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 cartridges), sample filtering and membrane extraction.

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

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-BSA 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.

**Results:**

- 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 3: Chromatograms for Anti-BSA and BSA by SEC-ICP-MS](image)

1. Anti-BSA (150KDa)
2. BSA (66KDa)
3. DTT (<1KDa)
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.

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)

![Graph showing peak area ratio of BA/BAEE for in-situ and pre-immobilised trypsin cartridges](image)

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

<table>
<thead>
<tr>
<th>Cartridge</th>
<th>Peak area ratio for BA/BAEE</th>
<th>%RSD</th>
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<tr>
<td>In-situ immobilised (n=5)</td>
<td>0.15 ± 0.01</td>
<td>7.0</td>
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<tr>
<td>Pre-immobilised (n=5)</td>
<td>0.50 ± 0.01</td>
<td>2.4</td>
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</table>

**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.](image)

**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.

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 immunoaffinity extraction.

1. **Condition buffer pH 4.5**
   - 500 μL @ 1000 μL/min
   - 10 mM MES buffer

2. **Activate with EDC/NHS**
   - 500 μL @ 300 μL/min
   - 0.1 M EDC/NHS (1:1)

3. **Wash buffer pH 4.5**
   - 500 μL @ 1000 μL/min
   - 10 mM MES buffer

4. **Immobilise ligand**
   - 250 μL @ 300 μL/min x 2
   - 50 ng/μL anti-BSA

5. **Wash buffer pH 4.5**
   - 500 μL @ 1000 μL/min
   - 10 mM MES buffer

6. **Endcap**
   - 500 μL @ 300 μL/min
   - 0.1 M ethanolamine pH 7.8

7. **Salt buffer wash**
   - 500 μL @ 1000 μL/min
   - 25 mM Tris, 10 mM CaCl₂

8. **Condition digestion buffer pH 8**
   - 500 μL @ 1000 μL/min
   - 25 mM Tris, 0.5 mM CaCl₂

9. **Load sample**
   - 100 μL @ 300 μL/min x 6
   - BSA sample in digestion buffer

10. **Salt buffer wash**
    - 500 μL @ 1000 μL/min
    - 25 mM Tris, 10 mM CaCl₂

11. **Elute captured protein**
    - 100 μL @ 300 μL/min x 6
    - 50 mM glycine pH 2

12. **Analysis**

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

1. **Condition buffer pH 4.5**
   - 500 μL @ 1000 μL/min
   - 10 mM MES buffer

2. **Activate with EDC/NHS**
   - 500 μL @ 300 μL/min
   - 0.1 M EDC/NHS (1:1)

3. **Wash buffer pH 4.5**
   - 500 μL @ 1000 μL/min
   - 10 mM MES buffer

4. **Immobilise ligand**
   - 500 μL @ 300 μL/min x 2
   - 50 ng/μL Trypsin

5. **Wash buffer pH 4.5**
   - 500 μL @ 1000 μL/min
   - 10 mM MES buffer

6. **Endcap**
   - 500 μL @ 300 μL/min
   - 0.1 M ethanolamine pH 7.8

7. **Salt buffer wash**
   - 500 μL @ 1000 μL/min
   - 25 mM Tris, 10 mM CaCl₂

8. **Condition digestion buffer pH 8**
   - 500 μL @ 1000 μL/min
   - 25 mM Tris, 0.5 mM CaCl₂

9. **Load sample**
   - 100 μL @ 300 μL/min x 30
   - Collect into LC vial

10. **Elute digested protein into LC vial**
    - 100 μL @ 1000 μL/min x 6
    - 25 mM Tris 10 mM CaCl₂ 10% ACN

11. **Analysis**
Chromatographic Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Details</th>
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<tbody>
<tr>
<td>System</td>
<td>Agilent Technologies 6510</td>
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<tr>
<td>Column</td>
<td>Accucore C18+ column (100 x 2.1 mm, 1.5 μm)</td>
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<tr>
<td>Flow rate</td>
<td>0.2 mL/min</td>
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</tbody>
</table>
| 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 temp  | 30 °C |
| QToF MS params | 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

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2Eprep Pty Ltd, Mulgrave VIC 3170, Australia

REFERENCES


μSPEED CARTRIDGE ORDERING INFORMATION

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<th>Part Number</th>
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<th>Description</th>
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<td>µSPEED</td>
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<td>µSPEED, Customisable Microreactor Carboxyl-3µm/120Å (Pkt 10)</td>
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<tr>
<td>01-10185</td>
<td>Cxyl-3µm/120Å</td>
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<td>3µm/120Å spherical bare silica packing. High purity silica for normal and hILIC applications</td>
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