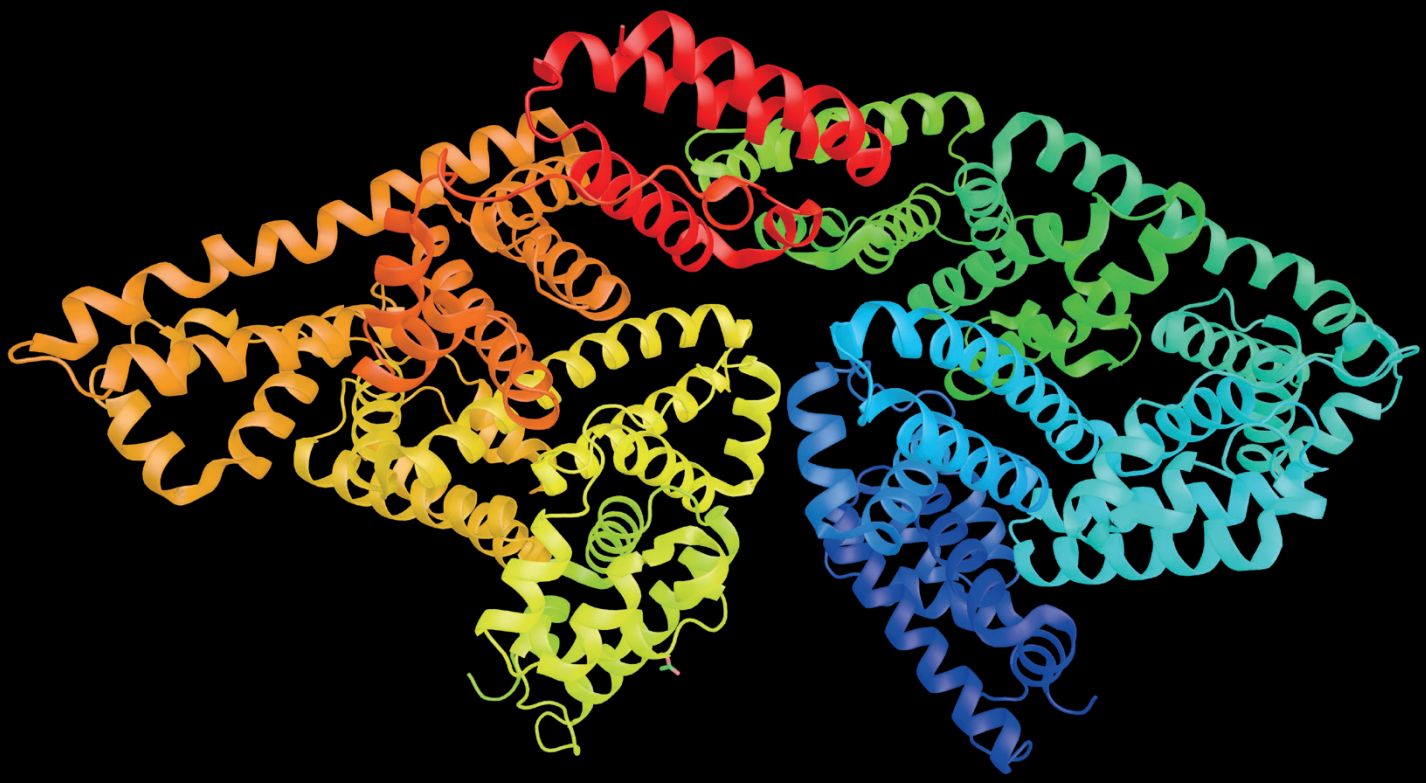
On-Column Digest Followed by SPE Desalting.

Application Note



UTILIZING THE UNIQUE DUAL CHAMBER DESIGN OF TECAN® NBE™ COLUMNS FOR RAPID AND SIMPLIFIED SAMPLE PROCESSING

TECAN.

INTRODUCTION

This application note demonstrates the analysis of trypsin-digested bovine serum albumin (BSA) with on-column digestion using Tecan's Narrow Bore Extraction (NBE) columns, followed by peptide clean-up by Solid Phase Extraction (SPE) utilizing Atlas® sorbent in the same column. Resulting recoveries were then compared to standard in-tube trypsin digestion followed by subsequent sample clean-up using the same Atlas sorbents in SPE columns.

NBE columns offer potential workflow advantages due to their unique anti-flow airgap between the reaction vessel and bottom chamber (which contains the SPE sorbent). This airgap prevents liquid from entering the sorbent chamber until positive pressure is applied. The empty upper chamber of the NBE column can be used independently of the lower chamber as a vessel for sample preparation. This upper chamber is widely used for assays utilizing enzymatic hydrolysis prior to SPE. The aim of these experiments is to demonstrate similar benefits when using NBE columns as the incubation vessel as well as the sample cleanup column for proteomics workflows, resulting in a simplified and fast sample processing procedure.

The Atlas SPE (Solid Phase Extraction) sorbent is a hydrophilic-lipophilic balanced (HLB) polymeric sorbent, which is an N-vinylpyrrolidone-divinylbenzene co-polymer. Atlas provides affinity for both polar and nonpolar analytes that allow the user to extract a variety of samples with ease. The divinylbenzene lipophilic backbone provides hydrophobic selectivity while the pyrrolidone hydrophilic chemistry will help increase interactions with polar functional groups of the analyte.

Bottom-up proteomics is widely used for protein identification and post-translational modification analysis. In this approach, proteins are first digested with an enzyme into peptides, and then analyzed by mass spectrometry. BSA was selected because of its common use as a test protein when developing bottom-up proteomic workflows.

MATERIALS AND METHODS

Protein Digestion and SPE Method

An in-solution tryptic digest of BSA was performed prior to SPE clean up. The digestion was carried out within the reaction vessel of the SPE column before de-salting the digested peptides utilizing the Atlas SPE sorbent. A comparison digestion was performed in a separate reaction tube and then transferred to the Atlas SPE for de-salting. Three technical replicate samples were used to evaluate the workflows, for both the on-column and off-column digestion experiments, as shown in Figure 3.

BSA protein (100 ug) was resuspended in 8M urea/50 mM ammonium bicarbonate solution. Disulfide bonds were reduced by the addition of TCEP for a final concentration of 5mM, and incubated at 37 °C for 30 minutes. After cooling at room temperature for 5 minutes, cysteines were alkylated by the addition of 200 mM iodoacetamide and an incubation in the dark, at room temperature for 30 minutes. The samples were then diluted to 1M urea, with the addition of 50 mM ammonium bicarbonate. Promega Sequencing Grade Modified Trypsin was added in an enzyme:protein ratio of 1:100, for the enzymatic digestion, and then incubated at 37 °C overnight. The digestion was stopped by the addition of 10% formic acid. Samples were then de-salted by the Atlas SPE sorbent in NBE columns (P/N 30174239, Table 1), utilizing the positive pressure and solvent dispensing of the Tecan Resolvex® A200[1] instrument. Compressed air supply to the Resolvex A200 was set to 80 psi and positive pressure was applied to the SPE columns for sample loading.

For the samples digested in the reaction vessel of the NBE SPE column (on-column), the reaction mixture was loaded onto the SPE sorbent bed by applying positive pressure to the SPE columns. The Atlas SPE sorbent required no pre-conditioning steps. For the samples that were digested in a reaction tube (off-column), the reaction mixture was manually transferred to the SPE columns and then subjected to positive pressure. The same Resolvex A200 SPE protocol was performed on both the on-column and off-column digestion experiments, for cleanup.

The samples were washed twice with 800 µL of 0.5% acetic acid. Peptide elution was achieved using 100 µL of 40% acetonitrile with 0.5% acetic acid, followed by 100 µL of 60% acetonitrile with 0.5% acetic acid. The combined eluates were dried down at 40 °C, under a nitrogen stream, for 30 minutes. Samples were reconstituted in 100 µL of 95:5 water:acetonitrile with 0.1% formic acid and analyzed by LC-MS/MS. SPE protocol parameters are outlined in Table 2.

Figure 1: Tecan Resolvex A200

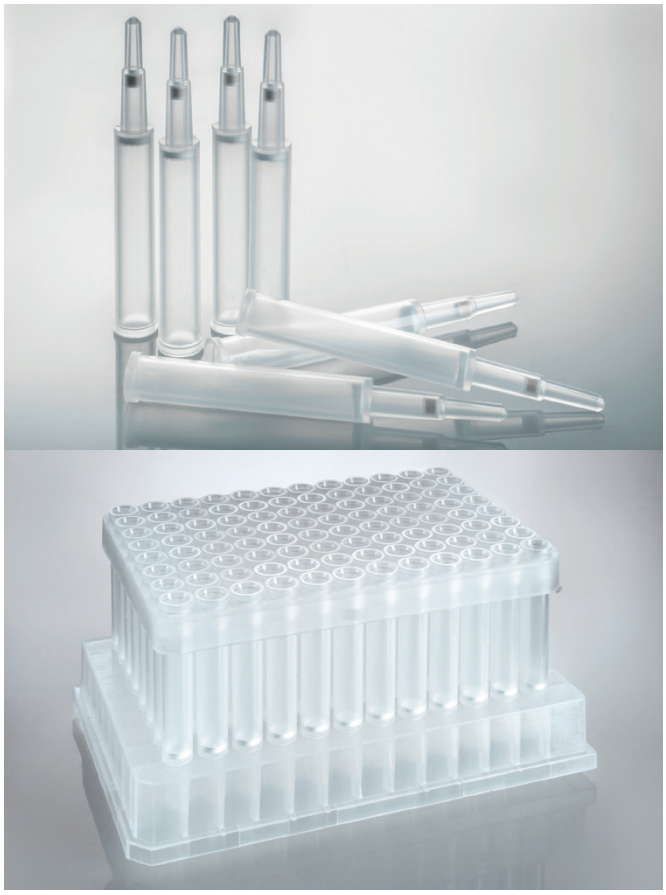


Figure 2: Tecan Narrow Bore Extraction SPE columns

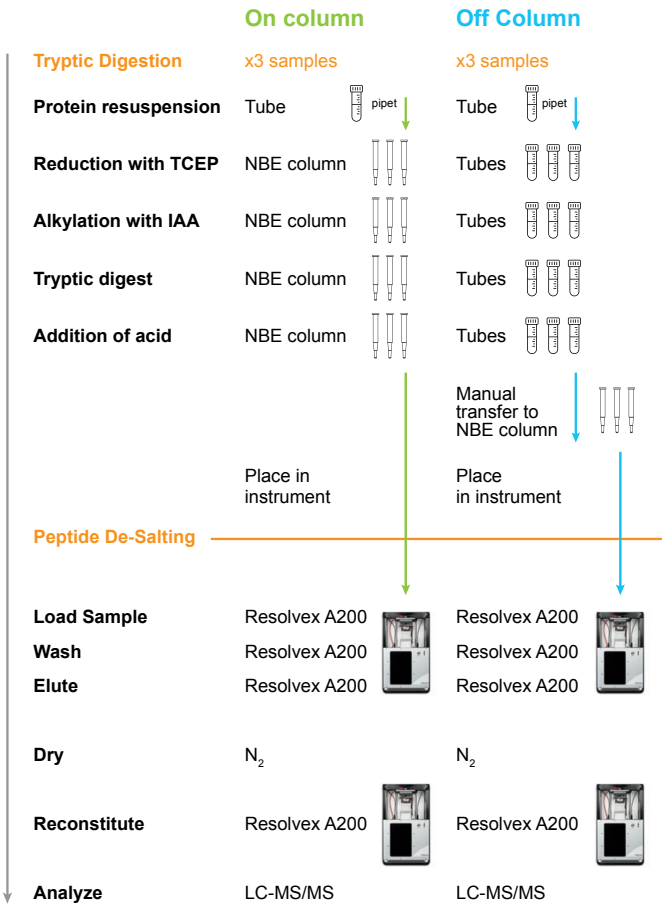


Figure 3: On-Column and off-Column tryptic digest and Narrow Bore Extraction SPE clean up workflows

Part Number	Format	Material Description
30174239	NBE 1mL	COLUMN NBE ATLAS 5MG _96/PK
30174237	NBE 96 Well Plate	PLATE NBE ATLAS 96 WELL 5MG _1/EA

Table 1: Atlas Narrow Bore Extraction SPE part numbers and formats.

Step	Description	A200 Pressure Profile
Load Sample	reaction mixture in column	High, 8%, 45 sec.
Wash	2X 800µL water with 0.5% acetic acid	High, 5%, 90 sec.
Elution	1) 100µL 40% acetonitrile with 0.5% acetic acid 2) 100µL 60% acetonitrile with 0.5% acetic acid	1) High, 5%, 30 sec. 2) High, 5%, 30 sec.
Dry Down	N_2, 40 °C, 30 min.	
Reconstitute	100µL 95:5 water:acetonitrile with 0.1% formic acid	

Table 2: Atlas Narrow Bore Extraction SPE protocol parameters

LC-MS/MS Conditions

Reversed-phase binary gradient chromatography was utilized for the separation of the BSA peptides. The summary of HPLC conditions is listed in Table 3. The overall method cycle time was 28 minutes.

LC Conditions		Gradient Conditions		
Column	Agilent Zorbax RRHD 300SB-C18, 2.1 x 150 mm, 1.8 µm	**Time**	**%A**	**%B**
Mobile Phase A	0.1% formic acid (aqueous)	0	97	3
Mobile Phase A	0.1% formic acid in acetonitrile	1	97	3
Column Temp.	55 °C	17	40	60
Autosampler Temp.	15 °C	17.5	0	100
Needle Wash	50:25:25 isopropanol: methanol:water	22	0	100
Injection Volume	1 µL	22.5	97	3
Flow Rate	0.3 mL/min	28	97	3

Table 3: HPLC conditions

Mass spectra were obtained for the selected BSA peptides, utilizing the Sciex ZenoTOF 7600 System in MRM-HR mode. Precursor masses were generated from Skyline (Ver. 21.2.565). The transition settings in Skyline were selected for 2+ and 3+ precursor charges and b and y fragment ions. The selected BSA peptides are shown in Figure 4. Precursor masses were used in the MRM-HR method, with the MS/MS set to a TOF range of 150-1500 Da. The general mass spectrometer parameters used are listed in Table 4. The selected fragments for each peptide were extracted from the data, using Sciex OS Analytics (Ver. 2.2.0.5738). Peptide and fragment information is shown in Table 5.

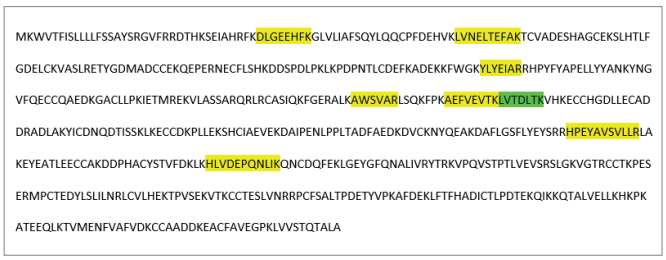


Figure 4: Selected peptides from bovine serum albumin

Parameter	Value
Source Temp.	500 °C
Ion Source Gas 1	55 psi
Ion Source Gas 2	60 psi
Curtain Gas	35 psi
CAD Gas	11 psi
Polarity	Positive
Spray Voltage	5500 V
Declustering Potential	80 V
Fragmentation	CID

Table 4: Sciex ZenoTOF 7600 mass spectrometry conditions

Peptide Sequence	Precursor ions (m/z)	Charge State	Product ions (m/z)	Ion Description	Collision energy (eV)
DLGEEHFK	325.4908	3+	415.1823 560.2827 430.2191 373.6770 431.2401	+3b4 +3y4 +3y7+2 +3y6+2 +3y3	18
LVNELTEFAK	582.3190	2+	951.4782 837.4353 708.3927 595.3086 670.3770	+2y8 +2y7 +2y6 +2y5 +2b6	28
YLYEIAR	464.2504	2+	764.4301 651.3461 488.2827	+2y6 +2y5 +2y4	28
AWSVAR	345.1901	2+	618.3358 432.2565	+2y5 +2y4	22
AEFVEVTK	461.7477	2+	851.4509 722.4083 575.3399 476.2715	+2y7 +2y6 +2y5 +2y4	27

Peptide Sequence	Precursor ions (m/z)	Charge State	Product ions (m/z)	Ion Description	Collision energy (eV)
LVTDLTK	395.2395	2+	676.3876 577.3192 476.2715	+2y6 +2y5 +2y4	20
HPEYAVSVLLR	428.5751	3+	757.4931 686.4559 587.3875 500.3555 527.2249 598.2620 697.3304 784.3624	+3y7 +3y6 +3y5 +3y4 +3b4 +3b5 +3b6 +3b7	20
HLVDEPQNLIK	653.3617	2+	841.4778 712.4352 691.3410 819.3995	+2y7 +2y6 +2b6 +2b7	30
	435.9102	3+	841.4778 712.4352 615.3824 487.3239 465.2456 594.2882 691.3410 819.3995	+3y7 +3y6 +3y5 +3y4 +3b4 +3b5 +3b6 +3b7	20

Table 5: Selected BSA Peptides: precursor ions, charge state, fragment ions, ion description and CE

DATA ANALYSIS

Data were reviewed using Sciex OS Analytics. The extracted fragment ion spectra for the selected BSA peptides were summed together in Sciex OS Analytics to generate a single composite peak area per peptide. Averages were calculated for the three technical replicates, for both the on-column and off-column experiments. The summed product ion responses from the MSMS spectrum from the on-column digestion were compared to the responses of the off-column digestion to evaluate the on-column digestion workflow. Representative chromatograms are shown in Figure 5.

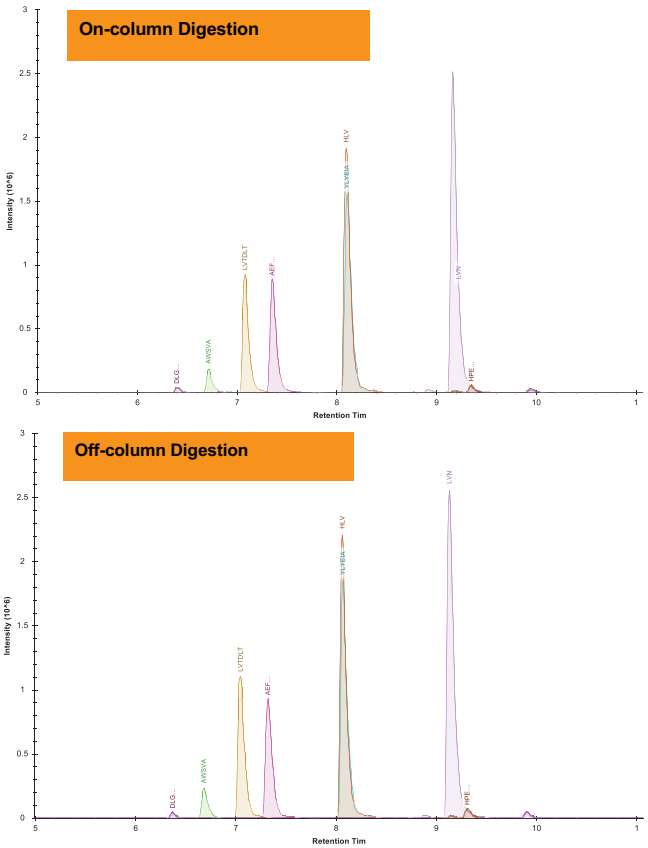


Figure 5: Chromatograms of peptide extraction, on-column and off-column.

RESULTS AND DISCUSSION

To evaluate the efficiency of the on-column digestion and Atlas SPE clean up workflow, peak areas of the selected BSA peptides were compared to that of the off-column digestion, as shown in Table 6. Three replicate digestion experiments were performed for both workflows, with three separate SPE extractions, without the use of an internal standard. By assessing the peak area responses, data was found to be comparable across both workflows. The %CV was calculated to evaluate reproducibility, represented in the error bars in Table 6. While specific peptide response varied between the on and off column digestions, the on-column workflow exhibited better precision and higher recovery for lower response peptides, DLGEEHFK and HPEYAVSVLLR, whereas higher variability was observed in the off-column experiments.

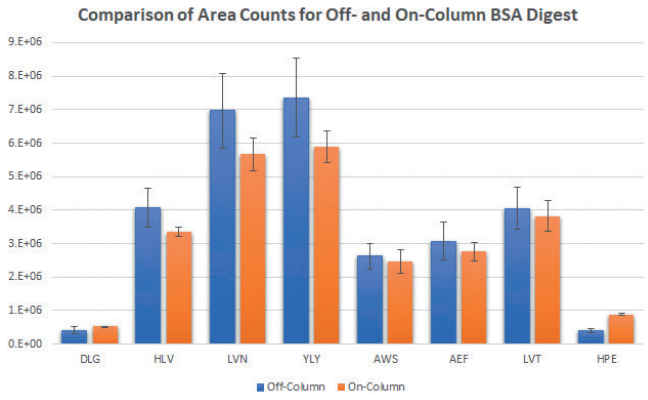


Table 6: Comparison of extracted BSA peptide peak areas for on-column and off-column tryptic digest workflows

CONCLUSION

The Tecan NBE columns and Atlas sorbents have been shown to provide good peptide recovery and desalting by SPE. Additionally, the ability to perform a tryptic digest within the column chamber by utilizing the unique dual chamber design and the anti-flow airgap of the column was demonstrated to be equivalent to standard digestion methods. By performing the digestion within the NBE column, contamination risk and sample loss is minimized by omitting extra manual pipetting steps needed to transfer the reaction mixture to the SPE columns.

Low volume eluates, made possible by the unique NBE format, allow for a significant reduction in dry times. Automating the SPE method using the Tecan Resolvex A200 streamlines the workflow, resulting in simple and efficient sample clean-up protocols.
By utilizing both the Tecan Resolvex A200 and the on-column digestion capabilities of the NBE columns, high-thoughput and automated methods for digestion and purification of proteolytic peptides can be performed.

REFERENCES

1) Refer to the link for instrument details. **https://diagnostics.tecan.com/positive-pressure-workstations?p=tab--3**

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