HIGH SPEED NANOSCALE UPLC SEPARATIONS COMBINED WITH OFF-LINE MALDI MS/MS FOR PEPTIDE ANALYSIS

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INTRODUCTION

- **Here we present a rapid LC-MS/MS method, which utilises a MALDI interface for a Q-Tof mass spectrometer**
- **MALDI in combination with high pressure nano-scale Ultra Performance Liquid Chromatography, reduces the complexity of the sample analysis.**
- **Successful fast LC-MS/MS data generated from *Escherichia coli* (*E. coli*) proteins separated by 2D PAGE is presented.**

Mass spectrometry has now firmly established itself as the primary technique for identifying proteins due to its unparalleled speed, sensitivity and specificity. Strategies commonly involve the digestion of the protein using a specific protease that cleaves at predictable residues along the protein backbone, providing small stretches of peptide sequence that are more amenable to analysis by mass spectrometry. When coupled with protein level fractionation strategies, this approach has proven highly successful in comprehensive protein identification and characterisation. A common approach to protein pre-fractionation is the use of 1 or 2 dimensional PAGE, coupled with LC-MS/MS. The downside of this approach is that an LC-MALDI experiment is required prior to analysis, with typical analytical HPLC run times of 45 minutes to 1 hour the amount of time required to analyses one top level sample can be prohibitive.

In this poster we describe the use of elevated flow rates combined with nanoscale columns packed with 1.7 µm particles for rapid peptide based separations using a high pressure nanoUPLC system. Increasing the flow rate of the separation from approximately 0.3 µL/min to 1.4 µL/min has the effect of increasing the pressure of the chromatographic system from 1950 psi to 5900 psi which allows the separation to be performed with a very rapid gradient over 8 minutes. The eluent is combined with matrix solution and spotted directly onto MALDI target plates using the CTC PAL spotter. This combined with an orthogonal acceleration time-of-flight mass spectrometer equipped with a 200Hz repetition rate Nd: YAG laser allows for the rapid characterisation of simplified protein mixtures.

Here we present data from a mixture of four protein digest standards to demonstrate the reproducibility of the method as well as data collected from 2D gel samples of *E. coli*.

Nanoscale UPLC Conditions

Samples were trapped on a 5µm Symmetry C18 180µm x 20mm column at a flow rate of 30µL/min for 0.5 min.

Nanoscale UPLC separations were performed on a 1.7 µm BEH 75µm x 50mm BEH column using a nanoACQUITY™ UPLC™ system (Waters Corporation). The column was maintained at 60ºC and a flow of 1.4 µL/min produced a typical back pressure of 5900 psi. A gradient over 2 minutes from 10% to 60% B was used where the mobile phase was A: 0.1% formic acid and B: MeCN. The total cycle time, injection to injection, was 8 minutes.

Matrix, alpha-cyano-4-hydroxycinnamic acid 2mg/mL (1:1 0.1% TFA:MeCN) was added at 3.6µL/min and mixed with eluent prior to spotting. The spotting time was 4-5 seconds per spot with a solvent delay of 2.1 minutes. The device used is shown in Figure 1.

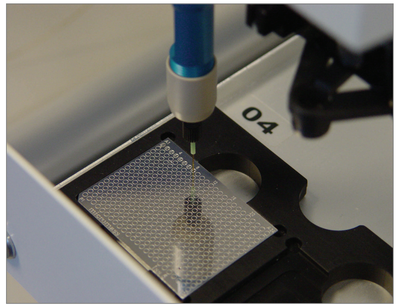

Figure 1. Automated application of eluent and matrix solution onto a 384 spot MALDI target plate.

MS Conditions

The Q-Tof Premier mass spectrometer was operated with a MALDI source. External calibration was initially performed using a PEG standard and the data were lock mass corrected post-acquisition using Glu-fibrinopeptibe B, to ensure high mass accuracy.
Data from LC separated samples were acquired in a two stage experiment. First MS data were obtained across the whole gradient, *i.e.* 12-24 MALDI spots. The MS information from one gradient was combined, non-peptide peaks such as matrix peaks were removed and a non-redundant MS/MS precursor list was produced using MALDI merge software.
In the second stage MS/MS data were acquired using the precursor list generated from MS data.

Samples

Experiments were conducted on Mix 1 (ADH, BSA, Enolase and Phosphorylase B) at a concentration of 50 fmol/µL with an injection volume of 1 µL.and on a 250 µg sample of a lyophilised *E. coli* protein sample (Bio-Rad, Hercules, CA) previously separated by 2D-gel electrophoresis. The proteins were visualised by Coomasie staining using a 0.08% Coomassie G250 (Merck, Darmstadt, Germany), 1.6% ortho-phosphoric acid 85%, 8% ammonium sulphate (Merck, Darmstadt, Germany) and 20% methanol (Merck, Darmstadt, Germany.
Gel spots were excised from the gel and deposited in one 96-well microtiter plate with 1-5 gel pieces per well. The gel samples were de-stained, reduced, alkylated, digested and 4.5 µL of the extracted peptide solutions were injected onto the UPLC column.

RESULTS

Initial experiments focused on the reproducibility and quality of the rapid UPLC separation when used in conjunction with a MALDI mass spectrometer. For this proof-of-principle work a mixture of four tryptic protein digests each at 50 fmol on column was used. Typical results are shown in Figure 2. Reproducibility in retention time was better than ±4 seconds (see Figure 2 (d)). Chromatographic peak widths were of the order of 4 seconds at base.

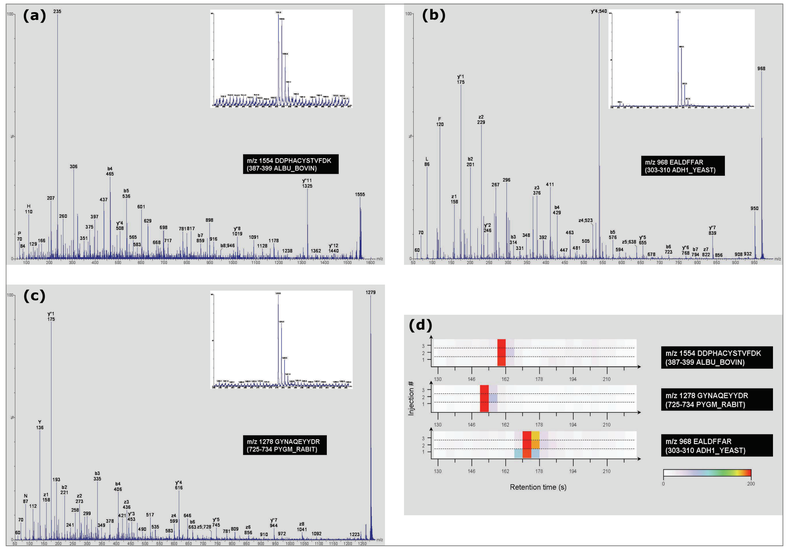

*Figure 2. Illustration of the chromatographic reproducibility of fast UPLC. **(a)-(c)** show examples of MS and MS/MS spectra of three representative tryptic peptides from a mixture of four tryptic digests which was separated over 1.5 minutes at a concentration of 50 fmol on column. **(d)** shows the reproducibility in retention time of the three peptides over three replicate injections.*

The feasibility of performing MS/MS analysis of peptides separated using rapid (1.5 min) gradients was tested on the 50 fmol digest mixture; *ca* 200 MS/MS spectra were obtained from a single LC run. Typical databank search results are shown in Figure 3.

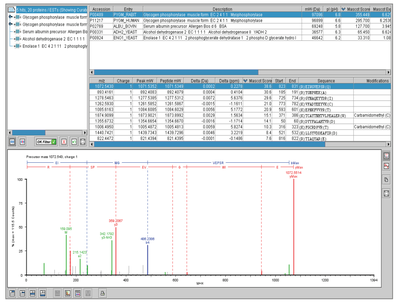

Figure 3. Typical databank search result for 50 fmol of a four protein test mixture separated using rapid nanoUPLC and mass analyzed by MALDI MS/MS

	Protein	Score	Peptides
Spot 1	DNAK_ECOLI:Chaperone protein dnaK (Heat shock protein 70)	307	16
	RS1_ECO57: 30S ribosomal protein S1	245	10
Spot 2	GLYA_ECOLI: Serine hydroxymethyl-transferase	180	12
Spot 3	GLYA_ECOLI: Serine hydroxymethyl-transferase	42	5
Spot 4	GLYA_ECOLI: Serine hydroxymethyl-transferase	37	4
Spot 5	TIG_ECOLI: Trigger factor	333	18
Spot 6	TRPB_ECOLI: Tryptophan synthase beta chain	113	6
Spot 7	ALF_ECOLI:Fructose-bisphosphate aldolase class 2	30	3
Spot 8	G3P1_ECO57: Glyceraldehyde-3-phosphate dehydrogenase A	42	4

Table 1. Proteins identified from MALDI MS/MS analysis of rapid nanoUPLC separated E. coli samples that had previously been 2D PAGE separated and tryptically digested.

Analysis of 2D PAGE separated protein samples

Eight *E. coli* samples were analysed using the methodology developed. Results obtained are summarized in Table 1. All eight gradients could be accommodated on a single 96 spot MALDI target plate and up to 32 gradients could be fitted onto a 384 spot target.

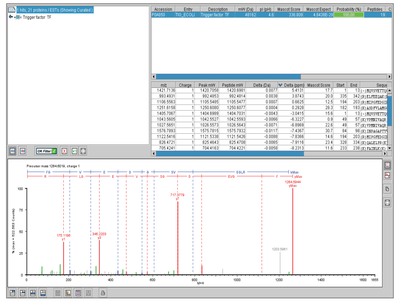

Figure 4. Databank search result for Spot 5 separated using rapid nanoUPLC and mass analyzed by MALDI MS/MS.

CONCLUSION

- **A rapid UPLC technique has been demonstrated for sample clean-up and reduction of complexity prior to MALDI MS/MS analysis.**
- **Excellent chromatographic reproducibility and resolution has been shown.**
- **The combination of MALDI MS/MS with nanoscale UPLC enables simple protein mixtures, such as 1D and 2D gel spots, to be analysed by LC-MALDI MS/MS in under ten minutes.**

ACKNOWLEDGMENTS

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