FACULTY OF SCIENCE
UNIVERSITY OF COPENHAGEN

Bioinformatics Master Thesis

Development of label-free quantification methods in proteomics

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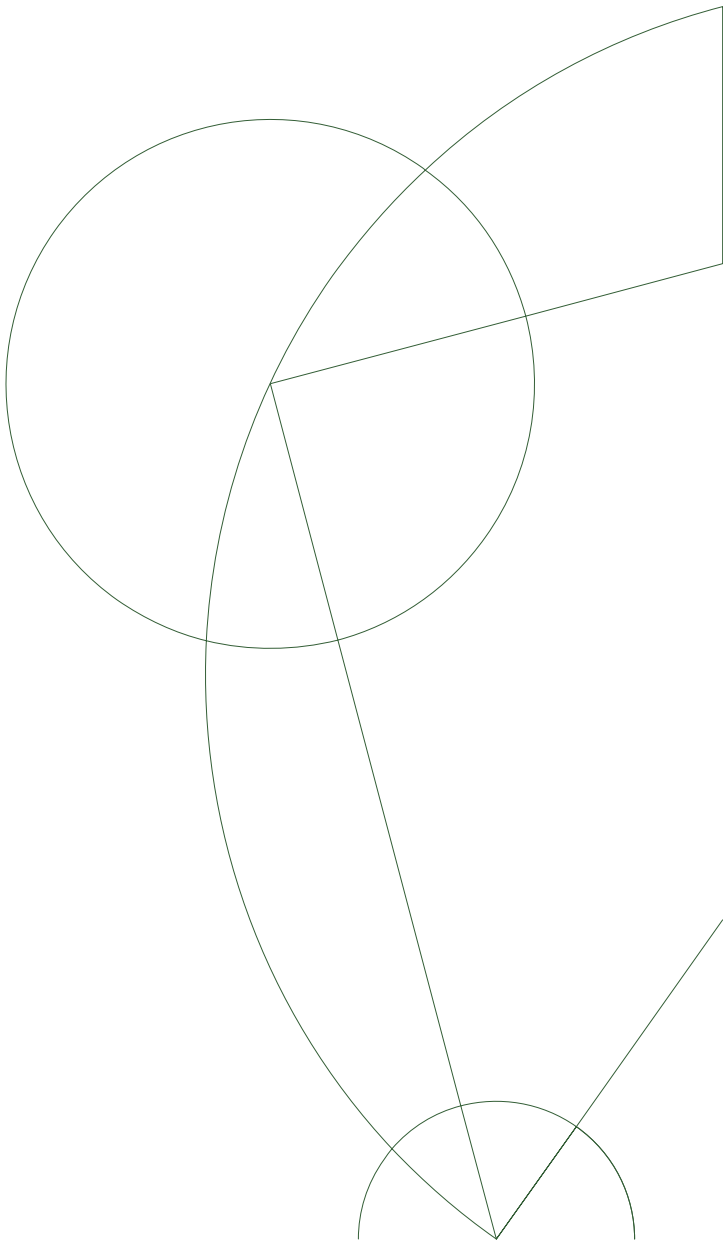

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Abbreviations

ESI Electrospray Ionization

FDR False Discovery Rate

FT-ICR Fourier Transform Ion Cyclotron Resonance

HPLC High pressure liquid chromatography

IT Ion Trap

m/z mass charge ratio

MALDI Matrix-Assisted Laser-Desorption Ionization

MS Mass Spectrometry

MS/MS Tandem mass spectrometry

MS1 first tandem MS analyzer

MS2 second tandem MS analyzer

NZ Novozymes A/S

Q Quadrupole

RT Retention time

SC Spectral Counting

TOF Time of Flight

TPP Trans Proteomic Pipeline

XIC Extracted Ion Current

Preface

Introduction

0.1 Aminoacids and proteins

Proteins represent the last link in the central dogma of biology, where information encoded in DNA, is transcribed to RNA for posterior translation into proteins at the ribosome.

Proteins are made up of 20 basic units, called aminoacids. All aminoacids share a common chemical structure, where a carbon atom (C_α) is covalently bonded to a hydrogen atom, a carboxyl group, an amino group, and last but not least, a radical, also called side chain of the aminoacid. The side chain differs between aminaocids and generates them from each other. A slight deviation from this pattern exists in proline, where the radical is bound to the nitrogen atom, making it an iminoacid. Even though the side chains are all different, they can be classified into four different groups: aliphatic, polar, positively charged and negatively charged (see figure 1).

Two aminoacids are joined together through the formation of a peptidic (covalent) bond between them. Such a linkage is formed by removal of the elements of water (dehydration) from the α-carboxyl group of one amino acid and the α-amino group of another [1]. The remaining α-amino and α-carboxyl groups are available for linkage to other aminoacids, and in this way peptidic chains or peptides can be created.

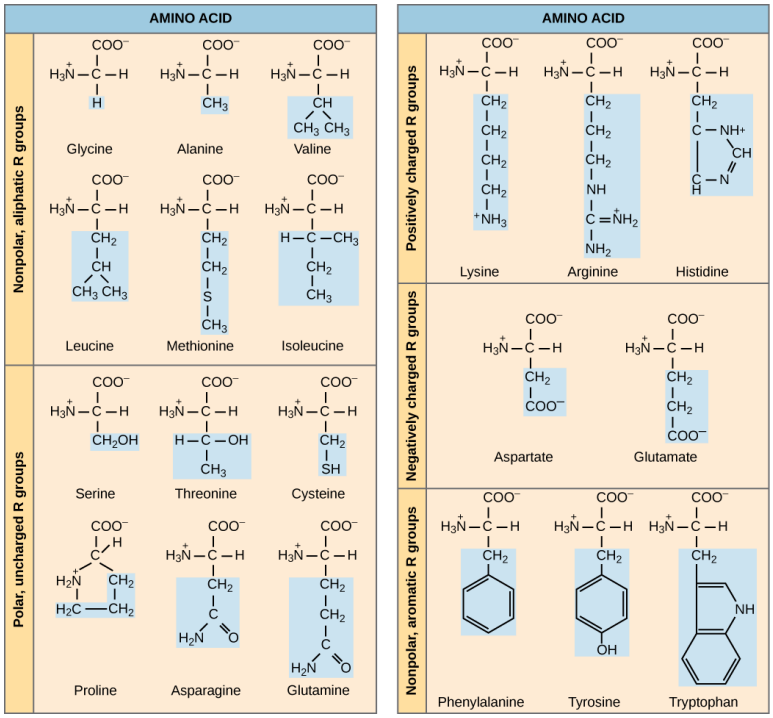


Figure 1 CAPTION AND REFERENCE

While there are 20 basic units that constitute the majority of naturally observable proteins, their side chains can be modified both by physiological processes and by experimental procedures cite. One frequent instance of such modifications is the oxidation of methionine.

0.2 The protein-focused biotechnology industry

Proteins carry out most of the cell's molecular functions, they work as molecular agents that can perform an extremely wide range of tasks. The advent of biotechnology has sought to take advantage of this power, either by using proteins as present in natural conditions (wild type) or engineered by humans. This potential economic activity is carried out by several biotech companies, including Novozymes A/S (NZ).

NZ is a company whose line of business consists of the development of enzymatic products performing chemical transformations in different industrial processes. The application of these products, instead of conventional chemical-based solutions, has the advantage that they require less chemical substances, potentially simplifying industrial processes, reducing their costs and their environmental impact. Notorious examples of such applications include waste-water treatment, household care and the baking industry.

The advancement of the way NZ does protein research is thus key to place the organization ahead of its competitors. The refinement of the currently used tools and the development of new ones could be of great significance for the company.

Protein research can be approached from different angles. This thesis exploited the combination of mass spectrometry (MS) and proteomics workflows (see chapter 1) for the qualitative and quantitative characterization of protein samples.

0.3 Objectives of the Thesis

In line with the goal of making NZ more competitive, this project aimed at the following objectives:

1. Develop an open-source, Linux based and easily deployable pipeline for the analysis of MS data, starting at the raw high-throughput data files and ending in the biological interpretation of the results.
2. Evaluate this pipeline with a benchmark dataset to assess if the pipeline is able to reflect the biological phenomena captured in the data.
3. Establish a label-free quantification probabilistic model that provides

relative abundance estimates and a measurement of their uncertainty based on the available data.

0.4 Structure of the Thesis

An overview over the MS and following computational data analysis steps is presented in 1. The pipeline development and its benchmark are explained in chapters 2 and 3, while the modelling problem is introduced in chapter 4. Finally, a conclusion of the work is given in chapter 4.

Chapter 1

Mass spectrometry and shotgun proteomics overview

Life systems consist of complex systems, meaning their behaviour cannot be easily explained by analyzing the individual elements alone. Moreover, they present multiple layers of complexity, given by the nature of the elements that make it up. The layer provided by proteins is one of them, and its study is called proteomics. It is a complex layer because thousands of different proteins can be present in a single cell at any time, and their exact composition and quantities constantly change, responding to the stimuli of the surrounding environment. The study of the protein-specific complexity is called proteomics. With proteomics, one endeavors to infer the protein composition of a sample, and eventually quantify its protein amounts.

The existing approaches are divided into two types of paradigms: top-down and bottom-up. In the top-down paradigm, intact proteins are directly used for the analysis. In the bottom-up paradigm (see figure **??**, the proteins are first cleaved into smaller parts, and these parts are then used for identification, characterization, and quantification. These smaller parts are called peptides. [2] Such peptides acquire physicochemical properties fitting the requirements of the downstream analytical methods, mainly the

mass spectrometer (MS), which performs the data acquisition. The bottom-up paradigm is most often used because peptides are much more suitable to analysis by mass spectrometry, as explained in 1.2.3. The top-down paradigm will be ignored in the rest of the manuscript.

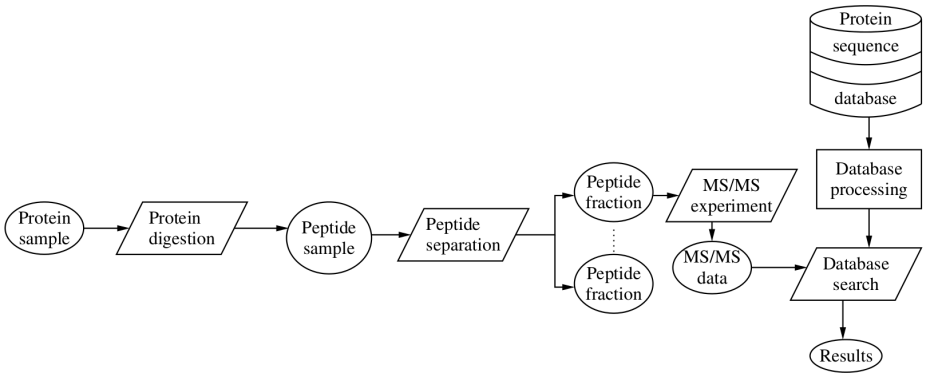


Figure 1.1 Overview of a standard bottom-up proteomics analysis. Figure 1.3 from [2].

MS is performed by means of a mass spectrometer, an ensemble of pieces of equipment that can acquire mass measurements for plenty of sample components. A detailed explanation of the sample processing required prior to MS is given in section , while an overview on mass spectrometers is given in section . The result of the MS analysis is a dataset that, with adequate computational analysis tools, is enough to perform the inference steps required to gather knowledge about the original protein sample. These inference steps can be condensed to the peptide and protein inference problems, explained in section . A third computational problem needs to be solved if quantitative, and not just qualitative information, is to be gained from the experiment. This is the quantification problem, explained in section CITE.

A summary of the bottom-up approach MS analytical pipeline is provided in the rest of the chapter. It can be divided into two main steps:

1. MS analysis and data generation. Sections 1.1 to 1.3.

2. Computational analysis of data. Sections 1.4 to 1.7.

MS analysis and data generation

1.1 Sample processing

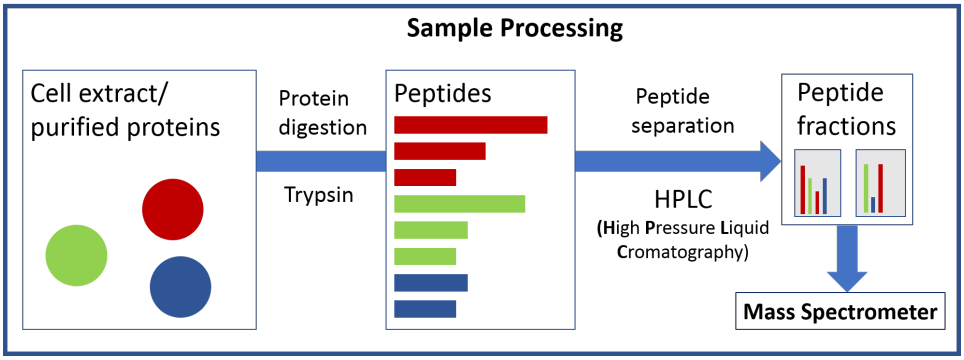


Figure 1.2 Overview of the sample processing step prior to mass spectrometry analysis. First, proteins are denatured and digested with a specific protease like Trypsin. This yields a peptide mix that is separated into peptide fractions that can be introduced in the mass spectrometer.

1.1.1 Protein digestion

An MS experiment starts with the generation of a protein sample from the biological system of interest. Proteins are then denaturalized so as to remove bias due to the divergent properties acquired by folded proteins. Then, proteins are subjected to digestion with specific enzymes, which cut the aminoacidic chains following a predictable pattern. Trypsin is the most frequently used for this task. It cuts peptidic bonds whenever a positively charged residue, either Lysine (K) or Arginine (R), lies on the carboxyl side of the peptidic bond. Since roughly 1/10 residues are either R or K, the average peptide length is 10 residues. As explained in 1.2.3, this length distribution is fitted to the resolution of the MS analyzer. Moreover, since R

and K are positively charged aminoacids, the resulting peptide is guaranteed in most cases to be able to capture at least one charge, thus making it visible in the spectrometer. All of these properties combined, together with its low prize, makes Trypsin the enzyme of choice in this step for most cases.

Even though enzymes are very specific, the cleaving process is far from perfect, as there could be: [2]

1. Missed cleavages
2. Unsuspected cleavages during the maturation/life cycle of the protein.
3. Unexpected cleavages occurring either in the wet-lab procedure of the proteolytic treatment.
4. Naturally occurring, intentionally or unintentionally induced chemical modifications.

Missed cleavages can happen due to steric impediments or the presence of specific aminoacids that can weaken the enzyme's function. This is the case of Trypsin whenever the residue on the other side of the peptidic bond is Proline. Altogether, a variability is created in the cleavage process that, though limited, needs to be taken care of in downstream analysis, as it could introduce biases in peptide observability.

The result of this process is a complex mix of peptides, made up by hundreds or thousands of different molecules, following a length distribution given by the cleavage sites frequency and each protein's aminoacidic composition. A peptide separation step is required before introducing the sample in the spectrometer.

1.1.2 Peptide separation

If presented with the problem of analyzing a mixture of peptides, the capacities of mass spectrometers are easily overwhelmed by a too complex mixture, resulting in the analysis of only a minor part of the total protein of the sample. This can be surmounted by independently analyzing different fractions of the sample over time. The required sample separation is achieved by High-Performance Liquid Cromatography (HPLC) methods, like reverse phase chromatography (separating on hydrophobicity) and strong cation exchange chromatography (separating on isoelectric point) [2].

During HPLC, the peptide mix loaded into a column containing a stationary and a solid phase. These phases create an environment where peptides interact differently based on their physico-chemical properties, set by the nature of the phases. The output of the column, called elute, will consist of subsets or fractions of peptides leaving the column at different retention times (RT) i.e the amount of time passed before the peptide is observed in the mass spectrometer. Therefore, the input to the machine will consist of a simplified mix of peptides at a time.

1.2 The mass spectrometer

The mass spectrometer is the ensemble of pieces of equipment analyzing a peptide mix such as those generated following the workflow enunciated in sections 1.1 and 1.1.2. It consists of three main parts: an ion source, a mass analyzer, and a detector (see figure 1.3).

[1] https://www.slideshare.net/joachimjacob/bits-introduction-to-mass-spec-data-generation

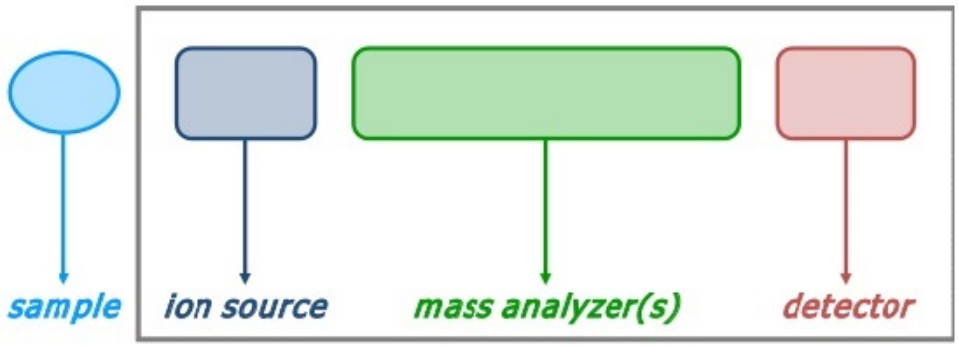


Figure 1.3 Schematic view of a mass spectrometer. Taken from [1]

1.2.1 The ion source

All mass spectrometers exploit the physical properties of mass and electric charge exhibited by the analyzed components. Ionization of the analytes is absolutely essential prior to any measurement, as analytes left uncharged will be unobservable to the equipment.

This step is performed in the ion source [2]. The most frequent ionization methods in proteomics are Matrix-Assisted Laser Desorption-Ionization (MALDI) and Electro Spray Ionization (ESI) [3]. Most peptides ionized by MALDI will acquire a single charge, whereas ESI can provide multiple charges (+2, +3, etc) too. Thus, the charge exhibited by an ion is not obvious when produced via ESI. Moreover, ESI can be run online with the right robotic equipment, while MALDI demands waiting time for vacuum generation. Finally, due to the chemical nature of the matrix components, MALDI ionizes more easily peptides containing aminoacids featuring aromatic rings (PYW), thus introducing a bias. Bias in ESI is less predictable. This is known as the competitive ionization problem [4].

The acquired charge yields a mass/charge (m/z) ratio, a property that is applied in the downstream component separation and measurement steps.

1.2.2 The mass analyzer

The plethora of ion separation methods is reflected upon the range of different analyzers available, mainly time of flight (TOF), Ion trap (IT) and quadrupole (Q). These apply different principles to perform the same task: separation (analysis) of the ion mix by the m/z ratio.

Moreover, two other analyzers exist which combine mass analysis with intensity measurement. These are Fourier Transform Ion Cyclotron Resonance (FT-ICR) and Orbitrap. They both register cylotron resonance frequencies that are Fourier transformed into the spectrum space. Remarkably, FT-ICR exhibits great resolving power, at the cost of high maintenance costs and difficult operability [2].

1.2.3 The detector

Detectors measure the intensity of an incoming ion signal. The ion's m/z ratio is known thanks to the previous mass analysis step. Performed for enough m/z ratios, the detector can produce a MS spectrum, which shows the intensity of ion current over an m/z range. Some topics in signal detection in MS need to be discussed.

On the one hand, the precision of the signal measurement is given by its mass resolution It is conventionally defined as the closest distinguishable separation between two peaks of equal height and width [5]. The resolution decreases as the m/z ratio increases because small increments in the m/z ratio become negligible at high m/z ratios. This is one of the reasons why proteins are better fit for analysis when digested into peptides, as m/z are reduced, thus increasing the mass resolution.

On the other hand, due to the natural occurrence of isotopes, particularly ^{13}C, the same peptide will induce the measurement of several signals with

very close m/z values. They constitute the isotopic envelope of the ion (see figure 1.4), and represent the signal created by peptides containing an increasing number of ^{13}C atoms. Every time a ^{12}C is replaced by ^{13}C, the mass increases by 1 Da. Even though the natural abundance of ^{13}C is 1.1 %, the sheer number of carbon atoms in a peptide makes it likely that at least one or even more carbon atoms will be ^{13}C, eventually driving the pure ^{12}C signal to comparatively small intensity values, and down to intensities below the background noise. Such event can be problematic if it entails that the ^{13}C peak is confused for the ^{12}C peak.

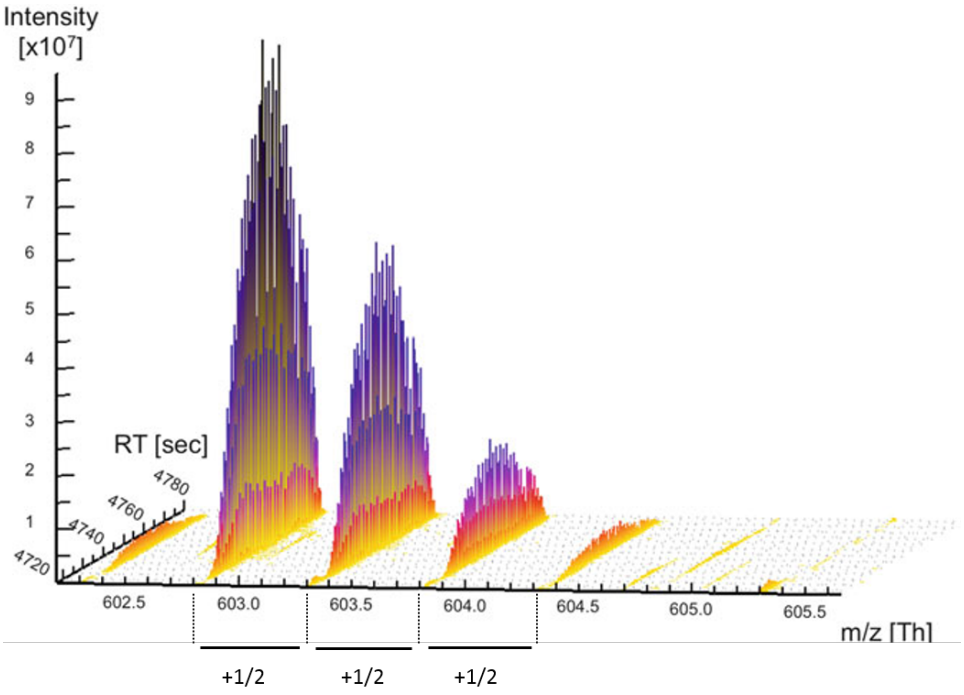


Figure 1.4 A doubly charged isotopic envelope with its monoisotopic ion measured at 602.8 Th. Each peak in the envelope is separated by 0.5 Th. This is explained by the peptide having 2 positive charges that make every extra Da in the ion mass account for 1/2 extra Th. Adapted from [3].

The resolution achieved by modern equipment allows for the distinction of each individual signal in most isotopic envelopes. Remarkably, the sepa-

ration across peaks in the envelope can be used to infer the charge of the peptide, as increases of 1 Da at charge 1 will induce a separation of 1 m/z, while at charge 2 it will be $1/2 = 0.5$ m/z, at 3 $1/3 = 0.33$ m/z, and so on.

It is up to the MS technician to decide on the best pieces of equipment according to their availability and particularities of the dataset.

1.3 Tandem MS workflow

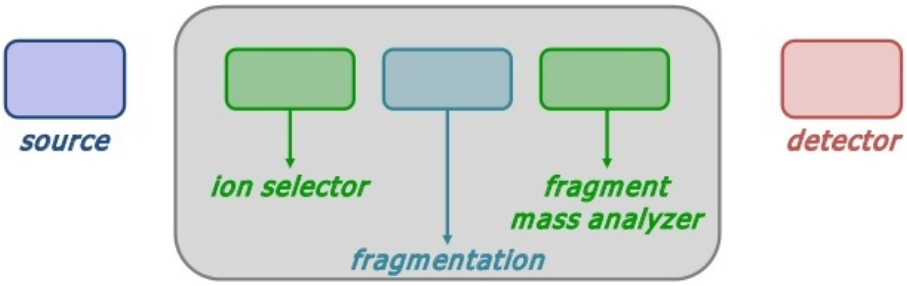


Figure 1.5 Illustration of the tandem MS workflow. The first spectrometer acts as an ion selector, that lets through ions with a given m/z ratio. The second spectrometer does not perform mass analysis, but instead provides the medium where peptide fragmentation occurs. Finally the third spectrometer records fragment mass spectra. Taken from [3].

Shotgun proteomics analyses make use of two or more mass spectrometers connected in series, giving rise to the so-called Tandem MS (MS/MS) workflow. In this setting, each mass spectrometer collects a different type of spectra and thus different information (see figure 1.6. An extra spectrometer, usually a quadrupole, is introduced in between.

- The first spectrometer records the intensity versus m/z ratio of the peptides eluting from the column at a given time and is used to filter

[3] https://www.slideshare.net/joachimjacob/bits-introduction-to-mass-spec-data-generation

ions exhibiting a selected m/z ratio.

- The ions filtered in the first spectrometer undergo fragmentation in the second spectrometer.
- The last spectrometer records the intensity versus m/z ratio of the fragments produced in the previous step. The produced spectrum can be use to read out the peptide sequence.

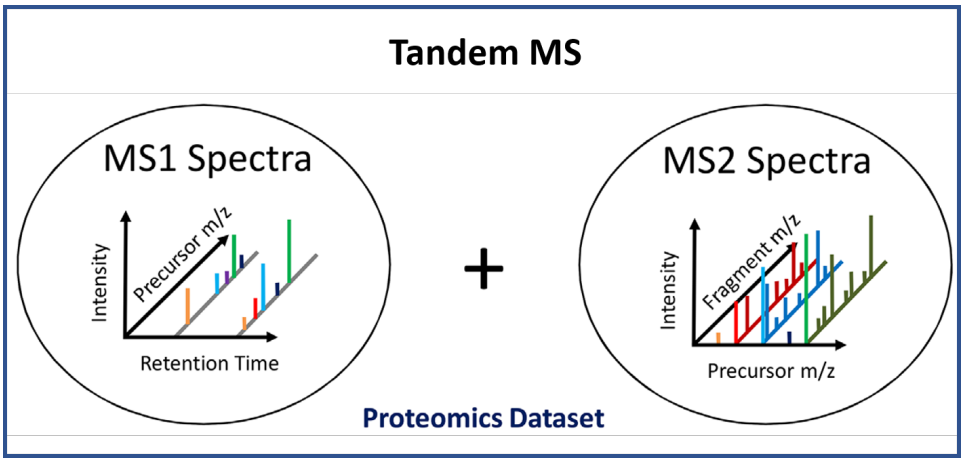


Figure 1.6 Illustration of the different spectra collected in tandem MS. MS1 spectra record precursor intensity vs m/z ratio at different times. MS2 spectra record the same magnitudes but the signal is generated by the fragments produced during fragmentation by the ion filtered in the first spectrometer. Altogether, they enable peptide identifications. Figure adapted from [6].

1.3.1 Fragmentation

The information that can be extracted from MS1 scans consists of the m/z ratio and retention time of the peptide ion, but not its sequence. Having the latter is paramount if the spectrum is to be matched to a theoretical peptide.

Fortunately, the peptide sequence of the ion can be inferred if fragmentation is performed. During peptide fragmentation, bonds along the peptidic

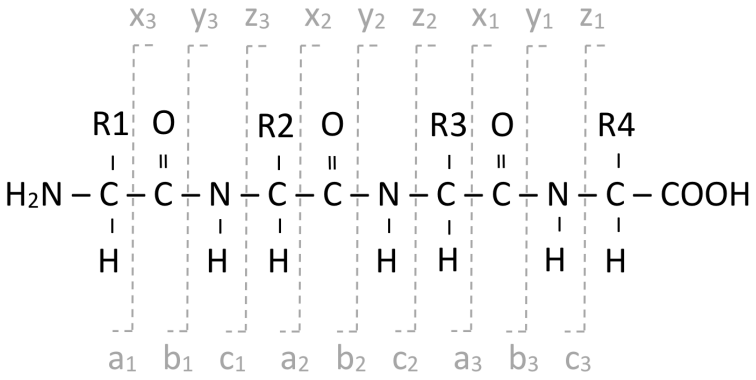


Figure 1.7 The common fragments and their relation to the peptide sequence can be organized into 2 groups of 3 series each. The abc fragments keep the N-terminal residue, while xyz keep the C-terminal one. Specific fragmentation techniques make fragments belonging to one series more likely than others. Other fragments are possible but much less likely. The fragment nomenclature was introduced in [7].

chain are broken, turning the peptide into smaller fragments. These fragments will consist of truncated versions of the original peptide at different positions, thus making it possible to read an m/z ratio difference between any pair of fragment ions. The difference can be exploited to infer which aminoacid is present in the longer fragment. If this process is repeated for enough pairs of contiguous fragments, with the right software, a sequence can be read from the MS2 spectrum. explained in 1.4.

Computational analysis

1.4 Spectra processing: search engines

Computational analysis of MS data starts with the matching of the spectra to a referene proteome (see figure 1.8). MS search engines are capable of performing the crucial step of peptide to spectrum matching (PSM). Dur-

ing this step, search engines perform *in silico* prediction of the peptides produced in the protein digestion step and their expected spectrum.

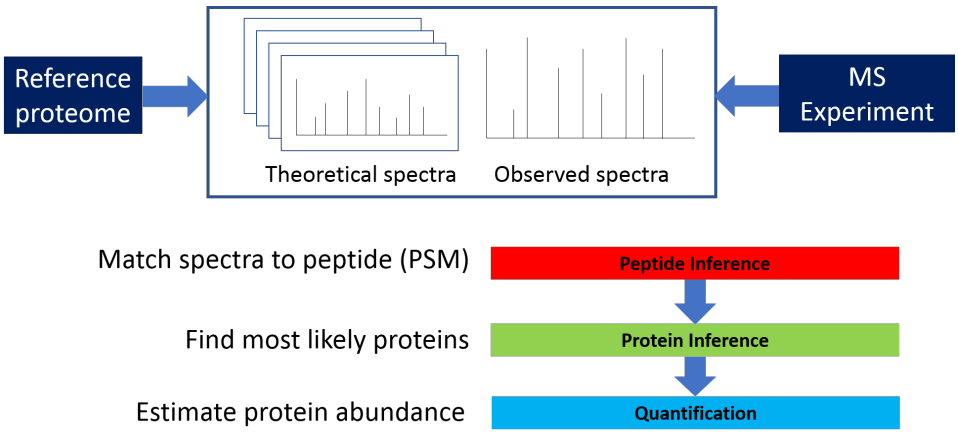


Figure 1.8 Diagram of the MS computational analysis. The PSM process maps the observed spectra to a list of peptides in the reference proteome that could have generated them. In other words, the software infers what peptides were introduced in the spectrometer. The analysis continues during protein inference and quantification.

Given the stochastic nature of the protein cleavage and spectra recording processes, the resulting spectra exhibit variability manifested in missing peaks or spurious ones. Furthermore, random (wrong) matches can be returned by the PSM process when running against a sufficiently big database. This translates to the generation of multiple matches, of which one, if any, will be correct. Therefore, the lists of matches need to be somehow ranked by goodness-of-fit. The issue is addressed by search engines through the deployment of statistical models that provide scoring systems. Assuming the correct protein is present in the database, a good scoring system should give the best score to the right peptide. Under this circumstances, if repeated for several peptides, enough evidence for the presence of individual proteins can be collected.

Multiple search engines exist that implement different matching and scor-

ing algorithms. The most modern ones include MS-GF+, MS-Amanda, Comet, X!Tandem or Andromeda. Notably, the results of each individual search engine can be combined to gather their strengths, at the expense of an increased computational cost and time [8].

1.5 Validation and quality control

The scoring systems implemented in search engines provide the best matches, but they are bound to contain false identifications. Nevertheless, these scores can be used to apply a filter that aims at minimizing the amount of errors.

A common filter is the false discovery rate (FDR), usually set to 1%, indicating that after the its application, only one out of a hundred filtered matches are expected to be false positives (wrong matches).

The most commonly used method to compute the FDR of a list of matches is the target-decoy search. Using this method, the search engine replicates the matching process, using the same spectra, but instead against a decoy database. The decoy database is generated by reversing or more generally applying a randomization technique upon the sequences present in the original database (target).

All matches to the decoy are by definition wrong. Since the basic properties of the decoy (size, composition, etc) remain identical to those of the target the amount of matches to the decoy exhibiting more than a given score s can be regarded as an estimate of the number of false identifications ($\hat{n}_{fp}$) in the list of target results exhibiting at least the same score. This is because the existence of shared properties entails that random matches are equally likely to happen in both databases [9]. Together with the number of PSMs passing a given score in the target ($n_{tp} + n_{tp}$), the FDR can be computed

using equation 1.1.

$$FDR = \frac{\hat{n}_{fp}}{n_{fp} + n_{tp}} \tag{1.1}$$

The equation tells us that we can compute the FDR at any score by counting how many decoy hits have a greater score ($\hat{n}_{fp}$), and dividing by the length of our target hits list. Thus, the score that makes the FDR equal to a predefined value, frequently 0.01 or 1 %, can be computed and used as threshold for the target hits.

The minimal FDR at which a given PSM is considered a valid match constitutes the PSM's . PEP is an estimate of the probability of a given PSM of being an incorrect assignment. Thus, the confidence is the probability of the PSM being a correct assigment [10].

The PSM confidence is defined as $1 - PEP$, where PEP stands for the Posterior Error Probability. Also known as local FDR,

1.6 Peptide and protein inference

Two steps in protein identification can be distinguished:

1. **Peptide inference**: infer the peptides present in the sample.
2. **Protein inferece *proper***: based on the inferred peptides, infer what proteins generated them. This is not trivial as peptides are degenerate and could map to more than one protein.

The result of the PSM step returns the inferred list of peptides. The ensemble of proteins most likely to have generated the list of peptides stemming from the filtered PSMs can be inferred using different algorithms. The degenerate nature of peptides is dealt with the Occam's razor principle, which

states that the most likely solution is the simplest one. Thus, protein inference algorithms aim at explaining the maximum amount of peptides using the least amount of proteins.

1.7 Protein quantification

The combination of all the aforementioned computational analyses yields a list of protein identities that reports the protein composition i.e qualitative information of the original sample. However, in most proteomics applications, quantitative data can be of great interest, as many biological phenomena are manifested mainly through changes in the protein abundances, rather than protein presence alone. For instance, cancer cells in response to a drug could modulate the abundance of several proteins without removing them from the cytosol or introducing new ones.

Protein quantification pipelines can be classified based on whether isobaric labelling was used (label-based) or not (label-free). These are explained in subsection 1.7.1. If the label-free approach is employed, more distinctions can be made based on:

- The proxy used for quantification: spectral counting (SC) or extracted ion currents (XIC). These are explained in 1.7.2
- The way the data are brought to the protein level from the peptide level: summarization-based vs. peptide-based.

1.7.1 Label-based and label-free approaches

Two paradigms exist in protein quantification: label-based and label-free. In label-based quantification, originally identical peptides from a number of different samples are made distinguishable by their masses via the incor-

poration of a label. All label-based methods simultaneously analyze several samples in each experiment, removing the difficulties associated with between-run variability [2]. The finite number of "plexes" available for a given label sets the limit to how many samples can be differentially quantified [11]. Different techniques, like Stable Isotope Labeling by Amino acids in Cell culture (SILAC) or Isotope-Coded Affinity Tags (ICAT), differ in the nature of the label and the way it is introduced. Remarkably, peptide labeling costs can be quite high. This, together with the limited amount of samples that can be compared makes a case for label-free quantification.

In the label-free quantification approach, peptides from different samples are not labelled differently and are thus distinguished by their presence in different, independent MS runs. In order to account for the introduced inter-run variability in peptide identifications and RT, a match-between-runs (MBR) processing step can be carried out (see figure 1.9).

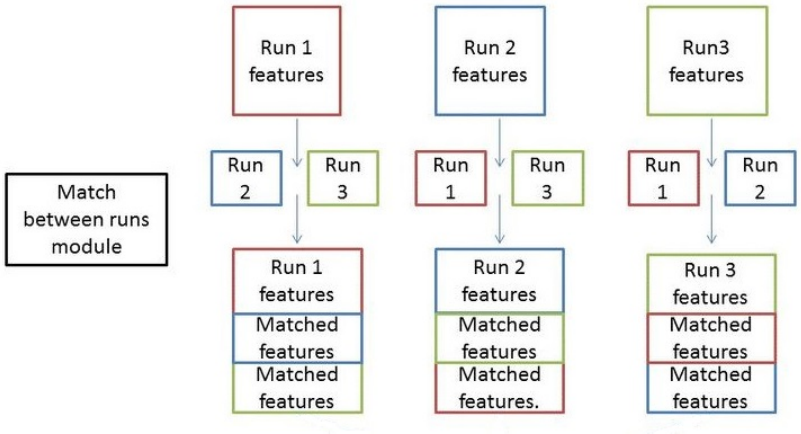


Figure 1.9 Illustration of the MBR step. **A** the information gathered from matches in replicate runs is put to use during a reanalysis of the spectra. Modified from supplementary information in [12].

This way, extra identifications are attained through a reanalysis of the spectra, factoring in the information collected in replicate runs. The RT and precursor mass of unidentified spectra in one replicate is matched to that

of identified spectra in the remaining replicates. As a consequence, more identifications with a lesser fraction of missing datapoints are achieved.

1.7.2 SC and XIC based quantification

Quantification can be spectral counting or XIC based.

On the one hand, spectrum counting based quantification is the simplest method in proteomics. It relies on the rationale that highly abundant peptides will have a higher intensity and are thus more likely to trigger the acquisition of MS/MS spectra. These methods have the advantage that they are very simple to implement and don't require any further data processing

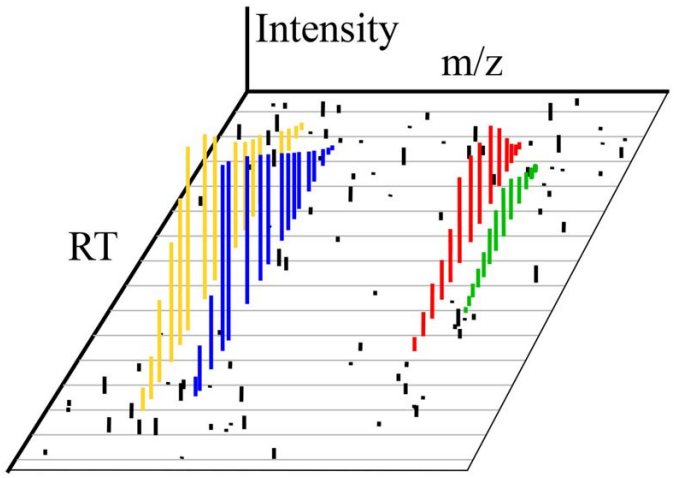
On the other hand, XIC based methods rely on intensity measurements at any level of the MS workflow as proxies for protein abundance. A wide range of algorithms are available to process these data and output estimates of protein abundance. All of them require an intensive preprocessing step, usually including (I) taking the log_2 intensity to make the data distributions symmetrical and thus make it fit for diverse parametric tests, and (II) quantile normalization to address between-runs variability in the intensity measurements. They can be classified in the bases of which MS level is used as proxy for the protein abundances and on whether or not a summarization step is performed to aggregate peptide-level data into protein-level data, or not.

As stated in [11], *although the abundance of proteins and the probability of their peptides being selected for MS/MS sequencing are correlated to some extent, XIC-based methods should clearly be superior to spectral counting given sufficient resolution and optimal algorithms. These advantages are most prominent for low-intensity protein/peptide species, for which a continuous intensity readout is more information-rich than discrete counts of spectra*. For this reason, only the XIC

approach will be regarded in the rest of the manuscript.

For a more robust XIC based quantification, a feature extraction step is usually executed to extract the apex intensity of the identified peak clusters, as shown in figure 1.10.

A



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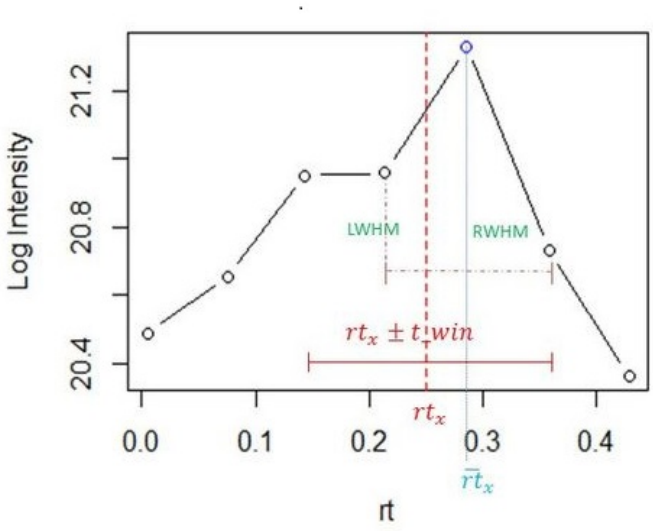


Figure 1.10 Illustration of the apex intensity extraction step.**A** Visualization of a 3D peak cluster on the RT / m/z plane. An ion current at the same m/z ratio, thus corresponding to the same precursor ion, is detected with varying degrees of intensity over a more or less narrow time window, spreading the signal over time. Taken from [13]. **B** A refinement analysis of these data attempts to fit a mathematical model of the signal over time and extract a representative measurement, such as the highest (apex) MS1 intensity. Modified from supplementary information in [12].

1.7.3 XIC-peptide-based models for label-free quantification

The data collected in the mass spectrometer refers to peptides originating from a latent protein composition, given by the original sample. However, the data interpretation requires the transfer of these peptide-level data into the protein level. This can be done by either (I) performing an aggregation of the peptide-level data, where a summary value of the peptide-level data is taken as representative for the protein-level data, or (II) performing the protein quantification directly at the peptide-level by means of linear regression models.

As stated in [14] *peptides originating from the same protein can indeed be considered technical replicates and theoretically should lead to similar abundance estimates. However, the summarization of the peptide intensities into protein expression values is cumbersome, and most summarization-based methods do not correct for differences in peptide characteristics or for the between-sample differences in the number of peptides that are identified per protein. This might introduce bias and differences in uncertainty between the aggregated protein expression values, which are typically ignored in downstream data analysis steps*.

It is for this reason that peptide-based models offer the statistical framework required to learn as much from the data as possible. This translates into improved results when compared to the other aforementioned methods [14]. This hypothesis is the motivation for the method explained in chapter 4.

Chapter 2

A label-free quantification proteomics pipeline

Summary

A pipeline making use of the set of tools published by the Compomics and StatOmics groups SearchGUI, PeptideShaker, moFF and MSqRob CITE THEM, was developed to support complete label-free protein quantification analyses using the most recent advances in the field with open-source software. The pipeline can be run on Linux computer clusters to perform (I) peptide to spectrum matching against a reference database, (II) quality control and filtering, (III) MBR and feature extraction, (IV) protein inference and (V) relative quantification. Its output can be passed to follow-up analyses in R or Python to get a biological interpretation of the results. A benchmark of its performance was accomplished using the proteome benchmark dataset published in [11]. The results exhibited an accuracy similar to those achieved by the MaxLFQ [11] software, excepting a bias produced by the sample fractionation of this dataset.

2.1 Introduction

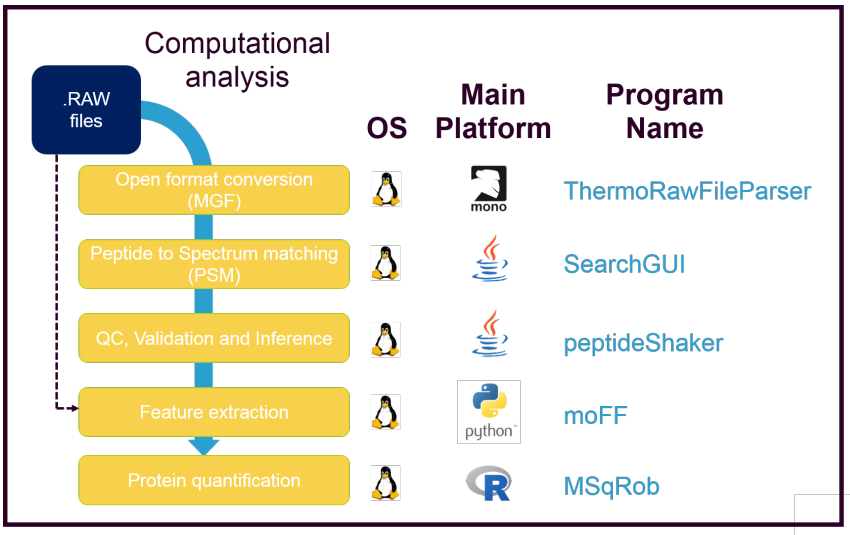


Figure 2.1 Pipeline

Several proteomics pipelines are available on the internet under different licensing conditions. Many are released as closed-source software, where information on how the program works is kept from the user. This is a serious drawback as it hinders the study of the implemented models and its customisation. Open-source, free alternatives, like the Trans Proteomic Pipeline (TPP) [15] or openMS [16] are nevertheless available for the community. MaxQuant, a freeware but closed-source proteomics analysis suite [17], has been extremely successfully adopted by the scientific community due to its ease of use and a comprehensive pipeline.

However, still only few of these tools have a fully Linux-supported, well-documented, command-line version available, which would make customised, automatic streamline analyses much easier to perform. As a GUI would not be required to run the pipeline, it would also be scalable to big datasets. Finally, having free licenses would imply that anyone, provided the technical knowledge, can perform the computational analysis independently, thus

speeding the data to knowledge turnover in NZ or any organization making use of it.

The development of a pipeline achieving these goals will be described in this chapter.

2.2 Materials and Methods

2.2.1 Data generation and loading

The proteome benchmark dataset from [11] was reanalysed starting at the output RAW files available at the PRIDE repository [1]. Briefly, the *Homo sapiens* and *E. coli (strain K12)* proteomes were mixed in 1:1 (condition L) and 1:3 (condition H) proportions, with 3 replicates for each combination. Moreover, each of the 3 replicates of the 2 conditions was analysed over 24 fractions. This experimental setting thus generated a total of $2 \times 3 \times 24 = 144$ RAW files. One file was missing in the repository. The ThermoRawFileParser CITE THERMORAWFILEPARSER program was used to convert RAW files to the open format MGF (Mascot Generic Format).

2.2.2 Decoy database preparation and search

The spectra saved in the MGF files obtained in the previous step were passed to the MS-GF+ search engine CITE by means of the SearchGUI `SearchCLI` tool CITE utility. The search parameters were set using the `IdentificationParametersCLI`. In order to account for potential post-translational modifications, the search was conducted allowing for the following variable modifications: oxidation of M and deamidation of N and Q. Moreover, C carbamidomethylation was set as fixed modification. The

[1] https://www.ebi.ac.uk/pride/archive/projects/PXD000279

enzyme was set to semispecific Trypsin, allowing for a non-tryptic cleavage on any side of the peptide. Up to two missed cleavages were allowed. The precursor tolerance was 10 ppm and the fragment tolerance 0.5 Da.

The target database was created by combining the Uniprot proteomes for *E. coli (strain K12)* (UP000000625) and *Homo sapiens* (UP000005640), downloaded in June 2018. The decoy database was created using the `FastaCLI` utility in SearchGUI by reversing all sequences in the target.

2.2.3 Quality control and validation

The SearchGUI results were filtered using the default built-in checks available in the PeptideShaker utility `PeptideShakerCLI` [2]. By default, the FDR was set to 1%. PEP and confidence statistics were computed using the PeptideShaker built-in algorithms. Output was extracted via the Default PSM report txt file, available in the `ReportCLI` utility.

2.2.4 Data refinement

The moFF command line utility CITE was applied to perform (I) match-between-runs and (II) extract MS1 apex intensity of each peak cluster. This required passing the original RAW files, together with the Default PSM report from PeptideShaker. Output was exported to a peptide summary file, containing one row per peptide and for every peptide, the detected apex intensity in each sample.

[2] https://github.com/compomics/peptide-shaker/issues/300

2.2.5 Quantification

Relative quantification was performed using the MSqRob utility by passing the peptide summary file from moFF. Prior to quantification, the data was preprocessed using the `preprocess_MSnSet()` function. In a nutshell, (I) MS1 apex intensities were log_2 transformed, (II) quantile normalized, (III) peptides belonging to protein groups that contained one or more proteins that were also present in a smaller protein group were discarded [18], and (IV) protein groups with only 1 peptide were dropped.

Once preprocessing is done, the ridge regression model with Huber weights and empirical Bayes estimation of protein variance, implemented in the MSqRob package [18], was fit to every protein individually. The peptide and fraction effects were considered random, while the condition was treated as a fixed effect. The significance of the treatment effect differences was assessed through a Student's T test, implemented in the `test.contrast()` function. Only protein groups that could be unambiguously mapped to one of the organisms were considered for the analysis.

2.2.6 Code implementation

2.3 Results

2.3.1 Thousands of spectra were identified and validated

The preprocessing of the RAW files produced by the mass spectrometer into the MGF open format enabled searchGUI to dispose of the registered spectra. PeptideShaker quality control and filtering capabilities (see figure 2.2) carried out the required search results validation. As expected, matches to the target and decoy exhibited similar score distributions at low score

values, while a divergence is observed at higher score values. Likewise, the m/z error was found to be closer to 0 on validated PSMs than on those which did not pass the 1 % FDR filter. The application of this filter implied that the FNR (false negative rate) was set to 5 %, i.e 5 out of every 100 discarded matches were estimated to be true positives.

The 1% FDR cutoff selected PSMs with a score higher than 78, which translated to a confidence of at least 65%.

The combination of both programs enabled the identification and validation (matching) of thousands of spectra with high confidence in all samples. However, when compared to the total amount of spectra available, the percentage of matched spectra was on average 37.4%, with a marked decrease starting at fraction 14 (see figure 2.3).

2.3.2 Protein inference

Total peptides	Unique peptides	Non-unique	Proteins	Protein groups
46595	27384	19211	25056	10039

Table 2.1 Protein inference results. The counts of the different molecular entities detected by peptideShaker is displayed.

A total of 46595 peptides were inferred to be present in at least one of the fractions. Of them more than 27 thousand mapped to a unique protein. However, a significant amount mapped to more than one protein. Such peptides are called non-unique peptides. The 25056 detected proteins were grouped into 10039 protein groups. Protein groups are peptide generating entities for which enough data to confirm the presence of at least one of them is available, but not to exactly assess which of them. Thus, protein inferene algorithms create them when a does not uniquely map to a protein

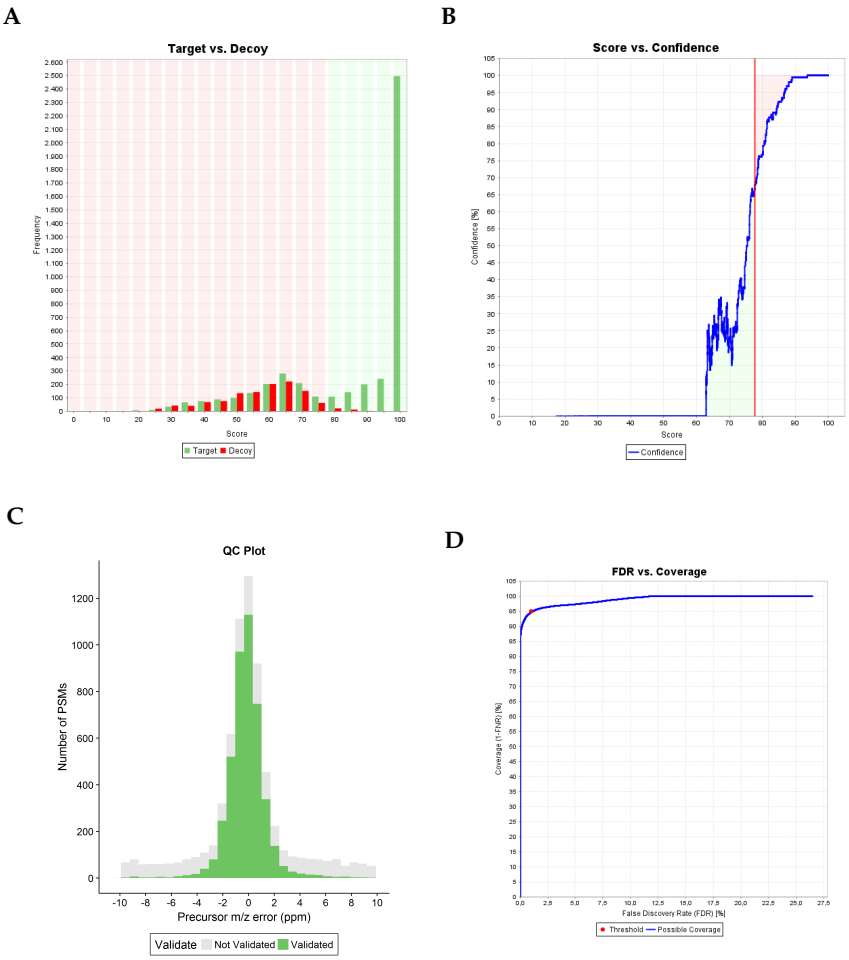


Figure 2.2 Quality control and validation of the 13th fraction of the third replicate in condition L. **A** Score distribution for matches to the decoy and the target databases. **B** Evolution of the PSM score with confidence. The implemented cutoff at FDR of 1 % is displayed with a red vertical line. **C** Distribution of the difference between the predicted and measured m/z values, segregated by validation status. **D** ROC curve built upon the number of false positives and negatives estimated from the decoy search. The cutoff is displayed as a red dot.

becuase it is not possible to distinguish which protein it truly comes from. Protein grouping decreased the amount of considered protein entities to 10039.

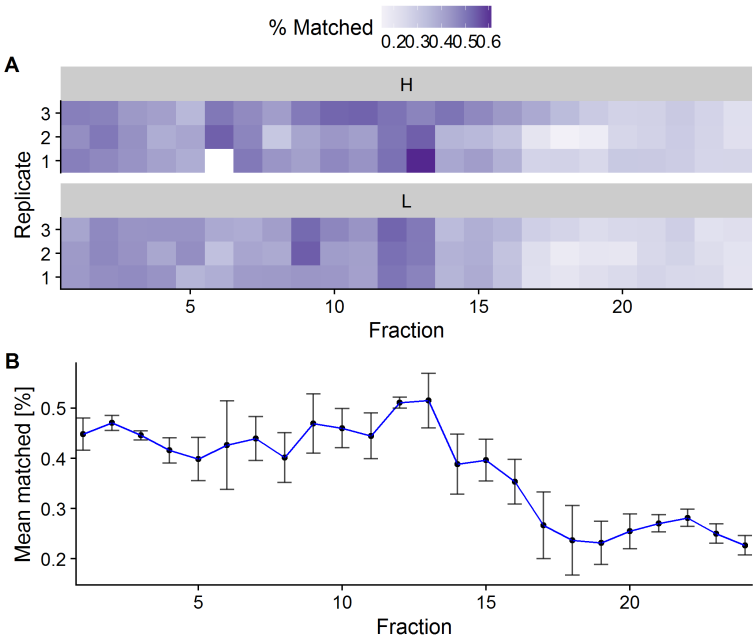


Figure 2.3 Percentage of matched spectra in all samples. The total number of spectra per sample ranged between 5894 and 20249. **A** Percentages are encoded with a blue palette, the darker, the higher, and viceversa. **B** The mean for each analysed fraction across conditions and replicates is displayed together with error bars to represent the standard deviation. The sixth fraction of the first replicate in condition H was missing in the PRIDE data repository.

Protein groups are very frequent due to several factors. For example, protein isoforms, consisting of protein sequences differing in potentially only one aminoacid, are very difficult to resolve. PeptideShaker executes a smart protein grouping by harnessing the annotation of proteins and classifying protein groups based on how well the annotation backs the protein group.

However, in the present analysis the quality of the protein group was not taken into account, as explained in the Materials and Methods.

2.3.3 MBR and Apex intensity extraction capacitate for accurate quantification

The match between runs step allowed for increased identifications by transferring successful matches between replicate runs. The results of this process for the 13th fraction of the L condition is shown in figures **??** and 2.4.

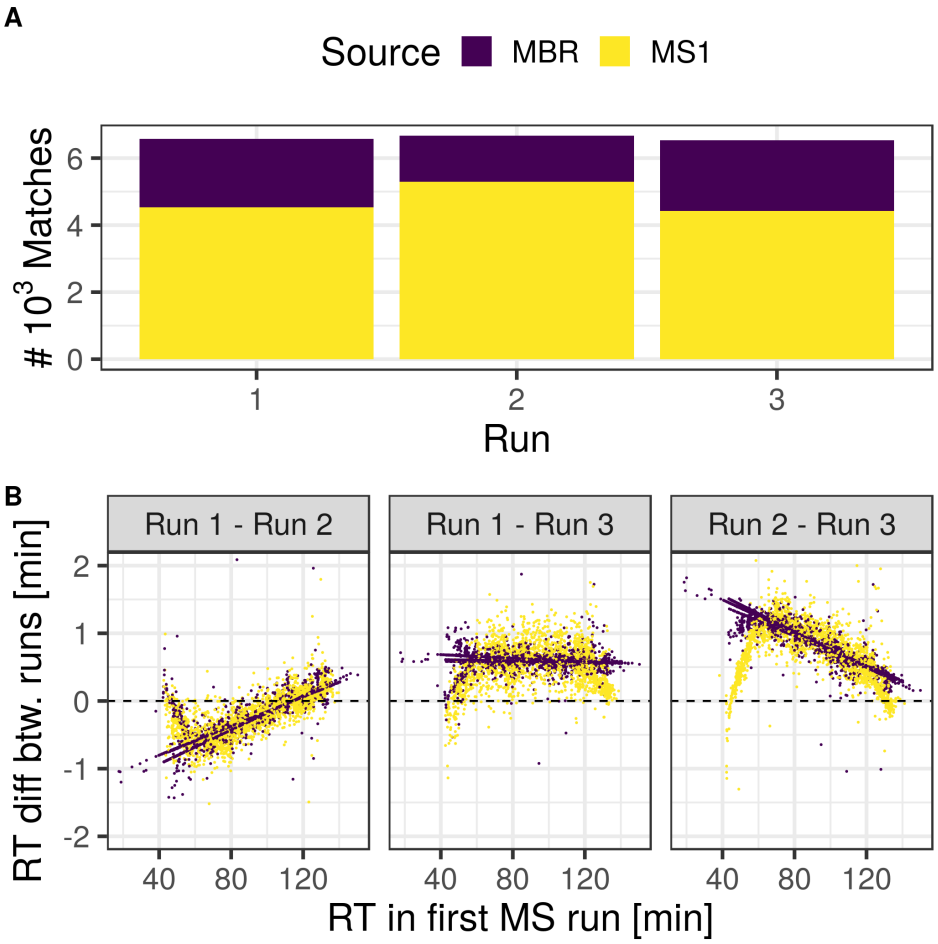


Figure 2.4 Match Between Runs with moFF. **A** Count of identifications on each run segreagated by source. More than 4k spectra were identified and validated by SearchGUI+peptideShaker. In this particular case, hundreds of new identifications were accomplished. **B** Match visualization. Every dot represents a peptide shared across 2 runs. The coordinate system illustrates its retention time on the first run on the x axis and the difference with the second run on the y axis. The color depicts whether the identification was carried out during the PSM process (MS1), or thanks to a cross-identification achieved by the MBR module (MBR).

Once as many identifications as possible were gathered, a refinement of the measured MS1 intensity can be implemented to select the apex of every

peak cluster, which yields robust intensity measurements for each sample (see figure 2.5).

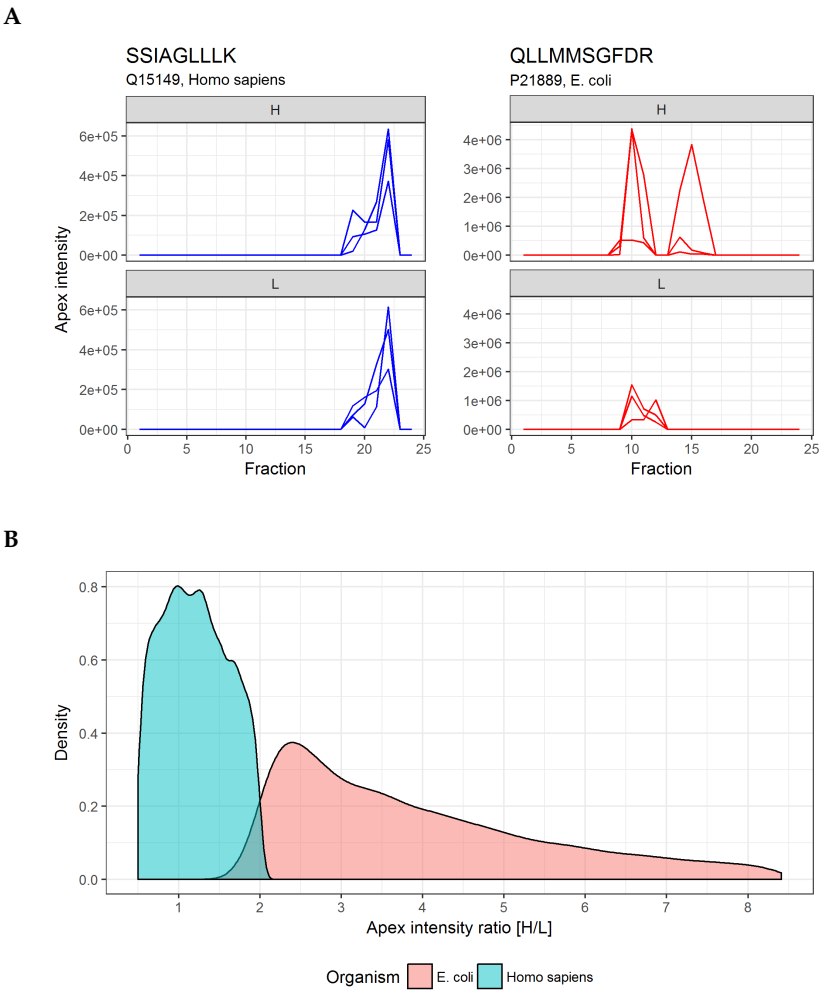


Figure 2.5 A The apex intensity profile across fractions for 2 different peptides, one from *Homo sapiens*, and one from *E. coli*. The figure illustrates the intrinsic intensity variability between technical replicates, particularly in the case of the *E. coli* QLLMMSGFDR peptide, as it was almost non-existent in one of the runs. **B** The expected pattern of overall similar intensities for the *Homo sapiens* data and 3-fold higher intensities for the *E. coli* data in condition H was observed, confirming an acceptable performance of the protocol.

2.3.4 Quantification

The extracted apex MS1 intensities were used as proxy for peptide abundance to estimate protein log_2(ratios), or *log2-fold-changes* across condition H and condition L. During quantification, it is important to take into account as many of the effects influencing protein quantification. In this case, besides the different H and L conditions, peptide, run and fraction effects could be distinguished. The MSqRob quantification engine treats effects differently depending on whether or not they are random, or fixed. Fixed effects should have a consistent impact in the ion currents measured in the spectrometer, whereas random effects are truly random and thus unpredictable.

The result of the process was the successful quantification of 5307 protein groups out of 10039 (see table 2.2).

The performance of the quantification can be evaluated thanks to the experimental design of the dataset. Since the *E. coli* proteome was mixed on a 3:1 ratio in condition H, a log2FC of $log2(3) = 1.58$ is expected for proteins coming from this organism. Likewise, human proteins should have a log2FC of 0. As such, the evaluation can be reformulated as a classification task were the quantification algorithm tries to distinguish between proteins coming from one or the other organism.

Some of the most frequent evaluations of the performance of a binary classifier are Receiver Operating Characteristic (ROC) and Precision-Recall (PR) curves [19], which demonstrate the performance of the algorithm at different cutoff values of a predictor variable. Together, they show the False Positive Rate (FPR), the False Negative Rate (FNR) and the precision exhibited by the algorithm.

	log2FC	qval	Protein	*Organism*
1	-4.76E-01	1.42E-24	P78527	*Homo sapiens*
2	6.80E-01	6.75E-24	P0A8V2	*E. coli*
3	8.50E-01	4.39E-22	P25516	*E. coli*
4	7.07E-01	2.05E-18	P63284	*E. coli*
5	1.04E+00	1.21E-17	P37095	*E. coli*
6	7.78E-01	1.51E-16	P13029	*E. coli*
7	8.89E-01	5.62E-16	P77804	*E. coli*
8	8.53E-01	1.72E-15	P23721	*E. coli*
9	-6.97E-01	1.41E-14	P09874	*Homo sapiens*
10	1.37E+00	1.41E-14	P37666	*E. coli*

Table 2.2 Results of the quantification pipeline. The 10 protein groups with the lowest *q-val* as estimated by MSqRob are shown. Notably, eight correspond to proteins belonging to *E.coli*. The two human proteins exhibited in both cases an absolute value of the log2FC below 1.

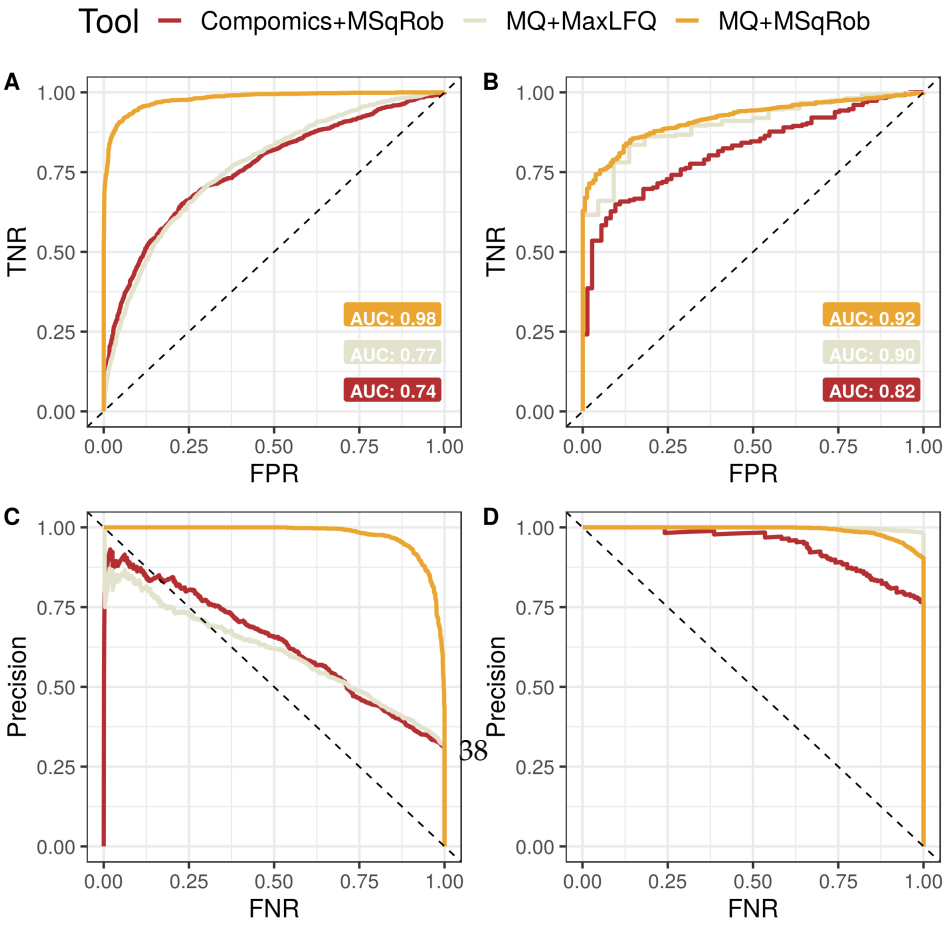


Figure 2.6 ROC and PR curves displaying the behaviour of 3 different

In the present problem, they can be used to show how good the *q-val* associated to a protein group is at separating the two proteomes. Thus, *E. coli* proteins are treated as positives, and those from *Homo sapiens* are treated as negatives. Moreover, a filter based on the log2FC can be applied to enrich the data in positives and facilitate the classification task. The result of this analysis is shown in figure 2.6). Remarkably, the pipeline presented in this manuscript (Compomics+MSqRob) achieved similar results to those resulting from using MaxQuant and MaxLFQ (MQ+MaxLFQ). Nonetheless, the latter performed clearly better when the log2FC filter was applied. In all cases, the combination of MaxQuant and MSqRob (Mq+MSqRob) was as good as MQ+MaxLFQ, if not much better.

Additionally, the accuracy with which each program assigns a log2FC estimate to each protein group, and how significantly different from the null value of 0 it was found to be, provides further insights on the pros and cons of each pipeline, as shown in figure **??**.

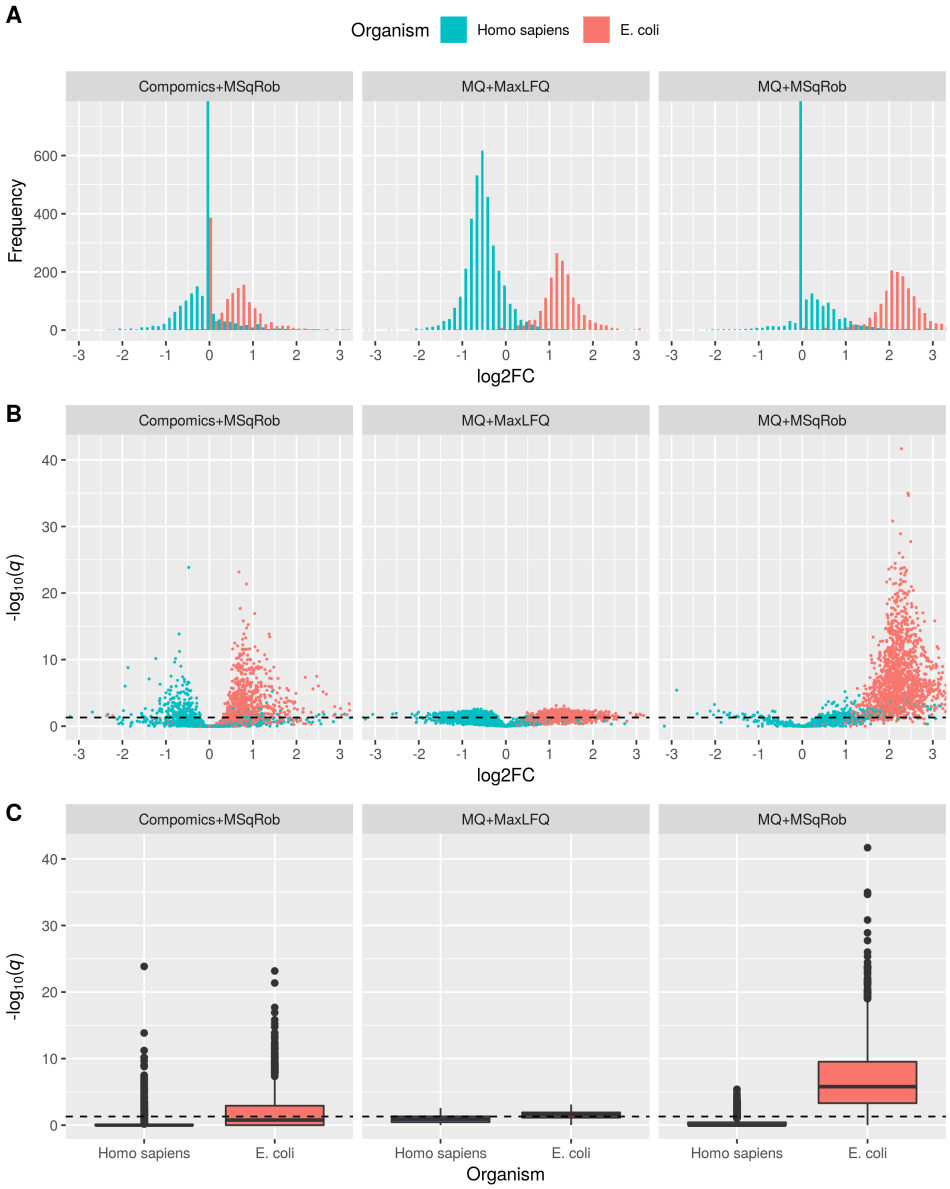

A
Organism
Homo sapiens
E. coli
Compomics+MSqRob
MQ+MaxLFQ
MQ+MSqRob
Frequency
log2FC
B
-log10(q)
C
Homo sapiens
E. coli
Organism

Figure 2.7

2.4 Discussion

2.4.1 Improvement of the PSM step

The low attained matching rate manifests existing room for improvement in the currently available tools. Remarkably, it has been shown that the combined usage of multiple search engines can increase identifications, since the different statistical frameworks implemented in each of them compensate each other's caveats. Furthermore, *de novo* search engines are available and supported by SearchGUI. Their usage could contribute to further improvements in the identification rate.

The most important reason why many spectra remain unidentified is the presence of post-translational modifications (PTMs), which exponentially increase the search space, forcing most workflows to discard many of the peptides featuring a PTM. New approaches to the problem are emerging, mainly machine learning methods for the handling of unexpected modifications CITE ralf gabriels. Moreover, the prediction of MS2 peak intensities patterns from peptide sequences promises to increase the amount of evidence available during the PSM process, thus boosting correct identifications [20] [21].

Finally, the extremely low matching rates in the latter fractions translates to decreased contributions to the number of identifications. This indicated that decreasing the number of fractions would not have had a major impact in the experiment's depth.

2.4.2 Improvement of the quantification step

The deployed relative quantification approach successfully manages to estimate the log2FC or thousands of proteins in the dataset. However, other

free tools are available to perform the same type of analysis.

2.5 Conclusion

Chapter 3

Pipeline benchmarking on NZ data

Summary

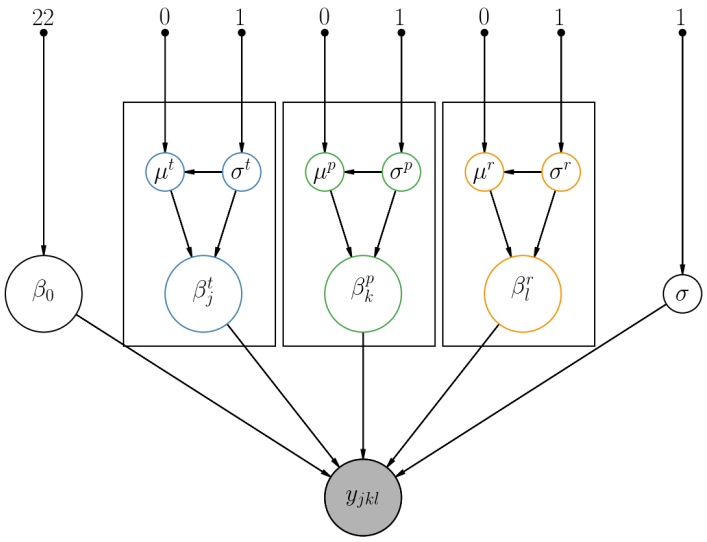
A benchmark dataset generated by NZ technicians was run through the pipeline presented in chapter 2 to showcase its performance. The experiment consisted of the application of two different treatments to THP-1 cell cultures. One group was subjected to an experimental procedure triggering the immunological response, while the other group was subjected to a negative control. The analysis of the resulting dataset through the aforementioned pipeline should thus reflect a change in the protein profile corresponding to an activation of the immune system in the first group when compared to the second. The results confirmed that the computational analysis successfully captured this response. It was concluded that the software can be used in future experiments where the expected biological phenomena is not known.

Chapter 4

BayesQuant: A Bayesian framework for the computation of pair-wise fold change estimates

Summary

The current label-free quantification methods, reviewed in section 1.7 all rely on frequentist statistics, which return point estimates of model parameters such as the estimate of the fold change across conditions. However, a Bayesian based approach to this problem is lacking in the literature. As a response to this shortcoming, a statistical model implemented in the probabilistic programming framework Pymc3 was developed and tested on the same benchmark dataset from chapter 2). The execution of the three steps required when doing Bayesian modelling, mainly (I) model implementation, (II) computation of posterior probabilities and (III) model checking, will be described for this particular problem in the present chapter, together with a discussion on its usability and its strengths.


22
0
1
0
1
0
1
1
μ^t
σ^t
μ^p
σ^p
μ^r
σ^r
β_0
β_j^t
β_k^p
β_l^r
σ
y_{jkl}

Conclusion

Appendix

```python
import pymc3 as pm
import numpy as np
import matplotlib.pyplot as plt
from theano import shared
import shutil
import os

def protein_model(observed_sh, feats_sh, x_treat_sh, x_pep_sh, x_run_sh, x_estimate_sh,
    n_peptides, model_name, n_draws=1000, n_chains=3,
    hierarchical_center=False, remove_backend=True, sequence=False):

    # Check working environment
    if not os.path.isdir("traces") or not os.path.isdir("plots/traceplots"):
        msg = "Please create a traces dir and a plots/traceplots dir before running this code"
        raise Exception(msg)

    if remove_backend and os.path.isdir(model_name):
        shutil.rmtree(model_name)

    # The number of proteins in this model is always one
    # i.e this model is fitted protein-wise
    n_prots = 1
    # The number of features is set to 9 for now
    # All peptides have 9 features, stored in feats_shared
    n_features = 9


    with pm.Model() as model:

        # Build a hyerarchical linear model of
        # log2(MS1 intensities) by accounting for:

            # Peptide effect
            # Run (batch) effect
            # Treatment effect
            # Remaining random effects

        # The difference in treatment effects is an estimate of the log2FC


        # Set a prior on the intercept
        intercept = pm.Normal("intercept", 22, 1)

        # Set a prior on the remaining random effects
        sigma = pm.HalfNormal('sigma', 1)

        ## Set priors on the peptide effect
        ################################################
        sigma_pep = pm.HalfNormal('sigma_pep', 1)
```

```
50
51          # Not using the sequence
52          if not sequence:
53              mu_pep = pm.Normal('mu_pep', mu=0, sd=sigma_pep, shape=(n_peptides, 1))
54
55          # Using the peptide sequence
56          else:
57              # sequence based modelling
58              mu_theta = pm.Normal('theta_generic', 0, sigma_pep, shape = 1)
59              theta = pm.Normal('theta', mu_theta, sigma_pep, shape = (n_features, 1))     # 9x1
60              theta_inter = pm.Normal('theta_inter', mu_theta, sigma_pep, shape = 1)
61              mu_pep = pm.Deterministic("mu_pep", theta_inter + feats_sh.dot(theta)) # n_peptidesx1
62
63
64          ## Set priors on the treatment and run effects
65          ################################################
66          sigma_treat = pm.HalfNormal('sigma_treat', 1)
67          mu_treat = pm.Normal('mu_treat', 0, sigma_treat)
68          sigma_run = pm.HalfNormal('sigma_run', 1)
69          mu_run = pm.Normal('mu_run', 0, sigma_run)
70
71          # Standard implementation of the hyerarchies
72          if hierarchical_center:
73              pep = pm.Normal("pep", mu_pep, sigma_pep) # n_peptidesx1
74              treat = pm.Normal('treat', mu_treat, sigma_treat, shape = (n_prots*2, 1))
75              run = pm.Normal('run', mu_run, sigma_run, shape = (n_prots*6, 1))
76
77          # Reparametrization to escape funnel of hell as noted in
78          # http://twiecki.github.io/blog/2017/02/08/bayesian-hierchical-non-centered/
79          else:
80              pep_offset = pm.Normal("pep_offset", mu=0, sd=1, shape = (n_peptides, 1))
81              pep = pm.Deterministic("pep", mu_pep + pep_offset * sigma_pep)
82              treat_offset = pm.Normal("treat_offset", mu=0, sd=1, shape=(n_prots*2, 1))
83              treat = pm.Deterministic("treat", mu_treat + treat_offset*sigma_treat)
84              run_offset = pm.Normal("run_offset", mu=0, sd=1, shape=(n_prots*6, 1))
85              run = pm.Deterministic("run", mu_run + run_offset*sigma_run)
86
87
88          # Model the effect for all peptides
89          # The sh variables consist of -1,0,1 matrices telling pymc3
90          # which parameters shall be used with each peptide
91          # In practice, the "clone" each parameter to fit the shape of observed_sh
92          # observed_sh is a n_peptides*6x1 tensor
93          # The first 6 numbers store the MS1 intensities of the first peptide in the 6 runs
94          # The next 6 those of the second peptide, and so on
95
96          estimate = pm.Deterministic('estimate', pm.math.sum(x_estimate_sh.dot(treat), axis=1))
97          treatment_effect = pm.Deterministic("treatment_effect", pm.math.sum(x_treat_sh.dot(treat), axis=
98          peptide_effect = pm.Deterministic("peptide_effect", pm.math.sum(x_pep_sh.dot(pep), axis=1))
99          run_effect = pm.Deterministic("run_effect", pm.math.sum(x_run_sh.dot(run), axis=1))
```

```
100
101         # BIND MODEL TO DATA
102         mu = pm.Deterministic("mu",
103             intercept + treatment_effect + peptide_effect + run_effect) #n_peptides*6x1
104         if hierarchical_center:
105             obs = pm.Normal("obs", mu, sigma, observed=observed_sh)
106         else:
107             obs_offset = pm.Normal("obs_offset", mu=0, sd=1, shape=(n_peptides*6,1))
108             obs = pm.Normal("obs", mu+obs_offset*sigma, sigma, observed=observed_sh)
109
110
111     print("Success: Model {} compiled".format(model_name))
112
113     with model:
114         # Parameters of the simulation:
115         # Number of iterations and independent chains.
116         n_sim = n_draws*n_chains
117
118         # Save traces to the Text backend i.e a folder called
119         # model_name containing csv files for each chain
120         trace_name = 'traces/{}'.format(model_name)
121         db = pm.backends.Text(trace_name)
122         trace = pm.sample(draws=n_draws, njobs=n_chains, trace=db,
123                           tune=2000, nuts_kwargs=dict(target_accept=.95))
124
125     # Save a traceplot
126     pm.traceplot(trace, varnames=["estimate"])
127     traceplot = "plots/traceplots/{}.png".format(model_name)
128     plt.savefig(traceplot)
129     plt.close()
130
131     return model
```

Bibliography

[1] David L. Nelson. *Lehninger : principles of biochemistry*. W. H. Freeman and Co,, New York :, 5 edition, 2008.

[2] L. Martens I. Eidhammer, K. Flikka and S.-O. Mikalsen. *Computational Methods for Mass Spectrometry Proteomics*. 2008.

[3] Hamid Mirzaei. *Modern Proteomics – Sample Preparation, Analysis and Practical Applications*, volume 919. 2016.

[4] Keqi Tang, Jason S. Page, and Richard D. Smith. Charge competition and the linear dynamic range of detection in electrospray ionization mass spectrometry. *Journal of the American Society for Mass Spectrometry*, 15(10):1416–1423, 2004.

[5] Alan G. Marshall, Greg T. Blakney, Tong Chen, et al. Mass Resolution and Mass Accuracy: How Much Is Enough? *Mass Spectrometry*, 2(Special_Issue):S0009–S0009, 2013.

[6] Kenneth Verheggen, Helge Ræder, Frode S. Berven, et al. Anatomy and evolution of database search engines-a central component of mass spectrometry based proteomic workflows. *Mass Spectrometry Reviews*, (April 2016):1–15, 2017.

[7] P. Roepstorff and J. Fohlman. Proposal for a Common Nomenclature for Sequence Ions in Mass Spectra of Peptides. *Biological Mass Spectrometry*, 11(11):601–601, 1984.

[8] David Shteynberg, Alexey I. Nesvizhskii, Robert L. Moritz, and Eric W. Deutsch. Combining Results of Multiple Search Engines in Proteomics. *Molecular & Cellular Proteomics*, 12(9):2383–2393, 2013.

[9] J. E. Elias and Gygi. S. P. Target-Decoy Search Strategy for Mass Spectrometry-Based Proteomics. *Current Protocols in Protein Science*, 604(SUPP.60):55–71, 2010.

[10] Alexey I. Nesvizhskii. A survey of computational methods and error rate estimation procedures for peptide and protein identification in shotgun proteomics. *Journal of Proteomics*, 73(11):2092–2123, oct 2010.

[11] Jürgen Cox, Marco Y. Hein, Christian A. Luber, et al. Accurate Proteome-wide Label-free Quantification by Delayed Normalization and Maximal Peptide Ratio Extraction, Termed MaxLFQ. *Molecular & Cellular Proteomics*, 13(9):2513–2526, 2014.

[12] Andrea Argentini, Ludger J.E. Goeminne, Kenneth Verheggen, et al. MoFF: A robust and automated approach to extract peptide ion intensities. *Nature Methods*, 13(12):964–966, 2016.

[13] Rob Smith, Andrew D. Mathis, Dan Ventura, and John T. Prince. Proteomics, lipidomics, metabolomics: A mass spectrometry tutorial from a computer scientist's point of view. *BMC Bioinformatics*, 15(Suppl 7), 2014.

[14] Ludger J.E. Goeminne, Andrea Argentini, Lennart Martens, and Lieven Clement. Summarization vs peptide-based models in label-free quantitative proteomics: Performance, pitfalls, and data analysis guidelines. *Journal of Proteome Research*, 14(6):2457–2465, 2015.

[15] Eric W Deutsch, Luis Mendoza, David Shteynberg, et al. A Guided Tour of the Trans-Proteomic Pipeline. *Proteomics*, 10(6):1150–1159, 2011.

[16] Marc Sturm, Andreas Bertsch, Clemens Gröpl, et al. Openms – an open-source software framework for mass spectrometry. *BMC Bioinformatics*, 9(1):163, Mar 2008.

[17] Jürgen Cox and Matthias Mann. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnology*, 26(12):1367–1372, 2008.

[18] Ludger J. E. Goeminne, Kris Gevaert, and Lieven Clement. Peptide-level Robust Ridge Regression Improves Estimation, Sensitivity, and Specificity in Data-dependent Quantitative Label-free Shotgun Proteomics. *Molecular & Cellular Proteomics*, 15(2):657–668, 2016.

[19] Andrew P. Bradley. The use of the area under the ROC curve in the evaluation of machine learning algorithms. *Pattern Recognition*, 30(7):1145–1159, jul 1997.

[20] Ufuk Kirik, Jan C Refsgaard, and Lars J Jensen. Improving peptide-spectrum matching by fragmentation prediction using Hidden Markov Models. *bioRxiv Bioinformatics*, 2018.

[21] Sven Degroeve, Lennart Martens, and Igor Jurisica. MS2PIP: A tool for MS/MS peak intensity prediction. *Bioinformatics*, 29(24):3199–3203, 2013.