Total Protein Assay as a Step in Peptidomics Analytical Frame Work

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INTRODUCTION

Peptidomics is the comprehensive quantitative and/or qualitative analysis of endogenously produced peptides in a biological sample. Endogenous neuropeptides are proteolytically processed *in vivo* from larger precursor proteins and serve as important bioactive molecules acting as hormones, neurotransmitters, neuromodulators, and biomarkers of certain pathology. The identification and functional characterization of naturally occurring peptides from model species like a mouse aids in understanding mammalian physiology, and ultimately, it may lead to the discovery of novel drugs.

A crucial step in peptidomics analysis is determination of tissue sample size needed for analysis. It has been shown that the amount of endogenous peptides correlates to the amount of protein content present in the tissue (1). To quantify the total protein concentration, a biochemical assay known as the BCA method is used. This assay produces a color change in the sample from green to purple, and the absorption of the sample can be measured using a spectrophotometer (3). With accurate information on overall peptide levels, more successful peptidomics are achieved.

OBJECTIVE

The purpose of this experiment was to use a colorimetric assay in combination with spectrophotometry to determine the tissue total protein content, and estimate the tissue sample size required for successful proteomics analyses of naturally occurring peptides in the mouse brain.

BACKGROUND: Spectrophotometry

Spectrophotometry is a method that measures how much light is absorbed by the sample. The measurement of light is acquired by a spectrophotometer which measures the intensity of light that is either absorbed or transmitted. Every compound is able to absorb or transmit light over a certain wavelength region, and this principle allows for the quantative analysis of compounds (2).

As seen in Figure 1, the light passes through the lens and into the monochromator where the light is broken down into its wavelength spectrum. The wavelength selector picks the selected wavelength which passes through the sample (2). Finally, the detector measures how much light was absorbed by the sample, and an absorbance readout is given.
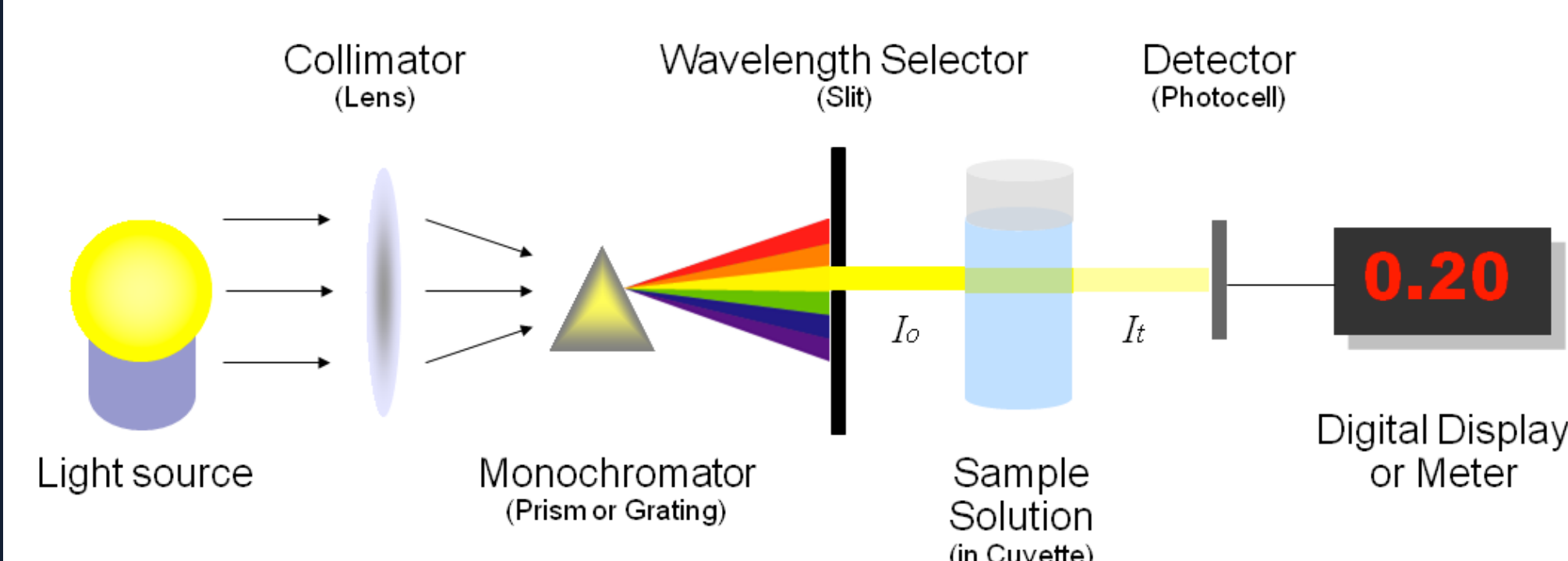

Figure 1: Components of a Spectrophotometer illustrated by Heesung Shim
Figure Source:
https://chem.libretexts.org/Core/Physical_and_Theoretical_Chemistry/Kinetics/Reaction_Rates/Experimental_Determination_of_Kinetcs/Spectrophotometry

METHOD: Peptide Extraction & Protein Assay

Mouse Brain Tissue Pulverization

The total protein assay was performed on mouse brain tissue, but in order to work with this tissue, it needed to be pulverized into a coarse powder for protein extraction using a tissue pulverizer and mallet as seen in Figure 2. To begin with, the tissue pulverizer is chilled in liquid nitrogen, for the extreme cold temperature of liquid nitrogen makes the tissue brittle. Afterwards, the frozen brain is placed inside the pulverizer. A mallet is used to hit the pulverizer which facilitates fragmentation of the frozen brain as seen in Figure 3.
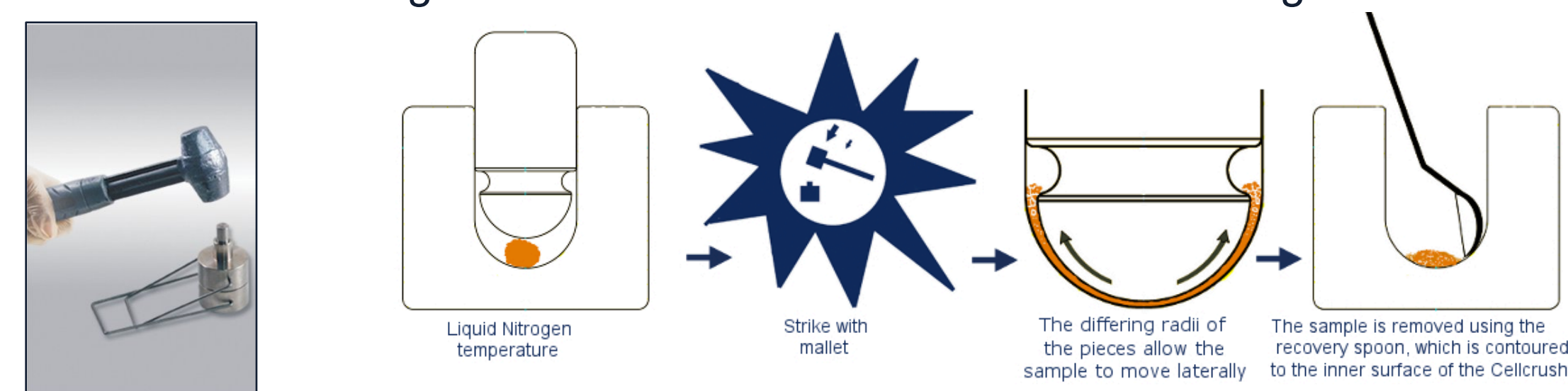

Figure 2: Mallet and tissue pulverizer
Figure Source:
https://assets.fishersci.com/TFS-Assets/CCG/product-images/F139975~p.eps-650.jpg

Figure 3: The sample is placed into the pulverizer, and the tissue is fragmented by the mallet.
Figure Source:
http://cellcrusher.com/tissuepulverizer/

Peptide Extraction:

For the extraction, centrifuge vials, powdered dry ice, and acidified acetone were used. The vials were weighed and placed onto dry ice to be chilled. Afterwards, the frozen powdered brain tissue was added to three separate, chilled centrifuge vials. Furthermore, 500 µL of acetone was added to each vial, and the vials were then allowed to warm up to room temperature. The vials were vortexed and then sonicated for five minutes. Finally, the vials were centrifuged at 1400 x g for 15 min so that the tissue could precipitate. The supernatant was removed and placed into a new set of vials. Furthermore, serial dilutions were performed for two out of the three supernatants. The serial dilutions and the supernatants were used for the BCA assay.

Bicinchoninic acid (BCA) colorimetric detection and quantitation of total protein

For this particular project, the Thermo Scientific™ Micro BCA™ Protein Assay Kit was used to assay the total protein in frozen, pulverized mouse brain tissue (Fig. 8). The BCA method uses the reaction of a cuprous ion with a bicinchoninic acid (BCA) molecule to produce a purple colored product (Fig. 4). More specifically, the Cu^{2+} ion is reduced to Cu^{+1} as seen in part 1 and 2 of Figure 5 (3). The Cu^{+1} binds with two BCA molecules to give the BCA/copper complex as seen in part 4 of Figure 5. This complex absorbs at a wavelength of 562 nm.

Using the kit, known concentrations of protein standards were diluted with water. Working reagent which facilitated the color change was added to both the standards and the unknown concentration of mouse brain samples. The samples and standards were incubated at 37°C to produce a colorimetric protein assay in a 384-well plate. Using the Epoch microplate spectrophotometer (Fig. 10), a wavelength of 562 nm was selected to interact with the samples, and the absorbance was measured.
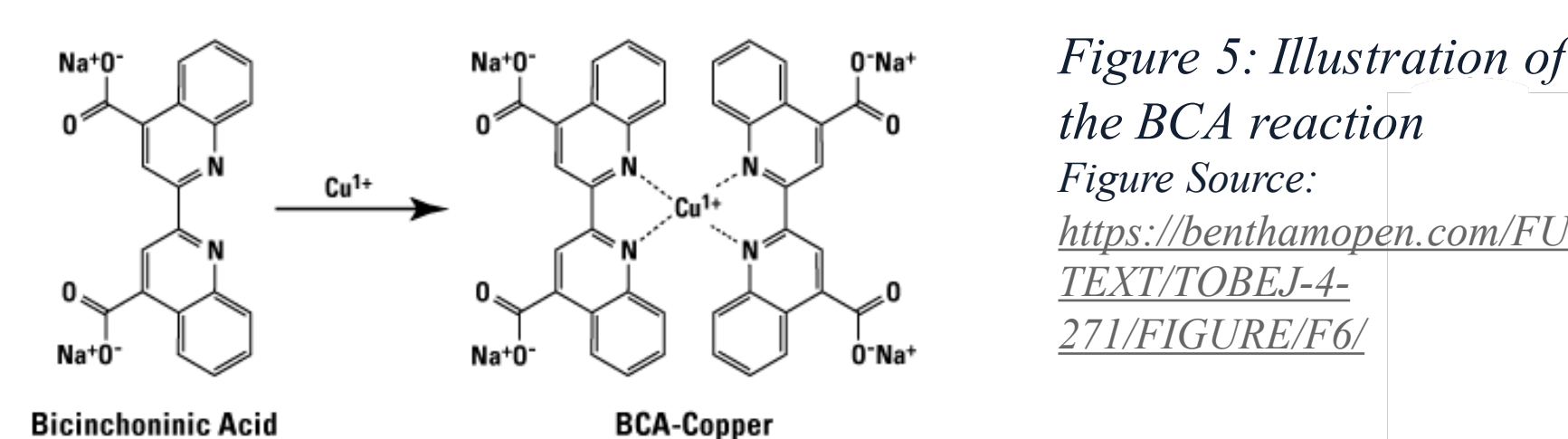

Figure 5: Illustration of the BCA reaction
Figure Source:
https://benthamopen.com/FULLTEXT/TOBEJ-4-271/FIGURE/F6/

Figure 4: BCA Reaction with Cu^{+1}
Figure Source:
https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/chemistry-protein-assays.html

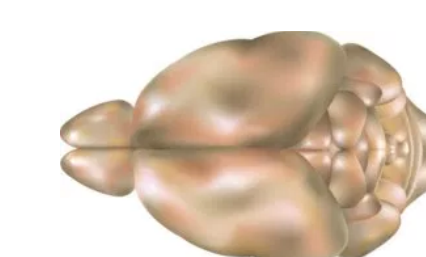## Experimental workflow: from tissue to peptide extract

Figure 6: Mouse Brain
Figure Source:
http://neurosciencenews.com/neuroscience-brain-size-cognition-143/mouse-brain-size/

Figure 7: Pulverization
Figure Source:
https://assets.fishersci.com/TFS-Assets/CCG/product-images/F139975~p.eps-650.jpg

Peptide Extraction

Figure 8: Preparation of samples and standards via Thermo Scientific™ Micro BCA™ Protein Assay Kit
Figure Source:
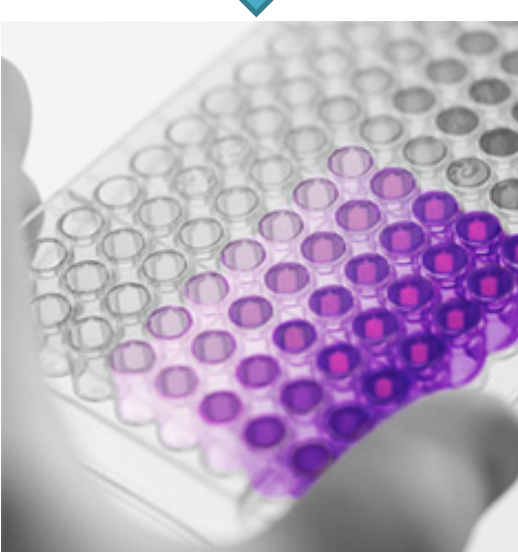*https://www.thermofisher.com/order/catalog/product/23235*

Figure 9: Incubation of standards and samples
Figure Source:
http://separations.co.za/products/protein-assay-kits/#s-tabs3

Figure 10: Measure absorbance via Epoch spectrophotometer

Citations

1. Guerrero et al. "Mechanistic Peptidomics…": *MCP* 13.12 (2014): 3343–3351. *PMC*. Web. 14 Apr. 2018.

2. Vo. "Spectrophotometry." *Chemistry LibreTexts*, Chemistry LibreTexts, 21 July 2016.

3. Smith et al. "Measurement of protein using bicinchoninic acid." Anal Biochem (1985) 150:76-85

Acknowledgements

RESULTS

Well ID	Well	Concentration (µg/mL)	Absorbance
BLK	E18		0.252
	E19		0.218
	E20		0.192
MB1	E21	124.56	1.414
	E22	130.421	1.471
	E23	66.59	0.85
	F9	83.45	1.014
	F10	85.907	1.03
	F11	83.76	1.017
	F12	46.87	0.658
	F13	47.89	0.668
	F14	47.38	0.663
	F15	48.72	0.676
	F16	55.19	0.739
	F17	56.42	0.751
	F18	39.98	0.591
	F19	45.45	0.615
	F20	41.93	0.61

Well ID	Well	Concentration (µg/mL)	Absorbance
STD1	D9	200	2.128
	D10	200	2.168
	D11	200	2.05
STD2	D12	40	0.524
	D13	40	0.759
	D14	40	0.875
STD3	D15	20	0.473
	D16	20	0.464
	D17	20	0.482
STD4	D18	10	0.314
	D19	10	0.308
	D20	10	0.311
STD5	D21	5	0.217
	D22	5	0.219
	D23	5	0.22
STD6	E9	2.5	0.184
	E10	2.5	0.185
	E11	2.5	0.182
STD7	E12	1	0.164
	E13	1	0.159
	E14	1	0.155
STD8	E15	0.5	0.149
	E16	0.5	0.147
	E17	0.5	0.149

Table 1: Absorbances of Unknown Mouse Brain Samples and of Known Concentrations of Standards

Absorbance vs. Concentration of Unknown Mouse Brain Samples (N=5) & Standards (µg/mL)

$y = 0.0097x + 0.2018$
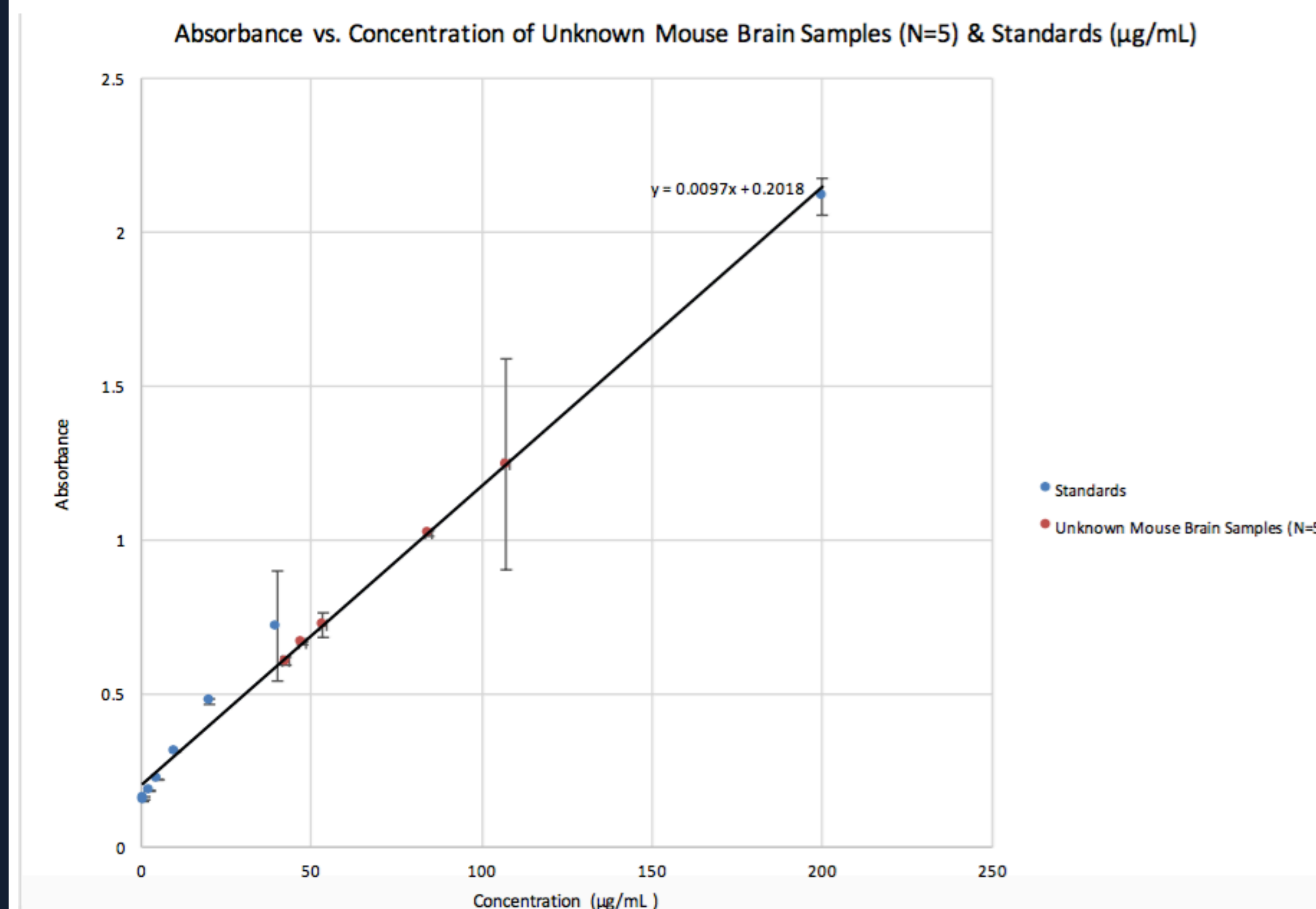
Graph 1: Plot of Absorbance of unknown mouse brain samples and standards
The graph shows a linear relationship with increasing protein concentrations and absorbance.
N: indicates the number of mouse brain samples analyzed.

CONCLUSIONS

1. The BCA/copper complex displayed stronger absorbances as the concentration of the protein standards and samples increased.
2. Using the absorbances of the unknown mouse brain samples, the total protein concentration was calculated using Beer's Law (A = εlc). The following concentrations (µg/mL) are the average values for the unknown mouse brain samples: 107.19 +/- 0.343, 84.37 +/- 0.009, 47.38 +/- 0.005, 53.44 +/- 0.04, and 42.45 +/- 0.013.
3. With the total protein concentration found for each of the unknown samples, the concentration of endogenous peptides can now be analyzed.
4. The brain tissue sample size can also be deduced for future peptidomics analyses since the total protein concentration corresponds to the concentration of endogenous peptides.