COMBINING A SUITE OF RAPID PROFILING LC-MS/MS METHODS AND ION MOBILITY WORKFLOWS TO INVESTIGATE THE METABOLOME OF PROSTATE CANCER PATIENTS.

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INTRODUCTION

Analysis of large cohorts of samples from a population is essential in identifying statistically significant markers of diseases like prostate cancer (1). Current diagnosis of prostate cancer is determined using a prostate specific antigen test (PSA), but lack of specificity of PSA to prostate cancer can lead to miss diagnosis (2).

Untargeted metabolomics and lipidomics are useful in determining unknown markers linked to specific conditions, but to fully profile a series of samples requires analysis using several complimentary methods. Therefore, conventional chromatographic separation methods can put a lot of pressure on instrument and personnel resources in addition to suffering from instrumental variation over the course of the analysis (3).

Here we describe the use of a suite of reproducible, complimentary rapid LC-MS/MS metabolic profiling methods, in the analysis of a cohort of serum samples from prostate cancer patients and controls.

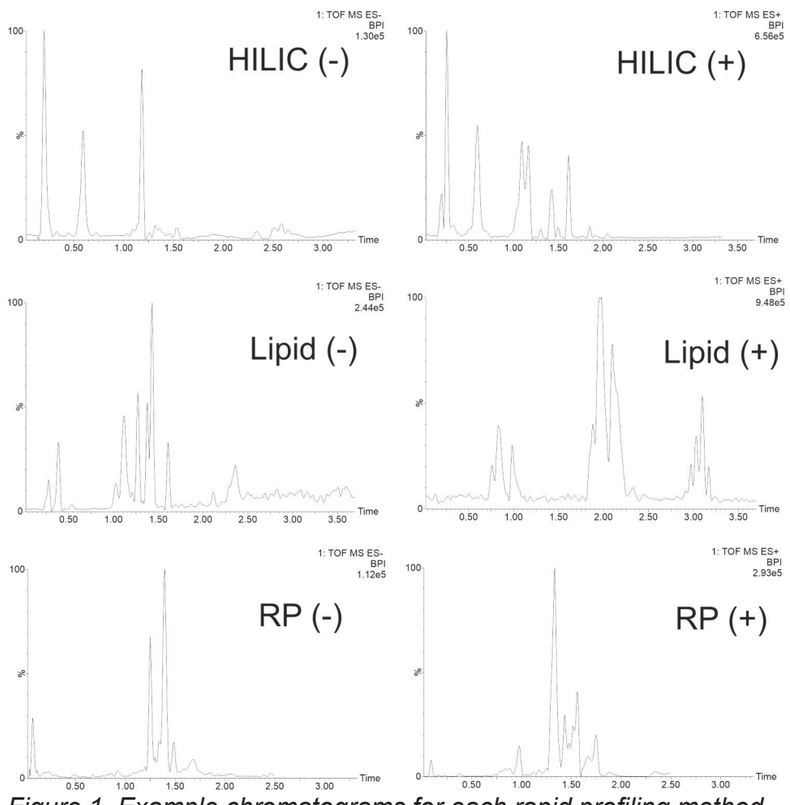

Figure 1. Example chromatograms for each rapid profiling method in positive and negative ESI mode.

METHODS

Rapid LC-MS/MS methods

The three rapid analytical methods employed used reversed-phase and a HILIC separation for polar metabolites and a reversed-phase separation for lipid profiling. Each method has previously been employed individually (4,5,6) and utilised analytical columns with a reduced internal diameter (1 mm). Furthermore, to reduce post column dispersion, a reduced flow ESI probe and reduced diameter peek tubing was also used. Each chromatographic separation was achieved in < 4 mins (Fig.1). Due to the reduction in run time, and the more compressed chromatography, in order to increase the method peak capacity, all acquisition was performed using ion mobility calibrated for collisional cross section (CCS).

The reversed-phase lipid separation was achieved using a Waters BEH C8 column with the small molecule separations using the Waters BEH amide and HSS T3 columns for HILIC and reversed-phase separation respectively.

Sample Preparation

For lipid analysis, each serum sample underwent protein precipitation using cold IPA. The samples were incubated for 2 hours at 2-8 °C prior to centrifuging at 13,000 rpm for 10 minutes. The supernatant was then analysed using the rapid lipid method.

For HILIC analysis, sample underwent protein precipitation using acetonitrile. The samples were incubated for 2 hours at 2-8 °C prior to centrifuging at 13,000 rpm for 10 minutes. The supernatant was then analysed using the rapid HILIC method.

For small molecule reversed-phase analysis, sample underwent protein precipitation using acetonitrile. The samples were incubated for 2 hours at 2-8 °C prior to centrifuging at 13,000 rpm for 10 minutes. The supernatant was then evaporated until dryness and reconstituted in water prior to analysis.

MS acquisition

All data was acquired on a Waters Synapt XS (Waters Corp, UK) using the HDMS^e data independent acquisition mode over the mass range of 50—1200 m/z. The mass spectrometer was calibrated using sodium formate and the ion mobility T-wave was calibrated for CCS using the Major

RESULTS/DISCUSSION

The serum samples (n=350) were acquired in triplicate with pooled quality controls (QC) samples and phenotypic pools for each group of diseased, benign cases and controls. In total, 1120 injections were acquired for each method and ESI polarity.

Compared to conventional analytical methods (~10—15 mins), the suite of rapid profiling methods enabled a reduction in acquisition time of the batch of samples by 2/3, reducing the burden on instrument and analyst logistics.

In order to assess the quality of each rapid profiling method, a selection of endogenous compounds (n=5) for each method were identified in the pooled QC sample, to determine the reproducibility and mass accuracy of the analysis. The data was processed using Progenesis QI (Waters Corp. UK) where the data was aligned, peak picked and normalised.

The response for each compound across the QC injections were plotted to demonstrate the consistency of the MS detection. Figure 2 highlights the response of the 5 compounds selected from the HILIC negative mode analysis and shows a stable response across the 29 QC injections throughout the batch of 1120 injections. All compound ions investigated for all acquisition modes had a %CV of < 30 % across the whole batch as summarised in table 1.

In addition to response, each compounds variation in mass accuracy (Fig. 3) and retention time (Fig.4) were also investigated across the QC injections and additionally summarised in Table 1. Out of the 30 compounds selected, 28 compounds have an average Mass error of less than 5 ppm, while all were below 7 ppm.

Retention time variation for the extracted compound ions deviated less than 8 seconds over the batch for all compounds bar one which had a variation of 11 seconds over the run. The

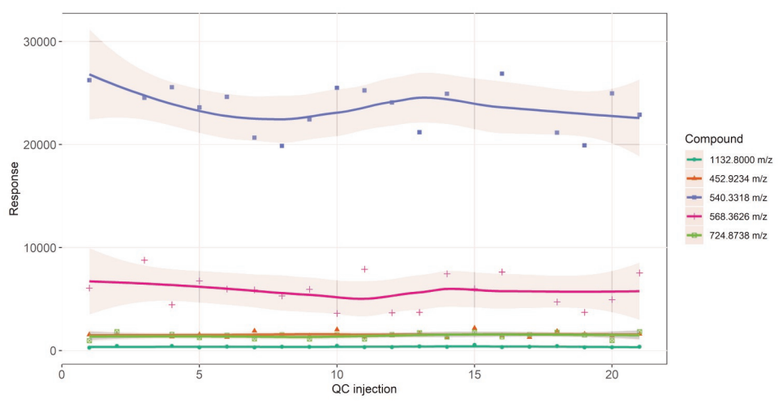

Figure 2. Representative scatter plot of example compound response across the pooled QC samples for the HILIC negative analysis.

Table 1. summary table of endogenous compound reproducibility across 29 QC injections from each of the rapid LC-MS/MS methods.

	Compound	tR (min)	tR Std Dev	tR % CV	Average Response	Response Std Dev	Response %CV	Average Mass Error	Mass error Std Dev
HILIC Negative	391.2853 m/z	0.25	0.007	2.67	166.15	22.02	13.25	-0.15	2.25
	566.3459 m/z	1.18	0.006	0.54	2842.17	303.71	10.69	1.43	2.61
	568.3624 m/z	1.17	0.006	0.51	3361.55	306.19	9.11	1.11	2.40
	540.3322 m/z	1.19	0.009	0.80	10514.71	1171.18	11.14	0.51	2.67
	271.0695 m/z	1.48	0.010	0.64	128.24	14.56	11.35	-0.51	2.74
HILIC Positive	284.2940 m/z	0.21	0.006	2.62	241.82	59.01	24.40	-0.69	3.41
	496.3403 m/z	1.19	0.015	1.26	49725.86	5244.06	10.55	4.83	2.08
	524.3718 m/z	1.17	0.018	1.55	17807.14	2232.71	12.54	0.65	2.54
	834.6005 m/z	0.55	0.043	7.78	13877.93	1048.06	7.55	0.16	2.03
	184.0739 m/z	1.12	0.022	1.92	326.43	75.93	23.26	-0.64	2.36
Lipid Negative	540.3319 m/z	1.35	0.120	8.90	21944.50	5279.72	24.06	0.83	2.42
	283.2643 m/z	1.82	0.135	7.43	6439.66	1009.50	15.68	3.20	2.21
	476.2791 m/z	1.17	0.100	8.57	1097.42	139.69	12.73	1.62	1.78
	769.0104 m/z	0.35	0.046	13.05	419.52	111.98	26.69	0.75	2.06
	305.0229 m/z	0.35	0.049	13.93	201.90	53.89	26.69	0.18	2.08
Lipid Positive	758.5692 m/z	2.20	0.190	8.64	512189.04	75463.13	14.73	4.20	1.96
	496.3400 m/z	1.00	0.143	14.33	147336.50	17371.75	11.79	5.11	2.25
	520.3380 m/z	0.93	0.140	15.11	81419.49	10340.42	12.70	6.13	2.16
	282.2798 m/z	1.14	0.146	12.76	40194.07	7377.55	18.35	4.80	2.07
	822.7538 m/z	3.17	0.144	4.54	17779.69	2127.93	11.97	2.24	2.17
RP Negative	540.3318 m/z	1.27	0.037	2.92	23574.88	2215.08	9.40	0.58	2.21
	568.3626 m/z	1.35	0.016	1.16	5793.07	1588.69	27.42	1.64	2.13
	452.9234 m/z	0.08	0.005	6.11	1558.34	256.03	16.43	-0.89	2.58
	724.8738 m/z	0.08	0.004	5.26	1447.46	278.57	19.25	-0.19	2.15
	1132.8000 m/z	0.08	0.004	5.26	372.38	67.98	18.26	0.51	1.89
RP Positive	444.4046 m/z	1.20	0.147	12.27	1049.68	291.91	27.81	0.46	2.44
	564.3600 m/z	0.89	0.088	9.88	579.77	187.67	32.37	-0.60	2.97
	768.4788 m/z	1.00	0.015	1.54	469.41	120.57	25.69	1.21	3.32
	393.2098 m/z	0.76	0.020	2.69	258.98	61.03	23.57	-0.74	4.30
	608.3861 m/z	0.85	0.018	2.15	463.74	100.05	21.58	-0.62	2.46

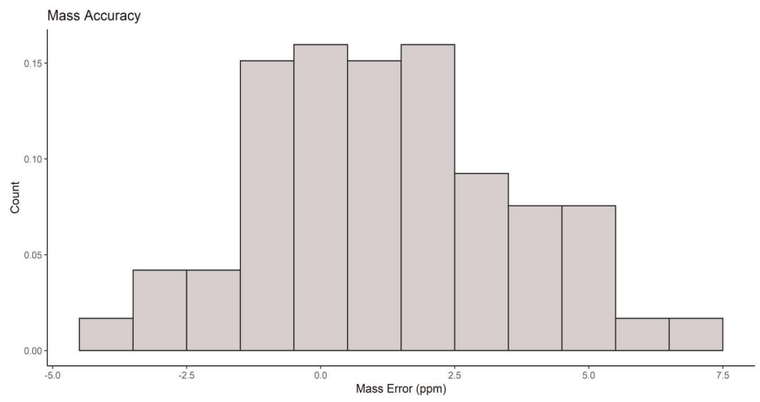

Figure 3. Representative histogram of mass accuracy for the lipid negative analysis.

Following database searches using HDMB and METLIN libraries, in addition to in-house CCS libraries for lipids and small molecules, a series of pharmaceuticals were identified including paracetamol, Ibuprofen in addition to the antihypertensive drug, deserpidine. For the endogenous compounds tentatively identified from the search, the coverage was assessed by grouping by class. Figure 5 highlights the class coverage of compounds from the HILIC negative analysis showing 1/3 of compounds being lipids and lipid like compounds. The next largest class of compounds were benzenoids at 20 % including many pharmaceuticals. Organoheterocyclic compounds like pyrimidine derivatives represented 16 % of the coverage, while organic oxygen compounds (e.g. carbohydrates, sugars) encapsulated 13 % of compounds.

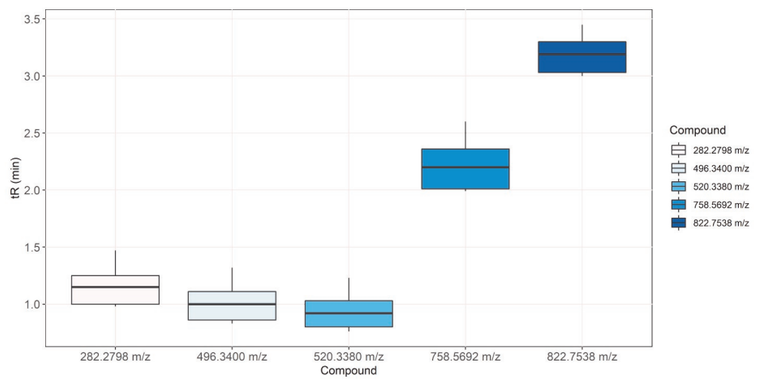

Figure 4. Boxplot of retention time variation for 5 endogenous compounds identified in the rapid lipid positive QC samples.

CONCLUSION

- **Untargeted metabolomic research for biomarker discovery requires accurate reproducible methods.**
- **The rapid suite of LC-MS/MS methods enabled serum profiling of 350 samples in a 1/3 of the time of conventional methods (totalling 6,720 injections).**
- **The methods were reproducible with stable analyte response, retention time and mass accuracy over a large batch of samples.**
- **The complimentary suite of methods enabled analysis of a broad selection metabolites across varying compound classes.**
- **These methods provide a reliable way of screening large cohorts of samples for disease research.**

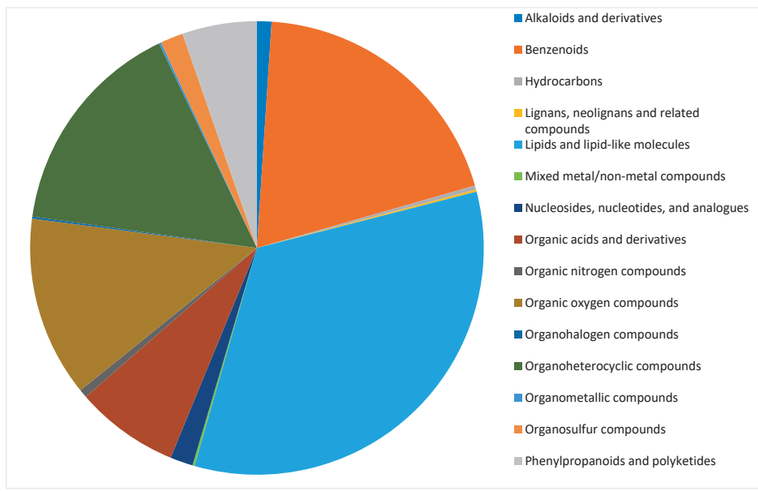

Figure 5. Pie chart of tentative compound class coverage following database searches for the HILIC negative analysis.

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