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ENHANCED MASS MEASUREMENT ACCURACY IN PROTEOMICS STUDIES BY LC-MS/MS WITH NANO LOCKSPRAY

Alan Millar, Steve Taylor, Jim Langridge, Therese McKenna, Steve Pringle, Robert Bateman, Kevin Giles, John Hoyes and Phil Young

Waters Corporation, Floats Road, Wythenshawe, Manchester, UK.

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OVERVIEW

- To describe the implementation of a dual sprayer nanoflow lockspray source on a Q-Tof mass spectrometer
- The source consists of a dual sprayer arrangement; one for the nanolitre/ min flow rate from the analyte, whilst the second is for the reference compound
- This facilitates routine exact mass measurement for nano scale LC-MS and MS/MS studies
- The focus of this poster is to detail the application of this source in proteomic studies

INTRODUCTION

Due to the complexity encountered in the analysis of proteins obtained from mammalian systems, the primary route for the identification and characterisation of the constituent proteins is electrospray (ESI) LC-MS/MS. The low endogenous levels and large dynamic range of proteins present in these samples dictate that nanoscale LC-MS/MS is often the method of choice due to the concentration dependant nature of the electrospray ionisation technique. This has led to nanoscale LC-MS/MS on a hybrid quadrupole orthogonal acceleration time of flight (Q-Tof) mass spectrometer becoming an established technique for high sensitivity identification and characterisation of proteins. Typically these experiments employ LC columns which have internal diameters of 75 µm, or less, operating at flow rates of approximately 200 nL/min. Whilst this set-up offers the optimum sensitivity it does not allow the post-column addition of an internal reference ion, as this would detrimentally effect the resolution of the LC separation, resulting in peak broadening.

The use of an internal reference is required to provide reliable high mass measurement accuracy. Here we report the use of a nanoflow lock spray interface to routinely provide enhanced mass measurement in the analysis of protein digests.

EXPERIMENTAL

Electrospray LC-MS/MS

- All data were acquired using a Q-Tof *Ultima* API, hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (www.micromass.co.uk).
- The analytical system used for the analysis consisted of a Micromass CapLC with a ten port valve attached directly to the Z spray source of the mass spectrometer, see **figure 1**.
- The HPLC was configured with a pre-concentration column, to allow large volume sample injection and a nanoscale analytical column. The trapping column was packed with 5µM Symmetry C18 (www.Waters.com) stationary phase (300µm ID x 5mm), whilst the analytical column was a 150mm x 75µM column packed with PepMap C18 material (www.lcpackings.com).
- A splitter was employed to provide a flow through the analytical column of 200 nL/min with the pump programmed to deliver a flow of 2 µL/min. The LC gradient ran from 5% to 60% acetonitrile plus 0.1% formic acid in 26 minutes.

MICROMASS MS TECHNOLOGIES

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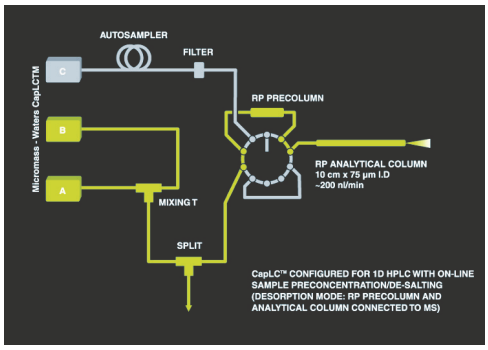

Figure 1. Schematic diagram of the nanoscale LC-MS/MS set-up

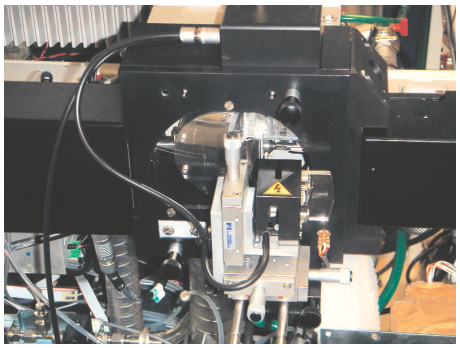

Figure 2. Photograph of the nano-lock spray source, consisting of dual sprayers for both analyte and reference spray

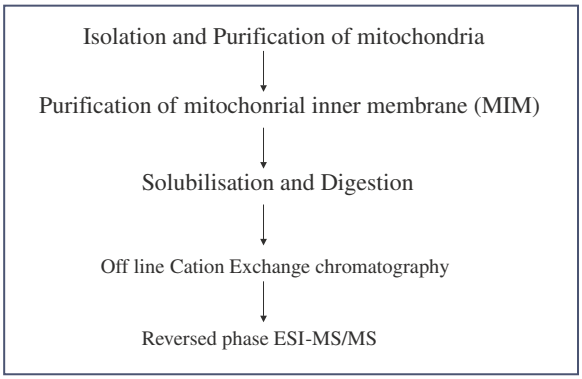

Figure 3. Flow diagram of experimental protocol for the preparation of the mouse mitochondrial inner membrane sample

Nanoflow lockspray

- The nanoflow lock spray interface consists of a dual sprayer, one for the analyte and one for the reference, see **figure 2**. Each spray is sampled individually by the mass spectrometer by means of an electronically controlled baffle plate.

- The software was configured such that the reference spray was sampled for one second in every ten. It is advantageous to separate the reference signal from the analyte signal in order to avoid ion suppression effects and possible interference to the lock mass from ions closely related in m/z. The reference ion used was the doubly charged ion of [Glu^{1}] - Fibrinopeptide B at m/z 785.8426.

- The mass spectrometer was operated in a data dependant acquisition (DDA) mode whereby following the interrogation of MS data, ions were selected for MS/MS analysis based on their intensity and charge state. Collision energies were chosen automatically based on the m/z and charge state of the selected precursor ions.

- The sample investigated in this study was a single fraction obtained from an off-line strong cation exchange (SCX) separation of a mouse mitochondrial inner membrane extract. The sample preparation methodology is summarised in **figure 3**.

DATA PROCESSING

- All data were processed using ProteinLynx Gobal server version 2.0 (www.micromass.co.uk). The processing consisted of automatically lock mass correcting the m/z scale of both the MS and MS/MS data utillising the lock spray reference ion.

- The MS/MS data was also MaxEnt III processed. The purpose of the MaxEnt processing was to simplify the MS/MS spectra by deconvoluting the data to the single charge state and de-isotoping the data. After processing the data was searched against Nrdb.

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RESULTS

- A preliminary list of the proteins identified from the LC-MS/MS analysis of the mouse inner mitochondrial membrane digest sample are presented in **Figure 4**. In total 25 proteins were identified.
- The RMS errors for all the matching peptides obtained from each protein was10.5ppm or better
- During a typical nanoflow LC-MS/MS experiment the tryptic peptides present exhibit a wide dynamic range and as such are detected by the mass spectrometer with varying signal intensities. This provides a challenge to achieving routine exact mass measurement as very intense peaks can cause the detector of the mass spectrometer to become saturated or move into 'dead time'
- Once a single ion has been detected there is a 'dead time' during which further ion arrival events will not be detected. The result is a non-linear response between ions detected vs ions arriving at the detector, this manifests itself in a shift to a lower mass being reported for that particular ion
- An algorithm termed digital dead time correction (DDTC) (www.micromass.co.uk) can be used to correct for this effect. An example of this is presented in **Figure 5** where a doubly charged ion at m/z 681.3 is shown both with and without DDTC applied during processing. The ion was assigned the sequence IQTQPGYANTLR during database searching.
- With DDTC applied the mass measurement error for the ion was -1.9 ppm whilst without DDTC applied the measured error was -19.8 ppm.
- An advantage of the tighter fragment ion mass tolerance is that it can reduce the number of ambiguous peptide matches for proteins that have been confidently identified from the database search result.
- To illustrate this two database searches were performed with the same data set, one with a MS/MS fragment tolerance of 0.05 Da and another with a tolerance of 0.01 Da.
- The matched peptides for the highest ranked protein, Carboamyl-phosphate synthetase 1, from both of database searches outlined above are shown in **Figure 6**.
- It can be seen that tightening the fragment ion tolerance has reduced the number of matched peptides to the identified parent protein. Close inspection of the MS/MS spectra that represent the peptides removed from the matched list reveal them to be of low quality, often being identified on the basis of a low number of ambiguous fragment ions. The tighter fragment ion tolerance acts as a filter to remove these poor quality matches.
- An additional advantage of the tighter fragment ion mass tolerance is that it can reduce the number of ambiguous protein identifications reported for a given database search. These proteins are typically represented by a low number of peptides with a poor correlation between the experimental and theoretical masses in the MS/MS data
- Reducing the number of false positives of this type reduces the amount of manual validation of the data set required and is invaluable in the analysis of complex protein samples by LC-MS/MS.

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Protein descriptions	No of matched peptides	RMS (ppm)
(NM_017072) carboamyl-phosphate synthetase 1 [Rattus norvegicu]	10	6.44
ADT2_MOUSE ADP,ATP carrier protein, fibroblast isoform (ADP/ATP translocase 2) (Adenine nucleotide translocator 2)	7	10.14
(NM_008831) prohibitin [Mus musculu]	6	7.43
(NM_001151) solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator)	5	7.88
ADT1_MOUSE ADP,ATP carrier protein, heart/skeletal muscle isoform T1 (ADP/ATP translocase 1) (Adenine nucleotide translocator	5	7.25
AF401758_1 (AF40175)	4	10.37
(NM_059526) ADP, ATP carrier protein	3	8.99
(AK005084) NADH dehydrogenase (ubiquinone) 1 alpha subcomplex,	3	3.39
(AK002960) ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1	3	6.80
CPSM_ranca carbamoyl-phosphate synthase mitochondrial precursor	3	8.18
(L31362) carbamyl	3	4.75
(NM_009941) cytochrome c oxidase, subunit IV [Mus musculu]	2	5.82
COX2_10418 (NC_001569) cytochrome c oxidase subunit II [Mus musculu]	1	6.98
(NM_053071) cytochrome c oxidase, subunit VIc [Mus musculu]	2	4.03
(NM_015829) solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 13 [Mus musculu]	2	2.72
(NC_003070) mitoch,	1	3.50
(NM_007507) ATP synthase, H+ transporting, mitochondrial F1F0 complex, subunit e [Mus musculu]	1	2.47
(NM_007747) cytochrome c oxidase, subunit Va [Mus musculu]	2	3.06
(NM_009437) thiosulfate sulfurtransferase, mitochondrial Mus musculu]	1	1.77
(NM_025567) cytochrome c-1 [Mus musculu]	1	6.39
(NM_007749) cytochrome c oxidase subunit VIIc [Mus musculu]	1	0.71
AAH06888 (BC006888)	1	6.70
(AK002517) data so	1	4.34
ATP0_OENBI ATP synthase alpha chain, mitochondrial	1	8.34
ATPK_mouse ATP synt,	1	7.09

Figure 4. Proteins identified from the nanoscale LC-MS/MS analysis of the mitochondrial membrane tryptic digest

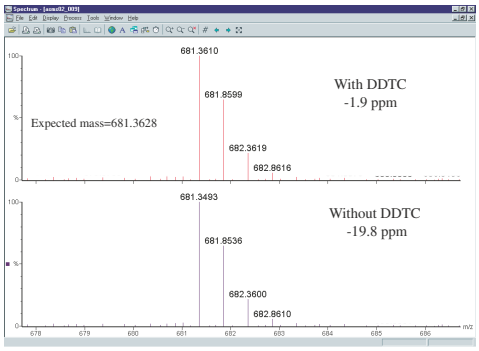

Figure 5. Doubly charged ion at m/z 681 processed both with and without the digital dead time correction algorithm

0.05 Fragment tolerance	0.01 Fragment tolerance
412.90	X
447.25	X
433.23	✓
453.24	✓
582.36	✓
889.47	✓
1140.11	X
480.76	✓
581.80	X
508.24	✓
649.83	✓
611.33	✓
794.39	X

Figure 6. Table of the matched peptides for the protein carboamyl-phosphate synthetase 1 reported in database searches performed with fragment ion tolerances of 0.05 and 0.01 Da

CONCLUSION

- The Nano lockspray source described provides a routine method of achieving enhanced mass measurement in LC-MS/MS experiments
- RMS errors for all the matching peptides constituting the identified parent proteins were 10.5 ppm or better
- Tightening the precursor and fragment ion tolerance reduces the number of 'false positive protein identifications' in the database search results
- A further effect of tightening the fragment ion tolerance is increased confidence in the proteins that are identified

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Author to whom all correspondence should be addressed:
Steve Taylor
Waters Corporation
(Micromass UK Limited)
Floats Road, Wythenshawe
Manchester, M23 9LZ
Tel: + 44 (0) 161 946 2400
Fax: + 44 (0) 161 946 2480
e-mail: steve.taylor@micromass.co.uk

WATERS CORPORATION
34 Maple St.
Milford, MA 01757 U.S.A.
T: 508 478 2000
F: 508 872 1990
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