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Automation of On-Resin Enrichment of S-Nitrosylated Proteins for Oxidized Cysteine-Selective cPILOT

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Abstract. S-Nitrosylation (SNO) is a cysteine post-translational modification that increases with normal aging and is present in Alzheimer's disease and other aging-related illnesses. Detection of SNO-modified proteins can be challenging; however, we previously developed a robust quantitative proteomics approach termed "Oxidized Cysteine-Selective combined precursor isobaric labeling and isobaric tagging (OxcyscPILOT)" that allows for detection of endogenous SNO-modified proteins. OxcyscPILOT involves enrichment of SNO-modified proteins using a thiol-based resin. This enrichment is performed manually, and wash steps with the resin require numerous stages and buffer reagents. The goal of this study is to transfer the manual protocol to an automated liquid handler system in order to reduce wash steps, increase sample throughput, and minimize experimental error. In order to accomplish this, we evaluated the Biomek i7 liquid handler automated workstation and a Positive Pressure ALP (PPA) apparatus to conduct automated on-resin enrichment. Our findings provide starting pressure conditions for the use of PPA in an automated OxcyscPILOT proteomics workflow that could be transferred to other robotic liquid handling systems.

I. Introduction

Due to its nucleophilicity and redox sensitivity, cysteine is one of the most susceptible amino acids to post-translational modifications (PTMs) (Gu & Robinson, 2016a; Gu et al., 2015). Cysteine is subjected to various oxidative PTMs, including nitrosylation, glutathionylation, palmitoylation, as well as formation of sulfenic acid and disulfide bonds (Couvertier, 2014).

Reversible cysteine modifications play important physiological roles such as modulating enzymatic catalysis, maintaining redox homeostasis, and conducting cellular signaling (Gu et al., 2015; Held & Gibson, 2014; Gu & Robinson, 2016b). One of these reversible cysteine modifications, S-Nitrosylation (SNO), occurs under oxidative stress and can be either neurodestructive or neuroprotective (Gu & Robinson, 2016b). An SNO post-translational modification is the result of the covalent addition of a nitric oxide (NO) group to a cysteine thiol; however, the specific mechanism of this addition has not been fully determined (Murray et al., 2012). SNO is believed to be highly regulated and occur at specific cysteine sites (Foster et al., 2009). SNO has important physiological roles related to maintaining protein activity, influencing protein conformation, and signaling apoptotic pathways (Gu & Robinson, 2016b). Under normal levels, nitric oxide contributes to the maintenance of normal neuronal activity; however, the normal aging process can trigger excessive production of reactive nitric oxide, leading to higher production of cysteine post-translational modifications such as SNO (Gu & Robinson, 2016b).

It has been widely accepted that oxidative stress plays an important role in Alzheimer's disease pathogenesis (Gu & Robinson, 2016a). For this reason, PTMs such as SNO, which are affected by oxidative stress, have become prevalent in studying

aging related diseases such as Alzheimer's. In Alzheimer's disease, SNO is dysregulated and plays a detrimental role in protein function, cell signaling, and apoptosis by affecting protein aggregation and misfolding, mitochondrial dysfunction, neuronal loss, and impaired metabolism (Gu & Robinson, 2016b; Dyer et al., 2019). Despite some studies into the role of SNO in Alzheimer's, many SNO-containing proteins demand further investigation, leaving the full role of SNO in Alzheimer's disease to be discovered (Dyer et al., 2019). Proteomic analysis of cysteine-enriched peptides coupled with reduction of oxidized thiols can be used to measure the oxidation states of cysteine, which is helpful for elucidating the role that oxidative stress plays in biology and disease (Gu & Robinson, 2016a).

Proteomics, the study of the entire set of proteins in cells, tissues, or organisms, offers techniques for the investigation of labile protein modifications such as SNO (Dyer et al., 2019). Proteomics increasingly contributes to our understanding of the roles that proteins play in biology and in diseases such as Alzheimer's and many other aspects of human health (Robinson et al., 2017; Arul & Robinson, 2019). The traditional approach of bottom-up proteomics requires digestion of proteins into peptides, which further increases the complexity of a sample (Gu et al., 2015). It is important to be able to determine differences in protein concentrations across numerous conditions, multiple time points, and in various tissues. This leads to a desire to increase the overall sample throughput in bottom-up proteomics.

Sample multiplexing is a technique in proteomics that allows the comparison of two or more sample preparations simultaneously within a single mass spectrometry (MS) injection (Shiio & Aebersold, 2006; Gygi et al., 1999). Sample multiplexing in proteomics increases the confidence of results due to the ability to compare and analyze high numbers of biological samples in tandem while also including an internal standard or quality control for normalization (Gu et al., 2015). Our laboratory previously developed the Oxidized Cysteine-Selective Combined Precursor Isotopic Labeling and Isobaric Tagging (OxcyscPILOT) method to study cysteine post translational modifications, specifically SNO. Previously, similar methods such as cPILOT have been used to increase the multiplexing capability of quantifying 3-nitrotyrosine-modified proteins (Robinson & Evans, 2012). In the OxcyscPILOT method, cell lysates or biological specimens are subjected to a bottom-up proteomics approach, which selectively captures SNO-modified and cysteine-containing proteins. OxcyscPILOT has three primary steps: (1) blocking of free thiols by a cysteine-reactive reagent, (2) enrichment of peptides containing SNO on a solid phase resin, and (3) isotopic labeling and isobaric tagging of enriched peptides on the solid phase resin. OxcyscPILOT approach offers the advantage of allowing total protein levels to be measured simultaneously across multiple biological replicates (Gu & Robinson, 2016b). This advantage also minimizes false-positive detection of biologically relevant SNO modifications and methodological error (Gu & Robinson, 2016b). OxcyscPILOT has two sample workup steps for cysteine isolation – total cysteine and SNO. The total cysteine technique aims to capture all cysteines initially present in the tissue homogenate, while the SNO technique aims to only capture the SNO-modified proteins. While the abundance of SNO-modified proteins in the samples is generally very low, the total cysteine technique, which yields higher peptide counts, can be used for protocol evaluation.

Cysteinyl-peptides can be enriched directly via the reactions of sulfhydryl groups, such as solid phase thiopropyl resin (Gu et al., 2015; Wang et al., 2006; Forrester et al., 2011). To enrich the samples for SNO-modified proteins in this experiment, a Thiopropyl Sepharose 6B Affinity Resin is used to capture the free thiol containing peptides (Couvertier et al., 2014). Dithiothreitol (DTT) is then used to cleave the sulfur bond on the reactive thiol group of cysteine in peptides and releases them for further analysis. Numerous sequential washing steps with buffer solutions remove peptides that were not bound to the resin – all non-cysteine containing peptides sequences – and then the cysteine-containing peptides are finally eluted. It should be noted that all of these steps are conducted using manual pipettors. Cysteine-containing peptide samples (SNO-enriched) are then analyzed using liquid chromatography coupled

to mass on an Orbitrap Fusion Lumos Tribrid™ Mass Spectrometer.

The aim of this project was to transfer the manual protocol for SNO-enrichment to an automated liquid handling device, the Biomek i7, and to evaluate the optimal pressures on a positive pressure apparatus (i.e., Positive Pressure ALP (PPA)) for efficient recovery of cysteine containing peptides. The values on the automated system were benchmarked to the previously optimized manual procedure. In the manual procedure, a centrifuge was used to push the wash buffers through the resin for peptide enrichment and recovery; however, because the Biomek does not have a centrifuge, the PPA was used. This goal of this experiment was to find a positive pressure that could be applied to the samples in the automated procedure that most resembles the centrifugation that serves the same purpose in the manual procedure. Results from this study are presented herein.

II. Materials

A. Animal Model

The animal model used was a male control mouse (C57/BLJ) liver.

B. Reagents

Reagents used were MS-grade Acetonitrile (ACN) *, Dithiothreitol (DTT)**, Ethylenediaminetetraacetic acid (EDTA)*, Formic Acid (FA)**, Iodoacetamide (IAM)**, NaCl**, Pierce Bicinchoninic Acid Protein Assay (BCA)*, Thiopropyl Sepharose 6B thiol-affinity resin*, Tris(hydroxymethyl)aminomethane(Tris)***, Urea*, and MS-grade H2O*.
* = Fisher Scientific, ** = Sigma-Aldrich, *** = Bio-Rad.

C. Equipment

Equipment used included the Biomek i7 liquid handler Automated Workstation (Biomek), a C18 RPLC column (75 µm i.d. × 23 cm, 100 Å 5 µm C18 stationary phase material), the Orbitrap Fusion Lumos Tribrid™ Mass Spectrometer (Lumos), and the Waters Oasis HLB C18 cartridges.

D. Buffers

Seven buffers were used in this experiment. They were a Coupling buffer 1 (CB1) composed of 50 mM Tris and 21 mM EDTA at a pH of 7.5, Digestion buffer composed of 20 mM Tris at a pH of 8.2, three HLB clean-up buffers (HPLC-grade ACN with 0.1% FA, HPLC-grade H20 with 0.1% FA, and 40:60 H2O:ACN with 0.1% FA), Resuspension buffer made with 50 mM Tris and 8 M urea at a pH of 8.2, and Wash Buffer (WB) made with 50 mM Tris and 1 mM EDTA at a pH of 8.0.

E. Software

Software used for analysis were Proteome Discoverer 2.1 and Microsoft Excel.

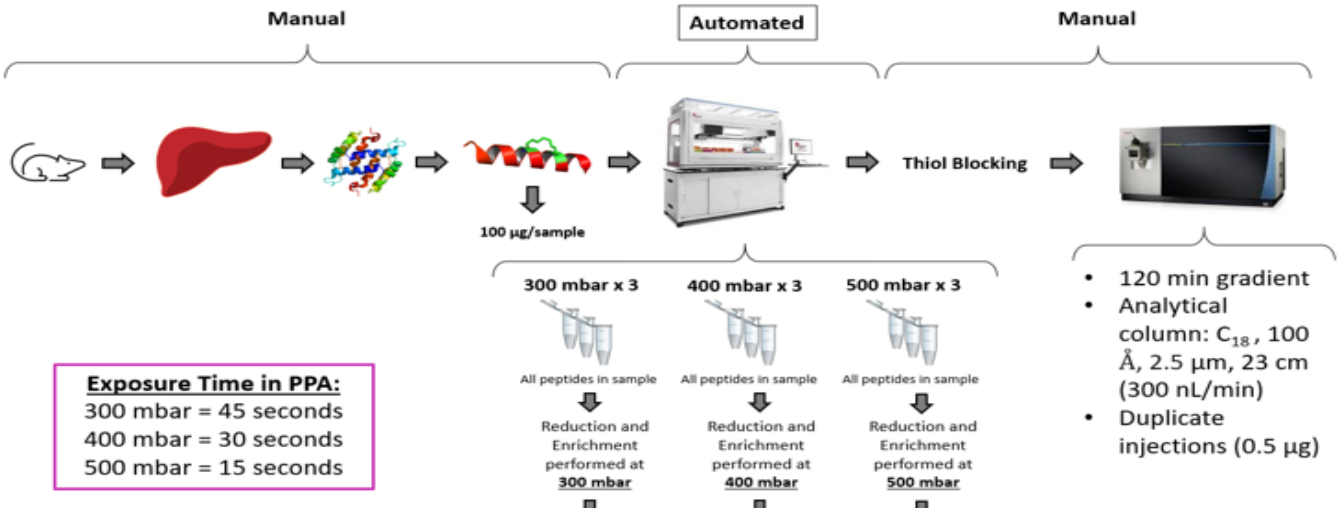

Figure 1: A workflow of the protocol. The experiment begins by manually preparing the sample from mouse liver homogenate to protein. Samples are divided into 100 µg samples and are digested overnight. They are then placed into the Biomek, which performs automated resin enrichment through a series of wash steps and pressure rinses. After the samples complete enrichment, they undergo manual thiol blocking and preparation for Lumos LC-MS/MS analysis.

III. Methods

A general workflow of the experiment is shown in Figure 1.

A. Animal Model

One male control mouse (C57/BLJ) was purchased from Jackson Laboratory and housed in the Division of Laboratory Animal Resources at the University of Pittsburgh. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Mice were fed standard Purina rodent laboratory chow ad libitum and kept in a 12 hour light/dark cycle. Liver tissue was harvested and stored at −80°C.

B. Sample Prep

i. Homogenization

Liver homogenate was centrifuged at 13,000 x g for 15 min at 4°C and supernatant was transferred to a clean 1.5 mL Eppendorf tube. The leftover insoluble portion of the homogenate was stored at -80°C. A bicinchoninic acid (BCA) assay was performed to determine protein concentration.

ii. Cysteinal reduction

A 1 M DTT in 50 mM Tris with 8M urea, pH = 8.2 was prepared. Nine aliquots containing 100 µg of protein and 1 µL of 1M DTT were prepared. 50mM Tris with 8M urea was added to bring each total sample volume to 100 µL.

iii.Tryptic Digestion

Samples were diluted tenfold with digestion buffer and trypsin was added in a 4% w/w enzyme/protein ration. Samples were incubated overnight in a 37°C water bath. 5 µL of FA was added to the samples to quench digestion. The pH of the samples was then tested with a pH test strip to ensure the pH was below 3. The samples were then cleaned using C_{18} HLB cartridges (10 mg) according to the manufacturer's protocol.

C. Enrichment

The *Manual Reduction and Enrichment* portion of the **C. Enrichment** section was not performed to obtain the data listed in the Results section. Instead, it is listed below to provide a comparison between the manual enrichment and the automated enrichment. It can be seen that the manual and automated enrichment protocols are identical except for the means of washing the resin. In the manual protocol, the resin and samples are washed via centrifugation. In the automated protocol, the resin and samples are washed via a positive pressure. The PPA exerted a pressure on top of the resin columns to force any wash buffer through the resin. Three pressures were evaluated in this protocol: 300 mbar, which allowed for a 45 second resin exposure to the solution; 400 mbar, which allowed for a 30 second exposure; and 500 mbar, which allowed for a 15 second exposure.

i. Manual Reduction and Enrichment

Water, CB1, and WB were degassed by sonication for 30 minutes. 100 mM of DTT was prepared in CB1 and 1 µL was added to the samples. An additional 20 µL of CB1 was added to the samples and incubated in 37°C water bath for 1 hour. Approximately 35mg of thiopropyl Sepharose 6B thiol-affinity resin was added to an empty 1.5 mL tube for each sample. 1 mL of degassed water was added to the resin and was allowed to sit at room temperature for 15 minutes. The resin was mixed with a 1 mL pipette and allowed to sit at room temperature for 15 minutes. The resin was then mixed again with a 1 mL pipette and allowed to sit at room temperature for an additional 10 minutes. The resin was then transferred to a 1 mL spin column held in a 2 mL centrifuge tube and centrifuged for 30 seconds at 1000 x g to remove water. The resin was then washed with 450 µL aliquots of degassed water two times under the same centrifuge conditions and then repeated two times with degassed CB1. 79 µL of CB1 was then added to the samples and the samples were transferred to the resin. The centrifuge tubes were capped and shaken on a vortex at a speed of ~800 rpm for 1 hour to enrich at room temperature. The spin column was then uncapped and placed into a clean 2 mL tube. The column was then centrifuged at 1500 x

g for 1 minute to remove unbound peptides. At 1000 x g for 30 seconds with 450 µL aliquots, the samples were sequentially washed one time with wash buffer, one time with 2M NaCl, one time with ACN w/0.1% FA, and one final time with WB.

ii. Automated Reduction and Enrichment

The *Automated Reduction and Enrichment* portion of the **C. Enrichment** section was performed to obtain the data listed in the Results section.

Water, CB1, and WB were degassed by sonication for 30 minutes. 100 mM of DTT was prepared in CB1 and 1 µL was added to the samples. An additional 20 µL of CB1 was added to the samples and incubated in 37°C water bath for 1 hour. Approximately 35mg of thiopropyl Sepharose 6B thiol-affinity resin was added to an empty 1.5 mL tube for each sample. 1 mL of degassed water was added to the resin and was allowed to sit at room temperature for 15 minutes. The resin was mixed with a 1 mL pipette and allowed to sit at room temperature for 15 minutes. The resin was then mixed again with a 1 mL pipette and allowed to sit at room temperature for an additional 10 minutes. The tray containing the resin columns was then transferred to the PPA where each workflow triplicate was exposed to its assigned pressure to remove water. The resin was then washed with 450 µL aliquots of degassed water two times under the same PPA conditions and then repeated two times with degassed CB1. Next, 79 µL of CB1 was added to each of the samples and the samples were transferred to the resin. The tray containing the resin columns was then transferred to a vortex plate, manually capped, and shaken at 800 rpm for 1 hour. Each workflow triplicate was then exposed to the PPA under its respective pressure to remove unbound peptides; the means by which this was accomplished is discussed in further detail in section 3.5 Optimization. Each workflow triplicate was washed one time with wash buffer, one time with 2M NaCl, one time with ACN w/0.1% FA, and one final time with wash buffer, being rinsed after each wash using the PPA.

D. Automation

The Biomek automated workstation is designed to perform liquid handling tasks hands-free. Programming the protocol into the Biomek is performed on the Biomek's user interface. To operate the Biomek, the workstation needs to be informed of what trays and reservoirs are inside of the machine, as well as where they are located. Locations within the Biomek are numbered similar to a chess board with numbers and letters denoting individual positions. The Biomek contains a rotating gripper, linear motion control, a 1mL multichannel pipetting head, and an open-platform design. Programming is performed in a procedural fashion; a command simply needs to be selected and placed into the order that the command is to be carried out in. Commands include "insert tips," "eject tips," "aspirate volume," "transfer tray," etc. It is possible to program loops, if/then statements, and other typical programming constructs into the protocol to ensure precise, accurate automation of the protocol. In this fashion, the Manual Reduction and Enrichment portion of the OxcyscPILOT protocol is transferred into the Biomek software.

i. Biomek Set-Up

The nine Eppendorf tubes containing the samples and the nine Eppendorf tubes containing the resin were then loaded onto the Biomek, along with three 96-deep well plates (buffer trays) that contained degassed water, CB1, WB, NaCl, and ACN w/0.1% FA. Each workflow triplicate was assigned its own buffer tray. Appropriate tip boxes were entered into the Biomek. The positions of all of the trays were programmed into the Biomek, and after a brief calibration period, the automated protocol began.

E. Optimization

In order to prevent each workflow triplicate from being subjected to the pressure of the others inside of the PPA, adjustable seals were used to seal the tops of the columns when the pressures of the other workflow triplicates were being used.

When all of the samples were sent into the PPA, the pressure was only able to act on one of the workflow triplicates; the other two were sealed. Once the samples were ejected from the PPA, the Biomek paused. The seals were manually changed to cover the sample that had just been washed and to uncover one of the other workflow triplicates. The Biomek then resumed its program - the pressure was updated, and the samples were then sent back in to the PPA. This pause/seal transfer/resume cycle repeated for each wash with the buffers so as to only expose each workflow triplicate to the pressure to which it was assigned; this was the only change from the OxcyscPILOT enrichment protocol.

F. Elution

20 mM of DTT in WB was prepared and 100 µL was then added to the samples.

i. Manual Procedure

The samples were capped and shaken on a vortex at ~800 rpm for 30 minutes at room temperature. The resin columns were uncapped, transferred to a fresh 2mL tube, and centrifuged at 1500 x g for 1 minute. The eluent was kept. The above was repeated twice more with fresh DTT each time and 10 minute shake times, collecting the eluent in the same tube. Finally, the above was repeated once with 80% ACN in place of DTT.

ii. Automated Procedure

The tray containing the resin columns was capped, transferred to a vortex plate, and shaken at ~800 rpm for 30 minutes at room temperature. The tray containing the resin columns was uncapped, placed on top of a collection tray, and both went into the PPA where each triplicate was exposed to its respective pressure using the pause/seal transfer/resume method. The eluent was kept. This process was repeated twice more with fresh DTT each time and 10 minute shake times, collecting the eluent in the same collection tray. Finally, the above was repeated once with 80% ACN in place of DTT. The eluent was then removed from the Biomek – the automated portion of the protocol was completed.

G. Free Thiol Blocking

1M IAM was prepared and 32 µL was added to the samples. The samples were then incubated in the dark at room temperature for 1 hour. The samples were pooled and dried to ~100 µL.

H. LC-MS/MS analysis

The samples were then prepared for MS analysis on the Lumos. The analytical column used for this was C18, 100Å, 2.5 µm, 23 cm, and allowed for a flow rate of 300 nL/min. Duplicate injections of 0.5 µg were used per sample to form technical replicates. A 120-minute gradient was used per technical replicate. Data from the Lumos was then

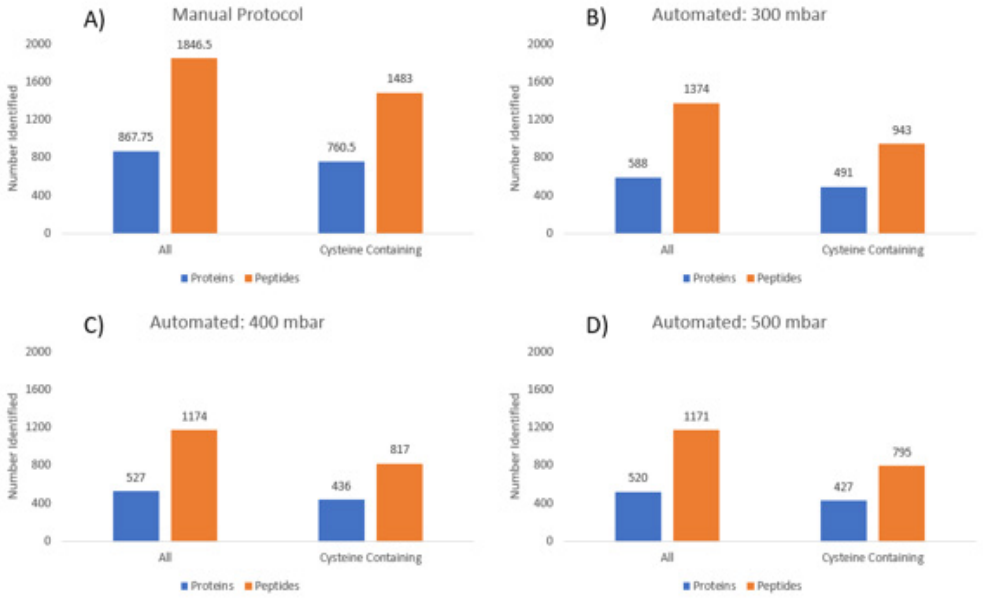

Figure 2: With each workflow triplicate, the aim was to obtain as many cysteine-containing proteins and peptides as was achieved with the manual procedure (A). Graphs (B), (C), and (D) show the proteins and peptides obtained from the sample sets that were administered 300 mbar, 400 mbar, and 500 mbar, respectively.

taken and analyzed on Proteome Discoverer.

IV. Results and Discussion

Samples were prepared manually before they were placed into the Biomek for peptide reduction and enrichment. By optimizing the pressure at which the PPA performed automated resin enrichment, we aimed to determine a specific pressure that is most efficient at recovering cysteine-containing peptides and produces similar results to an optimized manual procedure. This would allow full automation of the resin enrichment protocol using this optimum pressure. The automation of the resin enrichment protocol will allow researchers to work on other tasks such as sample preparation and data analysis while the Biomek is working.

Figure 2 shows the total number of proteins and peptides that were captured from each workflow triplicate as well as the number of those proteins and peptides that contained cysteine. The total number of proteins and peptides, as well as the cysteine-containing proteins and peptides from the manual protocol, are shown as well.

The resin capture efficiency (RCE) is a measure of the percentage of these proteins and peptides that contained cysteine. The RCE was calculated for each of the sample groups and compared against the manual RCE to determine the accuracy compared to previous results. This

Variables	Protein Ratio	Peptide Ratio
Manual	87.60%	80%
300 mbar	83.50%	68.60%
400 mbar	82.70%	69.60%
500 mbar	82.10%	67.90%

Table 1: Resin capture efficiency (RCE) of proteins and peptides. The RCE was determined by dividing the number of cysteine-containing proteins and peptides by the total amount of proteins and peptides found and converting to a percentage. All three automated triplicates had a lower RCE than did the manual procedure, averaging 4.8% lower resin protein capture efficiency and 11.3% lower resin peptide capture efficiency.

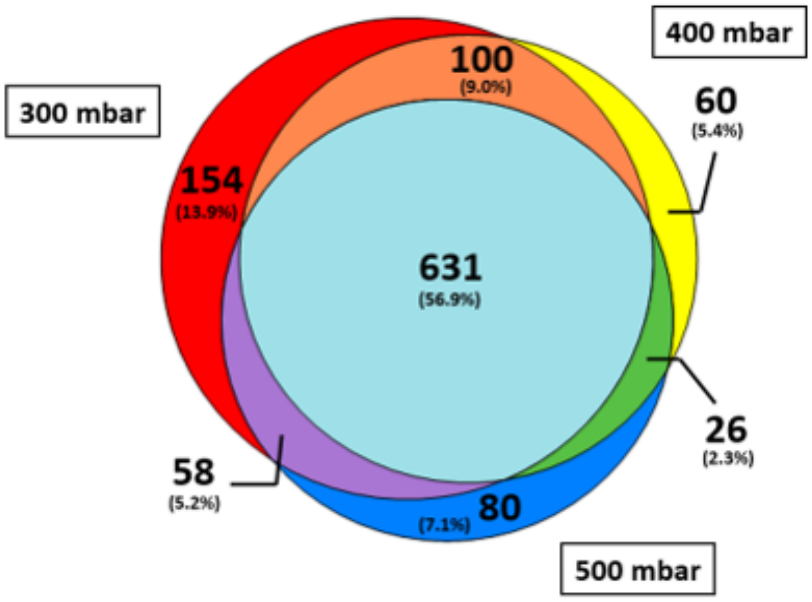

Figure 3: A Venn Diagram shows the distribution of the unique cysteine-containing peptides among the three sample sets.

comparison is shown in Table 1.

RCE quantifies the amount of cysteine-containing proteins and peptides as a percentage of the total proteins and peptides found. It essentially shows how good the resin was at doing its job (capturing cysteine-containing peptides and proteins). A high RCE indicates that the resin had sufficient contact time with the sample and was able to successfully capture a large amount of cysteine-containing peptides. The data show that the 300 mbar samples had the highest protein resin capture efficiency (83.5%) and the second highest peptide resin capture efficiency (68.6%). In total, 1109 unique cysteine-containing peptide sequences were found. A Venn-diagram (Fig. 3) shows the distribution of these peptide sequences among the sample groups.

Of the 1109 unique cysteine-containing peptide sequences found, 631 (56.9%) were found in all three sample sets, while only 294 (26.5%) unique cysteine-containing peptides were found in one of the three sample sets. Of the 1109 unique cysteine-containing peptide sequences found, the sample group that was administered 300 mbar in the PPA contained the highest amount – 943 peptide sequences (85.0%). The workflow triplicates that were administered 400 mbar and 500 mbar in the PPA contained 817 peptide sequences (73.7%) and 795 peptide sequences (71.7%) respectively. There were 631 peptide sequences (56.9%) found in all three sample groups.

V. Conclusion

The workflow triplicate that was administered 300 mbar of pressure produced the most efficient results and most resemble the manual procedure. Although the data from the 300 mbar samples is lower than the data from the manual enrichment, it still displayed better results than the 400 mbar and 500 mbar triplicates.

By investigating biomarkers of Alzheimer's disease, new methods may be developed as research progresses that allows for early diagnoses and prevention of the onset of Alzheimer's disease. As further research continues, it is more and more likely that automation will constitute a greater role than ever before due to its repeatability and the time it provides researchers to work on other tasks. The findings described in this study provide a good stepping-stone for the automation of experiments requiring a conversion from centrifugation to positive pressure.

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References

Arul AB, Robinson RAS. Sample Multiplexing Strategies in Quantitative Proteomics. Anal Chem. 2019 Jan 2;91(1):178-189. doi: 10.1021/acs.analchem.8b05626.

Couvertier SM, Zhou Y, Weerapana E. Chemical-proteomic strategies to investigate cysteine posttranslational modifications. Bba Proteins Proteom. 2014;1844(12):2315–30.

Dyer RR, Ford KI, Robinson RAS. The roles of S-nitrosylation and S-glutathionylation in Alzheimer's disease. Methods Enzymol. 2019; 626:499-538. doi: 10.1016/bs.mie.2019.08.004.

Forrester, M.T., Hess, D.T., Thompson, J.W., Hultman, R., Moseley, M.A., Stamler, J.S., Casey, P.J.: Site-specific analysis of protein S-acylation by resin-assisted capture. J. Lipid Res. 2011, 52, 393–398.

Foster MW, Hess DT, Stamler JS. Trends Mol Med. 2009;15:391–404.

Gu L, Evans AR, Robinson RA. Sample multiplexing with cysteine-selective approaches: cysDML and cPILOT. J Am Soc Mass Spectrom. 2015 Apr;26(4):615-30. doi: 10.1007/s13361-014-1059-9.

Gu L, Robinson RA. A simple isotopic labeling method to study cysteine oxidation in Alzheimer's disease: oxidized cysteine-selective dimethylation (OxcysDML). Anal Bioanal Chem. 2016 Apr;408(11):2993-3004. doi: 10.1007/s00216-016-9307-4.

Gu L, Robinson RA. High-throughput endogenous measurement of S-nitrosylation in Alzheimer's disease using oxidized cysteine-selective cPILOT. Analyst. 2016 Jun 21; doi: 10.1039/c6an00417b.

Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat. Biotechnol. 1999, 17 (10), 994– 9. DOI: 10.1038/13690

Held, JM; Gibson, BW Regulatory control or oxidative damage? Proteomic approaches to interrogate the role of cysteine oxidation status in biological processes Mol. Cell. Proteomics 2012 11 R111 013037.

Murray, C. I.; Uhrigshardt, H.; O'Meally, R. N.; Cole, R. N.; Van Eyk, J. E. Identification and Quantification of S-Nitrosylation by Cysteine Reactive Tandem Mass Tag Switch Assay. Molecular and Cellular Proteomics. 2012, 11(2):M111.013441.

Robinson, R. A.; Amin, B.; Guest, P. C. Multiplexing Biomarker Methods, Proteomics and Considerations for Alzheimer's Disease. Adv. Exp. Med. Biol. 2017, 974, 21– 48. DOI: 10.1007/978-3-319-52479-5_2

Robinson RA, Evans AR. Enhanced sample multiplexing for nitrotyrosine-modified proteins using combined precursor isotopic labeling and isobaric tagging. Anal Chem. 2012 Jun 5;84(11):4677-86. DOI: 10.1021/ac202000v.

Shiio, Y.; Aebersold, R. Quantitative proteome analysis using isotope-coded affinity tags and mass spectrometry. Nat. Protoc. 2006, 1 (1), 139– 45. DOI: 10.1038/nprot.2006.22

Wang, H., Qian, W.J., Chin, M.H., Petyuk, V.A., Barry, R.C., Liu, T., Gritsenko, M.A.,Mottaz, H.M., Moore, R.J.,Camp Ii, D.G.,Khan, A.H.,Smith, D.J.,Smith, R.D.: Characterization of the mouse brain proteome using global proteomic analysis complemented with cysteinyl-peptide enrichment. J. Proteome Res. 2006, 5, 361–369.