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PARALLEL MALDI –TOF POST SOURCE DECAY ANALYSIS OF A COMPLEX PROTEIN MIXTURE

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OVERVIEW

- A new MALDI post source decay (PSD) multiplexed (MX) technique is presented.
- This technique allows for the acquisition of PSD spectra from all precursor ions simultaneously, thus removing the serial nature of a conventional MALDI PSD experiment.
- Coupling this approach to an off-line HPLC separation, with deposition of the eluent onto a MALDI target plate, allows for comprehensive investigation of complex peptide mixtures.
- The new parallel MALDI PSD approach is compared and contrasted to MALDI MS/MS on a Q-Tof mass spectrometer.

INTRODUCTION

Liquid chromatography followed by mass spectrometry is the accepted approach for analysing complex protein mixtures. Electrospray ionisation has usually been the preferred technique as it allows comprehensive on-line LC-MS/MS to be performed. However in recent years there has been a growing interest in the use of MALDI ionisation for analysing complex tryptic digest mixtures that have been separated by off-line HPLC. This approach provides complementary information to that obtained by ESI, whilst deposition onto a MALDI target allows samples to be archived for further analysis. In this work a mixture of 15 standard proteins were digested by trypsin and separated by reverse phase HPLC, a separate mixture of *E. coli* cystolic cell fraction proteins were also digested. The eluent from these separations were spotted directly onto MALDI targets using a modified Waters 2700-MS spotting robot. Matrix solution was co-deposited with the HPLC eluent. In total 48 MALDI target wells (15 protein mix) and 24 MALDI target wells (*E. coli*) were analysed with the new Waters MALDI micro MX™ mass spectrometer and a Q-Tof Ultima MALDI™ mass spectrometer.

EXPERIMENTAL

Mass Spectrometry

MALDI micro MX

- The MALDI micro MX incorporates a MALDI source and axial TOF mass analyser with reflectron detector for recording of both MS and PSD MX data. Data were acquired in positive ion mode using automated software control. In MS mode, alcohol dehydrogenase (ADH) digest was used to generate a multi-point external calibration and subsequently an external lock mass correction using ACTH (18-39 clip) was applied. In PSD MX mode, data were calibrated using PSD fragments from ACTH (18-39 clip).

Q-Tof Ultima MALDI

- The Q-Tof Ultima MALDI instrument combines a MALDI source, with a quadrupole as the first mass analyser, which provides for selection of the peptide precursor ions. They are transmitted to a hexapole gas cell where they undergo multiple low-energy collisions to induce collision decomposition using defined collision energy. The product ions produced in the hexapole collision cell are mass measured using the orthogonal acceleration time-of-flight mass spectrometer.

Data were acquired in positive ion mode using the Data Directed Analysis™ (DDA™) software. Polyethylene Glycol (PEG) was used for an external multi-point calibration and subsequently an external lock mass using [Glu]^1-Fibrinopeptide b was applied. Ions were fragmented by collision induced dissociation (CID) to provide MS/MS product ion data.

Sample preparation

15 Protein mixture

- An equimolar mixture of 15 tryptic protein digests was produced containing: Phosphorylase B (rabbit), Serum albumin (bovine), Enolase (yeast), Lysozyme (egg white), Lactoperoxidase (bovine), Glucose oxidase (asp. niger), Carbonic anhydrase (bovine), Catalase (bovine), Alcohol dehydrogenase (yeast), Cytochrome C (horse), Fetuin (bovine), Myoglobin (horse), Alpha Lactalbumin (bovine), Beta casein (bovine), Alpha casein (bovine).
- Each protein was dissolved separately to 100 µg/mL in 0.1 M NH_4HCO_3 and then reduced with DTT (4 h at room temp) and alkylated with iodoacetamide (4 h rooms temp). Each was then digested with sequencing grade trypsin (Promega) for 4 h at 37 °C. The solutions were then diluted and mixed to give a final concentration of 100 fmol/µL.

E. coli cytosolic fraction

- An aliquot of 500 µL of K12 derived *Escherichia coli*, cytosolic fraction, was diluted in 0.1% RapiGest (Waters) in 25 mM ammonium bicarbonate, 0.5 M DTT , 1 M $CaCl_2$ and digested by adding 400 µg of Promega sequencing grade trypsin in resuspension buffer. After digestion at 37 °C for 14 hours, a second aliquot of trypsin was added and the digestion continued for an additional 4 hours. Finally, the sample was centrifuged at 12,000 g for 10 min. The resulting supernatant was collected and diluted five times in an aqueous solution containing 0.1%TFA, to a final protein concentration of 1 µg/µL.

MALDI LC conditions

- The complex tryptic digest mixtures (2 µL of the *E. coli* sample and 5 µL of the 15 protein mixture respectively) were injected and separated by reverse phase chromatography on a Waters CapLC™ system using a Waters NanoEase™ C18 column 150x0.075 mm. The flow rate was 8 µL/min, split to 200 nL/min pre-colum. Peptides were separated following a solvent gradient using mobile phase A (ACN/0.1% TFA in H20, 5:95) and mobile phase B (ACN/0.1% TFA in H20, 95:5): The gradient started with 5% of the organic mobile phase (B) and changed to 40 % B in 90 minutes.
- The entire flow from the HPLC column was spotted onto a 96 well MALDI target plate using the 2700 MS spotting device. A solvent delay of 12 minutes was applied before the beginning of the sample deposition. Each MALDI target position had 30 seconds of HPLC eluent applied, equivalent to 100nL. In parallel, alpha-cyano-4-hydroxycinnamic acid was co-deposited at a rate of1.8 µL/min.

PSD MX TECHNOLOGY

- In a conventional PSD experiment, one precursor ion at a time is selected to pass through an ion-gate and then disassociates via post source decay into fragment ions. In contrast, in a PSD MX experiment there is no ion gate and all precursor ions are transmitted and those precursors that fragment favourably by PSD are recorded.
- As fragment ions from different precursor ions are detected simultaneously, it is necessary to match the fragments to their associated precursor. This is achieved by acquiring two spectra, but at slightly different reflectron voltages. As fragments ions have a unique combination of mass and kinetic energy, which is related to the mass of the precursor. By measuring the shift in time-of-flight between the same fragment ion in the two spectra it is possible to determine the precursor of each fragment.
- Typically, one spectrum is acquired at the same reflectron voltage as for conventional PSD and is referred to as the Major spectrum. The second, or Minor, spectrum is acquired at a reflectron voltage approximately 4% lower.
- In both traditional and parallel PSD experiments, small low energy fragment ions do not penetrate as deeply into the back of the reflectron as their respective precursors and consequently are not as well focussed. This limitation is overcome by acquiring several Major spectra (commonly known as segments) at reduced reflectron voltages. A single fragment ion spectrum is then formed by "stitching" together the focussed regions of each segment.

RESULTS

Multiplexed PSD and Q-Tof data were acquired from a total of 48 wells containing the fractionated mixture of 15 standard proteins. The resulting fragment ion data were combined into a single peaklist file for each instrument and searched against the SwissProt protein database using Mascot (Matrix Science). Table 1 displays a summary of the database search results obtained. Figure 1 shows a comparison of the MS/MS data obtained from a tryptic peptide of Alpha lactalbumin using PSD MX and Q-Tof Ultima MALDI.

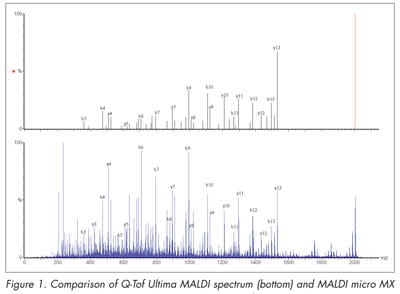

Figure 1. Comparison of Q-Tof Ultima MALDI spectrum (bottom) and MALDI micro MX PSD spectrum (top) of DDQNPHSSNICNISCDK ([M+H$^+$]= 2003.79 Da).

MALDI micro MX			Q-Tof Ultima MALDI	
Score	No. Peptides	Name of protein	Score	No. Peptides
509	34	BSA	1332	30
203	14	Enolase	431	14
132	8	Phosphorylase B	403	18
117	12	Alpha Lactalbumin	120	2
91	7	Myoglobin	221	5
76	3	ADH	330	10
62	3	Glucose Oxidase	129	5
59	5	Fetuin	223	4
58	4	Beta Casein	37	1
48	4	Cytochrome C	84	2
41	4	Lactoperoxidase	259	10
38	2	Carbonic Anhydrase	135	4
—	—	Catalase	123	3
—	—	Alpha Casein	95	3
—	—	Lysozyme	61	2

Table 1. Summary of identified proteins from 15 protein mixture.

- The MALDI micro MX correctly identified 12 of the 15 proteins (where a correct identification is defined as an ion score >37).
- A total of four proteins obtained ion scores greater than the identification level, but were incorrect (false positive identifications).
- The Q-Tof Ultima MALDI correctly identified all 15 proteins.
- However, for 6 of the proteins multiplexed PSD identified more peptides than the Q-Tof Ultima MALDI.

Figure 2 displays MS spectra from six consecutive HPLC fractions of the *E. coli* protein mixture. These spectra clearly show the efficiency of the HPLC separation in that there is little overlap of peptides between adjacent spots.

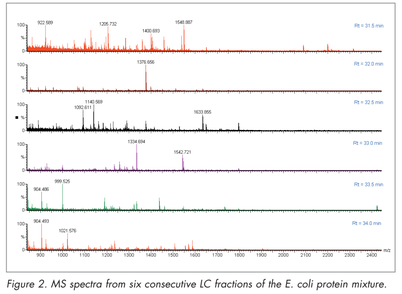

Figure 2. MS spectra from six consecutive LC fractions of the E. coli protein mixture.

Figures 3 and 4 are comparisons of the fragment ion spectra obtained using the Q-Tof Ultima MALDI (bottom) and MALDI micro MX (top) for two different peptides. The data in the two figures were obtained from the peptides DDVAFQIINDELYLDGNAR ([M+H$^+$]= 2180.04 Da) and EGVITVEDGTGLQDELDVVEGMQFDR ([M+H$^+$]= 2851.33 Da) respectively. Both figures show very similar fragmentation patterns however the noise level appears lower on the PSD MX spectrum as a result of the data processing algorithm designed to match fragments to parents.

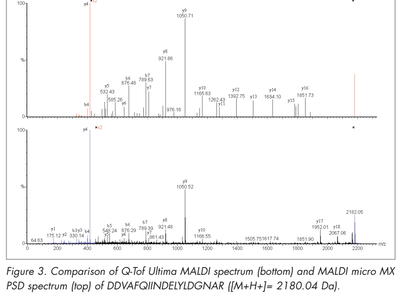

Figure 3. Comparison of Q-Tof Ultima MALDI spectrum (bottom) and MALDI micro MX PSD spectrum (top) of DDVAFQIINDELYLDGNAR ([M+H+]= 2180.04 Da).

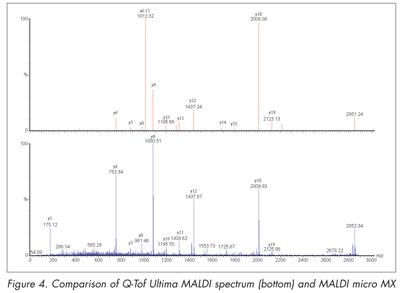

Figure 4. Comparison of Q-Tof Ultima MALDI spectrum (bottom) and MALDI micro MX PSD spectrum (top) of EGVITVEDGTGLQDELDVVEGMQFDR ([M+H+]= 2851.33 Da).

Results from the analysis for both the MALDI micro MX and the Q-Tof Ultima MALDI are summarised in Table 2. Entries in red illustrate a positive identification with a confident score. Those proteins with a yellow background show better identification using MALDI micro MX, whilst those with a grey background show better identification using the Q-Tof Ultima MALDI. Those identifications in black writing illustrate a tentative identification, with a low confidence level.

For each instrument, the fragment ion data from all 24 spots were combined into a single peaklist file, which was database searched using Mascot (Matrix Science).

Name of protein	MH+	Score		MH+	Score	Sequence
DCEA_ECOLI				929.47	22	FPJHEMR
				1085.56	6	RFPJHEMR
	1729.85	28				DGEDPGYTLYDLSER
	2180.92	109		2181.03	96	DDVAFQIINDELYLDGNAR
	895.51	6				HLPEPFR
TNAA_ECOLI				1100.51	1	YADMLAMSAK
	1102.55	54		1102.56	20	FAENAYFK
	1149.54	37+24		1149.55	37	GNFEDLEGLER
	1393.71	22+18		1393.71	22+38	KYDIPVVMDSAR
CH60_ECOLI	1845.88	20				DTTTIIDGVGEEAAIQGR
	2851.24	81		2851.32	83	EGVITVEDGTGLQDELDVVEGMQFDR
EFTU_ECOLI				1027.69	15	
	1218.52	1		1218.58	6	
	1376.68	32		1376.64	46+46+18+23	AFDQIDNAPEEK
	1793.91	5				TKPHVNVVGTIGHVDHGK
	1803.82	58		1803.89	17	GTINTSHVEYDTPTR
YIIJ_ECOLI				1752.85	77	NNISLSQEVQNAQHQR
OSMC_ECOLI	1140.58	26				GQAHWEGDIK
	2155.11	55				GTVSTESGVLNQGPYGFNTR
HDEB_ECOLI	1801.89	76				GGDTVTLNETDITQIPK
ACO1_ECOLI				1427.62	46	FGDDEAFEENVR
	980.52	10				DGLEDYIR
GLPK_ECOLI				1334.66	16+22	EFRPQETTER
	1410.79	32		1410.72	5	YIVAIDQGTTSSR
	1754.91	34		1754.89	16	AVVMDHIDANIISVSQR
WRBA_ECOLI				1257.64	36	QFSQEELSAR
DNAK_ECOLI	1149.58	16				KFEEIVQTR
	2623.28	33				MEIAQGQQHAQGQQTAGADASANINAK
FTSB_ECOLI				1216.57	35	GDWQNEVNVR
EFTS_ECOLI	1742.91	32		1742.87	23+35	VAALEQDVLQSYQHGAR
TALA_ECOLI	1702.88	28				KPMDPYVVEEDPGVK
				1457.66	55	WEHNQDAMAVEK
AHPC_ECOLI	1179.61	8				NGEFEIETEK
	1889.89	21				LGVDYYAVSTDTHFTHK
ODO2_ECOLI	1984.04	27				GQASIEEQGNNDALSPAIR
TIG_ECOLI	1576.82	26				INPAGAPTYVPGEYK
IF2_ECOLI	980.51	10				TSLEDYIR
	2646.12	14				WTDNAEPTEDSSDYIHYTTSQMAR
G3P1_ECOLI	1675.84	23				LVSWYDNETGYSNK
GAT2_ECOLI				1542.72	26	NYIEHFEATDPR
GLR2_ECOLI				1298.62	24	SAFDDFSTPAAR
KDGR_ECOLI				1293.67	20	VIFDNNYRPR

Table 2: Summary of the proteins identified from the analysis of the LC-MALDI target plate with MALDI micro MX and Q-Tof Ultima MALDI. Red Text = positive identification with a significant score. Black Text = tentative identification, with a low confidence level. Yellow background = better identification using MALDI micro MX. Grey background = better identification using the Q-Tof Ultima MALDI.

- The combination of the Maldi micro MX and the Q-Tof Ultima MALDI resulted in the identification of 14 proteins with significant confidence levels from the databank search.
- Of these 14 proteins, 5 proteins were common to the two techniques, whilst 3 proteins were only identified using MALDI micro MX. Conversely a further 6 proteins were only identified using the Q-Tof Ultima MALDI.
- Interestingly there were 7 further proteins that were identified using PSD MX with a score below the positive confidence level. Of these, two were confirmed by subsequent analysis on the Q-Tof Ultima MALDI.
- In addition, 4 proteins were identified using Q-Tof Ultima MALDI with a score below the positive confidence level, whilst analysis of these by MALDI micro MX was able to confirm one of these identifications with a high score.

These results indicate that the use of MALDI on a Q-Tof instrument and parallel PSD on an axial instrument can been seen as complementary techniques. The use of both techniques provides significantly better coverage of the proteins present than either technique alone.

The advantage of the Q-Tof Ultima MALDI instrument is the high mass measurement accuracy. The average mass accuracy on the 21 peptides confidently identified is 2.8 ppm. The mass measurement accuracy of the Q-Tof Ultima MALDI provides a considerable improvement in the specificity of the overall experiment. This enables significantly increased confidence to be obtained from the database search, or *de novo* amino acid sequence to be obtained in certain cases. As can be seen in this dataset, the MS/MS fragmentation pattern from one peptide is sufficient to positively identify a protein.

In comparison, the strength of the PSD MX acquisition is the parallel nature of the experiment. This has significant benefits in terms of automating the acquisition, and greatly simplifies the experiment. For example, the protein OSMC_ECOLI has not been identified with the Q-Tof Ultima MALDI but was confidently identified in the PSD MX experiment. The precursor ions at m/z 1140.6 Da and 2155.1 Da were not automatically selected using the Q-Tof Ultima MALDI as other more intense ions were present in the spectrum, and were selected for MS/MS.

The mass measurement accuracy of both conventional and parallel PSD (<0.5 Da RMS) is not as high as that obtained from the orthogonal acceleration time-of-flight instruments, however the parallel nature of the PSD MX experiment provides complementary information from multiple precursor and fragment ions which in turn enhances the specificity.

SUMMARY

- A novel parallel approach to PSD is presented.
- This allows for the acquisition of PSD spectra from all precursor ions simultaneously, thus removing the serial nature of a conventional MALDI PSD experiment.
- Valuable time and sample are not wasted acquiring data on peptides that do not fragment well by PSD.
- This approach simplifies the automated acquisition of PSD spectra and reduces the time required to generate data from multiple precursors.
- Coupling an off-line nanoscale HPLC separation in combination with deposition onto a MALDI target plate has significant benefits by reducing the sample complexity provided to the mass spectrometer.
- This approach has been compared favourably to data that was obtained from a MALDI source on a Q-Tof instrument
- The two MALDI techniques are complementary and the combination provides more information than either technique alone.

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