

## APPLICATION NOTE

## Preparing Triton-insoluble Membrane Proteins for iTRAQ Labeling and MALDI-MS/MS Analysis



PPS Silent® Surfactant

### INTRODUCTION

Recent advances in proteomics methods permit quantification of protein expression profiles between different experimental conditions. One such method is isobaric tagging followed by MS/MS analysis for relative and absolute peptide quantification. This quantification technology can be applied to the study of proteomics phenomena such as differential patterns of protein abundance associated with disease states, and up- and down-regulation of protein expression associated with experimentally altered phenotypes.

The complexity of the proteome requires subcellular fractionation procedures in order to enrich proteins of interest prior to the application of modern analysis methods such as isobaric tagging and tandem MS. However, techniques for enriching protein populations are largely biased in favor of soluble proteins. Researchers who wish to study less-soluble proteins must often use vigorous extraction procedures in order to disassociate proteins of interest from cell membranes, followed by additional treatments with aggressive detergents or other solubilizing agents to enable trypsin digestion. Unfortunately, the most effective extraction and solubilizing agents are often either destructive to the proteins of interest, or incompatible with downstream analysis procedures such as isobaric tagging, capillary chromatography, and mass spectrometry.

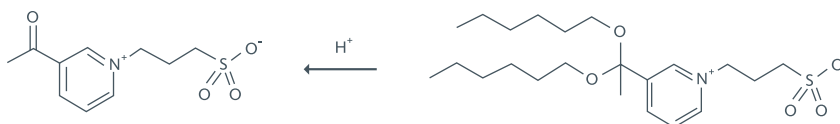
Among the less-soluble proteins of interest to researchers are those found within, or tightly associated with, pre- and post-synaptic membranes facing the synaptic junction. Proteins such as these are sometimes called “Triton-insoluble” in reference to their resistance to solubilization by the nonionic detergent Triton® X-100.

Joachim Uys, PhD, a neuroproteomics researcher in the Department of Neurosciences at the Medical University of South Carolina, studies the plasticity of the synaptic neuroprotein response to cocaine addiction in rats. Dr. Uys has developed a sample preparation protocol for iTRAQ 8-plex isobaric peptide tagging of the 0.5% Triton X-100 surfactant-insoluble fraction of the nucleus accumbens of cocaine self-administering rats. The protocol enables Dr. Uys to quantify relative expression differences among certain proteins of interest in the Triton-insoluble fraction.

This protocol uses PPS Silent® Surfactant, an aggressive, acid-cleavable detergent that enables recovery and solubilization of proteins in the Triton-insoluble fraction, and high-sensitivity mass spectrometry. Unhydrolyzed PPS in the sample retains its zwitterionic character and is removed during subsequent sample fractionation by strong cation exchange (SCX) HPLC.

## BACKGROUND

PPS Silent Surfactant (3-[3-(1,1-bisalkoxyethyl)pyridin-1-yl]propane-1-sulfonate) is a zwitterionic, cleavable surfactant capable of disrupting cell membranes and solubilizing proteins. At low pH, PPS hydrolyzes rapidly into cleavage products that have no remaining surfactant properties and do not interfere with mass spectrometry detection of proteins. When used to prepare biological samples for mass spectrometry, PPS Silent Surfactant is easily removed by acid hydrolysis or by cation-exchange column purification prior to sample ionization.



## OBJECTIVE

Develop an isobaric tagging protocol for Triton-insoluble brain tissue proteins from cocaine self-administering animals. Recovered, solubilized proteins must be compatible with trypsin digestion, iTRAQ labeling, SCX fractionation and subsequent nanoLC-MALDI-MS/MS analysis.

## PROTOCOL

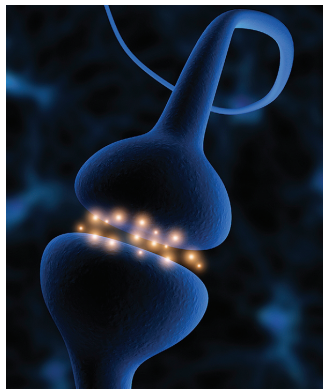
### SUBCELLULAR FRACTIONATION AND ENRICHMENT

Male rats were trained to self-administer cocaine for 10 days followed by 14 days of extinction training. Nucleus accumbens tissue samples of the cocaine self-administering animals were homogenized in 0.32M sucrose, 10mM HEPES (pH 7.4) and protease inhibitors. Nuclear and unhomogenized particles were removed by low speed centrifugation (900 x g for 10 min). The supernatant was then centrifuged at 12000 x g for 20 min. The resulting supernatant contained the cytosolic proteins and was removed. The pellet was resuspended in 1mM EDTA and 4mM HEPES (pH 7.4) and centrifuged at 12000 x g for 20 min and repeated once more to obtain a crude membrane fraction. The supernatant was removed and the pellet resuspended in 0.5% Triton X-100 in PBS, rotated for 15 min at 4°C and centrifuged at 12000 x g for 20 min. The resulting pellet contained enriched postsynaptic density and other detergent insoluble proteins. The pellet was solubilized in 1% PPS Silent surfactant in 500mM TEAB buffer and protein concentration measured using the BCA assay (Pierce). Each sample (50µg protein) was reduced in 5mM TCEP, alkylated with MMTS and digested overnight in trypsin (1:20, enzyme:protein ratio; Applied Biosystems). The resultant tryptic peptides were labeled with iTRAQ 8-plex reagents for 2 hours at room temperature and the contents combined.

### STRONG CATIONIC-EXCHANGE CHROMATOGRAPHY (SCX)

#### FRACTIONATION

The combined sample was acidified with 1% TFA and subjected to SCX fractionation on a Waters 600-MS HPLC system connected to a Waters 484-MS UV detector. A PolySULFOETHYL A™ column (200 x 2.1 mm I.D.,



5  $\mu\text{m}$ , 200  $\text{\AA}$ ) (PolyLC Inc., Columbia, MD) was used. Solvent A was 10 mM  $\text{KH}_2\text{PO}_4$ , 25% acetonitrile (ACN), pH 2.7–3.0; solvent B was similar to A but with the addition of 0.5 M KCl. A 40 minute gradient from 10% to 50% B, followed by 40 minutes at 50% B provided acceptable separation of the peptides. The flow rate used was 250  $\mu\text{L}/\text{minute}$ , and the elution of peptides was monitored by UV at 220 nm. Fractions were collected at 5 minute intervals. The fractions were completely dried in a SpeedVac Concentrator, and stored at  $-20\text{ }^\circ\text{C}$  until further fractionation by reversed phase HPLC.

#### REVERSED-PHASE CHROMATOGRAPHY (RP-HPLC) FRACTIONATION

15 SCX fractions were selected based on the UV analysis for further fractionation by RP-HPLC on an Ultimate-Switchos-Probot system (LC Packings, Sunnyvale, CA). The peptides were first loaded using the Switchos system on a C18 pre-column cartridge (5 mm  $\times$  300  $\mu\text{m}$  I.D. packed with PepMap 100, 5  $\mu\text{m}$ , 100  $\text{\AA}$  - (LC Packings)) using 2% ACN, 0.1% trifluoroacetic acid (TFA) in water at 40  $\mu\text{L}/\text{minute}$ . After 20 minutes of desalting, the peptides were eluted from the pre-column onto a C18 (150 mm  $\times$  100  $\mu\text{m}$  I.D., 3  $\mu\text{m}$ , 300  $\text{\AA}$ ) column (Micro-Tech Scientific, Vista, CA) using the Ultimate system at 600 nL/minute. Solvent A was 2% ACN, 0.1% TFA in water; solvent B was 85% ACN, 5% 2-propanol, 0.1% TFA. A 50 minute gradient from 12% B to 41% B at 600 nL/minute was used. Peptide elution was monitored at 214 nm.

#### MALDI FRACTION COLLECTION

The eluate from the reversed-phase HPLC separation was mixed at a 1:2 (eluant: matrix) ratio with an 8 mg/ml solution of  $\alpha$ -cyano-4-hydroxy-cinnamic acid (Bruker Daltonics, Germany) in 70% ACN, 0.1% TFA, and 0.15 mg/ml ammonium citrate being continuously delivered from the syringe pump of the Probot system. The mixture was spotted on stainless steel MALDI plates (ABI) in a 12  $\times$  33 pattern, every 10 seconds during the peptide elution phase of the RP-HPLC. Eight mass calibration spots were manually pipetted on the perimeter of the plate, and two mass accuracy verification spots were manually placed on the top center and bottom center of each run. Typically, 4 HPLC runs were collected per plate. The plates were kept in the dark until MS and MS/MS analysis.

#### MASS SPECTROMETRY ANALYSIS

MS and MS/MS analyses were performed on a 4800 Proteomics Analyzer matrix assisted laser desorption ionization (MALDI) time of flight (TOF)-TOF mass spectrometer equipped with the 4000 Series Explorer (Version 3.5) data acquisition software (Applied Biosystems).

#### PEPTIDE AND PROTEIN IDENTIFICATION

All MS/MS spectra from both iTRAQ experiments were searched using Protein Pilot 2.0 software with the Paragon search algorithm (Applied Biosystems). The protein sequence database used was the RefSeq protein sequence database for *Rattus Norvegicus* downloaded from the NCBI website (<ftp://ftp.ncbi.nih.gov/refseq>)

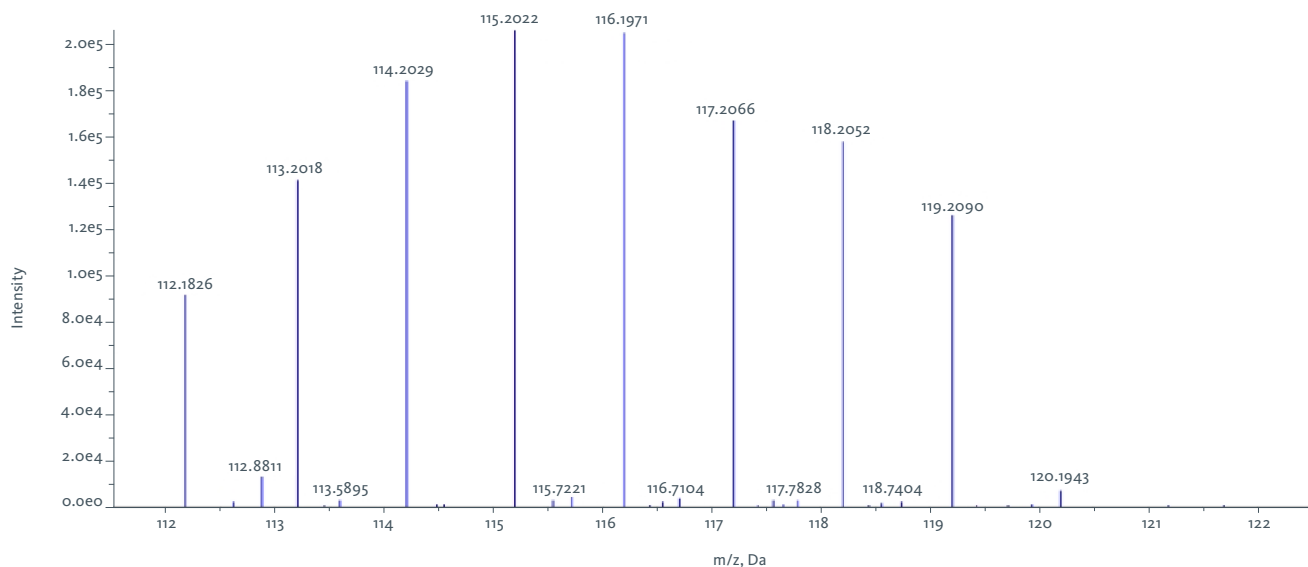
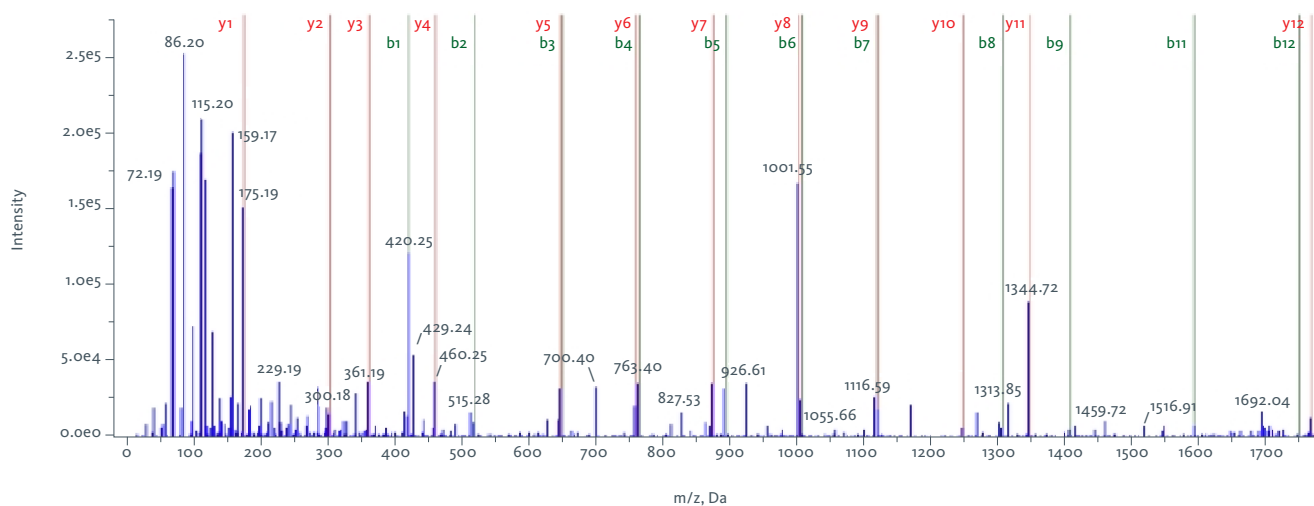
## RESULTS

## BETA SPECTRIN: DVEDEILWVGER

Residue	b	y
D	420.2396	1763.9117
V	519.3080	1344.6794
E	648.3506	1245.6110
D	763.3775	1116.5684
E	892.4201	1001.5415
I	1005.5042	872.4989
L	1118.5883	759.4148
W	1304.6676	646.3307
V	1403.7360	460.2514
G	1460.7574	361.1830
E	1589.8000	304.1615
R	1745.9011	175.1190

At a working concentration of 1%, zwitterionic PPS Silent Surfactant was sufficiently aggressive to recover and solubilize proteins of interest from Triton-insoluble fractions of nucleus accumbens tissues. PPS was easily removed by hydrolysis and SCX fractionation and therefore did not interfere with subsequent reversed-phase HPLC fractionation or mass spectrometry detection of proteins and isobaric tagging ions.

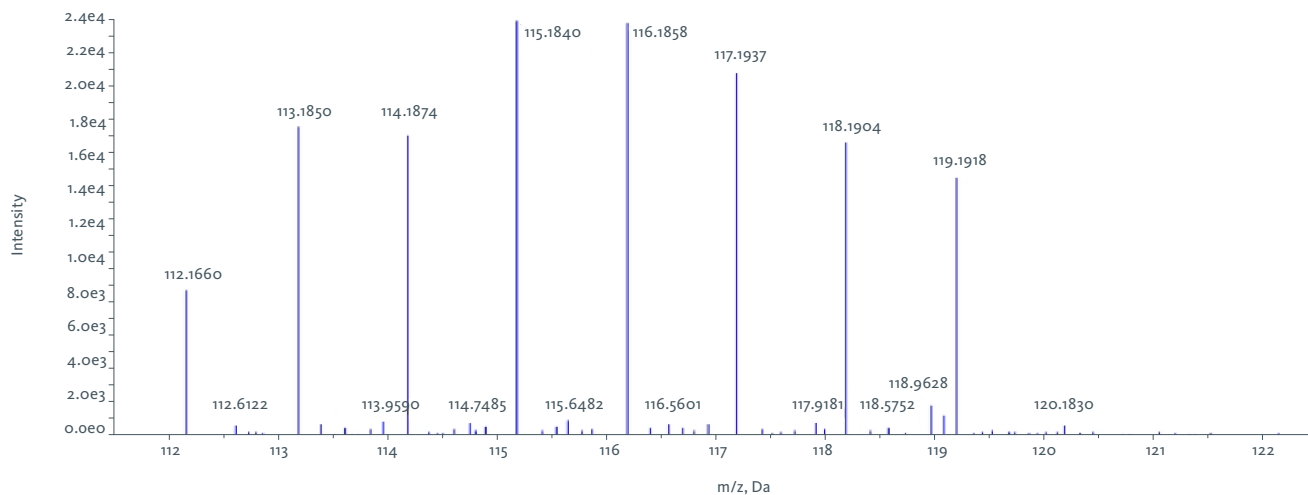
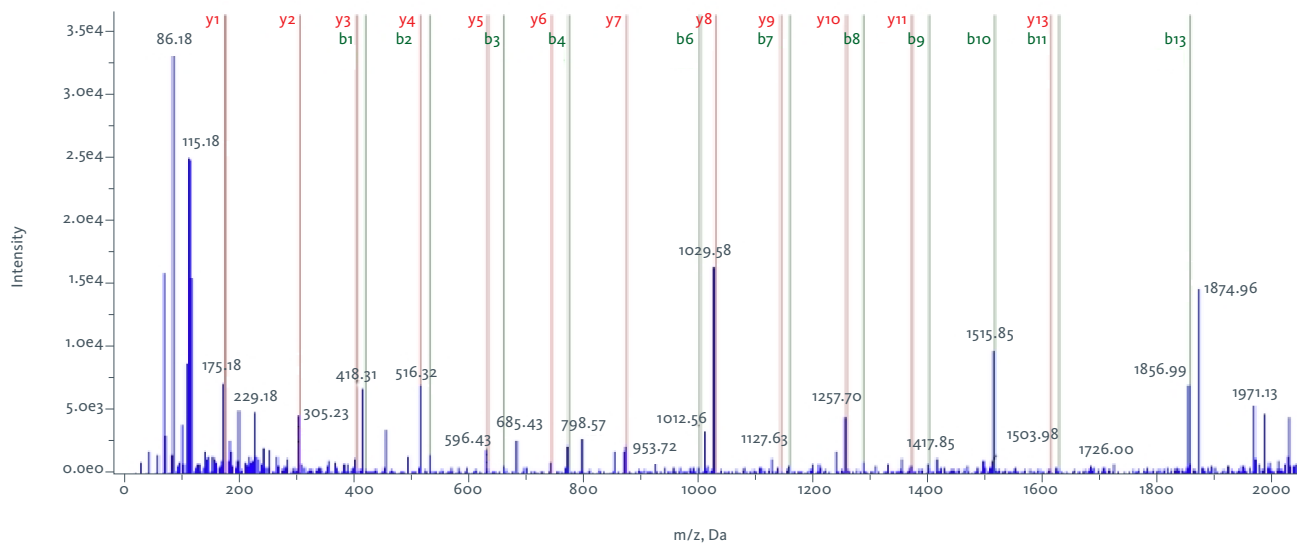
A total of 426 proteins were identified including membrane receptors, GluR2, mGluR3, scaffold proteins, Homer, SAP102, PSD-95, SynGAP, signaling proteins, CamKII alfa, beta and delta, PKA, extracellular matrix proteins: NCAM1 and presynaptic cytomatrix proteins, Piccolo.



Protein: Beta-Spectrin, size: 271 kDa, Beta-spectrin protein coverage of 55.6% was achieved. The peptide shown is DVEDEILWVGER. gi|61557085. Also shown are iTRAQ isobaric tagging ions, 113, 114, 115, 116, 117, 118, 119. iTRAQ ion 121 was not used.

**PICCOLO: ILQDIDRELDLVER**

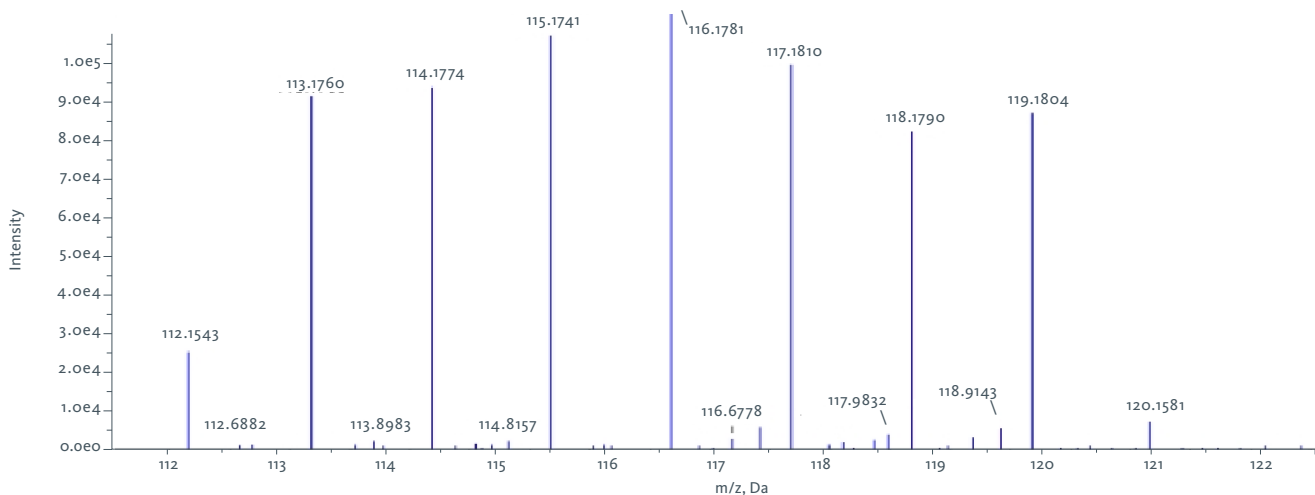
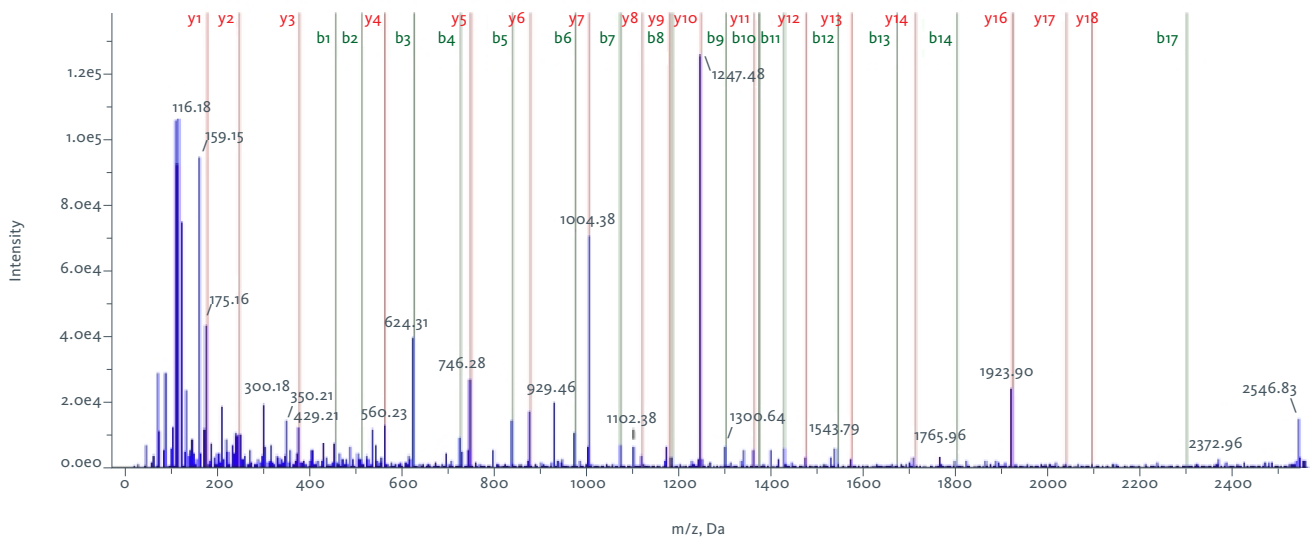
Residue	b	y
I	418.2967	2031.1387
L	531.3808	1613.8493
Q	659.4393	1500.7653
D	774.4663	1372.7067
I	887.5503	1257.6797
D	1002.5773	1144.5957
R	1158.6784	1029.5687
E	1287.7210	873.4676
L	1400.8051	744.4250
D	1515.8320	631.3410
L	1628.9161	516.3140
V	1727.9845	403.2300
E	1857.0271	304.1615
R	2013.1282	175.1190



Protein: Piccolo, size: 552 kDa, Piccolo protein coverage of 28.1% was achieved. The peptide shown is ILQDIDRELDLVER. [gi|10048483](#). Also shown are iTRAQ isobaric tagging ions 112–119.

**PSD-95: FGDVLHVIDAGDEEWQAR**

Residue	b	y
F	452.2810	2547.2570
G	509.3025	2095.9832
D	624.3295	2038.9617
V	723.3979	1923.9348
L	836.4819	1824.8664
H	973.5408	1711.7823
V	1072.6093	1574.7234
I	1185.6933	1475.6550
D	1300.7203	1362.5709
A	1371.7574	1247.5440
G	1428.7788	1176.5069
D	1543.8058	1119.4854
E	1672.8484	1004.4585
E	1801.8910	875.4159
W	1987.9703	746.3733



Protein: PSD-95, size: 95 kDa, PSD-95 protein coverage of 48.8% was observed. The peptide shown is VLHVIDAGDEEWQAR. gi|9665227. Also shown are iTRAQ isobaric tagging ions 112-119.

*"The proteins we study are tough to solubilize and digest, but PPS Silent Surfactant is strong enough to give us good digestions and recoveries. It is clear from our spectra that PPS Silent Surfactant does not interfere with subsequent labeling of peptides with iTRAQ."*



Joachim Uys, PhD  
Medical University of South Carolina  
Department of Neurosciences

## CONCLUSION

An aggressive detergent is required to enable recovery, solubilization, digestion, and analysis of hydrophobic Triton-insoluble proteins. Furthermore, the detergent used in this application must not interfere with the iTRAQ isobaric tagging protocol and must also be removable so that it does not contribute a high background signal to mass spectrometry readings. SDS is known to provide good recoveries of hydrophobic proteins, but is notorious for its interference with mass spectrometry analysis. Method development trials incorporating other denaturants such as guanidinium hydrochloride and urea were unsuccessful. PPS Silent Surfactant satisfied the surfactant reagent criteria for this application, enabling high recoveries of proteins of interest and the production of high-quality mass spectrometry data.

Significant advantages of using PPS with isobaric tagging are:

- 1) Excellent solubilization of 0.5% Triton X-100 detergent insoluble proteins, such as Bassoon and Piccolo, both of which exceed 400 kDa.
- 2) Proteins can be digested and tryptic peptides labeled with iTRAQ reagents in 1% PPS.
- 3) The zwitterionic properties of PPS allow it to be used in conjunction with SCX fractionation without prior removal (in contrast to anionic detergents such as Rapigest or SDS). The uncleaved PPS is removed by SCX prior to MALDI plate spotting.

## ACKNOWLEDGMENT

Data shown were produced at the Mass Spectrometry Facility at the Medical University of South Carolina.



protein  
discovery

Protein Discovery, Inc.  
418 S. Gay Street, Suite 203  
Knoxville, TN 37902  
www.proteindiscovery.com