49441 ABRF 2009 PRG Report

Summary: Ratios were measured for the proteins in the six samples with 2D RP/RP high/low pH chromatography and label-free relative quantitation. It was found that the following samples were duplicates; A=E, B=F, and C=D.

Sample preparation:
Samples were dissolved in 50 mM ammonium bicarbonate and 0.1% Rapigest prior to reduction with DTT and alkylation with iodoacetamide for in-solution digestion with trypsin. TFA was added to the samples to hydrolyze the Rapigest prior to analysis. 16 µg of tryptic digest was loaded for each 2D analysis in order to identify the spiked-in proteins at the lowest levels.

Instrumental conditions:
A nanoACQUITY UPLC system with 2D technology was used to perform separations in this study. The first dimension was a 300 µm by 5 cm column packed with 5 µm XBridge C18, with a 2 µL/min flow rate. The mobile phase for the first dimension was made up of 20 mM ammonium formate (pH 10) in water (A) and ACN (B). Six discontinuous steps were taken in the first dimension of separation. A 180 µm by 20 mm column packed with 5 µm Symmetry C18 was used to trap peptides between the two dimensions. Effluent from the first dimension was mixed with 20 µL/min, 0.1% formic acid in water prior to the trap column. The mobile phase for the second dimension was made up of 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B). A 75 µm by 150 mm analytical column packed with 1.7 µm bridged-ethyl hybrid (BEH) C18 was used as the second dimension column with a 90 min gradient from 5-40% B at 300 nl/min. A Synapt HDMS mass spectrometer was used to analyze the digests with MS^E.

Data analysis:
All data were processed with ProteinLynx Global Server version 2.3, with Identity^E informatics. Default parameters were used with a database made up of the human proteins from the UniProt database containing the spiked-in proteins and randomized versions of all proteins. Tolerances for mass accuracy were 10 and 20 ppm for precursor and fragment ions, respectively.

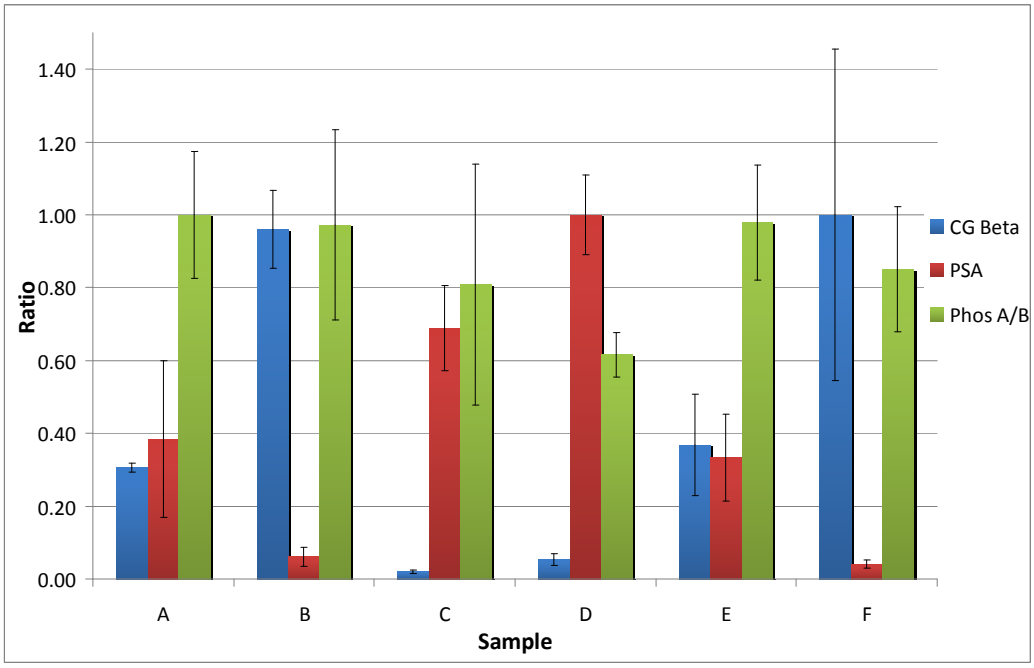

Figure 1. Protein ratios for the six samples, normalized to the sample in which the protein was most concentrated.

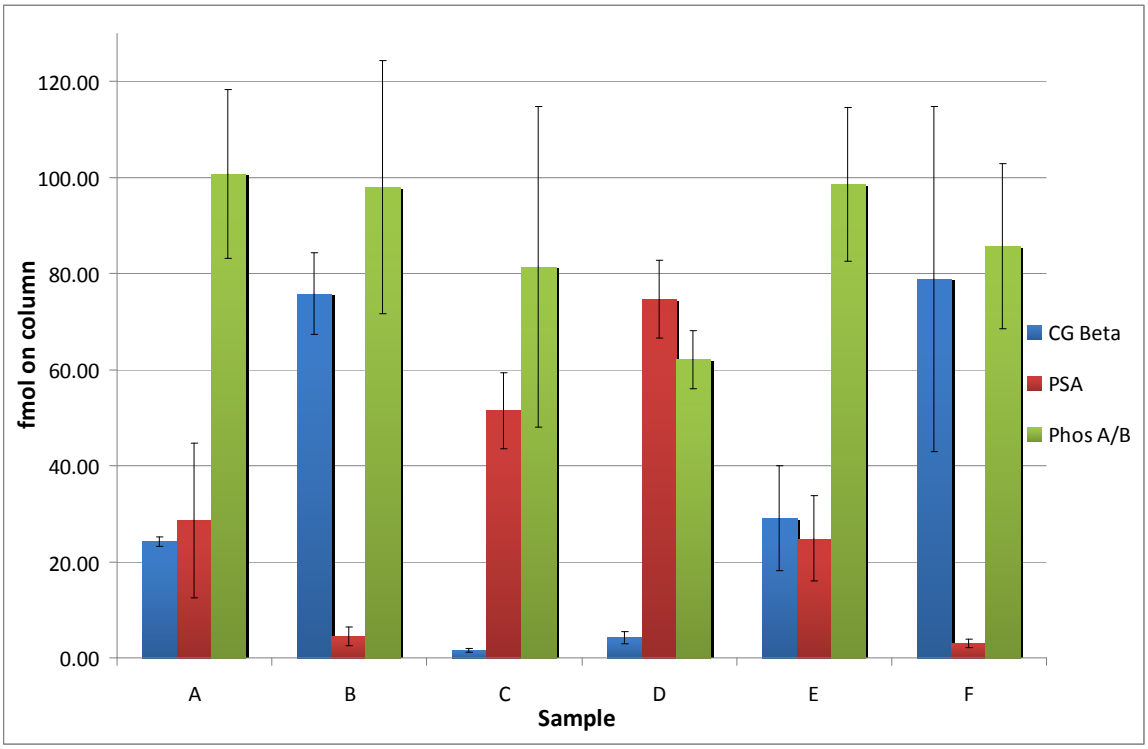

Figure 2. Amounts of each of the proteins in the six samples.

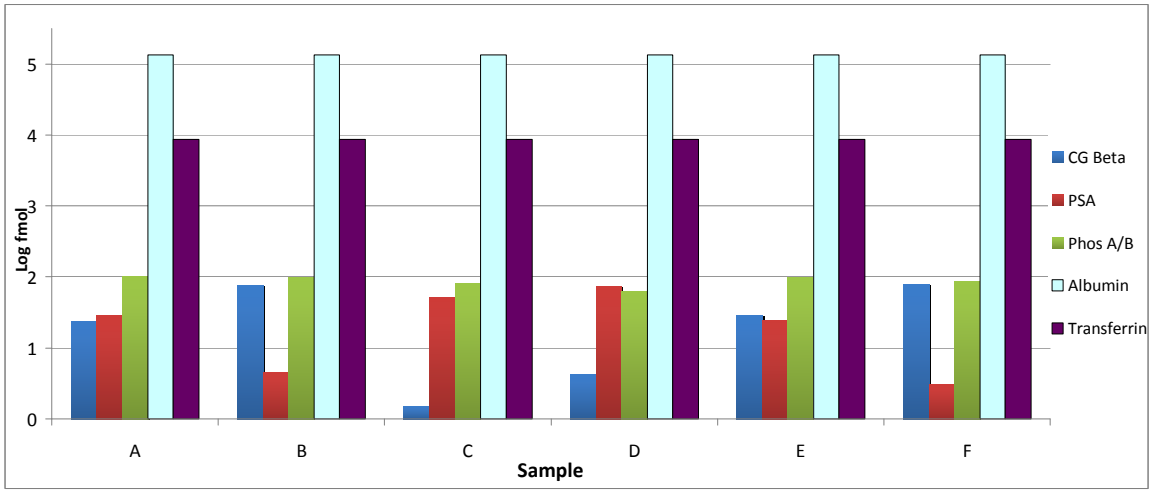

Figure 3. Amounts of each of the proteins in the six samples plotted on a log scale, including the approximate amount of serum albumin and transferrin injected with each analysis.

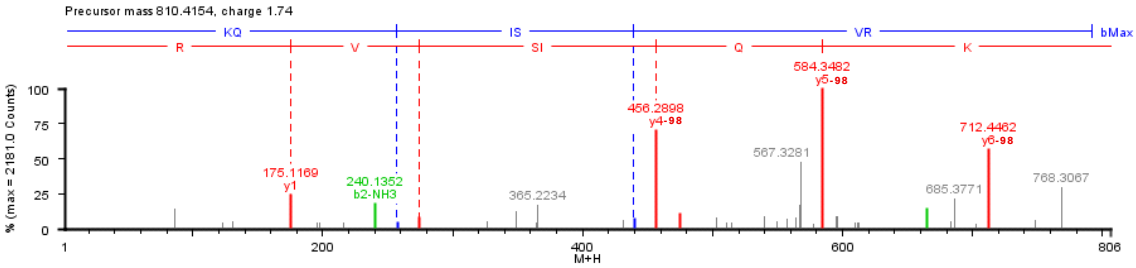

Figure 4. MS^E spectrum of the phosphopeptide from phos A (KQIS*VR).

Results

Relative protein ratios were calculated by measuring the peptide precursor peak areas in each sample for the spiked-in proteins. For phos A/B, five peptides were easily measured in all samples. For CG beta and PSA, two peptides were identified and then tracked by accurate mass and retention time down to the lowest levels, requiring that the peptides be found in the same fraction of the 2D separation each time. For some peptides, fragment ion peak areas were also measured and their relative intensities were used to confirm the protein ratios. The protein ratios, as normalized to the sample in which the protein was the highest, can be seen in Figure 1. The average relative standard deviation for three replicate injections for all samples was 26%.

Based on a known response from this particular Synapt (2200 counts per fmol), the fmol amount of each of the spiked-in proteins was calculated, to give an approximate absolute amount of each of the proteins loaded on column. The result of this calculation can be seen in Figure 2. In both Figures 1 and 2, it looks like the following samples are equivalent; A=E, B=F, and C=D. The fmol amounts of the spiked-in proteins varied from 1.5 fmol to 100 fmol. Since no depletion of the serum proteins was performed, this measurement was in the presence of up to 130 pmol of albumin and 8 pmol of transferrin (Figure 3), along with the rest of the serum proteins.

To identify the phosphopeptide in phos A, an enrichment using proprietary metal oxide was employed followed by 1D LC. It was not possible to measure the ratio of phos A/B due to the fact that the unphosphorylated version was not detected. The phosphopeptide in Phos A was measured with the following ratios: A (1), B (0.85), C (0.51), D (0.52), E (0.78), and F (0.68), as normalized to sample A, in which it was found to be the most abundant. The MS^E spectrum of the phosphopeptide from phos A (KQIS*VR) can be seen in Figure 4.