An assessment of peptide enrichment methods employing mTRAQ™ quantification approaches

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Abstract

The human plasma peptidome has potential in biomarker discovery not least because the plasma proteome is a challenging matrix due to its complexity and dynamic range. However methods to significantly reduce the amount of protein present in plasma whilst retaining the less abundant peptides present in plasma samples has been a major issue. Here we present a novel strategy which has been employed to assess the effectiveness of removing interfering proteins whilst retaining peptides of interest. In order to monitor peptide retention a spiked in digested protein, in this case a synthetic QconCAT™ protein, was employed. This enabled a variety of target analytes (peptides) to be monitored for their retention in liquid phase, providing a broader picture of peptide loss from each method assessed. The incorporation of mTRAQ™ labeling allowed the presence of each peptide to be monitored, and accurate peptide losses to be determined in a Selected Reaction Monitoring (SRM) assay, thus enabling an objective semi-quantitative conclusion to be drawn regarding the suitability of each method for protein removal and peptide retention. We also assessed a range of methods for retaining non-tryptic peptides in a plasma peptidomics workflow. From these data, we determined an optimal workflow for removing intact protein, whilst retaining peptides for MS-based analyses.

Introduction

Discovery of prognostic, diagnostic and surrogate biomarkers is an essential feature of biomedicine in the 21st century. The need to minimize invasive procedures means that plasma and urine are regarded as favored samples to monitor response to a disease or treatment. Thus plasma is being monitored for changes in protein levels as a response to drug treatments¹⁻³. However, plasma has a large dynamic range of proteins, spanning over ten orders of magnitude, with 22 proteins making up over 99% of the human plasma proteome⁴. In addition, plasma may contain large, intact proteins such as albumin at total concentrations of $\sim 70 \text{ mg/mL}$ ⁵. It has long been known that injecting large amounts of intact protein is detrimental to the performance of liquid chromatography systems⁶. This is especially true when using nano-LC systems. Furthermore, standard mass spectrometric procedures on tryptic digested protein samples would be inherently and majorly compromised by the protein (and therefore peptide hydrolysate) dynamic range. Thus in the search for potential biomarkers, profiling the human plasma peptidome has gained considerably in popularity over recent years⁷⁻¹¹. This approach would have benefit, for example, in considering the release of tumor proteins on treatment of a patient with targeted therapies such as tyrosine kinase inhibitors¹² or combination therapies including chemotherapeutic agents such as DNA damaging agents¹³. Several methods for the enrichment of smaller peptides and removal of intact proteins have been proposed¹⁴, including filtration⁴, precipitation¹⁴⁻¹⁶ and solid phase extraction using C₈ coated beads¹⁷. In this report we performed a relative comparison study between several of these methods using a range of peptide standards derived from intracellular proteins. We have assessed the efficiency of methods for the removal of intact protein from plasma samples. We have also determined the retention of peptides from the protein-depleted samples using a novel approach. The mTRAQTM approach was first used by Holzmann *et. al.*¹⁸ as an alternative to the AQUATM strategy to accurately determine the amount of MP1-p14 complex present in standard samples. mTRAQTM reagents are non-isobaric chemical tags similar to iTRAQTM, which once incorporated, lead to a mass shift of 4 Da between each label. Consequently, quantitative comparisons between a standard peptide and a sample peptide may be made, provided each is labeled with a different tag.

The versatility of mTRAQ™ labeling allows peptide quantification at both the MS1 (due to different precursor masses) and MS2 levels (due to different mTRAQ™ reporter ion masses). This offers clear advantages over iTRAQ™ as this only allows quantification to take place at the MS2 level. In order to assess the amount of peptide present, we have incorporated the use of mTRAQ™ labeled QconCAT™ peptides as surrogate standards, with subsequent selected reaction monitoring (SRM) analysis. In our protocol, the use of mTRAQ™ reagents enables a suite of standards to be prepared in order to assess the loss of target peptides from each enrichment method. Multiple target peptides for this analysis were generated from a trypsin-digested synthetic QconCAT™ protein. QconCAT™ was developed by Pratt *et al.*¹⁹ and involves generation of a synthetic ‘designer’ protein which, when digested, yields equimolar amounts of a number of peptide standards of interest with attention paid to MS ionization capability. Several QconCAT synthetic proteins have been developed for a variety of purposes²⁰⁻²². In this study, ten chemically distinct peptides were assessed for a broad assessment of protein depletion methods that retain target peptides more effectively.

Materials and Methods

Chemicals

All reagents, unless otherwise stated, were molecular biology grade or higher and purchased from BDH, Poole, UK.

The following chemicals were used throughout; HPLC grade water (Rathburn, Walkerburn, UK), HPLC grade acetonitrile (Fisher Scientific UK Limited, Leicestershire, UK), mass spectrometry grade formic acid (Fluka, Sigma-Aldrich, St. Louis, MO), and acetone.

SDS PAGE gels were prepared using 0.8% (w/v), bis-acrylamide solution protein and sequencing electrophoresis grade (Flowgen Bioscience, Nottingham, UK), Tris (Melford, Ipswich, UK), Sodium dodecyl sulfate, ammonium persulphate, tetramethylethyldiamine (TEMED) and glycine (all from Sigma, St. Louis, MO).

The chemicals used for silver staining were; analytical grade methanol (Fisher Scientific), acetic acid sodium thiosulphate, silver nitrate, sodium carbonate, $\geq 36.5\%$ formaldehyde in water (Fluka).

mTRAQ™ labeling reagents were purchased from Applied Biosystems (Foster City, CA). Tetraethylammonium bicarbonate 1.0 M, pH 8.5 and HPLC grade propan-2-ol were from Sigma. Trypsin was sequencing grade and its reaction buffer were supplied by Promega (Madison, WI).

Mass Spectrometry Analysis

All SRM-based peptide enrichment samples were analyzed on a 4000 Q-TRAP™ (Applied Biosystems, Foster City, CA) mass spectrometer coupled to a liquid chromatography system consisting of a Famos autosampler, a Switchos trap system and an Ultimate Plus pump (LC Packings, Dionex, Sunnyvale, CA). The analytical column was an Acclaim PepMap 100, 75 µm x 15 cm, C₁₈ column, particle size 3 µm size 100 Å (Dionex). The software used on the 4000 Q-TRAP™ was Analyst version 1.4.1 (Applied Biosystems). The control software for the liquid chromatography system was Chromeleon 6.50 SP4 Build 1000. Buffer A and the Switchos buffer both consisted of 97.9% water, 2% acetonitrile and 0.1% formic acid (v/v/v). Buffer B consisted of 79.9% acetonitrile, 20% water and 0.1% formic acid (v/v/v). The gradient was held at 8% B for 4 minutes. Following this, the % B was increased to reach 40% at 25 minutes. B was increased further to reach 60% at 35 minutes and held for 2 minutes, after which it was increased to 80% 0.1 minutes later and held for a further 5 minutes. Finally, the % B was reduced to 8% for 5 minutes at a flow rate of 320 nl/min. Q-TRAP™ ion spray voltage was 2300.0 V, interface heater temperature was 155.0°C, curtain gas was 20.0 and declustering potential was 60.0.

All shotgun peptidomic peptide enrichment samples were analysed on an LTQ Orbitrap Velos™ (Thermo Fisher Scientific, Hemel Hempstead, UK) coupled to a nanoAcquity UHPLC system (Waters, Manchester, UK). In this experiment, CID was performed in the linear ion trap on the top 20 precursors at 30,000 resolution (precursor m/z 300 – 2000) with a minimal signal of 5000 cps and a charge state of 2+ and greater after being detected in the orbitrap. Precursors were excluded for 30 seconds after two occurrences. Product ions were scanned over m/z 100.0 – 1700.0 at 7,000 resolution. Orbitrap source voltage was 1500.0 V and source temperature was 200.0°C. The software controlling the orbitrap was Xcalibur version 2.1 (Thermo). The nanoAcquity was controlled by MassLynx version 4.1. The loading flow was 15 µl/min and the gradient flow rate was 300 nl/min. Buffer A was 0.1% formic acid in water, and buffer B was 0.1% formic acid in acetonitrile. The loading buffer was 20 mM citric acid. The gradient increased from 3% B to

40% B over 90 minutes. Following this, the % B increased to 90% over 3 minutes where it was held at this level for 15 minutes. After this, the % B was reduced to 3% where it is held constant for 21 minutes. The analytical column was a 1.7 μm BEH130 C_{18} column, 75 μm x 250 mm (Waters) and held at a constant temperature (40°C).

Plasma Sample Collection and Preparation

Plasma was collected with informed consent from healthy volunteers and stored in 250 μl aliquots. Prior to analysis, each aliquot was centrifuged at 16,000 g at 4°C for 5 minutes. The supernatant was then removed and diluted four-fold with phosphate buffered saline (PBS). This was then filtered using a 0.45 μm ProteomeLab™ spin filter (Beckman Coulter, Fullerton, CA) centrifuged at 1,500 g at 4 °C for a further 30 seconds. The filtrate from each tube was removed, pooled together and vortexed for 30 seconds. From this stock, aliquots were taken (25 μl each) to prepare samples for analysis.

Sample Preparation to Determine Protein Depletion Efficiency

Precipitation methods

To an aliquot of plasma (25 μl), either one volume (25 μl) or two volumes (50 μl) of acetone or acetonitrile was added and the resulting mixture was vortexed for 10 seconds. The sample was then centrifuged at 9,300 g for 30 seconds. The supernatant was removed, dried to completion then resuspended in PBS (25 μl).

Filtration

An aliquot of plasma (25 μl) was diluted to a total volume of 1000 μl with PBS and placed in a 5 kDa molecular weight cut-off, 4 ml spin concentrator (Agilent, Santa Clara, CA). The filter was then centrifuged at 3,200 g at 10 °C for 30 minutes. The filtrate was transferred to a new tube. The residue was placed in a separate tube, and all samples were dried to completion. Each sample was then re-suspended in PBS (25 μl).

Solid phase extraction with C_8 coated magnetic beads

Samples were essentially prepared as described by Villanueva *et. al.* with appropriate scaling up to enrich 25 μl of plasma¹⁷.

All samples were prepared in triplicate. After incubating with 4x Laemmli buffer (2 μ l) at 100°C for 2 minutes they were loaded onto a 10 % acrylamide SDS-PAGE gel. SDS-PAGE analysis was performed according to standard procedures using a 10% acrylamide gel (Figure 1).

Silver Staining

Silver staining of all SDS-PAGE gels was performed as described by Shevchenko *et. al.*²³.

Peptide Standard Preparation

A QconCAT™ protein (200 pmol, 10 μ g) was digested by overnight incubation with trypsin (0.5 μ g) at 37°C. The digested sample was then split into two equal aliquots. Each was dried to completion and re-suspended in tetraethylammonium bicarbonate (1.0M, pH 8.5, 30 μ l). mTRAQ™ reagent was dissolved in propan-2-ol (35 μ l) and this was added to the sample. The mTRAQ™ container was rinsed with propan-2-ol (35 μ l) and this was also added to the sample. This mixture was vortexed for ten seconds and left at room temperature for a minimum of 2 hours. Solvent was then removed using a SpeedVac™. The samples were re-suspended in PBS to a concentration of 1 pmol/ μ l.

Sample Preparation to Monitor Peptide Losses

Plasma (25 μ l) spiked with 1pmol mTRAQ Δ_4 labeled peptides underwent either filtration or precipitation with two volumes of acetonitrile. Each depleted sample was collected and 1pmol Δ_0 labeled standards were added. The ratios of the mTRAQ™ reporter ions for each peptide were determined in order to assess peptide losses from each protein depletion method (Figure 2).

Figure 1. The workflow to assess which of the previously published methods being investigated in this study is the best at removing intact protein from plasma samples. This involves splitting a stock plasma sample into equal aliquots, and each aliquot enriched for peptides in a different way. Following this, the enriched samples were run on a 10% SDS-PAGE gel, along with an untreated plasma control sample. Following this, the gel was silver stained and the lane showing the least amount of staining corresponds to the method which is most efficient at removing intact protein from a plasma sample.

Figure 2. The workflow to investigate which enrichment method is best at retaining peptides in a plasma sample. This involves digesting a QconCAT™ protein with trypsin and splitting it into two equal aliquots. Each of these were labelled with a different mTRAQ™ tag. A series of standard samples containing only the two sets of mTRAQ™ labelled peptides in an equimolar amount were made in order to assess labelling efficiency and normalise enriched samples to these standard results. Following this, one set of mTRAQ™ labelled peptide standards (Δ₄) were added to a plasma sample and the sample was enriched. The Δ₀ labelled peptides were added to the enriched sample as a series of internal standards. All samples created in this study were then monitored by SRM using the transition to detect the mTRAQ™ reporter ion for quantification, and all other transitions for peptide identity. From this, the peptide losses from each enrichment strategy could be calculated.

SRM Generation

Six SRM transitions were designed for each target peptide per label for identification. This was done by performing a standard peptide identification experiment to obtain MS/MS data for each peptide. The MS/MS data was analyzed using the Mascot search tool to identify each peptide and find its retention time²⁴. All MS/MS spectra were manually checked to confirm peptide identity. The six most intense product ions were used for the Q3 values for the SRMs. Additionally, an extra transition was added to monitor the mTRAQ™ reporter ions for quantification (114 and 118 Da). A complete list of these transitions, along with their retention times, can be found in Table S-1 of Supporting Information.

Data Processing

SRM data was processed via MultiQuant software Version 2.0 (AB Sciex, Toronto, CA), where the quality of all peak integration was manually confirmed. Peak areas for the most intense transition for each peptide were compared between the Δ_0 and Δ_4 labeled samples and the ratio between the two calculated for each replicate injection. A $\log_{10}$ (average) ratio between the two labels was found for each target peptide by averaging the results for all standards. In order to determine the loss of peptide from each enrichment process, the $\Delta_4 : \Delta_0$ ratio for the enriched samples was compared to the average standard ratio. From these results, the percentage loss for each peptide, as well as the variance for each method was obtained.

All shotgun peptidomic data were processed by Proteome Discoverer 1.1 (Thermo) using the following criteria; Data were searched against the *Homo sapiens* proteome using the UniProt database, and a no enzyme specificity search. The strict and relaxed target false discovery rates (FDRs) were 0.01 (1%) and 0.05 (5%) respectively. Precursor mass tolerance was 15 ppm and fragment mass tolerance was 0.8 Da. Methionine oxidation as well as asparagine and glutamine deamidation were set as dynamic modifications. All peptides with an ion score ≥ 20 and assigned a protein accession number were counted as being identified, with one or more of these peptides being used to assign the identification of a protein.

Results & Discussion

Protein Removal

Plasma (25µl) was depleted for proteins using the methods described above and the enriched fraction collected. To assess remaining protein levels a silver-stained SDS-PAGE gel analysis was performed (Figure 3). The protein remaining in these enriched fractions varied with each depletion method. Sample treatments leaving the least intact protein remaining are both precipitation with two volumes of acetonitrile (Lane 6) and filtration with a 5 kDa molecular weight cut-off filter (Lane 7). These methods demonstrated good repeatability based upon replicate analysis (data not shown). In general, all of the methods investigated were found to be reproducible with the exception of C₈ solid phase extraction. In one sample preparation using C₈ solid phase extraction, the enriched peptide sample became a solid which could not be resuspended in any solvent.

Peptide Losses

Following the results shown in Figure 3, it was clear that the only two methods which efficiently remove intact protein are filtration and precipitation with two volumes of acetonitrile. Therefore, these two methods were taken forward to assess the amount and reproducibility of peptide recovery. A range of ten peptides was used to provide a more comprehensive overview on how peptides are retained from each depletion method. Peptides were selected with different lengths and amino acid sequences, meaning a range of peptides with a variety of physicochemical properties could be assessed.

A standard sample containing both sets of mTRAQ™ labeled peptides in equimolar amounts with no plasma background was analyzed a total of ten times throughout the experiment. These data confirmed that for all samples, the efficiency of each mTRAQ™ labeling was sufficient in both sets of target peptides¹⁸. Therefore, all plasma samples could be normalized against a set ratio generated from these known standards. From this, the loss of each peptide from each treatment method can be determined.

Figure 3. Silver stained 1D SDS-PAGE gel showing the amount of intact protein remaining in each sample after the depletion methods employed to remove protein. The high amount of protein present in lanes 2, 3 and 8 lead to inverted staining. This is suspected to be from the high amount of albumin present in these samples. The lack of staining in lanes 5 and 6 show that less protein is present in the samples enriched by filtration with a 5 kDa M_w cutoff and precipitation with two volumes of acetonitrile. These two methods will be examined further to compare their ability to retain peptides.

Table S-1 shows the SRM transitions for each peptide, along with their retention time and the collision energy used to fragment each peptide. Recently, Sherman and co-workers assessed the number of SRM transitions required to provide enough structural specificity to identify a peptide of interest²⁵. By incorporating the retention time and specific collision energy for each SRM peptide, four transitions provide enough information to identify each target peptide. However, due to the complex background of the plasma samples, it was decided to increase the number of SRMs for each peptide to six, this leads to coverage of approximately 80% of the total ion count of a peptide²⁶. The duty cycle time was increased to 300 ms per peptide, and the dwell time for each transition was kept at 50 ms. The use of an mTRAQ™ strategy allowed us to accurately quantify the degree of peptide losses as a result of the specific enrichment/isolation procedure (Figure 2) by using two transitions (one for each reporter ion) at a high collision energy (Table S-1). This increases the amount of mTRAQ™ reporter ion released upon fragmentation, increasing the sensitivity of this assay and providing more accurate results regarding the amount of peptide retained by each enrichment process²⁷. Data was generated from the most intense SRM transition for each target peptide (shown in bold in Table S-1), with the remaining five transitions used for identification purposes only.

The results in Table 1 show the percentage loss, as well as the cumulative variance (CV), for each target peptide when enriching plasma samples by filtration or precipitation with acetonitrile using three technical replicates for each method.

Acetonitrile precipitation retains more peptide than the filtration method for 8 out of the 10 target peptides, with recovery of the peptide AADDEPEYEDGR being similar in the filtration and precipitation methods (Table 1). In addition, the variance is lower in the majority of cases (7 out of 10) when using acetonitrile precipitation to enrich for peptides compared to filtration. Table 1 shows that only 3 out of 10 peptides have a CV < 20% when peptides from a plasma sample are enriched by filtration, as opposed to 8 out of 10 having CV's of < 20% when the sample is enriched by acetonitrile precipitation.

Table 1. Results showing the recovery of each target peptide, as well as the variation in recovery, from the two methods used to monitor peptide losses during sample enrichment.

Peptide Sequence	Filtration		Precipitation	
	Recovery (%)	Variance (%)	Recovery (%)	Variance (%)
AADDEPEYEDGR	29.1	17.5	27.5	19.6
AETIGEK	5.1	127.0	1.2	25.9
YEAVPADASSSSEVK	65.6	45.0	88.9	18.9
LRPLLEK	16.9	10.1	103.8	18.8
APGGEDEEEGVGGGGGGGELR	8.9	75.7	22.1	122.1
ASLTTPGSEYSSPSVISVSK	2.6	61.1	100.1	17.7
SWLPVVIK	0.4	63.8	68.6	0.7
LWIWEK	0.2	101.6	114.9	16.5
STGSFPFPYVLEPLGASPSETSK	1.8	4.9	92.7	18.4
AFYPASLSPPAAGTAASLSTALLR	0.6	60.8	105.9	18.9

It was noted that the variance in the recovery of peptide FSEISADK is very high when depleting plasma by filtration. In this case the response for the internal standard for this peptide was around 1 order of magnitude lower in one replicate when compared to the other two. However, other peptides from the same samples did not show this and it led to a high variance for this peptide in filtration. As such, this peptide was omitted from the data analysis at this stage due to this technical issue.

In Table 1, it can also be seen that we have recovery values reported to be greater than 100%. The percentage variance in these recoveries explains this. Therefore, it appears that the peptides LRPLLEK and LWIWEK have recoveries ranging from 85.0 – 100% and 98.4% – 100% respectively.

Due to the complexity of the sample and the high probability of interference from other peptides, MS1 quantification could not be performed in this study. However, the flexibility of mTRAQ™ allows quantification to be performed at both the MS1 and MS2 levels. Therefore, quantification was performed at the MS2 level, using one SRM transition to specifically monitor the diagnostic mTRAQ™ reporter ions, with a further six transitions being used to identify the peptides of interest.

Based on these data, precipitation with two volumes of acetonitrile was found to be the most effective method of remove intact protein and enrich for peptides in a plasma sample, from the methods examined in this study.

Retention of Endogenous Plasma Peptides

We next performed a standard peptide identification experiment using an orbitrap Velos to assess the total numbers of plasma peptides we could profile following enrichment by either acetonitrile precipitation or filtration. This experiment identified 354 peptides in precipitated samples and 14 in filtered samples. This equates to 95 and 9 degraded proteins identified respectively (using 1 unique peptide or more to identify a protein). This indicates that endogenous plasma peptides can still be profiled following enrichment with acetonitrile precipitation or filtration, with acetonitrile precipitation leading to a greater number of peptides and proteins being identified.

In addition, it appears that acetonitrile precipitation retains endogenous peptides in a more reproducible fashion. When using acetonitrile precipitation, 53% of endogenous peptides are seen in more than one replicate, with 34% of peptides being seen in all three replicates. This compares to 36% of peptides being

seen in more than one replicate, and 29% of peptides being seen in all three replicates when enriching plasma samples by filtration (Figure 4). Similar results are seen when assessing the reproducibility of protein identifications from each method, with 77% of proteins being identified in more than one sample when enriching plasma by acetonitrile, with 26% of proteins being identified in all three replicates. This compares to 50% of proteins being identified in more than one replicate when enriching plasma samples by filtration, with 20% being identified in all three replicates. This indicates that the plasma peptidome can be more comprehensively and reproducibly examined using acetonitrile precipitation enrichment as opposed to filtration.

(a)

(b)

(c)

(d)

(e)

(f)

Figure 4. Venn diagrams showing the number of peptides found in each replicate, along with the number of peptides found in more than one replicate when enriching plasma samples by (a) filtration, and (b) precipitation. (c) and (d) show the corresponding number of proteins identified in each replicate when enriching samples by filtration and precipitation respectively. The total number of peptides and proteins found by enriching plasma samples by filtration and acetonitrile precipitation, along with the number of peptides/proteins identified in both methods are shown in (e) and (f) respectively.

Conclusion

Whilst there have been many different methods designed to remove intact proteins from plasma to allow analysis of the plasma peptidome via nano-LC-MS experiments, not all methods work equally well. The use of a QconCAT™ protein digest allows the recovery of a range of known target peptides to be assessed, allowing a broader view on peptide losses from filtration or precipitation. The use of mTRAQ™ labeling coupled with SRM measurement allows quantification of the peptide losses and the reproducibility of each method to be accurately determined.

However in the first instance, protein removal is required. For precipitation experiments (using acetone or acetonitrile as a solvent), it is clear that increasing the volume of solvent used to precipitate proteins out of solution is more efficient for removing protein from plasma. This experiment also demonstrates that acetonitrile is a more effective solvent at removing proteins than acetone. C₈ enrichment proved to be the least effective at removing protein, as shown in Figure 3. Whilst filtration is comparable to precipitation with two volumes of acetonitrile in terms of protein removal, it offers lower peptide recovery with higher variance in these recoveries.

Of the two methods that demonstrate the best removal of proteins, filtration and precipitation with two volumes of acetonitrile, we show that peptides are more readily retained by using precipitation over filtration. This is shown for eight of the ten peptides examined here, with similar recoveries being seen for one of the other remaining peptides. This study also shows that in the majority of cases, the variances in precipitation are lower than in filtration, leading to a more reproducible recovery of the final peptide sample.

In addition, we have shown that both precipitation with two volumes of acetonitrile and filtration respectively retain non-tryptic peptides, allowing the endogenous peptidome of plasma to be profiled. From these results, we show that 95 peptides derived from known proteins can be identified with 95% confidence after enrichment with acetonitrile precipitation, and 9 after enrichment with filtration. These are based on ion scores of ≥ 20 , using only peptides with a protein accession number. One or more peptides unique to a protein were required to confirm the protein's identity.

Based on these results, we conclude that of the variety of methods examined here, the preferred way to enrich plasma samples for peptides whilst removing intact protein is to precipitate the sample with two volumes of acetonitrile. Whilst the number of endogenous, non-tryptic plasma peptides we identify is significantly lower than that seen in other studies²⁸, we were able to identify approximately 25% of the number of peptides identified by Tammen *et. al.* without the use of the additional fractionation step (giving greater sensitivity) used in that approach. We have therefore established a method for plasma peptidome analysis and recovery of peptides from protease assays in whole plasma using synthetic peptide substrates.

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Supporting Information Available: This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Supplementary Data Contains tables consisting of the SRM transitions used to determine peptide retention in this study (Table S-1) and the search results for endogenous peptides and proteins identified after enriching plasma samples with filtration or precipitation with two volumes of acetonitrile. In these search results, each replicate is colour coded , allowing them to be differentiated (red = replicate 1, green = replicate 2, blue = replicate 3).

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