Quality control in mass spectrometry-based proteomics

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Running title

Proteomics quality control

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Keywords

mass spectrometry, proteomics, quality control

Contents

I. Introduction **5**

II. Managing LC-MS variability through quality control **8**

A. Types of QC samples 8

B. Incorporating QC samples 11

C. Quality control throughout the experimental workflow 12

1. Sample preparation 12

2. Liquid chromatography 19

3. Mass spectrometry 22

4. Bioinformatics data interpretation 23

III. Conclusion **25**

Acknowledgments **26**

References **27**

Abstract

Mass spectrometry is a highly complex analytical technique and mass spectrometry-based proteomics experiments can be subject to a large variability, which forms an obstacle to obtaining accurate and reproducible results. Therefore, a comprehensive and systematic approach to quality control is an essential requirement to inspire confidence in the generated results. A typical mass spectrometry experiment consists of multiple different phases including the sample preparation, liquid chromatography, mass spectrometry, and bioinformatics stages. We review potential sources of variability that can impact the results of a mass spectrometry experiment occurring in all of these steps, and we discuss how to monitor and remedy the negative influences on the experimental results. Furthermore, we describe how specialized quality control samples of varying sample complexity can be incorporated into the experimental workflow and how they can be used to rigorously assess detailed aspects of the instrument performance.

I. Introduction

Proteomics is a crucial domain in modern biological and biomedical research (Aebersold et al., 2016). The current method of choice to identify and quantify complex protein samples is often liquid chromatography (LC) followed by mass spectrometry (MS). The importance of these techniques is exemplified by their use in large-scale research initiatives, such as the two recent attempts at providing a draft of the human proteome (MS Kim et al., 2014; Wilhelm et al., 2014) or the ongoing Human Proteome Project (HPP) by the Human Proteome Organization (HUPO) (Legrain et al., 2011; Marko-Varga et al., 2013; Lane et al., 2014; Omenn, Lane, Lundberg, Beavis, Nesvizhskii, et al., 2015; Omenn, Lane, Lundberg, Beavis, Overall, et al., 2016), where LC-MS techniques are used to identify, quantify, and characterize the human proteome.

As illustrated in figure 1, a bottom-up LC-MS experiment consists of multiple different stages. First, various sample preparation measures ensure that the biological samples are optimally suited for MS analysis. Typical steps include denaturation, reduction, and alkylation of the proteins. The denatured proteins are subsequently digested into peptides through proteolytic cleavage, typically by trypsin. Next, this peptide mixture is processed through liquid chromatography, which separates the peptides based on their hydrophobicity. After eluting from the column, the peptides are ionized to obtain a charge by electrospray ionization (ESI), and the derived spectra are generated in the mass spectrometer. Whether this is done in a data-dependent acquisition (DDA) or data-independent acquisition (DIA) manner, for a typical discovery experiment, in both approaches as many spectra as possible are generated through tandem mass spectrometry (MS/MS), whereas for a targeted experiment specific peptides of interest are exclusively monitored (Picotti et al., 2012). Finally, the generated spectral data is interpreted through various bioinformatics means (Nesvizhskii et al., 2007; Noble et al., 2012). Peptides can be identified from the mass spectra through sequence database searching (Eng et al., 2011), spectral library searching (Griss, 2016; Shao et al., 2016), or *de novo* sequencing (Medzihradszky et al., 2015); and the peptides can be mapped back to their originating proteins through protein inference (Huang et al., 2012). Additionally, protein quantification (Bantscheff et

al., 2012; Gonzalez-Galarza et al., 2012) and other advanced analyses may be performed.

As succinctly described above, performing a mass spectrometry experiment is an intricate process, and each of these different steps has to be optimized to acquire accurate and reproducible results. Unfortunately, despite the many recent technological and computational advances, the results of an experiment can still be subject to a large variability (Addona et al., 2009; Tabb, Vega-Montoto, et al., 2010). As represented in figure 2, this variability can originate from multiple sources (Piehowski et al., 2013): the different stages of an LC-MS experiment can each exhibit stochastic behavior and influence one another, contaminants can inadvertently be present (BO Keller et al., 2008), and the optimal computational interpretation is often not obvious (Bell et al., 2009). Furthermore, instrument drift and sample degradation can introduce a longitudinal variability (Bennett et al., 2015; Campos et al., 2015). Most notably, instrument interventions, such as a preventive maintenance, have a considerable influence upon the results (Bennett et al., 2015). Especially in regard to current large-scale studies this is of major importance, as measurements obtained at different times can only be correctly compared with each other if they were obtained under consistent and comparable conditions.

Therefore, in order to inspire confidence in the obtained results, it is of vital importance that appropriate quality assurance (QA) and quality control (QC) measures are taken to monitor and control the existing variability (Mann, 2009; Albar et al., 2013; Martens, 2013; Tabb, 2013). A systematic approach to quality control makes it possible to objectively assess the quality of a mass spectrometry experiment, and empirical quantitative measures enable the intra-study, intra-laboratory, and inter-laboratory comparison of the performance of mass spectrometry runs (Ivanov et al., 2013). As mentioned previously, these quality assessments are crucial to validate the results produced by long-term multi-site projects (Campos et al., 2015), such as the HUPO's Human Proteome Project (Legrain et al., 2011; Marko-Varga et al., 2013; Lane et al., 2014; Omenn, Lane, Lundberg, Beavis, Nesvizhskii, et al., 2015; Omenn, Lane, Lundberg, Beavis, Overall, et al., 2016) or the studies conducted by the National Cancer Institute (NCI): Clinical Proteomic Tumor Analysis Consortium (CPTAC) (Zhang et al., 2014; Slebos et al., 2015;

Tabb, Wang, et al., 2016). Also, the increased clustering of high-end research infrastructures such as mass spectrometers into institutional core facilities is pushing the need for systematic quality control (Meder et al., 2016). Since core facilities are often accessible on a fee-for-service basis, users want their samples to be analyzed according the highest quality standards. In case results are negative or below expectations, QC data can be shared with the user to for instance exclude low instrument performance. Ideally, through systematic monitoring, proteomics core facilities can define quality thresholds for every step in their workflow(s). In case these thresholds are not met, targeted corrective measures such as replacing key reagents or cleaning the mass spectrometer can minimize down-time and avoid precious sample losses, thereby increasing the overall performance of the facility.

With so many different factors that can impact the experimental results it is important to carefully consider the various influences independently of each other. To this end, for example, a Pareto chart is a helpful visualization technique, as it can be used to represent the contribution of each individual factor to the total variability (Bereman et al., 2014).

Here we will detail the origin of some common causes of variability that can influence the results of a mass spectrometry experiment and which steps should be taken to avoid them. Notably we will highlight how QC samples can be incorporated into the experimental workflow to systematically assess the instrument performance. Mass spectrometry is an advanced and versatile technique, and it can be used for a wide variety of applications. As a result, there is no definitive consensus on which QC methodology to employ (Martens, 2010), nor is it possible to establish a single uniform approach to quality control. Instead we will broadly review some of the representative QC approaches, discuss general considerations, and show how these steps can be used to monitor the various elements of an MS workflow.

Several computational tools exist to assist in this QC monitoring. Although such tools are instrumental in being able to obtain detailed quality assessments in a longitudinal fashion we will not describe them in detail here. Instead we refer the interested reader to recent work by Bittremieux, Valkenborg, et al. (2017) for an overview of available

computational QC tools and an evaluation of their technical requirements and capabilities.

II. Managing LC-MS variability through quality control

First, we will briefly introduce the different types of QC samples that can be employed to monitor the performance of a mass spectrometry experiment and how these samples can be incorporated within the experimental workflow. Next we will highlight some of the problems that can arise during the different stages of an LC-MS experiment, how they negatively influence the experimental results, and how QC methodologies can be used to detect these problems.

A. Types of QC samples

QC samples can range from a simple peptide mixture to a single protein digest to a complex whole-cell lysate, and each of these types of samples can be employed in a specific fashion to analyze the system performance.

Common simple peptide mixtures consist of a single protein digest (Köcher et al., 2011) such as bovine serum albumin (BSA), enolase, or cytochrome c; or a protein mixture containing a digest of a few proteins (Klimek et al., 2008; Bereman et al., 2014); for clarity we will further denote this type of QC samples as 'QC1'. Notably BSA is often used as such a sample because of its historical application in a variety of experimental procedures and its low cost. Furthermore, BSA is usually quite dissimilar from the protein content of the biological samples under consideration, which helps to minimize negative influence on the experimental results due to potential cross-contamination. QC1 samples are typically run on a very frequent basis, i.e. multiple times per day, to quickly evaluate the instrument performance, and they are especially of use to efficiently and systematically assess the LC performance based on observed peak widths and retention times (RTs). As running QC samples takes up valuable instrument time, there is a trade-off between time spent running them and time (and precious sample content) lost due to performing sample runs while the instrument was in a suboptimal state, leading to inferior results.

To minimize this trade-off, the QC1 samples are typically run using a short gradient so they can be performed on a frequent basis without unduly occupying an excessive amount of instrument time.

QC samples with a higher sample complexity, denoted 'QC2', consist of a whole-cell lysate, such as a yeast lysate (Paulovich et al., 2010; Beasley-Green et al., 2012), a HeLa cell lysate (Köcher et al., 2011), or a *Pyrococcus furiosus* lysate (Wong et al., 2013). QC2 samples are executed using settings equivalent to those for the biological runs to integrally simulate their performance. As this requires more instrument time than the simple QC1 runs, QC2 samples are carried out on a less frequent basis, typically once every day (Pichler et al., 2012). As instruments have been getting more powerful the importance of complex QC2 samples has increased. In contrast to the QC1 single protein digests, complex QC2 samples are used to primarily evaluate the MS performance. Given the sensitivity of current (Orbitrap) mass spectrometers, it is important to inject small amounts of QC samples (e.g. nanogram amounts of peptides; approaching the limit of detection) in order to sufficiently stress the machine and detect potential flaws (Köcher et al., 2011; Pichler et al., 2012). Using such low quantities has the additional advantage that it also helps in preventing or reducing cross-contamination of the biological samples by the QC samples. An important consideration to take into account when running QC2 samples is that ideally their characteristics should reflect those of the biological samples. For example, if phosphoproteomics experiments are conducted on a regular basis, it is important to not only perform a general quality assessment, but also to specifically evaluate the ability to detect phosphorylated peptides and proteins (Köcher et al., 2011). An example of a recent large-scale project where specialized complex QC samples were used is the CPTAC System Suitability (CompRef) Study (Edwards et al., 2015), whose objective it was to validate mass spectrometry protocols by the participants. The CompRef samples were compiled for use within the CPTAC cancer studies and consisted of human-in-mouse xenograft tumor tissue to closely resemble the biological samples. These CompRef samples were first used as a preliminary validation of the workflow during the System Suitability Study, and subsequently acted as QC samples during successive CP-

TAC studies to characterize human colon and rectal cancer (Zhang et al., 2014; Slebos et al., 2015) and to evaluate the longitudinal stability of quantitative proteomics techniques (Tabb, Wang, et al., 2016). Another example of a complex sample used in a recent high-profile, multi-site study is the hybrid QC sample used by Navarro et al. (2016) to benchmark software tools for label-free proteome quantification. This sample consisted of tryptic digests of human, yeast, and *Escherichia coli* proteins mixed in defined proportions to enable the evaluation of both precision and accuracy of label-free quantification, and it was used to assess and ultimately improve the performance of several software tools (Navarro et al., 2016).

Mixtures of synthetic peptides, denoted 'QC3', are a slightly different type of QC samples. Depending on the complexity of their composition, these mixtures can be run and evaluated individually, similar to the QC1 samples but with an even further simplified sample content, or they can be spiked into QC2 samples or even in real samples. By spiking a well-defined mixture into the biological samples quality control can be performed in parallel with the biological analyses and a direct link between the qualitative information and the experimental data can be established. Similarly, the synthetic peptides can be spiked into one of the above QC samples, typically a complex QC2 whole cell lysate, to combine the advantages of both types of samples into a single MS run (Paulovich et al., 2010). An important consideration when spiking synthetic peptides into other samples is that these peptides should not overlap with the original sample content. This can be avoided by using artificial, synthetically modified, peptides that are dissimilar from any naturally occurring peptides (Escher et al., 2012), or by isotopically labeling the synthetic peptides so that their mass is dissimilar from the mass of their naturally occurring peptide variants (Percy, Chambers, DS Smith, et al., 2013; Percy, Chambers, Yang, et al., 2013; Beri et al., 2015). These synthetic QC3 peptide mixtures are especially important to evaluate the performance of targeted approaches, such as selected reaction monitoring (SRM). To be able to consistently monitor the transitions of specific peptides and to optimally schedule SRM experiments chromatographic stability is an essential prerequisite, which can be evaluated using these well-characterized peptides as their transitions should exhibit

minimal run-to-run variation. Synthetic mixtures can be produced in-house or purchased from commercial vendors, and they are often composed in such a fashion that they can be used to examine specific performance characteristics, as we will discuss further.

B. Incorporating QC samples

As illustrated in figure 3, QC samples can be combined with the biological samples in several ways (Bereman, 2015). This is tightly linked to the experimental design (E Maes et al., 2016): how many controls, replicates, etc., are used cannot be considered independently from the use of QC samples.

Typically, simple QC1 samples are run at the start and end of each batch of experiments, or at least once a day in case of larger batches, as shown in figure 3a. Another approach is to systematically interleave the QC samples after each fixed number of biological samples, as shown in figure 3b. This limits the amount of sample loss that can occur due to an intermediate reduction in instrument performance. For example, Zhang et al. (2014) report that during their CPTAC study benchmark tumor xenograft samples were run after every five biological samples, and BSA samples were run after every ten biological samples. Further, as shown in figure 3c, a reference set detailing the expected performance might be required to statistically interpret the subsequent QC runs (Pichler et al., 2012; Bereman et al., 2014). This reference set can often be derived from historical high-quality data. In the absence of such measurements it might be necessary to run multiple QC samples successively prior to the start of an experiment. For example, when switching to a new QC standard sample or when employing a novel protocol the new data cannot be compared to the historical measurements and a reference set might need to be compiled explicitly. Likewise, if multiple (dissimilar) LC columns are combined interchangeably with the same MS instrument a separate reference set for each of the columns must be used, as performance characteristics are column- as well as instrument-dependent. Ideally, a standardized reference set would be provided by the proteomics community through analysis of the same QC sample(s) under comparable conditions. However, no consensus on QC samples currently exists and public data sources to store and share QC data are

lacking. Although such information is available for a few multi-site studies (Addona et al., 2009; Tabb, Vega-Montoto, et al., 2010; Abbatiello et al., 2013; Campos et al., 2015), as there exist numerous mass spectrometry applications no single QC strategy or reference set will likely be fully appropriate in all scenarios.

C. Quality control throughout the experimental workflow

As mentioned previously, a typical LC-MS experiment consists of several different stages. Broadly, this process can be divided in the four following phases (Köcher et al., 2011): (i) sample preparation, including proteolytic digestion of the proteins; (ii) peptide separation through liquid chromatography; (iii) mass spectrometry analysis; and (iv) computational data interpretation. All these steps can introduce significant variability that needs to be controlled in order to obtain reproducible results, so the ideal QC methodology should be able to assess the performance of each of these stages.

For each of these phases we will highlight potential sources of variability, and we will detail how structured quality control methodologies can be implemented to detect and control this variability.

1. Sample preparation

Sample preparation enables the analysis of complex biological samples by mass spectrometry techniques, and entails steps from the initial sample collection up to the proteolytic digestion and sample storage prior to the actual LC-MS analysis. As the results of an experiment depend on the initial sample quality this step is of vital importance to acquire trustworthy results (Bodzon-Kulakowska et al., 2007).

Due to the wide variety in sample origin and experimental applications, each with their specific peculiarities and points of attention, it is impractical to cover all existing sample preparation techniques. However, appropriate sample preparation steps for a bottom-up LC-MS experiment typically include denaturation with a chaotrope, reductant, and/or alkylating agent followed by tryptic digestion of the proteins before the resulting peptides can be further processed (Percy, Parker, et al., 2013; Dittrich et al., 2015). All these

different steps will introduce a certain degree of variability in the output results, which needs to be monitored and controlled. As there are often multiple ways to conduct a single step to optimize the sample preparation a careful performance evaluation of the alternative options is required. To this end isobaric labeling techniques, such as isobaric tags for relative and absolute quantitation (iTRAQ), can be employed (Glibert et al., 2014). Using iTRAQ each experimental condition under investigation can be differentially labeled, multiplexed, and analyzed simultaneously, after which the relative yield of each condition can be determined based on the associated reporter ions. This strategy minimizes inter-condition variation introduced during the LC-MS process and enables the usage of all data to compare different conditions, as opposed to the derived spectrum identification and quantitation information, which can be influenced by unexpected modifications and fragmentation.

Unexpected modifications During denaturation, the secondary and tertiary structure of the proteins are removed by interrupting their non-covalent bonds. Additionally, covalent disulfide bridges are cleaved via reduction, after which the proteins are alkylated to prevent the reformation of these disulfide bonds. A complete unfolding of the proteins is required to be able to achieve a full enzymatic cleavage into peptides, but these steps can also introduce unexpected post-translational modifications (PTMs) (Karty et al., 2002).

The chaotropic agent urea is often used for protein denaturation. An important consideration is that urea can cause artificial carbamylation (Stark et al., 1960). In aqueous solutions urea dissociates upon heating and over time. One of its degradation products is isocyanate, which covalently reacts with protein N-termini and ε-amine groups of lysines (and arginines to an extremely limited extent) to form carbamyl derivates (Kollipara et al., 2013). Prolonged incubation of protein samples in urea buffers can induce undesired carbamylation, which will occur at a higher rate if old urea or elevated temperatures are employed. Artificially introduced carbamylation is obviously detrimental for studies that investigate the effect of in vivo carbamylation, which has been related to protein ageing. However, general issues are that carbamylation hampers proteolytic digestion with trypsin, blocks amino groups from isotopic/isobaric labeling, and changes peptide

charge states, retention times, and masses (Sun et al., 2014). Therefore it is important to avoid the formation of urea-induced carbamylation during sample preparation. This can be done by minimizing the generation of cyanates or by removing active cyanates from the solution. Since urea only degrades in aqueous solution it should be prepared freshly (Kollipara et al., 2013). Other strategies involve maintaining the sample at a low temperature (Marier et al., 1964; Hagel et al., 1971), lowering the pH (Stark, 1965), or using a variety of buffers (Lin et al., 2004; Kollipara et al., 2013; Sun et al., 2014). To verify that unexpected carbamylation is not present in an excessive amount appropriate search settings during peptide identification should be used, i.e. a variable carbamylation PTM should be considered.

Another source of unexpected modifications comes from the alkylation step. Alkylation ensures that after disulfide bridges have been cleaved using a reductant the proteins remain unfolded by preventing reformation of the disulfide linkages. For this step, a commonly used alkylation agent is iodoacetamide (IAM). Through reaction with IAM a carbamidomethyl group is attached to cysteine residues to prevent these from reforming disulfide bridges, which results in a monoisotopic mass difference of 57.021 464 Da. A potential issue is that overalkylation with IAM will cause N-terminal carbamidomethyl modifications as well (Boja et al., 2001). Alternatively, alkylation can be done through carboxymethylation with iodoacetic acid (IAA), which adds a monoisotopic mass of 58.005 479 Da. Similar to IAM, overalkylation with IAA will result in N-terminal modifications (Woods et al., 2012). Therefore care has to be taken that the sample solution should not be overexposed to either IAM or IAA during alkylation, and appropriate search settings specifying the corresponding N-terminal modifications should be employed to verify this.

A prevalent modification that can easily be misinterpreted is the nonenzymatic deamidation of asparagines and glutamines to aspartates and glutamates respectively, whose rate can increase dramatically during prolonged incubations in digestion buffers at a mildly alkaline pH (Krokhin et al., 2006; Hao et al., 2011). As deamidation adds a monoisotopic mass of 0.984 016 Da, when not correctly considering this modification the

^{13}C peaks of non-deamidated peptides can be misassigned as monoisotopic peaks of the corresponding deamidated ones, although current high-resolution instruments are able to unambiguously distinguish these peaks. As before it is important to carefully perform the sample preparation and use the correct identification search settings to verify that unexpected modifications have not been introduced.

Suitable search settings are essential to correctly interpret the generated data. Importantly, any expected modifications as well as modifications that can be involuntarily introduced, as discussed previously, should be specified correctly. A recent analysis indicates that unexpected or unconsidered modifications account for missing identifications of a large proportion of unassigned spectra (Chick et al., 2015). Similarly, an analysis of 19 million spectral clusters based on previously unidentified spectra deposited in the PRoteomics IDEntifications (PRIDE) database (Vizcaíno et al., 2016) illustrates the extent to which unidentified spectra can be traced back to unexpected or unconsidered PTMs (Griss et al., 2016). As an illustration, figure 4 shows the most prevalent modifications detected in the MassIVE database.

Proteolytic digestion stability In a bottom-up LC-MS proteomics experiment proteins are not analyzed directly, instead they are cleaved into peptides through proteolytic digestion. For this task trypsin is currently the most frequently used protease (Vandermarliere et al., 2013). Advantages of trypsin are its low cost and its high cleavage specificity and activity. Furthermore, tryptic peptides have various desirable characteristics: their mass is within the preferred mass range for mass spectrometry analysis (based on an *in silico* digestion of all proteins in UniProtKB/Swiss-Prot (The UniProt Consortium, 2015) unique fully tryptic peptides have a median length of 12 amino acids and an interquartile range between 8 and 20 amino acids) and they are ideally suited to carry at least two defined positive charges (Steen et al., 2004).

At its most basic level, trypsin cleaves exclusively and systematically C-terminal of arginine and lysine, unless followed by a proline (Keil, 1992). Nevertheless, the formation of semitryptic and nonspecific peptides during protein digestion can still happen due to multiple reasons, although these peptides show a decreased repeatability (Tabb,

Vega-Montoto, et al., 2010) and they are often not considered during the subsequent bioinformatics analysis, resulting in missing or incorrect identifications (A Keller et al., 2002; Ma et al., 2009). Moreover, most notably for targeted and other quantification experiments consistency of the detectable peptides is of crucial importance.

There are many factors that can influence the digestion stability. One of these is the manner in which the preceding sample preparation steps were performed, and Proc et al. (2010) have shown that the choice of chaotropic agents, surfactants, and solvents significantly influences the digestion reproducibility. Other factors that have an influence are the temperature and the pH at which the digestion is carried out, the enzyme-to-substrate ratio, and the duration of the digestion. At a higher temperature the thermal denaturation of trypsin results in a loss of tryptic activity and autolysis (Finehout et al., 2005; Loziuk et al., 2013), while a lower pH improves trypsin stability over an extended digestion period (Loziuk et al., 2013). Meanwhile, although enzyme-to-substrate ratios reported in the literature range from 1 : 100 to as high as 1 : 2.5, Loziuk et al. (2013) have shown that at excessive enzyme-to-substrate ratios an "overdigestion" of peptides caused by increased tryptic autolysis occurs, which may lead to the generation of nonspecific and very small peptides. Similarly, Hildonen et al. (2014) recommend a limited digestion time, to avoid a complete digestion as this leads to an increased number of small peptides that are not LC-MS detectable. Furthermore, not all trypsin is created equally, with the origin of the trypsin an important source of variability. Comparisons have shown that the number of missed cleavages, semitryptic peptides, and nontryptic peptides can vary significantly based on whether the trypsin is of bovine or porcine origin (Walmsley et al., 2013) and between different commercial trypsins (Burkhart, Schumbrutzki, et al., 2012; Bunkenborg et al., 2013).

To assess the digestion performance it is important to monitor the extent of missed cleavages, semitryptic peptides, and nontryptic peptides. Ideally fully tryptic peptides should be preferred as their formation is more reproducible when the trypsin digestion is able to proceed to a state of equilibrium (Tabb, Vega-Montoto, et al., 2010). In some cases semitryptic peptides might be desired as well to generate more detectable peptides and

increase the protein sequence coverage (Hildonen et al., 2014). Furthermore it is important to take into account that digestion efficiency is protein- and sample-dependent (Fang et al., 2015). Therefore, there is no one-size-fits-all optimal digestion procedure; specialized protocols might be required to for example optimally monitor specific transitions in a targeted experiment.

DIGESTIF is a commercially available compound QC sample that can be used to evaluate the tryptic digestion efficiency (Lebert et al., 2015). The *DIGESTIF* standard is assembled from a protein scaffold and artificial peptides, with the amino acids flanking the cleavage sites of these peptides selected to either favor or hinder proteolytic cleavage. This allows to progressively monitor the digestion performance by checking which peptides are effectively generated compared to their theoretical cleavage specificity. Alternatively, to monitor the digestion performance Domon et al. (Gallien et al., 2014; Bourmaud et al., 2015) inserted QC samples at various moments during the experimental process. Prior to any sample preparation steps they start with a well-defined QC mixture of a few proteins, insert a first set of isotopically labeled peptides representing a subset of tryptic peptides of these proteins prior to digestion, and a second set of isotopically labeled peptides (with the same amino acid sequences but a different isotope pattern) prior to the LC-MS analysis. By comparing the relative intensities of the unlabeled peptides, originating from the initial proteins, and the labeled peptides from the first set of labeled peptides the digestion efficiency can be assessed. Further, through comparison with the intensities of the labeled peptides from the second set the overall recovery of the full sample preparation workflow can be evaluated.

Although trypsin is by far the most popular protease, employing another protease or performing a multi-protease protein digestion can have specific advantages (Tsiatsiani et al., 2015; Giansanti et al., 2016). A common alternative to trypsin is the combination of Lys-C and trypsin, which generates similar peptides and significantly reduces the number of missed cleavages (Glatter et al., 2012). Less frequently used proteases can be beneficial as well, for example to generate longer peptides for "middle-down" proteomics, although these proteases are usually not as thoroughly characterized as trypsin is, so care has to

be taken (Tsiatsiani et al., 2015). In these situations a consistent and systematic QC methodology assumes even greater importance.

Sample loss Differential recovery of peptides due to nonspecific adsorption is a potential source of unexpected sample loss during sample preparation, leading to a reduced reproducibility (K Maes et al., 2014). This sample loss can occur in all steps of a proteomics workflow, and care should be taken that suitable sample handling material is employed at all times. It should be taken into account that adsorption is peptide-specific (Goebel-Stengel et al., 2011), so optimized protocols might be required for specific situations. Furthermore, a systematic analysis of well-characterized QC samples can highlight signal loss.

The type of sample tubes that are used for peptide storage can result in a large variation in the results, with low-adsorption plastic tubes more suitable than regular plastic tubes or glass tubes (Bark et al., 2007; Kraut et al., 2009). In contrast, hydrophobic peptides exhibit an increase in recoverability for glass tubes (van Midwoud et al., 2007). Furthermore, the addition of other compounds to the sample solution can be used to reduce sample loss due to competition of adsorption with the peptides (Goebel-Stengel et al., 2011; Stejskal et al., 2013).

Adsorption does not happen exclusively to sample tubes; for example some peptides, including all sulfur-containing peptides, adsorb on the stainless steel injection needle as well (van Midwoud et al., 2007). As a rule, the more sample handling steps are undertaken, the more loss due to surface adsorption occurs (Magdeldin et al., 2014). Therefore, online and automated methods can help to reduce potential sample loss.

Contaminants Another important source of variability is the presence of contaminants in the sample (BO Keller et al., 2008; Weber et al., 2012; Hodge et al., 2013). Contaminants will compete with the spectra of interest during MS measurements and can cause ion suppression of low abundant peptides. Contaminants can often have seemingly innocuous origins, such as a lab member using a new perfume or wearing a wool sweater (BO Keller et al., 2008). It is important to be aware of potential sources of contaminants during all

sample preparation steps to avoid undue contamination.

Some contaminants can be traced back to a prior sample preparation step. For example, trypsin autolysis artifacts can be generated during protein digestion, or polymeric interferences can leak from plastics employed in the laboratory. Other contaminants can be involuntarily introduced into the sample. One of the most prevalent contaminants is keratin, which is omnipresent and can originate from skin, hair, dust, etc.

Total elimination of all contaminants is virtually impossible, but suitable procedures can help to minimize contamination. To prevent contaminants as much as possible it is important to always work in a clean lab environment, wear suitable lab clothes, and use specialized equipment for a single task exclusively. To be able to detect contaminants it is necessary to specify them in the identification search settings. A recent analysis of public data deposited in the PRIDE repository (Vizcaíno et al., 2016) indicates that a majority of commonly incorrectly identified spectra corresponds to contaminants such as albumin, trypsin, and keratin (Griss et al., 2016). The MaxQuant software (Cox and Mann, 2008) has functionality to automatically include a built-in database of contaminant sequences during sequence database searching (Cox, Matic, et al., 2009). Otherwise, lists of commonly observed contaminants are publicly available. The common Repository of Adventitious Proteins (cRAP) (`http://www.thegpm.org/crap/`) provides a resource of contaminant proteins, sourced from the Global Proteome Machine (GPM) (Craig, JP Cortens, et al., 2004). Both a fasta file for use in sequence database searching and a spectral library in the X! Hunter format (Craig, J Cortens, et al., 2006) are available. In addition, the PRIDE database (Vizcaíno et al., 2016) provides a spectral library of contaminants, generated through PRIDE Cluster (Griss et al., 2016).

2. Liquid chromatography

Prior to MS analysis peptides are typically processed using liquid chromatography to separate them based on their hydrophobicity. This adds a time dimension to the subsequently recorded MS data, which enables the mass measurement of individual peptides by spreading out the dense information content of a complex sample over the range of

the LC gradient, and which provides orthogonal information for the peptide identification (Pasa-Tolić et al., 2004).

The LC phase is typically subject to more variable influences than any other component of the LC-MS system (Barwick, 1999), and consequently it is the most common culprit of variability in the results of an experiment (Rudnick et al., 2010). A rigorous monitoring of the chromatographic performance is therefore essential. Useful QC metrics include the peak shape (width and height), as sharper peaks generate higher signal intensities and can reduce oversampling (Rudnick et al., 2010). A disproportionate level of signal intensity early or late in the gradient can indicate that the column should be serviced or replaced. An early signal can be caused by sample bleed, and a late signal can arise from peak tailing of either overloaded peptides or peptides with poor chromatographic behavior (Rudnick et al., 2010). The RT of known peptides and their elution order can be used to measure differences between early (hydrophilic) and late (hydrophobic) peptides in the chromatographic gradient (Rudnick et al., 2010; Abbatiello et al., 2013). Leakages are a problem that can be very hard to diagnose as tiny droplets can evaporate before they are spotted and localized (Noga et al., 2007). If a leakage occurs before the column it can be detected by examining the column pressure profile. However, if a leakage occurs between the column and the ion source the column pressure will remain similar to the standard pressure while there might still be a sensitivity loss and/or unstable operation of the ion spray as a smaller amount of liquid will exit from the column (Noga et al., 2007).

It is of vital importance to avoid cross-contamination due to sample carryover. Carryover happens when an analyte originating from a previously analyzed sample reappears during a subsequent injection, which will result in interference with the active measurements. Carryover can occur because of interactions between the sample and various materials it comes into contact with, as mentioned previously (Hughes et al., 2007). Other potential sources of carryover are mixing chambers, which are empty spaces within the column where consecutive segments of the sample mix together instead of being separated, and dead volumes, which are spaces in the LC system that are not swept by the mobile phase (Noga et al., 2007). Even the smallest irregularities in tubing connections can lead

to the generation of dead volumes where sample residues can be trapped (Mitulović and Mechtler, 2006). The presence of dead volumes can be diagnosed using the UV detector, which can be a useful tool for system diagnostics and troubleshooting (Mitulović, Smoluch, et al., 2003; Noga et al., 2007). Furthermore, both dead volumes and mixing chambers hamper the performance of chromatographic systems, as besides sample carryover, they can also give rise to peak broadening and prolonged elution times. To minimize or avoid carryover suitable column washing steps should be employed (Mitulović, Stingl, et al., 2009; Williams et al., 2012; Dolman et al., 2013). The level of carryover and the cleaning effectiveness can be tested by using blank injections between runs of different samples. Quality recommendations typically mandate that the response of any background component in a blank sample should be less than 20 % of the lower limit of quantification (LLOQ) (Jenkins et al., 2015).

Specialized QC samples can help to thoroughly monitor the performance of the LC system. By composing QC samples so that they contain peptides with varying hydrophobicities the elution profile of the LC gradient can be characterized and evaluated (Burkhart, Premsler, et al., 2011), as illustrated in figure 5. Notable are so-called indexed retention time (iRT) peptide standards. These peptides have standardized RTs spanning a wide gradient and can be used to normalize the RT of individual experiments (Escher et al., 2012). Although RTs can be predicted through computational modeling (Moruz et al., 2016), these predictions have a somewhat limited accuracy (Reimer et al., 2012). Instead, the reference RTs of the iRT peptides can be used to correct for variations in the RT of the other peptides detected in a single experiment or to align RTs across multiple experiments. Several QC standards containing iRT peptides have been proposed (Eyers et al., 2008; Burkhart, Premsler, et al., 2011; Beri et al., 2015; Lebert et al., 2015; Holman et al., 2016). These standards mostly vary slightly in the range of the LC gradient they can cover, but some standards have further advanced properties. For example, the previously mentioned *DIGESTIF* standard can additionally be used to evaluate the tryptic digestion performance (Lebert et al., 2015), while the *RePLiCal* standard consists of a synthetic protein that exclusively contains lysine-terminating peptides, which ensures that prote-

olytic digestion by both trypsin and Lys-C can be evaluated analogously (Holman et al., 2016).

3. Mass spectrometry

As peptides elute from the LC column their mass over charge is measured in the mass spectrometer.

Prior to the mass measurement peptides are ionized through ESI. The spray stability can be checked by monitoring for drops in the ion current, which can indicate spray sputter (Rudnick et al., 2010; Scheltema et al., 2012). Tryptic digests are expected to generate mainly peptides containing a 2+ charge, and a high rate of differently charged peptides can indicate ionization issues and will likely impact identification rates (Rudnick et al., 2010). Besides due to an unstable proteolytic digestion, as mentioned previously, partially tryptic peptides can also originate from in-source peptide fragmentation (JS Kim et al., 2013). It is possible to differentiate partially tryptic peptides originating from in-source fragmentation from other proteolytic-derived partially tryptic peptides as the former will have the same LC elution time as their parent peptides (JS Kim et al., 2013). To measure high-quality spectra sufficient signal should be present. Various parameters can influence the internal instrument behavior and these should be carefully optimized (Xu et al., 2009; Andrews et al., 2011; Kalli and Hess, 2012; Kalli, GT Smith, et al., 2013; Zhou et al., 2015). For example, for trapping instruments the maximum ion injection time and the automatic gain control (AGC) are interrelated instrument parameters that influence the signal-to-noise ratio, and the effective ion injection time can be monitored to detect problems with sample load (Kalli and Hess, 2012). By comparing the measured masses of known compounds, which can either be explicitly added reference standards or systematically observed contaminants, the mass accuracy can be evaluated (Rudnick et al., 2010). If the option is available, these known masses can further be used as lock mass during mass calibration if excessive mass deviations are observed (Staes et al., 2013). The dynamic range can be monitored if peptides are present in varying concentrations, as illustrated in figure 5. QC samples can contain distinct peptides in different concen-

trations (Burkhart, Premsler, et al., 2011) or isotopically labeled variants of the same peptide at different ratios (Beri et al., 2015). While the concentrations typically span two to four orders of magnitude, the ability to detect even the smallest concentrations indicates the capacity to detect low-abundant peptides over the observed extensive proteome dynamic range (Zubarev, 2013). Furthermore, the sensitivity of the MS instrument can be evaluated by employing only small amounts of QC samples, as mentioned previously.

4. Bioinformatics data interpretation

Although the wet laboratory workflow is often considered to contribute the most variability to the results of an MS experiment and multiple studies have aimed to improve and standardize existing protocols, the bioinformatics data interpretation can likewise introduce major errors that are a cause of irreproducibility (Bell et al., 2009). Already for the most fundamental task, mapping peptide sequences to spectra, there exist dozens of different search engines, each using a unique methodology, (possibly silent) assumptions, and peculiarities. Furthermore, even when using the same tool often different versions or parameter combinations can yield significantly dissimilar results. Although a careful evaluation can indicate the optimal search settings for a single tool (Fenyö et al., 2010; Vaudel et al., 2011), the high volume of the data generated by MS techniques and the complexity of the bioinformatics tools is a barrier for a mutual objective assessment (Yates et al., 2012; Gatto et al., 2016). The 'ground truth' for evaluation is typically not known and the introductions of novel tools regularly lack a sufficient comparison to the state-of-the-art methodology. Nevertheless, to inspire confidence in the acquired results a robust computational and statistical interpretation according to community best practices should always be performed before reporting novel biological findings (Serang et al., 2015).

In the previous sections we have already mentioned several evaluation criteria that should be investigated to detect specific problems. A benchmark of overall performance that is often monitored is the identification rate in terms of peptide-spectrum matches (PSMs), identified peptides, and identified proteins. This gives a quick insight into the performance of the whole experimental set-up and can indicate whether more detailed

quality assessments are required. Whereas for complex QC2 samples, such as a whole-cell lysate, the number of proteins is an often reported metric, for simple QC1 samples, consisting of only a single to a few proteins, the sequence coverage is usually more relevant. The appeal of these high-level QC metrics is that they give a quality assessment of the whole system in a single, easily interpretable metric. However, an MS experiment consists of multiple complex steps that are interrelated, and it might not be possible to identify the source of a decrease in performance based on only a single metric. Instead, sets of detailed QC metrics can be computed (Rudnick et al., 2010), highlighting individual performance aspects of the chromatography, the charge state distribution, the spectrum acquisition, etc. A disadvantage of these advanced QC metrics is that, unlike for the number of identifications or the sequence coverage where a higher value is usually better, their interpretation is often not straightforward and requires expert knowledge. Therefore, to establish value intervals of acceptable performance a high-quality reference set might be used, as described in section C (Pichler et al., 2012; Bereman et al., 2014). Furthermore, analyzing multiple metrics simultaneously requires a multivariate approach. Although this increases the complexity of the data analysis, recent research has shown some promising approaches for informed and automated decision-making based on multivariate sets of advanced QC metrics (Wang et al., 2014; Amidan et al., 2014; Bittremieux, Meysman, et al., 2016). Additionally, it does not suffice to investigate QC metrics for a single experiment in isolation. Instead, the longitudinal performance should be examined (Bennett et al., 2015). Through extensive monitoring of operation over time the technological passport of a mass spectrometer can be established, and based on these highly detailed and instrument-specific insights the reliability of the experimental results can be diagnosed. Although not necessarily related to their biological relevancy, this constitutes the bare essentials required to inspire solid confidence in novel scientific findings. Finally, currently QC metrics are mostly analyzed post hoc after the data acquisition has been completed. Instead, descriptive metrics should be monitored in a real-time fashion over the course of an experiment and spectrum acquisition should be halted automatically when an intermediate decrease in quality is observed to avoid the loss of precious sample

content (Scheltema et al., 2012). This requires advanced functionality to allow QC tools to directly interface with the MS instruments, which currently is largely missing.

III. Conclusion

Performing an LC-MS experiment is a highly complex activity and there exist a multitude of potential sources of variability that can influence the results and impact repeatability and reproducibility. We have tried to give an overview of some prevalent issues that can arise and how to detect them, but nevertheless we have only managed to cover the tip of the iceberg. Instituting a thorough QC methodology might initially seem like it requires a lot of effort and it occupies valuable instrument time without any immediate gains, but a systematic quality assessment pays off in the long run and is an indispensable prerequisite to inspire confidence in the acquired results. Especially in order to advance mass spectrometry techniques and use them as routine applications in a clinical setting a consistent analytical performance is a fundamental requirement (Martens, 2013; Smit et al., 2014; Wright et al., 2016).

Developments on both the experimental and computational front are needed to improve current QC methodologies, for which core facilities can act as an important driver (Lilley et al., 2011). As proteomics technologies have matured, core facilities have concentrated the cutting-edge technical expertise necessary to obtain high-quality results, and they form an essential means of providing this in an affordable manner (Meder et al., 2016). Maybe even more than research laboratories, core facilities have an incentive to support and develop robust quality assurance practices to demonstrate the quality of the generated data to their clients and stakeholders, and through their expert knowledge on a broad aspect of MS-based applications they are at the forefront of developing standardized QC workflows. Significant bioinformatics work is needed as well. All too often laboratories still only monitor detailed QC metrics in an empirical fashion when a malfunctioning is suspected, instead of on a systematic basis. This can be partly attributed to the relative absence of user-friendly tools and software suites that facilitate and encourage a methodical QC workflow. Although a few tools to compute advanced QC metrics exist (Bereman,

2015; Bittremieux, Valkenborg, et al., 2017), they remain underused in part due to their limited ease-of-use. Nevertheless, to make further progress objective metrics rooted in a solid bioinformatics foundation are mandatory. The end goal should not be to merely understand QC issues retrospectively, but also to prevent them from happening by timely suggesting solutions. Eventually the QC tools should ideally be tightly coupled to the MS instrumentation to make automated decisions on the fly, avoiding subjective and time-consuming manual quality assessments to increase the throughput.

Finally, because of these obvious advantages we expect that the importance of quality control will only increase in the (near) future. Currently QC information is often not included in scientific publications, which might lead to uncertainty on the conducted methodology. Instead, in the future reporting this information might become formalized, similar to existing guidelines mandated by journals (Taylor et al., 2007), and the QC metrics might become an integral part of a data submission to public data repositories (Eisenacher et al., 2011; Martens, 2016), with current work ongoing to provide the necessary technical basis for this goal (Bittremieux, Walzer, et al., 2017). Coupling comprehensive QC information to the experimental data will enable assessing the reliability of an experiment at a glance. Especially in light of some historical occasions where claims turned out to be exaggerated (Baggerly et al., 2005; Ezkurdia et al., 2015) and recent reports of the general reproducibility crisis in various scientific fields (Baker, 2016), an innate approach to quality control is mandatory to inspire confidence in and to advance the field of mass spectrometry-based proteomics.

Acknowledgments

W.B. is a postdoctoral researcher of the Research Foundation – Flanders (FWO). W.B., L.M., and K.L. acknowledge the support of the VLAIO grant “InSPECtor” (IWT project 120025). F.I. was supported by Odysseus grant G0F8616N from the Research Foundation – Flanders (FWO).

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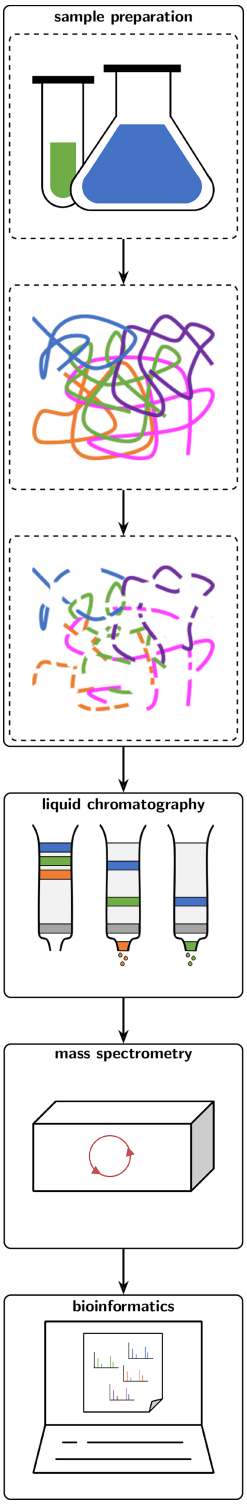


Figure 1: A typical LC-MS experiment consists of a sample preparation, a liquid chromatography, a mass spectrometry, and a bioinformatics stage. The sample preparation includes the proteolytic digestion of proteins into peptides. Next, consecutively the peptides are separated through liquid chromatography and measured through mass spectrometry. Finally, the acquired spectra are interpreted through bioinformatics means. From (Bittremieux, 2017d).

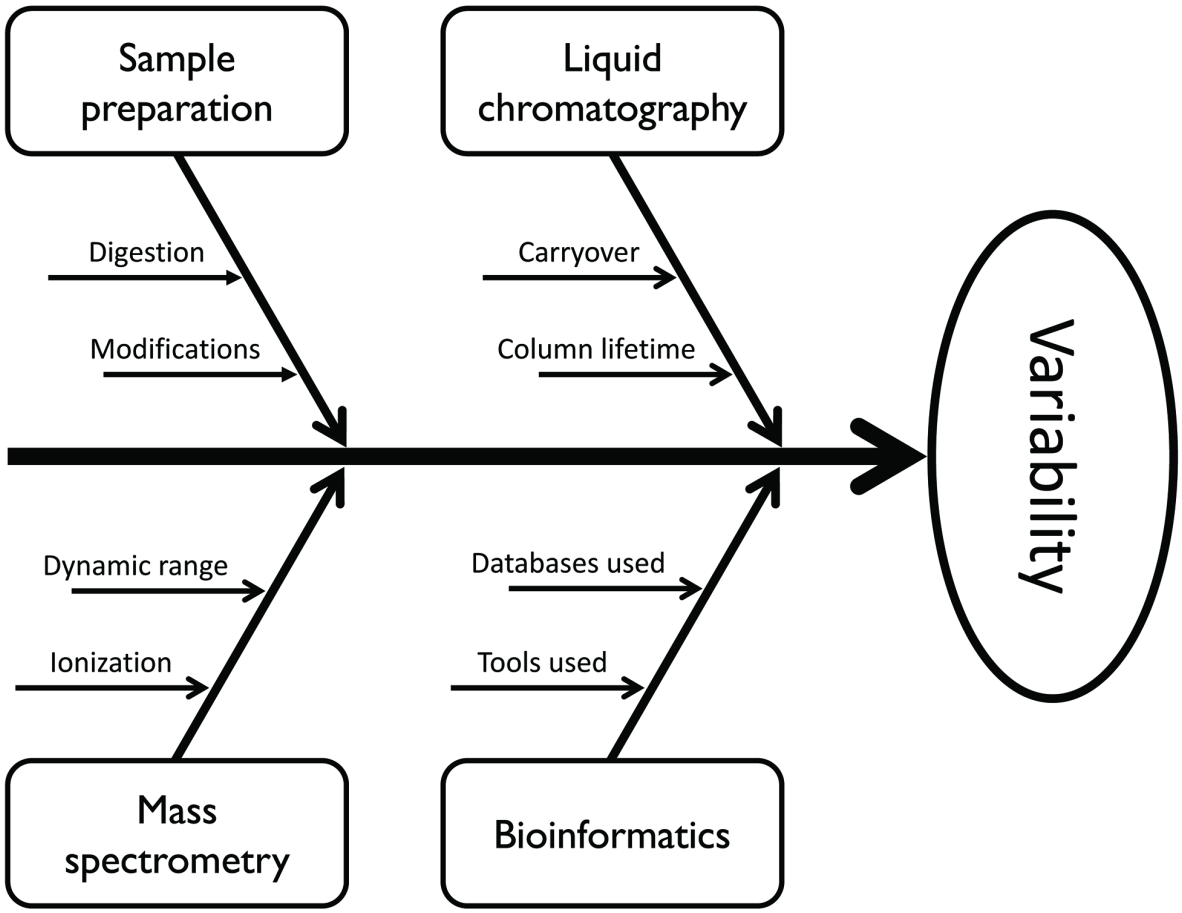
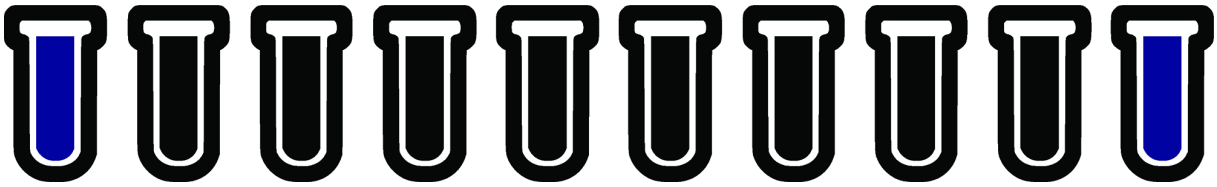
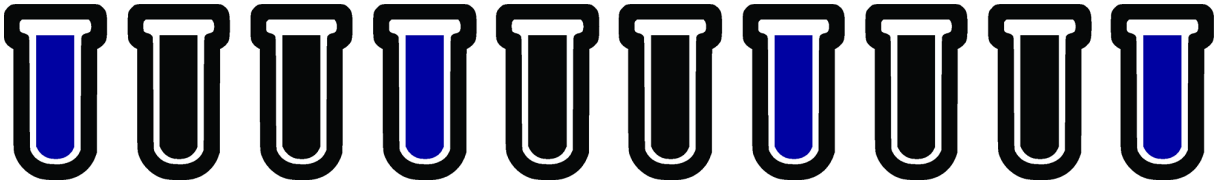


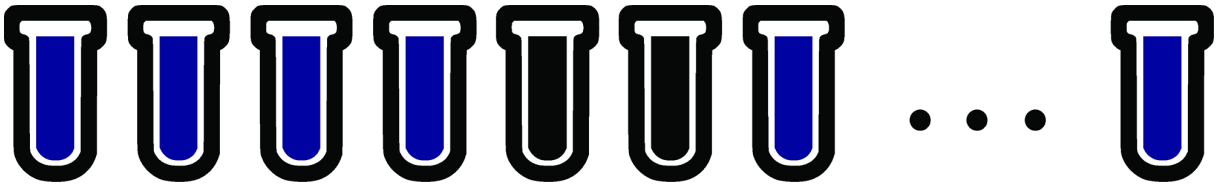
Figure 2: An Ishikawa diagram (non-exhaustively) highlighting some of the major sources of variability in each of the stages of an LC-MS experiment. These and other sources of variability will impact the results and should be considered in a comprehensive quality control workflow. From (Bittremieux, 2017c).



(a) QC samples are run at the start and at the end of a batch to assess the batch quality.



(b) QC samples can be interleaved with the biological samples within a single batch to detect an intermediate decrease in performance and avoid undue sample loss.



(c) A reference set of high-quality QC measurements is used as the basis to characterize the performance.

Figure 3: The experimental workflow can incorporate the QC samples (blue) through various combinations with the biological samples (black). From (Bittremieux, 2017b).

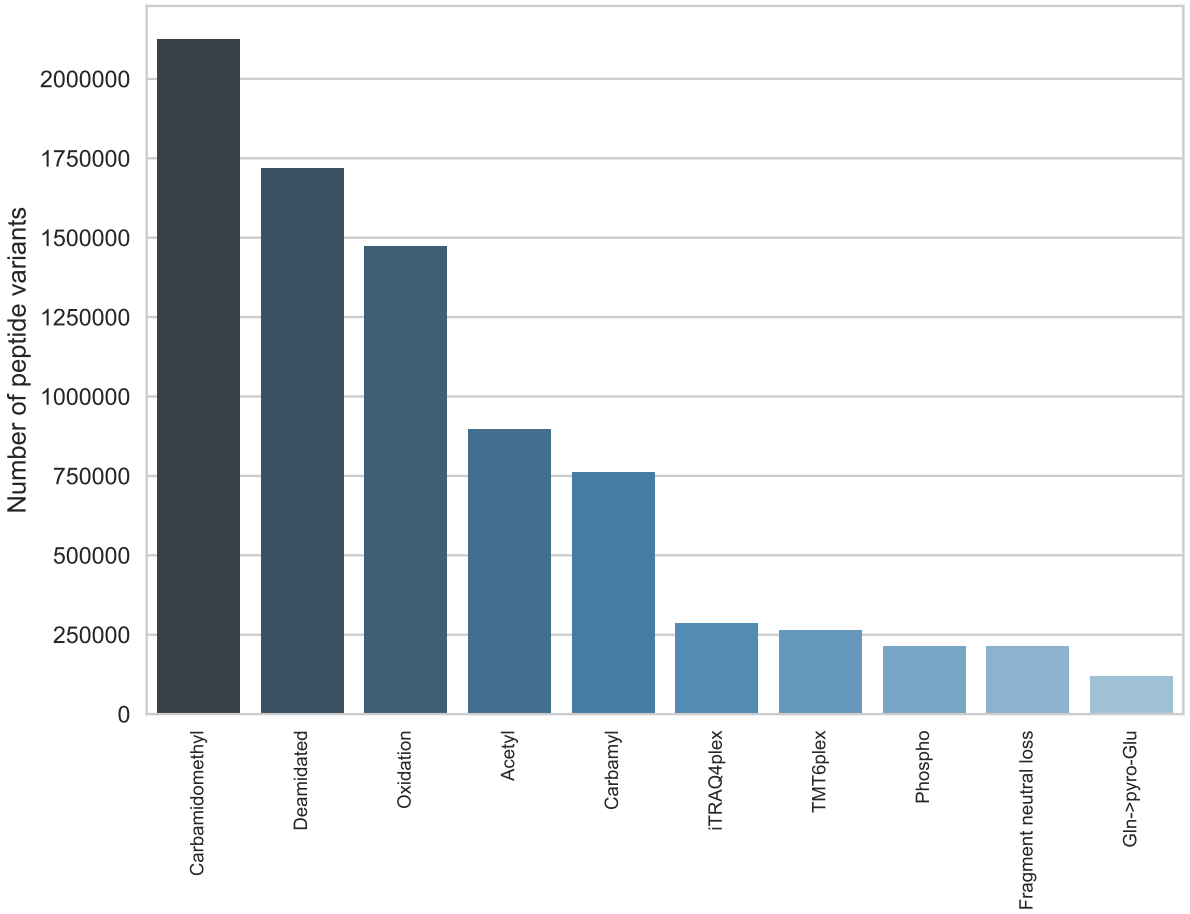


Figure 4: The most frequently observed modifications in the MassIVE database (June 26, 2017). Modification counts are based on the number of distinct peptide variants on which each PTM was detected, out of 10 773 204 peptide variants in total. From (Bittremieux, 2017e).

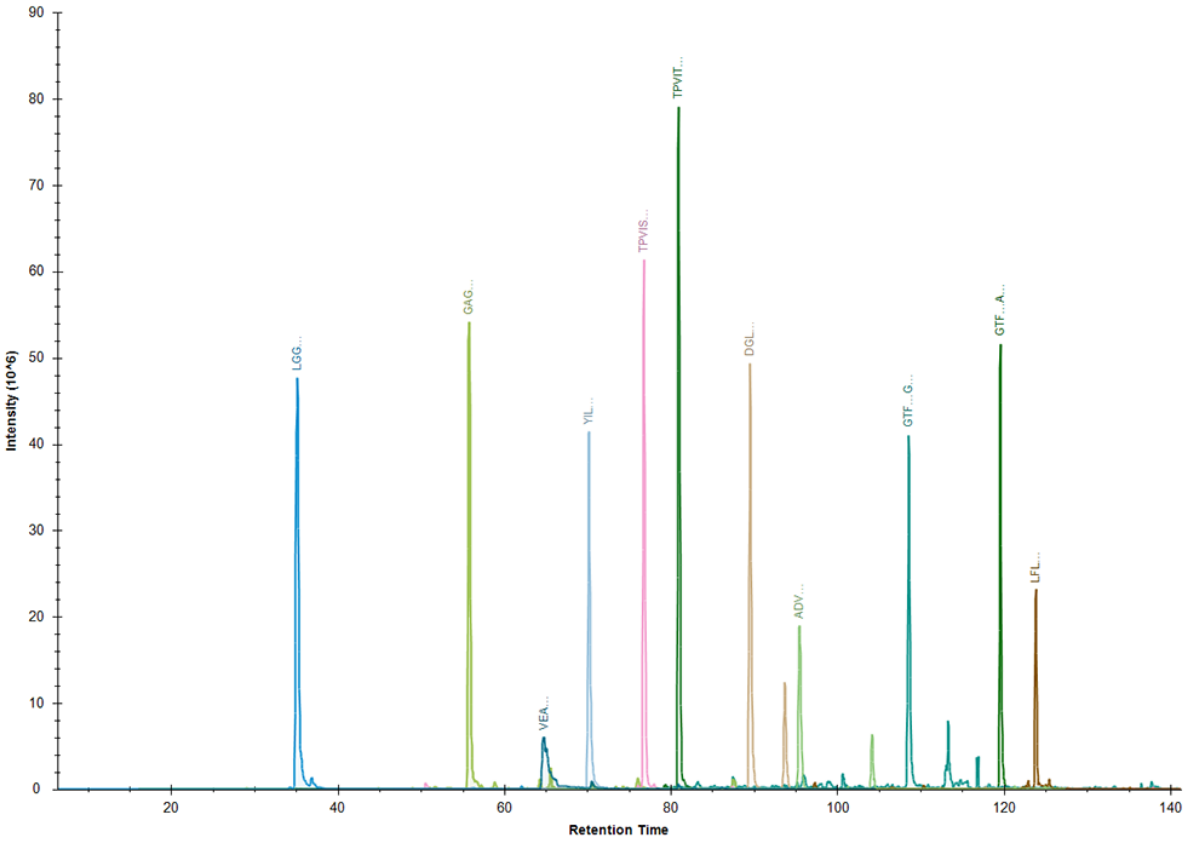


Figure 5: Depending on the composition of the QC samples the LC performance can be monitored using peptides that elute over the entire gradient, and the dynamic range can be monitored if peptides are present in varying concentrations. Depicted here is the Biognosys iRT standard which consists of eleven peptides with varying chromatographic retention (Escher et al., 2012). From (Bittremieux, 2017a).