Application

intelligent sample preparation

(w) www.eprep-analytical.com
(e) info@eprep.com.au

µSPEed | Application Note 2019

Immunoaffinity and enzymatic reactor micro-SPE cartridges for rapid protein isolation and digest

Pub No. 96-10017-01

INTRODUCTION

There is an increasing requirement for rapid identification and quantification of protein biomarkers in contemporary clinical laboratories. Customisable micro-solid phase extraction sorbent cartridges (µSPEed) for sample preparation prior to analysis by liquid chromatography-mass spectrometry provides a simple and rapid workflow for universal biomarker quantification with small sample sizes. A variety of ligands may be bound to generic customisable sorbents for targeted microSPE sample preparation.

EPREP, µSPEED AND CUSTOMISABLE CHEMISTRY CARTRIDGES

The introduction of the ePrep Sample Preparation Workstation (Figure 1) offers an innovative alternative to current manual and many automated analytical techniques, eliminating labour intensive processes to vastly increase precision and accuracy. Designed for automated liquid handling, ePrep's 'high' pressure capabilities allow automation for techniques such as micro solid phase extraction (µSPEed cartrdiges), sample filtering and membrane extraction.

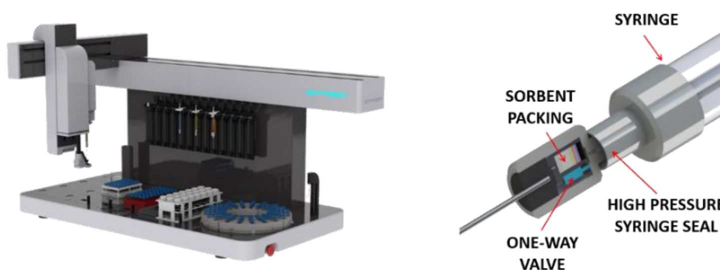

***Figure 1:** ePrep Sample Preparation Workstation and µSPEed cartridges with one-way check valves.*

µSPEed cartrdiges are design for automated microSPE. In conjunction with the ePrep Sample Preparation Workstation (Figure 1), they offer significant advancement over standard SPE cartridges using a 3 µm sorbent packed into an 8 µl (4.2 mg) bed volume, providing enormous separation power and high concentration factors in µl volumes. The operation of µSPEed involves liquid being draw through a one-way valve into the syringe. On dispensing, the valve closes directing the sample to flow through the sorbent bed.

Eprep has also developed µSPEed cartrdiges with customisable stationary phases for immobilized of biological ligands such as immunoaffinity or enzymatic micro-reactors in applications such as the isolation and digestion of proteins.

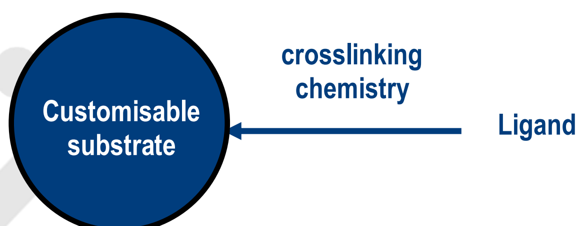

***Figure 2:** Ligands such as Enzymes and Antibodies can be immobilised to the customisable substrate*

This application covers an accurate and reproducible automated process for the isolation, pre-concentration, and digestion of proteins (biomarkers) at trace levels through immunoaffinity isolation and concentration and rapid and reproducible protein digestion. Immunoaffinity and digesting was performed using customisable Cxyl-µSPEed cartridges with chemistry modified through automated sequencing. Bovine serum albumin (BSA) as a model protein.

Cytochrome c, a more difficult protein to digest, was also tested with Trypsin-Cxyl-µSPEed Cartridges.

Immunoaffinity extraction of BSA using anti-BSA immobilized cartridges

An automated sample handling workflow was developed for the in-situ immobilization of anti-BSAonto onto Cxyl-µSPEed, using the workflow described in 'Methods' Figure 9. Effectiveness of the process was to check collecting samples at different steps of the workflow and analysed them by HPLC-SEC-ICP-MS. SEC was performed using an ACQUITY UPLC Protein BEH SEC column 1.7 µm, 4.6x300 mm (Waters, Milford, MA, USA) on an Agilent 8900 ICP-QQQ (Agilent, Santa Clara, CA, USA). MS/MS mode was used with O2 reaction cell gas for S determination 32→48.

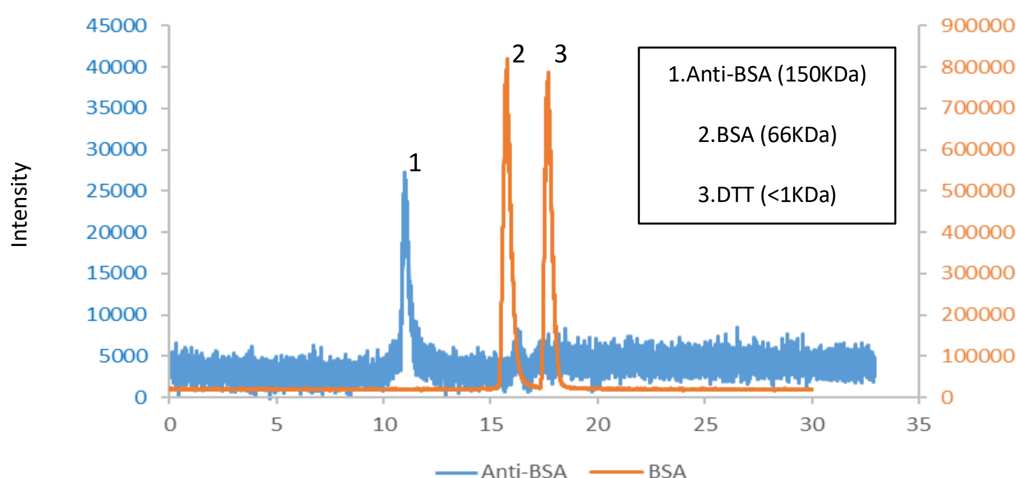

Figure 3: *Chromatograms for Anti-BSA and BSA by SEC-ICP-MS*

<u>Results:</u>

- Firstly, anti-BSA and BSA protein were analysed to ensure detection (see Figure 3). Anti-BSA was detected at 10 min, BSA at 16 min and DTT (present in the sample as it was used to reduce disulphide bridges) at 18 min.
- Trace levels of BSA were captured by the affinity cartridge.
- BSA was analysed by flow injection ICP-MS*. Figure 4 shows the presence of S containing analyte corresponding to BSA.

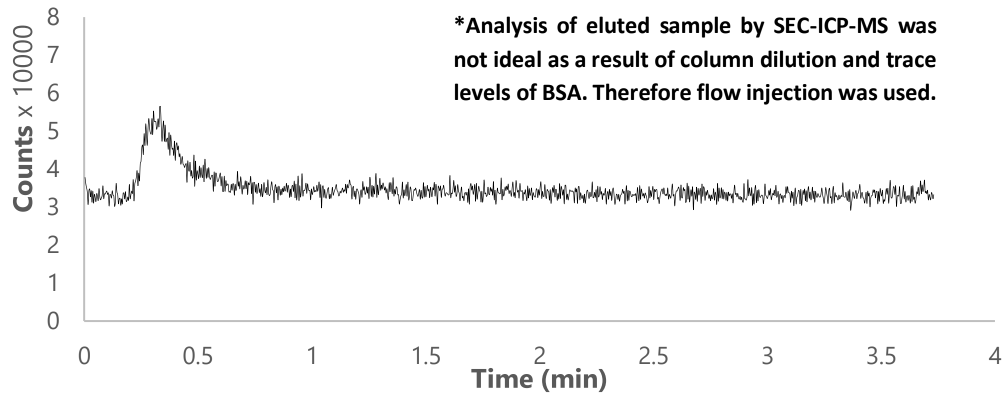

Figure 4: *Mass chromatograms for flow injection of affinity captured and eluted BSA from anti-BSA immobilised cartridge.*

PROTEIN DIGESTION USING TRYPSIN IMMOBILISED CARTRIDGES

Trypsin was immobilized onto novel support material in two ways, A) in-situ immobilization onto µSPEed-Cxyl cartridges and B) pre-immobilised onto material prior packing into µSPEed cartridges. 'Methods' Figure 10 describes the protein digestion workflow.

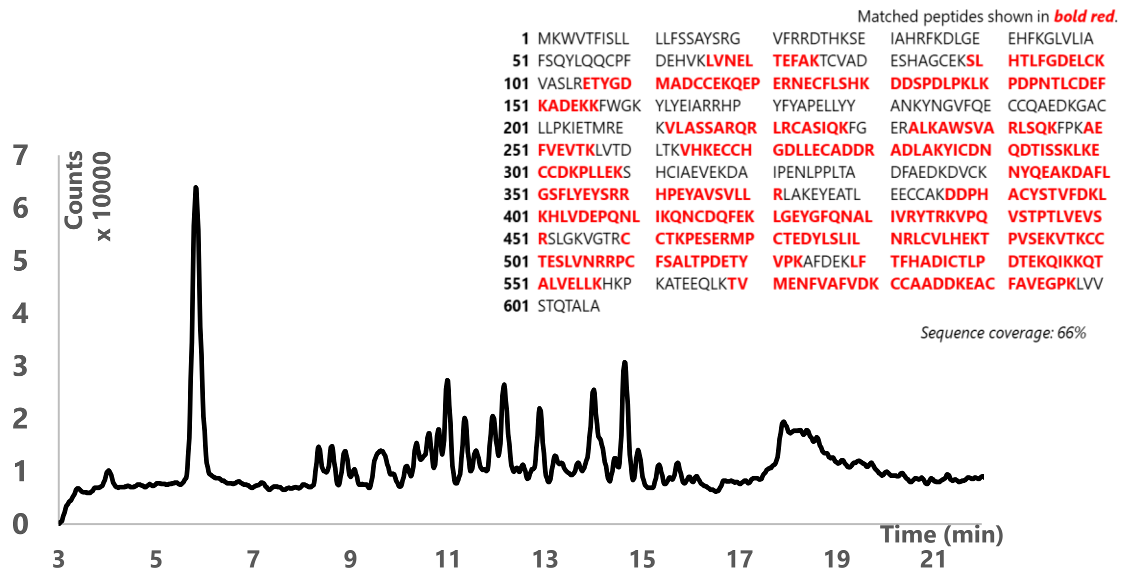

Figure 5: *Mass chromatogram of digested BSA peptides using pre-immobilised trypsin cartridges, and the sequence coverage from the Mascot database search. Injected: Peptides from 1.5 pmol of digested protein. Agilent Technologies 6510 LC-QTOF ('Methods' Table 2).*

Results:

- Digestion of BSA was achieved using in-situ and pre-immobilised trypsin cartridges (see Figure 5).
- Both cartridges digested BSA with minimal/no protein remaining.
- 1 µg of protein digested in 10 minutes – digestion times vary with amount of protein loaded onto cartridges.
- BSA coverage for pre-immobilised trypsin cartridges was 66% and 64% for in-situ immobilized cartridges

TRYPSIN CARTRIDGE REPRODUCIBILITY

Method Validation: Trypsin cartridge reproducibility was determined with benzoyl-L-arginine ethyl ester (BAEE) for cartridge in-situ immobilization and pre-immobilised material.

1 mM BAEE was passed through each cartridge with 5-minute digest and analysed by HPLC with a UV detector at 254 nm. The remaining undigested BAEE and resulting digest product BA peaks were integrated, and a peak area ratio of BA/BAEE is calculated for each cartridge (n=5 for in-situ immobilized cartridges, and n=5 for pre-immobilised trypsin cartridges)

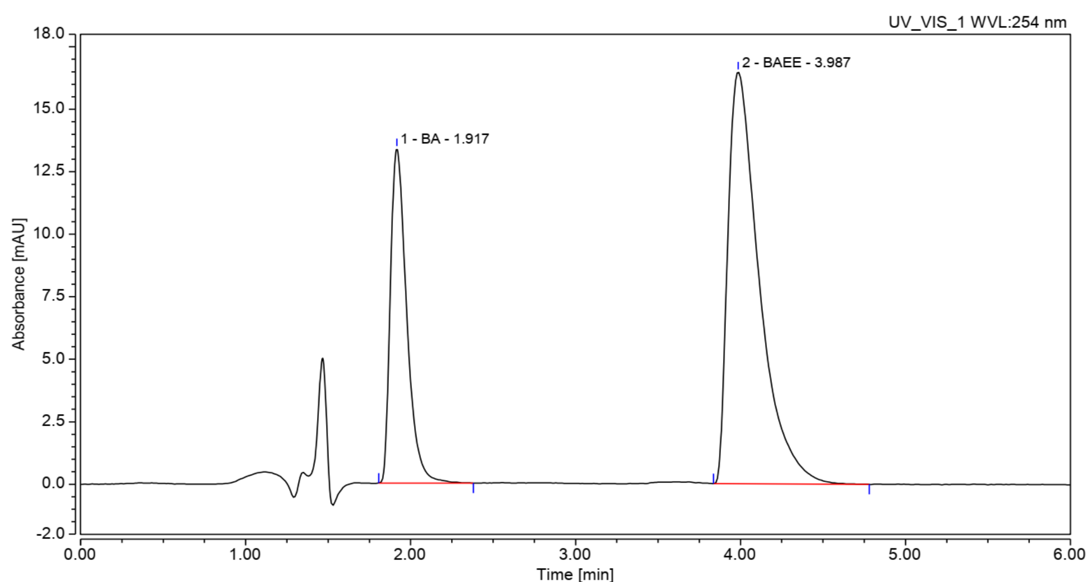

Figure 6: *Reproducibility of in-situ (n=5) and pre-immobilised (n=5) trypsin cartridges*

Cartridge	Peak area ratio for BA/BAEE	%RSD
In-situ immobilised (n=5)	0.15 ± 0.01	7.0
Pre-immobilised (n=5)	0.50 ± 0.01	2.4

Table 1: *Reproducibility of in-situ (n=5) and pre-immobilised (n=5) trypsin cartridges measure at 254nm.*

Results

- In the presence of trypsin, BAEE (4 min) is hydrolysed to BA (2 min) (see Figure 6).
- Both *in-situ* and pre-immobilised trypsin showed great digest reproducibility with RSD of 7.0% and 2.4% respectively (see Table 1)

CYTOCHROME C DIGEST

Cytochrome c was passed through the cartridge at ambient temperature with complete protein digestion in under 2 mins,

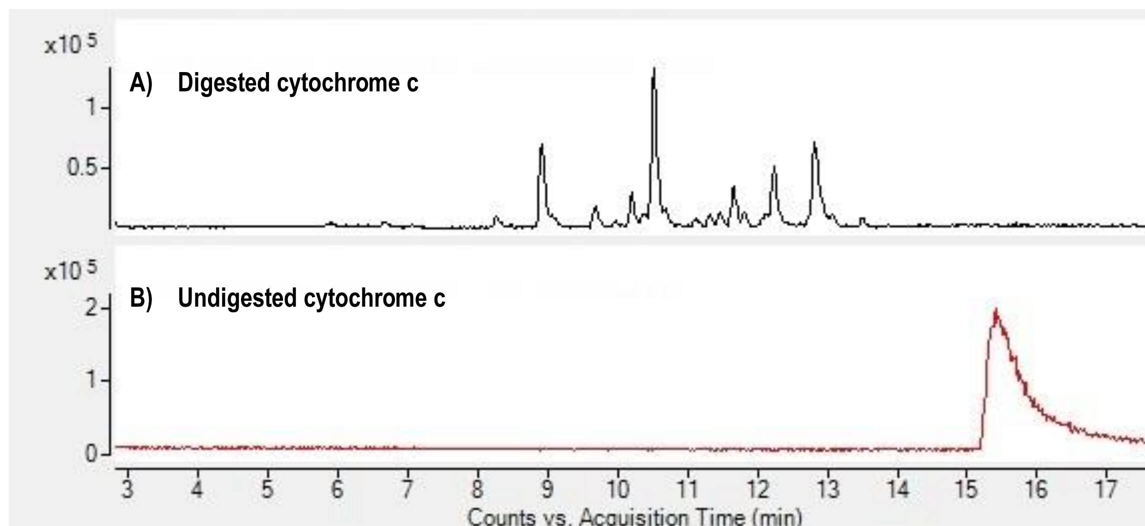

Figure 7: *TIC of A) cytochrome c digestion (~80 pmol) using trypsin immobilized micro-reactor cartridge and B) undigested cytochrome c. Chromatograms represent ~16 pmol loading on column (Accucore C18+, 100 mm x 2.1 mm, 1.5 µm). A. Correlation of mass spectral data with protein sequences by Vion UNIFI software.*

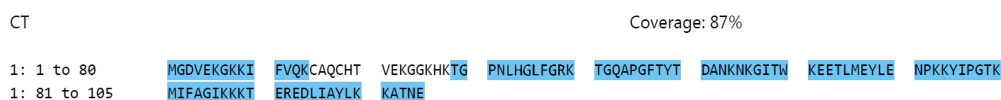

Figure 8: *Sequence coverage of BSA and cytochrome c analysed using the Waters Vion IMS QToF MS. The blue colour highlights peptides correlated with protein sequences.*

Results

- Digestion of cytochrome C in under 2 minutes, at ambient temperature.
- Cytochrome c, with 87% sequence coverages.

CONCLUSION

An automated µSPE method was successfully developed for the immobilization of Anti-BSA in-situ onto µSPEed cartridges allowing the isolation of trace levels of BSA protein. Enzyme reactor cartridges were developed using two modes of immobilization: in-situ and pre-immobilised methods. Both approaches show great digest reproducibility with RSD of 7.0% for in-situ and 2.4% for pre-immobilised cartridges.

Finally, we achieve a successfully BSA digestion in minutes using trypsin cartridges resulting in ~60% of sequence coverage. Cytochrome C was also digested in under 2 minutes with an 87% sequence coverage, at ambient temperature

Thus, µSPE cartridges present a novel, fast, automated and reproducible alternative to conventional protein digestion methods.

METHODS

Workflows - Immunoaffinity and Enzymatic Reactor

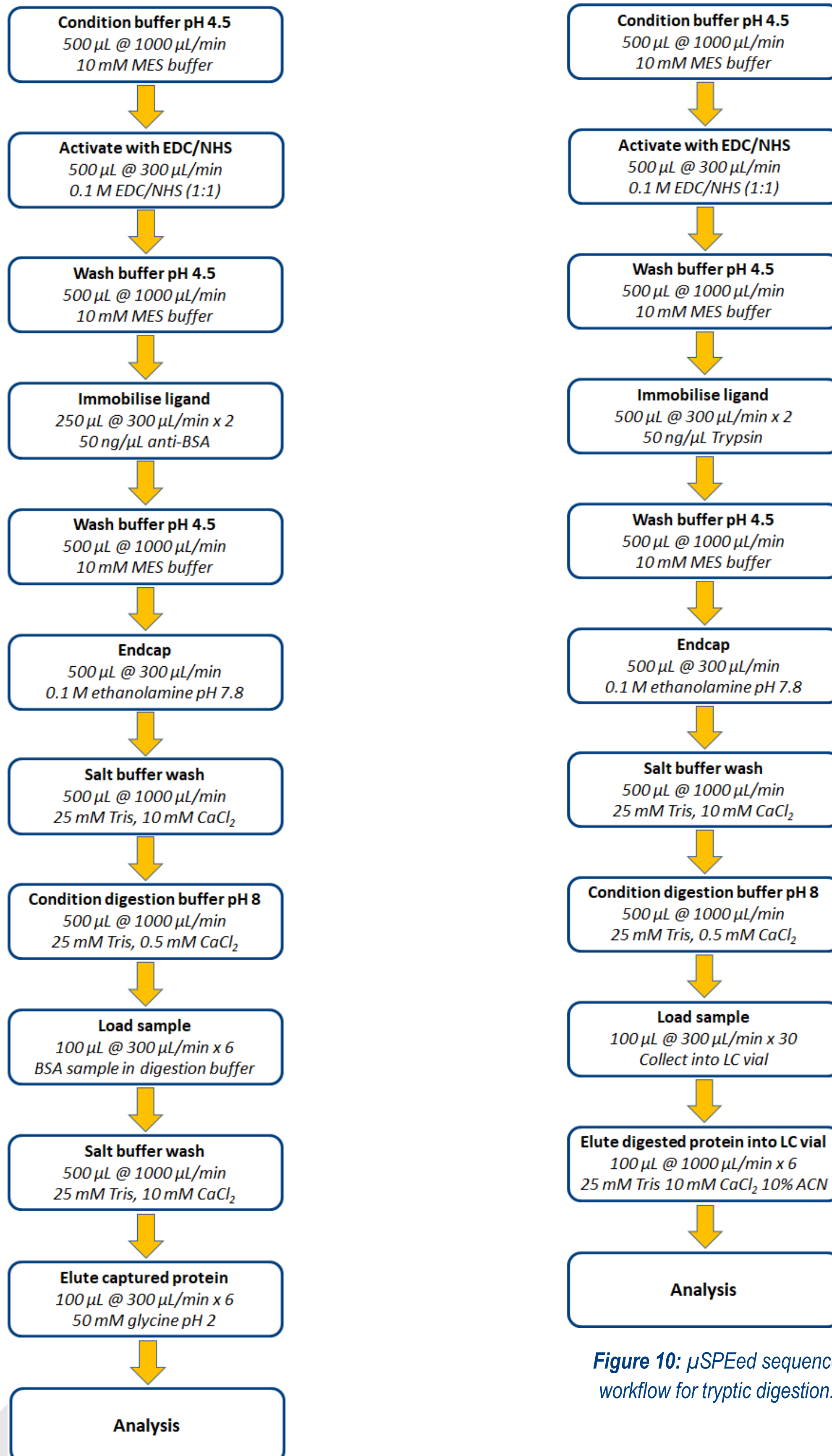

Figure 9: *µSPEed sequence workflow for immunoafiinity extraction.*

Figure 10: *µSPEed sequence workflow for tryptic digestion.*

Chromatographic Paratemeters

System	Agilent Technologies 6510
Column	Accucore C18+ column (100 x 2.1 mm, 1.5 µm)
Flow rate	0.2 mL/min
Mobile phase	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
Run time	5 min pre-equilibration and 30 min
Column temperature	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

Table 2: Chromatography and mass spectrometry parameters

ACKNOWLEDGMENTS

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