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The use of under-loaded C18 solid-phase extraction plates increases reproducibility of analysis of tryptic peptides from unfractionated human plasma

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Abstract

Bottom-up proteomics requires the digestion of proteins into peptides by processes that use salts for denaturing and buffering purposes. These salts need to be removed prior to mass spectrometry analysis to reduce ion-suppression; solid phase extraction (SPE) is a commonly used strategy. There are many commercially-available SPE sorbent types and sizes, which are generally provided with manufacturer recommendations for use, including protein loading capacity. We found that these general suggestions were often not ideal, and our data suggest that context-specific evaluation of sorbent type and amount can improve reproducibility. Specifically, the universal Oasis HLB sorbent provided better retention of the more hydrophilic peptides than the traditional C₁₈ reversed-phase SPE, but it did so at the expense of an increased loss of the more hydrophobic peptides. We found that increasing the amount of the C₁₈ sorbent beyond the manufacturer's guidelines decreased breakthrough (i.e., increased retention) of twelve hydrophilic, identifiable peptides without loss of hydrophobic peptides. This procedure was robust in a 96-well plate format.

INTRODUCTION

Technological advances in modern chromatography and high-resolution mass spectrometry, together with the development in bioinformatics tools for large-scale data analysis, have made label-free proteomics using LC-MS/MS a promising approach for biomarker discovery.^(1, 2) Human plasma arguably holds the biggest potential for the discovery of novel proteomic biomarkers.^(3, 4) Bottom-up proteomics, the most common approach,^(5, 6) utilizes enzymes such as trypsin to digest protein mixtures into their respective peptides. This process requires salts for denaturing and buffering purposes,^(7, 8) and these salts must be removed prior to analysis by LC-MS to avoid ion-suppression effects on the mass spectrometry. Solid-phase extraction (SPE) is often the method of choice for removing these salts.

When searching for low-level biomarkers in the complex human plasma sample, researchers often push the sensitivity and detection limits of the LC separation and mass spectrometry platforms, but the effectiveness and robustness of the SPE step is often overlooked. Here we

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evaluated several SPE sorbents and sizes to improve performance for the study of a tryptic digest of human plasma. Since the primary long-term goal in our studies is classification of study subjects by their diet, there were no specific targets for the SPE extraction. Thus, we aimed for maximizing comprehensive recovery of the plasma proteome. We are also interested in relatively small changes, and we thus place a premium on reproducibility. Therefore, rather than focusing on how much of a given protein or proteins was being recovered, we took an alternative approach -- to look at the sample loss, i.e., the compounds that broke through the SPE extraction. Our goal was to identify an SPE-based platform (column and method) that provided the minimal amount of breakthrough, i.e., the platform that had the fewest peaks that leaked through and/or lowest intensities of any peaks that leaked through. We also investigated the hydrophilic properties of any break-through peaks/peptides to better understand the retention capacity of different SPE columns.

The use of a 96-well extraction plate should help to reduce sample processing time and increase throughput, both of which will be essential for a large-scale biomarker discovery study. The use of 96-well SPE plates has been successfully applied to high-throughput quantification of drugs and metabolites from human body fluids.^(9, 10, 11) To our knowledge, however, there has been no study reporting its application to plasma proteomics. One major concern was the reproducibility of this approach/device when applied to a large-scale study containing hundreds to thousands of samples. At the end of the present study, we demonstrated the reproducibility of the device with our selected type of SPE using forty-five pooled human plasma samples.

EXPERIMENTAL SECTION

Immunoaffinity depletion

40 μ l aliquots of a commercially obtained pooled human plasma sample were loaded onto a 4.6 $\times$ 100mm MARS 14 column (Agilent, Palo Alto, CA). The MARS 14 column is designed to remove fourteen high-abundant proteins (albumin, IgG, α 1-antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, α 2-macroglobulin, α 1-acid glycoprotein, apolipoprotein AI, apolipoprotein AII, IgM, transthyretin, and complement C3). Together, these proteins constitute approximately 94% of the total protein mass of human plasma. Depletion was conducted using an Agilent 1200 HPLC system with the Agilent proprietary buffers A (binding, washing and equilibrating) and B (elution) following the manufacturer-recommended procedures. The flow-through fraction, which contains the medium and low-abundant proteins, was collected and stored in -80°C for use in the SPE studies described here. The quantity of protein contained in each flow-through fraction ($\sim 150\mu\text{g}$) was determined using the Coomassie Plus Protein Assay (Pierce, Rockford, IL). We focused on depleted plasma for these studies as depleted plasma is typically used by our lab and by others conducting serum/plasma proteomics studies to allow more in depth analysis of medium abundance proteins.

Trypsin Digestion

The flow-through fractions from depletion were first de-salted using AmiconUltra-4 filters (10kDa). The samples were then buffer-exchanged to 50mM triethylammonium bicarbonate (Sigma T7408) containing 6M guanidine HCl. Proteins were reduced in 5mM dithiothreitol for 1 hr at 37°C followed by alkylation with 10mM iodoacetamide for 15 min at 37°C in the dark. Following this reaction, 20mM of dithiothreitol was added to react with the remaining iodoacetamide for 15min at 37°C . The sample was then diluted 10-fold using 50mM triethylammonium bicarbonate. Proteolysis was initiated by adding 4 μg of Mass Spectrometry Grade trypsin in 0.5 $\mu\text{g}/\text{ul}$ solution (Promega, Madison, WI) to the mixture and

incubating it at 37°C for 16 hr. Upon completion of the digestion step, 20ul of 10% formic acid was added to stop the reaction.

SPE Procedures

Seven SPE products from Waters (Milford, MA) with different sorbents and/or sizes were compared in this study. They are: Oasis HLB μ Elution plate (2mg HLB sorbent), Oasis HLB 5mg, Oasis HLB 10mg, Oasis HLB 30mg, Sep-Pak C18 50mg, Sep-Pak tC18 50mg, and Sep-Pak C18 100mg. For all the SPE extractions, cartridges were pre-conditioned using 1ml acetonitrile, and equilibrated with 2ml water. The trypsin digest was loaded and washed with 1ml water. The flow through and the wash were collected and processed by a second SPE cleanup procedure to recover peptides not retained during the first SPE step. Elution was done with various amounts of 80% acetonitrile depending on the type/size of the sorbent (See Table 1). The elutes were then evaporated under vacuum and resuspended in 25ul of LC-MS mobile phase A (water containing 0.1% formic acid). For the 96-well plate study, we used an extraction plate manifold from Waters with an in-house built vacuum control setup.

LC-MS/MS Analysis

Approximately 24 μ g of the protein digest was loaded onto a 1.0 $\times$ 150mm UPLC column (BEH C18, 1.7 μ m particle). The LC separation was carried out with a 40min gradient of 1–41% B and 9min gradient of 41–99% B at a 30 μ l/min flow rate. Mobile phase A was water containing 0.1% formic acid; mobile phase B was acetonitrile containing 0.1% formic acid. Full scan spectra (m/z 300–2000) were acquired in the Orbitrap with a 60,000 resolution at m/z 400 followed by two data-dependent MS/MS events acquired in the LTQ. Dynamic exclusion was enabled with a 30 sec exclusion time window.

Data analysis

Peak detection was performed using Hardklör and Krönik. They are freely available software tools provided by the McCross group at the University of Washington^(12, 13). Hardklör deconvolutes and determines the monoisotopic mass and charge state of peptides; Krönik removes the redundancy in Hardklör results and returns a list of peptides detected in a LC/MS data file with their m/z values, charge states, and retention times.

MS/MS spectra were searched against the non-redundant human IPI database (version 3.47) using SEQUEST (Bioworks 3.3.1., Thermo Fisher Scientific). Carbamidomethyl cysteine (+57.0210 Da) was set as a fixed modification; oxidized methionine (+15.9949 Da) and N-acetylation (+42.0106 Da) were set as variable modifications. Searches were done with full trypsin digestion allowing three missed cleavages and a mass accuracy of 10ppm in MS mode and 1.0 Da in MS/MS mode. The false discovery rate (FDR) was estimated using a decoy database search in which a reversed sequence database was appended with the correct sequences. FDR was calculated as two times of the number of identifications from the reverse database divided by the total number of identifications.

The reproducibility study was carried out in SIEVE (version 1.3). The parameters used in SIEVE were: 0.8 min retention time window, 0.016 m/z window, and 20,000 maximum frames. The isotagger module was enabled. Once SIEVE generated a frame table, the result was exported to Excel for statistical analysis.

RESULTS AND DISCUSSION

Number of break-through peaks

Since the recovery efficiency from the SPE step is critical to our biomarker discovery study, we performed a side-by-side comparison of the performance of the three sorbents with various sorbent sizes as listed in Table 1. Three types of solid-phase extraction sorbents were tested in the present study: (i) Oasis HLB; (ii) Sep-Pak C₁₈, and; (iii) Sep-Pak tC₁₈. All three sorbents are suitable for desalting tryptic-digested protein samples. Oasis HLB is a universal sorbent that can be used for polar or nonpolar, acidic or basic compounds and which may thus provide better recovery for the more hydrophilic peptides. Sep-Pak tC₁₈ has overall similar retention capacity as C₁₈ sorbent, but the trifunctional binding chemistry is predicted to give tC₁₈ a better hydrolytic stability than C₁₈.

Since our goal was to maximize overall recovery of the plasma proteome, we characterized both the amount and the type of peptides that broke through each SPE extraction. This was assessed in the context of a two-step SPE process. The flow-through fraction of the initial SPE extraction was collected and this fraction was then run through a second SPE extraction. This enabled recovery and subsequent analysis of those peptides that broke through the first solid-phase extraction. Peak detection was performed using Hardklör and Krönik. The number of peaks detected in the "leak-through" fraction was counted only for peaks with a charge of 2 or higher (Table 2), because the typical tryptic peptide has a charge of 2 or higher.

Prior to trypsin digestion, our immunoaffinity-depleted plasma sample contained ~150µg of protein. The sorbent amount suggested by the manufacturer was 5mg, but we found that 5 mg of sorbent did not provide sufficient retention for our sample. Specifically, when we looked at the total number of peptides that leaked through columns with the Oasis HLB sorbent, it was clear that there was, not unexpectedly, an inverse relationship between the amount of the sorbent and the number of break-through peaks. The number of break-through peptides decreased from 11249 to 889 when the sorbent size increased from 2mg to 30mg. Our study showed that the 30mg of Oasis HLB sorbent had comparable but slightly lower capacity than the 50mg of C₁₈ and tC₁₈ sorbents (i.e., suggesting ~1.5× binding capacity under our conditions). When the amount of the C₁₈ sorbent used was doubled from 50mg to 100mg, the number of break-through peptides further dropped by half. These data indicate that neither the Oasis HLB-30mg nor the 50mg C₁₈ (and tC₁₈) sorbent was retaining all tryptic peptides. Thus, neither cartridge provided sufficient retention for our tryptic digested plasma sample, despite their theoretical capacities being well beyond the suggested sorbent amount.

Retention time distribution and intensities of the break-through peptides

To better understand the retention capacity/performance of the three SPE sorbents, we next divided the break-through peaks into several retention time sections. Since the C₁₈-100mg SPE column provided the best recovery, the first SPE extraction of the C₁₈-100mg column was used as a reference showing the typical retention time distribution of the tryptic peptides of human plasma. As shown in the reference sample in Table 2 (last column), the majority of the tryptic peptides (80%) eluted between 15 to 50min, so our main goal was to minimize the number of break-through peaks in this region while recovering as many hydrophilic peptides (i.e., those peptides that elute before 15min) as possible.

With the HLB sorbent, increasing sorbent amount was associated with a gradual decrease of the number of peptides in the 15–50min region that leaked through the initial SPE extraction (Table 2). Even with the Oasis HLB-30mg sorbent, however, the number of leak-through peptides in this region was still >10-fold higher than that observed with the three reverse-

phase sorbents studied. This comparison indicates that the reverse-phase sorbents (both C₁₈ and tC₁₈) provide superior retention of the more hydrophobic peptides, which make up, as noted above, the majority of our tryptic peptides. Conversely, use of the HLB columns was associated with a slightly better retention in the 0–10min region in comparison to the C₁₈-50mg and tC₁₈-50mg sorbents. These data indicate that the use of Oasis HLB did provide better retention for the more hydrophilic peptides (Table 2). Increasing the C₁₈-50mg and tC₁₈-50mg sorbents from 50mg to 100mg yielded a number of leak-through peptides in the 0–10min region comparable to the HLB columns (Table 2). When the amount of the C₁₈ sorbent was increased from 50 to 100mg, the number of break-through peptides eluted in the 15–50min region stayed constant, but the number of peptides that eluted in the 0–15min region dropped more than 50% (Table 2).

Together, these data indicate that increasing the amount of C₁₈ sorbent improved retention of the more hydrophilic peptides, while maintaining the excellent retention of the hydrophobic peptides. These data show that the use of the 100mg size of the C₁₈ sorbent enabled us to overcome the weakness of this C₁₈ sorbent when compared to the universal sorbent Oasis HLB and improve overall retention of our tryptic peptides. The analysis above describes experiments aimed at reducing breakthrough at the qualitative level; we were also interested in reducing breakthrough at the quantitative level. To illustrate this point, we plotted the intensities of the break-through peaks with their retention times from the three reversed-phase SPE columns tested (Figure 1). The C₁₈-50mg and tC₁₈-50mg columns showed overall similar retention capacity regarding both the number and intensity of break-through peaks in the entire chromatographic separation region. The C₁₈-100mg SPE column had fewer break-through peaks with lower intensity, and this difference is particularly noticeable in the 0–15min and 50–65min region. Regarding our primary target for minimizing break-through, the 15–50min region (highlighted in Figure 1 with dashed lines), all three silica-based SPE columns had few peaks and low peak intensities.

Identification of the break-through peaks

Based on our study, the C₁₈-100mg SPE cartridge (or the functionally equivalent tC₁₈-100mg) provided the best overall retention for our tryptic digested plasma sample. A SEQUEST search of the break-through peaks against the non-redundant human IPI database discovered only 12 hits with less than 1% false discovery rate (Table 3). Each of the peptides identified had a retention time between 5 to 10 min, i.e., the region in which the break-through peaks were found with the highest intensity (Figure 1). Peaks in other retention time regions were not identified with enough similarity to the database, possibly due to their low intensities. The majority of the identified break-through peptides had less than 50% recovery rate with the first SPE extraction due to their hydrophilic nature (Table 3). Since we would expect other peptides from these proteins within the main part of the chromatogram, these leaks are not a significant concern for this type of profiling proteomics.

Reproducibility of the 96-well extraction plate

To increase the throughput of the SPE extraction step for large-scale proteomics profiling, we selected the Sep-Pak tC₁₈-100mg 96-well extraction plate. As we demonstrated in our study, tC₁₈ has the same retention capacity as C₁₈ sorbent. The reproducibility of the extraction plate was investigated using 45 human plasma pool samples processed on 15 extraction plates. On each plate, three pool samples were spaced evenly by 93 individual plasma samples (i.e., every 31 individual human samples was followed by a pool sample). Data were analyzed in SIEVE (v1.3, 20,000 frames generated), and statistical calculations for frames with charge 2 or higher were performed in Excel. The reproducibility of the 96-well extraction plate was shown in three ways: reproducibility of the 20,000 frames for the pools within a given plate, reproducibility between the plates, and reproducibility of all the

human pool samples. Reproducibility within the extraction plate was the best (median CV = 21%, Figure 2). Reproducibility among the 15 tested extraction plates was essentially equivalent (median CV = 23%). The reproducibility of the 45 individual pool samples was slightly worse (median CV = 33%).

CONCLUSIONS

Our data suggests that context-specific evaluation of sorbent type and amount can improve reproducibility of bottom-up proteomics studies. In the present study, we compared seven different SPE columns to maximize comprehensive retention for proteome-wide tryptic-digested peptides from human plasma. We found that, compared to the traditional reversed-phase SPE, the universal HLB sorbent did provide better retention of the more hydrophilic peptides (eluted before 10min in a 65-min LC separation). The use of the universal HLB sorbent was, however, associated with a greater loss of more hydrophobic peptides (i.e., those eluted between 15 and 50min). In our case, we do not want to sacrifice the more hydrophobic peptides, which are often longer and provide better sequence information, for the shorter hydrophilic peptides. Therefore, reversed-phase sorbents C₁₈ and tC₁₈ were chosen over the Oasis HLB sorbent for our proteomics analysis. Capacity-wise, the 100mg column with C₁₈ sorbent provided the most thorough recovery with the least number of break-through peptides and the overall lowest intensities of break-through peaks. Database searching identified only 12 peptides in the break-through fraction of the SPE extraction using C₁₈-100mg column. All of these identified peptides eluted in the 5 to 10 min region. The 100mg tC₁₈ sorbent in the 96-well extraction plate format displayed equivalent reproducibility both within and between extraction plates (CV = 23%).

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Figure 1. Chromatographic distribution of breakthrough peaks

Intensities of break-through peaks in SPE extraction using C18-50mg (Δ), tC18-50mg ($\times$), and C18-100mg (solid circle) columns. Intensity was plotted in the log scale. Peaks eluted in 15–50min region were highlighted in a dashed box.

Figure 2. SPE Reproducibility

Histogram plots of the CV of 45 pooled human plasma samples processed on fifteen 96-well extraction plate with -100mg SPE sorbent. Blue bars show the within plate CV; red bar show the between plate CV; black bars show the CV of all the samples.

Table 1

List of the tested SPE columns and the elution volume for each column

SPE type	HLB- 2mg	HLB- 5mg	HLB- 10mg	HLB- 30mg	C ₁₈ - 50mg	tC ₁₈ - 50mg	C ₁₈ - 100mg
Elution volume	200 µl	200 µl	400 µl	800 µl	400 µl	400 µl	800 µl

Table 2

Retention time distribution of break-through peaks from each of the seven different SPE column types. As a reference, number of peaks in the first extraction of the C₁₈-100mg column was given in the last column of the table.

RT (min)	Number of Peaks (% of the total)							
	Second SPE							First SPE of C ₁₈ - 100mg
	HLB- 2mg	HLB- 5mg	HLB- 10mg	HLB- 30mg	tC ₁₈ - 50mg	C ₁₈ - 50mg	C ₁₈ - 100mg	
total	11249	2284	2056	889	673	654	328	14452
0-10	251 (2.2)	269 (11.8)	396 (19.3)	301 (33.8)	480 (71.3)	466 (71.2)	234 (71.4)	1124 (7.8)
10-15	1762 (15.7)	1175 (51.4)	1107 (53.8)	208 (23.4)	142 (21.1)	98 (15.0)	35 (10.7)	1655 (11.4)
15-50	9204 (81.8)	806 (35.3)	512 (24.9)	348 (39.1)	31 (4.6)	37 (5.7)	31 (9.5)	11570 (80.1)
50-65	32 (0.3)	34 (1.5)	41 (2.0)	32 (3.6)	20 (3.0)	53 (8.1)	28 (8.5)	103 (0.7)

Table 3
Peptide identification and their recoveries of the break-through peaks from the C₁₈-100mg SPE cartridge

<i>m/z</i>	RT (min)	<i>Z</i>	Peptide	Protein	Peak intensity		
					1st SPE	2nd SPE	% leak
362.1525	5.14	2	-CTEEGK.-	Beta-2-glycoprotein 1	9.89E+03	1.48E+05	93.75
344.8405	6.15	3	-GKQANTEER.-	gelsolin isoform a precursor	3.23E+04	8.91E+04	73.38
462.6990	6.48	2	R.MKDQCDK.C	clusterin isoform 2 preproprotein	1.18E+05	3.45E+05	74.50
374.1908	6.53	2	R.ANSAGATR.A	gelsolin isoform a precursor	3.97E+04	3.02E+05	88.39
454.6799	6.55	2	R.CNDQDTR.T	Fibronectin	3.01E+04	1.34E+05	81.68
417.1909	6.75	2	K.ENAEQSR.A	Antithrombin-III	1.99E+04	3.07E+05	93.91
470.7325	6.98	2	R.GTSSTTTTGK.K	Plasminogen	7.01E+03	1.50E+05	95.53
341.1527	7.60	2	K.SGSMGR.K	ITI heavy chain H4	9.17E+05	1.64E+06	64.17
413.6839	7.79	2	K.MGSSTSEK.T	Complement component C6	5.99E+05	5.69E+05	48.71
358.7186	8.16	2	-IDNVKK.-	Kininogen-1	5.28E+06	6.72E+05	11.29
335.6795	8.19	2	K.NGPPTK.T	Fibronectin	7.28E+05	2.71E+05	27.14
319.1434	8.16	2	R.ACCVK.S	Fibulin-1	2.68E+04	1.21E+05	81.86