A new approach to the analysis of intact MUPs (Major Urinary Proteins) from mice by CESI-MS

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Introduction

The Major Urinary Proteins (MUPs) of the house mouse, *Mus musculus domesticus*, are 19 kDa beta-barrel lipocalins that are involved in chemical communication between individuals. Many of them are excreted in urine where they play multiple roles, including coding of owner identity and transport, and slow release of bound volatile pheromones. It has been shown that mass spectrometric analysis of intact proteins, is capable of dissecting subtle structural differences between the members of this class of proteins. Moreover, mass spectrometric analysis of the intact proteins can contribute towards molecular phenotyping of MUPs[1, 2].

A couple of common approaches have been used for the analysis of intact MUPs by LC-MS. The first is direct infusion of desalted samples which have been purified using molecular cut-off filters[1]. The second used a C4 trap cartridge to first trap the proteins before eluting them with formic acid/acetonitrile at 10 µl/min[2] into the MS. These two approaches struggle to discriminate between proteins with small mass differences as charge envelopes would overlap in the LC-MS analysis. Another approach requires pre-fractionation by anionic LC to separate the proteins prior to LC-MS analysis[3]. This workflow is complex and can result in protein loss, negatively impacting the quantitation of proteins in the sample. Therefore, a technique which is able to separate proteins differing by only a few mass units without the need for pre-fractionation would be beneficial.

Capillary Electrospray Ionization (CESI) is the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into a single process in a single device (Figure 1)[4]. CESI-MS operates at low nL/min flow rates and offers several advantages. These include increased ionization efficiency and a reduction in ion suppression. CESI-MS separates analytes by their charge and size and is, therefore, a complementary separation mechanism to more traditional techniques, such as reverse phase LC. The purpose of this study was to investigate the use of CESI-MS in the separation of two desalted MUP extracts.

The study was designed to address two key questions. First, does the separation of MUPs by their charge and size by CESI-MS allow the detection of highly similar proteins by mass spectrometry. Second, can the analysis be performed directly on urine samples without the need for desalting to simplify the workflow and minimize the impact of the relative quantitation of the MUPs.

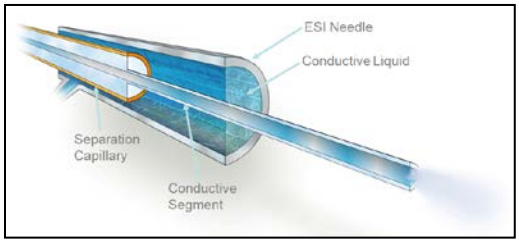

Figure 1: OptiMS® - Ultra low flow ESI Interface.

Materials and Methods

Chemicals: All chemicals were Reagent Grade and were purchased from Sigma Aldrich.
Sample Preparation: The MUPs samples were provided by Centre for Proteome Research in Liverpool University (https://www.liverpool.ac.uk/pfq). Samples were provided desalted into 50 mM ammonium acetate, pH 6.6 using Zeba columns and were at a high protein concentration (10 mg/mL). These extracts were further diluted 1 in 10 into 100 mM ammonium acetate, pH 6.6 which helped with isotachophoresis focusing on the separation capillary.

***CESI-MS method*:** Sample was injected by pressure (5 psi, 5 s) onto a Neutral OptiMS CESI cartridge (30 µm ID x 91 cm, polyacrylamide-coated) which was thermostatted using recirculating liquid coolant regulated at 20°C. For this analysis, the SCIEX TripleTOF® 5600+ mass spectrometer was fitted with the NanoSpray® III source. Gas 1 was not used and the temperature of the interface was set at 60 ºC. Ionization at very low flow rates occurs by simply applying the ionspray voltage (1800 V). The curtain gas was set at 4.2 psi (set automatically). The CE separation used the conditions shown in Table 1 with a background electrolyte (BGE) of 1% formic acid/10% isopropanol (pH 2.05) or 2% acetic acid / 10% isopropanol (pH 2.55). Data was acquired by a split period experiment. For the first period of 1 min the ionspray voltage was switched to 0 V (resulting in no ionspray), data was then collected at 1800 V for the next 36 min. In both periods the DP voltage was set to 100 V and data was acquired in a TOF-MS scan mode over the mass range 800– 2500 amu at a CE voltage of 10 V.

Action	Time (min)	Pressure (psi)	Direction	Voltage (kV)	Solution
Rinse	1.5	100	Reverse	0	10% Acetic acid
Rinse	3	100	Forward	0	0.1 M HCl
Rinse	5	100	Forward	0	BGE
Rinse	5s	5	Reverse	0	Sample Vial
Injection	10s	5	Forward	0	BGE
Separation	35	1.5	Forward	30	BGE
Voltage	2	10	Forward	1	BGE

Table 1: CESI separation conditions used for the detection of MUPs.

Results

In CE, proteins are separated according to differences in their intrinsic electrophoretic mobility. One of the ways to alter separations of proteins is the pH of the BGE as this affects the charge state of the protein. One of the first development experiments tried was to test the effect of the pH by comparing BGE containing 2% acetic acid/10% isopropanol (pH 2.55) with one containing 1% formic acid/10% Isopropanol (pH 2.05).

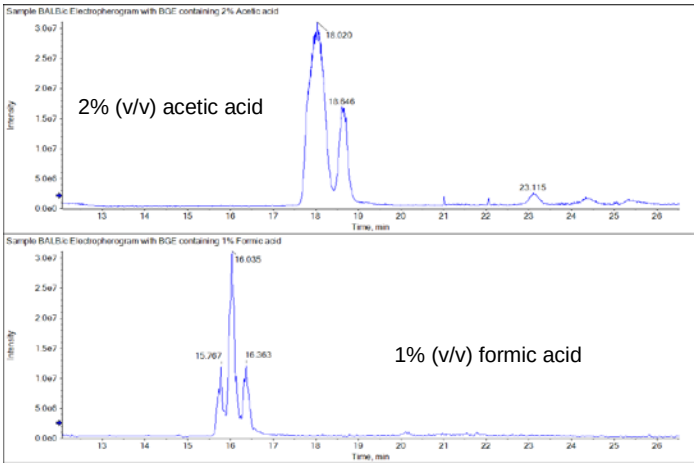

Figure 2: The effect of acid on the separation of MUPs.

Figure 2 shows the effect of changing the BGE pH. MUPs are acidic in nature with isoelectric points ranging from 4.3 to 4.5[5] , In theory changing the pH can improve separations and when the pH of the BGE was decreased, additional separation of the MUPs was observed. The proteins also migrated faster to the mass spectrometer speeding up the analysis. This combination of formic acid and isopropanol was used to compare two different MUP extracts.

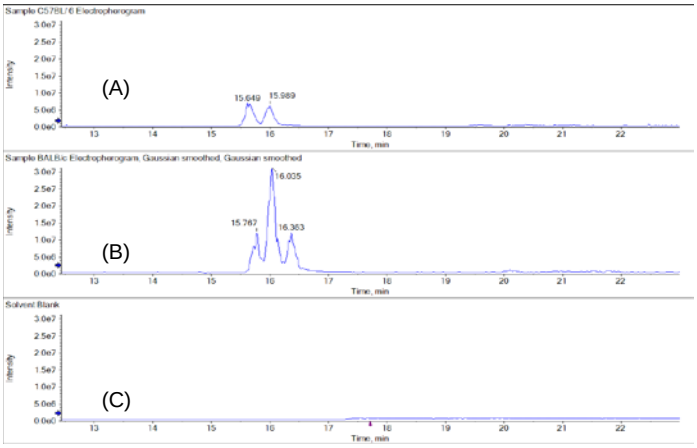

Figure 3: Comparison of samples C57BL/ 6 (A), BALB/c (B) with a solvent blank (C).

Figure 3 shows the total ion chromatogram of urine samples from two different mouse strains compared with a solvent blank and highlighted that CESI was capable of distinguishing the two different mouse strains. When the spectra of the peaks BALB/c and C57BL/6 were compared (Figure 4 and Figure 5) different proteins were separated and identified by CESI-MS (summarized in table 2). There are 5 MUPs which are identical in mass (18964 Da) two were separated by CESI-MS in sample BALB/c which would not have been possible by infusion analysis.

Sample C57BL/6			Sample BALB/c		
Protein MW (Da)	Migration time (min)	MUP [2]	Protein MW (Da)	Migration time (min)	MUP [2]
18708	15.6	10	18708	15.8	10
18645	15.6	7	18645	16.0	7
18893	16.0	20 (Darcin)	18693	16.0	1,12 or 2
18694	16.0	9,11,16,18 or 19	18694	16.3	9,11,16,18 or 19
18713	16.0	14			

Table 2: Major MUPs identified by CESI-MS experiments.

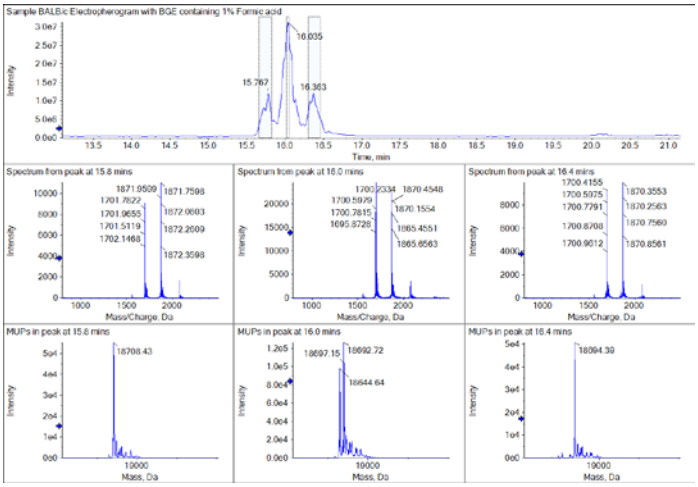

Figure 4: Spectra and de-convoluted protein profiles for sample BALB/c.

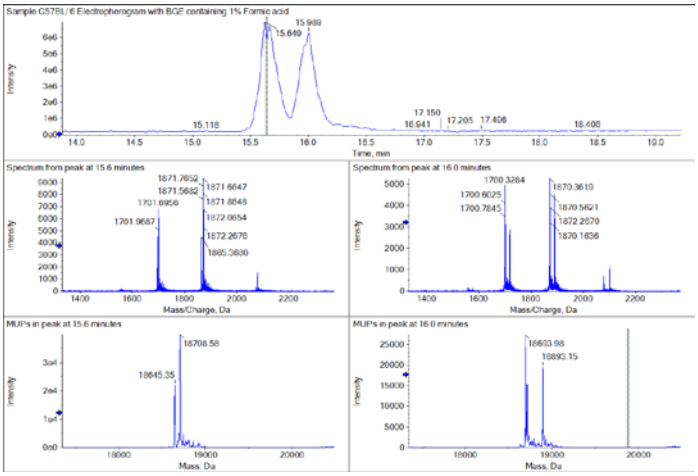

Figure 5: Spectra and de-convoluted protein profiles for sample C57BL/6.

Also oberved was a series of proteins of higher molecular weight, examples of which is shown in Figure 6.

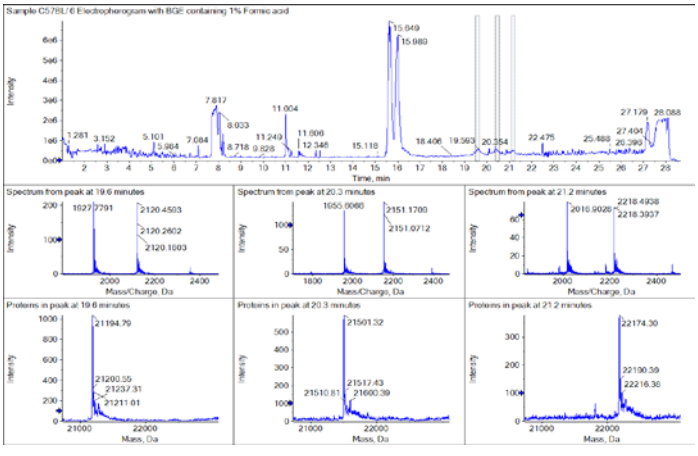

Figure 6: The identification proteins of higher molecular weights in sample C57BL/6.

The final variable investigated in this small study was the effect of salt on the intensity and protein profiles. The samples had been provided desalted so salt was added by mixing human urine with the previous desalted extracts. Each sample contained the same level of protein. In Figure 5 sample A contained no urine, sample B contained 10% urine and C 25% urine. The results show that the addition of human urine to a sample had a very low effect on both the intensity of the spectra obtained and the electropherograms observed.

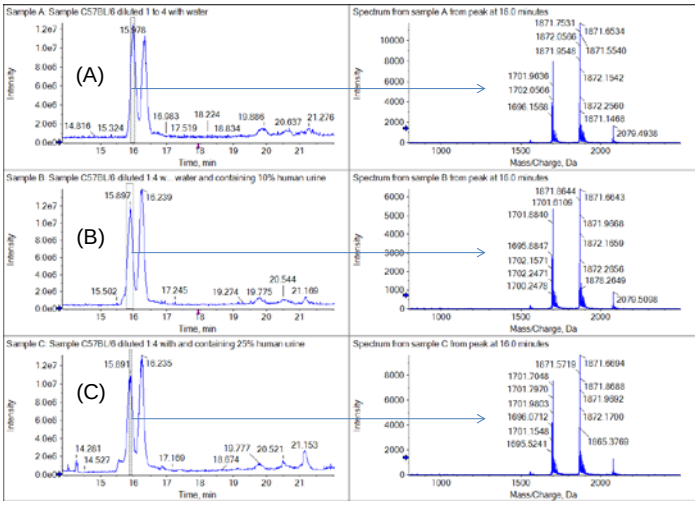

Figure 7: The effect of urine on MUP sample C57BL/6 protein profiles

Conclusions

A CESI-MS method for separation of intact MUPs has been developed. These initial results have shown that CESI-MS is capable of separating this class of proteins based on their charge and was capable of:

- Separating MUPs with the same molecular weight.
- Distinguishing different mouse strains by their MUP profiles.
- Detecting new MUPs of higher mass.

Future work is planned to further evaluate CESI-MS analysis of more complex crude extracts and to also evaluate the effect of salt concentration and sample pH on the overall separation efficiency.

References

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