# Immobilised Enzymes on Micro Solid-Phase Extraction Cartridges for Automated Protein Digestion

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**Introduction:** The rapid identification and quantification of biomarkers are of increasing significance in clinical settings, and this is achievable on small sample sizes by integrating the ePrep customisable micro solid phase extraction devices (µSPEed) with mass spectrometry. The µSPEed cartridges are comprised of a packed sorbent bed compartment, a unidirectional valve and a simple press fit connector for use with a digital syringe drive or an automated sample preparation workstation. The use of positive displacement digital syringes ensures precise and reproducible flow kinetics, providing the opportunity to perform time-dependent assays, such as speeding up protein digests.

Customizable µSPEed sorbents have been developed to immobilise ligands through crosslinking chemistry. Ligands possible for immobilisation include enzymes for micro-reactor cartridges, and antibodies for affinity separation cartridges. Here, cartridges immobilised with trypsin and pepsin are presented to digest proteins cytochrome c and bovine serum albumin (BSA) in minutes.

**Experimental:** Enzymes were immobilised onto the µSPEed cartridges (Figure 1) using carbodiimide crosslinking chemistry. The enzymes were conditioned to optimal pH for enzyme activity, and the protein solution is passed through the cartridge for digestion in minutes. The protein and peptides were analysed by liquid chromatography time of flight mass spectrometry (Agilent Technologies 6510 LC-QTOF), a peptide mass list is automatically generated using the Agilent Data Acquisition software and searched against the Mascot database [1].

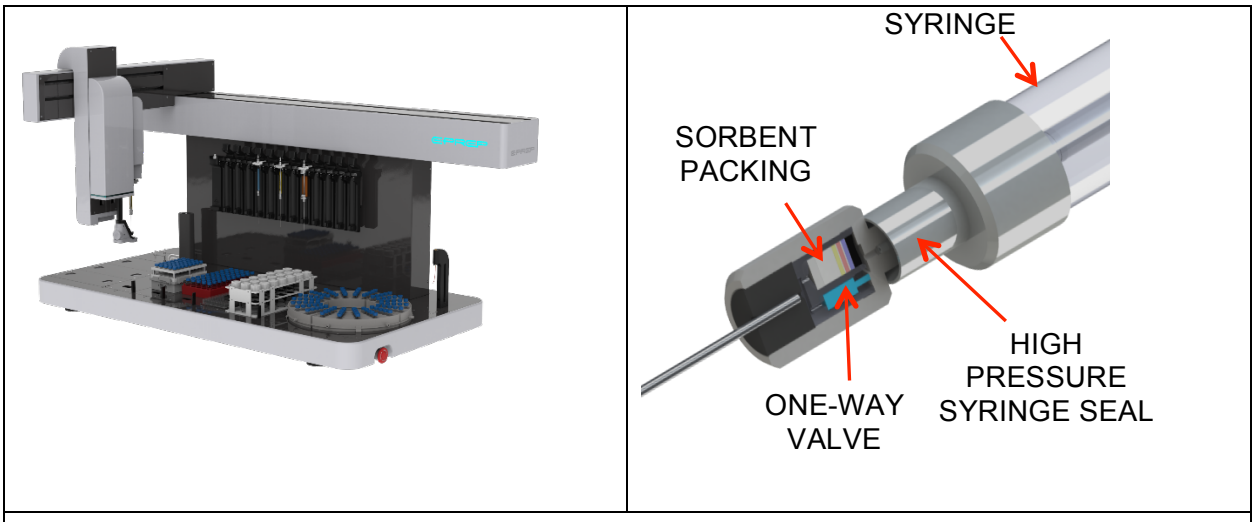


**Figure 1:** ePrep Automated Sample Preparation Workstation and µSPEed cartridges with one-way check valves for unidirectional flow.

**Results and Discussion:** Digestion of ~83 pmol cytochrome c using trypsin was achieved in 4 minutes (100 µL at a flow rate of 25 µL/min) at ambient temperature. The protein was efficiently digested as there was no undigested protein remaining (see Figure 1).

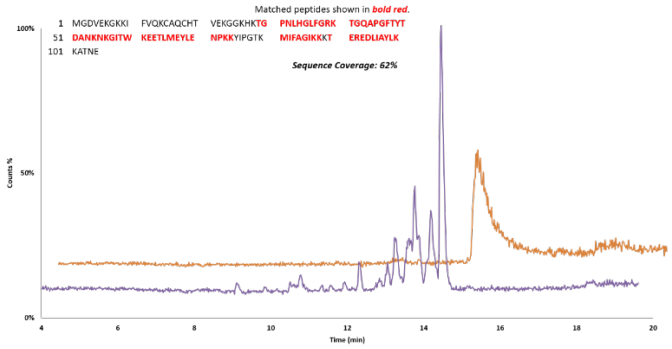


**Figure 2:** Mass chromatograms (Agilent Technologies 6510 QTOF) of undigested cytochrome c (orange), digested cytochrome c peptides (purple), and sequence coverage from the Mascot database search. Injected: 16 pmol of protein. Column: Thermo Fisher Scientific Accucore C18+ 2.1 x 100 mm. Mobile Phase: A: Water with 0.1% formic acid, B: Acetonitrile with 0.1% formic acid. Gradient: 5% B to 60% B over 25 minutes.

Digestion of BSA was also achieved using trypsin (Figure 3) and pepsin in 4 minutes with sequence coverages of 80% and 34% obtained respectively.

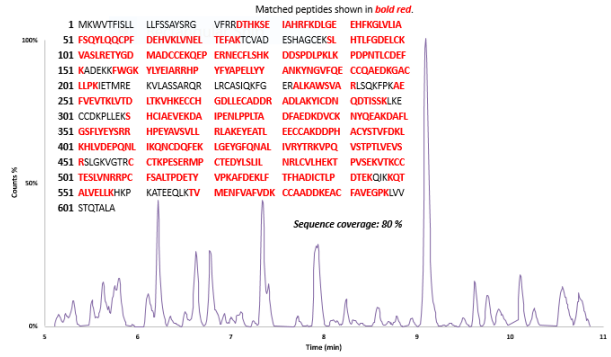


**Figure 3:** Extracted mass chromatogram of BSA digest with trypsin and sequence coverage from the Mascot database search. ~4 pmol of BSA (100 µL) was digested at a flow rate of 25 µL/min at ambient temperature. Injected: ~ 0.75 pmol of digested protein. Chromatography as Figure 2.

**Conclusion:** A workflow was developed for the immobilisation of ligands onto µSPEed cartridges. Pepsin and trypsin were covalently immobilised using EDC/NHS crosslinking chemistry. Bovine serum albumin and cytochrome C were digested in 4 minutes (100 µL at 25 µL/min) at ambient temperature using various enzymes. There was no undigested protein remaining supporting efficient digestion. The %sequence coverage is based on automated peptide mass list which is not optimised to extract all peptides. Future work will improve on chromatography columns to enhance LC-MS peptide recovery. The method workflow is being further optimised for faster digests and including other proteases.

**Acknowledgments:** This research is supported by the Research Training Scheme (RTS) funded by the Australian Government, UTS, and Eprep Pty Ltd.

1. Matrix Science, *Mascot Peptide Mass Fingerprint,* http://www.matrixscience.com.