Vol. 15, No. 1, 2024
ISSN 2233-4203/ e-ISSN 2093-8950
TECHNICAL NOTE www.msletters.org | Mass Spectrometry Letters

Optimization of Automated Suspension Trapping Digestion in Bottom-Up Proteomics via Mass Spectrometry

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Received November 29, 2023, Revised December 20, 2023, Accepted December 26, 2023
First published on the web March 31, 2024; DOI: 10.5478/MSL.2024.15.1.62

Abstract : The Suspension Trapping (S-Trap) method has been a prominent sample preparation technique since its introduction in 2014. Its capacity to induce protein aggregation using organic solvents has significantly improved protein purification and facilitated peptide identification. However, its full potential for automation has been limited by the lack of a suitable liquid handling system until recently. In this study, we aimed to enhance the automation of S-Trap sample preparation by optimizing the S-Trap digestion process, incorporating triethylammonium bicarbonate (TEAB) and $CaCl_2$. The utilization of TEAB buffer conditions in this innovative process led to a noteworthy 12% improvement in protein identification. Additionally, through careful observation of various incubation conditions, we streamlined the entire sample preparation workflow into a concise 4 hours timeline, covering reduction, alkylation, and trypsin incubation stages. This refined and expedited automated S-Trap digestion process not only showcased exceptional time efficiency but also improved trypsin digestion, resulting in increased protein identification.

Keywords : Mass Spectrometry, Automated Sample Preparation, Suspension Trapping (S-Trap)

Introduction

Proteomics is dedicated to the comprehensive identification of proteins, often encompassing the entirety of an organism's expressed proteins, known as the proteome.[1] Increased protein identification contributes to the improved statistical significance of mass analysis results and subsequently influences proteomic conclusions, making it crucial to achieve as accurate and extensive protein identification as possible.[2] This becomes especially salient in the context of metadata generation.[3] To fortify protein identification, an array of quantitative profiling methodologies has emerged, prominently cantered on mass spectrometry-based techniques, with a particular focus on the fusion of mass spectrometry and high-performance liquid chromatography (HPLC), commonly referred to as LC–MS, alongside tandem mass spectrometry (LC–MS/MS) for the discernment of peptides and proteins[4]. The rapid evolution of mass spectrometry-compatible protocols for protein and peptide separation has catalysed the development of innovative strategies optimized for the precise monitoring of numerous specific targets within exceedingly complex matrices, ensuring heightened sensitivity, specificity, and parallel assessment.[5] This confluence of technological advances has empowered modern mass spectrometers to generate copious volumes of information-rich data within relatively compressed time frames.[6]

In bottom-up proteomics, proteins should be fragmented into small molecule-weight peptides to be subjected to mass spectrometry analysis. Many research groups have tried to improve this digestion process by constructing automated protocols such as on-bead digestion, filter-aided preparation (FASP), and suspension trapping (S-Trap).[7] S-Trap is one of the latest methods that has been developed and applied in proteomics studied.[8] Suspension trapping is a reproducible, rapid, and simple digestion method that can effectively handle proteins even in minute quantities, in the low microgram or sub-microgram range.[9] The Suspension Trapping (S-Trap) method offers an efficient approach to preparing protein lysates containing SDS, significantly reducing the time required compared to the traditional protocols.[10] This innovative approach has been the subject of various studies aimed at optimizing proteomic sample preparation, further affirming its efficacy and utility within

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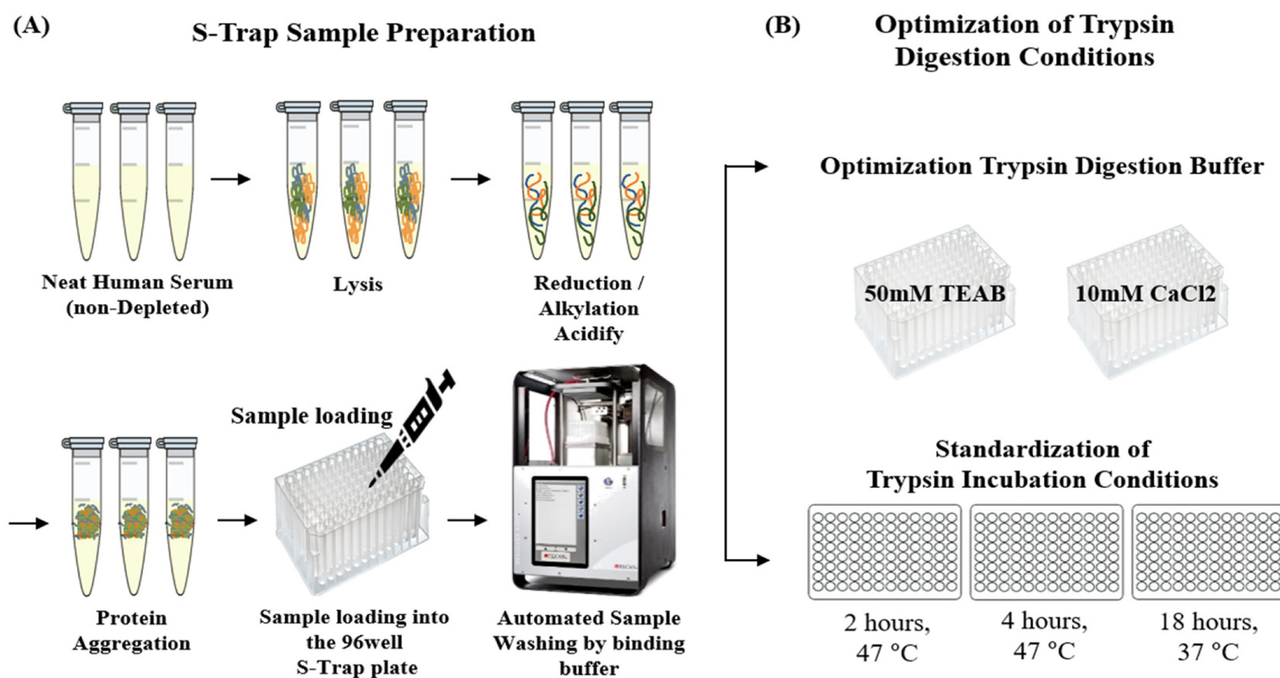

Figure 1. Experimental workflow to S-Trap digestion by automated platform. (A) Automated S-Trap digestion protocol. (B) Optimization of trypsin digestion buffer and trypsin incubation conditions.

the scientific community.[11–13] However, it is essential to acknowledge that, in comparison to some other sample preparation methods, the S-Trap digestion is the starting stage of an automated platform.

In this study, we enhanced the automated S-Trap digestion process with a robotic liquid-handling workstation. Building upon recent work to improve enzymatic digestion,[14] we conducted a comparative analysis of two digestion buffers, which are triethylammonium bicarbonate (TEAB) and $CaCl_2$, to optimize the trypsin digestion process using standard human serum. To determine the optimal conditions, we compared the recommended conditions provided by Protifi of 2 hours at 47°C[15] with the routinely used time conditions in bottom-up proteomics of 18 hours at 37°C.[16] Simultaneously, we conducted additional tests by incubating samples at doubling the initial 2 hours condition, examining whether the trypsin efficiency improved proportionally with doubling of incubation time. This study provides an enhanced automated S-Trap digestion platform by optimizing the trypsin digestion workflows.

Materials and methods

Reagent and chemicals

Human serum, chicken egg white albumin (A5503), sodium dodecyl sulfate (SDS), tris(2-carboxyethyl) phosphine (TCEP), triethylammonium bicarbonate (TEAB), phosphoric acid, iodoacetamide (IAA), formic acid, ammonium bicarbonate (ABC), and urea (ACS reagent, 99.0-100.5%) were procured from Sigma-Aldrich (St. Louis, MO, USA). Sequencing-grade modified trypsin was sourced from Promega (Madison, WI, USA). HPLC-MS grade water, acetonitrile,and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA).

Automated S-Trap sample preparation

Processing initiated with homogenizing 100 µg of human serum, followed by lysing with 5% SDS and 50 mM TEAB at pH 8.5. Protein concentration and quantity were determined using a Thermo Scientific BCA kit. Subsequently, 0.5 µL of TCEP was added, and the sample was incubated at 55°C for 15 minutes to reduce proteins. Alkylation was conducted by adding iodoacetamide at a final concentration of 40 mM and allowing the reaction in darkness at room temperature (RT) for 15 minutes. Post-alkylation, acidification was performed to reach a pH $\leq$ 1 using 27.5% phosphoric acid. Then, 350 µL of 100 mM TEAB with 90% methanol (MeOH) was added to aggregate proteins. The semi-automated Resolvex A200 apparatus (Tecan, Mannedorf, Switzerland) facilitated the 96-well S-Trap plate conditioning and washing with S-Trap binding/wash buffer by positive pressure within 2 min. The sample was loaded onto the filter in each well, undergoing an automated liquid handler for sample binding in the S-Trap column. Trypsin digestion followed at 47°C for 2 hours (enzyme to protein ratio 1:20, w:w). The digested samples were washed and eluted by automated procedures by 50 mM TEAB, 0.2% formic acid, and 50% ACN in 50 mM TEAB consistently, collected in Eppendorf tubes, and dried using a SpeedVac

apparatus. Desalted peptides were reconstituted in a 0.1% formic acid solution for LC-MS/MS analysis.

Manual S-Trap sample preparation

For comparison, manual S-Trap digestion was performed following Protifi's mini spin columns' instructions. Human serum underwent homogenization with 5% SDS in 50 mM TEAB. Subsequently, samples were reduced using 5 mM TCEP for 1 hour at 55°C and alkylated with 40 mM iodoacetamide in darkness for 40 minutes. After acidification and mixing with binding buffer, trypsin digestion was conducted at 47°C for 1 hour (protein-to-enzyme ratio 20:1, w/w). The digested peptides underwent elution by centrifuge 1 min, 4,000 rcf using the same buffer steps. Pool eluted peptides, dry, and resuspension for 0.1% formic acid.

Ovalbumin protein digestion

Ovalbumin quantified at 100 μg of protein, underwent an in-solution digestion protocol. The process involved reduction, alkylation, and dilution to adjust 8 M urea to 2 M using 1 M tris. Reduction was executed using 5 mM TCEP, succeeded by alkylation with 15 mM IAA. Trypsin digestion occurred at an enzyme-to-protein ratio of 1:20 (w/w) for 18 hours at 37°C. Following digestion, the samples underwent desalting and were reconstituted in water containing 0.1% formic acid. Subsequently, 10 ng of digested ovalbumin protein was injected into both manual and automated S-Trap digested samples for LC-MS/MS analysis.

Optimization of trypsin digestion buffer

The trypsin solution was diluted at a ratio of 1:20, and experimental conditions were established by loading each well with either 50 mM TEAB or 10 mM calcium chloride at a temperature of 47°C for 2 hours. Upon completion, an automated elution procedure recovered the digested samples, which were collected in Eppendorf tubes and dried using a SpeedVac apparatus. The resulting desalted peptides were reconstituted in a solution containing 0.1% formic acid for subsequent LC-MS/MS analysis.

Optimization of trypsin incubation conditions

Following established preparation conditions, trypsin was diluted at a 1:20 ratio, subjected to either 50 mM TEAB at 47°C for 2 hours, 4 hours, and 37°C for overnight (18 hours) An automated elution procedure recovered the digested samples, collected in Eppendorf tubes, dried using a SpeedVac apparatus, and reconstituted in a 0.1% formic acid solution for meticulous preparation before LC-MS/MS analysis.

Mass spectrometry and data analysis

The experiments were conducted using a nano liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) platform comprising a Thermo Fisher Scientific Q Exactive™ Hybrid Quadrupole-Orbitrap MS coupled with a Dionex Ultimate 3000 HPLC instrument (Sunnyvale, CA, USA). The mobile phase consisted of 0.1% Formic Acid (FA) in an aqueous solution (solvent A) and 0.1% FA in an 80% acetonitrile (ACN) solution (solvent B). A 75 min gradient set up for solvent B was used as follws: 4% (0-6 min), 4-28% (6-40%), 28-50% (40-52 min), 50-96% (52-52.1 min), 96% (52.1-60 min), 96-4% (60-60.1 min), 4% (60.1-75 min). The data-dependent acquisition was performed, and the top ten precursor peaks were fragmented with higher energy collisional dissociation and normalized collisional energy was 27. Ions were scanned at 70,000 in MS1 (the first level of mass analysis) and 17,500 in MS2 (the second level) over an MS scan range of 400-2000 *m/z* for both the MS1 and MS2 levels. The injection quantity was 1 ug. Peptide samples were introduced onto an Acclaim™ PepMap™ 100 C18 nano-trap column and subsequently underwent peptide separation on a PepMap™ RSLC C18 nanocolumn. These separations were conducted at flow rates of 3 μL/min and 300 nL/min, respectively.

Human serum data analysis and statistical interpretation

Thermo MS/MS raw files resulting from the analytical procedures underwent comprehensive analysis using Proteome Discoverer™ software (version 2.4). The *Human database (Release 2022.05)* was downloaded from Uniprot. A structured workflow was established, primarily involving peptide-spectrum match validation, employing the SEQUEST HT algorithm for precise database searching. The search parameters were precisely set: a precursor ion mass tolerance of 10 ppm, a fragment ion mass tolerance of 0.02 Da, and a maximum allowance of three missed cleavages attributed to the trypsin enzyme. Distinct considerations were made for peptide sequence modifications.

Static carbamidomethylation of cysteine residues (+57.012 Da), dynamic modifications such as methionine oxidation (+15.995 Da), and protein N-terminus acetylation (+42.011 Da) were included. These dynamic modifications were meticulously integrated within the Proteome Discoverer™ software platform during the analysis.The analysis underwent stringent filtering criteria, ensuring robustness. Peptides displaying a false discovery rate of below 1% were retained, with further refinement stipulating a minimum peptide length of at least 6 amino acid residues. This rigorous analytical approach aimed to ensure the precision and reliability of the identified peptides within the dataset. The mass spectrometry proteomics data have been deposited to the Proteome Xchange via the PRIDE partner repository with the data set identifier PXD047972.

Results and Discussion

Comparison of automated and manual S-Trap methods

To assess the efficacy of automated S-Trap digestion, we selected a human serum sample for processing in LC-MS/MS analysis. The results, listed in Table 1, compare manual

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Table 1. LC-MS/MS results of manual and automated S-Trap digestion methods

	PSMs*	Peptides	Protein Groups		PSMs	Peptides	Protein Groups
M*_01	5447	2395	193	A*_01	10002	3049	211
M_02	5053	2375	191	A_02	10461	3267	257
M_03	5258	2414	201	A_03	9603	2873	221
M_04	5195	2408	197	A_04	6261	2599	219
M_05	4175	2183	190	A_05	6514	2797	204
Average	5025, ± 443	2355, ± 87	194, ± 4	Average	8568, ± 1803	2917, ± 227	222, ± 18

*PSMs: Peptide-spectrum matched, M: Manual S-Trap digestion, A: Automated S-Trap digestion, ±: each sample's relative standard deviation.

Table 2. Peak area of two ovalbumin peptides in each run

GGLEPINFQTAADQAR	M_01	M_02	M_03	M_04	M_05	Average
	1.70E+07	1.95E+07	2.24E+07	2.21E+07	2.13E+07	2.04E+07
	A_01	A_02	A_03	A_04	A_05	Average
	3.67E+07	3.66E+07	4.56E+07	4.76E+07	3.36E+07	4.00E+07
HIATNAVLFFGR	M_01	M_02	M_03	M_04	M_05	Average
	3.56E+06	1.29E+07	1.28E+07	4.09E+06	3.57E+06	7.39E+06
	A_01	A_02	A_03	A_04	A_05	Average
	2.63E+07	3.91E+07	3.33E+07	2.74E+07	2.34E+07	2.99E+07

* M: Manual S-Trap digestion, A: Automated S-Trap digestion, E+N: The peak area multiplied by 10 to the power of N ($\times 10^n$)

and automated S-Trap digestion, revealing a notable enhancement in protein, peptide, and Peptide Spectrum Matched (PSMs) identification—approximately 14%, 24%, and 71%, respectively compared to the manual method.

To validate peptide recovery, we introduced 10 ng of standard ovalbumin protein, examining high-intensity peak areas for matched peptides in both manual and automated S-Trap digestion. Table 2 details the peptide peak areas for each digestion method. In the automated S-Trap method, the GGLEPINFQTAADQAR peptide area exhibited an approximately two-fold increase. Similarly, the HIATNAVLFFGR peptide in automated S-Trap digestion showed an increase of approximately 300% in the 3 peak area.

This improvement may stem from distinct elution processes. The manual S-Trap process involves centrifuge-driven elution and washing, causing repetitive drying and wetting of the filter. In contrast, the automated S-Trap digestion process maintains consistent pressure, rapidly delivering the solution. We posit that the automated S-Trap digestion could yield superior peptide recovery due to its improved eluting process.

Comparative analysis between 10 mM $CaCl_2$ and 50 mM TEAB buffers

To optimize the trypsin digestion buffer, we conducted a comparative analysis between two buffer conditions: 10 mM $CaCl_2$ and 50 mM triethylammonium bicarbonate (TEAB). The results, presented in Table 3, showcase the identification of proteins, peptides, and peptide-spectrum matches (PSMs) under each buffer condition. The utilization of the 50 mM TEAB buffer demonstrated an approximate 11% increase in protein identification, a 35% rise in peptides, and a 14% improvement in PSMs compared to the $CaCl_2$ buffer.

To assess trypsin efficiency, we analyzed missed cleavages and the number of tryptic termini (NTT). As depicted in Figure 2, the TEAB buffer exhibited a roughly 13% increase in un-missed cleavages (denoted as '0') compared to the $CaCl_2$ buffer. Figure 3 illustrates the count of fragmented peptide termini, revealing an increase of about 56.7% in peptides cut on both sides ('2') within the TEAB buffer condition. This result confirms an enhancement of 51.5% in total tryptic termini.

Table 3. LC-MS/MS results of $CaCl_2$ and TEAB buffer condition.

Samples	PSMs	Peptides	Proteins Groups
$CaCl_2$_01	5622	1478	230
$CaCl_2$_02	4328	1438	209
$CaCl_2$_03	4508	1549	253
Average	4819, ±572	1488, ±46	230, ±18
*TEAB_01	5298	1769	252
TEAB_02	5389	2135	262
TEAB_03	5738	2129	261
Average	5475, ±190	2011, ±2011	258, ±4

*TEAB: Triethylammonium bicarbonate

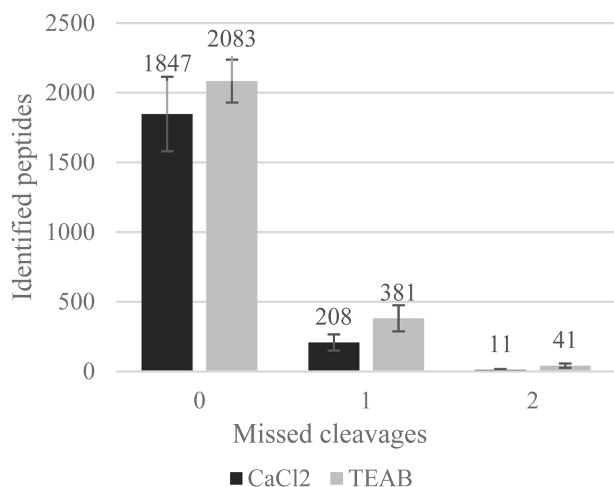

Figure 2. Comparison of missed cleavages between CaCl2 and TEAB buffer conditions.

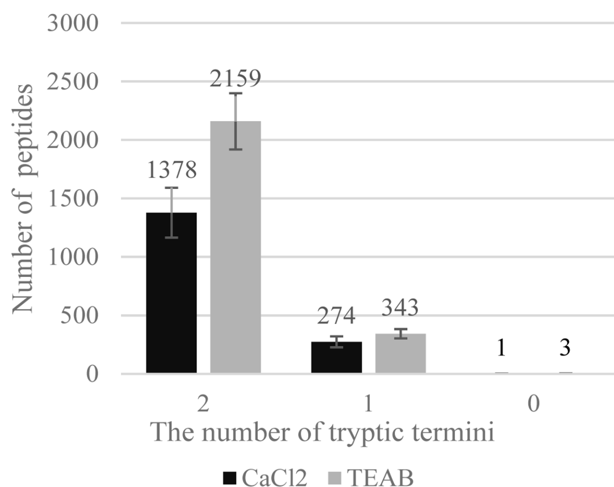

Figure 3. The number of tryptic termini of $CaCl_2$ and TEAB buffer conditions.

We hypothesized that different reactions might occur due to the use of an organic solvent. Prior research employed the $CaCl_2$ buffer in an in-solution digestion method without utilizing any organic solvent buffer. Our inference is that the TEAB buffer is more chemically compatible with organic solvents in the S-Trap digestion method.

Comparison of trypsin incubation conditions

To determine the optimal trypsin incubation conditions, three setups were explored: 1) 2 hours at 47°C, 2) 4 hours at 47°C, and 3) overnight (18 hours) at 37°C. The 2 hours incubation at 47°C resulted in the highest identification of protein groups, as depicted in Table 4. Conversely, overnight incubation at 37°C exhibited the highest identification of peptides and PSMs. Except for the 4 hours condition, both the 2 hours and 18 hours conditions yielded comparable identification results.

Table 4. LC-MS/MS results of 2 hours, 4 hours, 18 hours

	Protein Identification	Peptide Identification	PSMs
2h_1	255	3266	7790
2h_2	241	2115	5743
2h_3	222	3096	7316
2h_4	212	1745	4338
2h_5	228	3077	6586
2h_6	241	3226	6343
Average	233, ± 14	2754, ± 596	6353, ± 1116
4h_1	233	1907	4524
4h_2	218	1657	4308
4h_3	220	1488	4317
4h_4	227	1601	4378
4h_5	217	1533	5128
4h_6	237	1980	5555
Average	225, ± 8	1694, ± 185	4702, ± 474
18h_1	234	3126	6380
18h_2	225	3189	6475
18h_3	217	3273	6338
18h_4	216	3181	6365
18h_5	249	3169	6972
18h_6	242	3108	6203
Average	231, ± 12	3174, ± 53	6456, ± 244

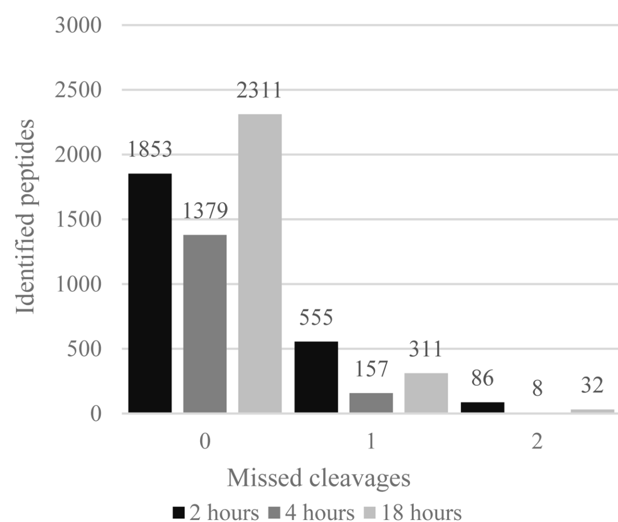

Figure 4. The missed cleavages of each incubation condition (2 hours, 4 hours, 18 hours).

To assess trypsin digestion efficiency, we evaluated missed cleavages and the number of tryptic termini (NTT). Figure 4 illustrates the missed cleavages of each condition. Recovery rates for no-missed cleavages were 75.1% for the 2 hours, 89.4% for 4 hours, and 87.1% for 18 hours. Although the 4 hours condition seemed to indicate a higher

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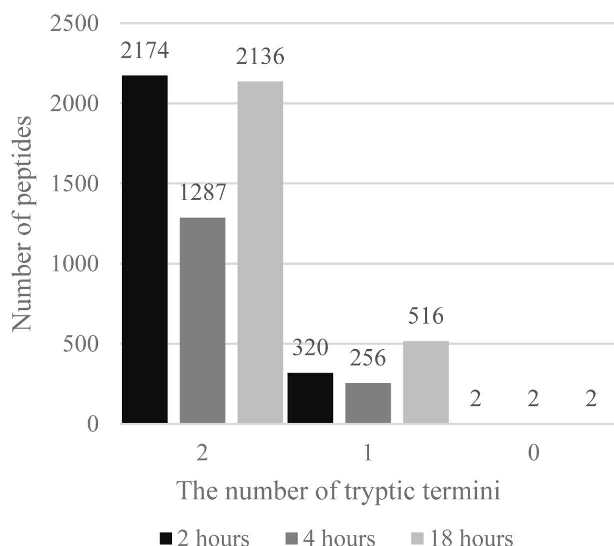

Figure 5. The Number of tryptic termini of different incubation conditions.

rate of peptide cleavage recovery, the total count of identified peptides was low, approximately 38%. Figure 5 illustrates the number of tryptic termini for each condition, revealing recovery rates of 87.1% for 2 hours, 83.3% for 4 hours, and 80.5% for 18 hours.

We believe that the 2 hours and 18 hours durations displayed efficiency in trypsin digestion and identification. The decreased identification in the 4 hours condition aligns with other research confirming that increasing trypsin incubation time does not linearly increase peptide identification. Additionally, considering the typical efficiency range of trypsin enzymes, roughly around 70-80%, it suggests that all conditions were reasonably well digested. However, for automated high-throughput sample preparation, we recommend the 2 hours incubation condition for its efficiency in both identification and speed.

Conclusions

The S-Trap digestion method has gained recognition in proteomic sample preparation, yet its integration with automation remains relatively nascent. This study aimed to enhance automated S-Trap sample preparation and refine trypsin digestion conditions. Comparing automated and manual S-Trap methods revealed superior performance in the automated approach, exhibiting enhanced protein identification. Notably, the automated S-Trap identified 222 proteins compared to 194 in the manual process. Additionally, the peptide area of ovalbumin quantified via spike-in increased approximately two-fold and 300% using the automated S-Trap. Moreover, in order to optimize the conditions of trypsin digestion, we comparison of buffer composition and incubation conditions. Under TEAB conditions, an 11.7% increase in protein identification was observed compared to $CaCl_2$. TEAB conditions exhibited high trypsin efficiency by missed cleavages and tryptic termini. While most incubation conditions showed similar protein identification and trypsin efficiency except for the 4 hours condition. However, for high-throughput sample preparation, we think the 2 hours condition is as the most suitable condition. Integrating a 2 hours digestion under TEAB conditions within an automated S-Trap workflow reduced the total sample preparation time to 4 hours. Consequently, the Automated S-Trap method showed a balanced improvement in both time efficiency and performance. This optimized workflow holds promise for potential application in high-throughput clinical sample preparation in the future.

Acknowledgments

This work was a grant from the National Research Foundation (NRF-2022M3H9A2086450) funded by the Korean Ministry of Science, National Research Foundation of Korea (NRF) (No. 2017M3D9A1073784), and ICT (MSIT) and Korea Evaluation Institute Of Industrial Technology (KEIT) (No. 20018578).

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