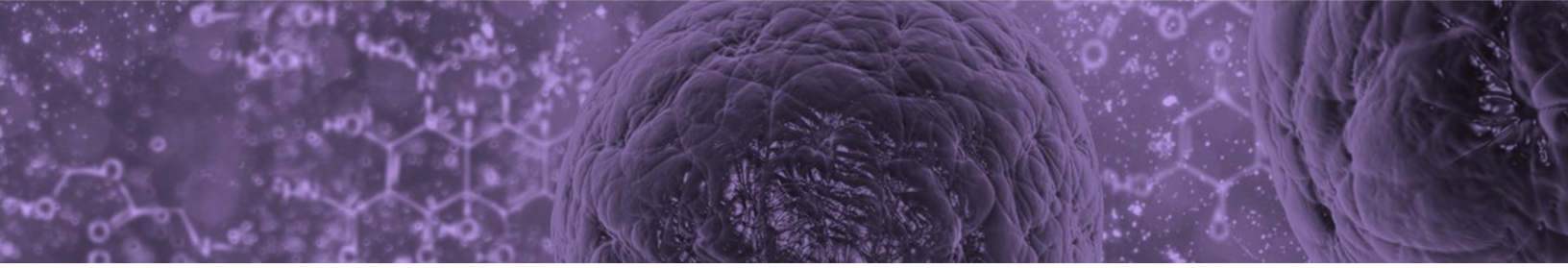

Application Note

nHance™ PCS

Next Generation Peptide Extraction

PEPTIDE CAPTURE AND RECOVERY FROM COMPLEX MATRIX

Keywords

Peptide enrichment, size exclusion, electrostatic interactions, charge-based affinity models, loading capacity, nanoporous surface, peptides, small proteins, 18th Annual Bioanalytical Land O Lakes Conference Poster Award recipient

Application Benefits

- Increased access to analytes in the range of 500 - 10,000 Dalton molecular weight range in complex biological samples
- Simple workflow, quick results
- Avoid common issues with critical targeting reagents

List of Abbreviations	
AA	Acetic Acid
ACN	Acetonitrile
FA	Formic Acid
IBC	Insulin Beta Chain
IS	Internal Standard
LC-MS	Liquid Chromatography-Mass Spectrometry
PA	Phosphoric Acid
PBS	Phosphate-buffered Saline

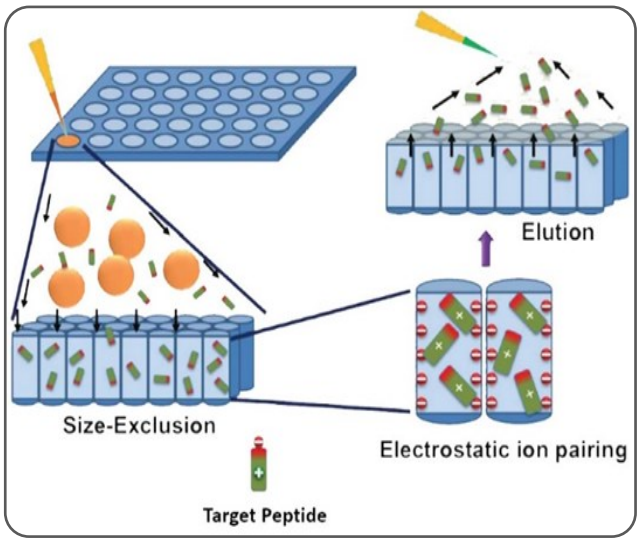

Figure 1: Schematic of the tandem effect of size exclusion and electrostatic ion pairing at play in the peptide capture achieved with the nHance™ 96-well plate.

Sample Preparation Workflow
Sample Pre-Treatment (Optional)
As needed (peptide specific. Refer to WFPCS1001)
Loading
Add and cover 20µL-100µL of sample solution to each well
Incubate 30 mins at room temp on slow-moving shaker/ rotation table
Use vacuum aspiration to remove contents from wells
Wash
Add 50µL washing solution to each well
Aspiration to remove washing solution from wells and repeat four (4) more times
Elution
Add eluent solution to each well
Pipet up and down 30 times over 30 seconds and withdraw eluent solution for analysis
Samples ready for LCMS

Figure 2: Protocol for using the nHance™ PCS plates. The pH modifier and the eluent are generated with the isoelectric point of the target peptide(s) taken into consideration.

INTRODUCTION

The industry standard for the quantification and characterization of large molecules such as peptides and proteins in complex biological matrices are ligand-binding assays (LBA) such as enzyme-linked immunosorbent assays (ELISA). An LBA requires targeting reagents that bind to specific structural regions of a molecule. Affinity based strategies can be very effective however, for some applications, the utility of an LBA is not well suited since the capturing reagent may not recognize subtle differences in peptide structures (e.g. phosphorylation, glycosylation,etc.).

To address this and other analytical needs, the biopharma industry adapted liquid chromatography mass spectrometry (LC-MS) as a complementary analytical tool. This technology provides specificity at the "detector-level" to elucidate the small but pivotal modifications. As such, analytical scientists can monitor multiple analogues and catabolites in parallel. Although mass spectrometry can address the specificity requirements for peptide/protein characterization and quantification, a protein/ peptide specific sample cleanup tool is still required when analyzing complex samples (blood, urine, serum, etc.). Approaches such as solid phase extraction (SPE) and immunoaffinity capture have been developed and their utility demonstrated. Although effective, these approaches can require complex pretreatment, lengthy method development, immunoaffinity based reagents, and are labor intensive and costly assays.

Agnostic to any bioanalytical approach (LCMS, ELISA, etc.), this nHance™ PCS device, a peptide enrichment device, provides increased (up to 5-fold greater) access to analytes in the range of 500 - 10,000 Dalton molecular weight range in complex biological samples. The nHnace™ PCS device accomplishes this through size exclusion and electrostatic interactions between the nHance™ PCS nanoporous surface and the peptide(s) of interest in approximately 30 minutes.

METHOD

The nHance™ Peptide Capture System is a revolutionary, patented nanotechnology developed specifically for peptide sample preparation and quantification. It eliminates many of the cumbersome steps associated with other approaches. The nanoporous coating at the heart of this system retains peptides within its nanopores based on size, electrical charge and retention time (see Figure 1). Unlike conventional sample preparation technologies on the market today, the peptides are first captured and then subsequently released after washing, in a quick and seamless elution step for subsequent LC-MS analysis (as outlined in Figure 2). It is truly a capture system for peptides and not a flow-through system based on retention time. As such, this approach allows sufficient time for both the size exclusion and electrostatic mechanisms of action to be fully utilized. Unwanted larger peptides and proteins and debris are removed during the washing procedures. Peptides of interest are extracted for subsequent identification and quantification absent of interference from the complex matrix.

In addition to the electrostatic and size exclusion properties, it is also critical to evaluate and understand the loading capacity of the nHance™ PCS device. The loading capacity of the nHance™ PCS was evaluated through the use of a representative peptide, insulin beta chain (IBC). Known concentrations of IBC were spiked into surrogate matrix (3% BSA in PBS), 100% serum, and serum at various dilution factors. The samples were then pretreated with acid and loaded onto the nHance™ PCS device. The IBC peptide was extracted as described in Figure 2 and the resulting samples were analyzed via LC-MS/MS as outlined in Table 1. Intact insulin was used as the internal standard to compensate for instrumental variability as shown in Figure 3.

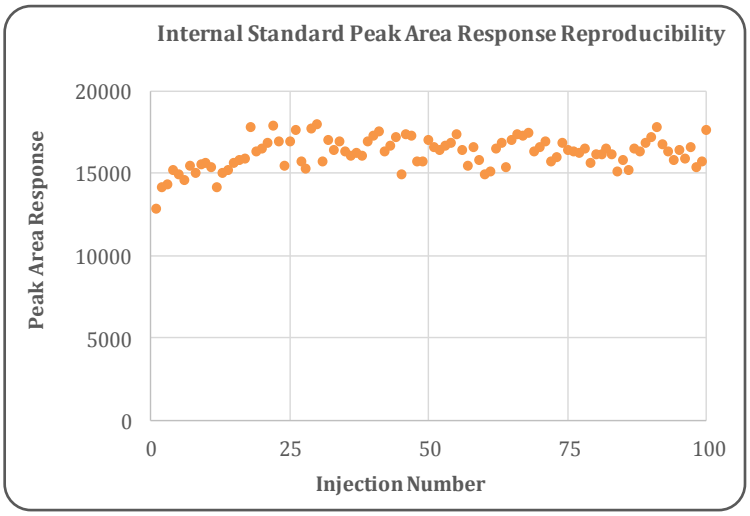

Figure 3: Experimental Reproducibility and Robustness

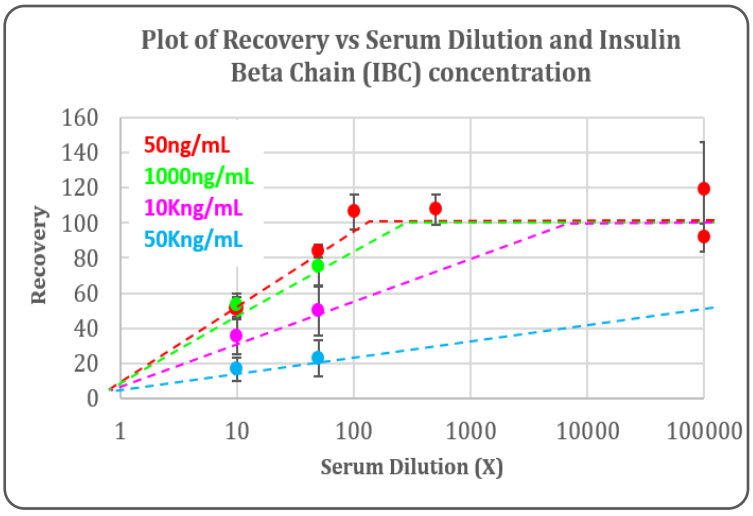

Figure 4: Plot of Recovery vs Serum Dilution and insulin beta chain (IBC) concentration. The maximum total loading (serum + peptide) that defines the operating point for 100% effective recovery for insulin beta chain (IBC) is shown here at 4 concentrations.

General	
Analyte	Bovine insulin B chain (Sigma Aldrich)
Internal Standard	Human intact insulin (Sigma Aldrich)
Surrogate Matrix	3% BSA in 1x PBS buffer
Complex Matrix	Human Serum (BioreclamationIVT)
Sample Pretreatment Paradigm	1% PA in water:ACN / 95:5 (v:v)
Sample volume	50 µL (treated samples)

Chromatography Settings							
Column type	Waters CORTECS C18 2.1x50 mm, 2.7 mm						
Column switching	0.8-2.4 min to mass spec, else to waste						
Column heater / oven temperature	40°C						
Mobile phase A	Water:1M AA:FA / 100:1:0.1 (v:v:v)						
Mobile phase B	ACN:Water: 1M AA:FA / 90:9:1:0.1 (v:v:v:v)						
Program	Gradient						
Time (min)	0	0.2	1.8	1.9	2.5	2.51	3.0
%B	20	20	50	95	95	20	20
Autoinjector Temperature	4°C						
Autoinjector wash solvent R0	ACN:Water:FA @ 20:80:0.1 (v:v)						
Autoinjector Wash solvent R3	ACN:Methanol:2-Propanol:FA @ 30:30:40:0.1 (v:v:v:v)						
Flow rate	0.5 mL/min						
Analysis time	~3.5 min						
Injection volume	10 mL						
Retention time	Insulin B-chain= ~1.53 min						
	Intact insulin (IS)= ~1.50 min						

Mass Spectrometer Settings (Recommended Values)	
Instrument	AB Sciex 6500
Source Temperature (TEM)	500°C
Collision Gas (CAD)	12 psig N_2
Curtain Gas (CUR)	20 psig N_2
Ion Source Gas 1 (GS1)	80 psig N_2
Ion Source Gas 2 (GS2)	80 psig N_2
Ion Spray Voltage (IS)	5500 V
Entrance Potential (EP)	10 V
Scan duration	3.0 min

Conditions for Analyte and IS		
	Insulin B Chain	**Intact Insulin (IS)**
Ionization Mode	TIS+	TIS+
Dwell Time (msec)	100	100
Declustering Potential (V)	120	120
Collision Energy (eV)	35	45
Collision Exit Potential (V)	10	10
Transition (m/Z)	874.8 —> 637.1	1162.5 —> 1391

Table 1: Analytical Method

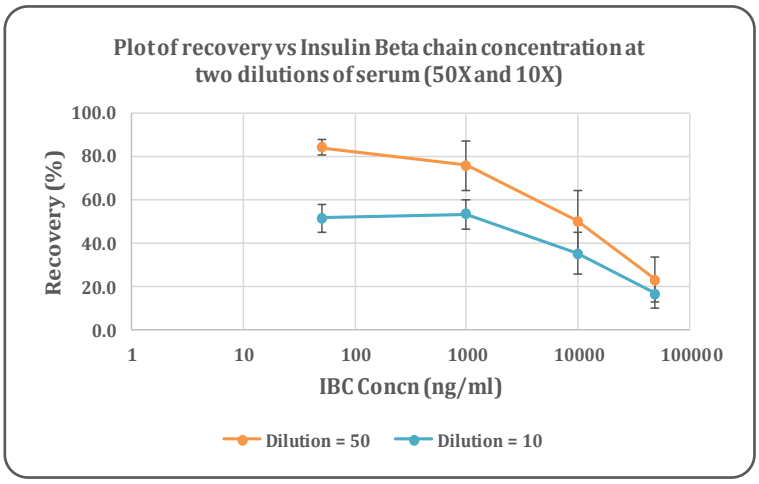

Figure 5: At a simple 10X dilution of serum, recovery is improved to 50% for a 50 ng/mL sample. Note that by equating the effective loading (at the same 50% Recovery) for the 10% serum dilution with 1 µg/mL IBC and the 50% serum dilution with 10 µg/mL IBC, the effective serum loading, L_{SERUM}, (in IBC equivalent concentration) may be obtained:

$$L_{SERUM}\ ,\ 10 + 1\ \mu g/mL = L_{SERUM}\ ,\ 50 + 10\ \mu g/mL \ \text{—>}\ L_{SERUM} = 112.5\ \mu g/mL$$

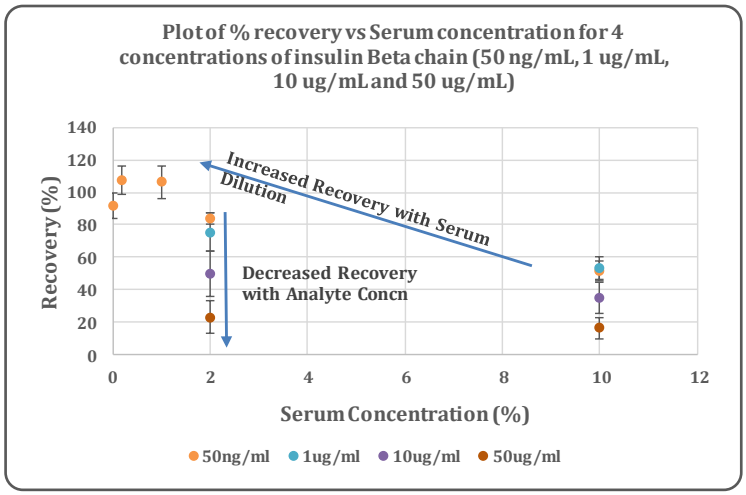

Figure 6: Note the increased recovery with Serum dilution and decreased recovery with increased IBC concentration.

RESULTS

The maximum total loading (serum + peptide) that defines the operating point for 100% effective recovery of the insulin beta chain (IBC) was identified by using an experimental matrix incorporating a minimum required dilution (MRD) approach crossed with target analyte spiking at increasing concentrations and analyzing the samples using LC-MS/MS (see Figure 4). For target analyte concentrations below 100 ng/mL, a simple 10X dilution of the serum matrix assured effective recoveries of >50% (Figure 5). Analysis of the recovery loss in the serum dilutions associated with various concentrations of the target analyte, including artificially high concentrations (1, 10, and 50 µg/mL), it was possible to establish an approximate equivalent loading for serum at about 100 µg/mL for IBC-like peptides. Loading capacity, as measured by %recovery of IBC, was observed to increase as dilution of matrix increased and decrease as IBC concentration increased (see Figure 6). Because the ~40 nm pores of the nHance™ PCS device remove proteins and other large serum components by size exclusion, this equivalent loading makes sense for the anticipated residual serum content that is made up of only small molecules.

CONCLUSION

The nHance™ PCS device is shown to be a multi-dimensional sample clean-up and small protein/ peptide enrichment tool. In conjunction with the charge-based affinity models for the nHance™ PCS interactions, this application note illustrates the importance of surface area models that can be combined with these new loading response models to provide a reliable starting point for assay method development.

In addition to being a highly flexible and tunable sample clean up and enrichment tool for peptides and proteins, the nHance™ PCS device has a greatly simplified workflow, is cost effective, requires no expensive reagents and further supports high-throughput analysis. The inherent innovation in this multi-dimensional approach has earned the founding scientists an award at the School of Pharmacy, University of Wisconsin -Madison at the 18th Annual Bioanalytical Conference.

FUTURE WORK

Please look for the follow up to this application note further demonstrating the size exclusion properties of the systems as well as the importance of understanding how non-specific binding of endogenous proteins can negatively affect the detection and quantitation of the peptide of interest. But no worries, nHance™ PCS device is here to help.

Application Support

Contact us today at appsupport@nhancetechnology.com to see how nHance™ PCS can simplify your peptide and protein sample preparation for LC/MS analysis. Our application team of seasoned bioanalytical chemists have significant experience in the use of LC/MS techniques for the detection and quantification of peptides and proteins.

Contact Information

Contact us today at info@nhancetechnology.com or visit us at www.nhancetechnology.com.

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