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Nano volume fractionation strategy for dilute-and-shoot injections in off-line loss-less proteomic workflows for extensive protein identifications of ultra-low sample amounts

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ABSTRACT

A proteomic workflow for a simple loss-less manual nano-fractionation (300 nL/fraction) for low µg sample amounts which avoids the need to dry down or transfer fractions to autosampler vials is shown to be feasible. It is demonstrated that the conventional procedure of drying samples down followed by reconstitution negatively affects the number of protein and peptide identifications. Furthermore, these losses seem to disproportionately affect hydrophobic peptides from the drying down and reconstitution step. By collecting and concatenating the fractions while the outlet of the column is submerged in a small predefined volume of 0.2% formic acid, the content of acetonitrile in the collecting vials was lowered such that it was compatible with direct injection for the online analysis. This additionally resulted in a time gain of approx. an hour for the total fractionation time. Acetonitrile concentrations up to 7.5% do not seem to compromise the chromatographic performance in the online analysis. Using as little as 2 µg digested HeLa lysate, approx. 7000 protein groups could be easily identified with 2 or more unique peptides. This was the case when fractionation was performed at pH 10 as well as at pH 5.5.

1. Introduction

High-pH or basic reversed-phase (BRP) fractionation (pH > 9) has been successfully implemented as part of proteomic workflows for more than a decade [1–3]. Such workflows are carried out to maximize protein identification in complex protein samples. They generally consist of a fractionation step after which each fraction is dried and reconstituted. Each fraction is then analyzed on nano-RP LC–MS. BRP fractionation is carried out off-line by using spin-columns protocols (Stage tips [4]), off-line by collecting fractions from a chromatographic column or in an on-line fashion [5]. The use of BRP fractionation with chromatographic columns in the first dimension has been shown to have better orthogonality with low-pH (pH > 4) reversed phase (RP) separations compared to low pH RP or SCX separations [6,7]. Additionally, it has been shown to outperform fractionation protocols using gel-based first dimension separations [8]. Although BRP × low-pH RP does not have the orthogonality as seen in HILIC × low-pH RP separations [3], this difference in orthogonality does not need to compromise the final coverage. By using the concept of concatenation of the fractions, analysis time can be reduced dramatically while maintaining high proteomic coverage [6,7,9]. Analysis of concatenated fractions yields chromatograms with evenly distributed peptide intensities even if both separation dimensions are not completely orthogonal [9]. For most chromatographists, it is counter intuitive to perform high-pH reversed phase separations on silica-based particles. However, solutions to the problems associated with silica instability at high pH have been described and addressed [10], and nowadays there are several silica based RP materials and columns available which exhibit good stability (>250 h) at high pH values.

One challenge associated with most of the fractionation protocols described is the relatively large amount of starting material needed to be able to perform experiments to unravel the deep proteome. To address this, efforts have been made to scale down the fractionation and concatenation allowing analysis of nano-gram (ng) to low micro-gram (µg) amounts of starting material. In recent

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work on nanoPOTS [11,12] it was shown that fractionation and concatenation was carried out with nano-volumes eluting from the 1st dimension. Fractions were collected and concatenated in nanowells. After collection, the aliquots were allowed to dry and then reconstituted in LC buffer before analysis [12]. To our knowledge these are the only studies dealing with nano-flow fraction collection. A similar principle was investigated by Kulak et al. [13] where a nano-fractionator was introduced to handle microliter fractions from a 250 µm ID capillary column. Also, here, a drying down and reconstitution step was necessary to make the concatenated fractions compatible with the on-line analysis. In most cases the drying down and reconstitution of samples seems inevitable, although it contributes to a peptide loss of on average 10−15% [14,15]. While BRP is becoming more common place in proteomics, there is still room for improvement to obtain the maximal amount of information from very small amounts of sample by optimizing the collection of nano-volumes and the downstream on-line analysis.

This paper explores the possibility of manual nano-flow acidic and basic-fractionation and concatenation using simple means. Combined with a downstream proteomic workflow that does not require any transfer of sample between vials or drying down and reconstitution of samples, this method provides true loss-less fractionation for maximal protein identification of samples containing a total amount of protein in the low microgram range.

2. Experimental section

2.1. Chemicals and reagents

Unless otherwise stated, chemicals used were of analytical grade. Pierce™ HeLa protein digest standard (20 µg/vial) from Thermo Scientific (Rockford, Il, USA) was used as complex sample.

2.2. Preparation of HeLa samples containing varying amounts of acetonitrile

A stock solution of 100 µg/mL HeLa was diluted with a 0.2% formic acid solution containing varying amounts of Acetonitrile (MeCN): 2.25 µL HeLa stock + 3.75 µL *x*% MeCN in 0.2% formic acid where *x* was either 0, 4, 8, 12, 16, 20 and 24%. This yielded after dilution 6 µL samples containing 37.5 µg/mL HeLa in 0, 2.5, 5, 7.5, 10, 12.5, and 15% MeCN. 4 µL of each sample was injected for analysis.

2.3. Fractionation conditions

Fractionation of the samples was carried out using a Proxeon Easy-nLC II (Thermo Scientific, San Jose, CA, USA). No column heating was applied (the backpressure during the fractionation was approx. 150–170 bar). A 100 µm I.D × 25 cm column with an in-house made Kasil frit and packed in-house with ReproSil-Pur C18-AQ 1.9 µm (Dr. Maisch GmbH, Ammerbuch, Germany) was used for separation. The flow was set to 200 nL/min. The gradient used was adapted from Kulak et al. [13]. In short, the basic reversed phase fractionation mobile phase A consisted of 2% MeCN in 20 mM NH4Ac pH 10, mobile phase B consisted of 80% MeCN in 20 mM NH4Ac pH 10. The following gradient program was carried out: 3–30% Solvent B (45 min), 30–60% B (17 min), 60–95% B (5 min), and finally constant at 95% B for 3 min. After this the gradient returned to 3% B in 10 min. *Re*-equilibration of the column (>15 column volumes) using starting conditions was performed for 30 min before injection of each sample. Samples were adjusted to pH 10 before injection.

Fraction collection was initiated from the moment the gradient began. Each fraction lasted for 90 s (300 nL) and was collected in a vial containing a defined volume of 0.2% FA.

All fractions were then pooled into a total of 8 samples according to the following concatenation scheme: fractions 1, 9, 17, 25, 33, and 41 were pooled; fractions 2, 10, 18, 26, 34, and 42 were pooled; and so on to create 8 concatenated samples (1.8 µL each) consisting of 6 pooled fractions.

The conditions for fractionation with the 250 µm column (250 µm I.D. × 360 µm approx. 20 cm in length; in-house packed using the same conditions as for the 100 µm column) were as follows: the flow rate was set to 1 µL/min, while the gradient was the same and fractions were concatenated in the same manner as for the 100 µm column. The difference was that the fractions were collected in dry Eppendorf tubes. After pooling of the fractions, the pooled samples were dried by SpeedVac and reconstituted in 0.2% formic acid.

2.4. Manual fractionation

During *column equilibration, sample pick-up,* and *sample loading* the flow from the fractionation column was placed in a vial marked “waste”. At the moment the gradient started to run ($t = 0$ min), the fritted side of the column was submerged in the aliquot of 0.2% formic acid in the first collection vial. It was placed such that there was little or no contact of the column with the walls of the vial. See Fig. 1.

After 90 s, the column was carefully transferred from the first collection vial, avoiding touching the walls of the vial, to be submerged in the aliquot of 0.2% formic acid in the second collection vial. This continued as described above (concatenation scheme). After the end of the fractionation the exit of the column was placed in the vial marked “waste” again.

In the case that (parts of) the samples needed to be dried down, Eppendorf vials were used for fraction collection. If the samples could be injected directly, autosampler vials were chosen for fraction collection.

2.5. Evaluation of the retention time performance at various pH values

The fritted column prepared for the fractionation (see above) was coupled to a PicoTip™ emitter (OD 360 µm, ID 20 µm, Tip 10 µm) from New Objective (Woburn, MA, USA). The analyses were carried out on a Proxeon Easy-nLC 1000 coupled online to an Orbitrap Elite mass spectrometer. The gradient used was the same as for the fractionation (see above). All mobile phases A consisted of 2% MeCN / 98% aqueous component, all mobile phases B consisted of 80% MeCN / 20% aqueous component. For pH 2 the aqueous component was 0.2% formic acid, for pH 5.5 the aqueous component was 20 mM ammonium acetate, for pH 10 the aqueous component was 20 mM ammonium formate. For this evaluation, 2 µL of 1 µg/µL HeLa standard was injected

2.6. Liquid chromatography-mass spectrometry

2.6.1. Chromatographic conditions

Gradient elution and sample injection was performed using a Proxeon easy-nLC 1000 (Thermo Scientific, San Jose, CA, USA). A 50 µm I.D × 25 cm column with a 10 µm electrospray tip (PicoFrit™ from New Objective, Woburn, MA, USA) in-house packed with ReproSil-Pur C18-AQ 1.9 µm (Dr. Maisch GmbH, Ammerbuch, Germany) was used for separation. The flow was set to 220 nL/min. Mobile phase A consisted of 2% MeCN in 0.2% FA, mobile phase B consisted of 80% MeCN in 0.2% FA.

For the 120 min gradient the following program was carried out: 2–6% mobile phase B (7.5 min), 6–25% B (82.5 min), 25–40% B (30 min) and to 100% B (1 min). The flow of 100% B was then kept constant for 15 min.

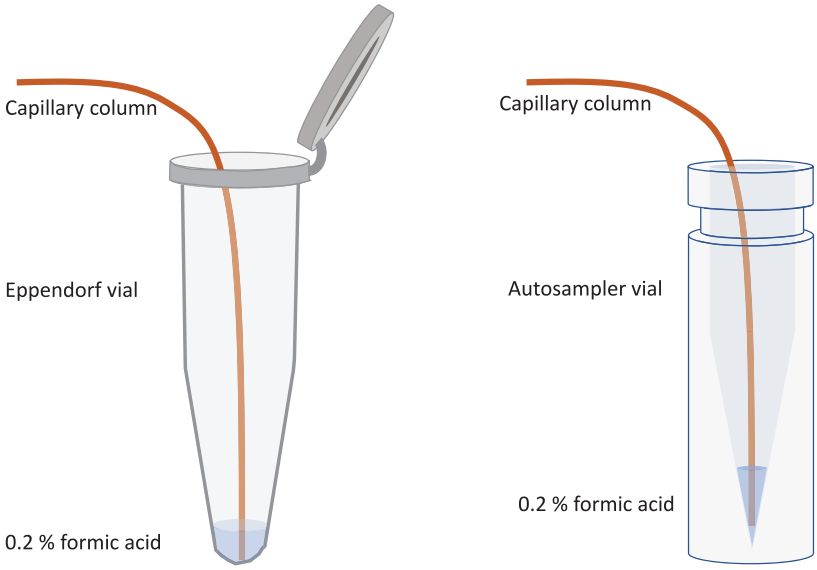

Fig. 1. Fraction collection can be carried out in an Eppendorf vial (A) where after the sample is dried down and reconstituted before it is transferred to an autosampler vial for analysis. Alternatively, collection can be carried out in the autosampler vial directly (B) without the need to transfer, dry down, or reconstitute samples, thus reducing sample loss.

For the 60 min gradient the following program was carried out: 2–6% mobile phase B (3.75 min), 6–25% B (41.25 min), 25–40% B (15 min) and to 100% B (0.5 min). The flow of 100% B was then kept constant for 7.5 min.

In both cases, re-equilibration of the column (>15 column volumes) using starting conditions was performed for 30 min before injection of the next sample.

2.6.2. Mass spectrometric conditions

Mass spectrometric detection was carried out either on an Orbitrap Fusion mass spectrometer or an Orbitrap Elite mass spectrometer (both Thermo Scientific, San Jose, CA, USA) operating in DDA mode.

Settings for the Orbitrap Fusion: Xcalibur version 4.1.50 and Orbitrap Fusion Tune application version 3.0.2041 were used to generate instrumental methods as well as to operate the mass spectrometer. A generic MS OT/ddMS2 IT HCD method was used as follows: The MS1 was operated in profile mode with a resolution of 120,000 and a scan range between 350 and 1500 m/z. The maximum ion injection time was 50 ms with an AGC target of 4e5. MS/MS was carried out in the ion trap operated in centroid mode, with a maximum injection time of 35 ms and an AGC target of 1e4. The isolation width was 1.6 m/z and the collision energy was 35%. Dynamic exclusion was set to 60 s and the overall cycle time was 3 s.

Settings for the Orbitrap Elite: Xcalibur version 2.2.SP1.48 and Thermo Tune Plus application version 2.7.0.1103 SP1 were used to generate instrumental methods as well as to operate the mass spectrometer. A generic MS OT/ddMS2 IT CID method was used as follows: The MS1 was operated in profile mode with a resolution of 120,000 and a scan range between 400 and 1600 m/z. MS/MS was carried out in the ion trap operated in centroid mode. The isolation width was 2.0 m/z, the collision energy was 35%, and the activation time was 10 ms. Dynamic exclusion was set to 90 s.

2.7. Data analysis

Raw data were analyzed with MaxQuant (version 1.6.1.0) [16,17] against a Human database (downloaded from UniProt on July 19th 2017, containing 93 591 sequences) and a contaminant database (245 entries). A decoy database was constructed by MaxQuant on-the-fly to determine the false discovery rate (FDR). Trypsin ([KR][^P]) was specified as the proteolytic enzyme with up to two missed cleavages. Carboxyamidomethyl modification of cysteine (57.0215 Da) was specified as a fixed modification. Variable modifications included oxidation of methionine (15.9949 Da) and protein N-terminal acetylation (42.0106 Da). Precursor mass tolerance was 4.5 ppm after recalibration in MaxQuant while fragment mass tolerance was 0.5 Da. Scores were thresholded to achieve a peptide and protein FDR of 1%.

3. Results and discussion

3.1. The effect of fractionation on peptide ID using small volumes and drying down fractions

In most fractionation workflows the separation is performed with capillary or micro flow, which due to the larger volume necessitates drying down the fractions by SpeedVac and subsequently reconstituting samples with a solvent compatible with the analysis in the second dimension (which typically is performed using nanoflow). Drying down the sample leads to loss of peptides [14] and potentially to loss of information. In the following section both the effect of drying down and fractionation on the type of peptides identified is investigated and discussed.

3.1.1. Comparison of single HeLa analysis (not dried) with the analysis of dried and reconstituted fractionated HeLa sample

Comparing the number of peptides identified in a single-shot injection of whole HeLa digest to that identified from the non-contiguously fractionated/concatenated samples shows that the majority of peptides were identified in the fractionated/concatenated sample (Fig. 2a). This was expected since the fractionation/concatenation reduces the sample complexity and increases the peak capacity as well as utilizes substantially increased instrument time. Additionally, it shows that most of the peptides identified in the single HeLa injection were also identified in the fractionated/concatenated sample. However, more than 6000 peptides were only identified in the single injection, representing about 16% of the total peptides identified from the non-fractionated sample. This was surprising since the same HeLa sample was used for both the fractionation and the single injection,

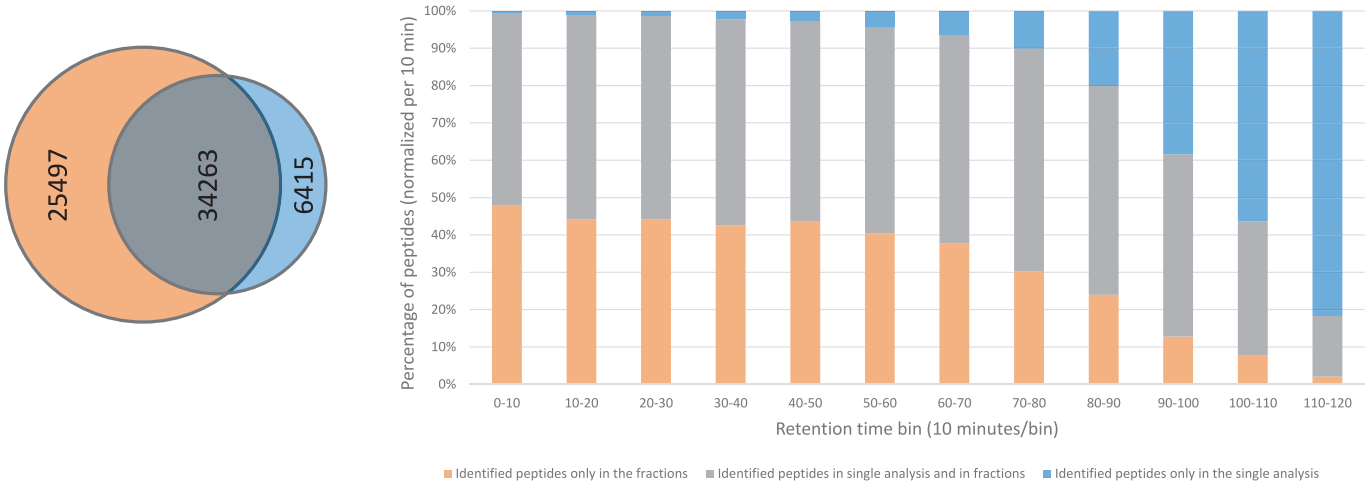

Fig. 2. (A) Venn-diagram showing the number of identified peptides in the fractionated/concatenated/dried/reconstituted samples (orange) and the single injected HeLa (blue). (B) The plot shows the percentage of peptides per 10 min analysis time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and we expected that nearly all the peptides identified in the single injection should also have been identified in the fractionated/concatenated samples. We therefore analyzed the properties of the peptides that were identified in solely the single-shot analysis versus those only identified in the fractionated/concatenated samples and those identified in both analyses and discovered a trend in which the peptides that were only identified in the single-shot analysis were primarily eluting very late in the online gradient (Fig. 2b). This suggested that the fractionation/concatenation procedure, which required drying down the samples in plastic microcentrifuge tubes and reconstituting in 0.2% FA prior to online LC–MS analysis, was primarily resulting in loss of the most hydrophobic peptides compared to the single-shot analysis.

3.1.2. Comparison of single HeLa analysis (not dried) with the single HeLa analysis which is dried down

We therefore analyzed whether a similar effect of drying down on the nature and number of identified peptides is seen with a HeLa standard which was dried down/reconstituted prior to the online LC–MS injection compared to a HeLa standard which was not dried down but injected directly. As expected, we observed a clear trend in which the highest percentage of the peptides only identified in the sample that was not dried down were present in the final portion (last 30 min of a total 120 min) of the online gradient (Fig. 3). These results support the notion that -hydrophobic peptides are preferentially lost during the lyophilization and reconstitution procedure, likely because very hydrophobic peptides remain stuck to the plastic walls of the microcentrifuge tubes and are extremely difficult to re-solubilize.

3.1.3. Comparison of a dried down and reconstituted fractionated HeLa sample with a diluted (non-dried down) fractionated HeLa sample

To further assess the impact of drying samples down and reconstituting after fractionation/concatenation, a final comparison was performed between identical fractions which were either collected in 0.2% formic acid, dried down and reconstituted before LC–MS analysis or just collected directly in 0.2% formic acid before injection for LC–MS. A basic reversed phase fractionation and concatenation of a HeLa sample was carried out and the fractions were collected in microcentrifuge tubes containing 8.4 µL 0.2% formic acid. After concatenation of 48 fractions, each of the 8 Eppendorf vials contained 10.2 µL. The content of each Eppendorf vial was mixed and split into two equal volumes, one volume to be dried down and reconstituted before injection while the other volume was injected directly.

The results for this comparison in Fig. 4 show the same trend as in Figs. 2 and 3: the share of hydrophobic peptides is relatively higher in the samples which were collected in 0.2% formic acid and injected directly compared to those which were dried down and reconstituted before injection. Moreover, 6273 more total peptides (approx. 10% more) were identified in the aliquots that were not dried down and reconstituted.

Fig. 5 shows the impact of fractionation and the effect of drying down on the number of protein IDs and the sequence coverage. The increase in proteome coverage achieved with fractionation is highly significant ($p \ll 0.01$), while the negative effect of drying down on the number of protein ID's is smaller, but still significant ($p < 0.01$). In single-shot injections of HeLa digest an average of 3231 protein groups ($n = 3$) were identified, while in the dried and reconstituted fractions an average of 6324 proteins ($n = 2$) were identified compared to the direct injection fractions where an average of 6640 proteins ($n = 2$) were identified (in all cases two or more counting only proteins with unique peptide sequences were required).

At the peptide level, the impact of fractionation on the number of peptide ID's is highly significant ($p \ll 0.01$), and while the impact of drying down on the number of peptides ID's is smaller, it is still significant ($p < 0.01$): in single injections an average of 21,518 peptides ($n = 3$) were identified, while in the dried and reconstituted fractions an average of 60,261 peptides ($n = 2$) were identified and in the direct injected fractions an average of 69,914 peptides ($n = 2$) were identified. For the sequence coverage the results were comparable: fractionation provided a highly significant increase in the sequence coverage ($p \ll 0.01$), whereas the effect of drying down is less substantial, but still significant ($p < 0.05$). The median protein sequence coverage for single injections was 13.2% ($n = 3$), for the dried down fractions it was 18.6% ($n = 2$), and for the diluted fractions it was 20.9% ($n = 2$).

Altogether these results show that there is, as expected, a tremendous information gain by performing fractionation compared to performing a single online injection using nanoflow. Moreover, it is clear that drying down and reconstituting samples prior to online LC–MS analysis has a smaller but significant

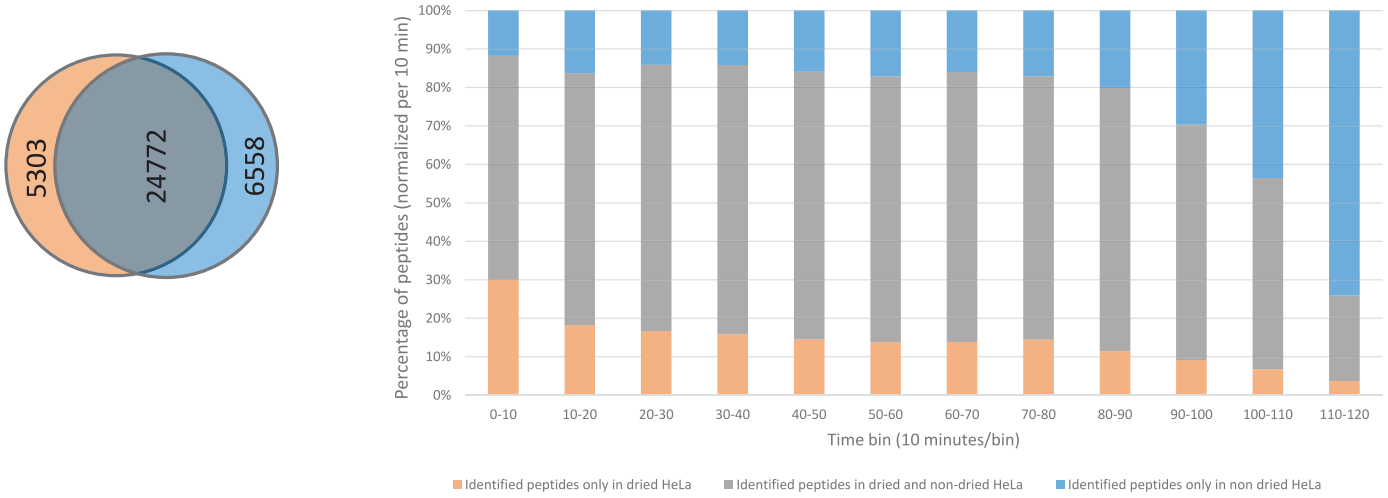

Fig. 3. A Venn-diagram showing the number of identified peptides in the dried and reconstituted single HeLa injections (orange, accumulated $n = 3$) and the non-dried single HeLa injections (blue, accumulated $n = 3$). The plot shows the percentage of peptides present in all three replicates per 10 min analysis time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

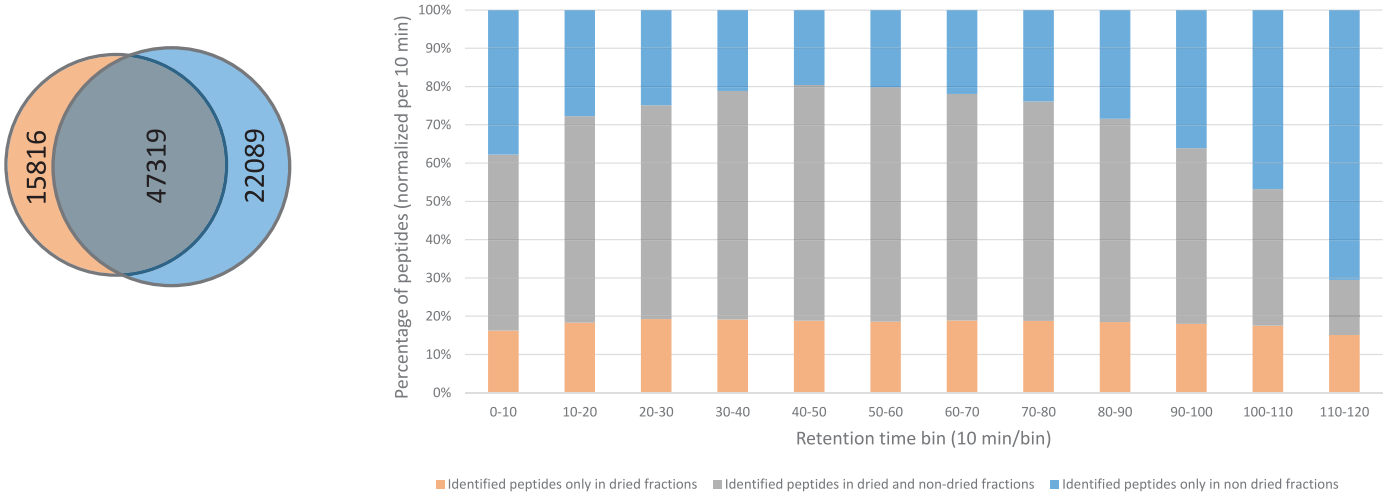

Fig. 4. A Venn-diagram of the number of identified peptides in the dried and reconstituted samples (orange, 48 fractions concatenated in 8 vials) and the non-dried directly-injected samples (blue, 48 fractions concatenated in 8 vials). The plot shows the percentage of peptides per 10 min analysis time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

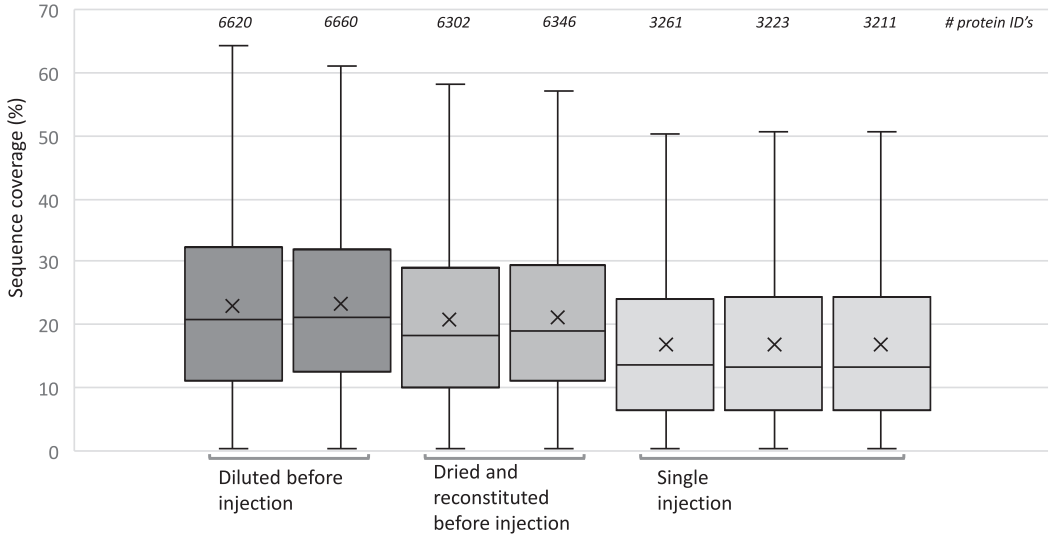

Fig. 5. Box and Whisker plots of the protein sequence coverage achieved by fractionation with or without drying down/reconstituting and by single injections of HeLa digests. The cross marks the median. Number of protein ID's (with 2 or more unique peptides) are shown above each plot.

Table 1
Calculated concentration of MeCN in a non-diluted pooled fraction (maximum) and after 5.55x dilution (dilution to 10 µL). The calculation is carried out for 48 fractions, 90 sec per fraction, and assumes that the amount of MeCN at the end of the column is the same as can be calculated from the gradient.

Concatenated fraction nr.	MeCN (maximum)	MeCN (after dilution to 10 µL)
1 (pooled fractions 1, 9, 17, 25, 33, 41)	21.7%	3.9%
2 (pooled fractions 2, 10, 18, 26, 34, 42)	22.1%	4.0%
3 (pooled fractions 3, 11, 19, 27, 35, 43)	25.6%	4.6%
4 (pooled fractions 4, 12, 20, 28, 36, 44)	27.8%	5.0%
5 (pooled fractions 5, 13, 21, 29, 37, 45)	29.8%	5.4%
6 (pooled fractions 6, 14, 22, 30, 38, 46)	30.6%	5.5%
7 (pooled fractions 7, 15, 23, 31, 39, 47)	25.7%	4.6%
8 (pooled fractions 8, 16, 24, 32, 40, 48)	20.6%	3.7%

negative impact on the number of peptide identifications, which is especially pronounced for highly hydrophobic peptides. In the following sections, the concept of sample dilution ahead of sample dry down and reconstitution is investigated.

3.2. Elimination of drying down and the consequences of sample dilution

From both the single-shot and fractionated analyses, we observe a disproportionate loss of hydrophobic peptides during the drying down/reconstitution process. Although there seems to be little or no effect of the drying down and reconstitution on the number of identifications at the protein level, this is only because it is unlikely that the only peptides identified for a given protein are all very hydrophobic. Avoiding the drying down step not only allows for more peptide identifications and higher sequence coverage, it also makes the workflow less complex. We suggest to do this as follows: as the volume per fraction exiting the 100 µm column is extremely low, dilution is carried out instead of drying down and reconstituting each fraction to change both the pH and the concentration of MeCN. In order to collect a volume of 300 nL (volume per fraction), the end of the column is submerged into an aliquot of 0.2% FA within a small microcentrifuge tube or autosampler vial. In this way the small droplet from the end of the column/capillary easily disperses into the much larger volume of FA already present in the vial. This diluted sample will not be dried down but can be injected directly (almost in its entirety) into the second dimension (online reversed-phase). To accomplish this, the amount of MeCN should be sufficiently low such that band broadening or peptide loss during the injection phase does not occur. Table 1 column 2 shows the percentage of MeCN present after 48 fractions of 90 s are concatenated into 8 vials with the gradient described above. The presence of the 0.2% FA during the collection of the fraction therefore allows for sufficient dilution of the MeCN content. In the case presented in Table 1, collection of 48 fractions (300 nL each) into 8 concatenated samples leads to pooled fraction volumes of 1.8 µL. When collected in aliquots of 8.2 µL 0.2% formic acid, this results in final MeCN concentrations of less than 6% in each sample to be injected in the second dimension (column 3). A direct consequence of diluting the fractions instead of drying down/reconstitution is time gain of an hour: the time between fractionation start and injection of the fractions in the second dimension is for the drying down/reconstitution work flow (for 48 fractions) approximately 180 min (approx. 50 min column equilibration, 72 min fractionation, 60 min for drying and reconstitution). For the dilution work flow this is approx. 120 min.

3.2.1. The effect of increasing the amount of MeCN in the sample on the chromatography (visual evaluation) and the peak intensity of the peptides

To investigate this, HeLa samples with varying amounts of MeCN between 0 and 15% were injected directly for LC–MS analysis with a relatively short gradient (60 min). The order of injection was from the highest MeCN concentration to the lowest. Fig. 6 shows typical chromatograms of these injections, which demonstrate a clear effect of the MeCN concentration on sample. For at least retention and separation. Especially the highest MeCN concentrations (12.5 and 15% MeCN samples), large portions of the chromatogram are affected. However, with only the most hydrophobic peptides having their retention and separation properties largely unaffected. On the other hand, having between 0–7.5% MeCN in the injected sample had minimal to no effect on all but the most hydrophilic peptides, whereas 10% MeCN had more pronounced effects early in the gradient but left the later portion of the gradient unaffected.

To better visualize the effect of MeCN present in the injected samples on peptide retention, we plotted the ratio of the peptide intensity at *x*% MeCN / peptide intensity at 0% MeCN at retention times across the entire gradient (see Fig. 7). In this plot, an intensity ratio of 1.0 indicates that the peptide intensity with the given MeCN concentration (*x*% MeCN) was identical to that observed in the sample without MeCN (0% MeCN). This analysis further demonstrates a strong, negative effect on the normalized peptide intensities in the 12.5% MeCN samples, which is most pronounced for peptides eluting earlier than 40 or 50 min in the 12.5 or 15% MeCN samples, respectively. On the other hand, there does not appear to be any significant detrimental effect on peptide intensity in samples containing 10% MeCN or less at any retention time, while there may actually be a slight benefit to more hydrophilic peptides eluting before 40 min when 5% MeCN is present in the injected sample. Altogether, these results suggest that having up to 10% MeCN in the sample does not have a significant effect on peptide chromatographic behavior. Given that we identified more peptides when a sample is not dried down and resuspended confirms that sample dilution is favorable over drying down and reconstituting, so long as the MeCN concentration present in the sample is less than 10%. This is especially favorable because it allows for fraction collection directly into LC–MS sample vials, thus avoiding loss of sample during sample transfer in addition to losses from drying down and reconstitution.

3.3. Evaluation of chromatographic performance in the nano-fractionation

Obtaining sharp and narrow peaks in the first separation dimension is considered to be advantageous since peak splitting during the fractionation caused by broad peaks will lead to lower peak intensities and more complex spectra of the fractions in the on-line analysis as more peptides will be spread across at least two consecutive fractions. To prevent overloading the column, which can be a major factor in the production of broad chromatographic peaks, we have not exceeded a total injection amount of 5 µg digested peptides on our in-house packed fractionation nano-columns. Although it is impossible

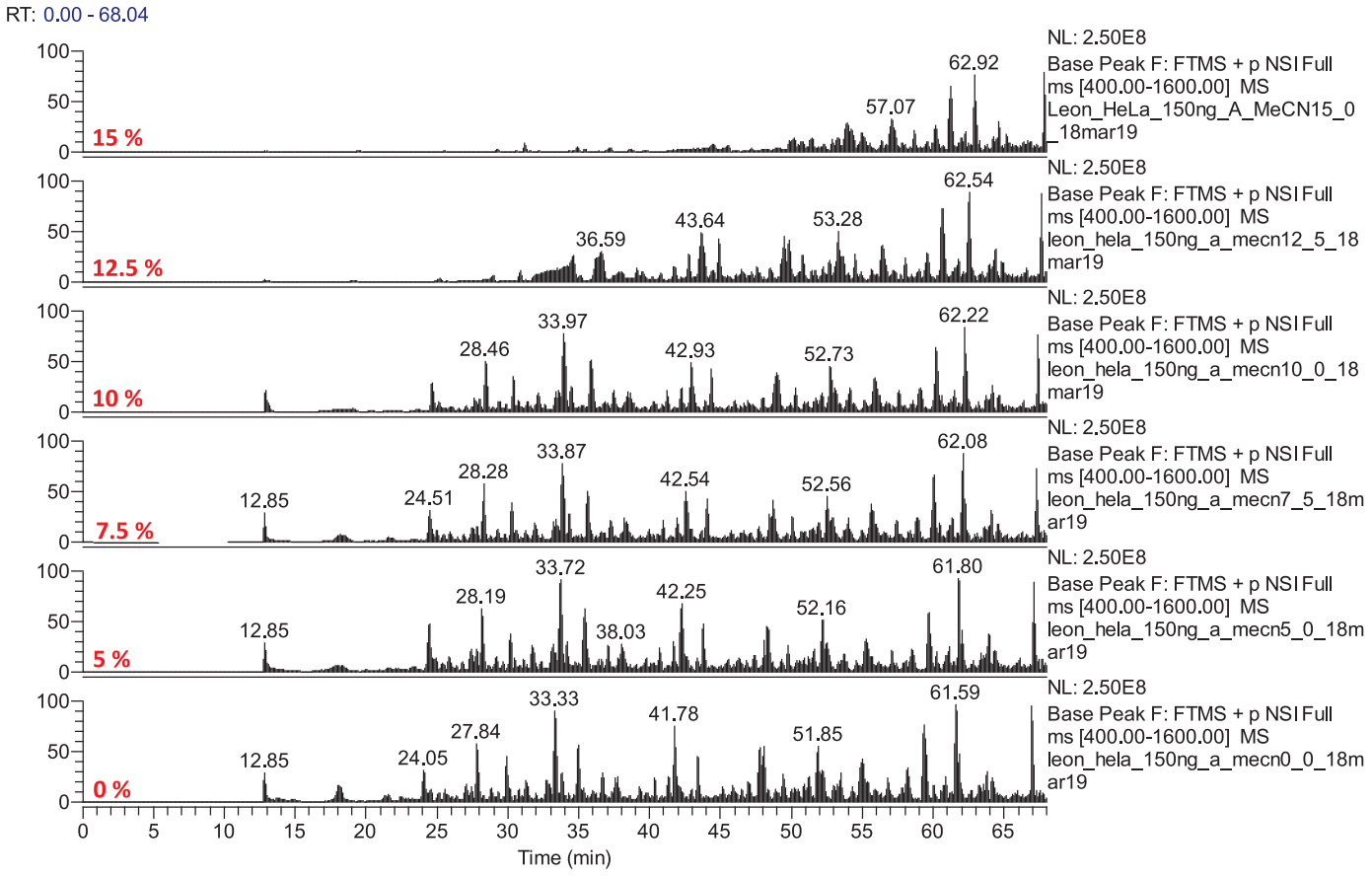

Fig. 6. Base peak chromatograms of HeLa samples (37.5 µg/mL) containing 0, 5, 7.5, 10, 12.5, and 15% MeCN (4 µL injected), all shown with a fixed scale at an intensity of 2.50E8.

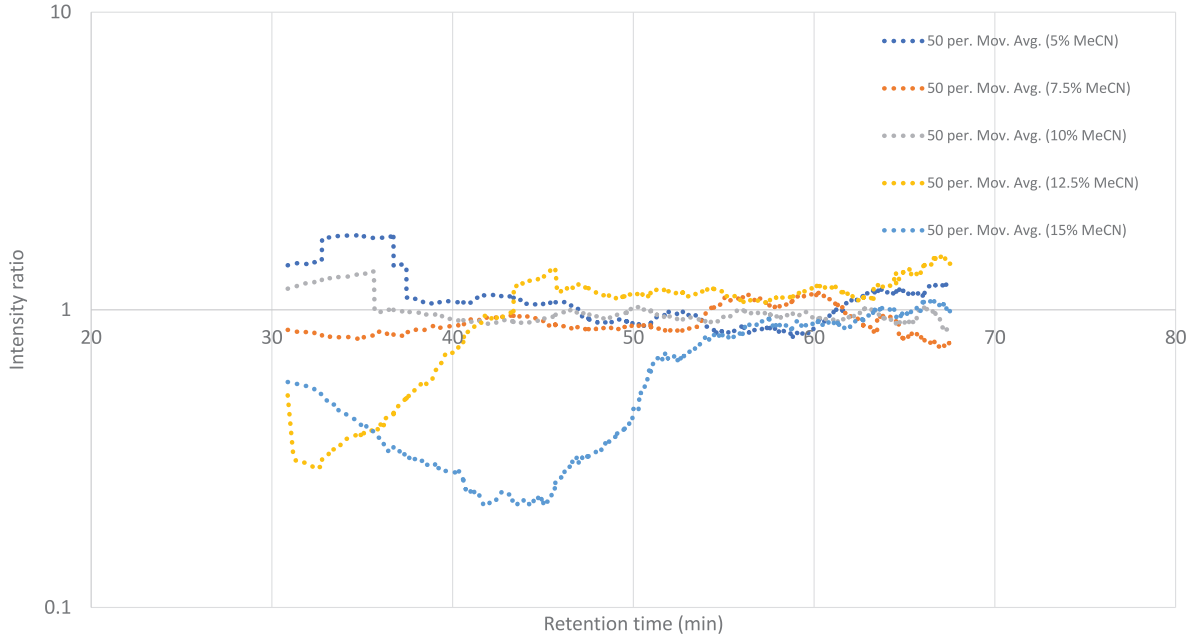

Fig. 7. Moving average (averaged over 50 points) of peptide intensities at 5, 7.5, 10, 12.5, and 15% MeCN normalized against the peptide intensities at 0% MeCN, plotted as a function of retention time. The plot shows data of 1000 randomly chosen peptides. Chromatography was carried out using the 60 min gradient.

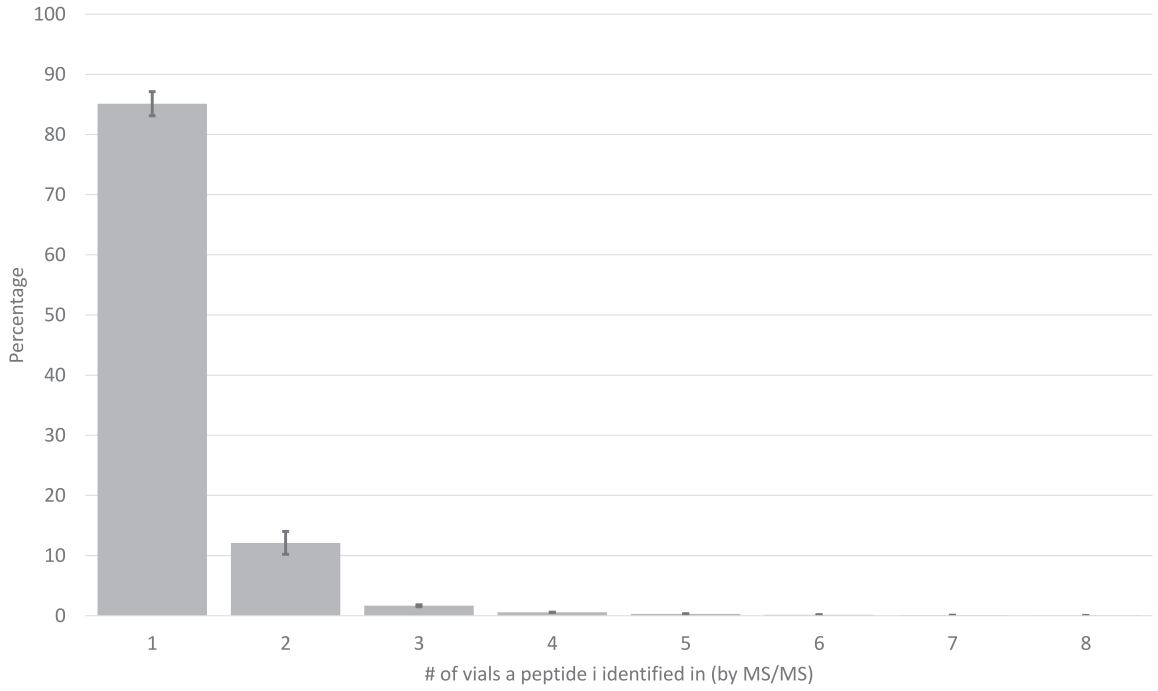

Fig. 8. The percentage of identified peptides present in only a single fraction, in two fractions, and so on up to all 8 fractions. The error bars are +/− absolute standard deviation ($n = 3$, fractionation carried out at pH 10).

to entirely avoid some peaks splitting into two fractions, the number of peaks eluting in three or more fractions should be minimal.

Fig. 8 shows a distribution for how many peptides were identified by MS/MS in a single collected fraction or in multiple fractions. As expected for good chromatographic performance, the majority of peptides (>80%) were identified in a single fraction by MS/MS, whereas approximately 12% were found in 2 fractions and only 4% of peptides were observed in 3 or more fractions. This data indicates that there is minimal peptide signal loss due to peak splitting during the nano-fractionation.

3.4. Evaluation of nano-fractionation at pH 10 and pH 5.5

In addition to the conventional basic (pH 10) reversed-phase fractionation, pH 5.5 was also tested for off-line reversed-phase nano-fractionation. The advantage of using pH 5.5 compared to pH 10 is the prolonged life-time of the fractionation column as well as the compatibility of this mid-pH with the fused silica lining sometimes used in the tubing of nano-HPLC pumps. Typically it is necessary to immediately replace the pH 10 mobile phases of the BRF fractionation system and flush the entire system and column with a low-pH buffer after sample fractionation is completed each day, as the basic pH 10 is corrosive to silica and may decrease the lifetime of system components as well as the column itself.

Another potential advantage of fractionation at pH 5.5 is that peaks tend to be narrower at this pH compared to pH 10. The median peak width at pH 2 is 23.6 s, at pH 5.5 this is 28.1 s and at pH 10 the peak width is 34.3 s. Narrower peaks should cause less peak splitting during fractionation, which in turn would improve peptide identifications as peak intensities are higher for peptides eluting in single fractions compared to those eluting in two or more fractions. Less peak splitting would also allow for collection of more cycles at pH 5.5 (and thus more fractions) within a given fractionation window, thus compensating for any lower orthogonality of fractionation at pH 5.5 compared to that at pH 10.

3.4.1. Direct on-line analysis of a tryptic HeLa digest at pH 2, 5.5, and 10

In order to evaluate the orthogonality of pH 5.5 and pH 10 (fractionation pH) with the typical on-line pH 2, the column used for fractionation was used for direct on-line analysis of 200 ng digested HeLa lysate. Fig. 9 shows retention times of the peptides for analyses carried out at pH 2, pH 5.5, and pH 10 plotted against their retention times at pH 2. Also shown are representative on-line chromatograms for each pH. All on-line analyses were carried out in positive mode. A correlation of 1 or close to 1 is defined as no or little orthogonality. The lower the correlation the more orthogonal the retention mechanisms are.

As expected, the correlation between multiple runs carried out at pH 2 (Fig. 9a) was close to 1. The correlation between pH 5.5 and pH 2 was approx. 0.91 (Fig. 9b), while the correlation between pH 10 and pH 2 was approx. 0.71 (Fig. 9c). The on-line chromatographic profiles at pH 5.5 and pH 10 both look satisfactory, with sharp peaks and a rather even distribution of peptides throughout the chromatogram. We did not make a comparison of peak intensities between these pH values, as the ionizability of the peptides will vary depending on the pH.

3.4.2. Nano-fractionation at pH 5.5 and on-line analysis at pH 2

To evaluate the true utility of performing nano-fractionation at pH 5.5 instead of pH 10, we completed an entire LC–MS analysis of concatenated fractions derived from pH 5.5 nano-fractionation of tryptic HeLa digest. Interestingly, despite its lower orthogonality, the number of protein groups (2 or more unique peptide sequences per protein) identified when nano-fractionation was performed at pH 5.5 with 2 µg injected (6741 protein groups) was essentially the same as we achieved with fractionation at pH 10 with 2 µg injected (6620 protein groups). Furthermore, the total number of peptides identified at pH 5.5 was even slightly higher than achieved with pH 10: 74,847 vs 69,607 respectively. Injecting 4 µg for fractionation at pH 5.5 resulted in 7177 identified protein groups (2 or more unique peptides per protein) and 86,594 peptide identifications. Therefore, we conclude that fractionation at pH 5.5 is a viable

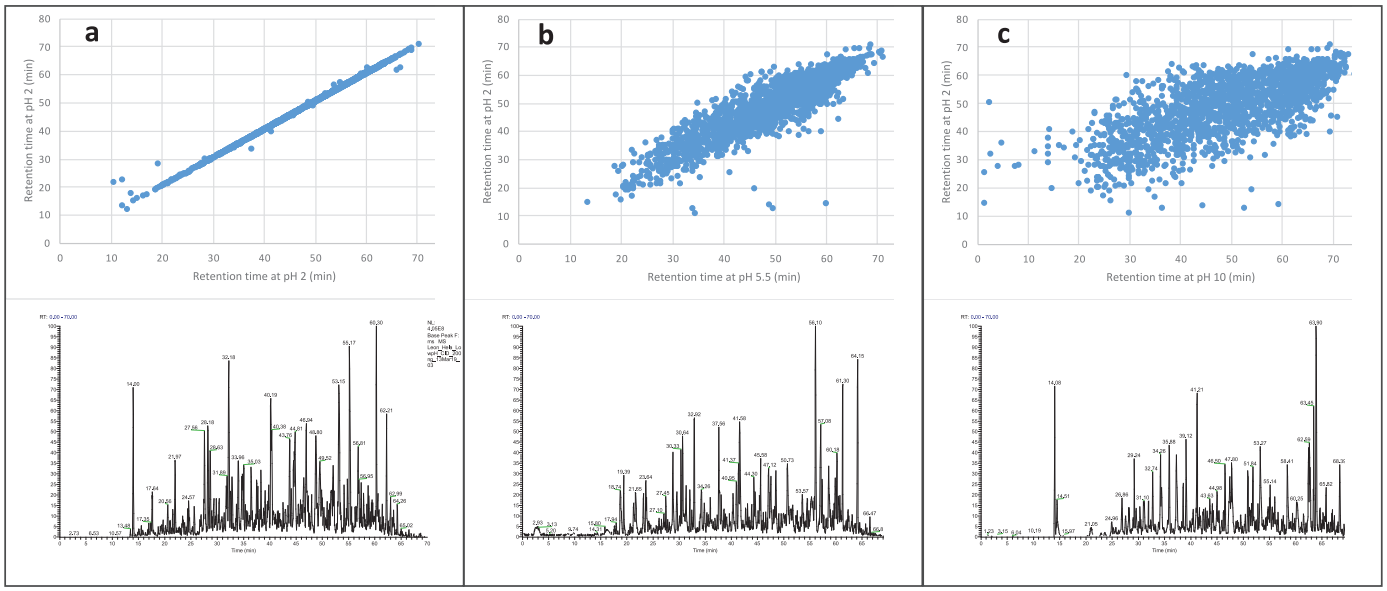

Fig. 9. The peptide retention times from on-line runs at various pH levels plotted against their retention times at pH 2. (a) pH 2 vs pH 2; (b) pH 5.5 vs pH 2; (c) pH 10 vs pH 2. Below each plot are typical on-line chromatograms for each pH: (a) pH 2; (b) pH 5.5; (c) pH 10.

alternative to conventional basic reversed-phase fractionation at pH 10, which has the added benefit of increased lifetime and stability of the column and HPLC system when silica tubing is used.

3.5. Study limitations and considerations

It should be noted that this study did not investigate different fractionation schemes or the effect of fractionating different amounts or sources of starting material. It is possible that varying the fractionation timing and/or the concatenation scheme might contribute to an improved number of identifications. Moreover, fractionating higher amounts of digested HeLa lysate would likely improve the number of identifications, although how much improvement could be achieved and at what amount of starting material would the system reach diminishing returns remains to be seen. Other studies have reported identifying up to 11,500 different proteins in HeLa lysates, albeit with a much higher amount of starting material than used in the current study [13]. Additionally, here we have only investigated C18 reversed-phase beads as the matrix for fractionation, but this could easily be replaced by alternative materials like graphene or HILIC to achieve comparable or even improved performance.

Setback of fractionation as described above is the manual skills needed to perform it. Since the exit of the column is transferred from one vial to another, there might be a chance of transferring peptides (on the outside of the column) to a next fraction. However, it will probably not affect the number of peptide and protein identifications. Cross contamination between injected samples will not occur as the exit of the column is washed between each fractionation.

Despite this, the primary goal and novelty of this study was achieved, which was to show that with relatively simple means, real loss-less nano-fractionation especially of very small amounts of starting material (2–4 µg) can be carried out while eliminating steps such as sample transfer and the drying down and reconstitution of fractions that typically result in significant peptide losses.

4. Conclusion

The current study has shown that basic reversed-phase nano-fractionation at a low flow rate (200 nL/min) by means of manual collection of volumes as low as 300 nL per fraction can achieve high numbers of identified proteins and peptides in complex digested HeLa lysates. This is possible through use of partially prefilled HPLC sample vials in which the end of the fractionation column is submerged in a small volume of 0.2% FA. Additionally, we found that drying down and reconstituting the concatenated fractions leads to significant peptide loss, especially of the most hydrophobic peptides. This can be circumvented by a simple dilution of the concatenated fractions to adjust the concentration of MeCN to values compatible with the on-line analysis. Dilutions of the MeCN content to 7.5% or lower do not affect the chromatographic behavior or the peptide intensities. Furthermore, this does not have much impact on the number of protein identifications but actually increased the number of identified peptides by around 10%, resulting in a slight increase in the average protein sequence coverage. This shows that the dilution strategy is as least as good as the drying down/reconstitution procedure while contributing to a simpler workflow and less sources of error because of its true loss-less nature. In addition to basic reversed-phase nano-fractionation, we found that mildly acidic reversed-phase nano-fractionation seems to be a viable alternative with the advantage that fractionation at pH 5.5 is more compatible with all the silica-based components in the workflow.

In order to further investigate the real potential of reversed-phase nano-fractionation, however, optimization of the fractionation and concatenation schemes should be carried out. It is expected that automation of the fractionation, eliminating factors like human error and carry over, will improve the results presented in this study even further. All in all, true loss-less nano-fractionation is a valuable tool to investigate the deep proteomes of samples that are only available in ultra-low amounts and should find utility for a number of proteomics studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] T. Nakamura, J. Kuromitsu, Y. Oda, Evaluation of comprehensive multidimensional separations using reversed-phase, reversed-phase liquid chromatography/mass spectrometry for shotgun proteomics, J. Proteome Res. 7 (2008) 1007–1011.

[2] N. Delmotte, M. Lasaosa, A. Tholey, E. Heinzle, C.G. Huber, Two-Dimensional reversed-phase × ion-pair reversed-phase HPLC: an alternative approach to high-resolution peptide separation for shotgun proteome analysis, J. Proteome Res. 6 (2007) 4363–4373.

[3] M. Gilar, P. Olivova, A.E. Daly, J.C. Gebler, Two-dimensional separation of peptides using RP–RP–HPLC system with different pH in first and second separation dimensions, J. Sep. Sci. 28 (2005) 1694–1703.

[4] H.-J. Lee, H.-J. Kim, D.C. Liebler, Efficient microscale basic reverse phase peptide fractionation for global and targeted proteomics, J. Proteome Res. 15 (2016) 2346–2354.

[5] P. Donato, F. Cacciola, E. Sommella, C. Fanali, L. Dugo, M. Dachà, P. Campiglia, E. Novellino, P. Dugo, L. Mondello, Online comprehensive RPLC × RPLC with mass spectrometry detection for the analysis of proteome samples, Anal. Chem. 83 (2011) 2485–2491.

[6] F. Yang, Y. Shen, D.G. Camp, R.D. Smith, High-pH reversed-phase chromatography with fraction concatenation for 2D proteomic analysis, Expert Rev. Proteom. 9 (2012) 129–134.

[7] Y. Wang, F. Yang, M.A. Gritsenko, Y. Wang, T. Clauss, T. Liu, Y. Shen, M.E. Monroe, D. Lopez-Ferrer, T. Reno, R.J. Moore, R.L. Klemke, D.G. Camp, R.D. Smith, Reversed-phase chromatography with multiple fraction concatenation strategy for proteome profiling of human MCF10A cells, Proteomics 11 (2011) 2019–2026.

[8] Z. Cao, H.-Y. Tang, H. Wang, Q. Liu, D.W. Speicher, Systematic comparison of fractionation methods for in-depth analysis of plasma proteomes, J. Proteome Res. 11 (2012) 3090–3100.

[9] R.C. Dwivedi, V. Spicer, M. Harder, M. Antonovici, W. Ens, K.G. Standing, J.A. Wilkins, O.V. Krokhin, Practical implementation of 2D hplc scheme with accurate peptide retention prediction in both dimensions for high-throughput bottom-up proteomics, Anal. Chem. 80 (2008) 7036–7042.

[10] J.J. Kirkland, J.W. Henderson, J.J. DeStefano, M.A. van Straten, H.A. Claessens, Stability of silica-based, endcapped columns with pH 7 and 11 mobile phases for reversed-phase high-performance liquid chromatography, J. Chromatogr. A 762 (1997) 97–112.

[11] Y. Zhu, P.D. Piehowski, R. Zhao, J. Chen, Y. Shen, R.J. Moore, A.K. Shukla, V.A. Petyuk, M. Campbell-Thompson, C.E. Mathews, R.D. Smith, W.-J. Qian, R.T. Kelly, Nanodroplet processing platform for deep and quantitative proteome profiling of 10–100 mammalian cells, Nat. Commun. 9 (2018) 882.

[12] M. Dou, Y. Zhu, A. Liyu, Y. Liang, J. Chen, P.D. Piehowski, K. Xu, R. Zhao, R.J. Moore, M.A. Atkinson, C.E. Mathews, W.-J. Qian, R.T. Kelly, Nanowell-mediated two-dimensional liquid chromatography enables deep proteome profiling of <1000 mammalian cells, Chem. Sci. 9 (2018) 6944–6951.

[13] N.A. Kulak, P.E. Geyer, M. Mann, Loss-less nano-fractionator for high sensitivity, high coverage proteomics, Mol. Cell. Proteom. 16 (2017) 694–705.

[14] A. Pezeshki, V. Vergote, S. Van Dorpe, B. Baert, C. Burvenich, A. Popkov, B. De Spiegeleer, Adsorption of peptides at the sample drying step: influence of solvent evaporation technique, vial material and solution additive, J. Pharm. Biomed. Anal. 49 (2009) 607–612.

[15] K.D. Speicher, O. Kolbas, S. Harper, D.W. Speicher, Systematic analysis of peptide recoveries from in-gel digestions for protein identifications in proteome studies, J. Biomol. Tech. 11 (2000) 74–86.

[16] J. Cox, N. Neuhauser, A. Michalski, R.A. Scheltema, J.V. Olsen, M. Mann, Andromeda: a peptide search engine integrated into the maxquant environment, J. Proteome Res. 10 (2011) 1794–1805.

[17] J. Cox, M. Mann, MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification, Nat. Biotechnol. 26 (2008) 1367.