Application

intelligent sample preparation

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µSPEed | Application Note 2018

µSPEed-Cxyl microreactor Cartridges – *in-situ* Trypsin immobilisation

Digests of Bovine Serum Albumin (BSA) and Cytochrome C in under 2 minutes

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INTRODUCTION

Appropriate sample preparation prior to analysis is crucial for efficient, accurate and precise measurement of target analytes for all analytical methods. Sample preparation is the most time-consuming step of analytical workflows, requiring significant consideration of chemical makeup of the analytes and their matrices.

Mass spectrometry is an increasingly viable alternative to immunoassays, but broad uptake is hampered by laborious and time-consuming sample preparation regimes. Quantification of proteins usually requires proteolytic digestion for production of smaller peptides which are amenable to targeted mass spectrometry analysis. This proteolytic digestion step is time consuming and often takes up to 24 hours. Identification and quantification of biomarkers is routine in the field of proteomics and are of increasing significance in clinical settings.

The introduction of Eprep's Sample Preparation Workstation offers an innovative alternative to out-dated analytical techniques, eliminating labour intensive processes to vastly increase precision and accuracy. This unit is designed for automated standard and sample dilution, and micro solid phase extraction (µSPE) that eliminates sample preparation bottle necks. The high pressure µSPEed Cartridges is a significant advancement over current SPE cartridges as 3 µm sorbents are packed into an 8 µl (4.2 mg) bed volume, providing enormous separation power and high concentration factors in µl volumes.

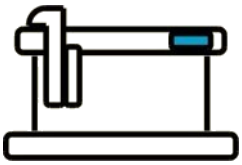
Eprep has developed customisable stationary phases for immobilised enzyme micro-reactors for digestion of proteins. This application note describes the use of ePrep's customisable µSPEed-Cxyl microreactor cartridges for ***rapid digestion*** at ambient temperature of bovine serum albumin (BSA, ~66 kDa) and cytochrome C (~12 kDa).[1] BSA is derived from cows and is the most common reference standard used in proteomic systems suitability and performance compliance checks. Cytochrome c is highly conserved across all species and is frequently used as a model protein for molecular evolution experiments.

METHODS

The µSPEed is a unique design of SPE cartridge for automated sample preparation in conjunction with the ePrep Sample Preparation Workstation (Figure 1). Typical operation involves loading a sample from a designated vial by drawing the sample into the syringe through the one-way check valve. The sample is loaded by reversing the

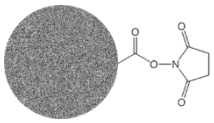
Figure 1: ePrep Sample Preparation Workstation and µSPEed cartridges with one-way check valves.

PROCEDURE



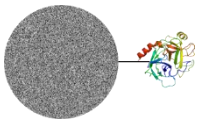
STEP 1 – Trypsin Immobilisation

µSPEed CONDITION:
200 µL @ 1000 µL/min Water x3



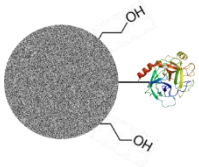
µSPEed EQUILIBRATE:
200 µL @ 500 µL/min
EDC/NHS recycled through x2

µSPEed WASH:
200 µL @ 1000 µL/min Water x2



µSPEed EQUILIBRATE:
200 µL @ 500 µL/min
Trypsin recycled through x2

µSPEed WASH:
200 µL @ 1000 µL/min Water x2

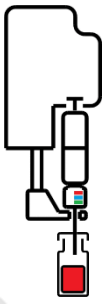


µSPEed EQUILIBRATE:
200 µL @ 500 µL/min
Ethanolamine recycled through x2

STEP 2 – Protein Digest

µSPEed EQUILIBRATE:
200 µL @ 1000 µL/min
50 mM Tris with 5 mM $CaCl_2$ x1
50 mM Tris with 500 µM $CaCl_2$ x3

uSPEed LOAD:
100 µL @ various flow rates
Protein flow through into a vial



uSPEed ELUTE:
100 µL @ 1000 µL/min
50 mM Tris with 5 mM $CaCl_2$ into the LC vial

Scheme 1: Digestion protocol for immobilised trypsin and protein flow through digestion.

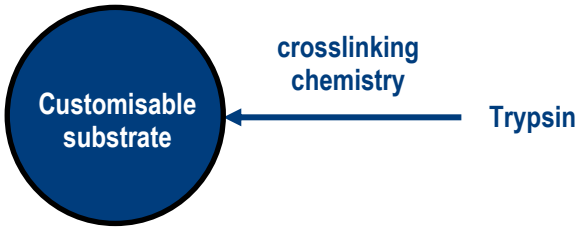
direction of flow by depression of the syringe, where the check valve is closed directing the sample to flow through the sorbent. The sample is eluted with an eluting solvent by repeating this process.

Solutions:

- 0.1 M EDC and 0.1 M NHS (1:1) in water
- 50 mM Tris buffer pH 8.0 with 500 µM $CaCl_2$
- 50 ng/µL trypsin* in water
- Digestion buffer: 50 mM Tris with 500 µM $CaCl_2$
- 10 ng/µL cytochrome c in digestion buffer
- 50 ng/µL reduced and alkylated BSA digestion buffer
- 100 mM ethanolamine pH 8.0

µSPEed Trypsin Digest Sequence:

STEP 1 – In-situ immobilisation of trypsin onto µSPEed-Cxyl cartridge



Trypsin was covalently immobilised in-situ onto µSPEed Cxyl cartridges by EDC/NHS chemistry. The material was dynamically end-capped with ethanolamine and conditioned to pH 8.0 with 50 mM Tris with 500 µM $CaCl_2$ for protein digestion.

STEP 2 – Protein digestion through the immobilised micro-reactor cartridge in under 2 MINUTES

The protein solution was cycled through the cartridge at various flow rates at ambient temperature, and the flow through collected into a vial for analysis. BSA (5 µg) and cytochrome c (1 µg) were loaded onto the cartridge for digestion according to the protocol shown in Scheme 1.

STEP 3 – Liquid chromatography time-of-flight mass spectrometry (LC-QTOF)

BSA and cytochrome c proteins and digestion mixtures were analysed by LC-QToF MS (Agilent Technologies 6510 and Waters Vion, Ion Mobility Quadruople time-of-flight mass spectrometer). Mass spectral data were correlated to protein sequences through the Waters Vion UNIFI Software.

* Trypsin concentration and loading can be optimised to minimise autolysis products.

Table 1: Chromatography and mass spectrometry parameters.

LC-MS conditions		
System	Agilent Technologies 6510	Vion IMS QToF MS
Column	Accucore C18+ column (100 x 2.1 mm, 1.5 µm)	Waters C18 (100 x 2.1 mm, 1.8 µm)
Flow rate	0.2 mL/min	0.3 mL/min
Mobile phase	A – Ultrapure water with 0.1% formic acid B – Acetonitrile with 0.1% formic acid	A – Ultrapure water with 0.1% formic acid B – Acetonitrile with 0.1% formic acid
Gradient	0 min: 5%B 5-25 min: Linear 5% to 60%, held for 5 minutes	0 min: 5%B 5-30 min: Linear 5% to 60%, held for 5 minutes
Run time	5 min pre-equilibration and 30 min	5 min pre-equilibration and 40 min
Column temperature	30 °C	30 °C
QToF MS parameters	Ionisation mode: Positive ion mode, ESI Vcap: 3500 V and drying gas flow of 5 L/min at 325 °C; Fragmentor voltage: 175 V Mass range: 400-2000 m/z	Ionisation mode: Positive ion mode, ESI Vcap: 2200 V and drying gas flow of 10 L/min at 120 °C Mass range: 350-1800 m/z

RESULTS AND DISCUSSION

A. Enzyme Immobilisation

The trypsin was immobilised on the cartridge in 5 minutes. µg quantities of trypsin were used for the immobilisation procedure to ensure saturation of the cartridge and high cartridge capacity. Minimal autolysis products of trypsin were detected after protein digests, which may be controlled via optimisation procedures depending on the required application.

B. LC-MS analyses of protein digests

Rapid digestion of BSA (5 µg) and cytochrome c (1 µg) were performed at ambient temperature. BSA was passed through the immobilised micro-reactor cartridge. Analysis of the digest by LC-MS showed excellent digestion efficiency with little or no residual BSA present (Figure 2).

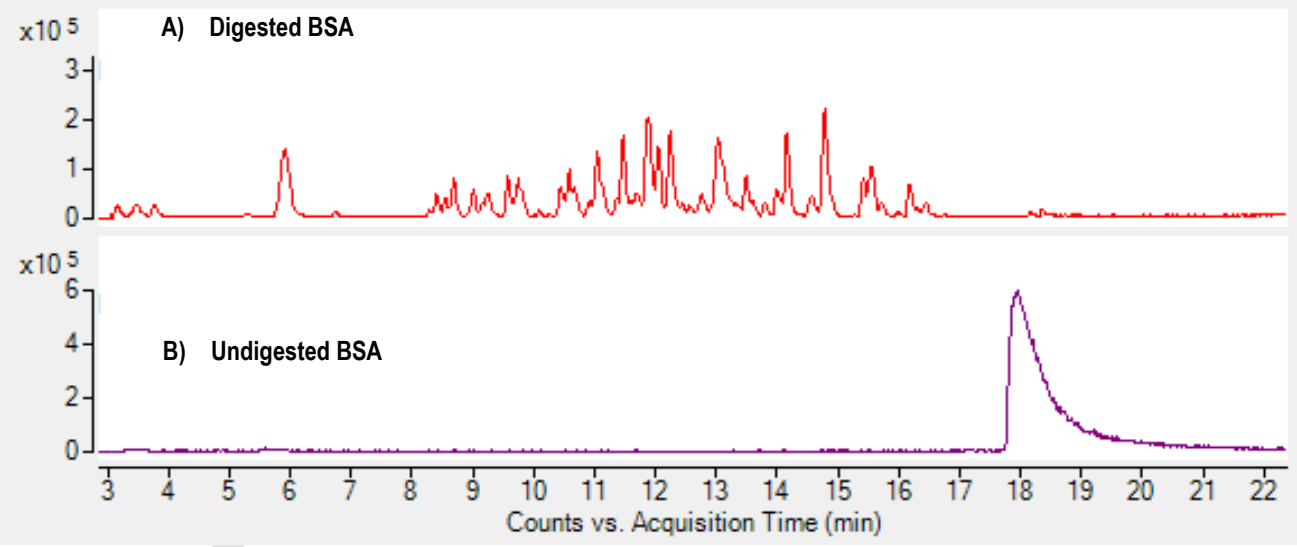


Figure 2: Total ion chromatograms (TIC) of A) BSA digestion (~40 pmol) using trypsin immobilised micro-reactor cartridge and B) Undigested BSA. Digestion time was 60 seconds (100 µL of protein at 400 µL/min cycled through the cartridge x4). Chromatograms represent ~7.5 pmol loading on column (Accucore C18+, 100 mm x 2.1 mm, 1.5 µm)

Similarly, cytochrome c was passed through the cartridge at ambient temperature with complete protein digestion in under 2 mins (Figure 3).

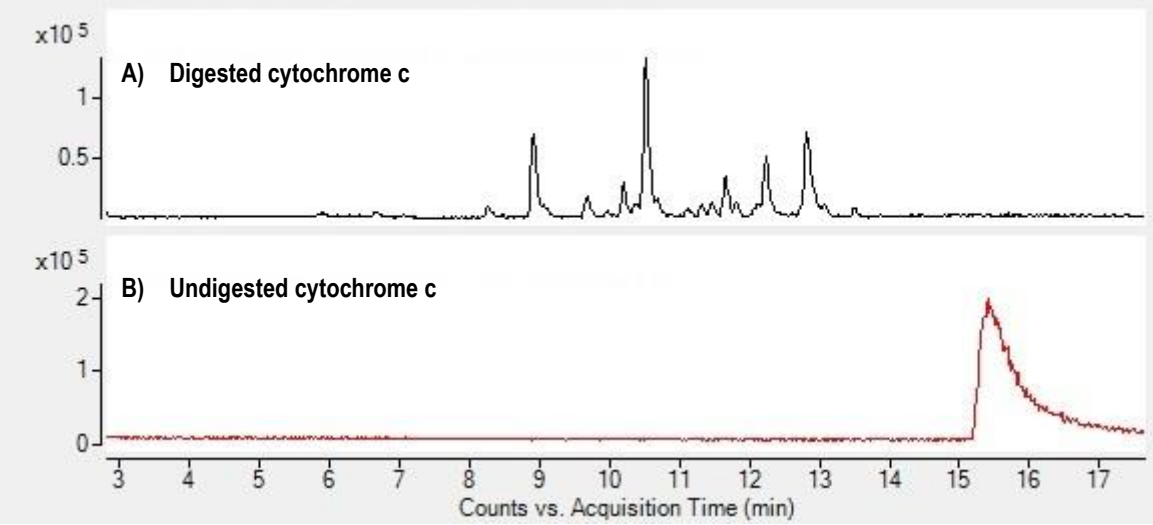


Figure 3: TIC of A) cytochrome c digestion (~80 pmol) using trypsin immobilised micro-reactor cartridge and B) undigested cytochrome c. Chromatograms represent ~16 pmol loading on column (Accucore C18+, 100 mm x 2.1 mm, 1.5 µm).

C. Correlation of mass spectral data with protein sequences by Vion UNIFI software:

Analysis of trypsin digests of BSA and cytochrome c on the Waters Vion QToF MS had sequence coverages of 94% and 87%, respectively (Figure 4).

BSA Coverage: 94%

1: 1 to 80	MKWVTFISLL	LLFSSAYSRG	VFRRDTHKSE	IAHRFKDLGE	EHFKGLVLIA	FSQYLQQCPF	DEHVKLVNEL	TEFAKTCVAD	
1: 81 to 160	ESHAGCEKSL	HTLFGDELCK	VASLRETYGD	MADCCEKQEP	ERNECFLSHK	DDSPDLPKLK	PDPNTLCDEF	KADEKKFWGK	
1: 161 to 240	YLYEIARRHP	YFYAPELLYY	ANKYNGVFQE	CCQAEDKGAC	LLPKIETMRE	KVLTSSARQR	LRCASIQKFG	ERALKAWSVA	
1: 241 to 320	RLSQKFPKAE	FVEVTKLVTD	LTKVHKECCH	GDLLECADDR	ADLAKYICDN	QDTISSKLKE	CCDKPLLEKS	HCIAEVEKDA	
1: 321 to 400	IPENLPPLTA	DFAEDKDVCK	NYQEAKDAFL	GSFLYEYSRR	HPEYAVSVLL	RLAKEYEATL	EECCAKDDPH	ACYSTVFDKL	
1: 401 to 480	KHLVDEPQNL	IKQNCDQFEK	LGEYGFQNAL	IVRYTRKVPQ	VSTPTLVEVS	RSLGKVGTRC	CTKPESERMP	CTEDYLSLIL	
1: 481 to 560	NRLCVLHEKT	PVSEKVTKCC	TESLVNRRPC	FSALTPDETY	VPKAFDEKLF	TFHADICTLP	DTEKQIKKQT	ALVELLKHKP	
1: 561 to 607	KATEEQLKTV	MENFVAFVDK	CCAADDKEAC	FAVEGPKLVV	STQTALA				

CT Coverage: 87%

1: 1 to 80	MGDVEKGKKI	FVQKCAQCHT	VEKGGKHKTG	PNLHGLFGRK	TGQAPGFTYT	DANKNKGITW	KEETLMEYLE	NPKKYIPGTK
1: 81 to 105	MIFAGIKKKT	EREDLIAYLK	KATNE					

Figure 4: Sequence coverage of BSA and cytochrome c analysed using the Waters Vion IMS QToF MS. The blue colour highlights peptides correlated with protein sequences.

CONCLUSION

- µSPEed microreactor cartrdiges were prepared with ePrep Sample Preparation Workstation in 5 minutes by immobilisation of trypsin onto µSPEed cartridges.
- Digestion of BSA was completed in 1 minute and cytochrome C in under 2 minutes, at ambient temperature.
- Complete digestion was observed for BSA and cytochrome c, with 94% and 87% sequence coverages, respectively.

ACKNOWLEDGEMENTS

Karen Duong[1], Dr Simin Maleknia[1], Andrew Minett[2], Prof Philip Doble[1],
[1]University of Technology Sydney - School of Mathematical and Physical Sciences, Faculty of Science.
[2]Eprep Pty Ltd, Mulgrave VIC 3170, Australia

REFERENCES

1. *Duong K. Maleknia S., Minett A., Bishop D., Doble, P. Immobilised Enzymes on Micro Solid-Phase Cartridges for Automated Protein Digestion. Proceedings of the 66th American Society for Mass Spectrometry Conference on Mass Spectrometry and Allied Topics, June 3-7, San Diego, USA; 2018.*

µSPEed Cartridge Ordering Information

Part Number	Code	Description
µSPEed Cartridges		
01-10185	µSPEed, Cxyl-3µm/120Å (Pkt 10)	µSPEed, Customisable Microreactor Carboxyl-3µm/120Å (Pkt 10)
01-10110	µSPEed, C18RPS-3µm/120Å (Pkt 10)	3µm/ 120Å ODS spherical silica packing with high acidic resistance suitable for general organic compound applications.
01-10115	µSPEed, Silica-3µm/120Å (Pkt 10)	3µm/120Å spherical bare silica packing. High purity silica for normal and hilic applications
01-10118	µSPEed, PFAS-3µm/120Å (Pkt 10)	3µm/120Å spherical mixed PFAS packing. Turned for PFAS analysis
01-10150	µSPEed, PS/DVB -3µm/ 300Å (Pkt 10)	3µm/ 300Å spherical, crosslinked polystyrene divinyl benzene
01-10151	µSPEed, PS/DVB RP-3µm/ 300Å (Pkt 10)	3µm/ 300Å Phenyl(RP) spherical, crosslinked polystyrene divinyl benzene
01-10155N	µSPEed, PS/DVB SAX-3µm/ NP (Pkt 10)	3µm/Non-Porous SAX spherical, crosslinked polystyrene divinyl benzene
01-10156N	µSPEed, PS/DVB SCX-3µm/ NP (Pkt 10)	3µm/Non-Porous SCX spherical, crosslinked polystyrene divinyl benzene