Protein Mass-Spectrometry

Introduction

Mass-spectrometry (MS) is used in a myriad of different scientific fields ranging from nuclear physics to medical diagnostics. The implementations of MS and the instruments used will vary very widely from one field to another. Even within the field of biology, mass-spectrometry is used in a wide range of applications, again each differing considerably in the instruments used and the information obtained.

Out of this very broad range of techniques, this part of the course will focus on one very specific mass-spectrometry application, namely peptide-fragmentation based analysis of protein samples.

At first sight, such a narrow focus may appear surprising for an introductory course on biological mass-spectrometry. But, peptide-fragmentation-based analysis of protein samples is such a powerful and widely used experimental technique that it accounts for a large fraction of mass-spectrometry experiments in biology.

Fundamental operating principle and components of a mass spectrometer

At its most fundamental level, mass spectrometry determines the mass of individual molecules by measuring how electrically charged versions of these molecules (ions) move when exposed to electric or magnetic fields in a vacuum.

A mass spectrometer (Figure 1) consists minimally of an **ion source**, which deposits an electrical charge on the molecule and transfers it from solution into the gas phase, a **mass analyzer**, which separates different ions based on their mass-to-charge ratio (m/z), and a **detector** that counts the ions passing the mass analyzer.

The mass filters/analyzers are constructed in such a way that ions of different m/z ratios are separated and hit the detector at different time points, or under different electromagnetic conditions of the mass analyzer (scanning). By recording the signal on the detector while scanning across a range of m/z ratios, a mass spectrum, that is to say the relative abundance of ions with different m/z ratios, can be recorded.

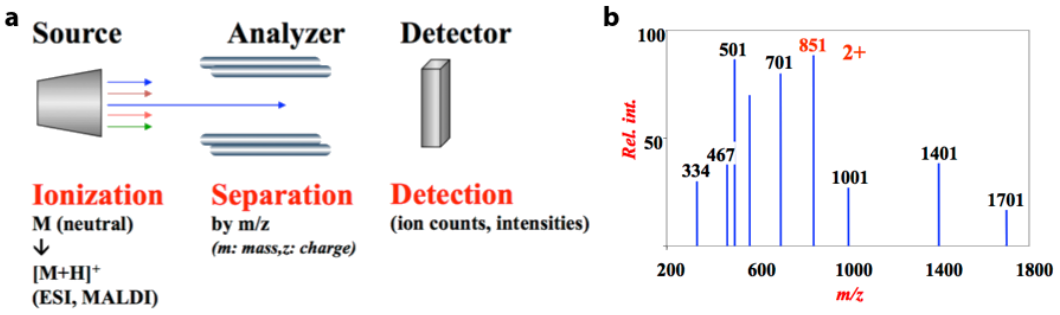


Figure 1 Simple schematic of a mass spectrometer **(a)**. The source deposits a charge on the sample molecules, transfers the resulting ions into the gas phase, and (unless a separate accelerator exists) accelerates them into a continuous molecular beam. This beam passes through the mass filter/analyzer section, which separates ions entering the system based on their mass-to-charge ratio (m/z). There are several physical implementations of mass filters/analyzers. The mass filter/analyzer is adjustable and can scan the window of m/z ratios that are allowed to pass across a wide range of values. By recording the intensity of the signal at the detector as a function of the m/z ratio that is allowed to pass through the mass filter, one obtains a mass spectrum **(b)**.

Ion sources

Biological molecules usually exist in aqueous solution. In order to analyze them in a mass spectrometer, these molecules need to be charged, isolated from the surrounding solvent molecules (i.e., water and salts) and transferred into the gas phase. These three steps are accomplished by the ion source. In modern mass spectrometers, two main types of ion sources are used: Electrospray ionization (ESI) and matrix-assisted laser desorption & ionization (MALDI). For the analysis of peptides from protein samples, ESI ion sources (Figure 2) are particularly popular and their physical principle is discussed here.

ESI ion sources spray a very fine stream of liquid out of a nozzle that is at a strong positive electrostatic potential relative to a plate at the opposite side of the injection chamber. As a result, the droplets leaving this nozzle carry an excess positive charge. On the way to the mass analyzer, which is in high vacuum, the droplets pass through a zone of dry gas that is also frequently heated. Under these conditions, the solvent in these droplets evaporates very quickly. This concentrates the charges on the droplet surface causing "coulombic explosions" that shatters large droplets into smaller droplets, which again shrink due to evaporation and explode again. This process continues until the solvent has evaporated completely and only the charged ions of the sample molecules remain[1].

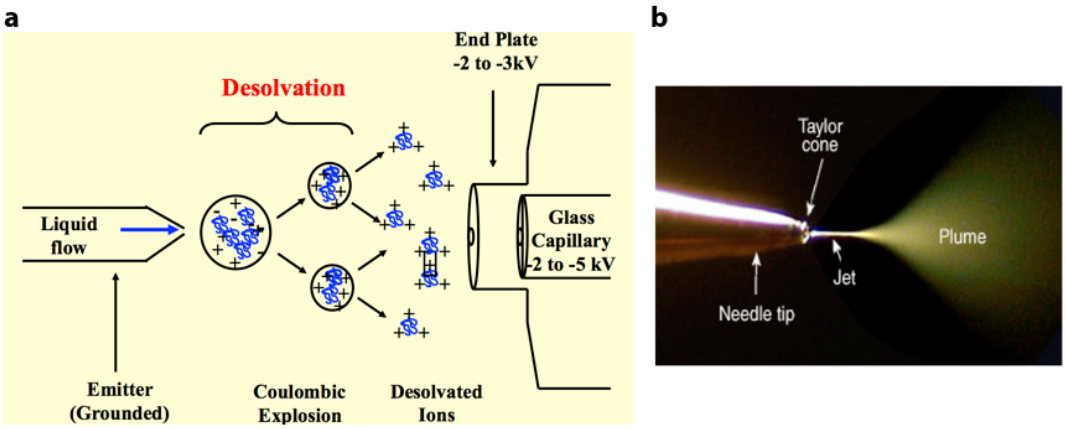


Figure 2 (a) Schematic representation of the processes in an electrospray ion source (ESI). A stream of positively charged droplets is sprayed from a fine nozzle into a sample chamber. The droplets are accelerated towards a plate at the opposite side of the chamber, which is at a strong negative potential. Desolvation of the droplet due to evaporation leads to repeated coulombic explosions of the droplets until only the charged sample molecules remain, which are pulled into the flight path of the mass spectrometer. **(b)** shows a photograph of the inside of an actual electrospray ion source. The electrostatic repulsion between the droplets leads to a splaying of the ejected droplets into a cone of droplets.

Mass filters/analyzers

A mass filter is a device that lets accelerated ions pass only if these ions have a particular *m/z* ratio that was specified by the experimenter. Several different types of mass filters exist that are based on rather different physical principles and that have different performance characteristics (more on that below). As an illustration of the physical principles that can be employed in such a mass filter, the function of a quadrupole mass filter is discussed in figure 3.

[1] Because the solvent molecules are neutral, they are not accelerated by the electrostatic field and are simply removed by the vacuum pumps.

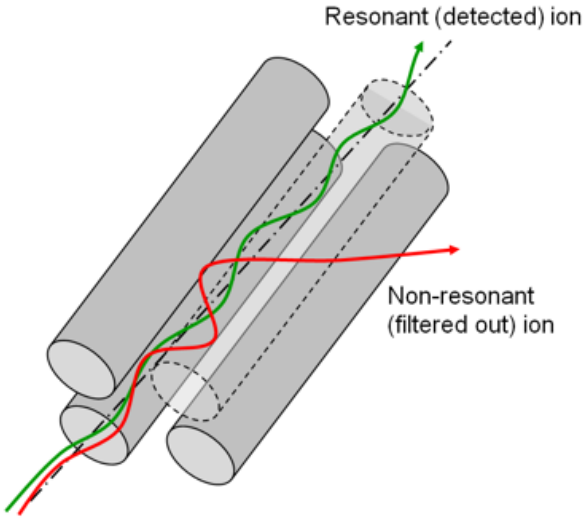


Figure 3 In a quadrupole mass filter, accelerated molecular ions pass through a channel surrounded by four metallic rods, to which a quickly oscillating electric field is applied. The resulting oscillating electrostatic field will set the ions in the channel on an oscillating path. Unless the oscillation of the field and the resulting oscillation of the ion resonate perfectly, the ions will veer off path, collide with the chamber walls, and will thus be filtered out. While the ion's frequency of oscillation is determined by its mass-to-charge ratio, the frequency of the quadrupole's oscillations can be adjusted electronically. This makes it possible to select and dynamically adjust the mass-to-charge ratio of the ions that are able to pass the filter.

Detectors

The final element in a mass spectrometer is the detector, with which the arriving ions are recorded. This occurs either by the ions physically striking the detector surface, or by the ions passing by the detector surface and the detector sensing the field of the passing ion. The actual number of ions arriving at the detector is rather small, so that the signal needs to be amplified considerably (e.g., by an electron multiplier) before it can be measured.

MS/MS spectrometers

Many modern mass spectrometers possess two or more mass filters that are arranged in series along the flight path of the ions. Between these two mass filters a **fragmentation stage** is inserted, in which the molecular ions are broken down into smaller pieces.

One of the most popular fragmentation stages is a collision-induced dissociation (CID) stage. In such a CID stage, the fast-moving molecular ions are allowed to collide with trace amounts of neutral gasses (often helium, argon or nitrogen). The kinetic energy of the collision is converted into very strong intra-molecular vibrations that can disrupt chemical bonds within the molecular ions. The actual fragmentation process is very complex and also depends on the site localization of the proton(s) captured during the ionization process. It is not discussed in detail in this course. The resulting fragments, provided they carry a charge, continue on their flight path while the neutral fragments are removed together with the collision gasses by the vacuum pumps. A range of other fragmentation methods have been developed. They have different properties than CID and are not further discussed in this course.

In a typical MS/MS experiment (Figure 4), the first mass filter selects ions of a particular m/z ratio that subsequently enter into the fragmentation stage, where they break down into fragments. These fragments are then analyzed in the second mass filter. By coordinating the scanning patterns for the first and the second mass filter, it is possible to select each of the sample ions, to fragment them, and to record a mass spectrum of the resulting fragment ions. The fragmentation pattern of a sample ion is often very characteristic and, together with the sample ion's m/z value, can be used to identify the chemical identity of the sample ion.

In practice, it turns out that the number of molecular ion types that arrive at the first mass filter is so large that it is not possible to record fragmentation spectra for every one of the molecular ions. The optimal strategy for selecting the sample ions that are fragmented and analyzed further depends very

much on the overall scientific question the experimenter wants to answer. This topic will be discussed in greater detail in the lecture.

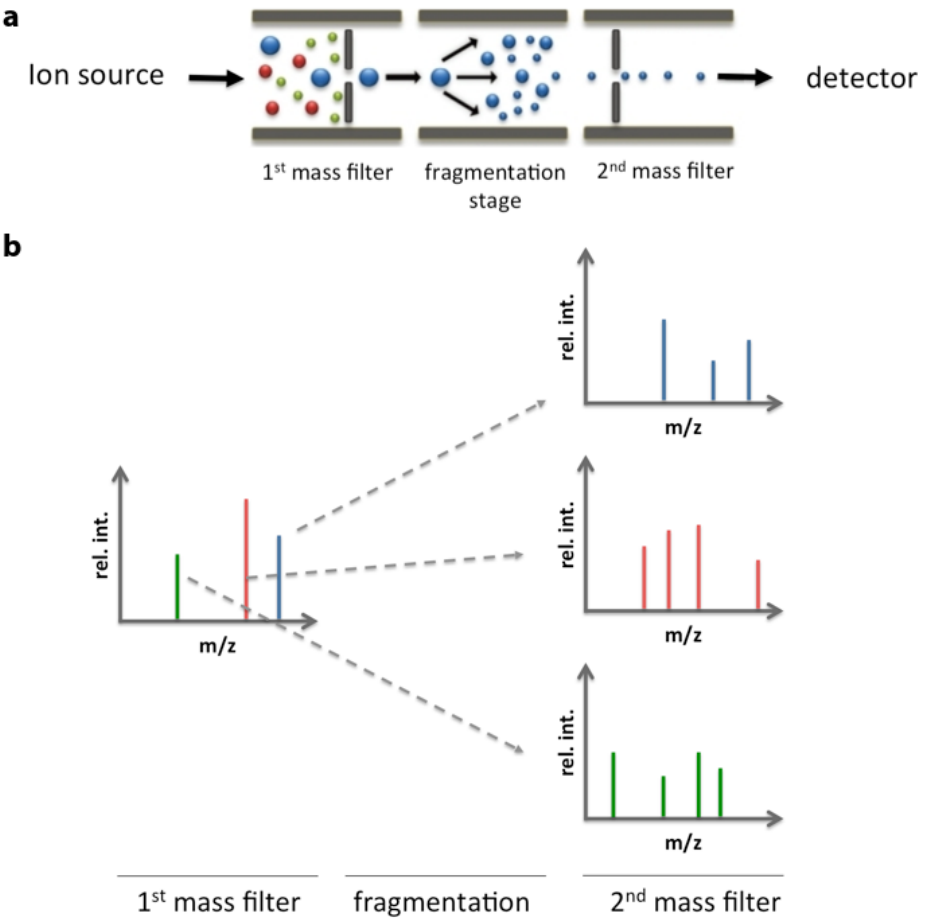


Figure 4 (a) Operating principle of an MS/MS mass spectrometer consisting of two mass filters and a fragmentation stage placed between these two filters. The first mass filter can select, based on their *m/z* ratio, which molecular ions enter into the fragmentation stage. The second mass filter is then used to filter the generated fragment ions according to their *m/z* ratio. **(b)** An MS/MS instrument therefore allows the selection of a molecular ion according to its *m/z* ratio in the first mass filter, and a further analysis of this ion by breaking it apart in the fragmentation stage and analyzing the resulting fragment ions in the second mass filter.

Features of mass spectra

As discussed above, a mass spectrum is a plot of the relative intensity of the detector signal as a function of the *m/z* ratio of the ions generating that signal. It is fundamental to the understanding of mass spectroscopy to realize, that one peak in the spectrum does **not** correspond to one molecular entity (e.g., an amino acid or a peptide). Multiple chemical entities may contribute to one peak and one molecular entity will typically generate multiple peaks in a mass spectrum.

Isobaric species

The mass spectrometer measures the mass of a molecular species, and this mass is determined by the sum of the masses of its atoms. Therefore, every molecular species that has the same atomic

composition (and charge) will contribute to the same peak in the mass spectrum, regardless of how the atoms in the molecule are arranged. Molecular species that have exactly the same mass are called isobaric species. The classic example of isobaric amino acids is leucine and isoleucine (Figure 5), which both contain 6 carbon, 13 hydrogen, 1 nitrogen and 2 oxygen atoms, but which have distinct chemical structures. Equivalently, the peptides Asp-Tyr-Pro-Lys-Glu-Thr and Tyr-Pro-Lys-Glu-Thr-Aps are isobaric because they have the same amino acid composition. However, please note that sequence and thus the biological significance of the two peptides is different.

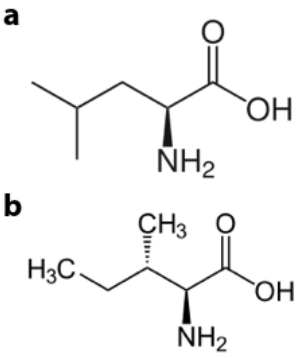


Figure 5 Example of two isobaric amino acids leucine **(a)** and isoleucine **(b)**. Both molecules have the same atomic composition ($C_6H_{13}NO_2$) and therefore have the same mass. Yet, they are chemically distinct and have different molecular properties.

Multiply-charged ions

A single type of molecular entity in the sample can generate multiple peaks in the mass spectrometer by adopting multiple charge states. In the ionization phase, one of two identical sample molecules may receive a single charge, while the other one may receive two charges (doubly charged ion), or even three or more charges (triple or multiply charged ion). Because the mass spectrometer actually measures the mass-to-charge ratio and not the mass, the doubly charged ion will appear in the mass spectrum at roughly half the *m/z* ratio of the singly charged ion, even though they originate from identical sample molecules. The actual *m/z* recorded for a doubly charged ion is $[M+2H]^{++}/2$.

Isotopes

Isotopes are atoms that contain the same number of protons and electrons and thus display the same chemical properties, but differ in their number of neutrons and therefore have different masses.

Atom	Mass	Rel. abund. (%)
carbon	12.000	98.900
	13.003	1.100
hydrogen	1.008	99.985
	2.001	0.015
nitrogen	14.003	99.630
	15.000	0.37
oxygen	15.995	99.760
	17.999	0.200
sulfur	31.972	95.020
	33.968	4.210

Figure 6 Table of the relative abundance and atomic mass of different elemental isotopes.

A list of naturally occurring isotopes for elements commonly found in biomolecules is shown in figure 6. Out of these elements, the isotopes of carbon are particularly important, because carbon is present

in virtually all biomolecules and possesses a relatively common isotope. Approximately one out of every hundred carbon atoms has an atomic mass of 13 and not 12. These ^{13}C carbon atoms have the same chemical properties as ^{12}C carbon atoms, and are therefore integrated into these biomolecules by random chance during the natural biosynthesis process.

The result is that a group of chemically identical molecules will actually contain some versions of these molecules, in which one, two, three or more of the ^{12}C carbon atoms are replaced by a ^{13}C carbon atom and will therefore have a correspondingly higher mass. Thus, even chemically pure compounds will consist of a mix of molecules with different masses. In a mass spectrum, this will be visible as a so-called isotope ladder (a.k.a. isotope distribution) of multiple peaks shifted relative to one another by one mass unit (Figure 7). The multiple ion species appear, because in a molecule with a number of carbon atoms the number of ^{13}C carbon atoms follows a Poisson distribution.

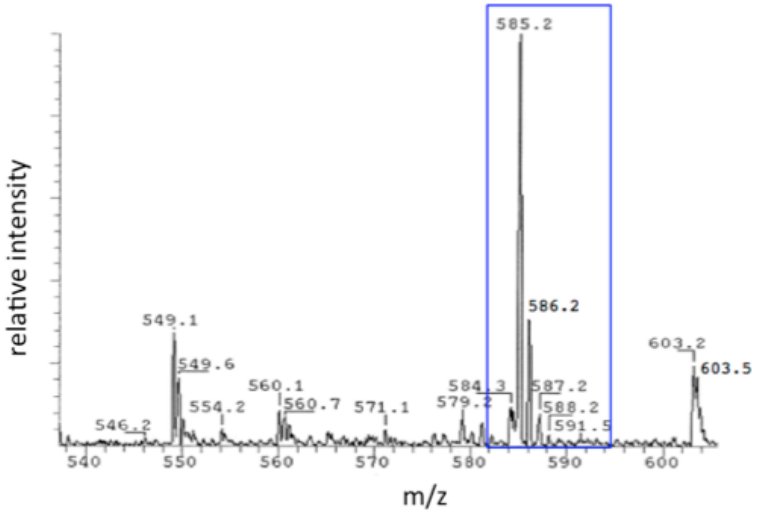


Figure 7 Zoomed-in view of a peptide mass spectrum showing the presence of a typical isotope series of mass peaks (blue box). Note how the masses of the peaks increase by one mass unit and decrease in intensity. These "isotope" peaks are caused by variants of the molecule that contain one, two, three etc. ^{13}C atoms.

Besides leading to relatively crowded spectra, the presence of these isotope ladders pose stringent requirements on the resolution a mass spectrometer needs to be able to achieve. If the resolution of the mass spectrometer is not sufficient to resolve these isotope ladders the peaks fuse and the resulting aggregate peak will have a mass value that is hard to interpret (Figure 8).

Sample preparation

One of the central tasks in mass spectrometry in biology is the analysis of the protein composition of living tissues or cell cultures, in particular how this composition changes in response to a perturbation. (e.g., a genetic mutation or a change in environmental conditions). For this, the proteins need to be extracted from the tissue and prepared for analysis in the mass spectrometer. Standard steps for extracting proteins from complex samples are the disruption of the tissue by physical means (blender, cryo-pulverization etc.), followed by chemical extraction steps using detergents and salts.

The proteins in the samples are then denatured (i.e., unfolded) and disulfide bonds in the protein are chemically reduced. To prevent disulfide bonds from reforming, the cysteine residues are chemically blocked.

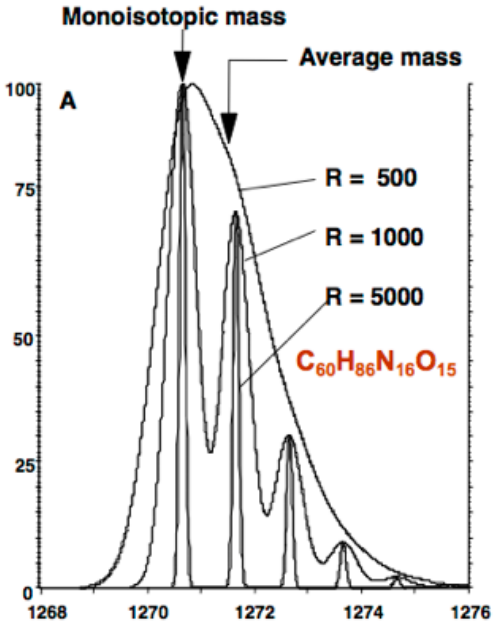


Figure 8 An isotope ladder in the mass spectrum of a biomolecule with the sum formula of $C_{60}H_{86}N_{16}O_{15}$, shown at three different resolutions (R=5000, 1000, and 500). As you can see, if the resolution is comparable to or greater than the *m/z* value of the ions being analyzed, the individual peaks of the isotope ladder can be separated. At lower resolution, the peaks of the isotope ladder fuse to an aggregate peak. Neither the peak mass nor the average mass of the resulting aggregate peak correspond to the *m/z* of any of the underlying ions, and the measured mass value becomes difficult to interpret.

Proteins are digested into peptides prior to MS analysis

While it is technically possible to analyze the masses of intact proteins in a mass spectrometer, this route is only pursued for very specialized applications. As it turns out, the physical and chemical properties of proteins vary so much from one protein to the next, that it would be difficult to develop one protocol that can be applied consistently across a wide range of different proteins.

Furthermore, as discussed above, the mass of a protein (or peptide) is not a unique identifier for the respective molecule and the determination of the amino acid sequence from the molecular ion of a large protein is very challenging. Oligopeptides, on the other hand, tend to have relatively similar physicochemical properties independent of the peptide's exact sequence. It is therefore possible to develop standard protocols that allow the handling and purification of the vast majority of peptides.

The standard strategy for analyzing complex protein mixtures by MS is therefore to digest the proteins into peptides with a length of approximately 10-20 amino acids, to analyze these peptide mixtures by mass spectroscopy, and to then infer the composition of the initial protein sample based on the observed peptide mix.

Protein digestion is performed by specific proteases

Proteins are cut into peptides using a well-controlled enzymatic digestion step. The most common protease used for this purpose is trypsin, which selectively cuts the peptide backbone of a protein on the C-terminal side of an arginine or lysine residue. This specificity, together with the frequency with which lysines and arginines typically occur in proteins, leads to peptides with a size of around 10-20 amino acids. Peptides of this size are short enough to be easily handled experimentally, yet they are long enough so that a peptide of this exact sequence is unlikely to occur multiple times in a proteome by pure chance.

The sequence specificity of trypsin further provides a useful constraint in the identification of a peptides sequence, because the C-terminal amino acid of the generated peptides has to be either an arginine or a lysine.

Peptide sequences are identified by MS/MS

The end product of the protein extraction and digestion step is a peptide mixture that may contain thousands to millions of different peptides that now need to be separated and identified via two-stage mass spectrometry (MS/MS) (Figure 9).

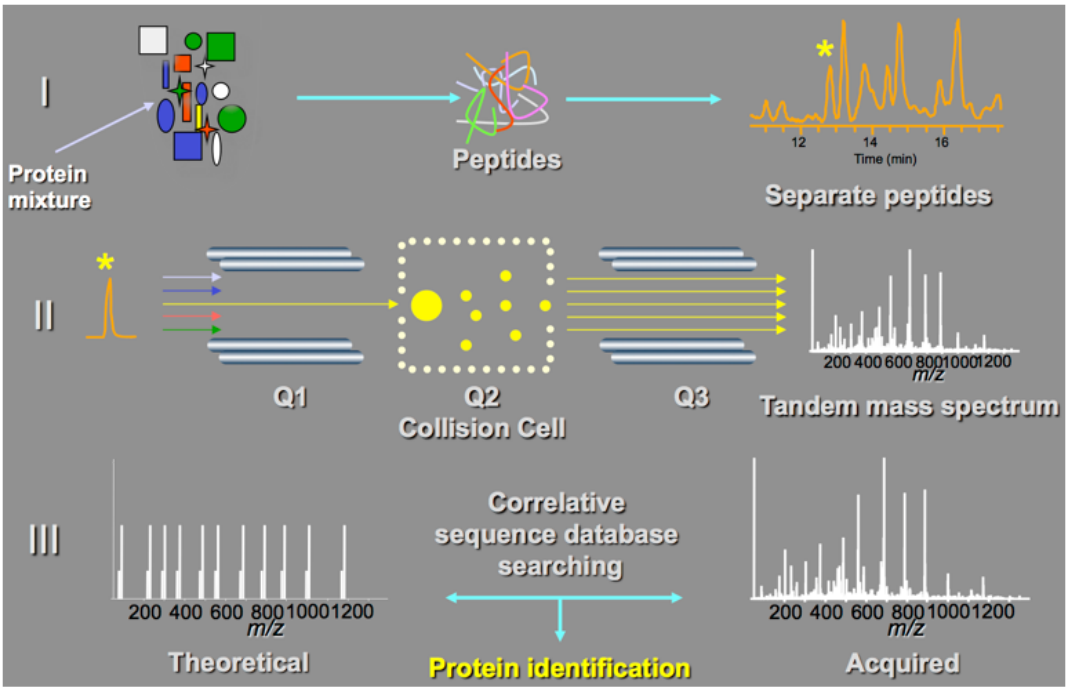


Figure 9 Workflow for using MS/MS to identify peptides in a complex mixture of protein-derived peptides. The workflow can be separated into three phases: **(I)** Sample preparation and pre-fractionation of peptides by HPLC. **(II)** MS/MS in a mass spectrometer equipped with two mass filters and (Q1 & Q3) and an interposed collision-based fragmentation stage (Q2). **(III)** Identification of individual peptides by matching their observed fragmentation ion spectrum to a database of all theoretically possible fragmentation spectra, predicted based on the sequence of the organism's protein-coding genes.

It would be possible to apply this complex mixture directly to the mass spectrometer, but it turns out that it is advantageous to pre-fractionate the resulting peptide mix before injecting it into the mass spectrometer. This pre-fractionation is achieved through high-pressure liquid chromatography (HPLC) of the peptide mixture on a reversed-phase column (Figure 10). In the most common configuration, the outlet of the HPLC column is directly connected to the ESI ionization stage of the mass spectrometer. The flow rate of the HPLC column is chosen such that the elution takes place over tens of minutes, which means that the composition of the peptides being eluted from the column changes much more slowly than the subsequent MS/MS-analysis step. This gives the mass spectrometer time to analyze the mixture of peptides arriving at the ionization stage in real time.

The first mass filter of the mass spectrometer analyzes the composition of the peptide ions and selects individual peptide species for further analysis

Despite the pre-fractionation step, the sample entering into the mass spectrometer at any given time will typically contain multiple different peptides. The masses and approximate abundances of these peptides are analyzed by the first mass filter. Then, this first mass filter is set so that only one of these peptides is allowed to pass to the subsequent analysis stages, which consists of the fragmentation of the peptide into smaller peptides. After this identification has taken place, the mass filter continues on

to the next peptide and so forth. During the time the mass spectrometer spends selecting a particular ion for fragmentation the other, concurrently present molecular ions are discarded.

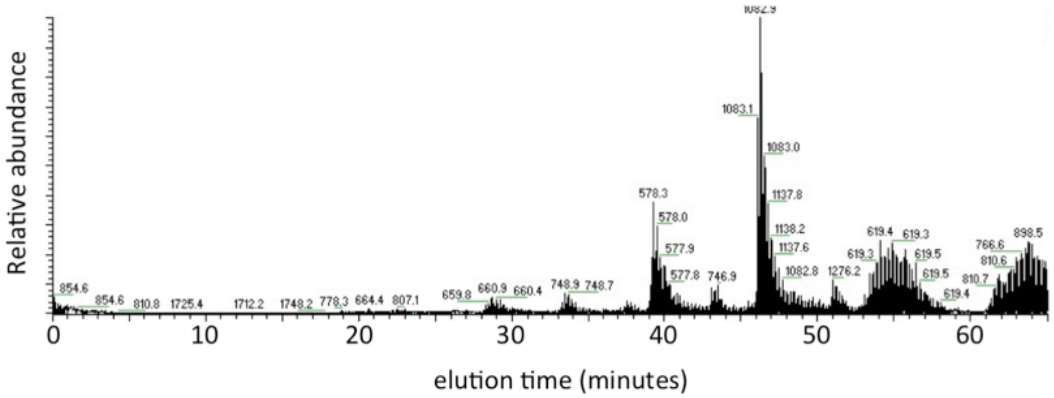


Figure 10 Elution profile of a complex peptide mixture from an HPLC column. The horizontal axis indicates the time of the elution, while the vertical axis indicates the amount of peptide being eluted from the column into the mass spectrometer. Prominent peaks in the elution profile are labeled with the mass of the dominant peptide species eluted at that time point.

Note that a typical protein sample from a tissue will contain so many different types of peptides, that a typical MS/MS experiment is unable to fragment and analyze all of them. The instrument software therefore selects only a fraction of the present peptides for further analysis. This usually does not present a major problem, because the presence of a protein in a sample can often be detected by the presence of one or two characteristic peptides per protein. Still, the strategy for selecting which of the peptides in a sample are analyzed further will vary, depending on the exact scientific question the experimenter wants to answer. Optimization of these strategies is the topic of active research.

Controlled collisions with inert gas atoms induces fragmentation of the peptide ions along the peptide backbone

Fragmentation of the peptide is usually achieved via the collisions of the highly accelerated peptide ions with inert gas ions inside a collision cell. This fragmentation process should not be imagined as a "random explosion" of the peptide into a multitude of random molecular shards. Instead, the conditions in the collision cell are chosen such that each peptide ion only experiences one collision, and that this collision results in one bond being broken. As it turns out, the peptide bonds in the backbone of peptide ions are far more susceptible to being broken by one of these collisions than any of the bonds in the amino acid side chains (Figure 11).

The breakages occur with comparable, but not identical, frequency at each one of the peptide bonds in the peptide. One might therefore find all possible fragments of a peptide that can be generated by breaking a single peptide bond (Figure 12). The mass differences between these fragments correspond to the masses of individual amino acids, which, in ideal cases, allows the sequence of the original peptide to be reconstructed. In practice, this direct reconstruction of the peptide sequence from the fragment ion sequence is rarely possible. By contrast, given a peptide's sequence, it is trivial to calculate its predicted fragment ion spectrum and to determine whether this predicted fragmentation spectrum matches the observed one.

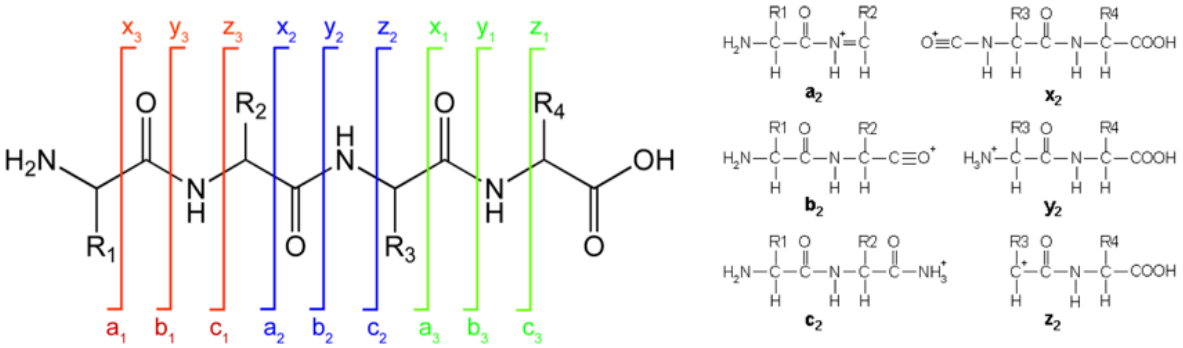


Figure 11 Fragmentation of a peptide ion following collision with an inert gas is most likely to occur along the peptide backbone. Each peptide unit contains three bonds where this fragmentation can occur, and for each of these breakages there is the possibility that the ion's charge ends up on the N-terminal fragment or on the C-terminal fragment. Fragmentation products where the charge ends up on the N-terminal fragment are called a-, b-, and c-ions while the products where the charge ends up on the C-terminal fragment are called x-,y-, and z-ions, respectively. Since neutral fragments are no longer accelerated, they are lost from the molecular beam and are not detected. In practice, a-,x- ,c-, and z-ions are rarely observed, and the fragmentation spectra of peptides are dominated by b- and y-ions.

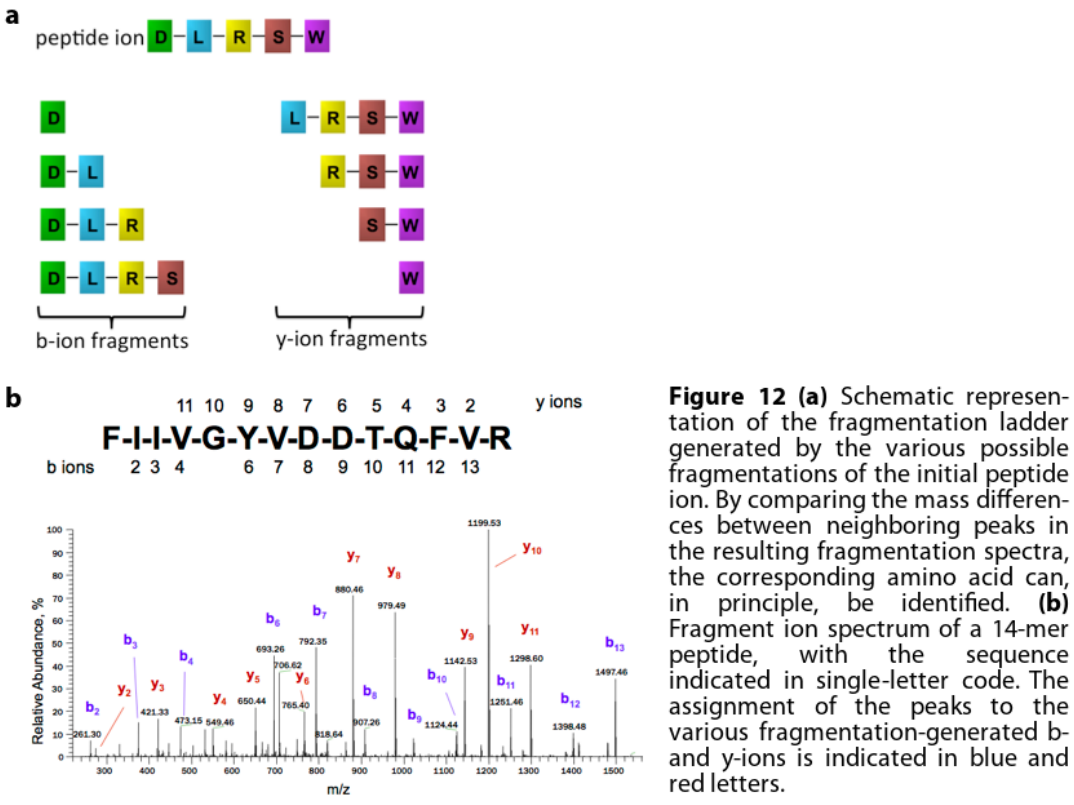


Figure 12 (a) Schematic representation of the fragmentation ladder generated by the various possible fragmentations of the initial peptide ion. By comparing the mass differences between neighboring peaks in the resulting fragmentation spectra, the corresponding amino acid can, in principle, be identified. **(b)** Fragment ion spectrum of a 14-mer peptide, with the sequence indicated in single-letter code. The assignment of the peaks to the various fragmentation-generated b- and y-ions is indicated in blue and red letters.

Observed fragmentation spectra can be matched against predicted spectra to identify peptides

Fragmentation spectra of peptides are rarely as clean and easy to interpret as the one shown in figure 12b. Often, only a few of the possible b- and y-ions are observed and the spectrum contains a large number of substantial noise peaks. Therefore, instead of trying to reconstruct the sequence of the peptide ion from the fragmentation spectrum, one takes the alternative route of predicting the fragmentation spectra of possible peptides and comparing these predicted spectra with the observed

fragmentation spectrum. For this, one uses the genomic information of the organism from which the sample is derived to predict the sequence of the proteins produced by this organism. The enzymatic digestion of those proteins into peptides is then simulated in the computer, and the masses for the b- and y-ions are calculated.

A scoring function is then used to assess the quality of the match between the observed and the predicted fragmentation pattern. These scoring functions have become increasingly sophisticated and take into account not only the quality of the match between the observed and calculated spectra, but also additional information such as the quality of matches with other candidate peptides or the likelihood that the peptide in question would be observed at that stage in the HPLC-elution profile. The end result of this analysis is a probability score that represents the probability with which the current peptide corresponds to a particular predicted peptide.

Identified peptides can be used to infer the presence of their precursor proteins in the original sample

With the information about the peptides contained in the sample in hand, one can then try to infer the presence of a particular protein in the original sample. Again, this process involves a series of statistical considerations that have been implemented in specialized software packages. In some cases, a single, highly unique peptide, that has been identified very clearly via its fragmentation spectrum, can be sufficient to establish the presence of a particular protein in the sample. This is the case if that specific peptide sequence occurs precisely once in the proteome tested. In other cases, several peptides need to be identified to make a reliable statement about the presence of a particular protein. It should be understood that in most cases, only a small fraction of the observable ions of a protein are actually observed; however, this number is generally sufficient to reliable infer the presence of a given protein. Using this strategy, modern mass spectrometers can reliably identify thousands of proteins per sample.

Where the analysis becomes more complicated is in cases of proteins with high sequence identity (e.g., proteins whose genes are derived from recent gene duplications), or cases of multiple splice variants of a particular protein. In those cases, it may be difficult to use the peptide-derived information to unequivocally infer the presence of a particular protein in the sample, since these different closely related proteins will generate many of the same peptides.

Quantification

The preceding section discussed the process of determining whether a particular protein is present in a sample or not. For many applications, this information is not sufficient. Instead, one wants to compare, for example, the relative abundance of a cell's different proteins before and after a perturbation. However, measuring the relative or even the absolute abundance of a protein via MS is a non-trivial task.

In MS, the intensity of the detector signal correlates poorly with the abundance of the protein in the sample

The intensity of the signal in the detector of a mass spectrometer depends on the efficiency of a number of physical processes that include the efficiency of ionization, the efficiency of fragmentation, and finally the efficiency with which an ion triggers a signal in the detector. The individual efficiencies of these different processes vary greatly across peptides, so that the overall efficiency of signal generation can vary across peptides by several orders of magnitude. In other words, the intensity of the detector signal generated by a peptide ion is a very poor indicator of the peptide's abundance in the sample.

Even for the same peptide the efficiency will vary from day to day and sample to sample, due to subtle drifts in instrument settings and due to interactions between the peptide and the sample matrix. For example, different peptide molecules compete for a limited number of charges in the electrospray droplets. A high abundance of a peptide that readily attracts charges may therefore deprive other peptides of a charge and prevent those peptides from being detected. This effect is called ion suppression.

Therefore, even relative intensities for signals, derived for the same peptide but from two different samples, are only a rough indicator of the peptide's relative abundance in the two samples.

Complex sample preparation steps hamper reproducibility of results

As we have seen above, the process from the original biological sample to the recording of the mass-spectrum involves many different steps that are also difficult to reproduce exactly for successive runs, thus further reducing the reproducibility of MS-based protein abundance measurements across multiple samples.

Selective isotope labeling of samples allows the simultaneous measurement of multiple samples and enables reliable comparison of protein abundance across samples

Currently, the most reliable strategies for comparing the abundance of proteins across samples via MS are based on stable isotope labeling. As discussed above, isotopes have the same chemical properties but different masses. Modern mass spectrometers are able to detect these inter-isotope mass differences reliably. Isotope-based labeling uses this fact to separately track chemically identical peptides from different sources in a single pooled sample. The general principle of isotope labeling is explained here on the example of SILAC (stable isotope labeling by amino acids in cell culture, Figure 13), but variations of this isotope labeling strategy can be applied to make quantitative measurements in a variety of experimental settings.

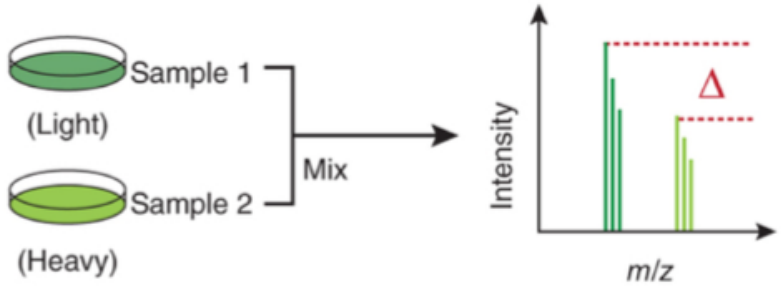
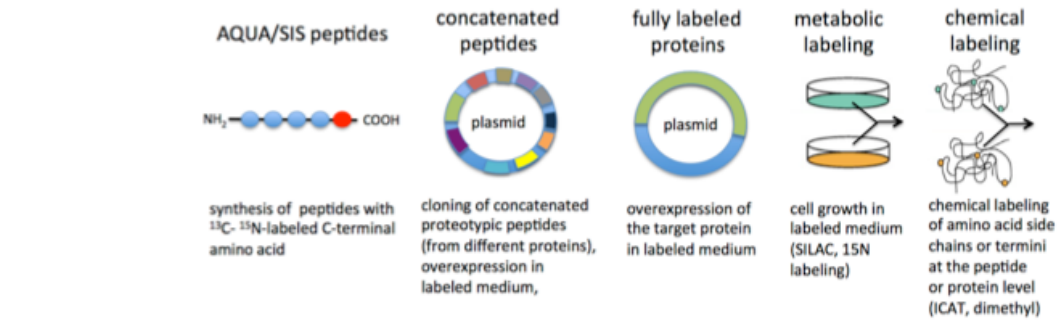


Figure 13 Figure 13 SILAC experiments allow the measurement of changes in the relative abundance of proteins in response to a system perturbation. Two identical cell lines are grown in cell culture using chemically identical growth media. But, the amino acids in the growth medium of one cell culture are replaced by their heavy-isotope versions. One of the two cell cultures is then perturbed. The cells from the two cultures are pooled and processed together. In the mass spectrum, the peptide ions stemming from the two cultures can be readily distinguished due to their mass differences, thus enabling the accurate detection of changes in the relative abundance of proteins between the two samples.

One of the standard systems biology experiments is the perturbation of a cell line (either by a mutation, a change in growth conditions, or the addition of a medicinal drug), followed by a measurement of how this perturbation has changed the relative abundance of different proteins in the cell. The SILAC experiment answers this question by growing two cell cultures in parallel. The first cell culture, which represents the unperturbed condition, is grown in a standard growth medium. The second culture is grown on a medium that is chemically identical to the growth medium used for the first culture, but contains variants of the amino acids that contain only heavy isotopes. This second culture is then exposed to the perturbation. The cells from the two cultures are then pooled and undergo the process of protein extraction, protease digestion and mass spectrometry as one single sample. However, in the mass spectrum, the signal from the ions stemming from the unperturbed and the perturbed sample can be readily distinguished due to their differing mass, thus allowing an easy and accurate comparison of the relative abundance of equivalent peptides in the two samples.

The same fundamental principle of isotope labeling is used in many variations (Figure 14) to enable the determination of relative and even absolute abundances of proteins and peptides. The isotope labels are either introduced by incorporating isotope labeled amino acids during natural protein synthesis or are chemically introduced after cells are disrupted. An alternative strategy is the spiking of the sample with isotope-labeled reference versions of specific peptides or proteins. These reference peptides can be either synthesized chemically or can be generated by recombinant expression in microorganisms grown on isotope labeled growth media. The disadvantage of these spiking strategies is that a specific reference peptide has to be generated for each of the peptides that is to be quantified. The advantage of the spiking strategy is that it the amount of labeled peptide that is added can be measured, which enables the determination of absolute concentrations.



relative quantification	+	+	+	+	+
absolute quantification	+	+	+	-	-

Figure 14 Comparison of different isotope-labeling strategies for the quantification of peptides and proteins in complex samples via mass spectrometry.