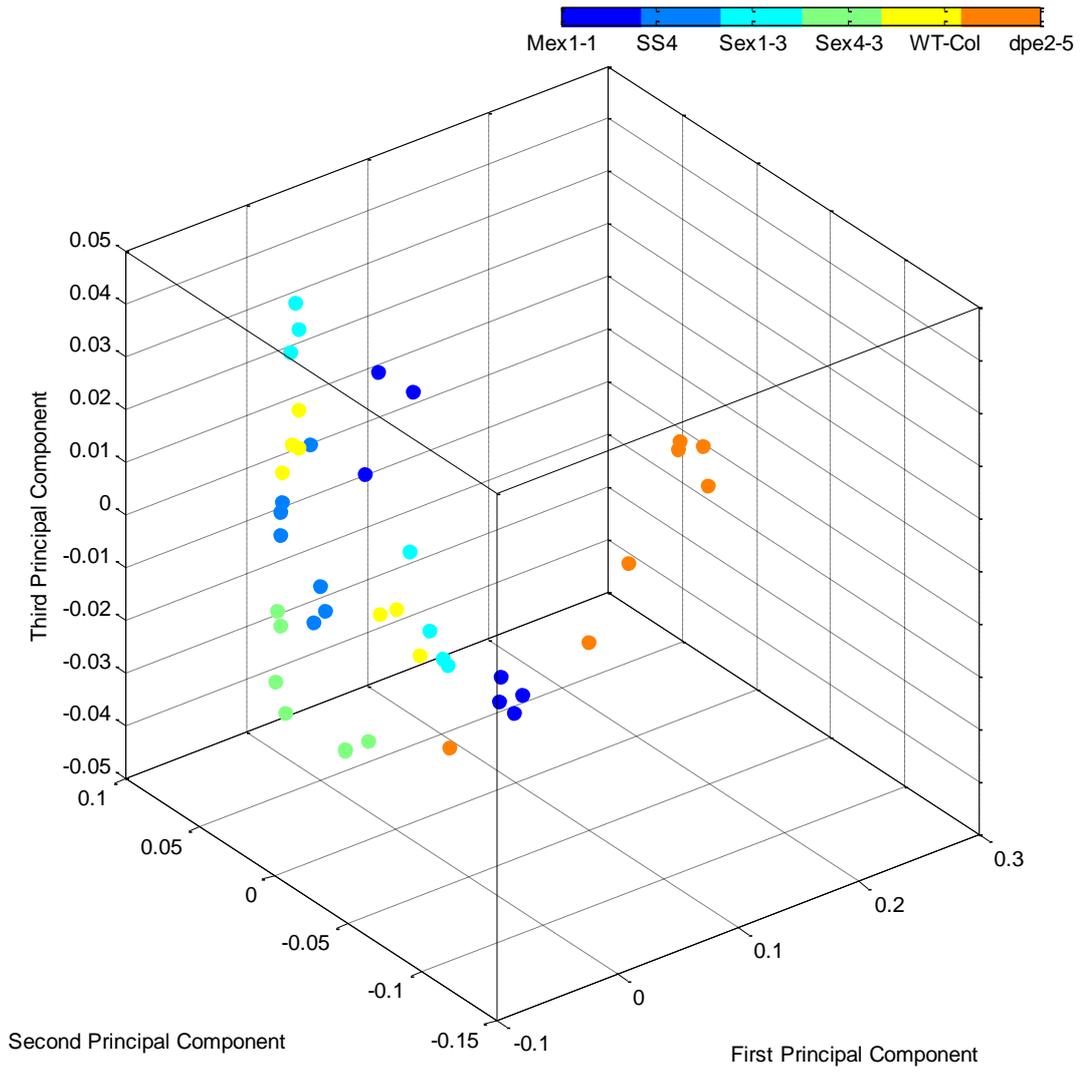
#### 5.4.2.4 Univariate Multiway Analysis of Variance (Anova-n)

In addition to multivariate analysis, univariate analysis, in the form of multiway-ANOVA, was carried out, as an alternative approach to identifying the most important metabolites for discriminating between genotypes, through an individual ranking method. As previously described, the starch dataset is dominated by a very strong batch effect, thus besides the biological variability due to the different genotypes, a univariate approach should also take into consideration the day of analysis as an additional factor of variance. I used here an ANOVA model (*anovan function* in Matlab) with two grouping variables: genotype and day of analysis. This model computes p-values for each of the two grouping factors, and performs multiple t-tests with Bonferroni adjustment to compensate for the multiple comparisons. By specifying the suitable type of sum-of-squares (TypeI), the calculations of the p-values in relation to the genotype (second term in *anovan function*) are performed on a fit that already includes the effect of day (first term in *anovan function*). In this way, finally a set of p-values that determine the most significantly different variables (mz-RT time pairs) due to genotype after compensating for the effect of day is obtained. The *p*-values of the 30 most significantly different values are shown on Table 5.4. The unique retention times identified here are 1467, 1137, 1418 and 1419 sec corresponding to fragments of maltose, myoinositol, sucrose and raffinose respectively.

This result is consistent with the outcome of the PLS-DA analysis (Tables 5.2 and 5.3). However fewer compounds were identified, and no information about the relationships between the mutants can be extracted; that is, there is no equivalent to the PLS scores plot which depicts the relative positions of groups and observations form one another in some chosen model space.



*Figure 5.32. Number of classification successes vs the number of PLS factors used in the PCA-DA method.*



**Table 5.4. The thirty most significantly different variables in ascending p-value order, identified by ANOVA-n**

| m/z ratio            | p-value (1.0e-011 *) |
|----------------------|----------------------|
| 'M130T <b>681</b> '  | >0.0001              |
| 'M259T <b>1467</b> ' | >0.0001              |
| 'M192T <b>1137</b> ' | 0.0001               |
| 'M266T1137'          | 0.0001               |
| 'M306T1137'          | 0.0001               |
| 'M305T1137'          | 0.0001               |
| 'M318T1137'          | 0.0001               |
| 'M362T1467'          | 0.0001               |
| 'M361T1467'          | 0.0002               |
| 'M265T1137'          | 0.0002               |
| 'M191T1137'          | 0.0002               |
| 'M434T1137'          | 0.0008               |
| 'M433T1137'          | 0.0010               |
| 'M291T1137'          | 0.0019               |
| 'M133T <b>1418</b> ' | 0.0024               |
| 'M147T1418'          | 0.0028               |
| 'M393T1137'          | 0.0035               |
| 'M117T1418'          | 0.0040               |
| 'M75T1418'           | 0.0053               |
| 'M145T1418'          | 0.0065               |
| 'M55T1418'           | 0.0073               |
| 'M73T1418'           | 0.0079               |
| 'M159T1418'          | 0.0112               |
| 'M319T <b>1419</b> ' | 0.0143               |
| 'M130T1418'          | 0.0144               |
| 'M367T1137'          | 0.0170               |
| 'M149T1418'          | 0.0178               |
| 'M97T1418'           | 0.0190               |
| 'M432T1137'          | 0.0242               |
| 'M104T1418'          | 0.0242               |
| 'M148T1418'          | 0.0302               |

Anovan thus has somewhat limited capabilities compared to multivariate methods. However, the method can be useful for comparative purposes, or could also be used as a variable selection method prior to the multivariate methods. For instance, in the whole data set of 1153 variables, only 451 variables passed the Bonferroni critical value  $p < 0.05/1153$ , forming a subset matrix of rank [42x451] that was subsequently used as input for PLS-DA analysis.

### **5.5. Summary**

This work has contributed to establishing relationships between the profiles of different *Arabidopsis* starch mutant genotypes. I have successfully pre-processed GC-MS data using an optimization method to select appropriate parameters for the data type, and shown that the different supervised classification modelling methods (multivariate and/or univariate) yielded similar results, and further, that characterisation using XCMS and/or AMDIS is both practical and fruitful. I have further seen that two PLS2 algorithms, NIPALS and SIMPLS, substantially differ after the fourth component, despite being thought of as nominally the same technique. It was clear that the data analysis is strongly affected by the batch effect in this dataset, and that this effect needs to be taken into account by whatever analysis method is adopted; hence, the unsupervised approach, HCA, did not perform well, and was only able to make very limited statements about the data of biological interest.

**CHAPTER 6:  
CONCLUSION**

## 6 CONCLUSION

This thesis has examined the holistic process involved in metabolomics studies of plant tissues, from data acquisition, through pre-processing and statistical analysis, to interpretation of the results in biological terms. The motivation for the work was specifically to address the implementation of the metabolomics “pipeline” at the John Innes Centre; that is, to handle LC- and GC-MS data acquired in the main from plant tissues. In the past, studies have largely focused on targeted analysis; manual analysis of spectra using proprietary software Chemstation, Agilent Technology; limited use of SIEVE; and statistical analyses mainly involving univariate, two-groups comparisons. However, there is increasingly the requirement to perform untargeted, metabolomics-scale analyses, with a greater range of options for data analysis and interpretation.

I suggested a **practical and functional software pipeline** for MS metabolomics data that comprises four main steps:

- **the pre-processing of raw MS data** using XCMS software
- **the pre-treatment of the data** via various scaling procedures (normalization, centring, variance scaling)
- **statistical analysis** using one of the statistics-oriented, open source programming languages (R, Matlab)
- **the annotation of metabolite signals** using on-line libraries

The key feature of this pipeline is that it is flexible and open-source. This implies that it avoids the disadvantages associated with instrument-specific software which are often expensive and non-transferable between machines. Moreover, all the methods and algorithms involved in the pipeline are transparent, which means they can be checked for correctness, or easily altered to adapt to different experiments/instruments.

I believe that this pipeline can be used extensively for the analysis of metabolomic experiments in the future, and will lead to fruitful results by helping to decode complex biological phenomena. Moreover, considering that the pipeline is flexible and adaptable to different technologies, with the right implementations it can be used

for merging data of different natures and structures. It is anticipated that a robust analysis of metabolomic data will be very important for the integration of metabolomics with other 'omic technologies, such as transcriptomics and proteomics.

All software elements in the pipeline are flexible and open source. Two programming platforms were employed for various different steps. The pre-processing step was conducted using XCMS software in the freely available 'R' environment. Pre-treatment and statistical analyses were conducted using 'R', and the commercial language, Matlab (The Mathworks, Inc). Comparisons and contrasts were made between alternative statistical methods, as well as across different implementations of the same method. Thus, the open source nature of both languages was fully exploited.

Some specific features of various components of the pipeline were investigated in detail, at the level of coding of the algorithms, and revisions and improvements were developed. For example, one element of the initial work was the revision of the default algorithms for PLS in R, which as of July 2009 did not provide cross-validated scores. It was possible to write a revised and updated routine to provide this functionality; eventually this may be uploaded to the 'R' project as a user contribution.

The statistical modelling step involves a choice of multivariate/univariate and supervised/unsupervised methods, with an emphasis on appropriate model validation. Particular attention was given to a commonly encountered chemometric method, Partial Least Squares Discriminant Analysis (PLS-DA). Consideration was given to different variants of the PLS algorithm, and it was shown these can impact quite substantially on the outcome of analyses. However, although methodological and even algorithm differences produced numerically quite different results, I found that the final outcomes of the alternative supervised modelling techniques in terms of biological interpretation were very similar.

Two particular experimental data sets have been examined in detail, both acquired from specimens of *Arabidopsis* wild-type and mutant plants. The first dataset

(HiMet, Chapter 4) of LC-MS data was used to demonstrate some considerations for specific steps of the pipeline, whilst the second dataset (Starch mutant analysis, Chapter 5) comprising GC-MS data was used for a thorough presentation of the pipeline.

In Chapter 4, I demonstrated that PLS-DA can be effectively used for the classification of a set of Arabidopsis mutants, and can make predictions on the identity of mutants with unknown functionalities (SMLines). I used this Chapter to introduce important pre-treatment steps and emphasize the importance of validation steps in the modelling process, which can avoid such phenomena as overfitting.

In Chapter 5, I presented XCMS as the software of choice for data pre-processing. XCMS by default is optimized for LC-MS analysis. A challenge was to thoroughly understand the various pre-processing functions as implemented within XCMS, rather than using the software as a black box, and to identify key parameters that should be optimized for the use of the software for GC-MS analysis. In this work I established non-default parameters for GC-MS analysis, concerning the chromatographic peak width (*fwhm*) and an across-samples grouping parameter (*bw*). In contrast with literature reports (Danielson et al., 2002) which assert that these parameters do not substantially affect the peak extraction process, I found that incorrect parameter settings could reduce the number of compounds identified by up to half.

An important observation regarding the data acquisition was that in all the data examined, experimental effects (batching, machine drift) had a considerable impact on the data. The observed batch effect (day-of-analysis effect) observed in starch mutants analysis (Chapter 5) suggested that a careful instrument operation is essential for the quality of the data. Nevertheless, multivariate analysis was in all cases able to generate models which were able to discriminate between the different groups (genotypes) under study, which indicates that the methods suggested are very powerful for the analysis of 'systematically noisy' data.

The output from the analysis of the starch mutants in Chapter 5 indicated key metabolites responsible for the difference between groups of samples. Some of the

putatively identified compounds were consistent with anticipated differences between sample types, confirming the effectiveness of the statistical approach. Additionally, some less anticipated compounds were identified as key discriminators. These results can be used as a strong indicator of the relationships underlying the particular pathways. However it should be emphasized that the data are taken from a single time point, and that the results from a single experiment can be used only as circumstantial evidence of the underlying relationships. Future experiments could involve a wider range of mutants, as well as measurements from different time points, the use of different extraction methods, and the combinations of different technologies. For example, a wider range of mutations would improve our understanding of the effect of genes on the metabolic phenotype by providing a better coverage of the metabolic effects of mutations. In addition, considering that variations in starch content can be observed throughout the diurnal cycle, investigating the metabolome at different time points during the diurnal cycle (e.g. at the end of the light period) and at different stages of plant growth would facilitate a better understanding of starch metabolism.

In conclusion, the use of metabolomics to decipher complex metabolic processes requires detailed understanding of the system under study, of the measurement technologies and their specific impact on the data produced, and of data handling and statistical techniques suitable for very large datasets. At this moment in time, this is far from an automated process that can reveal hidden patterns of biological interest just by feeding raw metabolomic data; instead, a thorough understanding of each of the steps involved in the pipeline by the researchers working with the data is crucial.

**CHAPTER 7:  
LITERATURE CITED**

## 7 LITERATURE CITED

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**APPENDIX A1:**  
**EXAMPLAR R AND MATLAB CODE**

## A1 Exemplar R and Matlab Code

The Matlab and R environments are conceptually similar but syntactically quite different. Some exemplar scripts that were written in the course of this study are presented here for purpose of illustration. The first of these is an in-house written version of PLS-DA - the NIPALS algorithm (Martens, 2001), which is not a core algorithm in standard Matlab (Matlab provides a routine based on the SIMPLS algorithm, only in separately purchased and/or third party toolboxes). The former is the near-equivalent algorithms with the one provided in R (for the PLS-R component; LDA step is a separate function).

It was noted in the course of this work that this standard R routine does not return cross-validated scores, even when implemented as a cross-validated modelling method. This is something of an oversight since PLS is a potentially overfitting technique. The third piece of code presented here is a revised component for the R tool, which incorporates full score cross-validation.

Note that the following scripts are annotated with comments (Matlab: green colour is used for comments- black and others for the main script; R: red colour is used for comments and black colour for scripts).

### A1.1 Matlab routines

#### A1.1.1 PLS-DA implemented with cross validation

```
% I. PRETREATMENT OF RAW DATA MATRIX (myx):
% MISSING VALUES
% myx contains two variables with large number of missing values
% that are discarded (columns 3 and 16).
myx=myx(:,setdiff([1:26],[3 16]));

% NORMALIZATION
% scaling each row of myx so that the sum of intensities for every
row is
% equal to unity
[n,d]=size(myx);
mysum=sum(myx,2);
Xan=myx./repmat(mysum,1,d);

% CENTERING
% column means are subtracted from each element of myx
myx=myx-(ones(size(myx,1),1)*mean(myx));
```

```

% VARIANCE SCALING
% each element of the meancentered myx is divided by its standard
deviation
myx=myx-(ones(size(myx,1),1) *mean(myx))./(ones(size(myx,1),1) *
std(myx)));

% II. GROUPING VARIABLES:

% TECHNICAL REPLICATES GROUPING
% When a dataset includes technical replicates
% the data should be sorted such as the technical replicates
% are grouped together
[temp1,temp2,temp3]=unique(myreps,'first'); % find unique sample
codes
[i1,i2]=sort(temp2); % arrange samples in ascending order
uniquereps=temp1(i2); % unique replicates in ascending order

% PLS GROUPING VARIABLE - DUMMY VARIABLE myy
% PLS is a supervised method, thus besides the data matrix myx
% a second matrix myy (dummy variable which includes grouping
% information for the several genotypes) is required
myg(strmatch('WT-Col',myy))=1;
myg(strmatch('act1',myy))=2;
myg(strmatch('fad2-1',myy))=3;
myg(strmatch('fae',myy))=4;
myg=myg';
my0=zeros(105,4);
my0(find(myg==1),1)=1;
my0(find(myg==2),2)=1;
my0(find(myg==3),3)=1;
my0(find(myg==4),4)=1;
% myg is a [105X1] vector with the grouping information
% my0 is the transformed myg as to consist only of the
% numbers 0 and 1; is used for computational reasons instead of myg

% III. PARTIAL LEAST SQUARE-DISCRIMINANT ANALYSIS ROUTINE

% Example of a leave-sample-out cross-validated PLSmult routine

ncomp=20; % number of components
allpreds=[]; % loadings matrix
alltestscores=[]; % scores matrix

% dimensionality reduction (see FigureX.XX): myx and myy
% matrices are transformed to the scores matrix alltestscores and
% the loadings allpreds respectively; the original variables will
% be reduced to 20 components

% cross validation: myx is split into a test set (testx) and a
training
% set (trainx); the equivalent testy trainy, and testg traing are
formed;
% the analysis is performed on the training sets and the test sets
are used
% for model validation; multiple rounds of cross-validation are
performed
% using every time one group of technical replicates as training
set;

```

```

% the routine stops when all unique samplecodes have been used as
training test
% exactly once

for j=1:length(uniqereps) % for each unique sample code

    %identify all observations from each samplecode, and extract
into test
    %segments; remainder form the training segment
    idx=strmatch(uniqereps(j),myreps);

    % test segments
    testx=myx(idx,:);
    testy=my0(idx,:);
    testg=myg(idx,:);

    % training segments
    trainx=myx(setdiff([1:length(myreps)],idx),:);
    % chose a scaling method between mean centering and variance
scaling
    % mean centering:
    trainx=trainx-(ones(size(trainx,1),1) *mean(trainx));
    % variance scaling:
    % trainx=trainx-(ones(size(trainx,1),1)
*mean(trainx))./(ones(size(trainx,1),1) * std(trainx));

    trainy=my0(setdiff([1:length(myreps)],idx),:);
    traing=myg(setdiff([1:length(myreps)],idx),:);

    % apply a pls algorithm "plsmult" on the training segment
    % for all components:
    %("plsmult" is an inhouse written routine; the equivalent
function
    % in the Matlab stats toolbox is "plsregress")
    [z,p]=plsmult(trainx,trainy,ncomp);

    %rotate the test segment into the PLS space:
    predg=[];
    for k=1:ncomp
        testz=testx-(ones(size(testx,1),1) *mean(trainx));
        %testz=testx-
(ones(size(testx,1),1) *mean(trainx))./(ones(size(testx,1),1) *std(tra
inx));
        testz=testz*p(:,1:k);
        assigns=discrim(z(:,1:k),traing,testz);
        predg=[predg,assigns];
    end

    % perform discriminant analysis using the in-house "discrim"
function
    % using subset of scores
    % (the equivalent Matlab stats toolbox function is called
"classify")

    allpreds=[allpreds;predg];
    alltestscores=[alltestscores;testz];
end

```

```
% work out the success rate as a function of number of components
used:
```

```
for j=1:size(allpreds,2)
    numcorr(j)=length(find(allpreds(:,j)-myg==0));
end
```

### A1.1.2 PLS – NIPALS algorithm

```
function [T,V,W,P]=plsmult(X,Y,ncomp)
% Usage: [scores,loadings]=plsmult(Xdata,Ydata,ncomp)
% Orthogonalised PLS for SEVERAL y-variable (training set only).
%     T = scores
%     V = loadings
% This routine centres (but does NOT variance-scale) X- and Y-data
% Scores by this method are UNCORRELATED
% Linear rotation matrix ("loadings") is V [=W*inv(P'* W)]
%
% where
% X is the matrix of data
% Y is the matrix encoding group membership
% ncomp is the maximum number of axes to calculate

[n,d]=size(X);
%[Y]=cent(Y);
%[X]=cent(X);
Y=Y-ones(n,1)*mean(Y);
X=X-ones(n,1)*mean(X);

W=zeros(d,ncomp);
P=zeros(d,ncomp);
T=zeros(n,ncomp);

for lp = 1:ncomp

    U1= Y(:,1);
    contnue=1;
    T0= zeros(n,1);

    while (contnue>0)

        W1= (X' * U1) * ((U1' * X * X' * U1)^(-0.5));
        T1 = X * W1;
        Q1 = (Y' * T1)/(T1' * T1);

        %There is a direct compromise between the PRECISION of the
        %scores/loadings and the size of the termination criterion
        value % this step is crucial for the consistency of the results when
        comparing the PLS- NIPLS with the PLS-SIMPLS algorithms mentioned as
        Tolerance in Figure 5.29.
        if (sum((T1-T0).*(T1-T0)) > (0.0000001/n))
            U1= Y * Q1 * (inv(Q1' * Q1));
            T0=T1;
        else
            P1 = (X' * T1)/(T1' * T1);
            contnue=0;
        end
    end
end
```

```

end

T(:,lp)=T1;
W(:,lp)=W1;
P(:,lp)=P1;

X= X - (T1 * P1');
Y= Y - (T1 * Q1');

end

V = W * inv(P' * W);

```

## A1.2 R routines

### A1.2.1 PLS-DA implemented with cross validation

```

# DATA PREPARATION AND PRETREATMENT IN R

getwd()
list.files()
library(pls)

oliveoil<-read.table(file.choose(), header=T, fill=TRUE)
attach(oliveoil)

greece<-oliveoil[1:10,]
italy<-oliveoil[11:27,]
portugal<-oliveoil[28:35,]
spain<-oliveoil[36:60,]

# creating a vector of responses (binary)
y<-cbind(c(rep(1, 10), rep(0,50)), c(rep(0,10), rep(1, 17), rep(0,
33)),
c(rep(0, 27), rep(1,8), rep(0,25)), c(rep(0, 35), rep(1, 25)))

# rownames, colnames
rownames(y)<-c(1:60)
colnames(y)<-c("Greece", "Italy", "Portugal", "Spain")

# creating a test set
ooTest.all<-rbind(greece[1:2, ], italy[1:3, ], portugal[1, ],
spain[1:5, ])
ooTest<-as.matrix(ooTest.all[, -c(1:2)])

# creating a training set
X<-as.matrix(rbind(greece[-(1:2), 3:ncol(greece)], italy[-(1:3),
3:ncol(italy)],
portugal[-1, 3:ncol(portugal) ], spain[-(1:5), 3:ncol(spain) ]))

test.rows<-c(1:2, 11:13, 28, 36:40)

Y<-as.matrix(y[-test.rows,])

```

```

# the training data frame
ooTrain<-data.frame(Y=I(Y), X=I(X))

# running pls
ooTrain.pls<-plsr(Y ~ X, data=ooTrain, validation="LOO")

# change colours
rainbow<-rainbow(4)
cols<- c(rep(rainbow[1], 10), rep(rainbow[2], 17),
         rep(rainbow[3], 8), rep(rainbow[4], 25))
trainCols<-cols[-test.rows]

# mean centering
X.cent<-apply(ooTrain$X, 2, meanCent)

meanCent<-function(x) {
  newX<-x-mean(x)
}

Y.cent<-apply(ooTrain$Y, 2, meanCent)

# centered training data
train.cent<-data.frame(Y=I(Y.cent), X=I(X.cent))

# PLS on centered data
train.c.pls<-plsr(Y~X, data=train.cent, validation="LOO",
method="oscorespls", ncomp=10)
# scores plot
plot(train.c.pls, "scores", comp=1:4, col=trainCols)

# pls on random data
x.random<-matrix(ncol=ncol(X.cent), nrow=nrow(X.cent),
data=rep(rnorm(ncol(X.cent)), nrow(X.cent)))

random.df<-data.frame(Y=I(Y.cent), X=I(x.random))
pls.rand<-plsr(Y~X, data=random.df, ncomp=4, validation="LOO",
method="oscorespls")

# VALIDATION DESIGN

# perform pls on a
ooTrain.pls<-plsr(Y ~ X, ncomp=4, data=ooTrain, method="oscorespls",
validation="LOO")

# ooTrain.pls$validation$scores
# extract scores into matrix
scoresList<-ooTrain.pls$validation$scores
scoreMat<-matrix(nrow=nrow(ooTrain), ncol=ncol(scoresList[[1]]))
rownames(scoreMat)<-rownames(ooTrain)
colnames(scoreMat)<-colnames(scoresList[[1]])
# makes a large matrix bound by rows (the rownames repeat and are
the observation IDs)
allScores<-do.call('rbind', scoresList)

```

```

# go through the original rownames
for (i in 1:nrow(ooTrain)){
  obsID.pattern<-paste("^",rownames(ooTrain)[i],"$", sep="")
# grep the indices from the rownames of the allScores matrix
  all.indices<-grep(obsID.pattern, rownames(allScores), perl=T)
# in the score Matrix, of observation i, store the average of the
scores
  scoreMat[i,<-apply(allScores[all.indices,], 2, mean)
}

# pls on random data
randData<-matrix(nrow=nrow(ooTrain$X), ncol=ncol(ooTrain$X))
randData<-apply(randData, 2, rnorm, ncol(randData))
dimnames(randData)<-dimnames(ooTrain$X)

rand.df<-data.frame(Y=I(Y), X=I(randData))
rand.pls<-plsr(Y~X, ncomp=4, data=rand.df, method="oscorespls",
validation="LOO")

# get the means of the validation scores
rand.scores<-rand.pls$validation$scores
rand.v.scores<-matrix(nrow=nrow(randData),
ncol=ncol(rand.scores[[1]]))

allRandscores<-do.call('rbind', rand.scores)

for (i in 1:nrow(randData)){
  obsID.pattern<-paste("^", rownames(randData)[i], "$", sep="")
  all.ind<-grep(obsID.pattern, rownames(allRandscores), perl=T)
  rand.v.scores[i,<-apply(allRandscores[all.ind,], 2, mean)
}

plot(rand.v.scores, type="p",col=trainCols)
# the algorithm is overfitting, potential pitfall of weak cross-
validation routine

```

### A1.2.2 PLS – A1.1.2 PLS – mvrCv function

```

# Changes to mvrCv function of package pls to ensure that cross
validated scores are returned
(notes from July 2009)
mvrCv<-function (X, Y, ncomp, method = pls.options()$mvralg, scale =
FALSE,
  segments = 10, segment.type = c("random", "consecutive",
  "interleaved"), length.seg, jackknife = FALSE, trace =
FALSE,
  ...)
{
  Y <- as.matrix(Y)
  dnX <- dimnames(X)
  dnY <- dimnames(Y)
  nobj <- dim(X)[1]
  npred <- dim(X)[2]
  nresp <- dim(Y)[2]

```

```

if (!is.logical(scale) || length(scale) != 1)
  stop("'scale' must be 'TRUE' or 'FALSE'")
if (is.list(segments)) {
  if (is.null(attr(segments, "type")))
    attr(segments, "type") <- "user supplied"
}
else {
  if (missing(length.seg)) {
    segments <- cvsegments(nobj, k = segments, type =
segment.type)
  }
  else {
    segments <- cvsegments(nobj, length.seg = length.seg,
type = segment.type)
  }
}
ncomp <- min(ncomp, nobj - max(sapply(segments, length)) -
1)
method <- match.arg(method, c("kernelpls", "widekernelpls",
"simpls", "oscorespls", "svdpc"))
fitFunc <- switch(method, kernelpls = kernelpls.fit,
widekernelpls = widekernelpls.fit,
simpls = simpls.fit, oscorespls = oscorespls.fit, svdpc =
svdpc.fit)
adj <- matrix(0, nrow = nresp, ncol = ncomp)
cvPred <- pred <- array(0, dim = c(nobj, nresp, ncomp))
# scores <- array(0, dim=c((nobj-1), ncomp, length(segments)))
# cvScores <-array(0, dim=c(nobj, ncomp, length(segments)))
cvScores<-list(length=length(segments))
if (jackknife)
  cvCoef <- array(dim = c(npred, nresp, ncomp,
length(segments)))
if (trace)
  cat("Segment: ")
for (n.seg in 1:length(segments)) {
  if (trace)
    cat(n.seg, "\n")
  seg <- segments[[n.seg]]
  Xtrain <- X[-seg, ]
  if (scale) {
    ntrain <- nrow(Xtrain)
    sdtrain <- sqrt(colSums((Xtrain - rep(colMeans(Xtrain),
each = ntrain))^2)/(ntrain - 1))
    if (any(abs(sdtrain) < .Machine$double.eps^0.5))
      warning("Scaling with (near) zero standard
deviation")
    Xtrain <- Xtrain/rep(sdtrain, each = ntrain)
  }
  fit <- fitFunc(Xtrain, Y[-seg, ], ncomp, stripped = FALSE,
...)
  cvScores[[n.seg]]<-I(fit$scores)
  if (jackknife)
    cvCoef[, , , n.seg] <- fit$coefficients

```

```

Xtest <- X
if (scale)
  Xtest <- Xtest/rep(sdtrain, each = nobj)
Xtest <- Xtest - rep(fit$Xmeans, each = nobj)
Ymeansrep <- rep(fit$Ymeans, each = nobj)
for (a in 1:ncomp){
  pred[, , a] <- Xtest %*% fit$coefficients[, , a] +
Ymeansrep
}
cvPred[seg, , ] <- pred[seg, , , drop = FALSE]

#print(seg)
#print(cvPred)
adj <- adj + length(seg) * colSums((pred - c(Y))^2)
}
if (trace)
  cat("\n")
PRESS0 <- apply(Y, 2, var) * nobj^2/(nobj - 1)
PRESS <- colSums((cvPred - c(Y))^2)
objnames <- dnX[[1]]
if (is.null(objnames))
  objnames <- dnY[[1]]
respnames <- dnY[[2]]
nCompnames <- paste(1:ncomp, "comps")
names(PRESS0) <- respnames
dimnames(adj) <- dimnames(PRESS) <- list(respnames, nCompnames)
dimnames(cvPred) <- list(objnames, respnames, nCompnames)
if (jackknife)
  dimnames(cvCoef) <- list(dnX[[2]], respnames, nCompnames,
  paste("Seg", seq.int(along = segments)))
list(method = "CV", pred = cvPred, coefficients = if (jackknife)
cvCoef,
  PRESS0 = PRESS0, PRESS = PRESS, adj = adj/nobj^2, segments =
segments,
  ncomp = ncomp, scores = cvScores)
}

```

**APPENDIX A2:**  
**SUPPLEMENTARY MATERIAL FOR CHAPTER 4**

**Table 4.4**

**Table 4.5**

**Table 4.6**

Table 4.4  
HiMet9 dataset

| Genotype | Sample Code  | Aminoacids  |             |             |             |              |             |             |             |             |             |             |             |             |
|----------|--------------|-------------|-------------|-------------|-------------|--------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
|          |              | A'          | AAA         | C'          | CIT         | D'           | E'          | F'          | G'          | GABA        | H'          | I'          | J'          | K'          |
| Yae2-1'  | 'sampil1170' | 1509.0622   | 63.30443661 | 5.3270504   | 237.5038996 | 2081.63826   | 4283.519368 | 128.6620017 | 505.231742  | 88.23193668 | 133.8898306 | 68.40327441 | 24.38750283 | 78.86202852 |
| Yae2-1'  | 'sampil1185' | 1317.397971 | 34.75073932 | 3.402072417 | 200.4573932 | 2119.278105  | 3494.323246 | 116.1079452 | 418.2750806 | 39.13258001 | 145.6611659 | 63.55248706 | 16.8616744  | 59.41865974 |
| Yae2-1'  | 'sampil1185' | 1224.157097 | 77.25853795 | 2.582379569 | 184.243085  | 1783.785743  | 3996.57424  | 95.05163187 | 375.9880147 | 359.6597122 | 106.3927155 | 61.08566554 | 16.69347295 | 63.15119489 |
| Yae2-1'  | 'sampil1185' | 1178.633981 | 52.62635356 | 7.78088637  | 274.25165   | 1974.803217  | 3112.561742 | 92.7745386  | 358.7326256 | 60.00351763 | 111.5763286 | 56.57334133 | 21.35546033 | 74.01598286 |
| Yae2-1'  | 'sampil1200' | 1172.197432 | 56.1179359  | 3.712088857 | 218.0042303 | 1507.406902  | 3067.324535 | 87.14958461 | 313.822392  | 170.2174076 | 105.085352  | 61.89947009 | 20.49672539 | 66.40027048 |
| Yae2-1'  | 'sampil1200' | 1597.798603 | 54.27713256 | 6.010486991 | 247.3502257 | 2220.648497  | 4239.910953 | 165.717215  | 547.5380477 | 65.147894   | 166.1993117 | 73.45872929 | 21.0448544  | 83.84622742 |
| Yae2-1'  | 'sampil1200' | 1300.564768 | 52.1320039  | 5.325386304 | 189.6961293 | 2270.423378  | 3618.468121 | 99.72498994 | 383.5338621 | 391.1580294 | 131.914845  | 63.61990974 | 19.76474778 | 82.55827867 |
| Yae2-1'  | 'sampil1215' | 1150.629994 | 34.16905147 | 1.479894509 | 152.848657  | 1260.4679    | 3196.073628 | 91.3380914  | 254.4947189 | 49.67278348 | 126.5471124 | 59.07601025 | 21.84183667 | 66.55729239 |
| Yae2-1'  | 'sampil1215' | 1141.293075 | 39.83253155 | 2.320472988 | 162.8498582 | 1767.517048  | 2844.468348 | 109.0151345 | 238.945618  | 177.9221043 | 102.6650189 | 53.56814294 | 19.53679508 | 60.32520239 |
| Yae2-1'  | 'sampil1215' | 1452.015992 | 45.79191121 | 5.413227938 | 232.428702  | 1985.413469  | 3421.365588 | 144.7060439 | 437.0380768 | 317.9149634 | 167.2805461 | 106.7590501 | 18.88568276 | 90.45039981 |
| Yae1'    | 'sampil1098' | 944.822972  | 35.24432883 | 1.245564976 | 107.8751767 | 1638.630553  | 3319.352197 | 98.92633835 | 184.7455663 | 51.26803177 | 127.641872  | 44.64322151 | 16.90486127 | 50.16448481 |
| Yae1'    | 'sampil1098' | 1126.238683 | 56.14678993 | 2.970519522 | 201.7085635 | 2084.454723  | 4495.043609 | 102.23744   | 248.978938  | 61.60291183 | 131.2200808 | 64.07455947 | 23.83633844 | 71.87599559 |
| Yae1'    | 'sampil1098' | 1027.952505 | 49.57827094 | 4.519314578 | 134.5727772 | 2078.865278  | 3952.618374 | 80.68604913 | 205.0881118 | 54.81284997 | 97.89540024 | 43.22638652 | 25.26634133 | 42.58708204 |
| Yae1'    | 'sampil1111' | 980.163873  | 28.86407593 | 3.495153884 | 144.1607509 | 1413.308314  | 2573.31798  | 99.90299527 | 238.2227806 | 45.63814081 | 114.5016266 | 48.75881975 | 24.79554935 | 40.14845652 |
| Yae1'    | 'sampil1111' | 1095.427867 | 59.86194581 | 2.477900579 | 141.8549044 | 2012.4253564 | 4349.197339 | 82.0402158  | 262.1832666 | 137.6951368 | 54.21603282 | 19.63422514 | 77.48344415 |             |
| Yae1'    | 'sampil1111' | 763.5292118 | 47.19480637 | 1.875031785 | 113.7976386 | 1593.783764  | 3464.499075 | 69.2594527  | 190.255892  | 49.47915682 | 85.9131327  | 32.45849362 | 11.3847997  | 41.89877011 |
| Yae1'    | 'sampil1126' | 1169.671442 | 39.74369694 | 3.501706923 | 157.0047024 | 1360.237724  | 3419.236147 | 69.93196152 | 181.0916519 | 47.63819559 | 125.2719816 | 49.68280404 | 23.83053118 | 46.01928002 |
| Yae1'    | 'sampil1126' | 1187.061123 | 38.68170181 | 3.954363527 | 234.8740201 | 1894.078792  | 4220.951812 | 95.70786958 | 210.0137681 | 45.24450063 | 108.4239766 | 47.7315246  | 27.90124947 | 47.7445741  |
| Yae1'    | 'sampil1126' | 1165.396941 | 37.91002786 | 2.680662845 | 186.3233113 | 1554.251154  | 3038.175752 | 93.22885666 | 181.8177642 | 116.1340474 | 51.47160347 | 19.87827872 | 57.44005651 |             |
| Yae1'    | 'sampil1141' | 1078.944121 | 48.12793211 | 4.948714925 | 186.0593906 | 1894.99261   | 4161.300521 | 120.9994684 | 177.3913817 | 79.23218739 | 112.526037  | 46.98517555 | 20.47415121 | 50.06167075 |
| Yae1'    | 'sampil1141' | 1109.297156 | 34.97582883 | 2.683080197 | 264.4880982 | 1749.450394  | 4443.610155 | 71.92086482 | 163.6645288 | 89.35764271 | 91.76050937 | 46.81173088 | 18.07155689 | 57.9469806  |
| Yae1'    | 'sampil1141' | 902.1116593 | 33.1883376  | 3.590712262 | 81.62199942 | 1231.50689   | 1946.094034 | 77.48531478 | 182.8970044 | 88.41205724 | 95.13921492 | 45.68572419 | 20.29345492 | 38.91173346 |
| Yae1'    | 'sampil1156' | 1055.509941 | 39.40201163 | 7.895397082 | 168.8874479 | 1745.456399  | 3688.921575 | 96.8601887  | 232.786354  | 50.61859892 | 107.373971  | 57.92144477 | 22.73847047 | 53.45872438 |
| Yae1'    | 'sampil1156' | 885.238661  | 31.70039284 | 1.541839274 | 117.839076  | 1473.826203  | 3114.042795 | 70.68184314 | 203.3210545 | 34.91673653 | 114.5976446 | 49.84109665 | 21.51924439 | 48.75013889 |
| Yae1'    | 'sampil1156' | 941.9778665 | 42.05841673 | 1.73084308  | 226.7646549 | 1937.198301  | 3403.784996 | 95.99167306 | 264.3826495 | 88.112655   | 115.4533458 | 55.23730694 | 11.32708883 | 54.14428898 |
| Yae1'    | 'sampil1171' | 1231.18043  | 39.28309784 | 3.106711449 | 292.0639442 | 2074.785061  | 4801.80012  | 108.1844032 | 371.4192286 | 108.0826709 | 159.2346999 | 61.20396614 | 22.19144786 | 67.90816428 |
| Yae1'    | 'sampil1171' | 1098.469502 | 53.35063798 | 4.02212083  | 283.1485697 | 1712.839279  | 3210.438026 | 107.249684  | 338.8107625 | 70.4391068  | 132.4300788 | 53.43117869 | 14.10090195 | 62.3940895  |
| Yae1'    | 'sampil1171' | 1066.873554 | 29.98905765 | 2.228740076 | 251.6869772 | 1742.302327  | 3260.201332 | 82.94510185 | 290.4289728 | 61.83226566 | 104.8050634 | 51.67061731 | 24.38807033 | 48.59652638 |
| Yae1'    | 'sampil1186' | 1042.984039 | 47.8075586  | 7.869335153 | 149.9000067 | 1726.741953  | 3523.395603 | 77.90980016 | 202.9840403 | 38.46933398 | 114.4490592 | 41.33803384 | 23.5733705  | 55.5798852  |
| Yae1'    | 'sampil1186' | 1179.238275 | 58.07801551 | 2.439946686 | 178.4589761 | 2218.426788  | 4359.65337  | 88.99021204 | 245.5763775 | 298.1029182 | 116.428776  | 45.45713957 | 20.36634321 | 68.31153743 |
| Yae1'    | 'sampil1186' | 1155.565816 | 60.99351223 | 2.7911467   | 208.3016485 | 1509.583761  | 3615.865374 | 92.7413403  | 213.4106828 | 55.7797602  | 115.9895193 | 55.51108775 | 18.61602719 | 62.3940972  |
| Yae1'    | 'sampil1201' | 1141.736547 | 45.38472529 | 6.187547459 | 145.5521295 | 1638.848724  | 3670.735053 | 100.5297436 | 246.042147  | 43.2595949  | 114.5744307 | 57.517913   | 25.74122719 | 54.12280352 |
| Yae1'    | 'sampil1201' | 1097.919284 | 64.1580674  | 4.387849088 | 223.955764  | 1840.689396  | 3771.584123 | 107.8143767 | 223.7654305 | 60.41216458 | 153.8613211 | 52.6358864  | 22.55920008 | 50.46750861 |
| Yae1'    | 'sampil1201' | 1008.022427 | 32.85927092 | 3.242793965 | 162.0790398 | 1392.846559  | 2754.805447 | 84.64337833 | 205.9582362 | 114.3312428 | 93.57250532 | 48.74854901 | 16.85818501 | 50.07412149 |
| Yae1'    | 'sampil1216' | 1003.857149 | 42.3079688  | 5.489795003 | 137.8723679 | 1859.363417  | 4580.505474 | 80.8339951  | 149.7563052 | 110.917249  | 120.1995588 | 54.28548988 | 20.07280189 | 60.90681528 |
| Yae1'    | 'sampil1216' | 1070.277016 | 39.57703562 | 3.166138719 | 157.1176429 | 1672.013414  | 2917.859776 | 69.26445853 | 132.6043736 | 121.474638  | 110.8905515 | 57.18223958 | 23.90477844 | 46.52471758 |
| Yae1'    | 'sampil1216' | 1122.466895 | 40.91186356 | 4.00343851  | 144.2520892 | 1927.407714  | 4212.121945 | 78.87194913 | 175.5562015 | 156.7406791 | 115.3416376 | 62.78240719 | 25.13985144 | 71.00416346 |
| WT-CoI   | 'sampil1099' | 1342.859029 | 65.6679109  | 6.393705747 | 166.8936676 | 2790.7953    | 4879.629424 | 115.3737008 | 236.7798008 | 496.5713678 | 122.5061903 | 77.70443558 | 33.10576385 | 82.83955665 |
| WT-CoI   | 'sampil1099' | 1323.540991 | 59.4035661  | 5.143158035 | 212.4790618 | 2193.832167  | 6877.624095 | 106.531796  | 231.856388  | 115.9465339 | 117.889093  | 72.44039438 | 17.63629641 | 79.70264354 |
| WT-CoI   | 'sampil1099' | 1191.307289 | 45.57824139 | 2.64292395  | 233.7680006 | 2141.921937  | 4314.708743 | 121.4268364 | 351.1352375 | 104.387721  | 127.0699948 | 51.14457809 | 19.80548894 | 76.67330708 |
| WT-CoI   | 'sampil1112' | 1095.726035 | 43.91963592 | 1.919065414 | 190.5583597 | 1737.278705  | 3723.575967 | 81.31615879 | 285.840986  | 72.25819968 | 105.038806  | 53.57972096 | 23.70355474 | 60.57625413 |
| WT-CoI   | 'sampil1112' | 1138.629436 | 32.43909925 | 2.480485718 | 128.2855912 | 1821.351698  | 2951.36801  | 76.9988781  | 351.6898581 | 80.3834267  | 131.2684048 | 55.47253286 | 22.61962398 | 53.43676669 |
| WT-CoI   | 'sampil1112' | 1119.28708  | 32.8077909  | 3.855620476 | 214.6967113 | 1713.767705  | 3816.187199 | 85.18048167 | 283.507951  | 95.6252111  | 110.3657041 | 57.32498946 | 23.19025355 | 68.69836796 |
| WT-CoI   | 'sampil1127' | 1249.108571 | 43.75153342 | 2.227701401 | 248.1702341 | 1902.086701  | 3974.424914 | 91.49165236 | 207.3986176 | 73.99153441 | 105.8015008 | 59.06183037 | 24.19254886 | 64.26351592 |
| WT-CoI   | 'sampil1127' | 1039.094793 | 51.37571623 | 5.418086233 | 186.6895783 | 1795.196699  | 4646.987254 | 62.50871816 | 193.5955268 | 79.22968473 | 107.1077138 | 58.13406403 | 14.7481731  | 52.06219286 |
| WT-CoI   | 'sampil1127' | 1014.596499 | 30.55279603 | 2.735640446 | 114.4141532 | 1249.37966   | 2388.297318 | 79.01825136 | 173.5697287 | 55.00073951 | 75.29458931 | 52.19253918 | 13.01732216 | 40.41780905 |
| WT-CoI   | 'sampil1142' | 836.278875  | 21.82347444 | 5.239482893 | 144.087446  | 1777.827267  | 3862.49969  | 60.59704962 | 169.7216953 | 37.28974874 | 65.19852572 | 36.7068721  | 14.34984229 | 39.53908462 |
| WT-CoI   | 'sampil1142' | 999.707386  | 45.69900229 | 3.214956856 | 162.1413289 | 1689.519286  | 3788.21281  | 94.98295145 | 215.8896646 | 41.13373905 | 91.65508944 | 48.81647617 | 22.02010874 | 63.18015156 |
| WT-CoI   | 'sampil1142' | 877.993977  | 41.5903942  | 3.78494728  | 204.4602939 | 1907.629827  | 3496.655775 | 70.69420034 | 221.8841594 | 41.91432189 | 78.51873887 | 42.04085806 | 26.4264287  | 43.05256277 |
| WT-CoI   | 'sampil1157' | 995.1754747 | 28.98923243 | 4.062310347 |             |              |             |             |             |             |             |             |             |             |

Table 4.4 (continued)  
HiMet9 dataset

| Genotype | Sample Code | Aminoacids  |             |             |              |             |             |             |             |             |             |             |             |             |     |     |  |
|----------|-------------|-------------|-------------|-------------|--------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-----|-----|--|
|          |             | 'A'         | 'AAA'       | 'C'         | 'DT'         | 'D'         | 'E'         | 'F'         | 'G'         | 'GABA'      | 'H'         | 'I'         | 'J'         | 'K'         | 'L' | 'M' |  |
| 'WT-Col' | 'samp11202' | 919.4327108 | 50.668246   | 2.022411286 | 102.7034449  | 1649.97764  | 3146.585851 | 68.96561825 | 239.7852546 | 70.88247995 | 105.7935301 | 42.20635766 | 22.76880425 | 47.0027225  |     |     |  |
| 'WT-Col' | 'samp11202' | 1232.753877 | 44.47871608 | 2.289011328 | 170.0878708  | 2135.836118 | 4485.157987 | 82.34485764 | 313.2705247 | 82.09470031 | 138.439664  | 56.58214986 | 21.50578536 | 56.5880055  |     |     |  |
| 'WT-Col' | 'samp11217' | 1006.566786 | 20.6945386  | 6.530069305 | 160.0414624  | 1636.513019 | 3493.974602 | 88.7029247  | 159.6563571 | 110.1948378 | 100.135339  | 64.08410978 | 15.03293001 | 61.2473949  |     |     |  |
| 'WT-Col' | 'samp11217' | 871.3684262 | 42.09598415 | 2.391794565 | 226.3898415  | 1205.040556 | 3169.48273  | 72.83712014 | 155.1322477 | 57.2685677  | 77.00401359 | 42.22634379 | 15.90184645 | 49.0162556  |     |     |  |
| 'fad2-1' | 'samp11097' | 1172.634374 | 36.19416189 | 3.829122386 | 82.0649127   | 1392.588684 | 3605.81363  | 87.69716483 | 228.9105507 | 87.45713728 | 117.4614121 | 50.99599169 | 35.43711029 | 64.24029361 |     |     |  |
| 'fad2-1' | 'samp11097' | 1250.98016  | 43.10383932 | 3.506142032 | 151.849199   | 1767.786773 | 2634.92781  | 81.76882145 | 237.4040278 | 52.08803798 | 160.534052  | 56.42021004 | 26.90787119 | 57.26850419 |     |     |  |
| 'fad2-1' | 'samp11097' | 1029.535342 | 63.75939868 | 2.530871393 | 115.244666   | 1953.891126 | 3159.909893 | 68.29880236 | 202.9572065 | 397.6697213 | 112.1713076 | 46.21961925 | 15.55690841 | 63.40651718 |     |     |  |
| 'fad2-1' | 'samp1125'  | 1166.498078 | 46.29023305 | 5.505788598 | 235.7459879  | 1804.973848 | 4609.894224 | 78.28785968 | 308.5754125 | 43.43348938 | 126.2627657 | 57.67251652 | 19.39352608 | 67.18879802 |     |     |  |
| 'fad2-1' | 'samp1125'  | 1489.441998 | 57.86323478 | 5.778543337 | 283.103662   | 2040.03946  | 4034.681848 | 118.0292969 | 606.8758302 | 65.9632465  | 183.705348  | 67.17897914 | 20.98255875 | 84.58666088 |     |     |  |
| 'fad2-1' | 'samp1125'  | 1234.61968  | 38.02307279 | 5.947076962 | 291.7286602  | 1987.155241 | 3144.498147 | 78.03879496 | 403.4364067 | 111.722191  | 122.6273117 | 63.70324979 | 23.4011589  | 54.10301692 |     |     |  |
| 'fad2-1' | 'samp11140' | 1376.0254   | 32.92723658 | 4.43116835  | 196.4862926  | 2021.270955 | 3401.253647 | 66.40203379 | 299.5319033 | 438.4116979 | 166.3870022 | 57.30982859 | 29.69640246 | 81.03495164 |     |     |  |
| 'fad2-1' | 'samp11140' | 1668.392743 | 48.42000155 | 5.299359854 | 320.7906897  | 2834.326977 | 4754.630201 | 92.5305712  | 476.8298009 | 620.3468665 | 204.5080153 | 78.73199969 | 34.78523488 | 94.93146237 |     |     |  |
| 'fad2-1' | 'samp11140' | 1580.954783 | 42.58113639 | 5.968485948 | 215.7208406  | 2654.060718 | 4010.55996  | 126.7543343 | 494.8357683 | 770.0440299 | 225.5110377 | 72.20318437 | 23.12828021 | 104.7545563 |     |     |  |
| 'fad2-1' | 'samp11155' | 1162.603872 | 40.74295584 | 2.548904945 | 164.8638663  | 1483.04354  | 3645.65033  | 108.6428586 | 258.1220174 | 47.9515281  | 106.0037601 | 67.2729248  | 25.94077051 | 68.62945454 |     |     |  |
| 'fad2-1' | 'samp11155' | 1089.389923 | 31.66430399 | 3.17189107  | 161.4879406  | 1343.519873 | 2229.784135 | 88.49388726 | 229.7089321 | 29.68157183 | 100.6637654 | 55.39304729 | 18.7927333  | 44.87368407 |     |     |  |
| 'fad2-1' | 'samp11155' | 1174.629602 | 43.78189151 | 5.678992085 | 156.4890390  | 1983.780066 | 4140.037767 | 101.1862246 | 299.8393243 | 44.26362429 | 123.434687  | 59.33362439 | 28.1865349  | 63.68910336 |     |     |  |
| 'fad2-1' | 'samp11170' | 1227.37555  | 53.01187631 | 4.684217523 | 205.9387697  | 1861.370016 | 3869.901052 | 109.8223589 | 376.9019479 | 55.410933   | 113.8790078 | 53.42877683 | 15.56160517 | 55.66586689 |     |     |  |
| 'WT-Col' | 'samp11217' | 1172.752636 | 43.17948638 | 2.410541992 | 189.105129   | 1634.732222 | 4204.087851 | 81.17741597 | 203.0013312 | 69.35589441 | 105.3240292 | 61.62297097 | 18.73490338 | 67.82102808 |     |     |  |
| 'fad2-1' | 'samp11170' | 1227.131127 | 41.92278714 | 5.816362979 | 167.8616811  | 1258.82087  | 2319.130222 | 89.38738549 | 338.7684059 | 38.27472278 | 101.4554693 | 56.17003255 | 23.05454032 | 47.21571843 |     |     |  |
| 'act1'   | 'samp11096' | 997.1447982 | 37.89680048 | 3.671520367 | 119.381423   | 2068.786543 | 5578.022356 | 105.2135012 | 189.2290271 | 121.5484433 | 95.18434078 | 56.03769113 | 23.46876964 | 51.3578079  |     |     |  |
| 'act1'   | 'samp11096' | 1111.874384 | 40.66023953 | 2.891321226 | 127.5938726  | 1872.302591 | 4531.148324 | 99.81399534 | 206.2745885 | 105.5565106 | 89.95094973 | 56.87675081 | 16.33774529 | 47.40311495 |     |     |  |
| 'act1'   | 'samp11096' | 989.0095834 | 34.17076491 | 3.14596236  | 90.88465106  | 1894.484502 | 4860.40038  | 90.90729779 | 192.802911  | 104.5131839 | 96.04029413 | 48.85025337 | 20.11985551 | 47.3999108  |     |     |  |
| 'act1'   | 'samp11110' | 1106.449709 | 46.43564334 | 3.746452171 | 93.06981791  | 1749.964578 | 3439.836718 | 96.24667483 | 303.3498101 | 78.17645226 | 99.06547433 | 65.62484767 | 25.33152758 | 48.94371177 |     |     |  |
| 'act1'   | 'samp11110' | 873.3667814 | 40.57321108 | 2.252808171 | 122.2033581  | 1358.11109  | 3644.680108 | 71.88600276 | 241.1642223 | 54.90261278 | 96.33004261 | 39.48773938 | 17.29880199 | 43.79374926 |     |     |  |
| 'act1'   | 'samp11110' | 1221.509495 | 65.31741212 | 2.524806759 | 126.9036685  | 2048.368104 | 5180.633575 | 99.58797641 | 272.1596272 | 115.7918625 | 130.8160672 | 55.04763407 | 19.58898663 | 57.95056296 |     |     |  |
| 'act1'   | 'samp11124' | 1230.869501 | 48.10107503 | 3.748830561 | 137.3148426  | 2169.582505 | 3902.295164 | 122.5623119 | 355.1612035 | 97.55159504 | 126.1697382 | 60.72389935 | 17.4746605  | 59.3596206  |     |     |  |
| 'act1'   | 'samp11124' | 1216.583204 | 25.56157799 | 1.545979352 | 146.2286286  | 2248.123362 | 3827.292016 | 92.0325205  | 268.4570298 | 151.320408  | 103.9228844 | 56.03809581 | 26.4164135  | 47.03870966 |     |     |  |
| 'act1'   | 'samp11124' | 997.3205147 | 46.9999544  | 4.744993134 | 86.952864548 | 1473.300488 | 3867.900312 | 91.7329772  | 221.6869281 | 112.2373897 | 91.52336854 | 47.53475136 | 33.04724207 | 46.41195992 |     |     |  |
| 'act1'   | 'samp11139' | 1138.374677 | 66.93886193 | 4.548879928 | 157.4698496  | 2012.99313  | 5083.122945 | 93.41282448 | 323.2290998 | 74.00373599 | 103.840799  | 62.52573809 | 27.15818526 | 64.42203846 |     |     |  |
| 'act1'   | 'samp11139' | 1118.753223 | 48.06856619 | 7.374204692 | 170.6343889  | 1985.925341 | 3921.342369 | 109.7015975 | 267.9501854 | 55.77134193 | 107.9380541 | 59.62611363 | 31.06911425 | 59.03920222 |     |     |  |
| 'act1'   | 'samp11139' | 1213.708839 | 45.50809774 | 2.897782227 | 180.4694967  | 1747.239596 | 3973.089693 | 109.7809332 | 299.6398009 | 117.4809455 | 101.3333964 | 67.63669368 | 25.35582875 | 60.4661488  |     |     |  |
| 'act1'   | 'samp11154' | 1045.801735 | 42.50088422 | 6.328845802 | 137.0184165  | 1926.768549 | 4521.609386 | 95.09368741 | 293.7734961 | 94.47813261 | 95.36993774 | 59.0935477  | 18.12888687 | 61.842303   |     |     |  |
| 'act1'   | 'samp11154' | 1101.8766   | 51.17755436 | 4.782267958 | 161.1535915  | 1916.618905 | 4847.630271 | 92.58151789 | 250.7779628 | 66.45820192 | 102.0010585 | 62.23728887 | 14.32007544 | 56.15836054 |     |     |  |
| 'act1'   | 'samp11154' | 1010.922732 | 35.4507471  | 2.970189437 | 125.4960351  | 1778.490145 | 4799.816901 | 89.59641044 | 186.508675  | 72.36140558 | 97.03061918 | 57.2041407  | 18.94289491 | 61.19242967 |     |     |  |
| 'act1'   | 'samp11169' | 1232.576852 | 56.19602379 | 3.102693518 | 144.3790274  | 1978.18496  | 4700.646822 | 110.5984471 | 238.9613319 | 88.82773964 | 113.2528695 | 46.24505894 | 18.7691346  | 55.8813961  |     |     |  |
| 'act1'   | 'samp11169' | 1029.821397 | 33.72216682 | 1.269590744 | 125.6720653  | 1566.593313 | 3097.571782 | 81.69260238 | 210.6397934 | 161.6639396 | 76.55530913 | 43.3706186  | 24.06821835 | 46.00034828 |     |     |  |
| 'act1'   | 'samp11169' | 1240.392806 | 31.26605449 | 1.932025636 | 152.9236784  | 2290.314602 | 4914.686085 | 123.0808939 | 263.9752841 | 40.08775843 | 106.4520438 | 66.3233104  | 29.60935508 | 60.62823199 |     |     |  |
| 'act1'   | 'samp11184' | 1278.089448 | 41.87765059 | 7.950361894 | 258.309622   | 2864.760132 | 5183.951842 | 111.8443934 | 399.6974807 | 69.73566972 | 130.5022245 | 52.43747298 | 29.5262021  | 51.69157949 |     |     |  |
| 'act1'   | 'samp11184' | 1037.824469 | 21.41013672 | 1.111700391 | 194.2626037  | 2018.568704 | 3776.436751 | 100.415907  | 283.7986045 | 113.9800593 | 72.40447357 | 53.26543019 | 26.77217393 | 40.58683304 |     |     |  |
| 'act1'   | 'samp11184' | 1491.749691 | 71.22092192 | 3.574098113 | 235.605572   | 2672.327324 | 6063.858788 | 141.0547397 | 477.3266516 | 101.9098639 | 129.0269557 | 63.77746707 | 25.71240354 | 63.40160036 |     |     |  |
| 'act1'   | 'samp11199' | 1041.666741 | 39.73506883 | 3.462362813 | 104.3162263  | 1658.795932 | 4151.078866 | 98.89793431 | 211.2626785 | 84.86471767 | 92.72613611 | 56.69189101 | 19.37615299 | 68.83607333 |     |     |  |
| 'act1'   | 'samp11199' | 865.4166429 | 23.9442894  | 1.772100274 | 130.8428208  | 1367.40567  | 3403.50305  | 92.96682048 | 182.8716366 | 63.37988092 | 82.01295131 | 59.09693898 | 14.96697779 | 62.05277418 |     |     |  |
| 'act1'   | 'samp11199' | 1109.414991 | 37.66634674 | 4.129726921 | 106.8764736  | 1945.460269 | 4432.881965 | 127.685114  | 269.0856873 | 86.41498035 | 93.61833668 | 60.1951563  | 33.1763023  | 81.50240137 |     |     |  |
| 'act1'   | 'samp11214' | 1251.612655 | 46.10883391 | 8.598997884 | 133.5107026  | 2239.163553 | 4579.720836 | 96.25102421 | 244.6989853 | 48.70163271 | 124.8202154 | 55.50751717 | 18.92237044 | 46.44415376 |     |     |  |
| 'act1'   | 'samp11214' | 1188.680618 | 44.89618311 | 4.013176849 | 112.9656118  | 1995.263828 | 4308.473992 | 95.24444076 | 217.7959677 | 98.0535247  | 108.1448489 | 59.16196384 | 23.40227511 | 60.19315884 |     |     |  |
| 'act1'   | 'samp11214' | 1004.224004 | 50.28392801 | 2.728283696 | 149.9233164  | 1898.860189 | 4642.479493 | 73.46799725 | 226.460218  | 79.00466853 | 92.26971862 | 48.48759336 | 20.77898088 | 53.84945622 |     |     |  |

Table 4.4 (continued)  
HiMet9 dataset

| Genotype | Sample Code | L           | N           | ORV         | P           | Q            | R           | S           | T           | V           | W           | Y           |
|----------|-------------|-------------|-------------|-------------|-------------|--------------|-------------|-------------|-------------|-------------|-------------|-------------|
| 'fad2-1' | 'sampp1170' | 70.75006504 | 1194.826144 | 10.90715478 | 358.2516234 | 8319.662185  | 348.7000995 | 2040.491466 | 1383.56094  | 247.4404612 | 37.55089923 | 34.09107276 |
| 'fad2-1' | 'sampp1185' | 64.3480548  | 1097.32851  | 8.46994875  | 325.192298  | 7757.175349  | 574.9213169 | 2064.57826  | 1250.401862 | 196.1929545 | 27.66639588 | 29.68951742 |
| 'fad2-1' | 'sampp1185' | 60.81308012 | 956.9226484 | 9.312087782 | 334.4531139 | 6150.8927788 | 214.1246664 | 1762.328852 | 993.1736519 | 176.920822  | 24.18830467 | 33.13394162 |
| 'fad2-1' | 'sampp1185' | 50.32111081 | 1005.520728 | 7.25941435  | 452.7527259 | 6721.231374  | 265.2697134 | 1861.030917 | 1075.578259 | 192.4641391 | 31.17134271 | 38.98207904 |
| 'fad2-1' | 'sampp1200' | 63.76806884 | 1022.471614 | 7.012698908 | 288.7792373 | 6244.117313  | 286.6688027 | 1406.361986 | 945.2961792 | 174.1516109 | 29.18840677 | 34.06334679 |
| 'fad2-1' | 'sampp1200' | 81.17287243 | 1303.954257 | 10.48953882 | 377.0247664 | 8025.892155  | 352.1170088 | 2110.2877   | 1405.613283 | 266.0961726 | 35.03083155 | 45.15611825 |
| 'fad2-1' | 'sampp1200' | 73.33265175 | 1159.12201  | 12.21512311 | 342.8944547 | 7061.809635  | 247.274275  | 1488.420527 | 1138.834242 | 194.5629964 | 42.36016441 | 39.0101     |
| 'fad2-1' | 'sampp1215' | 73.08149707 | 964.2385996 | 5.430093511 | 293.6271778 | 5071.459226  | 287.651057  | 1586.212193 | 1005.623196 | 185.5516256 | 30.7675177  | 34.26189955 |
| 'fad2-1' | 'sampp1215' | 58.75802517 | 850.5339947 | 8.886078899 | 235.8335626 | 4579.248272  | 290.4625611 | 1452.084638 | 919.4247023 | 163.9504079 | 30.45818023 | 25.93677836 |
| 'fad2-1' | 'sampp1215' | 116.1662588 | 1186.374625 | 12.37338525 | 379.7802051 | 6902.678298  | 352.9007621 | 1764.542013 | 1228.598669 | 276.6630604 | 53.67667709 | 41.12504906 |
| 'fae1'   | 'sampp1098' | 49.34202544 | 891.6587691 | 7.214085851 | 555.6368642 | 5588.880682  | 134.2839696 | 1367.524476 | 1094.453003 | 156.2629816 | 28.11679052 | 37.29246083 |
| 'fae1'   | 'sampp1098' | 55.29207256 | 1240.069015 | 5.896009351 | 823.5605338 | 8481.882981  | 158.8763004 | 1551.093347 | 1214.424438 | 213.5787964 | 29.951252   | 18.87967642 |
| 'fae1'   | 'sampp1098' | 44.97881045 | 855.9867857 | 10.53820592 | 510.8647195 | 6312.080062  | 95.19488132 | 1219.695191 | 939.2777217 | 158.635777  | 24.10503834 | 20.81275147 |
| 'fae1'   | 'sampp1111' | 52.42456943 | 902.3729335 | 4.926397397 | 537.262599  | 5952.890329  | 179.405285  | 1243.79293  | 943.7770282 | 186.7076272 | 30.22248813 | 20.20622906 |
| 'fae1'   | 'sampp1111' | 60.74176223 | 1114.34827  | 10.25769055 | 535.1698282 | 8036.54848   | 161.3176186 | 1479.172324 | 1101.862819 | 207.3615269 | 21.21420453 | 20.85113762 |
| 'fae1'   | 'sampp1111' | 41.30355313 | 755.2026719 | 9.056700563 | 395.1499223 | 5111.807876  | 71.99923112 | 1135.491874 | 751.9934816 | 154.591833  | 18.71295328 | 18.09737087 |
| 'fae1'   | 'sampp1126' | 60.98262143 | 980.7339564 | 6.407768249 | 556.2822631 | 5596.578034  | 212.7381258 | 1064.4123   | 906.4364607 | 196.1708615 | 27.73465122 | 30.1353947  |
| 'fae1'   | 'sampp1126' | 48.18261475 | 1089.689954 | 11.86430258 | 463.2568389 | 7130.435021  | 122.7393887 | 1257.257937 | 1030.802982 | 194.3633392 | 31.25136359 | 26.18828673 |
| 'fae1'   | 'sampp1126' | 50.91625277 | 1114.460645 | 7.111815965 | 535.3268384 | 5793.812431  | 141.8041314 | 1164.64704  | 997.3931066 | 169.8316271 | 34.17341263 | 22.04589722 |
| 'fae1'   | 'sampp1141' | 55.66494162 | 1009.782155 | 7.741491143 | 525.9779465 | 6145.871648  | 125.8693405 | 1478.727048 | 1168.737372 | 180.8931781 | 28.2036728  | 41.8552992  |
| 'fae1'   | 'sampp1141' | 55.06386211 | 1030.04178  | 16.22152538 | 428.2356967 | 6192.308065  | 155.9699944 | 1457.432766 | 1126.265683 | 208.8947579 | 21.41365554 | 20.24960284 |
| 'fae1'   | 'sampp1141' | 49.94117552 | 822.9350785 | 4.365290634 | 555.1469473 | 4500.71894   | 87.01592724 | 1046.259699 | 846.664812  | 144.5268924 | 24.5381441  | 18.25454522 |
| 'fae1'   | 'sampp1156' | 61.37521509 | 924.6717546 | 7.627476736 | 576.9254473 | 6638.841434  | 92.07524699 | 1747.893207 | 1129.392747 | 180.5874929 | 32.88808015 | 29.21292969 |
| 'fae1'   | 'sampp1156' | 53.34148085 | 809.1492736 | 3.761191911 | 427.7538161 | 5874.991201  | 115.2991206 | 1297.352188 | 978.271877  | 153.3526551 | 24.05202937 | 27.17571744 |
| 'fae1'   | 'sampp1156' | 63.35220581 | 838.6134212 | 9.126139463 | 676.4584693 | 5976.2236    | 91.88659693 | 1381.980385 | 1047.923295 | 245.6546111 | 26.59466094 | 26.8834041  |
| 'fae1'   | 'sampp1171' | 65.91872064 | 1249.296315 | 10.31263608 | 621.772411  | 8584.80443   | 221.1276926 | 1807.068765 | 1421.117897 | 243.532434  | 27.01820743 | 28.81716953 |
| 'fae1'   | 'sampp1171' | 60.67256693 | 1017.27759  | 7.055455838 | 605.5148141 | 8010.135341  | 179.9302475 | 1574.254257 | 1300.930394 | 229.7866702 | 37.58010937 | 27.6484242  |
| 'fae1'   | 'sampp1171' | 43.89324932 | 972.4515309 | 8.515225496 | 682.6540906 | 6608.612443  | 158.0484795 | 1303.576431 | 1099.558195 | 199.7011321 | 30.82044654 | 19.92163315 |
| 'fae1'   | 'sampp1186' | 45.55715587 | 983.9439077 | 9.624051436 | 404.6562244 | 5734.229543  | 108.2295509 | 1317.753948 | 986.3632309 | 185.9736611 | 29.69928808 | 29.24734923 |
| 'fae1'   | 'sampp1186' | 64.51925875 | 1121.695715 | 10.35829128 | 512.695392  | 6559.165455  | 143.1788598 | 1400.838416 | 1065.586011 | 230.5677252 | 24.09092252 | 33.02647236 |
| 'fae1'   | 'sampp1186' | 54.38390731 | 970.8316982 | 9.668790682 | 449.2984385 | 6463.495903  | 177.8756366 | 1411.191857 | 1076.426831 | 183.0131988 | 28.36606812 | 25.68424745 |
| 'fae1'   | 'sampp1201' | 59.92260456 | 1035.574291 | 8.788157726 | 570.2895704 | 6336.354949  | 122.5458823 | 1357.643164 | 1043.383797 | 155.984293  | 28.2871217  | 25.06846213 |
| 'fae1'   | 'sampp1201' | 50.38483549 | 1153.86362  | 9.792857846 | 461.557893  | 7927.46065   | 223.676434  | 1597.3921   | 1326.644275 | 195.2746252 | 37.01547126 | 31.08188292 |
| 'fae1'   | 'sampp1201' | 49.94359063 | 962.5622056 | 8.906467911 | 498.5173291 | 6234.691709  | 110.1350241 | 1495.439187 | 1057.825526 | 179.955378  | 23.84144355 | 23.60775599 |
| 'fae1'   | 'sampp1216' | 54.34793677 | 981.1150877 | 10.74260635 | 586.0411961 | 6279.184158  | 146.1889405 | 1266.153035 | 1003.449421 | 223.0264186 | 26.48719902 | 29.7979507  |
| 'fae1'   | 'sampp1216' | 56.26158609 | 906.1535249 | 5.487403548 | 632.7515979 | 4954.745321  | 97.14672994 | 1117.10306  | 1035.195285 | 213.16354   | 33.1877594  | 28.22432101 |
| 'fae1'   | 'sampp1216' | 63.89916265 | 1049.49521  | 7.886972893 | 768.864483  | 6772.89601   | 110.9733775 | 1308.0135   | 1223.91598  | 217.8502671 | 26.89134741 | 30.14646367 |
| 'WT-Col' | 'sampp1099' | 76.99144019 | 1510.191862 | 21.8345117  | 451.082784  | 8519.250767  | 251.417246  | 1396.140739 | 987.4504778 | 242.7491622 | 34.8453122  | 61.73364658 |
| 'WT-Col' | 'sampp1099' | 80.35343642 | 1436.918973 | 16.13613917 | 558.292026  | 7417.586944  | 186.3236605 | 1688.066286 | 1152.365921 | 269.0566179 | 36.84565924 | 30.40779125 |
| 'WT-Col' | 'sampp1099' | 67.58748096 | 1157.793905 | 11.05926764 | 499.289261  | 7000.62021   | 120.8056055 | 1657.820366 | 1124.63383  | 238.1607017 | 31.46956654 | 35.13172924 |
| 'WT-Col' | 'sampp1112' | 57.9293332  | 1132.054427 | 7.198126105 | 409.7354586 | 6889.264113  | 119.1084596 | 1340.772987 | 946.5501017 | 181.7786131 | 24.34553086 | 24.79487577 |
| 'WT-Col' | 'sampp1112' | 64.72757126 | 1093.577857 | 7.193919463 | 462.6717001 | 7211.853093  | 123.4536771 | 1461.163299 | 975.8866473 | 220.9180655 | 21.19584061 | 30.86862945 |
| 'WT-Col' | 'sampp1112' | 57.13648896 | 1144.625091 | 10.18158209 | 418.182079  | 7368.113539  | 107.1046216 | 1336.858171 | 957.3662891 | 206.337061  | 24.82301221 | 21.78994021 |
| 'WT-Col' | 'sampp1127' | 55.62684985 | 1234.150039 | 12.9983588  | 418.7717434 | 7597.877779  | 177.0277458 | 1247.373138 | 1039.743919 | 189.5058126 | 29.94047752 | 27.18830275 |
| 'WT-Col' | 'sampp1127' | 39.84207196 | 1105.724531 | 8.371038117 | 360.3346586 | 6500.24795   | 189.9298448 | 1098.258332 | 968.0229492 | 179.9721137 | 21.80225423 | 24.42624068 |
| 'WT-Col' | 'sampp1127' | 49.29545815 | 961.1953121 | 6.510948609 | 327.1541505 | 5171.516308  | 135.1346639 | 1085.826807 | 745.0536602 | 158.399843  | 16.08828191 | 19.07263022 |
| 'WT-Col' | 'sampp1142' | 48.42648313 | 831.350194  | 12.55434605 | 281.8512713 | 4334.36318   | 85.00027318 | 1060.052873 | 701.3930603 | 131.9959571 | 15.82069282 | 17.96818572 |
| 'WT-Col' | 'sampp1142' | 57.72067563 | 1008.628866 | 10.62640441 | 459.9179928 | 6350.367215  | 360.1219169 | 1150.294452 | 820.4058875 | 205.2339947 | 19.58006118 | 21.97020864 |
| 'WT-Col' | 'sampp1142' | 39.45605    | 823.5168915 | 10.64065479 | 365.1646473 | 5650.269554  | 123.4713065 | 1117.69763  | 605.3415322 | 130.4710336 | 20.8891054  | 16.90587209 |
| 'WT-Col' | 'sampp1157' | 56.82743528 | 1128.54461  | 7.050914252 | 399.9257397 | 6290.009878  | 242.7360498 | 1434.00225  | 928.8356919 | 178.250512  | 24.97372106 | 16.78155744 |
| 'WT-Col' | 'sampp1157' | 47.46036326 | 931.3809405 | 8.851365766 | 380.1127257 | 5010.213067  | 128.5608591 | 1087.046359 | 753.2117227 | 141.4782324 | 18.95917521 | 17.05614022 |
| 'WT-Col' | 'sampp1157' | 48.21703363 | 1069.512242 | 8.282511075 | 351.5565469 | 5831.36632   | 125.0957478 | 1188.966442 | 798.6510551 | 168.7973454 | 16.57701589 | 16.38945078 |
| 'WT-Col' | 'sampp1172' | 50.35218328 | 1385.518781 | 9.30689803  | 420.4395754 | 7204.012809  | 215.8339736 | 1398.457464 | 1103.04634  | 201.4426016 | 23.26226943 | 24.99356625 |
| 'WT-Col' | 'sampp1172' | 51.72994881 | 882.7433176 | 9.461213301 | 335.0600483 | 5114.922559  | 118.0472215 | 1127.848855 | 770.0886065 | 155.4140191 | 24.30159893 | 24.98126273 |
| 'WT-Col' | 'sampp1172' | 32.78441309 | 625.5775734 | 5.417237607 | 288.2392248 | 4696.063645  | 88.9639218  | 780.1969782 | 651.6423709 | 136.962516  | 17.76238575 | 19.79046539 |
| 'WT-Col' | 'sampp1187' | 51.49537275 | 1300.623186 | 17.67169551 | 526.3018471 | 8152.480926  | 146.6706531 | 1710.198255 | 1325.024975 | 240.040217  | 31.52119618 | 15.22170254 |
| 'WT-Col' | 'sampp1187' | 42.03766762 | 1056.001992 | 5.84892906  | 427.6835142 | 5423.710123  | 139.2366474 | 1213.977799 | 922.5976417 | 173.0351408 | 19.60811763 | 18.16920235 |
| 'WT-Col' | 'sampp1187' | 52.75950683 | 953.4       |             |             |              |             |             |             |             |             |             |

Table 4.4 (continued)  
HiMet9 dataset

| Sample   | Sample Code | 'L'         | 'N'         | 'ORN'       | 'P'         | 'Q'         | 'R'         | 'S'         | 'T'         | 'V'         | 'W'         | 'Y'         |
|----------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| 'WT-Col' | 'samp1202'  | 53.63742908 | 933.0938588 | 6.836813571 | 490.7834016 | 6099.662856 | 190.7537997 | 1247.497718 | 848.9924658 | 189.9086128 | 16.23245502 | 25.17935485 |
| 'WT-Col' | 'samp1202'  | 46.29908861 | 1195.198646 | 9.795260814 | 741.5265596 | 8301.424042 | 204.2494716 | 1593.249583 | 1167.13647  | 203.2859138 | 22.5326776  | 25.70778752 |
| 'WT-Col' | 'samp1217'  | 57.36310685 | 1143.462315 | 11.64639499 | 410.984549  | 6057.605611 | 151.7249676 | 1207.902327 | 958.5215011 | 177.0472403 | 24.01466381 | 35.98177514 |
| 'WT-Col' | 'samp1217'  | 48.85277042 | 885.0662777 | 9.192960236 | 333.2528495 | 4857.436954 | 89.71339329 | 1094.520014 | 801.5098611 | 158.6238364 | 28.15876972 | 26.08278391 |
| 'Yad2-1' | 'samp1097'  | 51.61978583 | 1546.118493 | 8.88106794  | 530.413042  | 5429.340267 | 631.0586137 | 1713.143032 | 952.4272346 | 166.1434327 | 22.62802941 | 32.50641726 |
| 'Yad2-1' | 'samp1097'  | 46.6076926  | 1615.907646 | 4.294888721 | 621.315467  | 7018.575875 | 521.7322312 | 1447.628211 | 1114.575665 | 185.2081831 | 27.40731121 | 32.02965241 |
| 'Yad2-1' | 'samp1097'  | 51.87246768 | 1325.311227 | 11.29071806 | 502.5805127 | 6108.898365 | 453.2723084 | 1387.884487 | 873.9831581 | 128.4985644 | 21.11923144 | 25.95418181 |
| 'Yad2-1' | 'samp1125'  | 74.08537356 | 1191.736936 | 12.96570204 | 263.3176859 | 7117.169294 | 529.3326487 | 1410.898827 | 925.1852508 | 185.7280881 | 26.59698352 | 24.93423276 |
| 'Yad2-1' | 'samp1125'  | 84.39358013 | 1446.60353  | 12.98017462 | 388.4613725 | 9553.665679 | 505.5015565 | 2040.217708 | 1198.036704 | 251.1590399 | 32.45393869 | 35.8164228  |
| 'Yad2-1' | 'samp1125'  | 54.55632328 | 1207.449271 | 9.925760049 | 296.0706009 | 6687.731639 | 310.6639556 | 1402.79638  | 892.5179116 | 185.5823054 | 24.27463292 | 25.06549597 |
| 'Yad2-1' | 'samp1140'  | 63.89636934 | 1333.149721 | 11.57452414 | 573.5083111 | 7014.057953 | 520.4582317 | 2019.712613 | 1175.794486 | 177.6018016 | 33.09428635 | 29.39641007 |
| 'Yad2-1' | 'samp1140'  | 89.23490707 | 1750.756118 | 13.49309178 | 754.6643362 | 9563.202765 | 825.7996773 | 2485.763343 | 1354.963739 | 288.1208641 | 24.73859548 | 35.30953757 |
| 'Yad2-1' | 'samp1140'  | 92.07376784 | 1611.003997 | 13.64825664 | 770.9046195 | 9783.06653  | 653.1752685 | 2656.611969 | 1556.268618 | 283.345579  | 32.83393699 | 41.3471026  |
| 'Yad2-1' | 'samp1155'  | 62.78860923 | 1279.225592 | 7.56072835  | 253.3717942 | 5700.640553 | 456.2658856 | 1729.905701 | 1099.333438 | 189.8822158 | 23.03153742 | 29.13859946 |
| 'Yad2-1' | 'samp1155'  | 49.74915811 | 913.5962049 | 4.134645279 | 281.5904189 | 5050.473872 | 201.9421428 | 1346.673678 | 1038.902781 | 191.043114  | 26.01827686 | 29.10277997 |
| 'Yad2-1' | 'samp1155'  | 62.40112561 | 1105.375435 | 13.16796413 | 256.3742836 | 6175.058912 | 440.2504197 | 1499.968737 | 1146.992952 | 196.4154391 | 31.39334186 | 35.60073989 |
| 'Yad2-1' | 'samp1170'  | 60.56228141 | 1060.783231 | 9.306772387 | 304.6761465 | 7129.977839 | 219.3549415 | 1659.257154 | 1062.15106  | 183.2658203 | 26.95340598 | 31.58926566 |
| 'WT-Col' | 'samp1217'  | 55.52030873 | 1238.539204 | 9.979638145 | 429.3446974 | 7243.355609 | 232.723774  | 1378.644925 | 990.3012072 | 190.1598654 | 26.01687902 | 22.72411469 |
| 'Yad2-1' | 'samp1170'  | 50.81700355 | 906.9871204 | 5.114480341 | 312.2218939 | 5188.631149 | 249.7414049 | 1381.243076 | 911.7863515 | 189.7124501 | 31.18043083 | 27.36328985 |
| 'act1'   | 'samp1096'  | 53.44983421 | 1237.782918 | 11.07145434 | 446.1839688 | 5059.364095 | 117.3006505 | 1746.82527  | 946.5260491 | 194.1228783 | 23.74850923 | 23.93684491 |
| 'act1'   | 'samp1096'  | 49.16092197 | 977.1361599 | 6.911490807 | 477.5769121 | 5387.18588  | 116.5081764 | 1652.475007 | 1057.384875 | 194.2094624 | 24.61707827 | 20.80813938 |
| 'act1'   | 'samp1096'  | 47.82275159 | 970.8724262 | 11.39971297 | 364.7642547 | 4882.795453 | 124.2426745 | 1491.24783  | 979.6007984 | 157.2536327 | 20.52758594 | 22.13647643 |
| 'act1'   | 'samp1110'  | 40.62787954 | 1336.74619  | 9.086518639 | 390.1490965 | 5482.548675 | 126.0830664 | 1583.77875  | 944.8010566 | 173.6774411 | 26.3013312  | 28.38292441 |
| 'act1'   | 'samp1110'  | 44.03252843 | 981.6465934 | 6.668040874 | 312.8210802 | 5365.028878 | 138.6164004 | 1281.765087 | 826.5291053 | 158.3660437 | 21.23454632 | 20.6772956  |
| 'act1'   | 'samp1110'  | 50.93597928 | 1375.049973 | 9.091853306 | 404.1420494 | 7458.743193 | 140.2136971 | 1604.830758 | 1075.207196 | 177.34153   | 24.85283294 | 37.8251175  |
| 'act1'   | 'samp1124'  | 59.25155105 | 1064.039506 | 9.290019896 | 518.3385533 | 5844.637196 | 170.2611904 | 1781.756132 | 1032.370758 | 210.9626166 | 26.65332604 | 38.09156258 |
| 'act1'   | 'samp1124'  | 42.08775779 | 1080.718601 | 8.268111519 | 422.8466242 | 5091.640058 | 242.8793963 | 1336.59746  | 857.6860445 | 168.6885015 | 17.81282856 | 21.72321189 |
| 'act1'   | 'samp1124'  | 41.98512244 | 977.746379  | 9.184064521 | 390.0804438 | 4472.117034 | 128.452632  | 1397.08989  | 858.7085051 | 153.7886275 | 29.24634641 | 24.30230543 |
| 'act1'   | 'samp1139'  | 57.85517143 | 1142.312655 | 11.57757311 | 551.8488367 | 6171.079584 | 144.9728535 | 1783.575095 | 1144.441353 | 194.16149   | 25.73925381 | 27.66503695 |
| 'act1'   | 'samp1139'  | 54.5963057  | 1183.415137 | 8.359607421 | 497.633883  | 6158.096795 | 237.0683273 | 1790.338381 | 1128.4081   | 187.4376238 | 30.02024653 | 40.28766585 |
| 'act1'   | 'samp1139'  | 54.65148368 | 1061.427117 | 7.37126305  | 486.4392682 | 5559.584139 | 184.8209842 | 1610.626676 | 982.235892  | 194.2559905 | 27.23569149 | 31.72232707 |
| 'act1'   | 'samp1154'  | 38.47818606 | 965.8531464 | 14.9639367  | 438.7970307 | 4679.221332 | 106.5272434 | 1845.170695 | 1069.90426  | 185.390291  | 22.03836078 | 28.6956543  |
| 'act1'   | 'samp1154'  | 58.63708489 | 980.2452379 | 13.49652891 | 388.7325054 | 4862.412102 | 165.9022313 | 1860.203828 | 1165.810002 | 198.3498813 | 17.06798404 | 26.69689666 |
| 'act1'   | 'samp1154'  | 48.7372421  | 1023.982841 | 12.46259878 | 334.0697979 | 4149.622128 | 174.608989  | 1803.371366 | 936.0479661 | 163.1883006 | 25.77968889 | 23.85419145 |
| 'act1'   | 'samp1169'  | 37.2490071  | 1075.870728 | 11.62170846 | 552.0821744 | 5968.805359 | 94.15937284 | 1837.279329 | 1081.348862 | 175.0859224 | 27.7565812  | 23.00920216 |
| 'act1'   | 'samp1169'  | 40.80601162 | 813.9468043 | 5.060093267 | 488.6974396 | 4035.912505 | 107.0015435 | 1270.205853 | 849.4831371 | 173.6171862 | 16.18327092 | 19.91663917 |
| 'act1'   | 'samp1169'  | 44.47080919 | 1116.795519 | 10.65389414 | 644.7325028 | 5942.002707 | 234.6927543 | 2186.415089 | 1270.873382 | 202.7367311 | 36.68656018 | 24.94110725 |
| 'act1'   | 'samp1184'  | 54.47775051 | 1167.882093 | 7.722326572 | 650.4363442 | 6392.010889 | 186.0630201 | 2410.187516 | 1343.999394 | 240.273731  | 35.45183241 | 21.61198892 |
| 'act1'   | 'samp1184'  | 45.06631624 | 799.2478016 | 7.470931303 | 550.7741661 | 4213.965367 | 127.5856765 | 1443.173825 | 838.364631  | 163.8975808 | 20.43130114 | 15.1539114  |
| 'act1'   | 'samp1184'  | 58.56832308 | 1189.444966 | 10.10385424 | 931.1501036 | 7411.9137   | 140.3542564 | 2697.522753 | 1349.386951 | 256.8392125 | 30.72709327 | 28.82231672 |
| 'act1'   | 'samp1199'  | 68.25169438 | 818.8251358 | 10.4969704  | 343.7336502 | 4568.618193 | 147.0223819 | 1434.192235 | 738.7296558 | 169.2241241 | 24.90859658 | 34.09166963 |
| 'act1'   | 'samp1199'  | 57.55738889 | 651.9230922 | 10.64501322 | 314.5794205 | 4074.676794 | 82.67922704 | 1313.406974 | 782.4465736 | 154.9668967 | 32.77084148 | 31.59104411 |
| 'act1'   | 'samp1199'  | 76.12447643 | 918.6203235 | 20.84265017 | 371.9263731 | 4570.099304 | 129.9940539 | 1836.957844 | 914.5775865 | 193.3206783 | 24.05064923 | 47.0919422  |
| 'act1'   | 'samp1214'  | 32.37884033 | 1054.487247 | 12.47989464 | 485.0840603 | 5489.879672 | 105.8326509 | 1759.129911 | 1110.615969 | 175.5919723 | 22.64761611 | 34.00901516 |
| 'act1'   | 'samp1214'  | 50.66790828 | 1014.821577 | 10.64590243 | 585.9244448 | 5460.01219  | 141.1069107 | 1726.753047 | 1015.823736 | 191.8647736 | 24.2118937  | 34.23920195 |
| 'act1'   | 'samp1214'  | 51.14015715 | 906.1999631 | 12.21877072 | 477.1718776 | 5485.688784 | 126.4269661 | 1403.071197 | 826.611216  | 188.7230336 | 19.24368652 | 25.83195964 |

Table 4.5

| Actual known group | SAMPLECODE | Predicted group by the PLS-DA model | Classification Result |
|--------------------|------------|-------------------------------------|-----------------------|
| 'act1'             | 'saml1096' | act1                                | CORRECT               |
| 'act1'             | 'saml1096' | act1                                | CORRECT               |
| 'act1'             | 'saml1096' | act1                                | CORRECT               |
| 'act1'             | 'saml1110' | fad-2                               | Incorrect             |
| 'act1'             | 'saml1110' | WT-Col                              | Incorrect             |
| 'act1'             | 'saml1110' | WT-Col                              | Incorrect             |
| 'act1'             | 'saml1124' | act1                                | CORRECT               |
| 'act1'             | 'saml1124' | act1                                | CORRECT               |
| 'act1'             | 'saml1124' | act1                                | CORRECT               |
| 'act1'             | 'saml1139' | act1                                | CORRECT               |
| 'act1'             | 'saml1139' | act1                                | CORRECT               |
| 'act1'             | 'saml1139' | act1                                | CORRECT               |
| 'act1'             | 'saml1154' | act1                                | CORRECT               |
| 'act1'             | 'saml1154' | act1                                | CORRECT               |
| 'act1'             | 'saml1154' | act1                                | CORRECT               |
| 'act1'             | 'saml1169' | act1                                | CORRECT               |
| 'act1'             | 'saml1169' | act1                                | CORRECT               |
| 'act1'             | 'saml1169' | act1                                | CORRECT               |
| 'act1'             | 'saml1184' | act1                                | CORRECT               |
| 'act1'             | 'saml1184' | act1                                | CORRECT               |
| 'act1'             | 'saml1184' | act1                                | CORRECT               |
| 'act1'             | 'saml1199' | act1                                | CORRECT               |
| 'act1'             | 'saml1199' | act1                                | CORRECT               |
| 'act1'             | 'saml1199' | act1                                | CORRECT               |
| 'act1'             | 'saml1214' | act1                                | CORRECT               |
| 'act1'             | 'saml1214' | act1                                | CORRECT               |
| 'act1'             | 'saml1214' | act1                                | CORRECT               |
| 'fad2-1'           | 'saml1170' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1185' | WT-Col                              | Incorrect             |
| 'fad2-1'           | 'saml1185' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1185' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1200' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1200' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1200' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1215' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1215' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1215' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1097' | WT-Col                              | Incorrect             |
| 'fad2-1'           | 'saml1097' | WT-Col                              | Incorrect             |
| 'fad2-1'           | 'saml1097' | WT-Col                              | Incorrect             |
| 'fad2-1'           | 'saml1125' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1125' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1125' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1140' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1140' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1140' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1155' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1155' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1155' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1170' | fad-2                               | CORRECT               |
| 'fad2-1'           | 'saml1170' | fad-2                               | CORRECT               |
| 'fae1'             | 'saml1098' | fad-2                               | Incorrect             |
| 'fae1'             | 'saml1098' | fad-2                               | Incorrect             |
| 'fae1'             | 'saml1098' | fae1                                | CORRECT               |
| 'fae1'             | 'saml1111' | fae1                                | CORRECT               |
| 'fae1'             | 'saml1111' | fae1                                | CORRECT               |
| 'fae1'             | 'saml1111' | fae1                                | CORRECT               |
| 'fae1'             | 'saml1126' | fae1                                | CORRECT               |
| 'fae1'             | 'saml1126' | fae1                                | CORRECT               |
| 'fae1'             | 'saml1126' | WT-Col                              | Incorrect             |
| 'fae1'             | 'saml1141' | WT-Col                              | Incorrect             |
| 'fae1'             | 'saml1141' | WT-Col                              | Incorrect             |

Table 4.5 (continued)

|          |             |        |           |
|----------|-------------|--------|-----------|
| 'fae1'   | 'sAMPL1141' | fae1   | CORRECT   |
| 'fae1'   | 'sAMPL1156' | WT-Col | Incorrect |
| 'fae1'   | 'sAMPL1156' | fae1   | CORRECT   |
| 'fae1'   | 'sAMPL1156' | fae1   | CORRECT   |
| 'fae1'   | 'sAMPL1171' | fae1   | CORRECT   |
| 'fae1'   | 'sAMPL1171' | fae1   | CORRECT   |
| 'fae1'   | 'sAMPL1171' | fae1   | CORRECT   |
| 'fae1'   | 'sAMPL1186' | fae1   | CORRECT   |
| 'fae1'   | 'sAMPL1186' | fae1   | CORRECT   |
| 'fae1'   | 'sAMPL1186' | WT-Col | Incorrect |
| 'fae1'   | 'sAMPL1201' | WT-Col | Incorrect |
| 'fae1'   | 'sAMPL1201' | WT-Col | Incorrect |
| 'fae1'   | 'sAMPL1201' | fae1   | CORRECT   |
| 'fae1'   | 'sAMPL1216' | WT-Col | Incorrect |
| 'fae1'   | 'sAMPL1216' | fae1   | CORRECT   |
| 'fae1'   | 'sAMPL1216' | fae1   | CORRECT   |
| 'WT-Col' | 'sAMPL1099' | fae1   | Incorrect |
| 'WT-Col' | 'sAMPL1099' | fae1   | Incorrect |
| 'WT-Col' | 'sAMPL1099' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1112' | act1   | Incorrect |
| 'WT-Col' | 'sAMPL1112' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1112' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1127' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1127' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1127' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1142' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1142' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1142' | act1   | Incorrect |
| 'WT-Col' | 'sAMPL1157' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1157' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1157' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1172' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1172' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1172' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1187' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1187' | fae1   | Incorrect |
| 'WT-Col' | 'sAMPL1187' | fae1   | Incorrect |
| 'WT-Col' | 'sAMPL1202' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1202' | WT-Col | CORRECT   |
| 'WT-Col' | 'sAMPL1202' | fae1   | Incorrect |
| 'WT-Col' | 'sAMPL1217' | fae1   | Incorrect |
| 'WT-Col' | 'sAMPL1217' | fae1   | Incorrect |
| 'WT-Col' | 'sAMPL1217' | fad-2  | Incorrect |













**APPENDIX A3:**  
**SUPPLEMENTARY MATERIAL FOR CHAPTER 5**

