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Sample Preparation and Relative Quantitation using Reductive Methylation of Amines for Peptidomics studies

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TITLE:

Sample Preparation and Relative Quantitation using Reductive Methylation of Amines for Peptidomics studies

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SUMMARY:

This article describes a sample preparation method based on heat-inactivation to preserve endogenous peptides avoiding degradation post-mortem, followed by relative quantitation using isotopic labeling plus LC-MS.

ABSTRACT:

Peptidomics can be defined as the qualitative and quantitative analysis of peptides in a biological sample. Its main applications include identifying the peptide biomarkers of disease or environmental stress, identifying neuropeptides, hormones, and bioactive intracellular peptides, discovering antimicrobial and nutraceutical peptides from protein hydrolysates, and can be used in studies to understand the proteolytic processes. The recent advance in sample preparation, separation methods, mass spectrometry techniques, and computational tools related to protein sequencing has contributed to the increase of the identified peptides number and peptidomes characterized. Peptidomic studies frequently analyze peptides that are naturally generated in cells. Here, a sample preparation protocol based on heat-inactivation is described, which eliminates protease activity, and extraction with mild conditions, so there is no peptide bonds cleavage. In addition, the relative quantitation of peptides using stable isotope labeling by reductive methylation of amines is also shown. This labeling method has some advantages as the reagents are commercially available, inexpensive compared to others, chemically stable, and allows the analysis of up to five samples in a single LC-MS run.

INTRODUCTION

"Omics" sciences are characterized by the deep analysis of a molecule set, such as DNA, RNA, proteins, peptides, metabolites, etc. These generated large-scale datasets (genomics,

transcriptomics, proteomics, peptidomics, metabolomics, etc.) have revolutionized biology and led to an advanced understanding of biological processes[1]. The term peptidomics began to be introduced in the early 20th century, and some authors have referred to it as a branch of proteomics[2]. However, peptidomics has distinct particularities, where the main interest is to investigate the naturally generated peptides content during cellular processes, as well as the characterization of biological activity of these molecules[3,4].

Initially, bioactive peptide studies were restricted to the neuropeptides and hormone peptides through Edman degradation and radioimmunoassay. However, these techniques do not allow a global analysis, depending on the isolation of each peptide in high concentrations, time for the generation of antibodies, besides cross-reactivity possibility[5].

Peptidomics analysis was only made possible after several advances in Liquid chromatography coupled mass spectrometry (LC-MS) and genome projects that delivered comprehensive data pools for proteomics/peptidomics studies[6,7]. Moreover, a specific peptide extraction protocol for peptidomes needed to be established because the first studies that analyzed neuropeptides globally in brain samples showed that detection was affected by the massive degradation of proteins, which occur mainly in this tissue after 1 min post-mortem. The presence of these peptide fragments masked the neuropeptide signal and did not represent the peptidome *in vivo*. This problem was solved mainly with the application of fast heating inactivation of proteases using microwave irradiation, which drastically reduced the presence of these artifact fragments and allowed not only the identification of neuropeptide fragments but revealed the presence of a set of peptides from cytosolic, mitochondrial, and nuclear proteins, different of degradome[6,8,9].

These methodological procedures allowed an expansion of the peptidome beyond the well-known neuropeptides, where hundreds of intracellular peptides generated mainly by the action of proteasomes have been identified in yeast[10], zebrafish[11], rodent tissues[12], and human cells[13]. Dozens of these intracellular peptides have been extensively shown to have both biological and pharmacological activities[14,15]. Furthermore, these peptides can be used as disease biomarkers and possibly have clinical significance, as demonstrated in cerebrospinal fluid from patients with intracranial saccular aneurysms[16].

Currently, in addition to the identification of peptide sequences, it is possible through mass spectrometry to obtain data of absolute and relative quantitation. In the absolute quantitation, the peptide levels in a biological sample are compared to synthetic standards, while in the relative quantitation, the peptide levels are compared among two or more samples[17]. Relative quantitation can be performed using the following approaches: 1) "label free"[18]; 2) *in vivo* metabolic labeling or 3) chemical labeling. The last two are based on the use of stable isotopic forms incorporated into peptides[19,20]. In label-free analysis, the peptide levels are estimated by considering the signal strength (spectral counts) during the LC-MS[18]. However, isotopic labeling can obtain more accurate relative levels of peptides.

Many peptidomic studies used trimethylammonium butyrate (TMAB) labeling reagents as chemical labeling, and more recently, Reductive Methylation of Amines (RMA) with deuterated and non-deuterated forms of formaldehyde and sodium cyanoborohydride

reagents have been used[11,21,22]. However, the TMAB labels are not commercially available, and the synthesis process is very laborious. On the other hand, in the RMA, the reagents are commercially available, inexpensive compared to other labels, the procedure is simple to perform, and the labeled peptides are stable[23,24].

The use of RMA involves forming a Schiff base by allowing the peptides to react with formaldehyde, followed by a reduction reaction through the cyanoborohydride. This reaction causes dimethylation of free amino groups on N-terminals and lysine side chains and monomethylates N-terminal prolines. How proline residues are often rare on the N-terminal, practically all peptides with free amines on the N-terminus are labeled with two methyl groups[23–25.]

PROTOCOL:

The following procedure for peptide extraction and reductive methylation was adapted from previously published procedures[24–27]. This protocol followed the guidelines of the National Council for Animal Experimentation Control (CONCEA) and was approved by the Ethics Commission for Animal Use (CEUA) at Bioscience Institute of Sao Paulo State University. The protocol steps are shown in **Figure 1**.

NOTE: Prepare all aqueous solutions in ultrapure water.

1. Peptide extraction

1.1 Cell culture

1.1.1 Cultivate SHSY5Y cells in a 15 cm dish at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium containing 15% fetal bovine serum and 1% Penicillin-Streptomycin.

1.1.2 Use 2–3 plates for each sample. Grow the cells to 100% confluence.

1.1.3 After treatment intended, wash the cells twice with phosphate-buffered saline. Next, add 10 mL of Phosphate-buffered saline, scrape the cells and collect into a 15 mL tube.

1.1.4 Centrifugate at 800 x *g* for 5 min and remove the supernatant. Resuspend the pellet in 1 mL of ultra-purified deionized water at 80 °C.

1.1.5 Transfer the contents of the tube (cellular lysate) to a 2 mL microfuge tube.

1.2 Animal tissues (mainly nervous tissue):

1.2.1 Anesthetize wild-type male adult *Danio rerio* (zebrafish) with a lethal dose of MS 222 (100 mg/L) and immediately subject it to 8 s of microwave radiation to inactivate peptidase and protease.

NOTE: A household-type microwave oven can be used. A 900 W microwave was used for 8 to 10 s at full power. The microwave used must be able to raise the brain temperature to > 80 °C within 10 s. Reproducibility in heating between samples would also be benefited by placing the tissue in the same location in the microwave.

1.2.2 After heat-inactivation, collect the whole brain in a 2 mL microfuge tube and freeze at −80 °C until analysis.

1.2.3 Resuspend the tissue sample in 1 mL of ultra-purified deionized water at 80 °C. Sonicate the tissue with a probe using 30 pulses (4 Hz) of 1 s.

NOTES: For liver and kidney tissues, use a mechanical homogenizer at 10,000–30,000 rpm for 20 s. For muscular tissues, grind the tissue in liquid nitrogen using a porcelain crucible and pestle. The following steps are the same for the cellular lysate or the homogenate tissue.

1.3 Incubate the cellular lysate or homogenate tissue at 80 °C for 20 min. Next, cool it on ice for 10–30 min.

1.4 Add 10 µL of 1 M HCl stock solution for each 1 mL of sample volume to obtain a final concentration of 10 mM. Mix by vortexing for 20 s and further incubate on ice for 15 min.

NOTE: Before acidifying, ensure the sample is completely cooled to avoid breaking the peptide bonds caused by acidification at elevated temperatures.

1.5 Centrifuge the cellular lysate or the homogenate tissue at 12,000 x *g* at 4 °C for 15 min. Collect the supernatant in low binding protein microcentrifuge tubes and store it at -80 °C.

1.6 Clean the ultrafiltration devices (10 kDa cut-off filters) by adding water and centrifuge at 2300 x *g* for 3 min. Repeat this step two more times.

1.7 Place the supernatant in the pre-washed 10 kDa cut-off filters and spin according to usage guidelines in a refrigerated centrifuge at 4 °C. The flow-through represents the peptide extract.

1.8 Desalt the samples on reversed-phase cleanup columns according to the manufacturer's instructions using acetonitrile (ACN) and trifluoroacetic acid (TFA) solutions as described below:

1.8.1 Equilibrate the column with 1 mL of 100% ACN.

1.8.2 Wash the column with 1 mL solution of 5% ACN with 0.1% TFA.

1.8.3 Load the complete volume of the sample in the column.

1.8.4 Wash the column with 1 mL solution of 5% ACN with 0.1% TFA

1.8.5 Elute the peptides from the column with a 1.8 mL solution of 100% ACN with 0.15% TFA in protein low binding microcentrifuge tubes.

1.9 Dry the sample completely in a vacuum centrifuge. Set the concentration method for organic solvents and temperature at 30 °C. The concentration time is monitored on display.

1.9.1. Store the samples at −80°C until the next step.

2. Peptide quantification with fluorescamine

NOTE: The amount of peptide can be estimated using fluorescamine at pH 6.8 as previously described[11,28]. This method consists of the attachment of a fluorescamine molecule to the primary amines present in the lysine (K) residues and not of the N-terminal peptides. The reaction is performed at pH 6.8 to guarantee that the fluorescamine reacts only with the amino groups of the peptides and not with free amino acids. The fluorescamine is measured by using a spectrofluorometer at an excitation wavelength of 370 nm and an emission wavelength of 480 nm.

2.1 Prepare different concentrations of the standard peptide (0.05, 0.1, 0.15, 0.2, 0.3, 0.5 and 0.7 µg/µL) and store the aliquots at -20 °C.

NOTE: The peptide 5A (LTLRTKL) is suggested since it has a known composition and concentration.

2.2 Prepare fluorescamine stock solution (0.3 mg/mL) in acetone. Aliquot quickly in microcentrifuge tubes (1 mL), seal using parafilm, and store at -20 °C in the dark.

2.3 Prepare 0.2 M Phosphate buffer (PB) at pH 6.8.

NOTE: Prepare 0.2 M PB by adding 0.1 M phosphate buffer pH 6.8 (26.85 mL of Na_2HPO_3 1M) and 0.1 M phosphate buffer pH 6.8 (23.15 mL of NaH_2PO_3 1M) to 250 mL of water

2.4 Resuspend the peptide samples in 100–200 µL of ultra-purified water.

2.5 Pipette 2.5 µL of the standard peptide concentrations and samples onto the white 96-well plate for fluorescence assays in triplicate. Add 25 µL of 0.2 M Phosphate buffer.

2.6 Add 12.5 µL of fluorescamine with a multichannel pipette. Homogenize gently for 1 min on the orbital rotator shaker.

2.7 Next, add 110 µL of water with a multichannel pipette to stop the reaction.

NOTE: Transfer the fluorescamine stock solution and ultrapure water to two reservoirs to pipette these solutions with a multichannel pipette.

2.8 Adjust the following reading parameters on the spectrofluorometer: Read the samples from the top, excitation wavelength at 370 nm, and emission wavelength at 480 nm.

2.9 Read the plate on the spectrofluorometer.

3. Reductive methylation of amines labeling

NOTE: This isotopic labeling method is based on the dimethylation of amine groups with deuterated and non-deuterated forms of formaldehyde and sodium cyanoborohydride reagents. The final product of this reaction adds 28 Da, 30 Da, 32 Da, 34 Da, or 36 Da to the final mass of each peptide at each available labeling site (lysine or N-terminal). This reaction produces an m/z difference in the peptides labeled with different forms observed in the MS spectrum (**Table1**).

CAUTION: Proper safety equipment should be used to handle these compounds, and care should be taken to minimize exposure. Procedures with formaldehyde and sodium cyanoborohydride reagents should be performed in a fume hood because they are very toxic (including weighing the sodium cyanoborohydride). During the quenching reaction and acidification, a toxic gas (hydrogen cyanide) may be generated.

3.1. Prepare the following fresh solutions from the stock or reagents on the day of the procedure in ultrapure water:

3.1.1. Dilute the stock of 37% Formaldehyde (CH_2O) to 4%.

3.1.2. Dilute the stock of 20% Formaldehyde Deuterated (CD_2O) to 4%.

3.1.3. Dilute the stock of 20 % Deuterated C^{13} Formaldehyde ($^{13}CD_2O$) to 4%.

3.1.4. Prepare 0.6 M NaBH3CN.

3.1.5. Prepare 0.6 M NaBD3CN.

3.1.6. Prepare a solution of 1% Ammonium bicarbonate.

3.1.7. Prepare a solution 5% Formic acid.

NOTE: Regarding the relative quantitation of peptides, as different experimental schemes can be performed depending on the number of labels used, attention is necessary during the chemical labeling procedure. It is recommended to separate small aliquots of the labels in separate racks with the respective samples to be labeled to reduce the potential for human error in adding the wrong reagent to the sample tubes.

3.2. Prepare each sample containing up to 25 µg of the peptide. Samples must not contain Tris or Ammonium Bicarbonate.

NOTE: The quantities described below are sufficient for each sample in a volume of 100 µL. Proceed with steps 3.3–3.8 in a fume hood.

3.3. Add 1/10th volume of 1 M TEAB to the samples (final concentration of the solution 100 mM of TEAB). Check the pH with a pH indicator paper; it must be between 5–8. Adjust with HCl or NaOH if needed.

3.4. Add 4 µL of non-deuterated, deuterated Formaldehyde or C^{13} deuterated Formaldehyde according to the established labeling scheme. Mix for 5 s by vortexing.

3.5. Add 4 µL of $NaBH_3CN$ (0.6 M) or $NaBD_3CN$ (0.6 M) according to the established labeling scheme. Mix for 5 s by vortexing.

3.6. Incubate in a fume hood for 2 h at room temperature, mixing every 30 min.

3.7. Repeat steps 3.4 and 3.5. Incubate the samples in a fume hood overnight at room temperature.

3.8. Add 16 µL of Ammonium bicarbonate (1%) and mix by vortexing. Place the sample on ice, add 8 µL of formic acid (5%), and mix by vortex for 5 s.

3.9. Combine the samples, adjust the pH to 2–4 and desalt the combined samples on reversed-phase cleanup columns as previously described in step 1.8.

3.10. Dry the sample completely in a vacuum centrifuge. Set the concentration method for organic solvents and temperature at 30 °C. The concentration time is monitored on display.

3.11. Store the samples at -20 °C.

4. Liquid chromatography and mass spectrometry

4.1. Perform LC-MS analysis using a nanoHPLC system coupled to a MS instrument compatible with methyl tags.

NOTE: A variety of MS instruments are compatible with RMA labeling. A nanoHPLC system coupled to Orbitrap is typically used to perform LC-MS analysis through a nanoelectrospray ion source. First, the sample is loaded into a precolumn and peptides separated in an analytical column. The elution of peptides is performed using a linear gradient of 5%–45% acetonitrile, in 0.1% formic acid, during 90 min, with a flow of 200 nL/min. The mass spectrometer is set to function in data-dependent mode. Each full scan is acquired at an intensity of 10–30 eV, 2.3 Kv, and then the ten highest peaks are selected for collision-induced dissociation (CID) fragmentation. The injection time is set on the ion trap at 100 ms, and the Fourier Transform (FT)-MS injection is fixed with a resolution of 1000 ms 30,000 at m/z 300–1800. A minimum of 5000 counts and a dynamic exclusion of 70 s is used to perform fragmentation scanning.

5. Relative quantitation of peptides

NOTE: The MS spectra are analyzed in the mass spectrometer software. Peak groups of labeled peptides with different tags are identified in the MS spectra. The relative quantitation is calculated by the intensity of each monoisotopic peak. Each treated group is compared to the respective control group.

5.1. Right double-click on the raw sample file to open the spectrum analysis software. Load the Retention time (RT) and MS spectrum (EM) chromatograms in two tabs, top and bottom, respectively.

5.2. Right-click once sequentially on the **Display** and **Mass** options icons in the software toolbar and set the mass precision to four decimals.

5.3. Position the mouse cursor anywhere on the RT tab. Look for the retention time of the corresponding ion to be analyzed and click the right mouse button. The MS spectrum of the selected time will automatically be shown in the EM tab.

5.4. Position the mouse cursor anywhere on the EM tab. Look for the ions to be analyzed.

5.5. Right-click and hold in an adjacent region to the left near these ions. Then, drag the mouse to the right at the desired range to zoom the region of interest.

5.6. Keep the mouse positioned on the EM tab and click on the right or left keyboard arrows to define the range of ions to be analyzed.

5.7. Position the mouse cursor the RT tab again at the beginning of the desired time interval.

5.8. Right-click and drag the mouse until the chosen time value. Leave the button. The accumulated intensity of the ions will automatically be shown on the EM tab.

5.9. Collect the m/z, z, and ion intensity data on a spreadsheet.

NOTE: The monoisotopic mass of each peptide without added methyl groups is calculated from the following formula:

$$Mass\ unmodified\ peptide = (m/z_a \times z) - (C_a \times T) - (1.008 \times z)$$

m/z_a is the observed mass to charge value for the monoisotopic peak for each peptide labeled with different combinations of tags (a =1, 2, 3, 4 or 5, corresponding to the sample number).
z is charge state.
C_a is the monoisotopic mass of a pair of methyl groups:
For a=1, Ca = 28.0313 (the net addition of two CH3 groups to the primary amine)
For a=2, Ca = 30.0439 for two CHD2 groups

For a=3, Ca = 32.0564 for two CD2H groups
For a=4, Ca= 34.0690 for two CD3 groups
For a=5, Ca = 36.0757 for two 13 CD3 groups
T is the number of pairs of methyl groups incorporated into the peptide. This can be calculated from the following formula when five tags are used: $T=z*(m/z_5 - m/z_1)/8$. For peptides that contain a single primary amine and therefore are labeled with only two methyl groups present peak overlaps on the MS spectra when adjacent labels are used. The peak intensity of each labeled peptide can be corrected using the equations described by Tashima and Fricker[25].

6. Peptide Identification

6.1. To identify peptides, analyze the MS/MS data using a database search engine[29,30].

6.2. To calculate the false discovery rate (FDR) using the decoy fusion method, search a decoy database.

NOTE: The search parameters generally used are no enzyme specificity; precursor mass tolerance set 15–50 ppm; fragment ion mass tolerance of 0.5 Da; variable modifications: reactive amines from Lys residues and N-terminus of the peptides isotopic methylated labels (L1 (+28), L2 (+30), L3 (+32), L4 (+34) and L5 (+36)), oxidized methionine (+15.99 Da) and acetylation (+42.01 Da).

6.3. Then, sort the peptides by their average of local confidence to select the best spectra to annotate and filter them by FDR ≤5%.

[Place **Figure 1** here]

REPRESENTATIVE RESULTS:

The results obtained from the runs carried out on the mass spectrometer are stored in raw data files that can be opened in the mass spectrometer software. In the MS spectra, it is possible to observe peak groups representing labeled peptides according to the labeling scheme used, ranging from 2–5 labels. For example, in **Figure 2**, pairs of peaks detected in a chromatographic time are represented in an experiment where only two isotopic labels were used in two different samples in the same run. **Figure 3** shows other possibilities of positive results, using 3 and 4 different labels in each LC-MS run. When using 4 or 5 labels in an LC/MS run, there may be an overlap of peaks of the labeled peptides with the different tags that need to be corrected to obtain the real intensity value of each peak **(Figure 4)**.

The isotopic labeling can also be used to show substrates and products *in vitro* for a given protease or peptidase, as shown in **Figure 5**. Finally, different software can be used to identify the labeled peptides, such as Peaks Studio or MASCOT. These software applications were created for proteomic analysis; therefore, the protein quantitation data should not be considered for peptidomics analysis, and each labeled peptide identified within the reliability parameters must be checked and then quantified. If the peptides have been successfully detected and labeled, these programs will provide a list of identified

peptide sequences containing the labels. **Figure 6** is shown an example of the identification of a peptide sequence done by the program. In this case, only 3 different forms of the labels (L1, L3, and L5) were used to label three different samples separately, which were then mixed and analyzed by mass spectrometry in a single run.

FIGURE AND TABLE LEGENDS:

Figure 1: Peptidomic studies workflow. Steps of peptide extraction and Reductive Methylation of amines.

Figure 2: MS spectrum representative of a chromatographic time accumulated in a typical labeling experiment using reductive dimethylation of amines. In (**A**), the red arrows indicate the presence of peak pairs of different peptides labeled with 2 isotopic forms (L1 and L5) for comparison between two different samples (S1 and S2). In (**B**), an enlarged image of an MS spectrum of the same peptide showing different m/z because of the use of labels. In this case, there was no variation in the peak intensity for this peptide present in these samples.

Figure 3: Representative MS spectrum of labeled peptides with reductive methylation of amines using different numbers of tags. (**A**) A triplex labeling was used. It is possible to observe an MS spectrum of a peptide present in 3 different samples (S1, S2, and S3) labeled with L1, L3, and L5 tags, respectively. In this case, the level of the labeled peptide with L5 was twice the level observed for the same peptide labeled with L1 and L3 tags. (**B**) A quadripex labeling was performed using the L1, L2, L3, and L4 labels. In this case, control samples (S1 and S3) were labeled with L1 and L3, respectively, and compared with two experimental samples (S2 and S4) labeled with L2 and L4 labels. No significant differences were observed for this peptide between the samples.

Figure 4: Representative MS spectrum of a labeled peptide presenting a peak overlap. This figure shows the MS spectrum of a peptide of charge 3, mass of 2098.87 Da with a single primary amine available for labeling. The difference between the labeled peptides is only 2 Da, causing an overlap when 4 or 5 labels are used in the same LC/MS run. Using a model based on cubic polynomial equations, it is possible to correct these overlaps between the labels. In the graph, the red bars show the average of the intensity values adjusted for this peptide in two runs. The black bars show the average of the overlapping intensity values. After correction, this peptide showed little variation in intensity between samples (red bars).

Figure 5: Representative MS spectrum of labeled peptides with reductive methylation of amines in a proteolysis study. Here, peptide extracts were incubated with 200 nM and 20 nM neurolysin to characterize their substrates and products. For confirmation of the result, two LC/MS runs with Forward and Reverse labeling strategies were performed. In the second run the position of the samples is changed in the labeling procedure in relation to the scheme used in the first run. In A, it is shown a peptide that does not change (NC) in the presence of the neurolysin enzyme. In B, a peptide that disappeared in the presence of a high concentration of the enzyme (S2) and that showed a small reduction in the low concentration of the enzyme (S4) in both forward and reverse labeling. This peptide is considered a substrate (SB) of the enzyme. In C an example of a peptide that was

considered a product (PD), because its concentration was increased (S2 and S4) in the presence of the enzyme.

Figure 6: Representative MSMS spectrum and identification of a labeled peptide performed by a database search engine. In this example, it is possible to observe 2+ ions with m/z 672,3802, 676,4045, and 680,4241 corresponding to a peptide of the same mass labeled with the L1, L3, and L5 methylated forms, respectively. This sequence ADQVSASLAKQGL was identified through the MS/MS spectrum as a fragment of the microtubule-associated protein tau isoform X1. This peptide has the N-terminal and a lysine available as labeling sites adding a mass difference of 8 Daltons between the labeled peptides. The MS spectrum of this peptide is shown in **Figure 3A**.

Table 1: Reagents of a typical experiment using the indicated combination of deuterated and non-deuterated forms of formaldehyde and sodium cyanoborohydride.

DISCUSSION:

In most peptidomics studies, one of the critical steps is, without doubt, the sample preparation that should be carefully performed to avoid the presence of peptide fragments generated by proteases after a few minutes post-mortem. The initial studies on brain extracts prepared from non-microwaved samples showed a large number of protein fragments to be present in the 10-kDa microfiltrates. Different approaches have been described to avoid peptide spectra from protein degradation: focused microwave irradiation animal sacrifice[6,8], cryostat dissection followed by a boiling extraction buffer[31,] and post-sacrifice microwave-irradiation of tissue using a household type microwave oven[9,26]. For cell culture and some tissues, the protease inactivation can be done directly by adding water at 80 °C. However, some samples, such as nervous tissue, can be more sensitive to post-mortem change, and protease inactivation by microwave irradiation has been indicated as a choice method. Furthermore, another important point during the peptide extraction is to make sure that extracts are ice-cold before adding acid to prevent acid-labile bonds from breaking, like cleavage of Asp-Pro bonds[26].

Different strategies can be used for the relative quantification of peptides, but none of them can be considered completely ideal. To choose the method to be used, the researcher must consider factors such as availability of hours of use in the mass spectrometer, commercially available and costs of labeling reagents, and ease in analyzing the data obtained[17,25,26,32]. The label-free method has been widely used but requires many hours in the mass spectrometer. It is necessary to inject technical replicates for each sample and depends on chromatographic reproducibility among the samples. Other chemical labeling reagents, for example, ITRAQ (isobaric tags for relative and absolute quantitation) and TMT (tandem mass tag), are expensive and only provide quantitation of peptides selected for MS/MS analysis[25,33,34].

The major limitation of relative quantification through chemical labeling using RMA is the overlap that occurs for some peptides when performing a protocol using 4 or 5 labels at the same run resulting in mass differences of 2 Da for peptides with a single primary amine and 1 Da for peptides with N-terminus proline and no internal lysine residues. However, Tashima and Fricker (2018) developed a model to correct isotopic overlapping based on

cubic polynomial equations[25], which get the correct intensity of the labeled peptides in the samples. Furthermore, not all peptides can be seen by RMA. For example, some peptides lack an N-terminal free amine due to acetylation, pyroglutamylation, or another modification. If internal lysines are also absent in these peptides, they will not be labeled by the RMA reagent and will appear on the m/z spectra as unquantifiable single peaks[27]**.**

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Figure 1

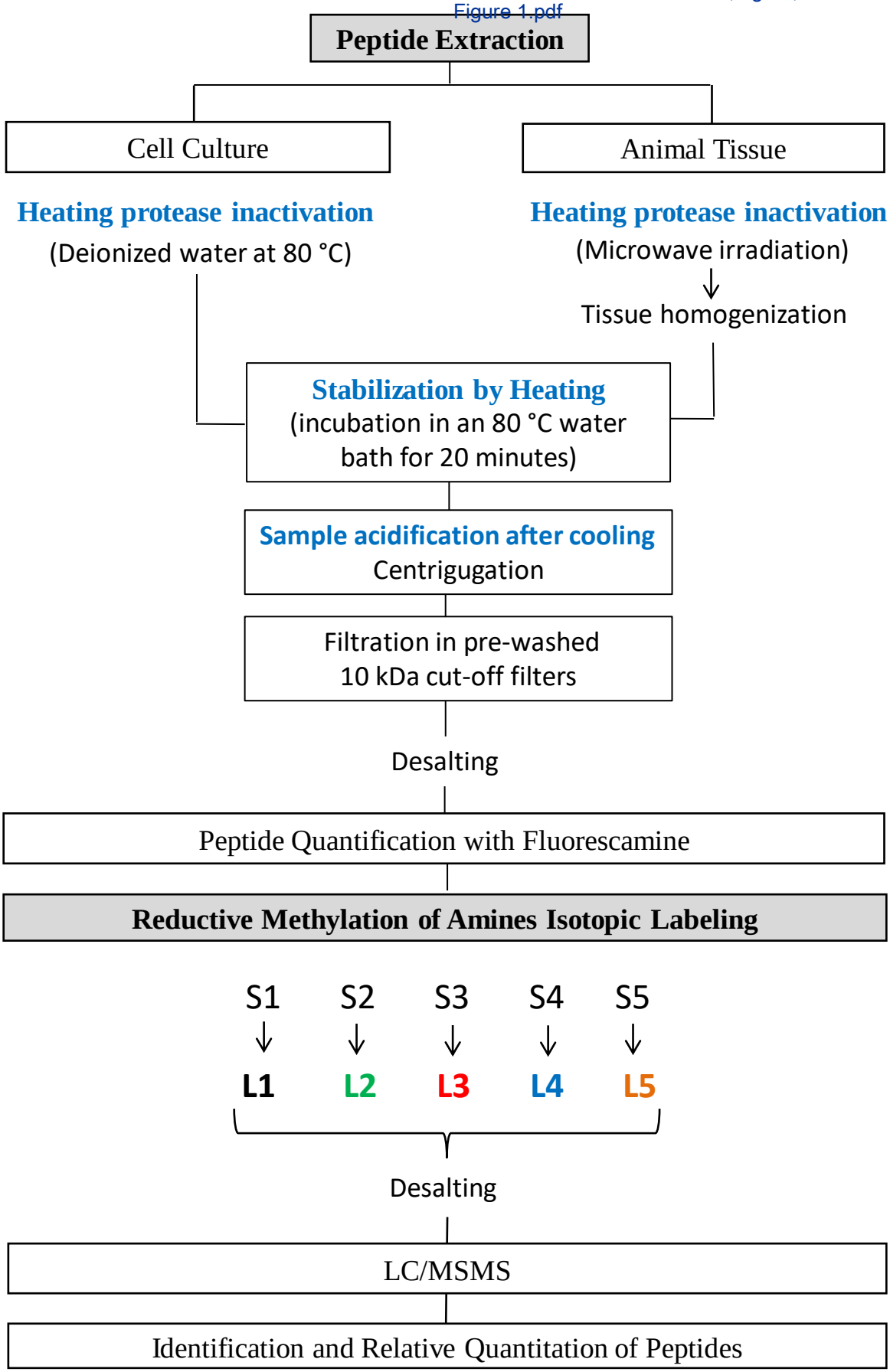

Figure 2

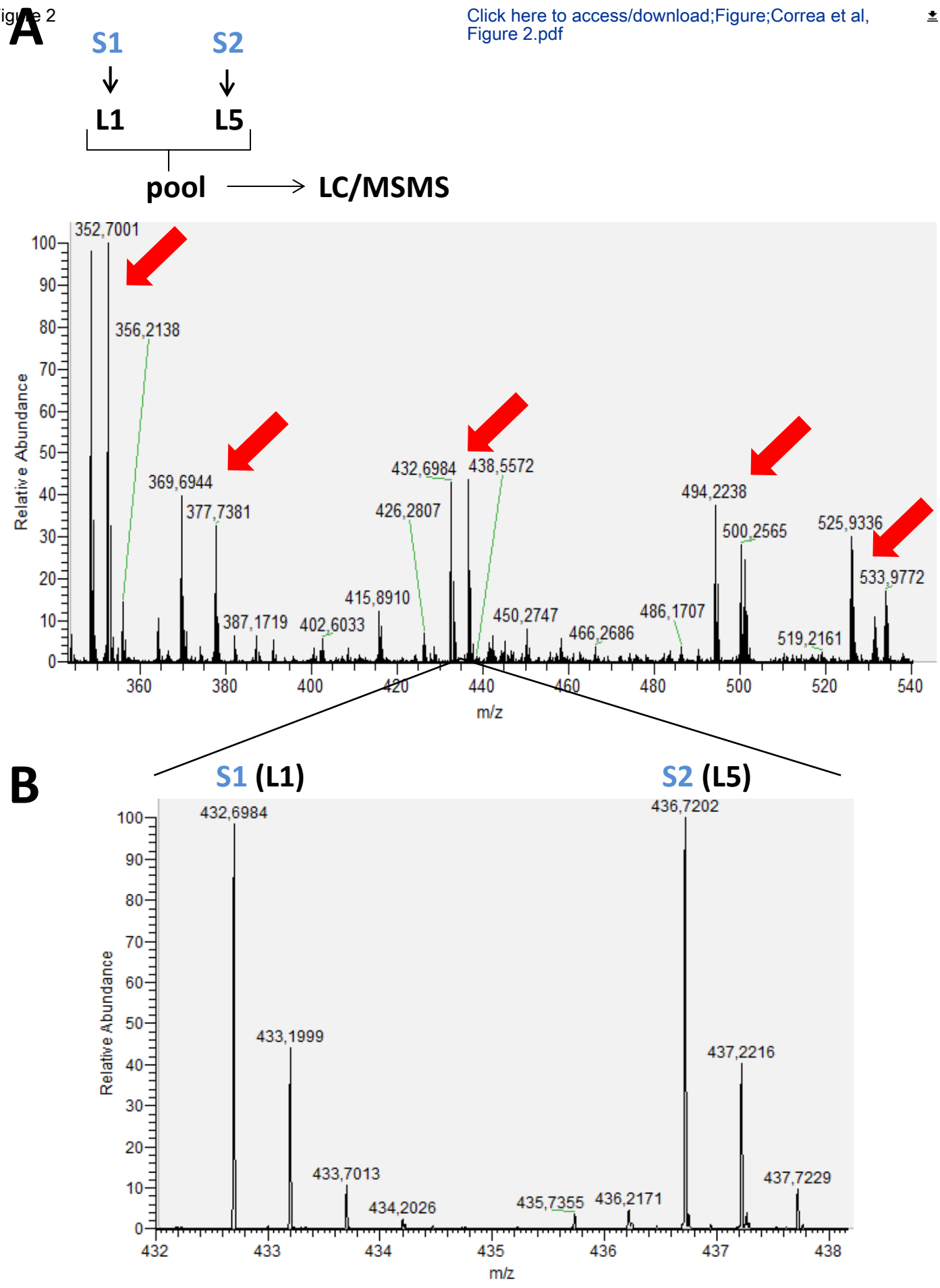

Figure 3

Click here to access/download;Figure;Correa et al, Figure 3.pdf

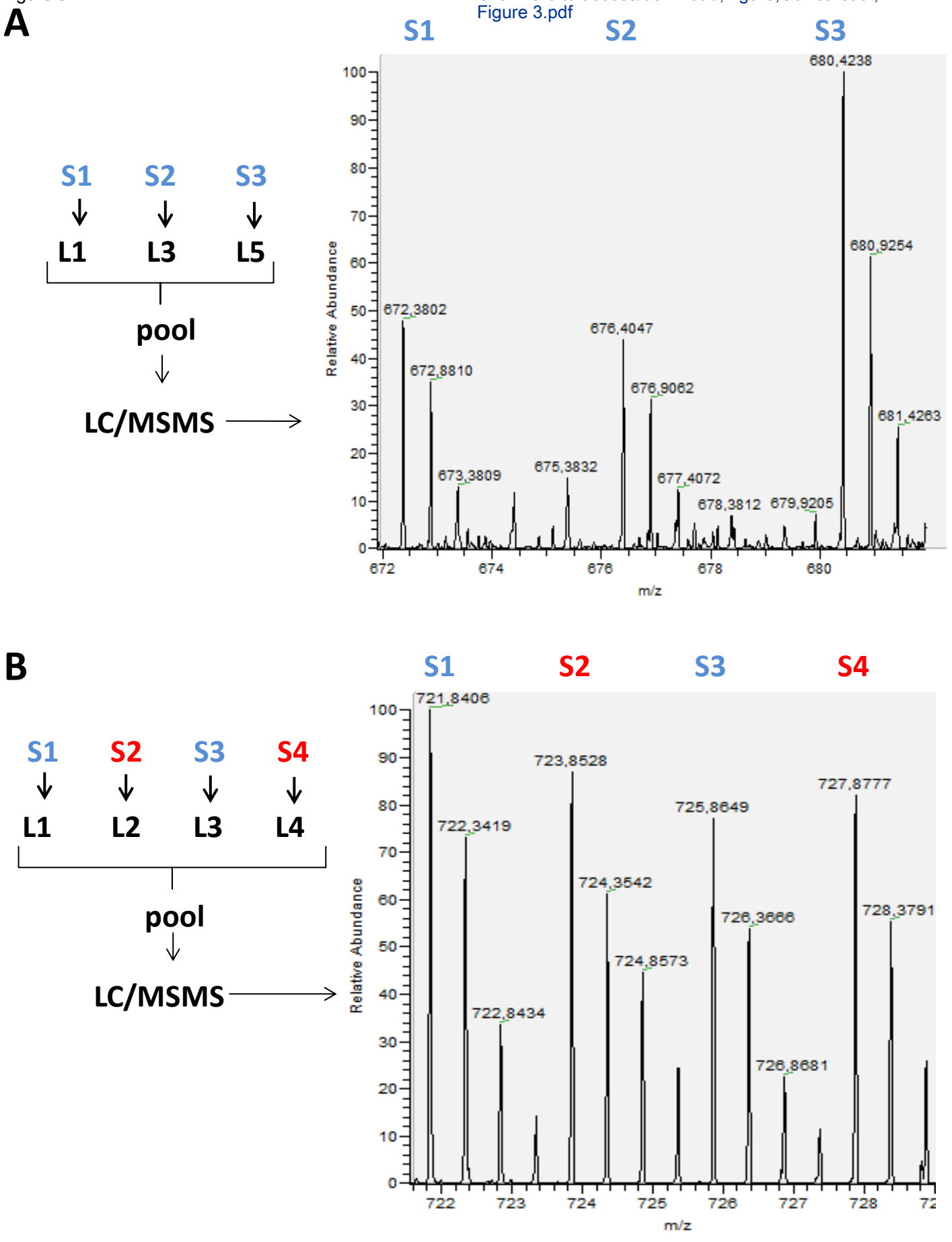

Figure 4

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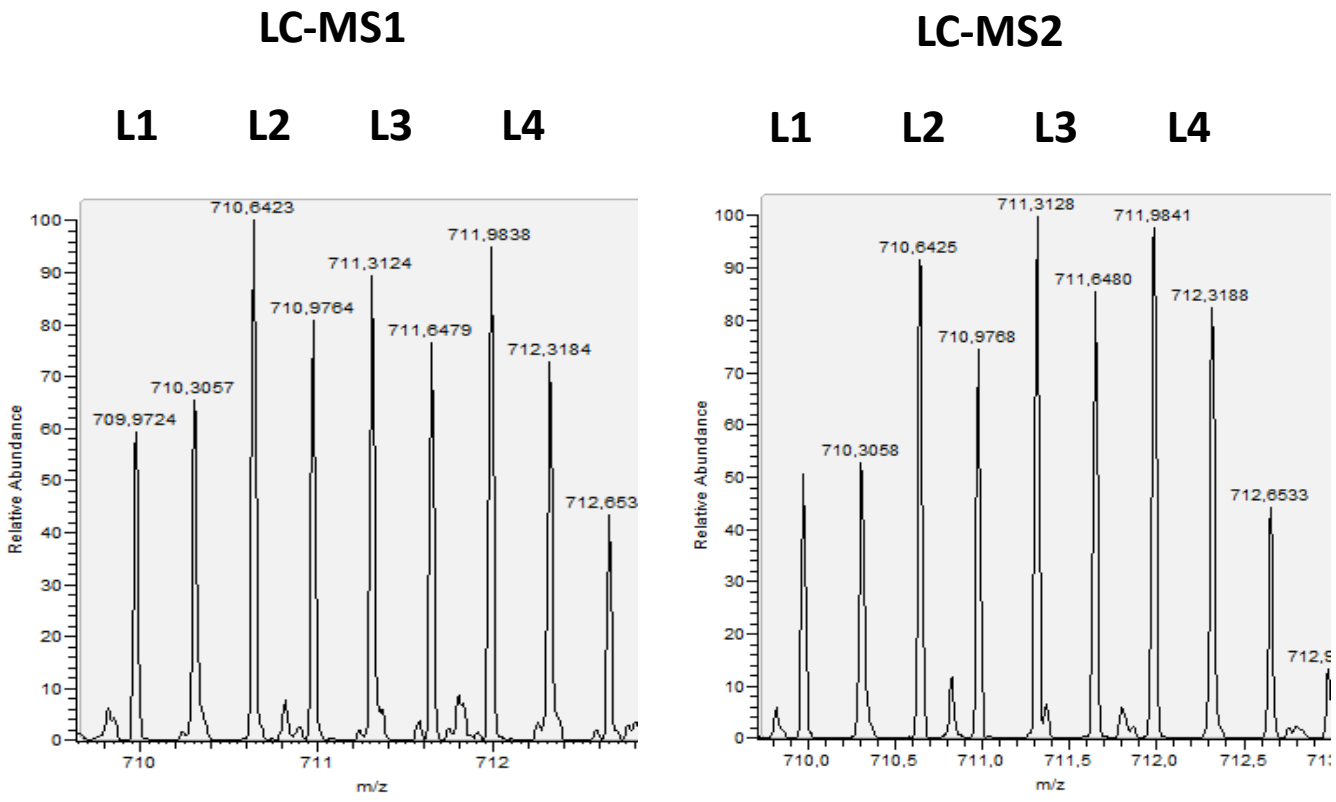

Group	Elute Time (min)	m/z CH3	m/z CH2D	m/z CHD2	m/z CD3	z	#Me	Observed Mass
LC/MS1	44.2	709.973	710.642	711.312	711.984	3	2	2098.87
LC/MS2	44.2	709.973	710.642	711.312	711.984	3	2	2098.87

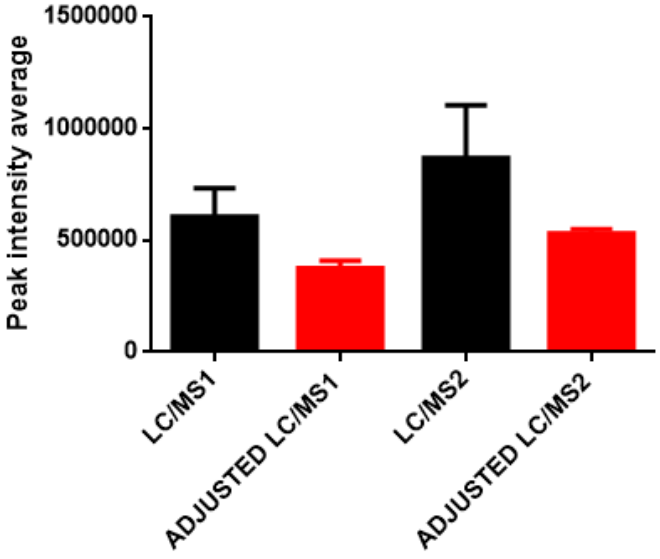

Figure 5

Click here to access/download;Figure;Correa et al, Figure 5.pdf

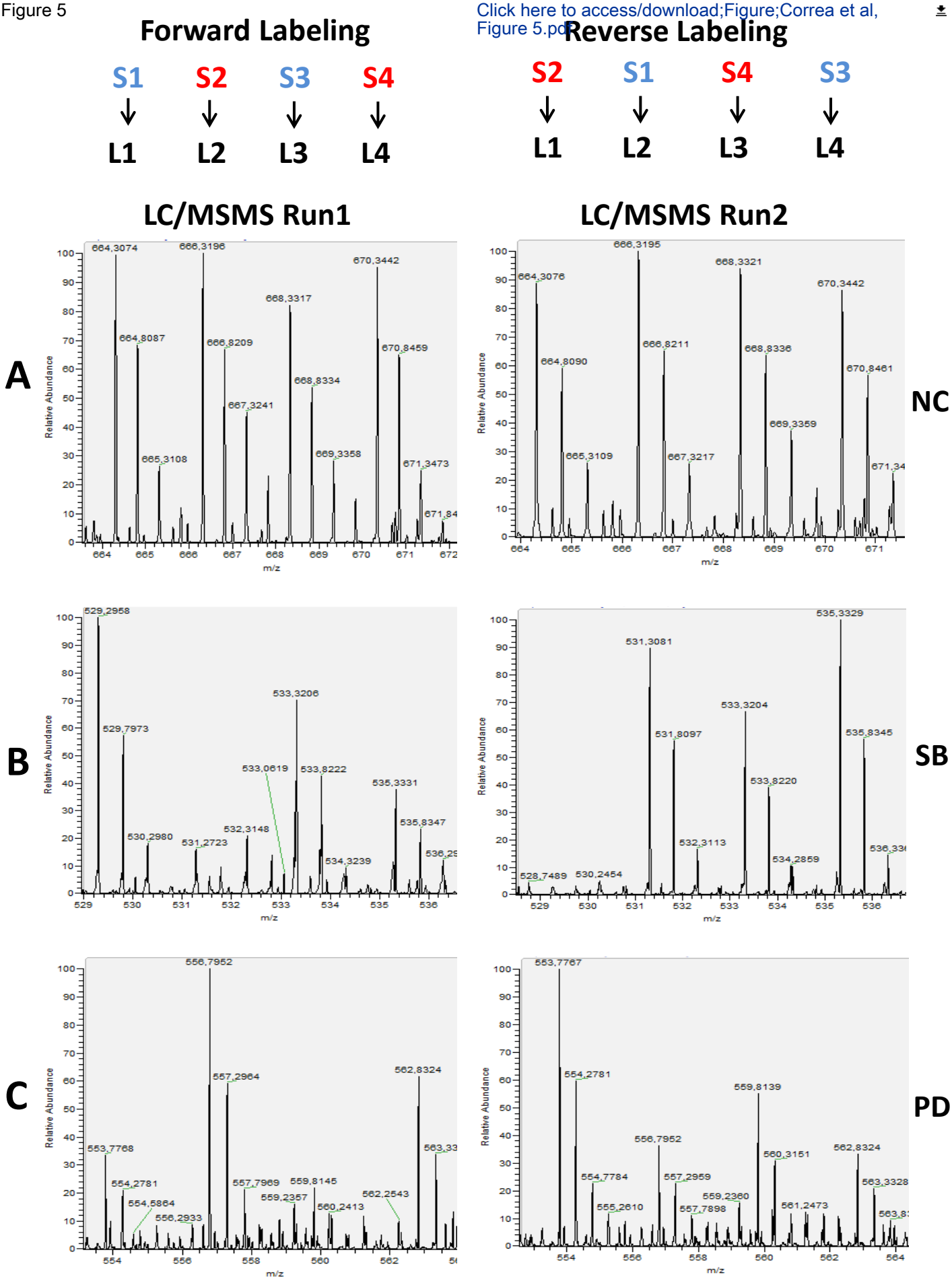

Figure 6

Click here to access/download;Figure;Correa et al, Figure 6.pdf

Peptide	Unique	-10lgP	Mass	Length	ppm	m/z	RT	Area Sample 1
A(+36.08)DQVSASLAK(+36.08)QGL	•	44.59	1358.8344	13	-0.5	680.4241	30.94	2.14E7
A(+32.06)DQVSASLAK(+32.06)QGL	•	34.53	1350.7958	13	-0.9	676.4045	30.98	9.5E6
A(+28.03)DQVSASLAK(+28.03)QGL	•	31.48	1342.7456	13	0.2	672.3802	31.40	1.05E7

>XP_021329127.1 microtubule-associated protein tau isoform X1

```
481  PARKASVPSK AKAGAGATPE KKAGDAKTKT AGAKPQGVGA KIPAASRMEQ RRTGPGSIER ADSPKTPDRS GCSSPASRSS
561  TPGQQVKKVA VVRTPPKSPG SLRSRAQIAP VAPMPDLKNV KSKIGSTENL KHQPGGGKIQ IVHKKIDLSN VQSKCGSKAN
641  IHHKPGGGNV EIKSEKLDFK AQSKVGSLEN IGHVPGGGQR RIESHKLNFR DQAKARTDHG ADIVCKSPDI STDGSPRRLS
721  NVSSSGSLNM TDSPQLSTLA DQVSASLAKQ GL
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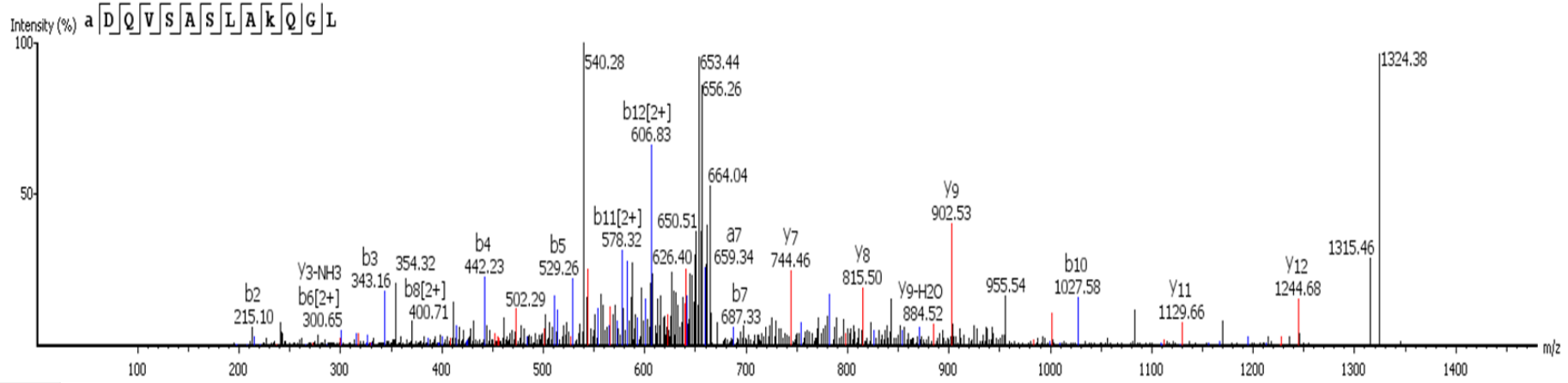

Ion Match

#	b	b-H2O	b-NH3	a	a-H2O	a-NH3	b (2+)	a (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	100.08	82.07	83.05	72.08	54.07	55.05	50.54	36.54	A(+28.03)					13
2	215.16	197.25	198.20	187.11	169.10	170.08	108.05	94.05	D	1244.68	1226.58	1227.79	622.69	12
3	343.27	325.39	326.34	315.26	297.16	298.14	172.08	158.08	Q	1129.61	1111.69	1112.51	565.44	11
4	442.29	424.28	425.36	414.32	396.32	397.36	221.19	208.12	V	1001.56	983.61	984.57	501.35	10
5	529.26	511.27	512.24	501.35	483.55	484.38	265.37	251.13	S	902.53	884.57	885.50	451.70	9
6	600.31	582.29	583.27	572.33	554.14	555.28	300.28	286.31	A	815.49	797.79	798.45	408.41	8
7	687.34	669.32	670.30	659.60	641.33	641.96	344.17	330.33	S	744.50	726.45	727.43	372.73	7
8	800.42	782.52	783.39	772.42	754.63	755.39	400.48	386.38	L	657.43	639.26	640.11	329.37	6
9	871.21	853.68	854.43	843.46	825.45	826.38	436.23	422.23	A	544.18	526.33	527.01	272.19	5
10	1027.51	1009.57	1010.55	999.69	981.57	982.56	514.37	500.29	K(+28.03)	473.32	455.04	456.17	237.24	4
11	1155.53	1137.63	1138.61	1127.81	1109.43	1110.35	578.20	564.34	Q	317.24	299.37	300.28	159.09	3
12	1212.68	1194.57	1195.63	1184.66	1166.57	1167.43	606.63	592.53	G	189.12	171.11	172.10	95.06	2
13									L	132.10	114.09	115.07	66.55	1

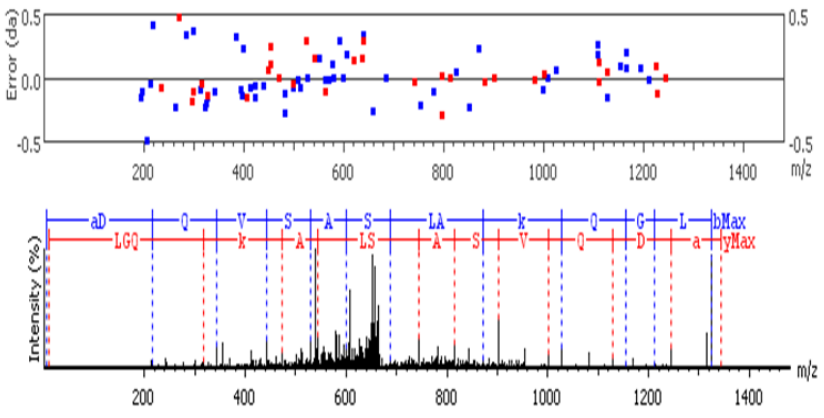

Table 1

Sample	H_2CO 2 x 4 µL	D_2CO 2 x 4 µL	$D_2{}^{13}CO$ 2 x 4 µL	$NaBH_3CN$ 2 x 4 µL	$NaBD_3CN$ 2 x 4 µL	Label code
1	X			X		L1
2	X				X	L2
3		X		X		L3
4		X			X	L4
5			X		X	L5

Additional Mass (Da)
28.0313
30.0439
32.0564
34.069
36.0757

We wish to thank the reviewers for their useful comments. We have revised the manuscript according to their comments. Point by point replies to all reviewers' and editorial comments can be found below.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
It was checked.
2. Please provide an institutional email address for each author.
Claudia Neves Correa: claudia.neves@unesp.br
Louise Oliveira Fiametti: louise.fiametti@unesp.br
Maria Eduarda Mazzi Esquinca: eduarda.mazzi@unesp.br
Leandro Mantovani de Castro: leandro.mantovani@unesp.br
3. Please revise the following lines to avoid previously published work: 22-23, 73, 81-85, 108-109, 224-225, 228-238, 242-246, 271-275, 277-278, 356-359, 373-375, 382-385. Please refer to the iThenticate report attached.
The lines were checked and changed.
4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).It was checked and corrected.
5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials
For example: LoBind, LTQ-Orbitrap Velos, n Easy-nLC II nanoHPLC, Thermo Fisher Scientific, Phenomenex, Jupiter®, Xcalibur, PEAKS, etc..It was checked and corrected.
6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.It was checked and corrected.
7. For SI units, please use standard abbreviations when the unit is preceded by a numeral throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2. It was checked and corrected.
8. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks It was checked and corrected.
9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.It was checked and corrected.
10. Line 95-97: Please specify the type of cells grown. Are the cells purchased or isolated? Please mention. Please specify the conditions of incubation to grow cells
It was checked and the information added.

11. Line 101-103: Cell lysate? Does it mean the contents of the tube? Is there any centrifugation step?
12. Line 105-106: Please specify the ages/ sex, etc. of the zebra fish used in this study.
It was checked and the information added.
13. Line 112: Please specify what tissue is dissected. Please specify the organs.
It was checked and the information added.
14. Line 113-114: Please specify the volume of water. How is the tissue sonicated and homogenized? Please specify the conditions for sonication. Was it probe sonication or bath sonication? It was checked and the information added.
15. Line 115: Maximum speed? Please be specific or provide a range.It was checked and the information added.
16. Line 118-120: Please specify the volume of acid added. Vortexing for how long? It was checked and the information added.
17. Line 129-135: Please correct the step numbers.
It was checked and corrected.
18. Line 137/214: Please specify if there are any specific settings used in the vacuum centrifuge.
It was checked and the information added. line 140
19. Line 166-167: Homogenize on a spectrofluorometer? Please rephrase the sentence to add clarity. “Adjust the readings” – what does this mean? What is it adjusted to? It was checked and the information added. line173-174
20. Line 180-189: Please add the statement of caution.It was checked and the information added.
21. Line 265-280: Please ensure that the Protocol section consists of numbered steps. We cannot have non-numbered paragraphs/steps/headings/subheadings.It was checked and the information added.
22. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.It was checked and the protocol steps for the video were highlighted.
23. Please ensure that the references appear as the following: [LastName, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.
It was checked and corrected.
24. Figure 4: Please include the unit of peak intensity average in the Y-axis.
It is just a numeric value.
25. Table 1: Please revise “µl” to “µL”.
It was checked and corrected.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript described a sample preparation method for the quantification of peptides. A heating treatment was involved to minimize the protease activity, this might be the only novelty in this work. The resultant peptides were labeled with isotope formaldehyde and sodium cyanoborohydride, and analyzed by LC-HRMS. Since there are lots of reported works using multi-plex dimethyl labeling for proteomic research, some important references should be included. Moreover, this manuscript was not well organized. I'm afraid a major revision is needed before considering acceptance in JoVE.

Major Concerns:

1. Remove some generation descriptions of proteomics in Abstract and Introduction.

Answer: Thank you for the comment. We have removed generation descriptions of proteomics in Abstract and Introduction.

2. The results between with and without preheating treatment should be presented.

Answer: Thank you for the comment. This manuscript does not intend to bring novelty to the field of peptidomics, because protocols for rapid inactivation of proteases by heating or microwave radiation, as well as those for labeling by reductive dimethylation, have already been established and used in many studies, mainly for the characterization of peptidomes, as cited throughout this manuscript. However, most of these previous studies used isotope labeling with TMAB tags, which present a complicated synthesis. Our objective in organizing this manuscript was to show the step-by-step preparation of a peptide extract and its subsequent isotopic labeling by RMA, which has some advantages such as stability, it is commercially available and relatively cheap.

3. The figures of the mass spectrum should be redone in Origin/Excel, and present in sufficient resolution.

Answer: Thank you for the comment. MS images are extracted directly from the chromatograms as is. It is very difficult to improve resolution and size. However, we removed from the images some information not necessary.

4. Some figures, such as Fig 6, could be moved to supporting Information.

Answer: We would like to keep the figures in the Representative Results section for the reader to better visualize the results described.

5. The format/layout of the manuscript should follow the authors' guide.

Answer: Thank you for the comment. We follow the format/layout of the manuscript described in the authors' guide.

We would like to thank you for your comments. We hope to have reached the expectations.

Reviewer #2:

Manuscript Summary:
The focus of the paper is interesting in the sense that it addressing the problem of digestion of protein samples prior to sample mass spectrometry insertion. Where the major modification of the structure and biochemical properties caused by such digestion are known to be minimal to affect the analysis, but the possibilities of it could cause inaccurate analysis shouldn't be negated.
As an alternative to protease digestion, the authors suggested heat-inactivation instead coupled with cost-saving RMA labelling.

Major Concerns:
Line 51-".....which occur mainly in this tissue already 1 min postmortem"....the sentence doesn't make sense, maybe should be written as "....which occur mainly in the tissue after 1 min postmortem."????
Answer: Thank you for the comment. The paragraph was modified.

Line 53 and 70 please italic the in vivo
Answer: Thank you. The expression in vivo was changed to italic.

Methodology: Need to rewrite the whole methodology section into past tense, and also must be in paragraph format, not in points form.
Answer: We follow the format/layout of the manuscript described in the authors' guide.

Figure 2 B, replace the figure to include the last peak i.e. 437.7229
Answer: Thank you.The figure was replaced and included the last peak 437.7229.

line 268 Tashima and Fricker ....please add in the year in bracket i.e. Tashima and Fricker (XXXX)
Answer: Thank you for the comment. Missing information has been added.

We would like to thank the reviewer for your comments. We hope to have reached the expectations of improving your major question.