Novel On-Line Multidimensional Low-Flow LCMS Setups for Comprehensive and Fast Proteome Profiling

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ABSTRACT

Online 2D low-flow LC-MS/MS is a powerful separation tool capable of significantly increasing peptide and protein identifications in challenging sample matrices by significantly reducing complexity.
Here we propose a simple, online-2D reversed phase x reversed phase approach which affords deep proteome coverage within typical nano-flow LCMS analysis time frames.
The setup is based on Thermo Scientific™ UltiMate™ 3000 RSLCnano system coupled to a Thermo Scientific™ Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. The separation of peptides in the first dimension was achieved using a high pH stable Thermo Scientific™ PepSwift™ Monolithic Capillary Column. After 1st dimension fractionation, peptides were separated on a Thermo Scientific™ EASY-Spray™ LC Column at low pH.
This online 2D approach is easy to setup and provides excellent separation robustness in both the first and second dimensions, rendering it highly applicable to the routine analysis of complex biological matrices.

INTRODUCTION

Despite many recent technical advances, proteomics researchers are still forced to choose between "long, deep and slow" vs "short, shallow and fast" when it comes to running their bottom-up proteomics LCMS analyses. Thus necessitating a tradeoff between throughput and depth of proteome coverage and / or quantification.
Due to the complexity of the human proteome coupled with the increasing demand for high throughput, there is a clear requirement for additional "quick but comprehensive" approaches for the analysis of such samples. To this end, on-line and off-line 2D and multidimensional separation methods have drawn substantial attention during recent years. The tendency for the combination of strong cation exchange (SCX) with RP chromatography for peptides fractionation has been replaced by a combination of high pH reversed-phase (RP) chromatography followed by low pH reversed-phase LC MS/MS. This is explained by significantly better efficiency of peptide separation under reversed-phase conditions that is more important than the better orthogonality of SCX-pH.
Offline high-pH reversed-phase fractionation is a standard approach for reducing sample complexity after labelling or for comprehensive proteome profiling [1]. However, offline 2D, has several disadvantages, e.g. low throughput, and the requirement for high amounts of sample. It is also prone to sample loss during fraction collection and transfer between fractionation devices and analytical instruments.

The work presented here is a proof-of-principle study that demonstrates the feasibility of a simple online high-pH low-pH low-flow RP x RP separation approach that helps to reduce sample complexity, can easily be adopted to the number of required fractions and doesn't require manual manipulation of fractions during the analysis.

MATERIALS AND METHODS

Online 2D low-flow LC System

The UltiMate 3000 RSLCnano system comprised of an NCS-3500RS, NCP-3200RS, WPS-3000TPLRS and VWD-3400RS modules (Figure 1) was configured as an online 2D setup based on the schematic shown in Figure 2. Only one 10-port 2-position switching valve installed in the column compartment is required to run the online 2D configuration. The micro-flow loading pump Integrated in the NCS-3500RS module was used to dilute and acidify the eluate from the first dimension to ensure proper retention of peptides prior to separation in the second dimension. A PepSwift Monolithic Capillary Column (200 µm x 250 mm, PN 164542) was used to separate peptides in the first dimension. These are then concentrated onto one of two 300µm x 5mm trap cartridges (P/N 160454). An EASY-Spray column (75 µm x 150mm, 3µm, P/N ES800A) was subsequently used to separate peptides in the second dimension. It was coupled with a Q Exactive HF-X HRAM mass-spectrometer equipped with an EASY-Spray source operated in Full MS mode.
In the first dimension the mobile phase A was water with 10 mM ammonium bicarbonate and mobile phase B was 80% ACN with 10 mM ammonium bicarbonate. The separation was performed at 1.5 µL/min. A Tee-piece was used to combine the eluate from the 1st dimension with an 8.5 µL/min flow of 0.05 % trifluoracetic (TFA) in water make-up flow from loading pump which was used to acidify the eluate and transfer peptides onto one of two trap cartridges (1 or 2, Figure 2).
In the second dimension mobile phase A was water with 0.1% formic acid (FA) and mobile phase B was 80% ACN with 0.1% FA. The peptides were separated at 0.6 µL/min. Thermo Scientific™ Pierce™ HeLa Protein Digest Standard at a concentration of 200ng/µL, 5 µL per injection.

Data Acquisition and Analysis

Data were acquired with Thermo Scientific™ Xcalibur™ 4.1 software. The RSLCnano system was controlled with Thermo Scientific™ Standard Instrument Integration (SII) software for Xcalibur™. Data were processed using Thermo Scientific™ FreeStyle™ software v. 1.5.

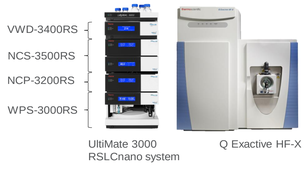
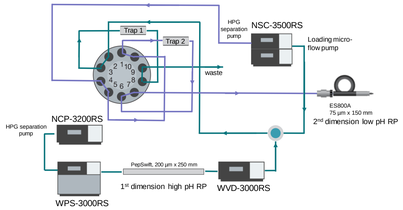


Figure 1. On-line 2D UltiMate 3000 RSLCnano system coupled with Q Exactive HF-X mass-spectrometer

Figure 2. Schematic of the fluidic setup for the on-line 2D high pH RP x low pH RP configuration

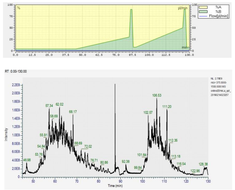


RESULTS

State-of-the-art LC-MS platforms for shotgun proteomics, optimized for sensitivity and resolution are typically associated with long analysis times, long washing and equilibration steps and long sample loading times. We developed an online 2D RPxRP method that splits the sample into fractions that are independently analyzed with HRAM MS within the time frame of a typical nanoLCMS experiment (ca. 2 hours). The sample is separated within 90 min in the 1st dimension resulting in 2 fractions that are automatically collected onto trap cartridges and analyzed with low-flow LCMS in the 2nd dimension (Figure 3, 4). Utilizing a relatively short column in the second dimension and elevated flow rate (0.6 µL/min) we were able to achieve high MS utilization for this experiment. We previously showed that this type high throughput nanoLC approach adopted here for the second dimension permits a throughput of 180 samples analyzed per 24 hours [2].

Figure 3. Gradient for high pH reversed-phase peptide separation in the 1st dimension on a monolithic polymeric capillary column including time stamps for fractionation (indicated by valve switching)

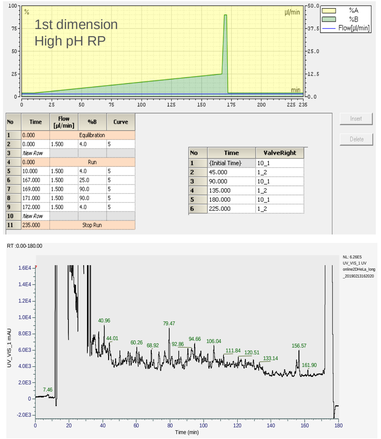
Figure 4. Gradient for the low pH reversed-phase peptide separation of 2 fractions in the 2nd dimension and corresponding TIC profiles



ONLINE 2D LC WITHIN TYPICAL NANOLCMS ANALYSIS TIME FRAME

The goal to achieve deeper proteome coverage requires longer analysis times to allow the MS to collect a sufficient number of fragmentation spectra, larger sample loading amounts to improve dynamic range, and more fractions to reduce sample complexity. We tested a 180 min gradient in the 1st dimension that allows to collect and analyze 4 fractions in ca. 4 hours in the second dimension (Figure 5, 6). It is well known, that high pH x low pH RP x RP separations have limited orthogonality as separation in both dimensions is strongly dependent on peptide hydrophobicity. Thus, to improve MS utilization we programmed 2nd dimension gradients with a staggered increase of mobile phase B portion at the start and end of the gradient to ensure that peptides are well distributed over the 2nd dimension in each fraction (Figure 6, 7).

Figure 5. Gradient for high pH reversed-phase peptide separation in the 1st dimension, time stamps for fractionation and corresponding UV chromatogram



No	Time	Flow [µL/min]	%B	Curve
1	0.000		Equilibration	
2	0.000	1.500	4.0	5
3	flow #1ow			
4	0.000		Run	
5	10.000	1.500	4.0	5
6	167.000	1.500	25.0	5
7	169.000	1.500	90.0	5
8	171.000	1.500	90.0	5
9	172.000	1.500	4.0	5
10	flow #1ow			
11	235.000		Stop Run	

No	Time	ValveRight
1	(Initial Time)	10_1
2	45.000	1_2
3	90.000	10_1
4	135.000	1_2
5	180.000	10_1
6	225.000	1_2

Figure 6. Gradient for low pH reversed-phase peptide separation of 4 fraction in the 2md dimension on ES800A column

In order to check the overlap of peptides between adjacent fractions, we investigated extracted ion chromatograms for 6 peptides from high-abundant proteins in HeLa cells (Table 1). Peptide peaks were symmetric in each fraction.

This shows that high quality separations can be obtained for each fraction and hydrophilic as well as hydrophobic peptides. We did not observe significant overlap for selected peptides between fractions.

Figure 7. TIC profiles of 4 fractions and EIC for peptides distributed over 4 fractions (see Table 1 for more details)

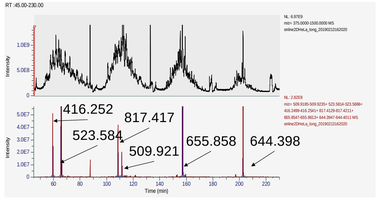


Table 1. Chromatographic characteristics for 6 selected proteotypic peptides of HeLa cell lysate distributed among the 4 fractions

Peptide	Protein	Acc. No.	m/z	Ret. time, min	PW base, min
[K].VDNDENEHQLSLR.[T]	Nucleophosmin	P06748	523.584	59.5	0.9
[K].STELLIR.[K]	Histone H3.2	Q71DI3	416.252	65.7	1.2
[K].VNQIGSVTESIQAck.[L]	Beta-enolase	P13929-1	817.417	108.5	0.9
[K].SLTNDWEDHLAVK.[H]	Heat shock protein HSP 90-beta,	P08238	509.921	111.4	1.4
[K].TVTAMDVVYALK.[R]	Histone H4	P62805	655.858	157.1	0.9
[KR].VTIAQGGVLPNIQAVLLPK.[K]	Histone H2A type 2-C	Q16777	644.398	202.58	0.9

ROBUSTNESS OF ONLINE 2D SEPARATIONS

Multidimensional methods are often criticized for insufficient reproducibility of results. The developed online 2D approach provides reproducible separation profiles in both the first and second dimensions (Figures 8, 9) with retention time precision on a par with one-dimensional separations. This level of reproducibility will allow not only deep proteome profiling, but also the development of scheduled targeted LCMS methods for complex samples.

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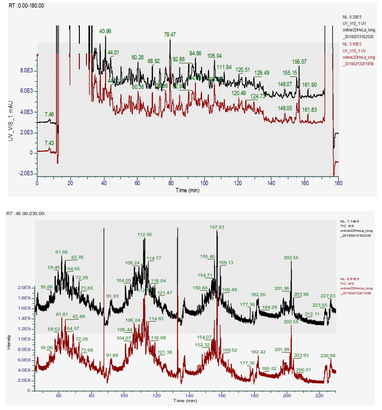
Figure 8. Overlay of UV traces and TIC profiles for 2 cosequtive replicates of online 2D reversed phase x reversed phase separation of 1 µg HeLa cell lysate protein digest



CONCLUSIONS

We developed a novel and simple approach for multidimensional low-flow LCMS analysis of complex samples that provides high reproducibility of results and that can be utilized for separations within a typical nanoLCMS experimental time frame.

We show how the resolving power of the UltiMate 3000 RSLCnano system coupled with the latest state-of the art HRAM MS technology and advanced phase column chemistries can be combined to create a new standard in the speed and depth of proteome profiling.

The developed online 2D approach represents a promising alternative to long one-dimensional separations for shotgun proteomics as well as targeted analysis in complexes matrices.

REFERENCES

1. RSLCnano pre-concentration nano LC kit (P/N 6720.0310) in the Ultimate 3000 RSLCnano Standard Applications Guide. Hyperlink

2. Boychenko, A.; Pynn, C.; van den Berg, B.; Arrey, T, N., Baynham, M.; Decrop, R.; Ruehl, M. High-throughput capillary-flow LC-MS proteomics with maximum MS utilization. TN 72227. Hyperlink

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