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ABSTRACT

Offline high-pH reversed-phase fractionation is widely used to reduce sample complexity in proteomic workflows. This is due to the semi-orthogonality and high peak resolution of the two separations. Offline 2D fractionation, however, is low throughput and requires several manual manipulations and is prone to sample losses. To address these issues, we developed an online two dimensional high-pH – low-pH reversed-phase-reversed-phase (2D RPRP) LC-MRM method whereby hundreds of peptides can be quantified in a single LC-MS/MS injection. The method allowed the reproducible and sensitive quantitation of a test panel of 367 peptides (168 proteins) from undepleted and non-enriched human plasma. Of these, we were able to detect and quantify 95 peptides (29 proteins) by 2D-RPRP that were not detectable by 1D LC-MRM-MS. Online 2D RPRP resulted in an average increase of roughly 10-fold in sensitivity compared to traditional 1D low-pH separations, while improving reproducibility and sample throughput relative to offline 2D RPRP by factors of 1.7 and 5, respectively, compared to offline 2D RPRP. This paper serves as proof-of-concept of the feasibility and efficacy of online 2D RPRP at analytical flow rates for highly multiplexed targeted proteomic analyses.

1. Introduction

LC/MRM-MS-based bottom-up quantitative proteomics is an increasingly implemented technique for the quantitation of potential protein biomarkers, and human plasma is the most common sample matrix for determining biomarkers of diagnostic value [4,6,13,14,17,19]. Despite this, due to the inherent complexity and wide range of protein concentrations in plasma [2] traditional 1D-LC-MS does not provide sufficient sensitivity for quantitation of low-abundance disease-relevant protein biomarkers in undepleted plasma [16]. To address this fundamental limitation, immuno-affinity or immuno-depletion strategies are routinely utilized to increase the sensitivity for specific analytes, but immuno-enrichment mass spectrometry is time consuming, expensive to develop, and not immediately amenable to a high degree of multiplexing. Immunoaffinity techniques also depend on the availability of a consistent supply of antibodies that can capture the antigen of interest in sufficient quantities to be detected [12]. Immunodepletion strategies typically introduce sample-to-sample variations which are not amenable to the reproducible protein quantitation that is needed in a clinical environment [19]. Sample prefractionation using orthogonal (or complementary) separation approaches is useful for simplifying the plasma matrix while avoiding the high costs and problems associated with immuno-enrichment methodologies or reproducibility issues endemic to depletion strategies [1,6,13,15,18,20,21].

2D-LC/MRM using high-pH offline prefractionation has been demonstrated to be advantageous in large-scale bottom up quantitation of the plasma proteome – usually outperforming other prefractionation techniques like strong cation exchange (SCX) [3,13,14,21]. Despite this, offline prefractionation is time-consuming and peptide recoveries may be adversely affected by adsorptive losses during the fractionation, pooling, or lyophilization stages due to stochastic adsorption to labware [9]. By performing on-line high-pH reversed-phase prefractionation rather than off-line, these problems may be mitigated. Therefore, our goal in this paper was to determine the feasibility of performing online 2D RPRP at analytical flowrates for highly multiplexed targeted protein

Abbreviations: SIS, stable isotope labeled standard; MRM, multiple reaction monitoring; 2D RPRP, two dimensional reversed – phase-reversed-phase.

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quantitation, and to compare the throughput and reproducibility of the online 2D RPRP method to the offline 2D RPRP method.

2. Experimental details

2.1. Plasma samples

Human plasma was acquired from BioreclamationIVT (Westbury, NY, USA) in K2 EDTA vials and stored at −80 °C until use. Plasma samples were acquired from healthy donors (ages 18 to 50), who provided informed consent.

2.2. Standard peptides

For the assessment of peptide recovery and the initial scaling experiments, we used a small test panel of 32 stable isotope labeled internal standard (SIS) peptides from the PeptiQuant Daily QC kit (MRM Proteomics, Victoria, Canada). Subsequent validation of the chromatographic method was done using a larger panel of 400 target peptides. All peptides were selected because of their relevance as either confirmed or putative disease biomarkers. In both panels these peptides covered a wide range of hydrophobicities and isoelectric points (pI), as well as endogenous concentrations (in normal plasma). Peptides were pre-selected to be amenable to MRM analysis and to be unique in the human proteome using our PeptidePicker [10,11] software package, and have been previously quantified in undepleted and non-enriched human plasma [13]. Peptides were synthesized by the University of Victoria-Genome BC Protein Centre (University of Victoria, British Columbia, Canada) using Fmoc chemistry according to established protocols [7]. The synthesized peptides were purified by reversed-phase HPLC and characterized by MALDI-TOF-MS. Peptide purities were then assessed by capillary zone electrophoresis and underwent amino acid analysis to assess purity. Peptides were synthesized with either $^{13}C/^{15}N$-labeled lysine (K + 8) or arginine (R + 10) from Cambridge Isotope Laboratories (Andover, MA, USA).

Optimal collision energies for all measured SIS peptides were empirically determined by direct infusion ESI-MS/MS. according to established protocols developed at the University of Victoria- Genome BC Protein Centre [4].

2.3. Sample processing

Human plasma was subjected to reduction, alkylation, tryptic digestion, solid phase extraction (SPE), and vacuum concentration, essentially as previously described [14]. In brief, 20 μL of human plasma was diluted 10-fold in 25 mM ammonium bicarbonate (pH 8.0). Plasma proteins were denatured by adding sodium deoxycholate (1% final concentration) and tris(2-carboxyethyl)phosphine (TCEP) (5 mM final concentration) and incubating at 60 °C for 30 min. Cysteine thiols were alkylated to prevent disulfide bond reformation by adding 100 mM iodoacetamide (10 mM final concentration) and incubating in the dark at 37 °C for 30 min. Unreacted iodoacetamide was quenched

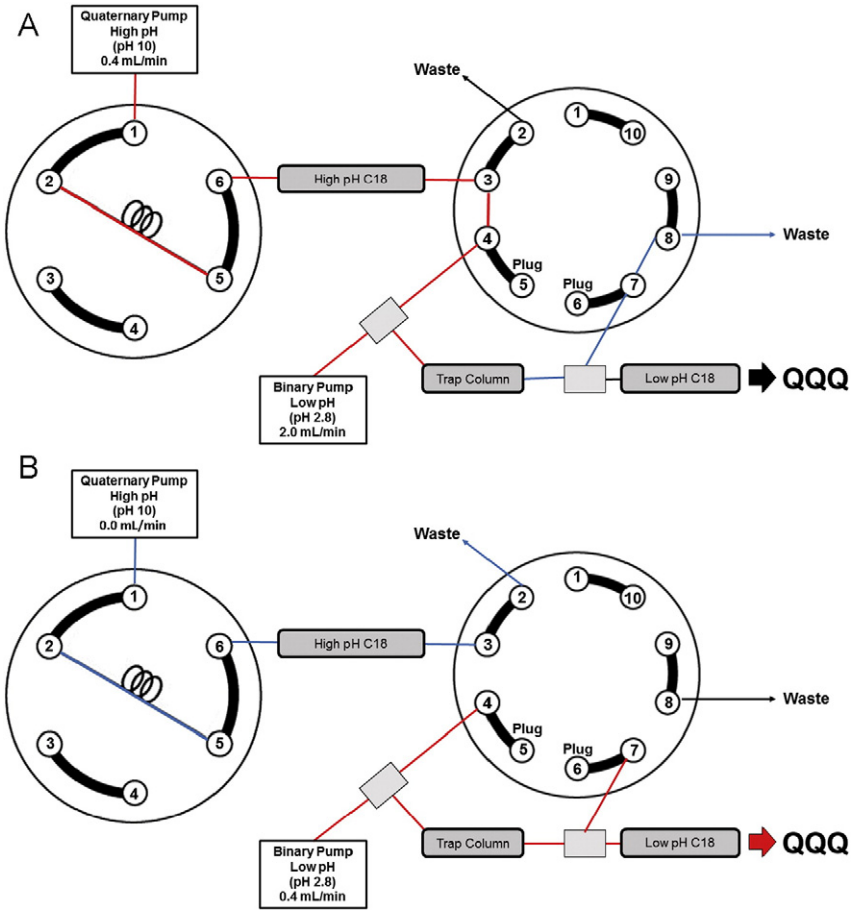

Fig. 1. Schematic of valve configuration for online 2D reversed-phase – reversed-phase. A) Peptides are separated in the first dimension under basic conditions using a Waters BEH C18 4.6 × 50 mm 2.5 μm C18 column. Eluted peptides are diluted in acidic aqueous mobile phase prior to being trapped on an Agilent Infinity Lab Poroshell EC-C18 column. B) Peptides are eluted from the trapping column under acidic conditions and further separated using an Agilent RRHD UHPLC (Zorbax Eclipse Plus 2.1 × 150 mm 1.8 μm) analytical column prior to MRM-MS.

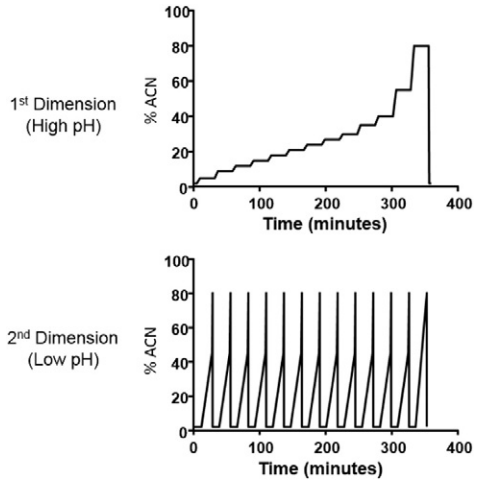

Fig. 2. Gradient and fractionation schematic for online 2D-RPRP method. Plasma is initially loaded onto the first dimension column (pH 10 – buffered by a constant infusion of 10% mobile phase C- 100 mM ammonium hydroxide), then in a discontinuous, step-wise fashion, the first dimension gradient is run and held for 2 min prior to switching valve position from load to analytical mode. In the second dimension, peptides are separated using a linear reversed-phase gradient under acidic conditions. Although these gradient parameters were sufficient for our test panel, further optimization of the gradient could theoretically be performed to increase throughput and multiplexing capacity.

by adding 100 mM dithiothreitol (DTT) (10 mM final concentration) at 37 °C for 30 min. Proteins were then digested with TPCK-treated trypsin (Worthington, Lakewood, New Jersey, USA) at an enzyme-to-substrate ratio of 1:20 (75 μg trypsin: 1500 μg plasma proteins). Digestion proceeded overnight at 37 °C. Immediately prior to the termination of tryptic digestion, samples were spiked with SIS peptides at a ratio of 1:140 (picomoles of SIS: μg protein). Digestion was subsequently quenched by adding 10% formic acid (1% final concentration). Sodium deoxycholate was pelleted by centrifugation at 18,000 ×*g* for 2 min. The supernatant, which contained the tryptic peptides, was desalted by SPE (Waters, Millipore) according to the manufacturer's protocol and concentrated in a vacuum centrifuge at 4 °C. Peptides were reconstituted in water with 0.1% formic acid prior to LC/MRM-MS analysis.

2.4. 1D reversed-phase LC-MS/MS

Peptide measurements by 1D LC-MS/MS were conducted using an Agilent 1290 Binary UHPLC system interfaced with an Agilent 6495 triple quadrupole mass spectrometer which was equipped with a standard flow Jetstream ESI source operated in positive ion mode. Peptides were separated using a 1 h reversed-phase gradient on an Agilent Zorbax Eclipse C18 analytical column (2.1 × 150 mm 1.8 μm) under acidic conditions (pH 2–3) prior to MS detection in dynamic MRM mode (Supplementary Table 1). For data used in peptide quantitation, retention times (RT) were scheduled and dynamic MRM measurements were acquired in 1-minute retention time windows. All precursor/product pairs were acquired using empirically optimized collision energy values. Criteria for method duty cycle included a minimum dwell time of 10 ms and a total cycle time <1000 ms. Details of the instrument and gradient parameters can be found in the Supporting information.

2.5. Offline high pH reversed-phase peptide fractionation

Reverse calibration curves were prepared by digesting human plasma as described above and spiking varying amounts of an equimolar mixture of SIS peptides into 6 separate samples, corresponding to 6 SIS peptide dilution levels. SIS peptides and their concentrations in each replicate are described in the Supplementary Material. Each of the plasma samples were subjected to high-pH reversed-phase fractionation and subsequent 1D LC-MRM analysis essentially as described previously [14]. Briefly, 500 μg of plasma tryptic digest was loaded onto a Waters XBridge Peptide BEH C18 column (4.6 × 150 mm, 130 Å, 5 μm) and fractionated by basic (pH 10) reversed-phase chromatography at a flow rate of 1 mL/min using an Agilent 1260 LC system equipped with a fraction collector. Mobile phases A, B, and C were water, acetonitrile, and 100 mM ammonium hydroxide in water, respectively. C was maintained at a constant value of 10% of the solvent composition (corresponding to a constant infusion of 10 mM ammonium hydroxide). Specific gradient conditions are included in the Supplementary material. Fractions were collected every 30 s over a 30 min gradient. After fractionation, every 12th fraction was pooled (e.g., pooled fraction 1: 1, 13, 25, 37; pooled fraction 2: 2, 14, 26, 38, etc.), and each pooled solution was lyophilized to dryness for a total of 12 pooled fractions, corresponding to the first 24 min of the gradient. The fractionated samples were

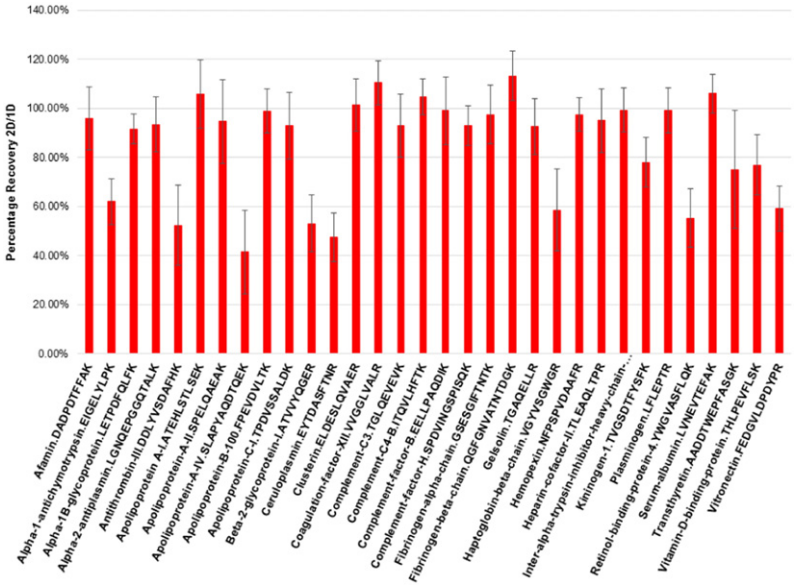

Fig. 3. Recovery comparison between a 1D and 2D RPRP. Percentage recovery was assessed by comparing relative ratios of peak responses (areas) from equivalent triplicate injections of an equimolar mix of 35 SIS peptides (250 fmol on column) over the course of 5 days. Error is represented as ± the standard error of the mean (SEM).

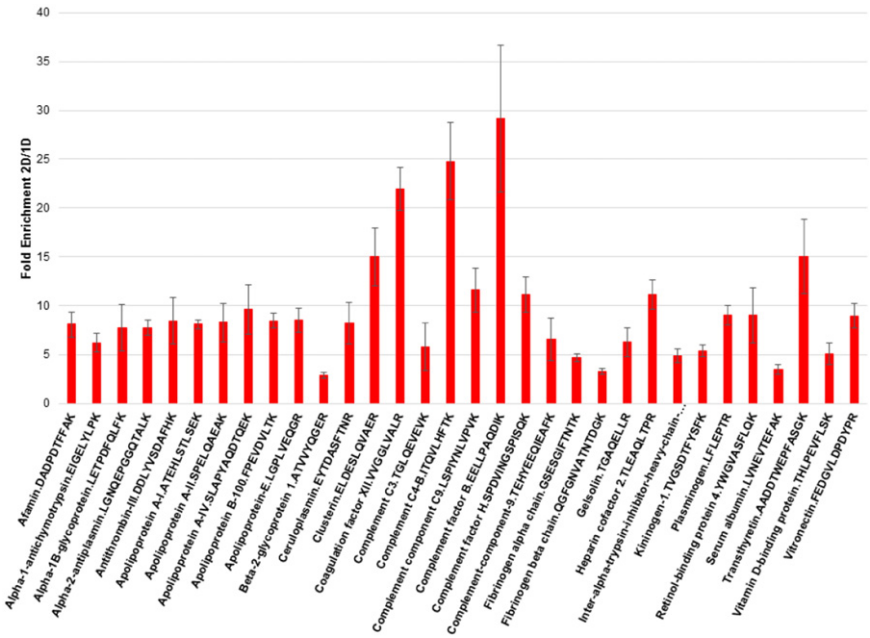

Fig. 4. Comparison of total peak area response 2D vs 1D. To assess the extent to which peak area ratios are enhanced by increasing the initial sample loading amount we compared plasma protein loading amounts of 25 μg by 1D to 500 μg of plasma protein by 2D. Enrichment was taken as the average interday ratios of peak areas by 2D to 1D. Error bars represent ± the standard error of the mean (SEM).

reconstituted in water containing 0.1% formic acid prior to analysis by 1D LC-MS/MS as described above.

2.6. Online 2D high-pH reversed-phase – low-pH reversed-phase liquid chromatography

LC separation of peptides prior to MRM-MS was conducted by using both high-pH and low-pH reversed-phase separations in an online manner by splitting the chromatographic separation process into 13 cycles of high-pH separation and subsequent low-pH analytical separations. See Fig. 1 for a representation of the valve positions and general plumbing used for the separation. With the injector in the load position, peptides were loaded and separated on a Waters Xbridge BEH 4.6 × 50 mm 2.5 μm C18 column under basic conditions, at a flow rate of 0.4 mL/min (10 mM ammonium hydroxide, pH 10) (Fig. 1A). The peptide-containing eluent from the first dimension was first diluted with acidic mobile phase (0.1% formic acid, final pH 2–3) by installing a junction where the solution is added with an Agilent 1290 UPLC binary pump (2 mL/min). Peptides are then trapped using an Agilent Infinity Lab Poroshell 120 EC-C18 column (4.6 × 50 mm 2.5 μm particle size). With the valve in the analytical position, trapped peptides are eluted from the trapping column and separated using an Agilent Rapid Resolution C18 column (2.1 × 150 mm 1.8 μm) under acidic reversed-phase conditions at a flow rate of 0.4 mL/min prior to MRM analysis (Fig. 1B). For a schematic overview and detailed descriptions of the gradients and the timing of the valve switching refer to Fig. 2 and Supporting information Table 1 respectively. In total, 13 high-pH fractions were collected online and further separated by second dimension low-pH reversed-phase gradients. The total analytical run time for the method was 360 min, however this time could be shortened depending on the number of high-pH fractions employed, as well as the 1st and 2nd dimension gradient lengths depending on the analyte(s) of interest and the degree of multiplexing required.

2.7. MRM-MS analysis of plasma peptides

SIS peptides and unlabeled endogeneous (END) peptides were analyzed in the MRM mode on an Agilent 6495 triple quadrupole instrument equipped with a Jetstream ESI source, operated in positive ion mode. All MRM transitions were previously optimized using Masshunter Optimizer and screened for interferences in buffer as well as in plasma tryptic digest matrix. Screening for interferences with the endogenous peptide signals was performed by monitoring the top 3 most intense MRM transitions (previously determined during the optimization of the corresponding SIS peptides). Endogenous peptides were screened for interferences using the following criteria: A) retention times must be an exact match (RT matches to 0.1 min) between SIS and endogenous peptide channels, B) co-eluting peptide fragment ion ratios were compared between the SIS and endogenous peptides, C) Peptides with a dot product (comparison of relative ratios of transitions between endogenous and SIS peptide signals) below 0.9 were discarded. All tentatively accepted peptide detections were further inspected visually to ensure similar peak shapes. Detailed description of instrument parameters as well as MRM transitions for all measured peptides can be found in the Supporting information.

2.8. Data analysis

All MRM data was analyzed using Skyline [8] (Skyline Daily 64 bit, version 3.6.1.10279, http://bit.ly/2jvJKrW). After ensuring accurate

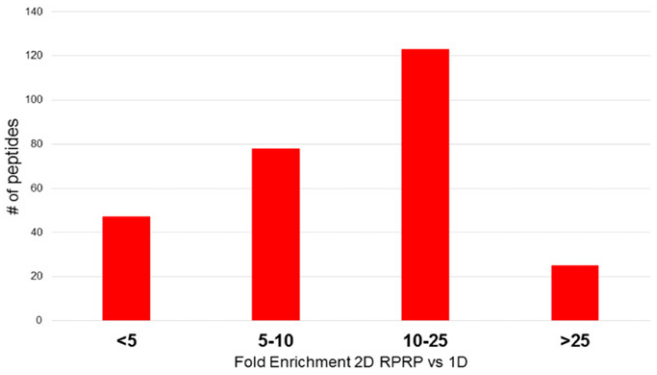

Fig. 5. Distribution of fold enrichment for all quantified peptides as relative ratio of responses of total peak area peptides in plasma digest (2D/1D).

peak selection and integration, peak-related information (i.e., retention time, dot product, and total peak areas) was extracted. While qualification of the peptide responses was determined by calculating the average relative ratios (discussed in the preceding section), quantitation of the interference-free peptides was accomplished by taking the product of a peptide's relative response (as a END/ SIS ratio) and the SIS peptide concentration.

3. Results and discussion

3.1. Description of the online 2D high-pH – low-pH reversed-phase-reversed-phase method

In order to increase the sensitivity of LC/MRM based peptide quantitation in highly complex matrices, it is often necessary to use

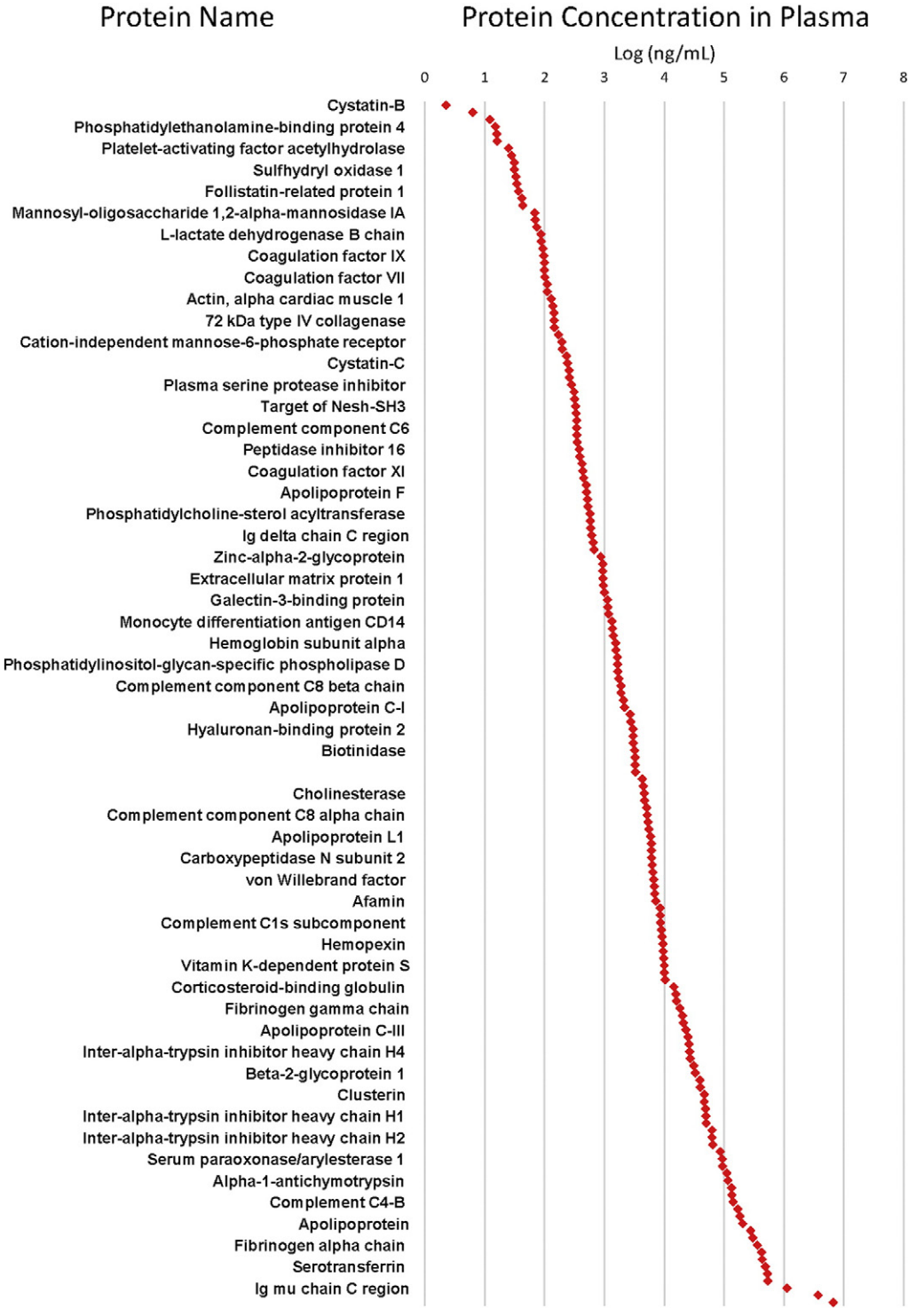

Fig. 6. Peptide quantitation summary 2D RPRP - MRM.

complementary techniques and separations. To address this need, we examined whether high-pH - low-pH-RPRP LC-MS could be performed in an online manner. We used a test panel of 400 peptides to show proof of concept of the online 2D method for highly multiplexed and sensitive MRM measurements of potentially disease relevant plasma proteins. The offline high pH prefractionation protocol was adapted to an online method, as illustrated in Fig. 1.

In this workflow, SIS and/or plasma peptides are separated in two stages 1) basic prefractionation and 2) acidic analytical separation. The first separation is performed under alkaline reversed-phase conditions at a flow rate of 0.4 mL/min using a quaternary pump to supply a constant infusion of 10% of 100 mM ammonium hydroxide (10 mM final concentration, pH 10). Next, the eluted peptides are diluted 5-fold with acidic mobile phase from the binary pump, in a T-junction (Fig. 1A). This results in the eluted peptides being diluted in the aqueous mobile phase and the pH being neutralized prior to capture on a trapping column (Agilent Poroshell 4.6 × 50 mm 2.5 μm). After the 1D separation, the flow is diverted using a 10-port valve so that the quaternary (high pH) mobile phase is diverted to waste and the binary pump is used to elute peptides from the trapping column and separate them on the analytical column (Agilent Zorbax Eclipse Plus C18 2.1 × 150 mm 1.8 μm) (Fig. 1B). Since all analytes are separated in an online fashion, this new method is significantly faster than similar offline prefractionation methods since no additional sample cleanup or concentrations steps are required. Based on established methods [14], offline fractionation requires considerable time (approximately 30 h) and manual sample manipulation since samples must be fractionated, pooled, lyophilized, and reconstituted prior to injection and analysis by 1D LC-MRM. Conversely the online method circumvents all of these steps. The total run time of the online method is 6 h, but the method could easily be employed as a heart-cutting technique with much shorter run times for smaller panels of target peptides. Optimization of online 2D-RPRP mainly involved optimization of the separation in the first dimension (high-pH) and selection of an appropriate trapping column since the analytical portion had been rigorously optimized and tested earlier. We decided to use shorter 4.6 mm i.d. × 50 mm column rather than the longer 4.6 × 150 mm column routinely used in offline basic reversed phase fractionation. This was done to allow lower flow rates and shorter equilibration times for the high-pH column and to reduce the amount organic solvent in the eluate of the first dimension which could adversely affect the binding of the eluted proteins to the trapping column, and switching to a smaller column allows the use of lower flow rates in the first dimension, thereby resulting in higher dilution ratios at the first junction (where the high-pH eluate and the acidic mobile phase from the binary pump are mixed). This allows for sufficient dilution of the organic solvent for peptides to be trapped. Online 2D LC configurations have been employed in the past for RP-RP separations predominantly at lower flow rates within the context of untargeted discovery workflows and for the quantitation of single peptide targets [5,15], but to our knowledge this is the first time that an online 2D RPRP method has been developed and validated at analytical flow rates for highly multiplexed quantitation of targeted proteins. Although the high-pH gradient was initially established in order to separate a much larger panel of SIS peptides (>1300), many of these fractions were unnecessary for our final panel of 400 target peptides and could have been consolidated to shorten the overall run time.

3.2. Initial validation of online 2D RPRP LC-MRM platform

Initial testing of the 2D RPRP method was conducted using a small panel of 32 peptides, which corresponded to a subset of SIS peptides included in a commercially-available kit of quality control peptides (PeptiQuant Daily QC kit, MRM Proteomics, Victoria, Canada). These peptides were selected in part due to their physiochemical parameters which spanned a wide range of hydrophobicities and pIs, as well as their relatively high endogenous abundances which would allow them to be detected in human plasma. Peptide recovery was assessed by measuring the relative ratios of SIS peptide response (total peak area ratio) from an equivalent injection (200 fmol of equimolar SIS mix) on both 1D RP and 2D RPRP systems (see Supporting information for a list of peptides and their MRM transitions). The decision to assess the recoveries of the SIS peptides in buffer instead of in plasma matrix was mainly made to limit potentially confounding matrix effects, because simplification of the sample matrix by high-pH prefractionation should theoretically decrease ion suppression and thus lead to artificially high recoveries from 2D vs 1D. The average recovery for the 2D RPRP method was 92.6% compared to the 1D method (Fig. 3). The average analytical precision for the 2D method was a 7.16% CV, vs 6.21% for the 1D method.

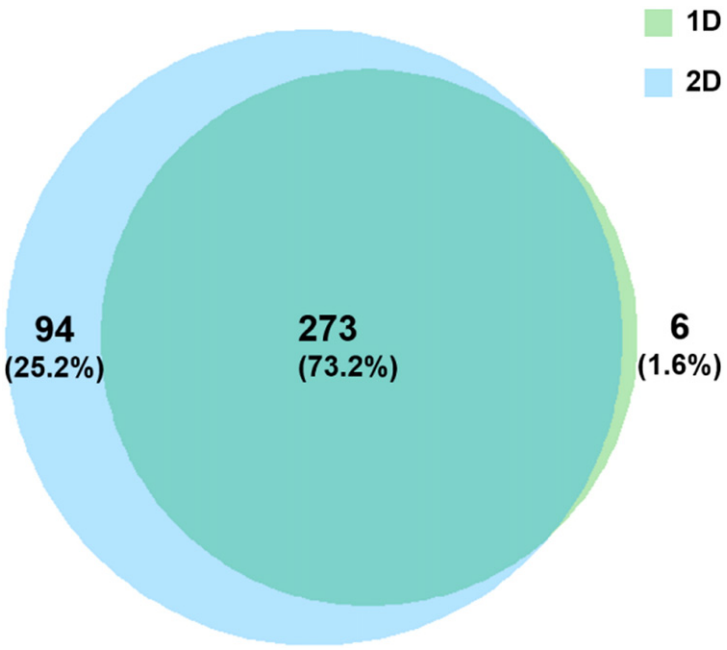

Fig. 7. Venn diagram comparison of quantified peptides 1D vs 2D.

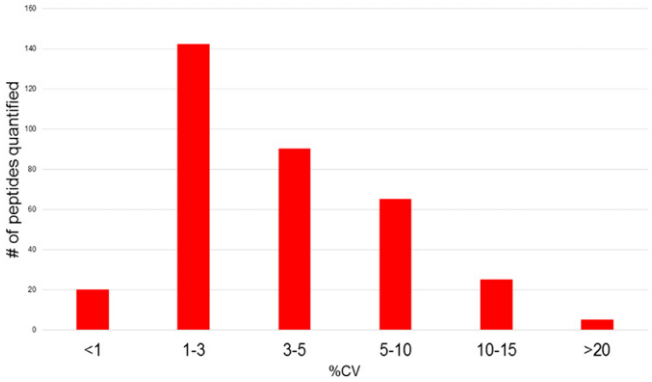

Fig. 8. Distribution of percent coefficient of variation for the 367 quantified peptides by 2D RPRP.

One of the main advantages of the online 2D RPRP method is the greater loading capacity in the first dimension, resulting from the use of a higher capacity column, which allows for a theoretical enrichment of nearly 20-fold compared to the typical loading amounts on smaller-capacity UHPLC columns which are frequently used for plasma proteomics at standard flow rates (approximately 25 μg protein equivalent by 1D LC-MS vs 0.5 mg protein equivalent by online 2D RPRP 0.5 mg was the maximum amount of protein that we attempted to load given the binding capacity of our 1st dimension column). Human plasma has a typical protein concentration of 60–80 mg/mL, and thus is not a protein-limited sample matrix which makes it amenable to increased loading to improve the detection of low-abundance target peptides. The peptide response scaling or enrichment capacity was determined by comparing the relative responses of higher initial loading amounts of SIS peptides in 2D RPRP (200 fmol for 1D vs 5 pmol for 2D on column).

Since online 2D RPRP affords higher initial loading capacities (500 μg protein equivalent vs ~25 μg for 1D), even peptides with lower recoveries (<50%) could still be enriched by the 2D RPRP method (Fig. 4). For example, Apolipoprotein A–IV peptide SLAPYAQDTQEK which had an average recovery of 41.3% (CV 10.0%) was enriched 9.6-fold. Median enrichment from this assay was approximately 8.25-fold (CV 18.2%). When the same assay was applied to a larger panel of plasma peptides, a large majority of peptides showed enrichment ratios of 10–25 fold when plasma digests were scaled from 25 to 500 μg of protein equivalent on column (Fig. 5).

To further assess the efficacy of the online method, online 2D RPRP was compared to offline high-pH reversed-phase peptide fractionation (offline 2D) in terms of sensitivity and interday-reproducibility. To make these comparisons, 6-point reverse calibration curves were separated in triplicate by either offline or online 2D RPRP. The offline high-pH reversed-phase prefractionation resulted in a total of 12 fractions per sample replicate, each of which was subsequently analyzed by 1D LC-MS/MS in MRM mode to quantify 65 SIS peptide targets. This resulted in a total of 216 injections by offline 2D compared to 18 by the online method. Data from these curves was used to assess both the lower limit of quantitation (LLOQ) as a measure of sensitivity, and the interday reproducibility of each method as a %CV between a minimum of three technical replicates spanning 5 days of instrument run time. After undergoing interference screening, peptide LLOQs were defined as being the lowest point within the linear dynamic range of the curve and having a CV of <25%.

Sensitivity was comparable between the two methods with the average being 14.17 fmol/μL of plasma by online vs 13.65 fmol/μL by offline 2D (see Supplementary material). Additionally 27 (41.5%) of

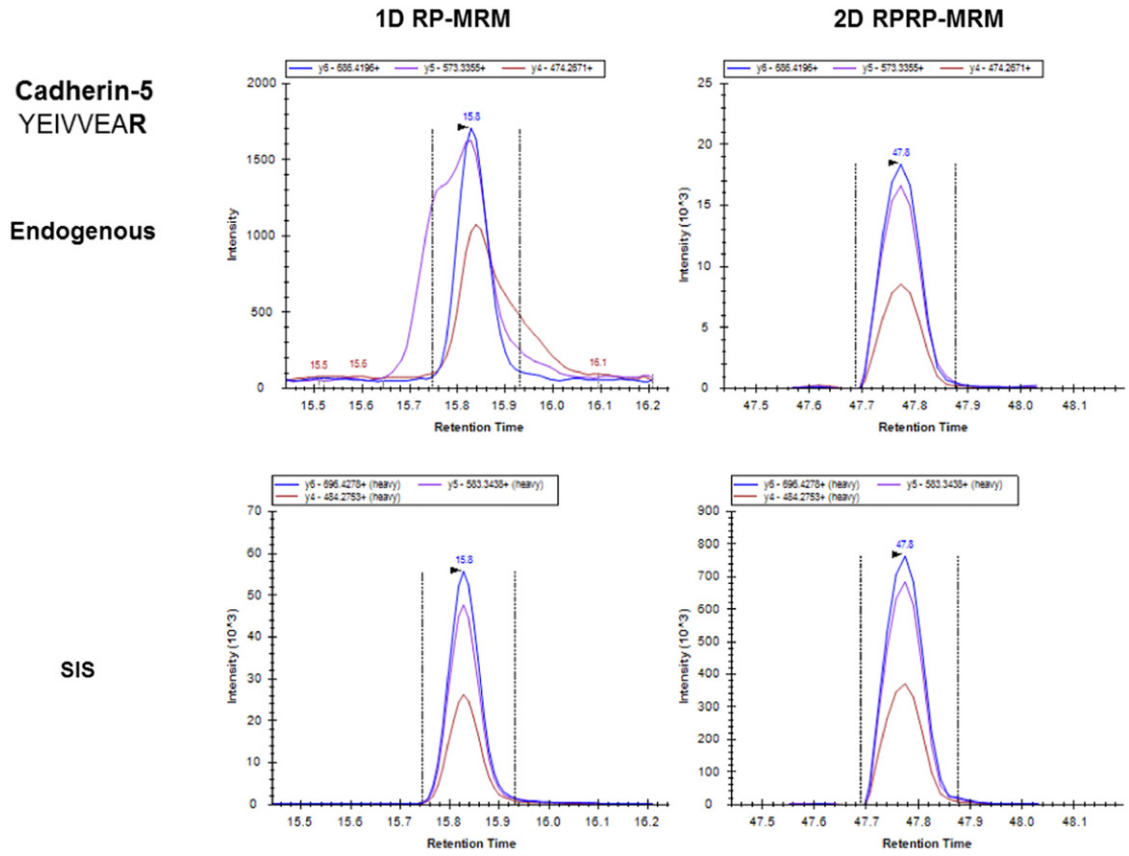

Fig. 9. Example chromatogram comparing 2D to 1D for Cadherin-5 peptide YEIVVEAR. Comparison of the peak area ratios from 2D to 1D shows a roughly 15-fold increase in sensitivity for cadherin-5 peptide YEIVVEAR. By 1D this peptide has obvious peak shape distortions in 2/3 transitions indicating interferences however the peak can be resolved without interferences by 2D RPRP.

the 65 peptides had the same LLOQ between the two methods, while 22 (33.9%) and 16 (24.6%) had a lower LLOQ by online and offline 2D respectively (Supplementary material - Fig. S1). For peptides with different LLOQs between the two methods, the average magnitude of this difference was 3.27-fold. Interday reproducibility was assessed as the %CV of the LLOQ between a minimum of 3 technical replicates. The average interday %CV for online 2D RPRP was 7.46% compared to 12.94% by offline 2D RPRP representing a 1.7-fold increase in reproducibility (Supplementary material – Fig. S2). LLOQs and %CVs for the 65 monitored peptides can be found in the Supplementary material.

These results indicate that offline and online 2D RPRP afford similar sensitivity, but greater reproducibility is obtained with the online method. The difference in reproducibility is likely attributable to the automated nature of the online method. In comparison, offline fractionation requires many manual and time consuming steps, including sample pooling, lyophilization, reconstitution, and transfer between plates or vials. Each of these manual manipulations could introduce error due to sample losses by adsorption to sample vials and tubing, inconsistencies in pipetting, and other sources of human error. In comparison, the online method only requires the user to reconstitute the sample prior to injection, which is far less labor intensive and much less time consuming.

3.3. Application of online 2D RPRP method to quantification of endogenous plasma proteins

As a proof-of-principle experiment for the use of online 2D RPRP for highly multiplexed protein quantitation MRM assays, we evaluated the method on a panel of 400 target peptides. This panel corresponds to peptides with a large range of physiochemical parameters which were known to span >7 orders of magnitude in concentration range – from low ng/mL to mg/mL of protein in plasma. First, peptide retention times were measured by unscheduled MRM analysis using a pooled equimolar SIS standard containing all 400 peptides. Scheduled dynamic MRM was then used to assess the detectability of the endogenous peptide targets from the equivalent of 500 μg of plasma protein, by measuring at least 3 transitions per target peptide. All qualified peptides had to meet the following criteria: a) peaks corresponding to the endogenous peptide must co-elute with their corresponding SIS peptide, b) at least 2 of the 3 transitions had to be detected, c) the transition ratios must be similar (dot product >0.9) and d) the peaks must have similar shapes. To rule out potential false positives due to incomplete incorporation of $^{13}C/^{15}N$ in our reference SIS peptides, we performed parallel digests with 50-fold less SIS. If the level of endogenous peptide remained unchanged, the peptide was validated as being interference free.

367 qualified peptides, corresponding to 168 proteins, were quantitated with a single 2DRPRP-MRM method (Fig. 6). This compared favorably to the 279 peptides (corresponding to 140 proteins) that were quantifiable by three separate 1D RP LC-MRM-MS analyses (Fig. 7). Notably, 94 peptides (corresponding to 76 proteins) were quantified by 2D RPRP but not by the 1D method (see Supplementary material - tables for more information). Assessment of the inter-day reproducibility was also favorable, with the majority of peptides having an average %CV of <5% (Fig. 8).

The improved sensitivity of the online 2D RPRP method compared to the 1D method is attributable to the following: 1) the semi-orthogonal nature of the high-pH separation in the first dimension sufficiently simplifies the sample matrix allowing for better ionization, 2) the increased loading capacity of our system allows for “brute force” scaling as a means of increasing the amount of analyte on column – thus improving overall signal, For example, the cadherin-5 peptide YEIVVEAR SIS showed a roughly 15-fold increase in concentration between the 2D and the 1D methods, but the protein was not quantifiable by the 1D method, due to interferences in 2 of the 3 transitions (Fig. 9).

The protein concentrations determined by the 2D RPRP method spanned 7 orders of magnitude – from the low ng/mL to mg/mL protein concentration ranges. The median inter-day CV ($n = 5$) was 3.39% for peptides quantified by the 2D method which was comparable to that determined by 1D method, which had a median CV of 2.24% (Fig. 8). In general, the peptide concentrations determined by the 2D RPRP method correlated well with those measured by 1D LC-MRM (slope = 0.975, $R^2 = 0.94$) (Supplementary material - Fig. S3). For the remaining 33 peptides that either failed qualification due to interference, or were simply not detected in the endogenous sample, we hypothesize that this can be attributed to at least two issues. The first is that our online method did not make use of special pooling strategies as employed in other offline methods to improve separation orthogonality in the first dimension. Secondly, due to the orthogonal behavior of peptide retention between the high- and low-pH separations, it is possible that some peptides eluting from the high-pH column even after organic solvent dilution will show less retention.

4. Conclusions

Due to the inherent complexity of the human plasma proteome there is an obvious need for additional matrix simplification methodologies to help ease the analytical challenge of measuring low-abundance putative protein biomarkers. This paper serves as a proof-of-concept for the feasibility of performing online high-pH low-pH RP-RP separation to help reduce the complexity of the sample and to allow better analytical sensitivity than traditional 1D LC-MRM based measurements. Compared to the offline 2D RPRP method published previously [13], the online method is significantly faster even in its current length (6 h for 13 fractions compared to ~30 h for offline fractionation and subsequent 1D analyses).

In a few cases, offline 2D RPRP resulted in better sensitivity than the online method, despite having higher %CVs at the LLOQ. For example, carboxypeptidase N catalytic chain peptide SIPQVSPVR was 20-fold more sensitive by offline 2D RPRP (see Supplementary material – tables). This could be due to better separation orthogonality as a result of the strategic pooling of non-adjacent fractions by the offline method which is not possible with online 2D RPRP. Despite this, the online 2D RPRP method provided equal or greater sensitivity for the majority of peptides analyzed. Additionally, the online method is more amenable to high-throughput analysis of multiplexed peptide panels since considerably less manual sample manipulation is required. Compared to the offline 2D RPRP method published previously [13], the online method is significantly faster even in its current length (6 h for 13 fractions compared to ~30 h for a single sample analyzed by offline fractionation and subsequent 1D analyses).

Additionally, the online method demonstrated excellent robustness as evidenced by lower inter-day CVs relative to offline 2D RPRP. Furthermore, although not investigated here, we performed SPE clean-up on our samples post-digestion (so that the same digests could be injected by both 1D and 2D). However, because the first dimension separation effectively desalts the sample it is possible this step could be skipped as well thus saving additional time and cost. In future work, we will adapt this method to smaller more precise “heart-cutting” methods for specific target peptides of biological interest in addition to comprehensive lists of proteins – since methods targeting these smaller panels of analytes can be developed and optimized much more quickly.

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Transparency document

The Transparency document associated with this article can be found in the online version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2017.07.018.

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