RAPID IDENTIFICATION AND CHARACTERISATION OF TRYPTIC PEPTIDES USING HIGH LINEAR VELOCITY NANOBORE UPLC MALDI MS/MS AND ION MOBILITY SEPARATION

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OVERVIEW

- **Here we present a rapid LC-MS/MS method, which utilises MALDI interfaces on Q-Tof and Synapt mass spectrometers.**
- **MALDI in combination with high pressure nano-scale Ultra Performance Liquid Chromatography, reduces the complexity of the sample analysis.**
- **Ion Mobility Separation (IMS) was used as an orthogonal technique to separate co-eluting isobaric peptides.**
- **Fast LC-MS/MS data generated from *Escherichia coli* (*E. coli*) proteins separated by 2D PAGE is presented.**

INTRODUCTION

Here we describe the use of elevated flow rates combined with nanoscale columns packed with 1.7µm particles for rapid peptide based separations using a high pressure nanoUPLC system. Increasing the flow rate of the separation from approximately 0.3µL/min to 1.4µL/min has the effect of increasing the pressure of the chromatographic system from 1950psi to 5900psi which allows the Chromatographic run time to be compressed to 8 minutes injection to injection. Conventional HPLC run times for this type of experiment are typically 45 minutes to 1 hour. The eluent is combined with matrix solution and spotted directly onto MALDI target plates using a spotting robot. 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. A novel ion mobility separation device was used to separate isobaric peptides.

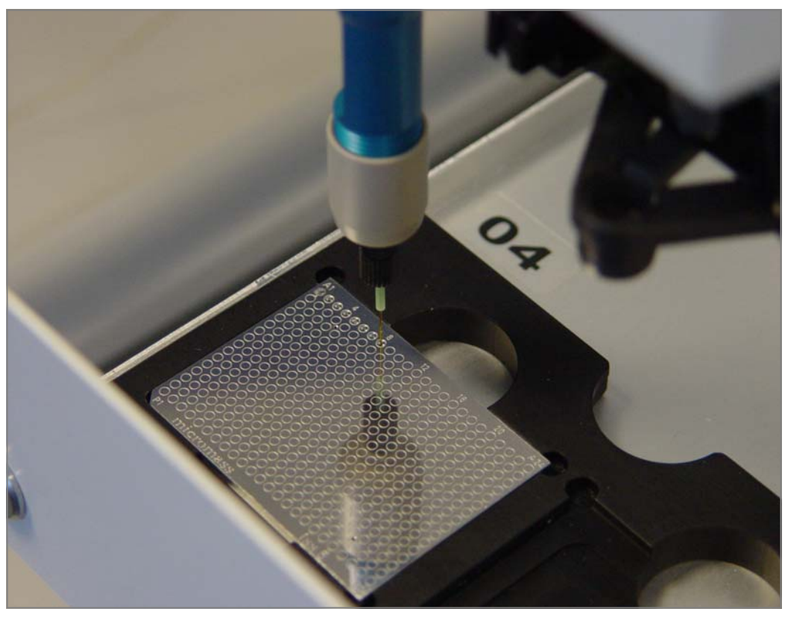

Figure 1. Automated application of eluent and matrix solution onto a 384 spot MALDI target plate.

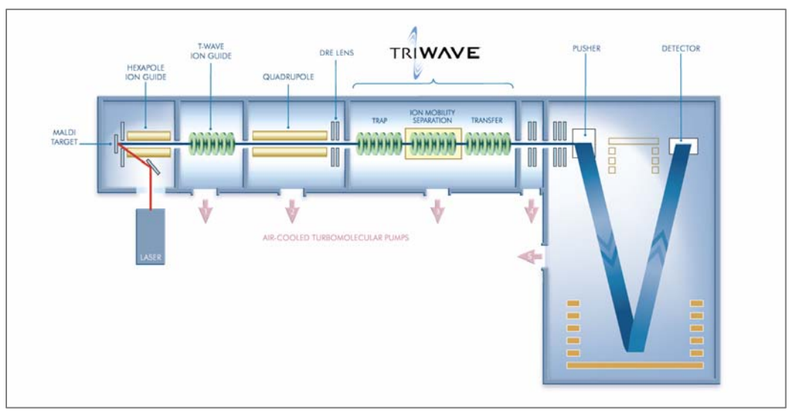

Figure 2. Schematic representation of the MALDI Synapt mass spectrometer used for ion mobility (IMS) experiments.

METHODS

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 1.7µm BEH 75µm x 50mm BEH column using a nanoACQUITY UPLC system (Waters, Milford MA). The column was maintained at 60ºC and a flow of 1.4µL/min produced a typical back pressure of 5900psi. A gradient over 2 minutes from 10% to 60% B was used where the mobile phases were 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.

MS Conditions

The Q-Tof Premier mass spectrometer (Waters, Manchester UK) 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-fibrinopeptide 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 ions 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. Finally, IMS-MS and IMS-MS/MS of UPLC fractionated peptide mixtures were acquired on a MALDI Synapt mass spectrometer (Waters, Manchester UK) equipped with a TriWave IMS device (see Figure 2).

Samples

Experiments were conducted on an equimolar mixture of tryptic ADH, BSA, Enolase and Phosphorylase B digests at a concentration of 50fmol/µL with an inject volume of 1µL, and on 250 µg of 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 4.5µL 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 50fmol on column was used. Typical results are shown in Figure 3. Reproducibility in retention time was better than ±4 seconds (see Figure 2 (b)). Chromatographic peak widths were of the order of 4 seconds at base.

The feasibility of performing MS/MS analysis of peptides separated using rapid (1.5min) gradients was tested on the 50fmol digest mixture; *ca* 200 MS/MS spectra were obtained from a single LC run. Typical databank search results are shown in Figure 4.

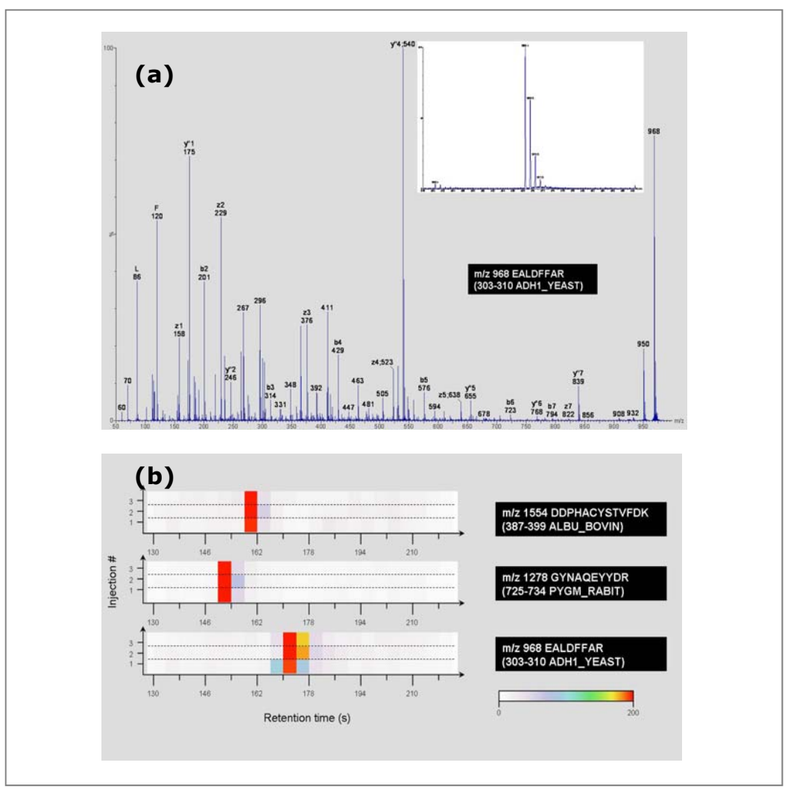

*Figure 3. Illustration of the chromatographic reproducibility of fast UPLC. **(a)** shows an example of MS and MS/MS spectra of a representative tryptic peptides from a mixture of four tryptic digests which was separated over 1.5minutes at a concentration of 50fmol on column. **(b)** shows the reproducibility in retention time of three peptides over three replicate injections.*

Figure 4. Typical databank search result for 50fmol of a four protein test mixture separated using rapid nanoUPLC and mass analyzed by MALDI MS/MS

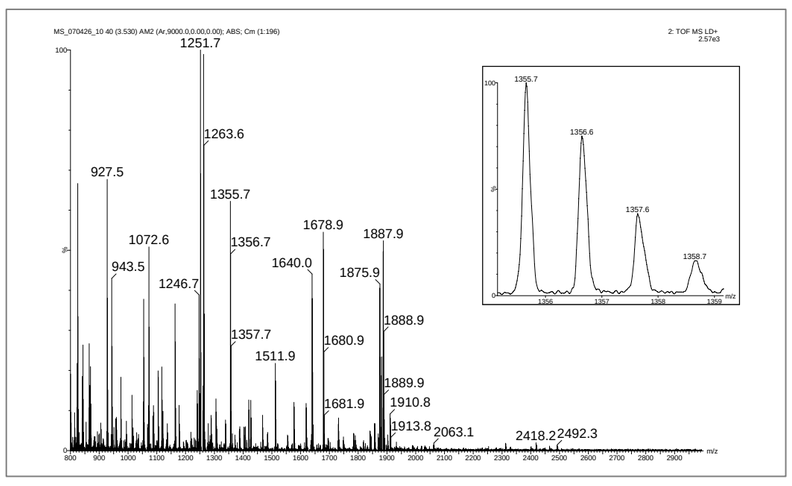

Figure 5. MS spectrum of UPLC fraction (Fraction 10) containing co-eluting isobaric tryptic peptides. Isobaric peptides cannot be distinguished (see inset).

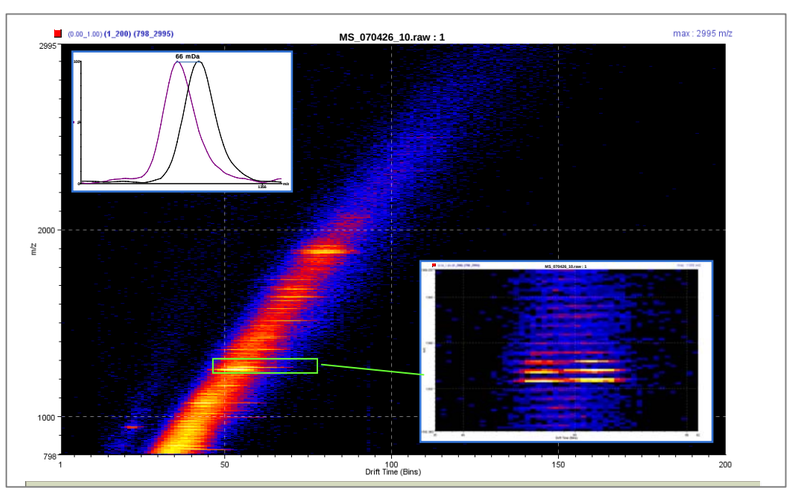

Figure 6. IMS-MS plot of Fraction 10 co-eluting peptides have been highlighted and can be seen to separate in the drift dimension.

Separation of co-eluting isobaric peptides

IMS MS analysis of the 50fmol digest mixture showed that sometimes isobaric peptides co-eluted in the same UPLC fraction. These peptides could not be distinguished in the conventional MS spectrum, Figure 5, but are clearly separated in the Driftscope IMS view, Figure 6. By performing an IMS-MS/MS experiment (Figure 7), it was possible to assign fragment ions to their precursors as they exhibit the same drift time, see Figure 8. Figure 9 shows that after IMS-MS/MS it was possible to assign both peptides, whereas in a conventional MS/MS experiment only one peptide could be identified.

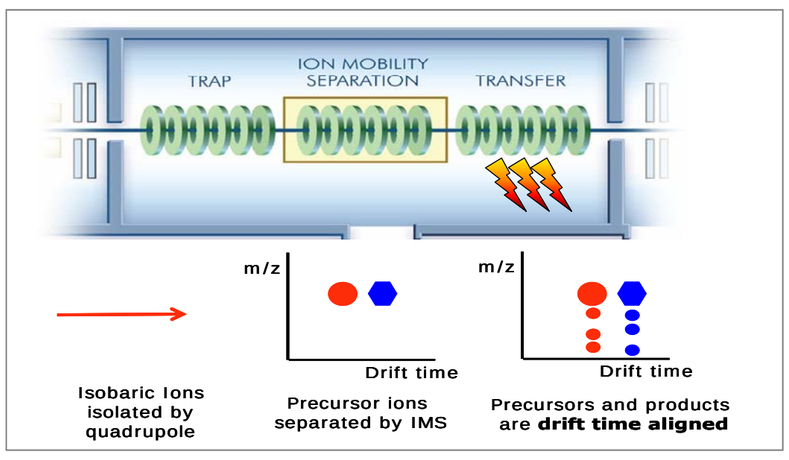

Figure 7. IMS MS/MS is used to separate isobaric species in the drift time dimension. Fragments and precursors are drift time aligned.

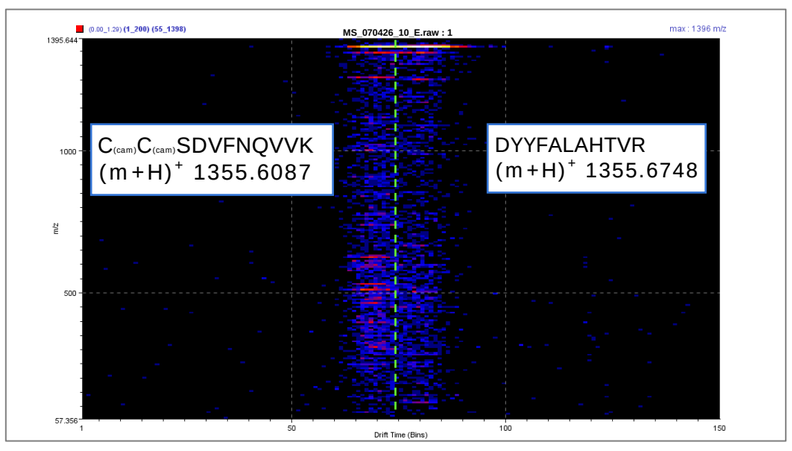

Figure 8. Peptides and their fragments line up according to the mobility of the precursor.

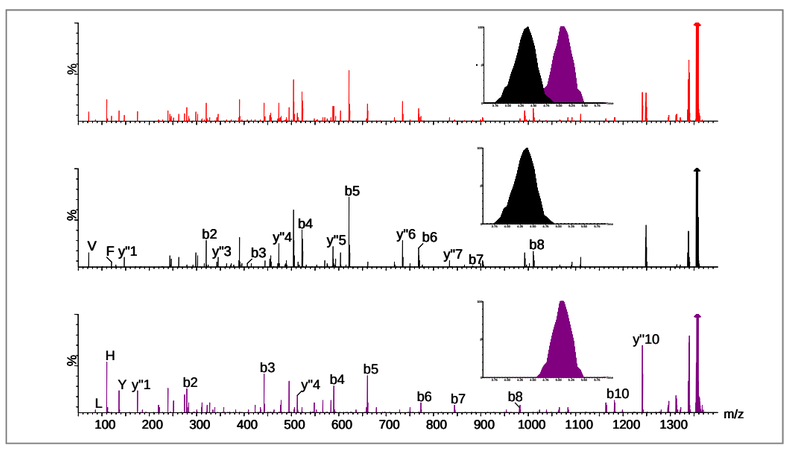

Figure 9. (a) conventional MS/MS spectrum, only one peptide can be identified (b) and (c) after IMS separation both peptides can be assigned.

Analysis of 2D PAGE separated protein samples

Eight *E. coli* 2D gel spots were analysed using the fast LC MALDI method 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.

	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 hydroxymethyltransferase	180	12
Spot 3	GLYA_ECOLI: Serine hydroxymethyltransferase	42	5
Spot 4	GLYA_ECOLI: Serine hydroxymethyltransferase	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.

CONCLUSION

- **IMS-MS and IMS-MS/MS made it possible to separate and characterise co-eluting isobaric peptides which could not be analysed using conventional MS/MS.**
- **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.**
- **UPLC separations with a total chromatographic cycle time of 8min injection to injection were performed.**
- **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.**

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