Sensitive Detection of Peptides in Cytochrome C Digest Using Nano LC with Micro Pillar Array Columns (µPAC™) and Microsaic Real-Time 4500 MiD® Mass Spectrometer

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Introduction

Liquid chromatography coupled with mass spectrometry is a powerful analytical tool for detection and identification of chemicals in complex mixtures. With the low flow capability of Nano LC-MS, higher resolution, ion suppression tolerance and ionisation efficiency is obtained which gives greater chemical sensitivity [1]. The low flow capability of nano-LC couples perfectly with the microfluidics of the Microsaic 4500 MiD® mass detector. The reduced solvent, nitrogen and power consumption of the Microsaic 4500 MiD® provides a lower cost and greener solution for mass detection.

The Microsaic 4500 MiD® is a miniaturised single quadrupole mass detector designed with the chemist in mind. By utilising micro-electro-mechanical systems (MEMS) technology, Microsaic have successfully miniaturised the key components of a mass spectrometer. These patented chip-based technologies are called ionchip®, spraychip® and vac-chip™. The Microsaic 4500 MiD® retains the performance of a conventional MS system but is considerably smaller and less complex to operate and maintain with a substantially reduced cost of ownership. The 4500 MiD® has been designed with the vacuum system, electronics and computer all inside one box meaning the instrument can be installed in places where no other MS can be easily deployed and no floor pump is needed.

PharmaFluidics' µPAC™ technology (micro Pillar Array Column) is a unique and novel approach to a chromatographic support structure and builds upon micromachining chromatographic separation beds into silicon, with exceptional properties that result in excellent chromatographic performance with high-resolution and high sensitivity [2]. The inherent high permeability and low 'on-column' dispersion obtained by the perfect order of the separation bed makes µPAC™ based chromatography unique in its kind and offers several advantages compared to conventional column technologies (packed beds and monoliths). The peak dispersion originating from heterogeneous flow paths in the separation bed is eliminated (no A-term contributions) and therefore components remain much more concentrated (sharp peaks) during separation. The freestanding nature of the pillars also leads to much lower fluidic back pressure allowing the use of very long columns. These exceptional properties result in excellent chromatographic performance with high-resolution and high sensitivity. With a flow rate range between 0.1 and 1.5 µl/min, these columns are ideally suited for a variety of applications where direct interfacing with ESI-MS is needed for sensitive detection and identification of chemicals in complex mixtures.

The compact nature of the Microsaic 4500 MiD® MS system and the combination of low pressure requirements and high separation resolution that PharmaFluidics µPAC™ LC columns can offer have made this combination a perfect fit for performing targeted proteomics studies in an environment where technical resources are limited. Reversed phase separations of cytochrome c tryptic digest are routinely employed for the quality control of HPLC column performance towards proteomics applications. This application note describes the Nano LC-MS analysis of cytochrome C digest using a PharmaFluidics µPAC™ column under various elution programs. The mass spectrometer used was the Microsaic 4500 MiD® alongside the standard UV detector for comparison.

Experimental

The Microsaic 4500 MiD® (Figure 1) was coupled to a Nano LC system (Thermo Fischer Ultimate 3000 nano RSLC), fitted with a Micro Pillar Array Column (PharmaFluidics, 200 cm µPACTM, Figure 2). The Nano LC-MS analysis of 8 peptides present in a cytochrome c tryptic digest at different concentration levels was carried out under the conditions outlined in Table 1. The 4500 MiD® was set to acquire the data in selected ion monitoring (SIM) mode, to produce data with the optimum sensitivity. The peptides of interest, along with the corresponding molecular weights, charge states and m/z ratios are reported in Table 2.

Figure 1: 4500 MiD® mass detector used in the analysis of peptides

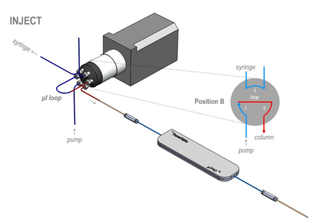

Figure 2: PharmaFluidics 200 cm C18 µPAC™ column

Table 1: System settings for the analysis

Mass detector	Microsaic Systems 4500 MiD®
Ion Mode	Positive
Scan mode	SIM
SIM count time	100 ms
LC Module	Thermo Fischer Ultimate 3000 nano RSLC
Pump solvent A	Water, 0.1% FA
Pump solvent B	Acetonitrile, 0.1% FA
Gradient profiles	Linear gradient separation 1 to 40 % Acetonitrile in 15-30-60-120-240 min
Column	200 cm µPAC™
Pump flow rate	1 µl/min
Injection volume	1 µl

Table 2: Cytochrome c digest peptide peak selection

Peak n°	Amino acid sequence	Theoretical mass (monoisotopic)	m/z value observed	Charge state
1	Ac-GDVEK	588.3	589.8	+1
2	KYIPGTK	805.5		
3	YIPGTK	677.4	339.8	+2
4	IFVQK	633.4	318	+2
5	KTGQAPGFSYTDANK	1538.8	529.6	+3
6	TGQAPGFSYTDANK	1455.7	729.8	+2
7	MIFAGIK	778.4	390.4	+2
8	TGPNLHGLFGR	1167.6	390.4	+3
9	GEREDLIAYLKK	1433.8	478.6	+3
10	EDLIAYLKK	963.5	483.2	+2
11	GITWGEETLMEYLENPKK	2137		
12	GITWGEETLMEYLENPK	2008.9	672	+3

RESULTS AND DISCUSSIONS

One of the main aims of this evaluation was to determine the detection sensitivity of the mass detector in comparison with the UV detector. This was done by performing Nano LC-MS analysis of a tryptic digest of cytochrome c (8 different peptides) at varying concentrations (from 10 to 500 fmol/µl), collecting both the UV and MS data as shown in Figure 3. The signal-to-noise (S/N) ratios of both UV and MS detection are shown in Figure 4 for a selected set of peptides. It is clearly demonstrated that UV detection of peptides becomes problematic at concentrations below 100 fmol/µl, with the S/N ratios dropping below 5, while the MS detector still provides sensitive detection with sharp and distinctive peaks and S/N ratios above 10 for concentrations as low as 25 fmol/µl. Based on the data collected, the 4500 MiD® MS is on average 40 times more sensitive than the UV detector.

The peak width (4σ) of the peptide eluting at around 15.5 min (YIPGTK, m/z 339.8) was calculated at 0.11 min when MS was connected directly to the column. In comparison, the peak width was 0.19 min when the MS was used in tandem after UV detector, as shown in Figure 5. When used in tandem, an increase in peak width can be expected with the introduction of an extra connecting union, which can be a very delicate practice in nano LC analyses. On average, peak widths were reduced 1.5 times by connecting the column directly to the MS system. In combination with an increase in peak shape symmetry, this will yield even higher S/N ratios. Therefore MS detection alone was used later for the evaluation of the peak capacity of the µPACTM columns.

The separation performance of LC columns is often expressed as peak capacity, a parameter that takes both peak width and the time frame in which a separation occurs into account (equation 1). By plotting peak capacity as a function of the gradient time, a fair comparison towards chromatographic performance per time unit can be made between column types and conditions. For each condition, peak capacities were calculated according to equation (1) using the peak width of 7 baseline separated tryptic peptides (peptides 3,4,5,6,7,9 & 10, listed in Table 1).

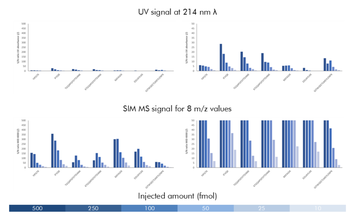

Figure 4: Cytochrome C digest S/N ratio for selected peptides

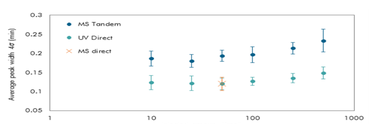

Figure 5: Average peak width comparison between UV and MS

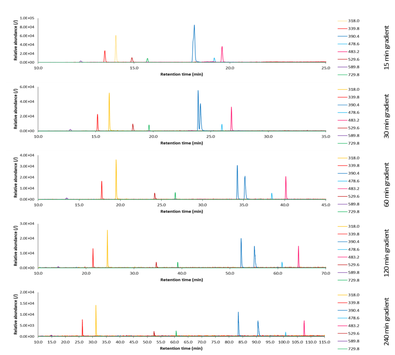

Figure 7: SIM MS chromatograms obtained for 9 peptides in a cytochrome c digest sample.

A significant gain in peak capacity can be observed when comparing the MS data obtained in this study to data previously obtained with UV detection. This gain can be attributed to the fact that MS detection allows operating the µPAC™ column under optimal sample loading conditions. For UV detection, a minimum sample concentration of 500 fmol/µl is needed to yield sufficient S/N ratios for long gradient (>120 min) separations, hereby slightly overloading the µPAC™ column. MS detection allows generating high quality chromatograms with only a fraction of this sample load. Therefore column overload can be avoided by working with sample concentrations down to 50 fmol/µl.

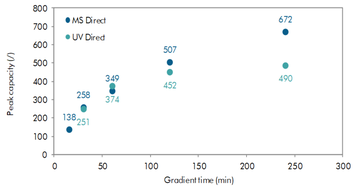

Figure 6: Peak capacity on a µPAC™ column.

$$(1) \quad n_c = 1 + \frac{t_g}{(\sum_1^i 4.\sigma_i)/i} = 1 + \frac{t_g}{\bar{w}_{13\%}}$$

MS data obtained for the gradient separation of 50 fmol cytochrome c digest are shown in Figure 7. SIM traces for 8 m/z ratios which correspond to tryptic peptides 1,3,4,5,6,7,9 &10 in Table 1 have been monitored. By increasing the solvent gradient time from 15 to 240 minutes, an increase in separation resolution is clearly accomplished. This increase in resolution becomes tangible when looking at the SIM MS trace obtained for m/z ratio 390.4. The cytochrome c digest sample contains 2 tryptic peptides which are detected at the same m/z ratio, but have different charge states. The peptide with amino acid sequence MIFAGIK has a molecular weight of 778.4 and is doubly (2+) charged, whereas the peptide with sequence TGPNLHGLFGR has a molecular weight of 1167.6 and is triply (3+) charged. In the 15 min gradient separation, both peptides are co-eluting and detected as 1 relatively broad peak. By extending the gradient time to 30 minutes, these two peptides are gradually getting separated from each other. As from 60 min gradient times, baseline separation is observed for these two peptides, indicating longer separation time is needed when separating complex mixtures.

CONCLUSIONS

The Microsaic 4500 MiD® proved to be an easy-to-use mass spectrometer, with the ability to effortlessly couple to a Nano LC system for LC-MS analysis. This report indicates the 4500 MiD® was able to detect low fmol quantities, whereas, the UV detection of protein digest samples becomes problematic when less than 100 fmol is injected (S/N<5). The 4500 MiD® MS system is on average 40 times more sensitive than UV detection and allows proper detection of protein digest samples down to 10 fmol.

Excellent peptide peak widths (down to 0.11 min) can be obtained with minimum dead volume in the connection to the 4500 MiD® MS system. Protein digest separations can be monitored at optimal sample loading concentrations for µPAC™ columns with the 4500 MiD® MS system, enabling higher peak capacity values than observed with UV detection.

The addition of the 4500 MiD® for Nano LC-MS analysis greatly improves the ability of peptide identification during each LC run, which is demonstrated in this report by the Nano LC-MS analysis of a cytochrome c digest. The compact nature and the microfluidics of the 4500 MiD® makes it an ideal upgrade for existing nano LC instrumentation. In combination with µPAC™ column technology, the 4500 MiD® system allows performing high resolution analyses of peptide mixtures in an environment where analytical resources are limited with 40 times more sensitivities than the UV detection.

ACKNOWLEDGEMENT

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REFERENCES

[1] Steven Ray Wilson et al, Nano-LC in proteomics: recent advances and approaches, Bioanalysis, August 14(2015), 1799-1815

[2] W. De Malsche, J. Op De Beeck, S. De Bruyne, H. Gardeniers, G. Desmet, Realization of 1 × 106 Theoretical Plates in Liquid Chromatography Using Very Long Pillar Array Columns, Anal. Chem. 84 (2012) 1214-1219

About Microsaic

Founded in 2001, Microsaic Systems plc (AIM: MSYS) is a high technology company which develops point-of-need mass spectrometers, designed to improve the efficiency of chemical and biological workflows.

Microsaic's core products, the compact MiD series of mass detectors, are designed to integrate seamlessly with a wide range of third party OEM equipment providers, or to be standalone. At the forefront of our design ethos is to deliver fast, easy to use, powerful mass spectrometry (MS) performance.

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