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Characterization and usage of the EASY-spray technology as part of an online 2D SCX-RP ultra-high pressure system

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ABSTRACT:

Ultra-high pressure liquid chromatography (UHPLC) systems combined with state-of-the-art mass spectrometers have pushed the limit of deep proteome sequencing to new heights making it possible to identify thousands of proteins in a single LC-MS experiment within a few hours. The recently released EASY-spray technology allows one to implement nano UHPLC with straightforwardness. In this work we initially characterized the EASY-spray containing a 50 cm column containing <2 µm particles and found the system allowed 3000 proteins to be identified in 90 minutes. We then asked the question whether a fast and sensitive online 2D SCX-RP UHPLC-MS/MS workflow, could compete with 1D long gradient analyses, using total analysis time- versus proteome coverage and sample used as benchmark parameters. The 2D LC-MS strategy consisted of EASY-spray system that had been augmented by the addition of an SCX column. The conversion was made facile since no additional valves were required and by the use of components

containing viper fittings. We benchmarked the system using a human cell lysate digest (<10 µg). The 2D SCX-RP UHPLC-MS/MS workflow allowed the identification of almost 37000 unique peptides and 6000 proteins in a total analysis time of ~7 hours. On the same system a 1D RP UHPLC-MS/MS workflow plateaued at only 20000 peptides and 4400 unique proteins and required approx. 8 hours of analysis time. Furthermore, the 2D workflow could continue to increase the proteome coverage with longer analysis times, in fact with a 21 hour analysis we identified 56600 unique peptides and >7500 proteins. We report, here, that with this fast online SCX-RP UHPLC-MS/MS workflow, the proteome coverage can be substantially extended without significantly compromising analysis time and sample usage.

INTRODUCTION:

A major goal in mass spectrometry based proteomics is the *complete* characterization of the proteome that is to detect/observe all proteins in all their proteoforms. The main analytical obstacles to reach this goal can be classified into three areas; lack of sensitivity, complexity and insufficient dynamic range. At present most in-depth analyses of proteomes employ 'shotgun strategies';[1] where all proteins are extracted from the cells, digested with trypsin, whereafter the resulting complex peptide mixture is subjected to a form of liquid chromatography (LC)-mass spectrometry (MS) analysis.[2] These two symbiotic analytical technologies (liquid chromatography and mass spectrometry) have improved immensely in the last decade. Mass spectrometry has advanced in many aspects, i.e. new peptide sequencing techniques, sequencing speed, mass resolution, sensitivity and dynamic range in ion detection.[3-12] The advances in MS, particularly speed, have meant that the overall performance of a proteomic LC-MS analysis would progress with increased resolution power of the upstream separation approach. Furthermore, higher resolving power would increase the chance of detecting low-abundant peptides, including those that are very similar in mass to charge (*m/z*) ratio. In single dimensional (1D) LC-MS experiments, for which normally C18 materials are used, longer columns with smaller internal diameter (ID) and particle size have been introduced to increase the resolving power, even though such modifications lead to a dramatic increase of the back pressure on the system.[13] Jorgenson *et al.* pioneered ultra-high pressure liquid chromatography (UHPLC) using long columns with smaller ID and sub-2 µm particles.[14, 15] Subsequently, Smith and co-workers provided compelling evidence of the utility of UHPLC for the proteomics community.[16] Nowadays, UHPLC systems which can hold up pressures of about 1000 bar have become commercially available and have been implemented in proteomics workflows by an increasing number of laboratories. Illustratively, Kocher *et al.* showed that a peak capacity of almost 700 (with a 10 hour gradient) can be achieved through the use of 50 cm columns and 2 µm particles.[17] One-dimensional RP-based UHPLC combined with the latest MS/MS technologies have extended the achievable depth in proteome coverage. Several groups have demonstrated that a few thousand proteins can now be detected in a single LC-MS/MS run using UHPLC with long columns, <2 micron particles and extended gradients.[18-22]

There is a very direct link between proteome depth and peak capacity of the chromatographic system.[17] This relationship highlights the need for even higher peak capacities since full proteome analyses (in terms of the number of proteins and peptides) are still not being achieved. Although the current 1D LC-MS/MS workflows are powerful, it is still evident that no single dimensional approach has the right requirements to achieve this ultimate goal. Offline multidimensional approaches, ideally using orthogonal separation principles,[23, 24] provide the ideal way of dramatically increasing the overall peak capacity, but typically that comes at the expense of sample loss and total MS analysis time.[25]

The pioneering work of Yates and co-workers[26] whereby they coupled SCX to RP LC-MS/MS analysis ('MudPIT'), both in offline and online workflows, became the template for many 2D LC-MS/MS approaches. In MudPIT, the peptides bind first onto the SCX material and are eluted to the RP through several steps of solvent injections containing increasing concentrations of volatile salt. In between the salt steps a gradient of ACN is applied for the separation of the peptides that became bound to the RP material.[26] The original MudPIT approach has been further developed, for instance, by introducing a thriphasic version of the capillary containing RP-SCX-RP particles or using mixed phase ion exchange columns.[27, 28] Here, the first RP operates as a trap, thus avoiding the sample loss during the needed offline desalting step prior to LC-MS/MS analysis.[29] Yates and co-workers also demonstrated a MudPIT system operating in the UHPLC regime [30] several years before UHPLC systems were commercialized.

Despite the excellent performance of MudPIT, offline systems are still more popular because they are perceived as less difficult to build and control. In valve switching approaches, the two columns are not directly connected, allowing higher flexibility in the choice of the solvents. Additionally, first dimension SCX columns with an ID bigger than the RP second dimension column can be employed, so that a higher (and more appropriate) amount of sample can be loaded.[25, 31] Often, both in MudPIT and column switching approaches, a RP-based trap column is placed in between the SCX and RP columns used for the peptide separation. This allows to concentrate and desalt the peptides eluted from the SCX column, adding extra flexibility and giving the possibility to use solvents containing either volatile[32] or non-volatile salts.[33] Gebler *et al.*[34] and more recently, Kislinger *et al.*[35] demonstrated that commercial systems can be modified without too

much effort into a 2D LC system where salt plugs were injected directly from the auto-sampler. In this way, the setup could be simplified because the solvent containing the salt does not need to be delivered by additional pumps.

Here, we demonstrate that the EASY-spray design can easily be augmented into a 2D LC configuration. We extend the platform by introducing an SCX trap (with very few new connections) and by using nanoViper fittings. Our design requires no extra pumps for the release of the SCX solvents, and can be switched rapidly from 1D to 2D and *vice versa*. We also show that this fast online 2D SCX-RP UHPLC-MS/MS workflow remarkably extends the proteome coverage remarkable when compared to 1D approaches, notably without compromising analysis time and sample usage.

EXPERIMENTAL SECTION:

Materials

Complete Mini EDTA-free protease inhibitor cocktail and phosStop phosphatase inhibitor cocktail were obtained from Roche Diagnostic (Mannheim, DE), lysyl endopeptidase (Lys-C) from Wako (Richmond, VA, USA) and trypsin endopeptidase from Promega (Madison, WI, USA). iodacetamide, dl-dithiothreitol (DTT), ammonium bicarbonate (AMBIC) and ammonium acetate were purchased from Sigma Aldrich (Steinheim, DE). Urea, formic acid (FA) and dimethylsulfoxyde (DMSO) were obtained from Merck (Darmstadt, DE). HPLC grade acetonitrile (ACN) was obtained from Biosolve BV (Valkenswaard, NL). Bradford protein assay was purchased from BioRad Laboratories (Hercules, CA) and high purity water was obtained from a Millipore Milli-Q system (Billerica, MA).

Preparation of the HEK293 digest

HEK293 cells were re-suspended in lysis buffer composed of 8M urea, 50 mM AMBIC pH 8,
1 tablet of PhosStop phosphatase inhibitors, and 1 tablet of complete Mini EDTA-free protease inhibitor cocktail. The cell lysate was sonicated 3 times on ice. After, centrifugation at 20000 g at 4°C for 20 minutes the soluble protein fraction was separated from the insoluble protein fraction. The soluble fraction was collected and the protein concentration of the lysate was determined by a Bradford protein assay. Proteins were reduced with 2 mM DTT at 56°C for 25 minutes, followed by alkylation with iodacetamide (4 mM) at room temperature for 30 minutes in the

dark. After the alkylation step the digestion was carried out with a first step of Lys-C for 4 hours at 37°C with a protein to enzyme ratio of 75:1 (w/w). Subsequently, the sample was diluted 4 times with 50 mM AMBIC to a urea concentration of 2 M. The second step of digestion was performed with trypsin overnight at 37°C with a substrate to enzyme ratio of 100:1 (w/w). After digestion the sample was acidified with 10% FA.

1D LC setup and experiments

LC separation was performed with an EASY-spray system (Thermo Scientific, Odense, DK) consisting of a 50 cm, 75 µm ID PepMap RSLC, C18, 100 Å, 2 µm particles which was connected to an Easy-nLC Ultra UHPLC system (Thermo Scientific, Odense, DK).

The spray emitter was set at 1.9 kV and the column was heated to 30 °C. The sample was picked up from the auto-sampler vial plate and loaded into the loop (Figure 1A) using the Pump S at 20 µl/min with solvent A (99.9% water, 0.1% FA). During the sample pick up, 5 µl and 1 µl of solvent A were respectively used to equilibrate the pre-column and the analytical column at the controlled back pressure of 700 bar. The sample was loaded (Figure 1B) from the loop on the back-flushed trap column (Thermo, PepMap RSLC, C18, 100 Å, 5 µm particles packed in 5 mm trap column with 300 µm ID) and the trap was connected to waste for the time needed to wash with 20 µl of solvent A. The pre-column, analytical column equilibration and the loading were back pressure controlled, therefore the time employed to perform these steps can be prone to little variance. After the loading/washing step the back flushed trap column was then switched online with the analytical column (Figure 1C) and a gradient of solvent A and B (99.9% ACN, 0.1% FA) was started.

The gradient for the separation ranged from 7 to 30% of solvent B respectively in 22, 37, 67, 157, 214, 334, 454 and 574 minutes at a flow rate of 150 nl/min. After the gradient, the column was washed for 2 minutes by increasing the buffer B concentration to 100% followed by conditioning the system with 93% buffer A for at least 15 minutes. All the steps led to an analysis time of ~45, ~60, ~90, ~180, ~240, ~360, ~480 and ~600 min. Total analysis time includes washing steps, pre-column equilibration, analytical column equilibration and loading, which in each run approximately took 20-25 min.

With analysis times of 45, 60, 90 and 180 minutes lengths, 1 µg of protein digest was injected while for the longer runs of 240, 360,480 and 600 minutes, 4 µg were injected in order to have a similar total ion current in every run.

2D LC Setup and automated 6 salt plugs experiments

The 2D experiments were performed using the same UHPLC system, trap and analytical column as described for the 1D experiments (Figure 1). In order to implement a 2D setup we added a SCX trap column (Luna, 5 µm, 100 Å, 100 µm ID, 5 cm, Phenomenex, Utrecht, NL) (Dashed box in Figure 1) in between the sample line and the W valve (Figure 1). (An SCX material with similar performance to the one used in this work, is also commercially available and can be purchased as a readymade column from Thermo Scientific). In all experiments 10 µg of HEK293 digest was used. The sample was transferred from the auto-sampler to the sample loop (Figure 1A) with solvent S (0.05% FA and 5% DMSO). The sample was loaded on the SCX trap column (Figure 1B) with 25 µl and at 1 µl/min. Typical flow rates are an order of magnitude higher for loading but we found that decreasing the flow rate for the SCX trapping helped to quantitatively trap the peptides on the SCX material. The peptides that did not bind the SCX trap column (flow through) were trapped on the back-flushed reversed phase trap column. Switching the W valve switch connects the trap column with the analytical column and a gradient of solvent A and B, as described above for the 1D experiments, was applied for the separation (Figure 1C). We used 37 min gradients for short 2D experiments and 157 min gradients for the long 2D experiments. The above described steps were repeated for each of the injected salt steps. The six salt plugs contained ammonium acetate at concentrations of 5mM, 10 mM, 20 mM, 50 mM, 100 mM, 500 mM, 5% of ACN[36] and 0.1% of FA. Each injection contained 18 µl of salt solution (Figure 1A) which was transferred on to the SCX trap column. Total volume of sample loading (Figure 1B) was 40 µl (i.e. 18 µL of salt and 22 µL of solvent A) to ensure an extensive washing. The total analysis time for the 2D short experiment amounted approx. to ~420 min while for the long 2D experiment to ~1260 min.

Mass Spectrometric Conditions:

Mass spectra were acquired with an Orbitrap Q-Exactive mass spectrometer (Thermo Scientific, San Jose, CA) in a data-dependent mode, with automatic switching between MS and MS/MS scans using a top 10 method, where the 10 most abundant precursors were chosen for fragmentation in every MS scan. MS spectra were acquired in positive mode at a resolution of 35000 with a scan range going from 350 to 1500 m/z. For the full scans the AGC target was set to $3x10^6$ ions and maximum injection time to 250 ms. The precursor ions selected for MS2 scans were then fragmented by high-energy collision dissociation (HCD) with the energy set at 25 NCE. Ion selection was performed at 1.5 m/z and the intensity threshold was set to 4.2×10^3 with charge exclusion of z=1 ions. The MS/MS spectra were acquired with fixed first mass of 180 m/z, resolution of 17500, AGC value of $5x10^4$ ions and maximum injection time of 120 ms. The dynamic exclusion varied in each method due to the chromatographic performance. Increasing gradients generated wider peak widths, thus in each method the dynamic exclusion was changed accordingly to the average peak width. For the methods with 45, 60, 90, 180, 240, 360 480 and 600 minute analysis times, the dynamic exclusion was set to 10, 15, 20, 30, 40, 40, 40, 50 and 50 s respectively.

Data Analysis:

Each raw data file was processed and quantified by Proteome Discoverer (version 1.3.0.339, Thermo Scientific). Top N Peaks filter was selected, where the 10 most abundant peaks in a mass window of 100 Da alongside a signal-to-noise threshold of 1.5 were parsed. All generated peak lists were searched using Mascot software (version 2.4.1 Matrix Science). Data were searched against the UniProt-SwissProt version 2010-12 for Human. The database search was performed with the following parameters: a mass tolerance of ±50 ppm for precursor masses; ±0.05 Da fragment ions, allowing two missed cleavages and cysteine carbamidomethylation as fixed modification. Methionine oxidation, N-terminal Acetylation, phosphorylation on serine, threonine and tyrosine were set as variable modifications. The scoring of phosphorylation sites of the identified phosphopeptides was performed by the phosphoRS[37] algorithm (version 2.0) implemented in Proteome Discoverer. The enzyme was specified as trypsin while the fragment ion type as ESI-QUAD-TOF. Percolator[36, 38] calculated the target FDR with a strict cut-off of 0.01.

The identified and quantified peptides were first filtered for high confidence (FDR below 1%) then all the results were combined and further filtered with the following criteria: Mascot ion score of at least 20 on peptides and proteins, maximum peptide rank 1, maximum search engine rank 1 and pRS isoform confidence probability of at least 75%. Precursor ion area detection node was added for the 1D 180 and 600 min triplicates and the 2D experiments (1260 min and 420 min total analysis times), in order to obtain the area under the curve of the proteins as sum of the three most intense peptides of a given protein.[39, 40]

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org) via the PRIDE partner repository[41] with the dataset identifier PXD000705. Reviewer account: username: reviewer37669@ebi.ac.uk password: UatCrcr1. To access the data please visit: http://tinyurl.com/od4hwsm

RESULTS AND DISCUSSION:

Evaluation of the performance of the EASY-spray platform in a 1D workflow with back-flush configuration:

The EASY-spray technology[42] consists of a plug a play source and a single piece that contains an integrated (and disposable) analytical column, column heater and emitter (Figure 1). The configuration used in this work contained a 50 cm, 75 µm ID analytical column (Acclaim PepMap RSLC C18, 2 µm) that was kept at 30°C. In order to decouple the SCX from the RP analytical column on the commercial EasyLC system, and therefore increase the flexibility of our 2D system, we opted to use a valve switching configuration requiring the RP trap column to operate in back-flush mode (Figure 1), which is already a well-established configuration in proteomic workflows.[43-45] We took a human cell lysate digest to benchmark the performance and assessed variable gradient times ranging from 22 to 574 min (see materials and methods). All these 1D analyses were performed in triplicate. The results as given by the number of unique peptides and proteins detected are represented in Figure 2 and the Table S-1. For instance, using a 157 minute gradient (~180 min analysis time) we identified, on average, 14575 peptides per run and 3520 proteins, while extending the gradient time to 574 min (~600 min analysis time) led to an increase in

identifications to 24367 peptides and 4626 proteins. The data, including column peak capacity, follows closely the results by Kocher et al.[46], unsurprising since the column in the EASY-spray platform is constructed of the same packing material, by the same manufacturer and has the same dimensions. Moreover, these results are in line with recently reported data of alternative UHPLC designs[18, 19, 42, 46] from which we conclude that 1D back-flush configuration shows a highly competitive performance. We further tested the stochastic nature of data dependent acquisition (DDA) in MS analysis, wherein peptide fragmentation analysis is triggered on the most abundant ions in the full scans. This behavior is evident in our data, as when combine the triplicate analysis of the ~180 min runs (i.e. ~540 min total analysis time), we increased our coverage to a total of 24367 peptides and 4369 proteins. Similarly 37805 peptides, and 5646 proteins could be detected combining the data of three 600 min runs (i.e. 1800 min total analysis time) (Table S-2).

As also shown by us[18] and others[12, 18, 20, 46] and visualized by our data in Figure 2, in a 1D LC-MS/MS approach using ultra-long gradients the amount of detected "new" unique peptides and proteins levels off rapidly, certainly beyond 214 min of gradient time in our system. These data indicate that other dimensions of separation are needed to increase the proteome coverage.

Evaluation of the EASY-spray platform in a 2D SCX-RP workflow

Our aim was to design an automated online multidimensional UHPLC workflow, requiring minimal facile changes, having competitive performance in parameters such as proteome depth, total analyses time and sensitivity. In our design no extra pumps are needed to supply the salt solution as the plugs are injected directly from the auto-sampler (Figure 1A). In this system, switching from the 1D to 2D setup is straightforward, as just an SCX trap column is connected via a nanoViper fitting using a zero dead volume connector (dashed box Figure 1). After an initial evaluation we found that the optimal salt plug concentrations and compositions were consistent with previous MudPIT experiments,[26, 30, 35] unsurprising since we were utilizing an SCX column of similar composition and dimensions. To further evaluate the system we used aliquots (10 µg) of the same HEK293 digest. The 2D configuration was tested with two types of analyses; one set were aimed at evaluating the performance when employing short (37 min) second dimension RP

gradients, while the second set of experiments used longer RP gradients (157 min). Total analysis times for the two 2D SCX-RP experiments were ~420 and ~1260 min, respectively.

The MS analyses of the flow through revealed relatively few regular unmodified tryptic peptides indicating good retention by the SCX material (Figure 3). Moreover, a more careful observation of the peptides eluted in the flow through and 5 mM salt plug, showed that the majority contained either an acetyl group on the N-terminus or a phosphate group (Figure S-1), in agreement with earlier observations by Alpert *et al*.[47] Salt plugs of 10, 20 and 50 mM ammonium acetate produced highly complex peptide mixtures, causing identification rates to be essentially controlled by the length of the reversed phase gradient. The number of unique peptides and proteins identified per fraction, and cumulatively, are summarized in Figure 3. Each subsequent salt plug produced diminishing returns. Pleasingly, we observed that the peak capacity of the RP column (above 400 for 157 min gradients using peak widths at 4σ or at 13.4% peak height) remained intact after the injection of salt plugs and typical for this material and column dimensions[17]. Peptides identified in multiple fractions, numbered no more than 20% of any fraction, suggesting a mild issue with carryover or poor fractionation. Based on this data, we attempted a third experiment where we chose a gradient length that is linked to the complexity of the salt plug. We chose a 37 min gradient for the 0 and 5 mM fractions, 214 min for the 10, 20, 50 and 100 mM fractions and 157 min for the 500 mM salt plug, for a total analysis time of ~1260 min.. Unfortunately, optimal gradient for each fraction had a negligible effect on the end result (Figure S-2).

In the short 2D experiment we identified 36943 unique peptides and 5958 proteins while with the long 2D experiment we identified 56600 unique peptides and 7565 proteins (Figure 3, Table S-3). These results are not only favorable in total analysis time compared to the triplicate 1D LC-MS/MS analyses (~540 and ~1800 min) described above, but also in the achieved proteome depth. Using ~1260 min of total analysis time our 2D LC-MS/MS approach identifies 34% more proteins than the ~1800 min triplicate 1D LC-MS/MS approach. See Table S-4 for an overview of all results in the context of sample amount, time and configuration.

We next investigated the overlap in identified peptides comparing one-to-one the cumulative data of the three short and long analysis time triplicate 1D experiments, with the short and more

extended 2D experiments, respectively (Figure S-3). The choice of 1D runs was based on analysis time. We not only identified, in the extended 2D LC experiment, the majority of proteins and peptides found in the two 1D experiments, but we also significantly increased the number of peptides and proteins identified (Figure S-3C,D,G,H). This performance of the longer gradient time 2D experiment is likely due to the combined effect of fractionation and longer RP gradient used i.e. a larger effective peak capacity. It is worthy to note that even the short version 2D experiment could rival with the long-gradient 1D combined triplicates (Figure S-3 A, B, E, F).

To further demonstrate the advantageous effect of the online multidimensional UHPLC separation we next investigated the proteins abundances as reflected by the ion currents of their top-3 peptides.[39, 40] In Figure 4 the intensities of the proteins are plotted as extracted from the data out of the 2D and 1D experiments. The dots at the top represent proteins only observed in the 1D experiment while the dots at the right side of the plots represent proteins uniquely identified in the 2D experiment. Pleasingly, in general the data points follow a straight line, indicating that the derived protein abundance is in agreement among the different experiments. However, it is clear from this data that high abundant proteins have typical ratios close to 1:1, while most of the medium to low abundant proteins have higher estimated abundance in the 2D experiments, making them appear above the straight line. This observation is likely the results of the higher resolution obtained in the 2D approach, which decreases ion suppression elevating[48, 49] ion currents for peptides co-eluting with high abundant ones in the 1D workflow. Figure 4 also provides the dynamic range of the different experiments calculated as the difference in the intensity between the highest abundant protein and the lowest. As expected the highest dynamic range belonged to the long 2D experiment followed by the short 2D one. The 1D 600 min triplicate showed a similar dynamic range to the short 2D experiment despite its much longer analysis time, while 1D 180 min triplicate performed poorest. It seems that a single 2D experiment of similar analytical time outperforms a set of 1D experiments. Moreover, the mass spectrometer itself is a key component which heavily influences overall proteome performance. Recent improvements in mass spectrometers such as those found in the Orbitrap Fusion[50] will also have a major influence. We envisage that these mass spectrometers will equally benefit (in

terms of proteome coverage boost) when the peak capacity of the separation is increased by switching from 1D to 2D LC setups.

CONCLUSION:

Here, we demonstrate that the EASY-spray system is a powerful and versatile platform. We report on a plug and play online multidimensional SCX-RP UHPLC system that does not require additional pumps for the elution of the salt plugs and is capable of identifying over 7500 proteins when using a Q-Exactive. A major benefit of our setup is that minimal changes are required to modify the 1D LC-MS system. Therefore, we believe this system can be easily constructed by any proteomics laboratory.

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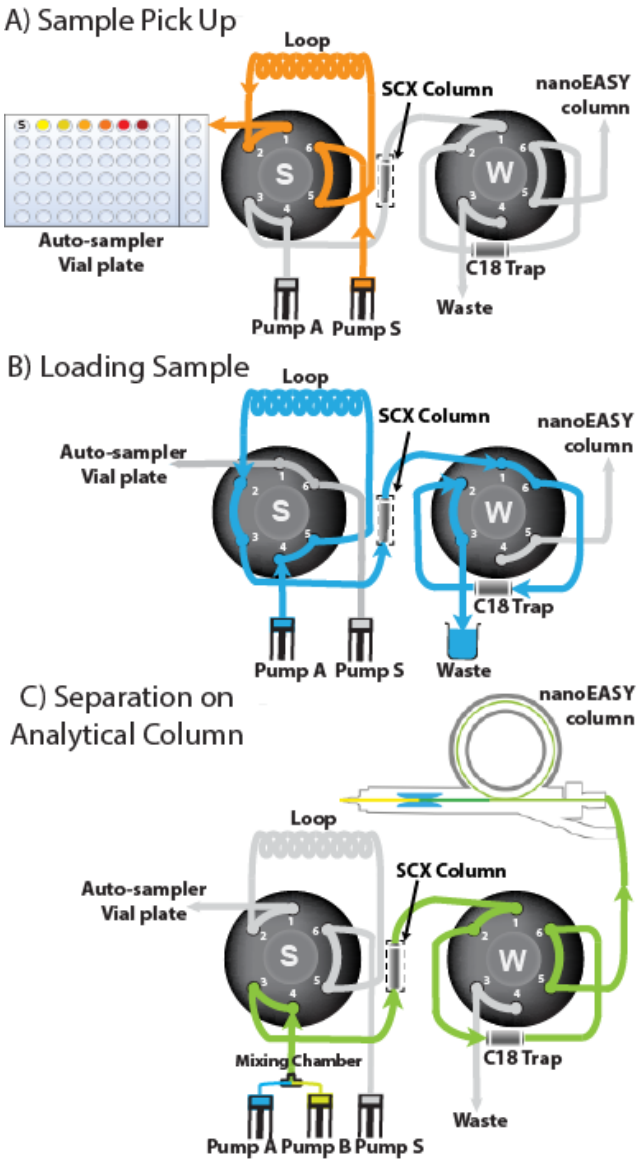
FIGURES

Figure 1



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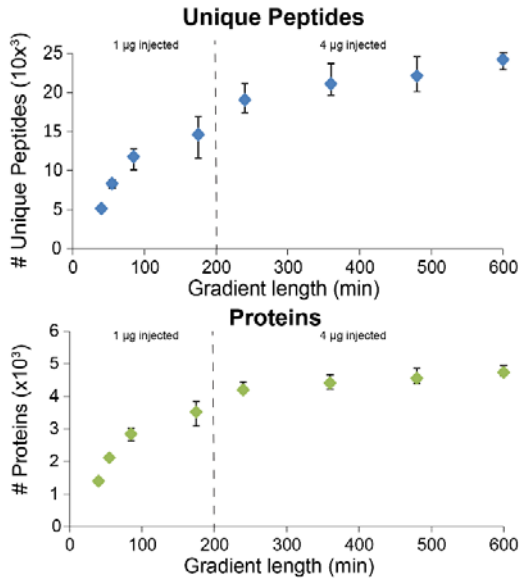

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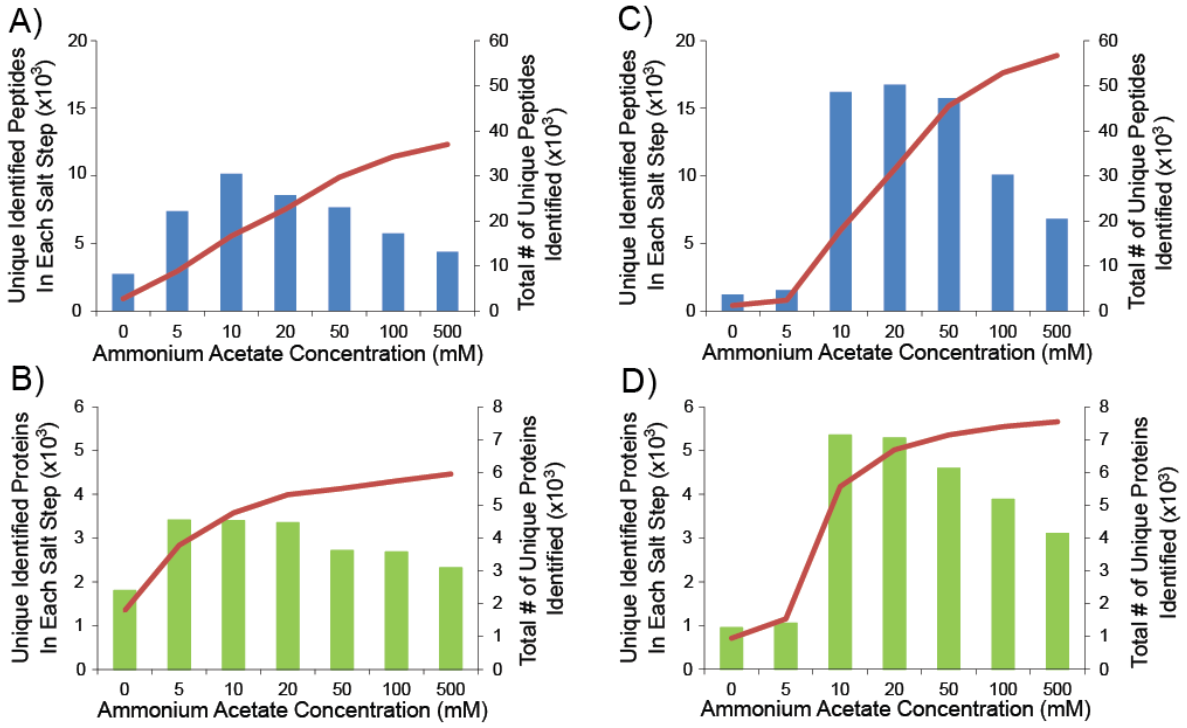
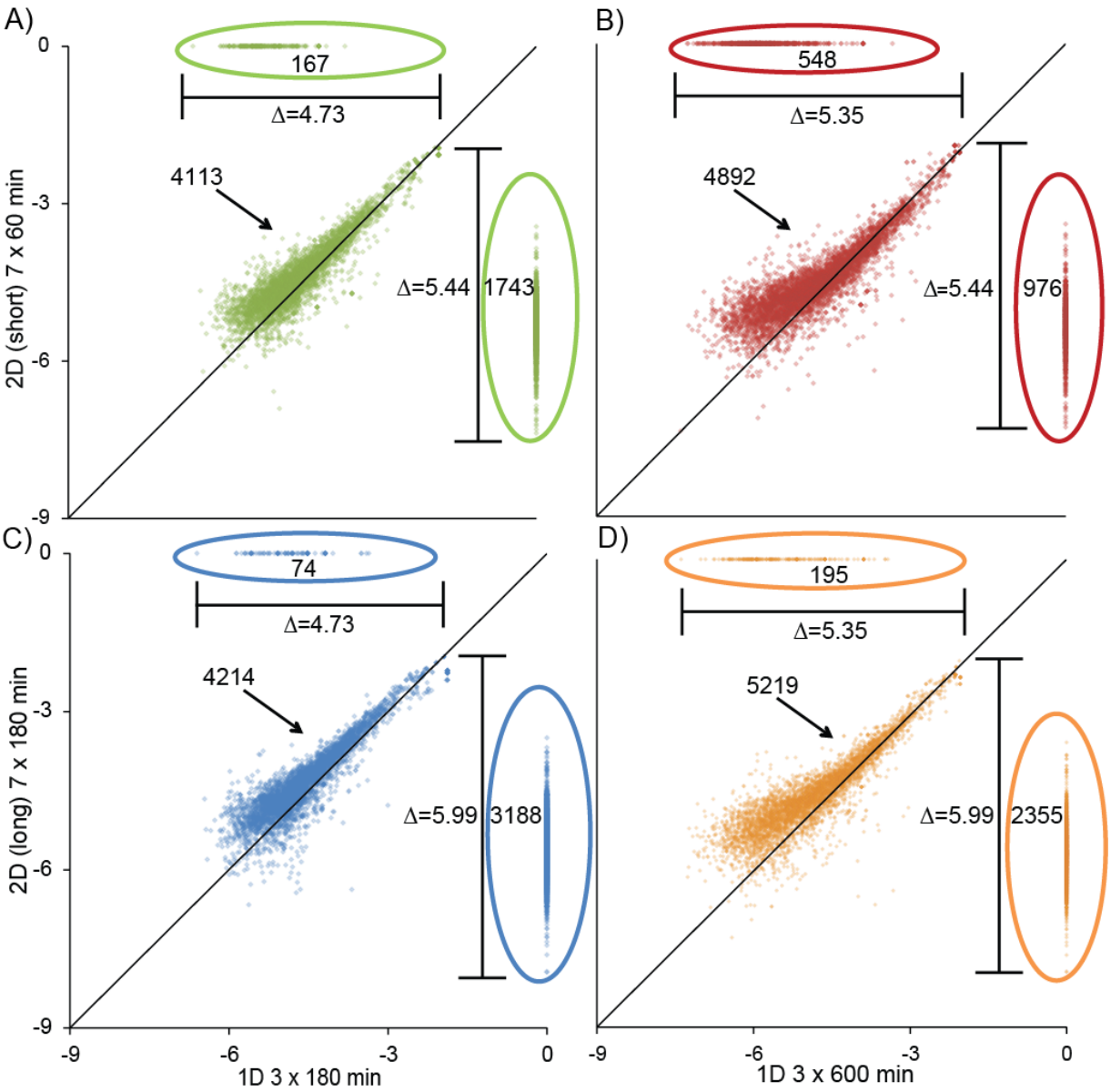


Figure 4:



CAPTIONS:

Figure 1:

Description of the 1D and 2D EASY-spray configurations. An SCX trap column (dashed box) between valve S and W is the difference between the 1D and 2D setups. A) Sample pick up: the sample or the salt plugs are picked up from the auto-sampler vial plate and loaded on the loop using pump S. B) the sample is carried from the loop to the C18 trap in the 1D fashion and from the loop to the SCX column for the 2D strategy. In the 2D setup the salt plugs are subsequently injected, populations of peptides with increasing net charges are displaced from the SCX column and bind the C18 trap. C) The peptides are then eluted from the back-flushed C18 trap column and separated on the nanoEASY-spray column with a gradient of A and B solvents.

Figure 2:

Average number of peptides and proteins identified in the 1D triplicates. The top graph reports the number of unique peptides identified (Y axis), an average of three replicates, for each of the gradient lengths applied (X axis). The graph on the bottom represents the average number of unique proteins identified, from three replicates, for each applied gradient length.

Figure 3:

Total and per fraction number of unique peptides and proteins identified in the 2D experiments.

A) The plot shows for the 2D short experiment (420 min analysis time) the number of unique peptides identified for each salt plug injected (0 mM to 500 mM) and with the red line the cumulative number of unique peptides identified.

B) Number of unique proteins for each salt plug and the cumulative number of unique proteins identified is presented for the 2D short experiment.

C) Number of unique peptides for each salt plug and the cumulative number of unique peptides identified is showed for the 2D long experiment (1260 min analysis time).

D) The number of unique proteins for each salt plug and the cumulative number of unique proteins identified is indicated for the 2D long experiment.

Figure 4:

Normalized protein intensities (Log_{10}) for the 2D and 1D combined triplicate experiments.

The plots show on the Y axis the normalized intensity (Log_{10}) of proteins quantified with the 2D short (A,B) and long (C,D) experiment and on the X axis the normalized protein intensities in the logarithmic scale for the 1D 180 (A,C) and 600 min (B,D) combined triplicates. The clouds in the middle of the graphs are the overlapping quantified proteins among the 2D and 1D experiments. On the X axis (top part) the graphs show the number of proteins uniquely quantified by the 1D 180 min (A,C) and 600 min (B,D) combined triplicates while on the Y axis (right side) the number of proteins only quantified with the 2D short (A,B) and long (C,D) experiment are showed. The horizontal bars illustrate the dynamic range of the 1D 180 min (A, C) and 600 min (B,D) triplicate experiments while the vertical the dynamic ranges for the 2D experiments.