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Development of Multiplex Mass Spectrometry Methods for Probing the Response to Copper Toxicity in the Blue Crab

By
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Lloyd Smith, Professor, Chemistry

Chapter 8

WISL Chapter

Written for the Wisconsin Initiative for Scientific Literacy to describe this thesis work for a broader audience.

Over the last five years of graduate school, I have been asked "So what is it that you do?" more times than I can count. Although I find it easier to answer that question for people with a background in my field, it is arguably more important to answer the question for those unfamiliar with proteomics, analytical chemistry, etc. I wrote this chapter not only to prevent seeing the look of confusion on my parents' faces (the same one I have when my dad tries to explain what pass interference is), but also to learn to present my work more succinctly and successfully to broader audiences. As scientists, it is important to acknowledge the importance of communicating results and delivering a product to the people that supported our research (i.e., taxpayers). I am thankful for the help and feedback provided by the Wisconsin Initiative for Science Literacy, especially Elizabeth Reynolds, Cayce Osborne and Bassam Shakhashiri.

Crustacean Model Organisms

The human nervous system is incredibly complex and includes not just the brain, but also the spinal cord and nerves throughout the body. This system is comprised of specialized cells called neurons that allow the different parts of the body to communicate with one another. Communication between different parts of the body is an incredibly broad function, explaining the complexity of the nervous system and the reasons it is so challenging to study. Studying the nervous system is important, however, as it provides researchers with more information about many neurological disorders like dementia, Parkinson's disease, and depression.

To better understand the human nervous system researchers often start with a simpler model organism. Where some labs study mice, rats, etc., our lab studies

crustaceans, specifically the blue crab, *Callinectes sapidus*. Blue crabs have well-defined nervous systems with far fewer neurons than humans, making it significantly easier to study them. Although it is simpler, the overall processes in the crustacean nervous system are similar to humans and other organisms, so researchers can apply the techniques used and developed in this work to more relevant biological problems in humans.

Understanding the Nervous System

There are many ways that neurons communicate with one another to send signals. This can be done with electrical signals, similar to circuits, or chemically. With chemical signaling, one can think of neurons as buildings and the connections between neurons as roads. In this analogy, the chemical signals are the cars and pedestrians traveling on the roads to different buildings. These chemical signals are released due to a stimulus, such as people leaving their homes to go to work because of the time of day. Trying to study how specific neurons communicate with each other in relation to the entire system is incredibly difficult as there are too many (up to billions depending on the organism) interactions to feasibly study. Instead, looking at the overall trends in signaling is not only easier, but can still provide information on how different biological states affect signaling. By comparing signaling in a healthy organism and one with a particular disease like Alzheimer's disease, we can start to uncover what is going wrong at a molecular level. Using the traffic example, trying to observe differences between a few pedestrians in New York City is difficult, but if an entire subway line is closed, there

will be changes in the overall traffic across the city that are observable. This information can provide researchers with new tools for understanding, diagnosing, and treating diseases.

There are many different types of chemical signals that are created in and released by neurons, but one of particular interest to our lab is neuropeptides. Neuropeptides are simply peptides that originate in the neuroendocrine system. They can exert their signaling effects locally at adjacent neurons, or they can be released into the circulating fluid and exert their effects at distant neurons throughout the body.[1] Neuropeptides have been shown to be involved in different diseases, stress response, wake-sleep cycles, and many more biological processes.[2–4] As a result, their dysregulation–when their concentration levels are out of balance–has been implicated in different neurological disorders.

Improving the Detection of Neuropeptides

As mentioned before, neuropeptides are peptides originating in the neuroendocrine system, and peptides are simply chains of amino acids. Amino acids are typically viewed as the building blocks of peptides and proteins; there are 20 different amino acids, and the exact number of them in a particular chain (peptide) and the order of them in that chain is critical to their structure and the role they play in the body. Amino acids are assigned letter codes, so peptides can be thought of as strings of letters to make a word, except the alphabet only has 20 letters. Like letters in a word, the amino acids in a peptide and the order of them is important. Adding a letter to a

word (e.g, RIDE to PRIDE) changes its meaning, as does rearranging the letters of a word (e.g., RIDE to DIRE).

Techniques to analyze neuropeptides must be capable of determining the neuropeptide sequence–its amino acid content and order of those amino acids–to discern different neuropeptides. Mass spectrometry (MS) is a technique that allows the researcher to determine the sequence of a neuropeptide based on its mass. These instruments act almost as molecular scales, allowing the mass of a peptide to be observed. The peptide is then broken apart and the pieces are "weighed" as well. By combining the information from the mass of the intact peptide as well as its fragments, an analytical instrument (i.e., mass spectrometer) and computer output a spectrum of masses corresponding to different fragments. Researchers, either manually or using data processing software, can determine the peptide sequence from the spectrum based on the unique combination of peaks in it. An overview of this is given in **Figure 1**. What makes MS such a powerful technique is that it can be used to determine the sequence (identity) and relative expression (quantity) of all neuropeptides in a single analysis. This allows researchers to not only see which neuropeptides are present, but also how they are changing between two or more samples, such as stressed vs unstressed or diseased vs healthy samples.

When analyzing neuropeptides, MS can provide quantitative information using different strategies.[5] By tagging the neuropeptides, the samples can be analyzed at the same time and the relative peak intensities in the mass spectrum can be used to determine the amount of neuropeptide from each starting sample. This not only allows

more samples to be analyzed in the same amount of time, but also reduces variation caused by the instrument, improving accuracy. One type of labeling is known as isotopic labeling. In these methods, a part of the neuropeptide is labeled with a chemical tag, like formaldehyde to cause a shift in the mass of the peptide. Different channels of the same tag have different isotopes of certain atoms; these isotopes are the same chemical element but differ in mass from one another due to differences in the number of neutrons they have. For example, ^{12}C and ^{13}C are both carbon atoms, but one has a mass of 12 and the other has a mass of 13 (from the mass of an additional neutron compared to ^{12}C). The different channels with different isotopes therefore increase the mass of the neuropeptides by a different amount. By labeling Sample A (e.g., diseased) with one tag and Sample B (e.g., healthy) with another tag, there will be a mass difference and two peaks will be observed. The relative signal of these peaks is then indicative of the concentrations of that neuropeptide in the biological samples (visually explained in **Figure 2**). We have applied isotopic labeling to study changes in neuropeptides across four samples.[2]

Another labeling method, known as isobaric tagging, is like isotopic labeling, but each channel of the tag incorporates the same mass. Neuropeptides from Sample A and Sample B will appear identical, but when they are analyzed by their fragments (also known as MS^2), reporter ions form for each channel. These reporter ions are unique to each tag and result in peaks that can be used for quantification. In other words, these diagnostic ions report the relative signal from each sample and correlate to quantitative differences between the two samples.

To compare isotopic and isobaric labeling, it is important to remember that they have the same goal: quantify relative differences between samples in a single analysis. Isotopic labeling achieves this by adding tags of different masses to the sample, and isobaric tagging adds tags with the same mass, but the isobaric tags result in distinct fragments (reporter ions) for the different channels. This can be thought of like voting in an election. In Scenario A (analogous to isotopic labeling), each person that votes receives an "I voted" sticker, but the number of stickers they receive is indicative of the candidate they voted for. Votes are tabulated by counting the number of voters that have one sticker, two stickers, etc. Conversely, in Scenario B (isobaric labeling), everyone receives one sticker, but the color of the sticker is dependent on the selected candidate. Votes are then tabulated by comparing the number of people with blue stickers, red stickers, etc. The primary advantage of the isobaric label is that there are fewer peaks in the spectra (or stickers handed out), so data-interpretation is less complicated. By minimizing complexity, isobaric tagging allows for more samples to be analyzed at the same time to improve throughput. **Figure 3** shows the main differences between isotopic and isobaric tagging. While many isobaric tags exist, in the Li Lab, we use a custom isobaric tag known as DiLeu (*N,N*-dimethyl leucine).[6]

In most MS experiments, data is collected in a manner known as data-dependent acquisition (DDA). In a DDA experiment, an initial spectrum is collected without fragmentation, known as an MS^1 or precursor scan, and subsequent scans are collected in which the top peaks of the precursor scan are fragmented and analyzed (MS^2 scans). The initial scan measures the mass of the intact peptides in the sample, and then the

instrument (depending on the data it just acquired, hence data-dependent acquisition) selects the peptides with the greatest signal in that spectrum to be fragmented and analyzed further. DDA methods allow many samples to be quickly and easily analyzed and many neuropeptides can be identified from each sample. Not all neuropeptides, however, are going to be the peaks with the highest signal, so they can often be omitted from the MS^2 scans. By not selecting these neuropeptides for MS^2 scans, the instrument can only output information about the intact mass. If we again compare neuropeptides to words, the intact mass is incapable of distinguishing between RIDE and DIRE. Additionally, the reporter ions required for quantification rely on the instrument fragmenting the intact neuropeptides and generating MS^2 information–trying to quantify the neuropeptides without MS^2 information would be like having a person with colorblindness tabulate the votes in the earlier analogy. By omitting neuropeptides from the MS^2 scans, the mass spectrometer prohibits researchers from confidently identifying and quantifying neuropeptides. To address this, we have demonstrated how we can adjust the various DDA parameters to increase the number of relevant spectra collected. This work led to a 3-fold increase in the number of neuropeptides we could identify and quantify in a single analysis. **Figure 4** summarizes the results of the optimization by showing how each iteration increases the number of identified neuropeptides. We then applied these optimized methods to the study of copper toxicity to gain insights into how crabs survive influxes of copper in the ecosystem.[7]

Related Protein Studies

We have shown neuropeptides to be dysregulated in response to copper toxicity, but there are likely other molecules involved. One of particular interest are metallothionein proteins (MTs). Proteins are structurally similar to peptides, made up of the same amino acids, but they are much larger and have more dynamic structure and function. MTs are of interest because they bind metals, like copper, in the body so they can be transported.[8] Copper is a necessary nutrient in the body, but also toxic at high levels; MTs help keep the amount of copper in the body at a desirable range. Large influxes of copper are therefore likely to cause a change in the amount of MTs observed.

Using the same DiLeu isobaric tag that was used for the neuropeptide studies, we developed a method for quantifying MTs using MS. This presented some challenges as MTs are larger than most peptides and have more labeling sites that need to be taken into account. Additionally, the proteins are high in cysteine, a specific amino acid that gives the protein their metal-binding properties. These cysteine components are able to interfere with the DiLeu labeling process, so they need to be modified by chemical reaction to prevent side reactions during the labeling. The work presented in this thesis demonstrates the effectiveness of using a modified DiLeu labeling strategy to measure relative amounts of intact metallothionein proteins. Typically, because they are much larger than peptides, proteins are often split into smaller pieces before analyzing them, a process known as digestion. Digesting the proteins makes it easier for the instrument to accurately analyze the proteins, simply because it is easier to measure differences between small molecules compared to large molecules. Although effective,

this digestion process can lead to losses in information, such as differences between similar metallothionein proteins. By measuring the proteins intact (without digestion), researchers gain more information about the system. The labeling method developed here is one of the first to demonstrate the ability to use isobaric tags to quantify changes in intact proteins. Briefly, proteins are chemically labeled with four different channels (distinct tags) of DiLeu, similar to the neuropeptide experiments. In real biological samples, protein concentration can vary greatly. We created a sample to mimic this that had set amounts of labeled protein from each channel; channels 116, 117, and 118 were present in this sample at concentrations 5, 10, and 20 times greater than channel 115 respectively. each channel were pooled together to create a sample that would yield reporter ions of different, but known, relative intensities. Comparing the observed signal intensities to the theoretical intensities (1:5:10:20), the accuracy of the method could be determined (summarized in **Figure 5**). We observed accuracy >80%, demonstrating the method is suitable for future biological applications.

Research Impact and Future Goals

This research aims to develop the tools to better study neuropeptides and related biomolecules involved in different biological processes. Specifically, this work creates a framework for studying these important biomolecules with improved speed and accuracy by incorporating DiLeu isobaric tags. The developed methods are applied to the study of crustacean neuropeptides to show relative changes after exposure to an environmental stressor like copper toxicity. These methods are transferable to other

biological problems, however, and can have impact in clinical research to study neurological disorders like Alzheimer's disease and Parkinson's disease. By expanding the tags to other targets, like proteins and neurotransmitters, there are even greater possibilities for discovering new ways to diagnose and treat disease.

Acknowledgements

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Figures

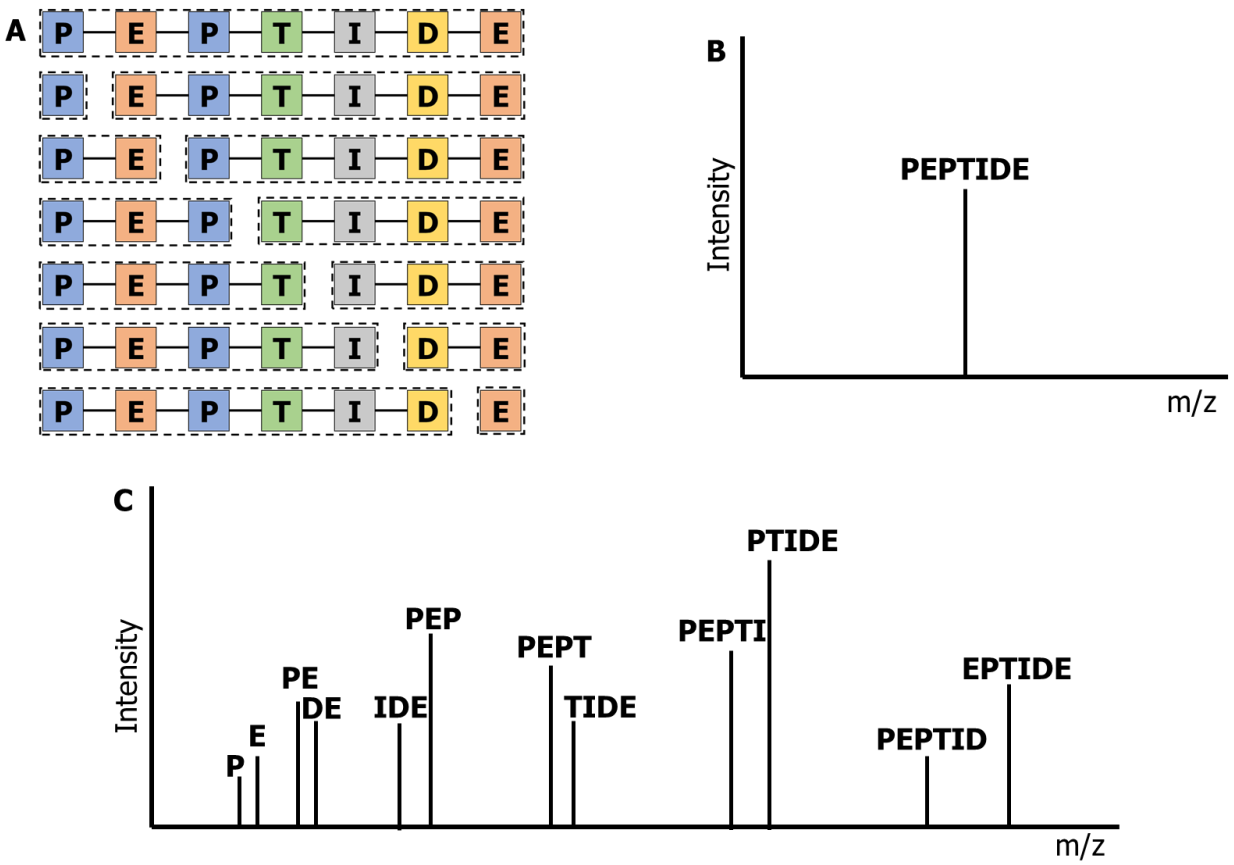


Figure 1: An example MS analysis of a peptide with sequence PEPTIDE. **A** shows the sequence of the peptide with dashed lines around the fragments that are shown in **B** and **C**. **B** is the MS^1 or precursor mass spectrum where the intact mass is observed. **C** is the MS^2 spectrum where the fragments from **A** are observed.

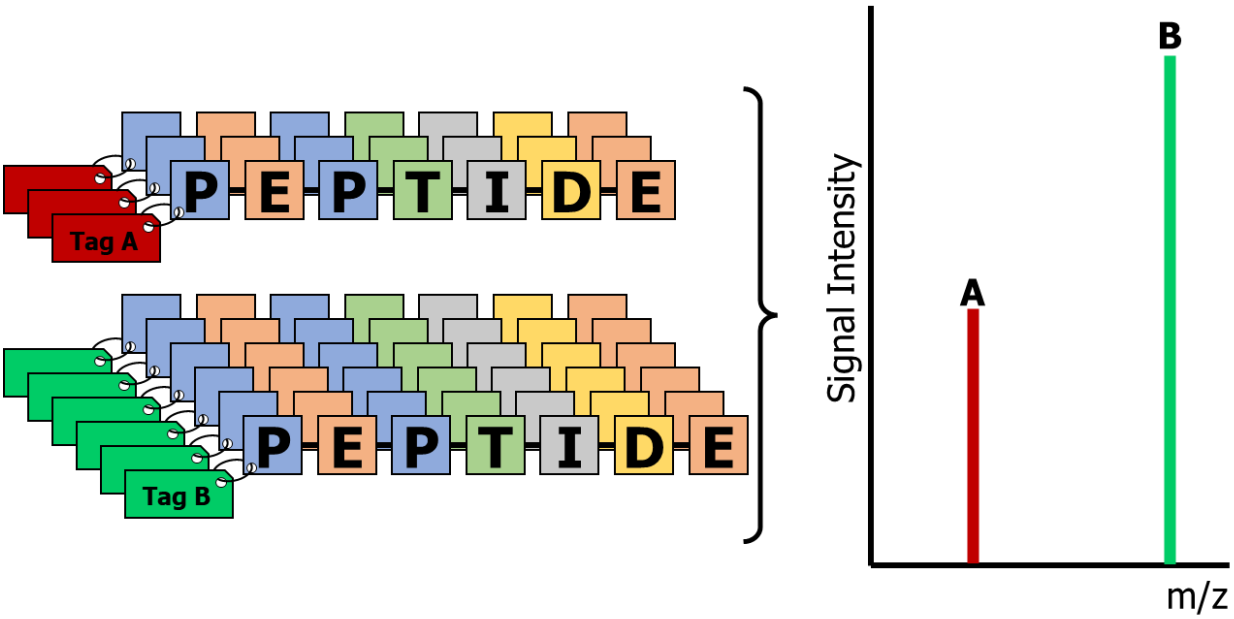


Figure 2: Quantification of labeled samples. The peptides are labeled with different tags depending on the samples they came from. Different tags yield different signals and can be distinguished by mass spectrometry. In this example, the signal from Sample B is twice this signal of Sample A as the amount of peptide is doubled from Sample A to B.

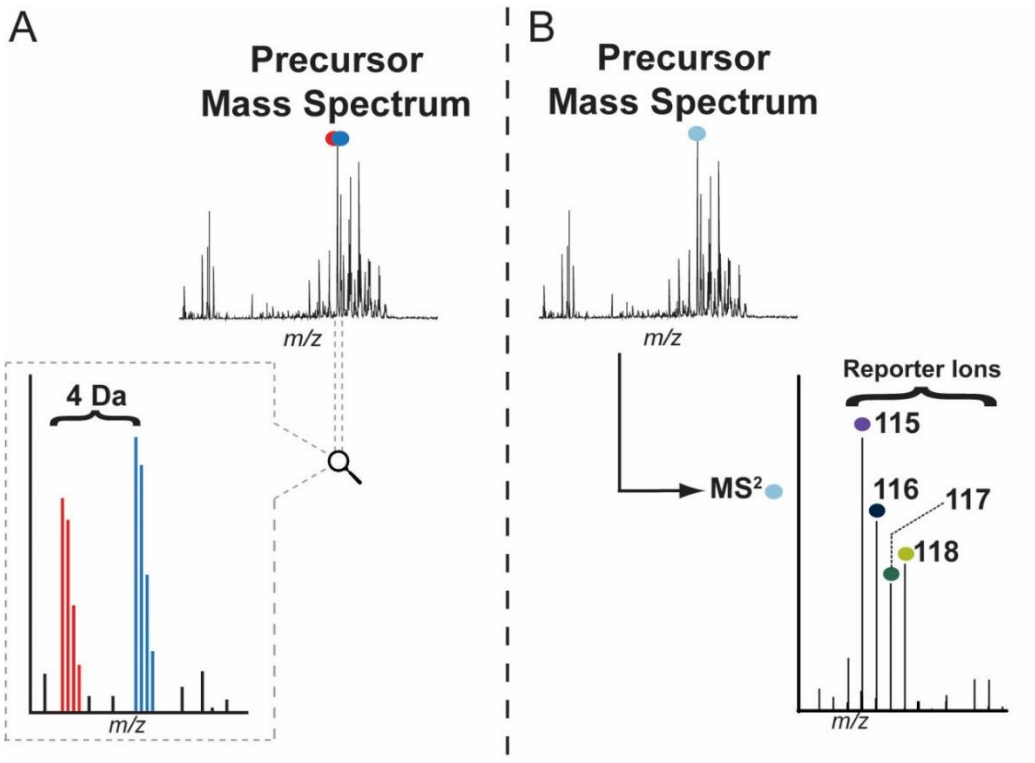
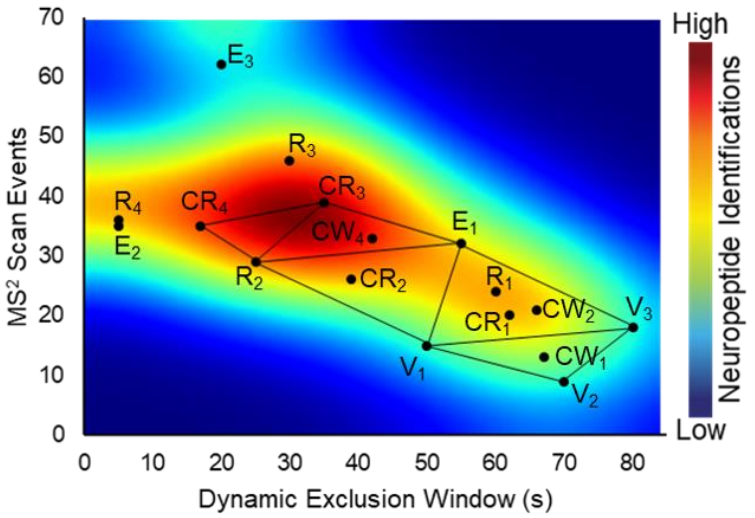


Figure 3: Comparison between isotopic and isobaric labeling. In isotopic labeling (**A**), samples have different masses added to them to create a mass shift between molecules of interest (analytes). In isobaric labeling (**B**), the same mass is added, but analytes are distinguishable at the MS^2 level where unique reporter ions form.



Simplex	Vertex	Dynamic Exclusion Window (s)	MS^2 Scan Events	Neuropeptides Identified
1	V1	50	15	29
	V2	70	9	25
	V3	80	18	27
2	R1	60	24	29
	E1	55	32	41
	CR1	62	20	35
	CW1	67	13	24
3	R2	25	29	88
	E2	5	35	81
	CR2	39	26	63
	CW2	66	21	54
4	R3	30	46	54
	E3	20	62	53
	CR3	35	39	72
5	R4	5	36	64
	CR4	42	33	55
	CW4	17	35	66

Figure 4: Optimization of DDA parameters. Two DDA parameters, the dynamic exclusion window and MS^2 scan events, were optimized stepwise. With each iteration, new conditions were tested, moving away from the conditions that had worse performance. The optimization of these parameters resulted in a 3-fold increase between the initial, unoptimized parameters, and the optimized parameters. The optimum region is shown on the heatmap in the red region (region with more identified

neuropeptides). The table below the heatmap shows the more specific numbers for each point.

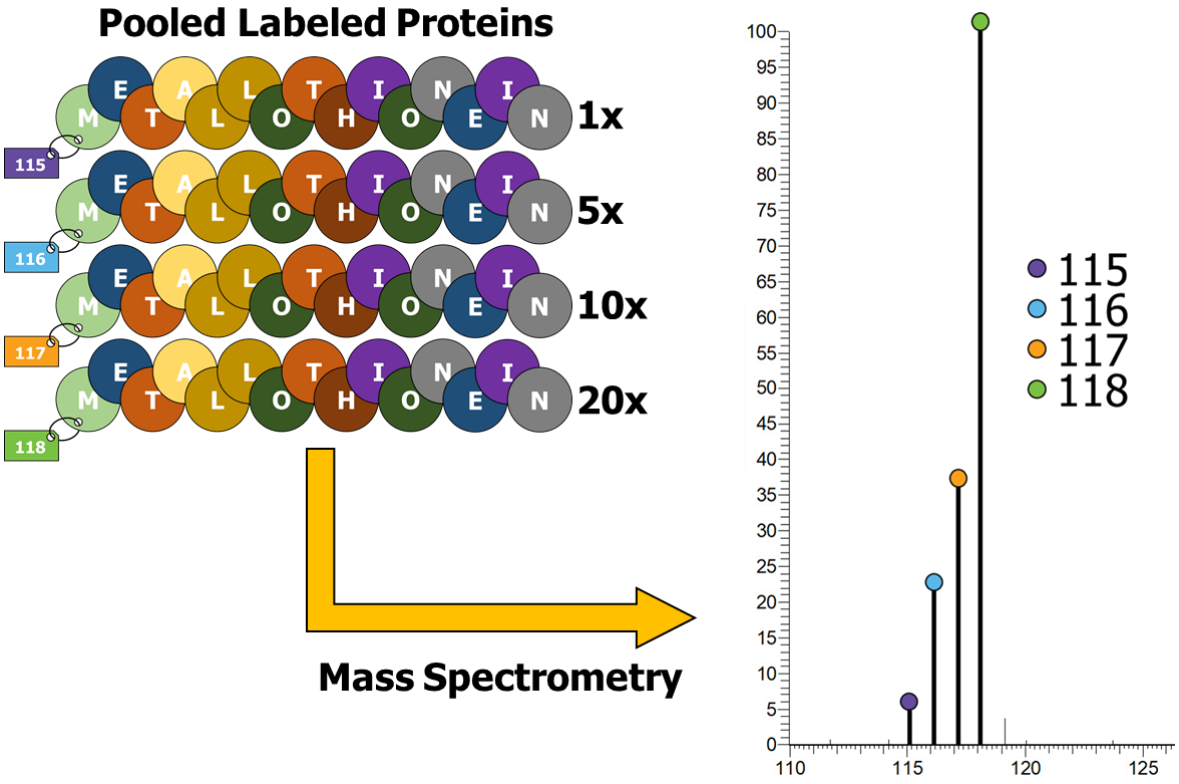


Figure 5: Example mass spectrometry of labeled metallothionein. Proteins labeled with different tags (115, 116, 117, or 118) were combined in different amounts (1:5:10:20) (shown left). MS analysis of the pooled sample showed reporter ion intensities that are within 80% of the expected values (shown right). The high accuracy of the method demonstrates feasibility for applications to future biological applications.