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A SYSTEMATIC STUDY INTO HPLC AND MS REPRODUCIBILITY IN THE ANALYSIS OF COMPLEX PROTEIN TRYPTIC DIGESTS

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OVERVIEW

- In previous studies we have shown that relative quantification of peptides, across samples, can be accomplished by LC-MS, without the use of isotope labelling.
- In this poster, we have investigated the chromatographic and mass spectrometric reproducibility that can be obtained for the analysis of complex peptide mixtures by LC-MS.
- To enable this comparison we have used automated algorithms to compare and contrast the peptide ions that could be matched across multiple injections.

INTRODUCTION

It has become increasingly clear that relative quantification of protein expression changes is important in modern biology. Several current approaches have been developed that utilise stable isotope labelling of samples in combination with tryptic digestion, separation and subsequent analysis by mass spectrometry [1-2]. However, previously we described an LC-MS strategy [3] where quantification is achieved via normalisation of the LC-MS datasets and comparison of the peptide intensities, (of the observed tryptic peptides), across samples is performed. In this type of experiment, it is desirable to perform replicate injections and this places a requirement upon good chromatography, especially in terms of retention time reproducibility. In addition, exact mass measurement of the eluting ions is required as well as the generation of reproducible and reliable peak intensity, or area, calculations for the eluting tryptic peptides. The ability to measure the mass to charge ratios of ions accurately, between injections and across samples, increases the confidence that the same ions have been matched from each sample injection.

In this poster, we have investigated the reproducibility of an LC-MS system to determine the levels that are required, and that may be expected. In particular we have investigated chromatographic reproducibility at the 75µm scale on the clustering of known peptide ions from run-to-run using an automated algorithm. In addition, we have studied the effect of mass spectrometer resolution on the mass measurement accuracy obtained and the number of peptide species that may be confidently assigned.

METHODS
LC Conditions

- Waters® nanoACQUITY UPLC™ system (direct flow Nanoscale LC) configured in trapping mode with the columns as detailed below:
  Trapping Column: Waters Symmetry® C18, 5µm, 180µm x 20mm.
  Analytical Column: Waters Atlantis® dC18, 3µm, 75µm x 100mm.
- Solvent Compositions: A 99.9% water + 0.1% Formic Acid
  B 99.9% acetonitrile + 0.1% Formic Acid
- Sample loading; Injected and washed on trap with 100% A, at 5µL/min for 3 min.
- Gradient 1: 3 to 40% B in 30minutes at a flow rate of 250nL/min, followed by a high organic wash and re-equilibration.
- Gradient 2: 3 to 40% B in 90minutes at a flow rate of 250nL/min, followed by a high organic wash and re-equilibration.

Mass Spectrometry

- All MS data was acquired on a Waters Micromass® Q-Tof ™ Premier mass spectrometer (Waters, Manchester, UK), operated in positive ion electrospray mode. The MS was fitted with a NanoLockSpray™ source to provide exact mass measurement.
- The reference probe of the NanoLockSpray source was set up to continually infuse a solution containing Glu-Fibrinopeptide b $[M+H]^{2+}$=785.8426 amu; continuously delivered at 500nL/min by the auxiliary pump of the nanoACQUITY UPLC system. The reference mass was sampled every 30 seconds in both acquisition modes described below;
- MS Acquisition Parameters 1:
  Tof MS acquisition, m/z 350—1990 in 0.5 seconds
- MS Acquisition Parameters 2:
  'Expression' (alternating low/ elevated collision energy), m/z 50—1990 in 1.5 seconds. Low CE = 4eV, elevated CE = 15, linearly ramped to 40eV during the scan.
- Data was acquired with the oa-Tof operating in either the V-Optics™ of operation, at a resolution of >10,000 FWHM, or in the W-Optics™ mode of operation with a resolution of >17,500 FWHM.

Samples

Sample 1: 50fmol of a mixture of four standard tryptic digests: Alcohol Dehydrogenase (ADH), Bovine Serum Albumin (BSA), Yeast Enolase and Phosphorylase B (Waters MPDS).
Sample 2: 2µg of a Human Serum tryptic digest.
Sample 3: 0.5µg of an *E. Coli* tryptic digest, containing 50fmol Yeast Enolase.(Waters MPDS).

Bioinformatics

- The LC-MS data contains the exact masses of all detectable peptide molecular ions.
- In the Expression experiment (MS acquisition parameters 2), the elevated energy data provides fragment ion information, relating to the amino acid sequence of the peptide. Hence protein identifications can be obtained by submitting this data to a protein database.
- Interrogation of the acquired raw data, via an automated algorithm [3], extracts all of the relevant peptide information, including; the de-isotoped monoisotopic exact mass, HPLC retention time and peak intensity/area for each detectable peptide. This is known as an Exact Mass Retention Time (EMRT) signature.
- Subsequent comparison of these EMRT signatures across injections (replicates) and conditions (different samples) is performed, and this provides a list of the EMRT "clusters". The algorithm used to match the EMRT's can take account of variability in retention time reproducibility, and mass measurement accuracy from experiment to experiment.
- The data from replicate runs can be interrogated to provide information about the total number of ions and, from this, the number of clustered ions replicating across injections can be calculated.

RESULTS
LC-MS Reproducibility

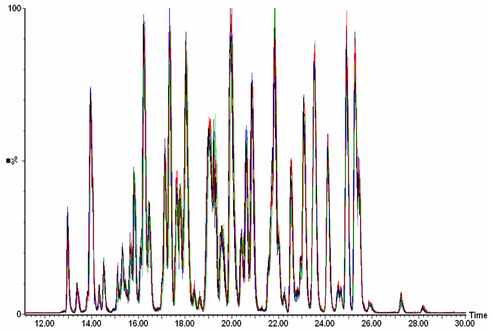

Figure 1 Overlaid Base Peak Intensity (BPI) chromatograms obtained from six replicate injections of sample 1, the four-protein typtic digest mixture. This data was obtained with the nanoACQUITY UPLC running gradient 1 and a 75µm column.

System Reproducibility

Forty injections of sample 2 were made onto a 75µm analytical column over an 80 hour timeframe, with a total of 80µg of digested serum injected. Gradient 2 was used to separate this complex sample and the resulting dataset was divided into two sample 'conditions' for comparison. The EMRT's matching in more than 75% of runs (a total of 3569 ions) were considered and the system reproducibility examined.

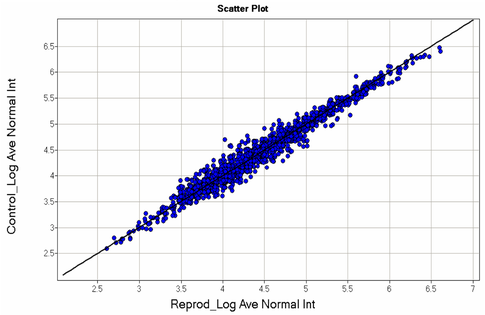

Figure 2 Plot of the averaged normalised intensity (ln) for the matched peptide (EMRT) components between the two serum conditions; injection 1-20 vs injections 21-40. Over 3,500 matching EMRT's are displayed.

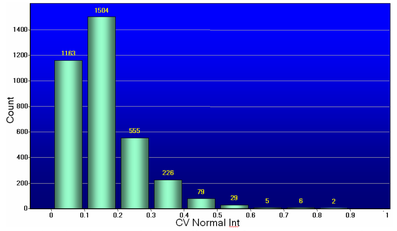

Figure 3 The distribution of the coefficient of variance (CV), of the intensities of the EMRT's, from 40 repeat injections of sample 2. The data shows that 75% of the ions have CV's of less than 20%.

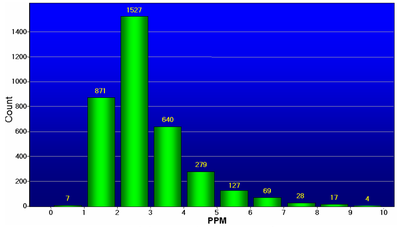

Figure 4 Mass precision of the matched EMRT's from 40 repeat injections of human serum. The measurements for 67% of the matched ions differ by less than 3ppm.

Mass Analyser Stability

An important factor in the matching of ions across multiple runs and over extended time periods is the stability of the mass analyser, both in terms of resolution and mass measurement accuracy.

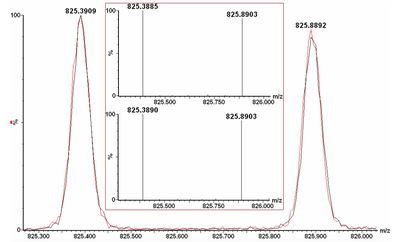

Figure 5 Shows the stability of TOF MS resolution. Displayed is a doubly charged ion (FWHM = 19500) from a tryptic peptide originating from an *E.coli* protein. This data was obtained from repeat injections of sample 3, 28 hours apart. **Figure 5 (inset)** displays the lockmass corrected doubly charged ion. The mass measurement difference observed over the 28-hour period is 0.5mDa or 0.6ppm.

V to W Mode Comparison

Five injections of sample 3 were performed, in both the V and W-Optics, to compare the protein identifications obtained from the LC-MS system. Protein identifications were achieved by processing the raw data and submitting to databank searching.

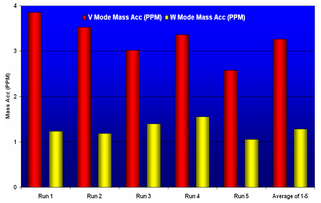

Figure 6 A comparison of the mass accuracies obtained in V-Optics vs W-Optics mode. Plotted is the mass accuracy for the two highest scoring peptides in the top ten proteins identified in each injection. The average error for the V-mode is 3.3ppm whilst the W-mode is 1.29ppm.

	V Mode		W Mode	
Injection	No. Peptides	Score	No. Peptides	Score
1	17	313	15	426
2	15	300	15	417
3	18	325	14	505
4	15	218	17	487
5	16	335	16	490
	Total = 81		Total = 77	

Figure 7 A comparison of the databank search results obtained from the V and W-Optics data, from 5 repeat injections of sample 3. The total number of peptides is slightly lower in the W-Optics mode, however, the improved mass measurement accuracy in W-mode leads to considerably higher protein scores and hence more confident identification. The peptides not apparent in the W-Optics mode, but identified in V-Optics, are of low intensity and provide little fragment ion data.

CONCLUSIONS

- The nanoACQUITY UPLC system exhibits excellent chromatographic reproducibility.
- This LC reproducibility, with CV intensities for the majority of ions of around 20%, in conjunction with excellent mass analyser performance (precision 3ppm and drift of less than 0.6ppm over 28 hours), is important in allowing the EMRT's to be generated and matched between runs that may be separated by long periods of time.
- 'W-Optics mode, where the mass spectrometer resolution is >17500 FWHM, leads to better mass measurement than 'V-Optics' mode at a resolution of >10000 FWHM. This consequently leads to more confident assignments of protein identifications.

REFERENCES

[1]Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R.; Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* 1999 Oct.17(10): 994-9
[2]T.P. Conrads, K. Alving, T.D. Veenstra, M.E. Belov, G.A. Anderson, D.J. Anderson, M.S. Lipton, L. Pasa-Tolic, H.R. Udseth, W.B. Chrisler, B.D. Thrall and R.D. Smith, *Anal. Chem.* **73** (2001), p. 2132
[3]T. McKenna et al; Proceedings of the 52nd ASMS conference, Nashville, TN

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