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Low-flow On-line 2D LCMS Hyphenated with HRAM MS for Comprehensive Proteome Profiling

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ABSTRACT

Purpose: Develop an automated on-line 2D setup and corresponding low-flow LC-MS/MS methods for deep proteome profiling of plasma and cell digest.

Methods: The developed on-line 2D 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 captures on trap columns and separated on a Thermo Scientific™ EASY-Spray™ LC Column at low pH.

Results: We propose a simple, online-2D reversed phase high pH x reversed phase low pH approach which affords deep proteome coverage within typical nano-flow LCMS analysis time frames, provides the ability to use long LC-MS/MS runs with high sample loading without negative effects on the peak shape and peak width. 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The tendency for the combination of strong cation exchange (SCX) with RP chromatography for peptides fractionation has been replaced by a combination of high pH reversed-phase (RP) chromatography followed by low pH reversed-phase LC MS/MS.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.

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

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 (100 µm x 250 mm, PN 164543) was used to separate peptides in the first dimension. These are then concentrated onto one of two trap columns (75µm x 20mm). 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 or DDA 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 0.3 µL/min. A Tee-piece was used to combine the eluate from the 1st dimension with a 2.7 µL/min 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 columns (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 0.4µL/min. Thermo Scientific™ Pierce™ HeLa Protein Digest Standard at a concentration of 200ng/µL or 1.0µg/µL and plasma protein digests were used to test online 2D LC-MS methods with total length of 135min (2 fractions), 225min (4 fractions) and 405min (8 fractions) (Table 1).

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 and Thermo Scientific™ Proteome Discoverer™ software.

RESULTS

State-of-the-art LC-MS platforms for shotgun proteomics, optimized for sensitivity and resolution are typically associated with a long analysis times, long washing and equilibration steps and long sample loading times. We developed an online 2D high pH RP x low pH RP method that splits the sample into fractions that are independently analyzed with HRAM MS. The sample is separated within long gradient in the 1st dimension resulting in multiple 45 min fractions that are automatically collected onto trap columns and analyzed with low-flow LCMS in the 2nd dimension (Table 1). Utilizing a relatively short column in the second dimension and elevated flow rate (1.3 µL/min for loading, washing, equilibration and 0.4 µL/min for separation) we were able to achieve high MS utilization for this experiment. We previously showed that high-throughput nanoLC approach adopted here for the second dimension permits a throughput of 30 samples analyzed per 24 hours [2].

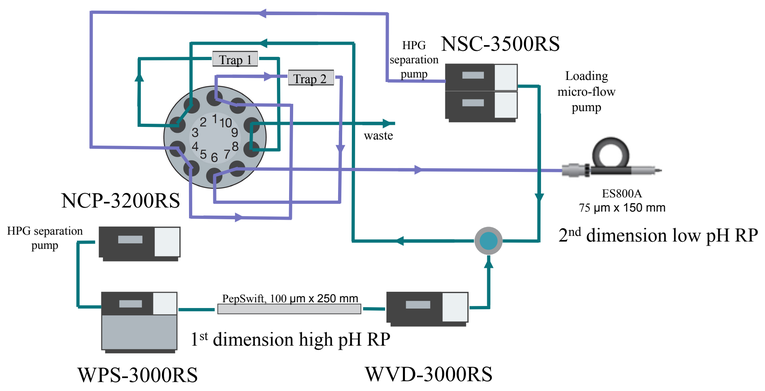
Table 1. On-line 2D LC-MS/MS methods that were tested in this work for cell lysate and plasma digest proteome profiling

Number of fractions	1st dimension gradient length	2nd dimension gradient length	Total run length	Tested samples
2	70 min	45 min	135	Plasma
4	160 min	45 min	225	Plasma, HeLa
8	340 min	45 min	405	Plasma, HeLa

ONLINE 2D LC SETUP AND METHODS

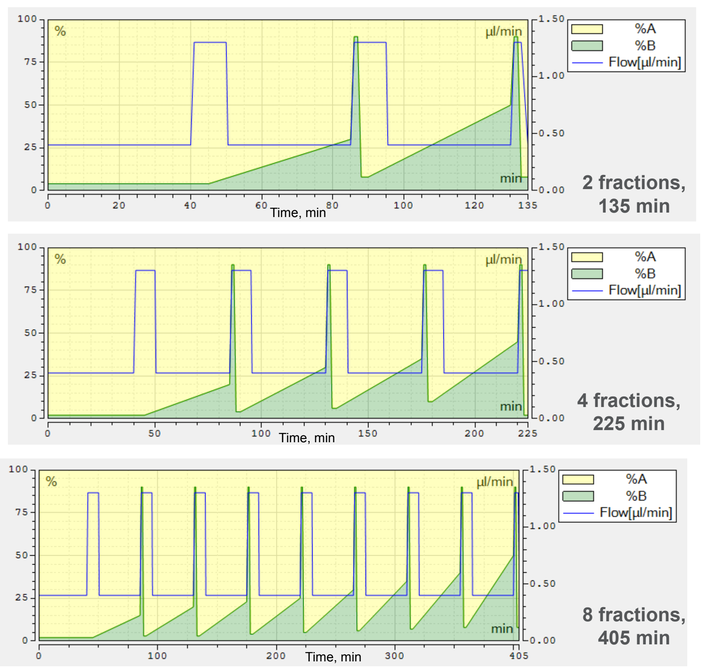
The goal to achieve deeper proteome coverage requires longer analysis times to allow the MS to collect a sufficient number of fragmentation spectra, larger sample loading amounts to improve dynamic range, and more fractions to reduce sample complexity. However, longer runs result in peak broadening and sensitivity reduction. Additionally, the loading capacity of nano columns is limited that in combination with the dynamic range of MS instruments limited to around 5 orders of magnitude doesn’t allow to get deep into the proteome of complex biological samples, e.g. biofluids. Here we developed an online 2D LC fluidics configuration that can be used for automatic comprehensive analysis of complex samples. After a sample is loaded onto the 1st dimension polymeric monolithic column and separated with high pH mobile phases the effluent is diluted with low pH flow from the micro flow loading pump and collected onto one of the two trap columns. At the same time, the previously collected fraction is separated on a short EASY-Spray column that is directly coupled to HRAM MS (Figure 2). This setup can be used for generating of LCMS method with virtually any required number of fractions that are automatically collected and analyzed without any manual steps required.

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



We tested online 2D LCMS methods that separate a sample into 2, 4 or 8 fractions. 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 staged 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 3).

Fig. 3. Gradients for low pH reversed-phase separation of 2, 4, 8 fractions in the 2nd dimension on ES800 EASY-Spray 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 2). Peptide peaks were symmetric in each fraction as well did not experience the increase in peak width with the retention time. 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.

The concentrating of each fraction onto the trap column ensures that even for very long runs with multiple fractions the quality of separation and sensitivity will be similar to 1D separations.

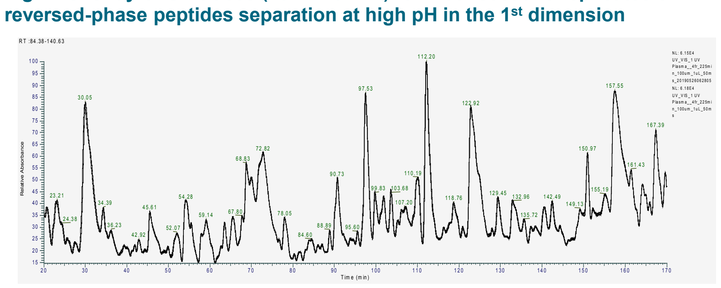
Table 2. Chromatographic characteristics for 6 selected proteotypic peptides of HeLa cell lysate distributed among the 4 fractions

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 (Figure 4) 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.

Fig. 4. Overlay of UV traces (20 – 170 min) for 2 consecutive replicates of reversed-phase peptides separation at high pH in the 1st dimension



DEEP CRUDE PLASMA ANALYSIS

We tested comprehensive online 2D LCMS methods by analyzing crude plasma digest that was prepared using the simple procedure that doesn’t incorporate protein reduction and alkylation (Figure 5). The increased number of fractions as well as higher loading results in the improved proteome depth that can be revealed without any manual sample handling procedures (Table 2). An excellent separation quality was observed for peptides on each fraction even when the high amount of plasma protein digest were loaded onto the column (Figure 6).

The main challenge with analysis of plasma or serum samples is that the same 100 to 200 protein groups can be confidently and identified in crude samples. The depletion of high abundant proteins allows looking deeper into the proteome. However, immunodepletion very often has low reproducibility and unpredictable distribution of proteins between fractions of high- and low-abundant proteins.

This proof-of-principle study shows that almost 400 proteins can be identified within only 8 fractions method. The further optimization of LC and MS parameters would result in even deeper proteome coverage the most complex biological sample (Table 3).

Figure 5. Simple sample workup for crude plasma extract analysis

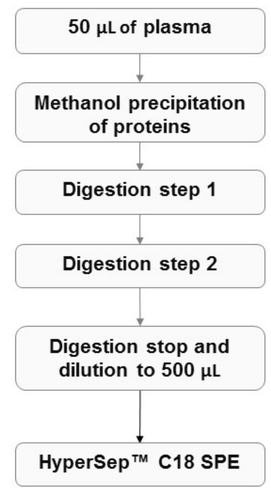


Figure 6. UV profile for 1st dimension and TIC profiles for 2nd dimension for plasma samples separated into 2, 4, and 8 fractions.

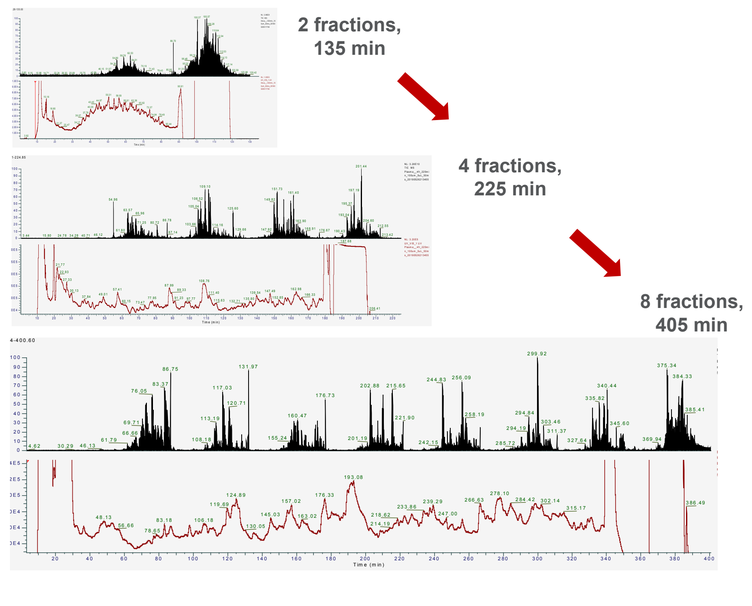


Table 3. The number of proteins, peptides and PSMs identified in crude plasma using online 2D RPxRP methods with 2, 4, and 8 fractions.

Number of fractions	Injection volume	Protein groups	Peptide groups	PSMs
2	1	206	1943	6453
4	1	265	2215	8895
4	2	285	2726	11468
4	5	317	3152	14826
8	10	394	4366	24500

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 of complex biological matrices without manual sample handling.

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 with high dynamic range of targets.

REFERENCES

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

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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