# Automated Peptide Desalting Using Dispersive Pipette Extraction Tips for Increased Protein Identifications

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integrated micro-chromatography systems

## INTRODUCTION

Desalting peptides is an essential procedure for improving LC-MS/MS analysis (1-3). Removing salts utilizes solid phase extraction (SPE), which has relied on cartridge formats. These formats have been used for routine sample preparation and the microspin columns have been developed to miniaturize and speed up the desalting processes. However, these procedures are not readily adapted for automated liquid handlers. Here, we present automated, dispersive pipette extraction technolog y in IMCStips® for robust and high-throughput proteomics analysis. Several different resins were compared in spin column versus dispersive pipette (4). Specific peptides were monitored for optimal loading and recoveries for both SPE formats (spin vs tip), and total proteome analysis was assessed on cell lysates to determine total protein IDs for both extraction processes.

## MATERIALS AND METHODS

HEK293T cells were lysed with 8 M Urea, 75 mM NaCl, 50 mM $NH_4HCO_3$, 1 x protease and phosphatase inhibitor mixture. The samples were digested with trypsin (1:200, trypsin:lysate) overnight at 37 °C. For automatic sample processing, we developed a method for VIAFLO 96 from Integra and other high-throughput automatic liquid handling system (***Figure 1, 2***).

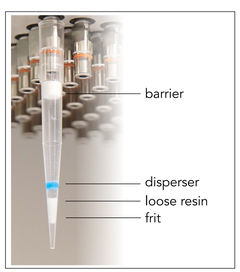


***Figure 1.*** *Components in dispersive pipette extraction, IMCStips*

*Figure 2. Desalting IMCStips with RP resin on a VIAFLO96 from Integra*

To measure desalted peptide quantity, we used UPLC TSQ-Endura triple quadrupole mass spectrometry with optimized conditions. The area under the curve of enriched samples were compared with the equal amount of exogenous standard mixture.

For global peptide identification followed by desalting, we used Q-Exactive mass spectrometer coupled with Ultimate 3000 nano-UHPLC system. MS spectra were acquired by data dependent scans consisting of MS/MS scans of the twenty most intense ions from the full MS scan with dynamic exclusion option which was 10 seconds. Spectra were searched using Sequest HT algorithm within the Proteome Discoverer v2.1 (Thermo Scientific) in combination with the human UniProt protein FASTA database (20,193 entries, December 2015).

| | Quantification | Identification |
|---|---|---|
| Mass spectrometer | TSQ Endure, Thermo Fisher | Q-Exactive Thermo Fisher |
| Liquid chromatography | Vanquish UPLC | Ultimate 3000 nano-UHPLC |
| Mobile phase A | 0.1% formic acid (F.A.) in water | 0.1% F.A. in water |
| Mobile phase B | 0.1% F.A. in acetonitrile | 0.1% F.A. in acetonitrile |
| LC gradient | 5% - 35% B for 15 minutes | 2% - 30% B for 170 minutes |
| Trap column | N/A | Acclaim PepMap 100 ($C_{18}$, 5 µm, 100 Å, 300 µm X 5mm) |
| Analytical column | Syncronis $C_{18}$, 100 X 2.1 mm, 1.7 µm | Acclaim PepMap RSLC ($C_{18}$, 2 µm, 100 Å, 75 µm X 15 cm) |
| Column oven temperature | 40 °C | Room temperature |

## RESULTS

Automatic sample preparation using VIAFLO96 from Integra and a high-throughput automatic liquid handling system was optimized for 10 mg or 20 mg desalting resins in 1 mL IMCStips and compared with spin-column (Sigma-Aldrich) extraction. The total desalting process on the VIAFLO96 took less than 10 minutes with minimal hands-on time (**Table 1**).

**Table 1. Desalting Protocol using 1 mL IMCStips**

| Steps | Process | Solvent | Aspiration µL | Volume µL | Repeat # | Duration minutes |
|---|---|---|---|---|---|---|
| 1 | Activation | 100 % ACN | 600 | 800 | 2 | 0.6 |
| 2 | Condition | 70% ACN, 0.1% F.A. | 400 | 800 | 3 | 1.0 |
| 3 | Equilibrate | 1% TFA | 400 | 800 | 3 | 1.0 |
| 4 | Bind | 1% TFA | 400 | 500 | 10 | 3.3 |
| 5 | Wash 1 | 0.1% TFA | 400 | 800 | 3 | 1.0 |
| 6 | Wash 2 | 0.1% F.A. | 400 | 800 | 3 | 1.0 |
| 7 | Elution 1 | 70% ACN, 0.1% F.A. | 400 | 400 | 3 | 1.0 |
| 8 | Elution 2 | 70% ACN, 0.1% F.A. | 400 | 400 | 3 | 1.0 |
| | | | | | Total | 9.9 |

We established selected reaction monitoring (SRM) method for TSQ Endura triple quadrupole mass spectrometer to test recovery of exogenous peptide standards on our $C_{18}$ and divinylbenzene (RP) IMCStips (**Table 2**).

**Table 2. Selected Reaction Monitoring Transition of the Peptide Standards**

| Sequence | Name | Parent Mass m/z | Product Ion 1 m/z | Product Ion 2 m/z | Collision Energy V |
|---|---|---|---|---|---|
| RPPGFSPFR | Bradykinin | 354.2 | 506.3 | 419.2 | 15.7 |
| DRVYIHPFHL | Angiotensin I | 432.9 | 647.4 | 619.4 | 18.7 |
| DRVYIHPF | Angiotensin II | 349.5 | 513.3 | 371.2 | 15.6 |
| NVIQISNDLENLR | Leptin | 509.9 | 644.4 | 531.3 | 21.7 |
| RPVKVYPNGAEDESAEAFPLEF | ACTH18-39 | 822.4 | 505.3 | 981.0 | 33.5 |
| DRVY*IHPF | Angiotensin II | 376.2 | 371.2 | 756.3 | 15.0 |
| IKNLQS*LDPSH | Cholecystokinin | 444.6 | 340.2 | 455.2 | 25.0 |
| DFNKFHT*FPQTAIGV | Calcitonin | 601.3 | 757.8 | 814.4 | 15.0 |

**Phosphorylated amino acid.*

First, we compared five spin columns including two competitor's material with five tips including two 1:1 combined materials with RP resin for non-phosphorylated peptides (**Figure 3**). Then, we compared the recovery of phosphorylated peptides and found that the IMCStips method out performed the spin column method (**Figure 4**).

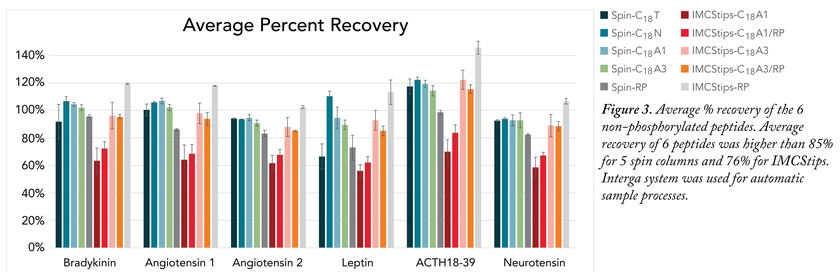


***Figure 3.*** *Average % recovery of the 6 non-phosphorylated peptides. Average recovery of 6 peptides was higher than 85% for 5 spin columns and 76% for IMCStips. Intergra system was used for automatic sample processes.*

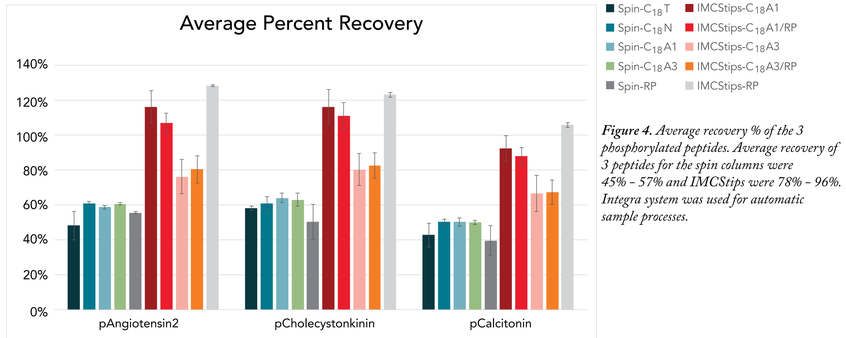


***Figure 4.*** *Average recovery % of the 3 phosphorylated peptides. Average recovery of 3 peptides for the spin columns were 45% - 57% and IMCStips were 78% - 96%. Integra system was used for automatic sample processes.*

With optimized liquid handling protocol on a high-throughput automatic liquid handling system, the average recovery of 6 non-phosphopeptides was greater than 84% on 6 type of combinational IMCStips with $C_{18}$A1, $C_{18}$A3, and RP resins (Figure 5).

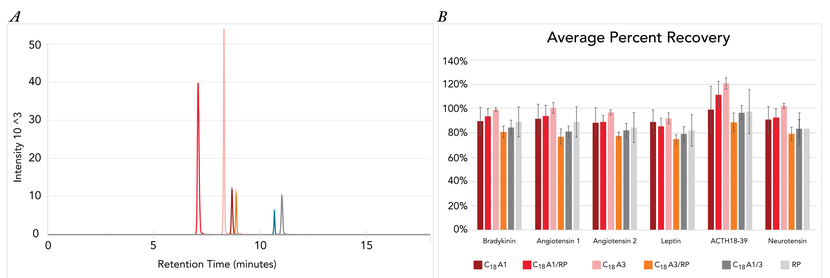


***Figure 5.*** *Average recovery % of the 6 peptides. A) Automatic AUC integration using Skyline software for target peptides, B) Average recovery of 6 peptides was greater than 84%. A high-throughput automatic liquid handling system was used for automatic sample processing*

To test desalting for the global proteomics analysis, we compared five desalting materials ($C_{18}$, $C_{18}$A1, graphite carbon black-1, graphite carbon black -2, RP) with spin column and tip formats. We identified 2358 ± 187 (mean ± SD) proteins with 10 mg spin columns and 2737 proteins with 10 mg RP IMCStips (Figure 6).

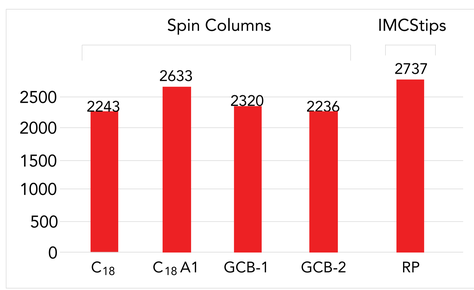


***Figure 6.*** *Comparison of the protein identification using spin columns and RP IMCStips.*

To evaluate the physicochemical property of enriched peptides, we compared the grand average of hydropathicity (GRAVY) values. There was no significant differences of GRAVY values of the of identified peptides between four different materials (Figure 7).

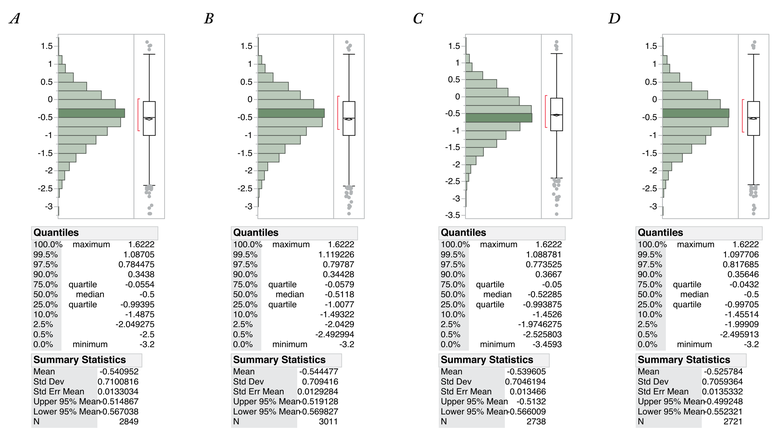


***Figure 7.*** *Histograms of GRAVY hydrophobicity values of the identified peptides from naïve and three different IMCS desalting tips, A) Naïve peptides, B) $C_{18}$A1, C) $C_{18}$A3, D) RP.*

## CONCLUSIONS

Mass spectrometer has become a mainstream analytical tool for a broad range of applications. One of the major bottlenecks in mass spectrometry is having the ability to process many samples in a consistent and reproducible manner. This consistency should leverage an automated liquid handling system that eliminates many of the errors stemming from monotonous manual operations. Here, we explored several different resin types with dispersive pipette extraction technology on an automated liquid handler. This approach demonstrates faster workflows while exhibiting higher recovery efficiencies than traditional spin column formats. Furthermore, the screening of several different reverse phase resins ($C_{18}$ silica, graphitized carbon black and wet-able polystyrene) was done to determine the most effective resin type for routine desalting and peptide enrichment. Based on the work, IMCStips packed with wet-able polystyrene crosslinked with divinylbenzene (noted as RP) showed consistently high recoveries of the control peptides, phosphopeptides and higher protein IDs from cell lysates. The peptide recovered using the RP resin showed little or no statistical variance from the peptides recovered using $C_{18}$ resins as indicated by GRAVY values. The flexibility and high throughput capabilities of IMCStips for proteomics applications show relative ease of processing large number of samples while maintaining highly consistent operations.

## REFERENCES

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**Abbreviations:**
ACN: Acetonitrile; AUC: Area under the curve; $C_{18}$A1: $C_{18}$ 100 Å resin; $C_{18}$A3: $C_{18}$ 300 Å resin; $C_{18}$N: $C_{18}$ spin column from vendor N; $C_{18}$T: $C_{18}$ spin column from vendor T; RP: wettable polystyrene cross linked with divinylbenzene; F.A.: Formic acid; GCB: Graphitized carbon black; TFA: Trifluoroacetic acid.

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