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# Absolute Quantification of the Model Biomarker Prostate-Specific Antigen in Serum by LC−MS/MS Using Protein Cleavage and Isotope Dilution Mass Spectrometry

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Protein cleavage-isotope dilution mass spectrometry (PC-IDMS) can be used to quantify proteins, with an isotope-labeled analogue of the peptide fragment used as an internal standard. Here, we investigate use of a standard LC−MS/MS platform for quantifying a model biomarker directly from serum by this technique. We synthesized a peptide (IVGGWECEK) identical to the N-terminal tryptic fragment of PSA but with each glycine containing two $^{13}C$ atoms and one $^{15}N$ atom. PSA-free human serum was denatured with urea followed by the introduction of PSA standard and the stable isotope labeled internal standard peptide. The sample was then proteolyzed with trypsin and subjected to quantification using LC−MS/MS on a triple quadrupole mass spectrometer. A linear least squares calibration curve made from five different concentrations of PSA added to serum and digested (each made in triplicate and randomly injected three times) had a mean slope of 0.973 (SE = 0.023), intercept of −0.003 (SE = 0.022), and $R^2$ of 0.971. Recovery of calibrators ranged from 70 to 85% with a mean run-to-run CV of 13% and a mean within-run CV of 5.7%. PC-IDMS is a promising technique for quantifying proteins covering a broad range of applications from standardizing immunoassays to monitoring post-translational modifications to quantifying newly discovered biomarkers prior to the development and implementation of an immunoassay, just to name a few. Issues surrounding the application of PC-IDMS for the absolute quantification of proteins include selection of a proteolytic fragment for quantification that can be cleaved and isolated reproducibly over a broad dynamic range, stable isotope labeled synthetic peptide standards that give consistent results, and LC−MS/MS methods that provide adequate sensitivity and reproducibility without creating impractical analysis times. The results presented here show that absolute quantification can be performed on the model biomarker PSA introduced into denatured serum when analyzed by LC−MS/MS. However, concerns still exist regarding sensitivity compared to existing immunoassays as well as the reproducibility of PC-IDMS performed in different matrixes.

**Keywords:** LC−MS/MS • absolute quantification • serum • biomarker • prostate-specific antigen • protein

## Introduction

The production of a serum biomarker assay for a particular disease follows a natural progression from the discovery phase, where a potential biomarker is found to have sufficient specificity, to the quantification phase where a biomarker found to have suitable positive predictive value is used for clinical diagnostics.[1] Prior to using a biomarker for clinical applications, whether as a diagnostic marker, predictive marker, or a prognostic marker, a study is performed whereby the concentration of the biomarker is determined in samples from patients whose disease state is known along with cohorts from a control population, typically as part of a randomized blind study (for a review see ref 2). A comprehensive statistical analysis of the results from such a study can lead to a calculated range of concentration values for the biomarker in individuals afflicted by the disease. Physicians can then use these values to aid in the interpretation of clinical data. The primary tool for determining the concentration of a biomarker in serum is the immunoassay. Although immunoassays are an inexpensive and effective means of acquiring quantitative data on biomarkers in serum, different vendor assays can lead to different values for the same patient sample. Efforts to normalize immuno-

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assays quantifying the same biomarker have increased as agencies given the task of overseeing the data produced by different clinical laboratories begin to insist upon more consistent measurements.

Mass spectrometry is currently one of the most prominent analytical techniques owing to its long history as a fast, rugged, accurate, and precise analytical methodology. Although recent advances in ionization techniques, such as ESI[3] and MALDI,[4] have led to numerous publications describing the use of mass spectrometry for protein *identification*, there are a limited number of studies applying mass spectrometry as a tool for *absolute* protein *quantification*[5,6] (where the term 'protein' is a generic term typically referring to a polypeptide with an average molecular weigh greater than 5 kDa). This has not been the case for smaller molecules ranging in average molecular weight from roughly 50 Da to 5000 Da. In fact, mass spectrometric techniques such as LC−MS/MS have been used extensively to perform absolute quantification on small organic compounds from complex mixtures for decades (for a review see refs 7 and 8). However, absolute quantification of an intact protein from a complex mixture by LC−MS or LC−MS/MS, without some form of pre-fractionation or immunoaffinity chromatography, is currently not widely practiced. There are a number of issues related to limitations in instrumentation and ionization process, whether it be ESI or MALDI, that make intact protein quantification from *complex mixtures* impractical at this time.

Alternatively, intact proteins may be cleaved into fragments using either chemical or enzymatic means for the purpose of quantification, much in the same way proteins are cleaved for the purpose of identification. An early example of the use of proteolysis coupled with MS for the purpose of quantification is the work by Desiderio and co-workers who developed methodologies for quantifying beta-endorphin (average MW = 3463 Da) from human pituitary.[9] This same group also developed MS quantification techniques for neuropeptides using internal standard peptides that were either nonlabeled,[10] labeled via enzymatic means,[11] or by using stable isotope labeled amino acids and peptide synthesis.[12] In addition, many of the MS studies were performed in parallel with RIA's to assess the sensitivity of MS and to evaluate MS as a tool to provide specificity to RIA data and as an orthogonal method for normalizing RIA data.[13,14] Since that time, other researchers have performed IDMS on peptides as a means of comparing immunoassay to mass spectrometric techniques.[15,16]

Barr and co-workers were the first to describe the use of proteolysis coupled with MS for the absolute quantification of a protein, a BCR certified apolipoprotein A-1 standard (CRM293, average MW 28 079 Da) that was proteolyzed with trypsin and quantified using a stable isotope labeled internal standard peptide with LC− flow-FAB MS/MS.[17] Their results suggested that protein cleavage coupled with isotope dilution mass spectrometry (PC-IDMS) was a viable approach as a methodology for standardizing measurements of specific proteins in the clinical laboratory. The premise of this technique is simple; a peptide that is cleaved from an intact protein can serve as a representation of the concentration of the intact protein when cleavage is complete since a 1:1 molar ratio will exist between the intact protein and the cleaved peptide. The response of the labeled internal standard peptide is used to calculate the concentration of the unlabeled peptide via IDMS. IDMS has been in use for over 35 years (for a review of IDMS, see Leenheer and Thienpont[18]) and continues to be a powerful technique for the quantification of a wide variety of molecules. By measuring the responses of collisionally dissociated fragment ions from the internal standard and native peptides using LC−MS/MS (typically done on a triple quadrupole mass spectrometer running in selected reaction monitoring mode), the absolute concentration of a single protein, or multiple proteins, in a complex mixture can be determined.

Other groups have described the use of PC-IDMS for the absolute quantification of proteins such as membrane proteins and proteins with post-translational modifications,[19,20] but to the best of our knowledge the study presented here represents the first example of PC-IDMS used for the absolute quantification of a protein directly from serum without the use of immuno-affinity chromatography. This study was performed to evaluate the ability of PC-IDMS to provide accurate, reproducible data on the concentration of a protein found in serum without purification using a standard LC−MS/MS platform with a fast LC gradient (4 min elution time, 8 min total run time flow rate 250 $\mu$L/minute). PSA was chosen as the model protein since it can be obtained in a purified form and human serum can be obtained that does not have detectable amounts of PSA. Serum samples were denatured prior to the addition of PSA and internal standard to preclude any alteration in their concentration by intrinsic proteases and protease inhibitors such as $\alpha$-2-macroglobulin. Although the use of a denaturant prior to the addition of PSA prohibits the use of this methodology for the direct measurement of PSA from serum, the results presented here affirm the specificity and reproducibility of PC-IDMS for the absolute quantification of PSA from digested serum matrix.

## Materials and Methods

**Stable Isotope Labeled Peptide Standards.** The peptide IVGGWECEK was synthesized in the Mayo Proteomics Research Center (Rochester, MN) on an ACT 396 Multiple Peptide Synthesizer (Advanced ChemTech, Louisville, KY), using recommended procedures for DIC (1,3-Diisopropylcarbodiimide) activation and coupling. The starting resin was preloaded lysine-Wang resin. The stable isotope labeled $^{13}$C, $^{15}$N-Fmoc-Glycines (Cambridge Isotope Laboratories) were coupled in the peptide sequence at positions 3 and 4 to give a total molecular mass shift of 6 Da from the nonlabeled peptide and a monoisotopic molecular weight of 1020.19 Da. The peptide was cleaved from its solid support with a cleavage cocktail containing 5% phenol, 5% water and 2.5% TIS (triisopropylsilane) in TFA (triflouroacetic acid). The peptide was purified by reverse phase HPLC on a Jupiter $C_{18}$ column (250 × 21.2 mm, Phenomenex, Torrance, CA) in 0.1% TFA/water with a 50 min gradient going from 900 mL/L water−100 mL/L acetonitrile−containing 1 mL/L trifluoroacetic acetic acid pH 3.0, to 200 mL/L water−800 mL/L acetonitrile containing 1 mL/L trifluoroacetic acetic acid pH 3.0. The molecular weight of the purified product was verified by ESI−MS on a PE SCIEX API 165 Mass Spectrometer (Applied Biosystems, Foster City, CA), and was found to comprise the expected monoisotopic mass of 1020.2 Da. The purified peptide was subjected to total nitrogen analysis performed at Scientific Research Consortium, Inc. (St. Paul, MN) and was found to be 78% peptide by mass with a CV value of 2.7%. The concentration of internal standard peptide in stock solutions was calculated using the purity value from the total nitrogen analysis times the mass of the peptide weighed using a Cahn C-31 microbalance (Thermo Cahn, Madison, WI).

**PSA Protein Standard.** A purified PSA standard purchased from Research Diagnostics, Inc. (RDI, Flanders, NJ) was used for all experiments. The certificate of analysis provided by the manufacturer stated that the protein was >96% pure and contained 3.04 g/L of PSA as determined by radial immunodiffusion (RID). The concentration of the standard was checked by diluting it with Tandem-R PSA zero diluent followed by measurement using the Beckman Coulter Access Hybritech PSA immunochemiluminescent assay (ICMA) and was found to have a concentration 4.91 g/L. The concentration of PSA obtained by ICMA was used for all subsequent calculations involving the PSA standard. The concentration of PSA in serum from a normal female human donor, used for all spiking experiments, was found to be below the level of detection using ICMA. The serum was obtained from the Department of Transfusion Medicine at the Mayo Clinic and was acquired using IRB approved protocols. The average mass of the intact PSA standard was determined using LC−ESI−FT-ICR MS and was found to be 28 456 Da. The total protein concentration in the serum used was 80 g/L determined using a BCA Protein assay (Pierce, Rockford, IL). Western blot was also performed on PSA samples separated using SDS-PAGE on a 4−20% gradient gel. The gel was transferred to nitrocellulose and PSA was visualized using an anti-PSA rabbit polyclonal primary antibody (DAKO A/S, Glostrup, Denmark) and a goat anti-rabbit horseradish peroxidase conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). Detection of PSA was carried out using chemiluminescent substrate (Pierce, Rockford, IL) and imaging film (Kodak, NY). The PSA standard material was kept at 4 °C as instructed by the manufacturer while the serum was kept at −80 °C prior to use.

**Digestion of Female Serum Spiked with PSA.** A total of 600 μL of serum was thawed for each set of experiments then denatured with urea by adding solid urea (289 mg) directly to the serum to a concentration of 6M followed by vortex mixing for 30 s. An aliquot of the stable isotope labeled peptide IVGGWECEK derived from a 1 g/L stock solution was added to a 500 μL aliquot of denatured serum (an aliquot was removed from the original volume of denatured serum since there is a volume increase with the addition of solid urea to the sample) so that the final concentration of internal standard peptide was 3.5 μmol/L in the denatured serum. PSA was then added to the denatured serum at five different concentrations; 0.07 μmol/L (2 mg/L), 0.433 μmol/L (12 mg/L), 2.6 μmol/L (74 g/L), 4.3 μmol/L (122 mg/L), and 6.5 μmol/L (184 mg/L) with each concentration made in triplicate.

Each denatured serum sample was diluted 1:1 with 100 mM ammonium acetate pH 8.0 followed by reduction and alkylation of cysteine residues. Reduction was carried out for 30 min at 30 °C using a concentration of 10 mM dithiothreitol (DTT) in each denatured serum sample followed by alkylation using 30 mM iodoacetamide with the reaction to taking place in the dark at room temperature for 30 min. Reduced and alkylated serum samples were then diluted further with 100 mM ammonium acetate to reduce the urea concentration to 2 M. Each denatured serum sample was then digested with TPCK treated bovine trypsin (Sigma-Aldrich Company, St. Louis, MO) for 12 h at 37 °C in a shaking water bath using an enzyme to substrate w/w ratio of 1:10 at a pH of 8.0. The trypsin was preactivated in 50 mM acetic acid pH 4.0 heated at 30 °C for 15 min prior to addition to the sample. After 12 h the samples were acidified with TFA at a concentration of 20 mL/L to a pH < 2. The denatured serum samples were then centrifuged at 14 000 × *g* for 30 min. Digests using Lys-C followed the same digestion procedure as the tryptic digests.

**LC−MS/MS Conditions.** Tryptic peptides were separated on a 2.1 × 50 mm $C_{18}$ Higgins Analytical TARGA PEEK cartridge column running at 250 μL/min using a Shimadzu LC10-ADvp HPLC system. A linear gradient was used starting with 999.5 mL/L $H_2O$ containing 0.5 mL/L acetic acid pH 3.5 and ending with 999.5 mL/L methanol containing 0.5 mL/L acetic acid pH 3.5 with a total run time of 12 min. Denatured serum samples were loaded into 96-well plates and an injection volume of 10 μL was loaded onto the column via a CTC Analytics LEAP autosampler.

The effluent from the LC column was directly coupled to the ESI source of a Micromass Quattro Micro triple-quadrupole mass spectrometer running in positive ion-mode. Conditions for the peptide IVGGWECEK, with the cysteine residue carboxyamidomethylated, were optimized for SRM experiments using the precursor-fragment ion transition of the doubly charged intact peptide IVGGWECEK $[M+2H^+]^{2+}$ to the singly charged $y_7''$ ion GGWECEK $[M+H^+]^{1+}$. The transition for the unlabeled peptide was 539.5 > 866 Da, while the transition for the labeled peptide was 542.5 > 872 Da. The resolution on each quadrupole was set to less than unit resolution or a resolution setting of 13 for both low and high in the instrument settings page. The areas associated with the response from each set of transitions, at the same retention time as the labeled peptide run without serum present, were used for quantification.

SCX chromatography was carried out using a Michrom BioResources, Inc. (Auburn, CA) MAGIC 2002 HPLC and a Michrom BioResources Magic Bullet SCX cartridge column at a flow rate of 200 μL/min. A gradient was run from 100% Mobile phase A (900 mL/L water with 5 mM $KH_2PO_4$ pH 3.0 and 100 mL/L acetonitrile) to 100% Mobile phase B (900 mL/L water with 5 mM $KH_2PO_4$ pH 3.0 and 100 mL/L acetonitrile with 500 mM KCl) over 30 min and fractions were collected every minute in a 96-well plate. The fraction eluting at 9 min (roughly 12% Mobile phase B) was found to contain the internal standard peptide and this fraction was used for quantification.

**Data Analysis.** The peak area responses recorded for each denatured serum sample digest analyzed were found by using QuanLynx software found in the main instrument operation software MassLynx 3.5. The concentration of PSA in each denatured serum digest was determined by setting the internal standard concentration constant and allowing the software to calculate the concentration using the ratio of the labeled to unlabeled peptides. A Microsoft Excel spreadsheet was used to perform linear least squares regression analysis (LLSRA) and ANOVA calculations.

## Results and Discussion

**Specificity and Quantification.** Digests of serum with PSA and the internal standard stable isotope labeled peptide (referred to as IS for Internal Standard) added were analyzed by LC−MS where the instrument was scanned from 400 to 1000 Da. The experiment was done to evaluate the intensities and the number of the proteolytic peptides eluting at the same time as the IS and the N-terminal tryptic peptide from PSA (referred to as tpPSA for **t**ryptic **p**eptide PSA) since there are nearly 500 confirmed proteins in serum,[21] and thus the probability that other peptides would coelute with the IS and the tpPSA was very high. The base peak ion chromatogram from this experiment is displayed in Figure 1 demonstrating the complexity of the peptide mixture with the resolution obtained by using a

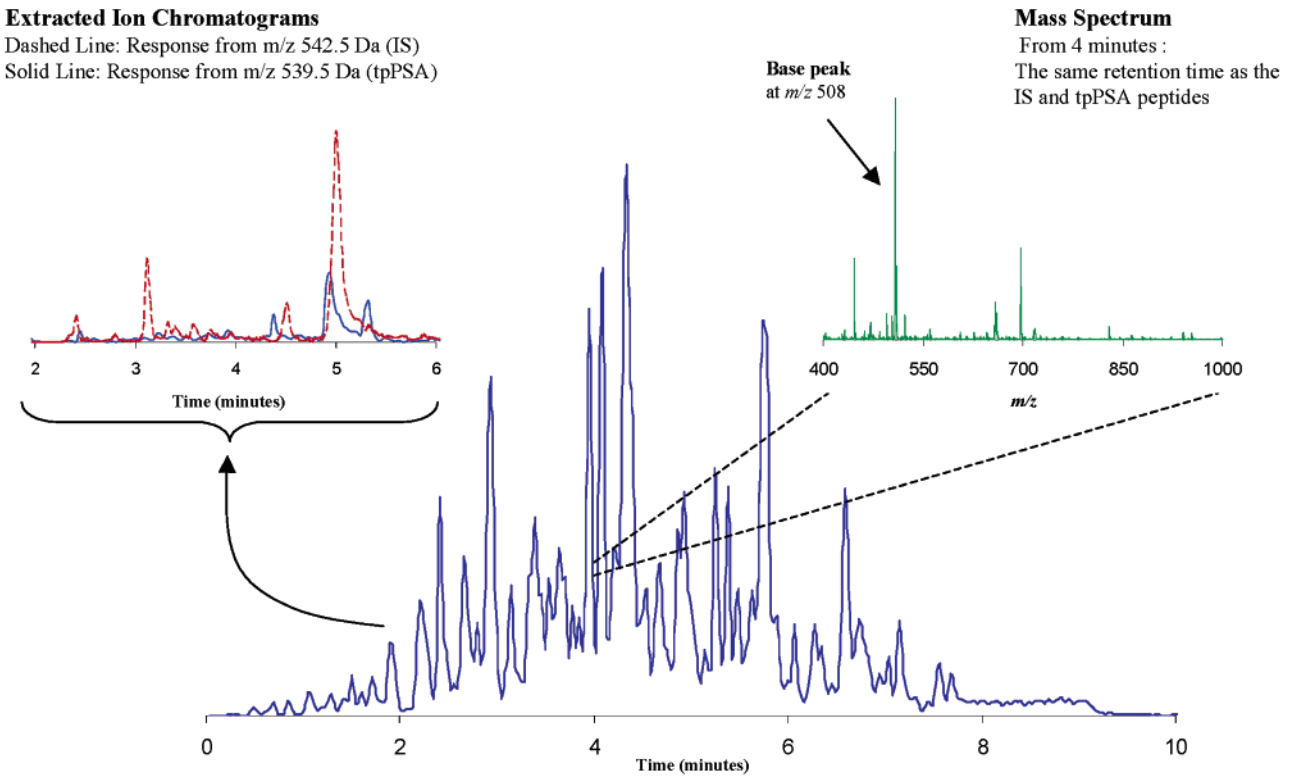


**Figure 1.** Base peak ion chromatogram from the LC−MS analysis of a digest of denatured serum with PSA added at a concentration of (12 mg/L), 2.6 $\mu$mol/L showing the complexity of the tryptic peptides formed by digesting raw serum. The inset on the left shows an overlay of two extracted ion chromatograms. The dashed line represents the extracted ion response from all ions with an *m*/*z* of 542.5 Da, which is the mass of the intact IS peptide, and the solid line represents the extracted ion response from all the ions with an *m*/*z* of 539.5 Da, which is the mass of the intact tpPSA peptide. The chromatogram demonstrates that there are no distinct peaks with the same elution time of approximately 4 min that would represent the IS and tpPSA peptides and could be used for quantification. The inset on the right shows a mass spectrum from the retention time for the internal standard peptide and the tpPSA at 4 min demonstrating the number of components that elute at the same time as the two peptides where the base peak is shown at 508 Da.

relatively high-flow, fast gradient LC system. The inset on the left side of Figure 1 is an overlay plot of two extracted ion chromatograms taken from an LC−MS/MS run from a digest of PSA added to denatured serum. The dashed trace represents the response detected for ions with $m/z$ = 542.5 Da, which is the same mass as the intact IS peptide, whereas the solid trace shown represents the response detected for ions with $m/z$ = 539.5 Da which is the same mass as the intact tpPSA peptide.

The results reveal that the LC conditions used were not adequate to completely resolve the various components in the digest and thus quantification using SIM alone would most likely be inadequate. These results further establish the recognized need for tandem mass spectrometry for absolute quantification when performing PC-IDMS in complex mixtures. The inset on the right shows a mass spectrum from an elution time of approximately 4 min, which is the elution time for the internal standard and the tpPSA peptides. The spectrum demonstrates that there are a number of components that elute at the same time as the two peptides with greater response. As evident from the mass spectrum there are a large number (approximately 50 discernible peaks) of other components present coeluting with the IS and tpPSA and the response of the base peak at $m/z$ 508 Da in this single mass spectrum is nearly 100 times greater than that of the IS and the tpPSA.

Figure 2 is an example of the responses observed from an LC−MS/MS run performed on a triple quadrupole mass spectrometer utilizing SRM for an injection of the same digest of denatured serum with PSA added as shown in Figure 1. The mass spectrometer was set to record only the response from specific fragment ions resulting from the collisionally induced dissociation of selected precursor ions (i.e., the IS and the tpPSA) as described in the Methods. The response from the tpPSA (top) and the IS (bottom) illustrate the dramatic reduction in complexity brought about by the selectivity gained using SRM. The integrated peak areas for each peptide at the retention time of 3.97 min (the known retention time for the IS peptide standard analyzed alone in buffer) are used to generate a calibration curve which can be used to evaluate the response of the IS and tpPSA peptides over a range of concentrations. The chromatogram for the tpPSA peptide clearly shows another peptide that shares the same transition at a retention time of 4.23 min. However, since this same peak was not observed in the chromatogram from the IS peptide and thus represents another component in the mixture that shares the same transition as the tpPSA peptide. These results demonstrate the benefit of having an internal standard present which is critical to the success of the methodology since the IS can compensate for fluctuations in the ionization process and the response of the instrument over time. The ability to have a reproducible elution time for the analyte is also critical. In this study, the calculated CV for the retention time from 55 injections was 0.87% (data not shown). The coupling of the IS with reproducible LC retention times, along with the specificity of SRM, enable the quantification of the IS peptide and the tpPSA peptide even in the presence of other components with the same transitions.

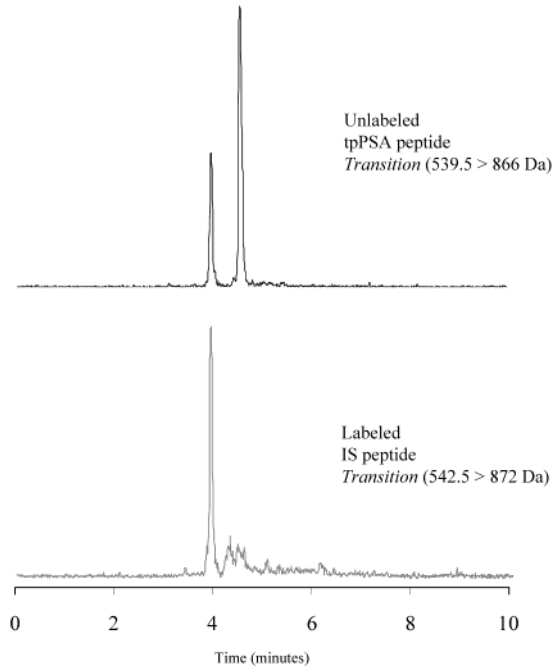


**Figure 2.** Ion chromatogram from the LC−MS/MS analysis of the same digest of denatured serum with PSA added as shown in Figure 1. The chromatograms illustrate the dramatic reduction in complexity and increase in selectivity brought about by the use of SRM. The peak observed at 4.23 min in the chromatogram from the tpPSA peptide is another peptide sharing the same transition since a corresponding peak is not observed in the chromatogram for the IS peptide.

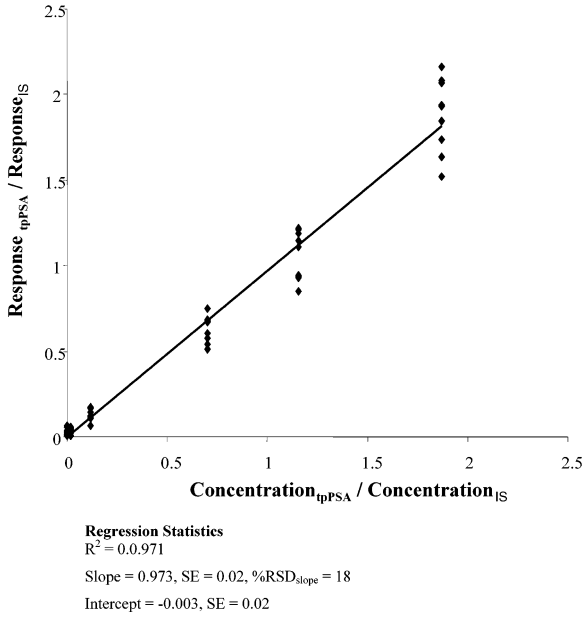


**Figure 3.** Linear least squares regression analysis using SRM data from the digest of denatured serum with PSA added. The graph shows the results from regressing the ratio of the tpPSA peptide response/IS peptide response vs the ratio of the their known concentrations. For an ideal system the slope of the line should be equal to unity, which is within the calculated error of the slope. The limit of detection (LOD) for the digest in serum was calculated using standard regression statistics and was found to be 0.16 $\mu$mol/L or 4.5 mg/L.

The linear regression shown in Figure 3 was derived from the LC−MS/MS analysis of denatured serum tryptic digests where 5 different PSA concentrations were made in triplicate with three injections made per denatured serum sample resulting in 9 data points per concentration. The *x* and *y* values used for the linear regression analysis in Figure 3 represent ratios of the known concentrations of PSA and the IS peptide (*x*-axis) and the observed responses for PSA and the IS peptide (*y*-axis). The statistics associated with the linear regression analysis are shown next to the calibration curve. For an ideal system the slope of a line derived from the ratio of the response of PSA/IS (shown as $\text{Response}_{\text{tPSA}}/\text{Response}_{\text{IS}}$ on the *y*-axis in Figure 3) vs the ratio of the concentration of PSA/IS (shown as $\text{Concentration}_{\text{tPSA}}/\text{Concentration}_{\text{IS}}$ on the *x*-axis in Figure 3) should be equal to unity and should intersect the origin. The observed slope of the line in Figure 3 is 0.973 (SE = 0.023) with an intercept of −0.003 (SE = 0.022). The $R^2$ value calculated from the LLSRA was 0.971 signifying that the response is linear for the tpPSA peptide derived from the PSA added to denatured serum over the concentration range evaluated. The LOD for the digest in serum was found by setting *y* equal to the response calculated using the upper 95% confidence interval value at the *y*-intercept and calculating a concentration value for *x* using the LLSR equation for the calibration curve. Using this approach the LOD was found to be 4.5 mg/L (1.6 $\mu$mol/L), which is higher than the lowest calibrant at 2.0 mg/L (0.07 $\mu$mol/L).[22] Upon inspection, control digests had minimal background for each of the two SRM channels, further demonstrated by the value of −0.003 observed for the intercept.

**Reproducibility.** Additional digests were performed that focused on the variability associated with the digestion step in the absence of serum background. The first experiment compared replicate digests containing IS and a synthetic peptide with the same sequence as the IS *without* stable isotopes vs replicate digests containing IS and PSA. The digests containing uIS peptide are not affected by trypsin since no cleavage takes place as compared to the digests with PSA where cleavage must take place in order for the tryptic peptide from PSA to be observed. These experiments were performed in 100 mM ammonium carbonate buffer followed by reduction, alkylation, and tryptic digestion with each sample containing the same amount of IS. Table 1 shows the CV values from these experiments along with the variation contributed by the digestion process as a percentage of the total variance. The finding that the percent variation due to digestion is higher for the digest performed in buffer vs the 'process digest' performed on the uIS indicates that the act of digestion of PSA contributes the majority of run-to-run variability.

**Table 1.** Comparison of Variation between Replicate Analyses for 'Process' Digests Containing Synthetic Native and Labeled Peptides vs Digests with PSA Added to Denatured Serum along with Labeled Peptide[a]

| | IS digest in buffer (%) | PSA digest in buffer (%) |
|---|---|---|
| $\text{CV}_{\text{run-to-run}}$ | 3.0 | 5.0 |
| $\text{CV}_{\text{within-run}}$ | 1.7 | 2.1 |
| $\%\text{variation}_{\text{due to digest}}$ | 22 | 84 |

[a] The CV values labeled run-to-run are calculated using the mean response derived from triplicate injections of the 9 or 10 replicate samples while the CV values labeled within-run are calculated using the CV value from triplicate injections of the 9 or 10 replicates. $\%\text{Variation}_{\text{due to digest}} = \text{ICC} = (\text{BMS} - \text{WMS}/\text{BMS} + (n-1)\text{WMS})$. ICC = interclass correlation coefficient; BMS = between -run mean square; WMS = within −run mean square; *n* = number of replicate injections = 27 or 30.

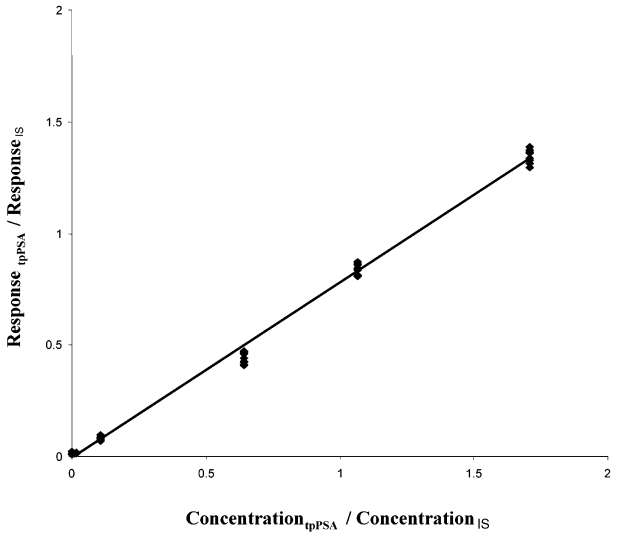


**Figure 4.** Linear least squares regression analysis using SRM data from the digest of PSA added to 100 mM ammonium acetate buffer pH 8.0. The graph shows the results from regressing the ratio of the tpPSA peptide response/IS peptide response vs the ratio of the their known concentrations. The limit of detection (LOD) for the digest in buffer was calculated using standard regression statistics and was found to be 0.04 $\mu$mol/L or 1.2 mg/L.

To further evaluate the effect of the matrix on the digestion process, a set of calibrators with the same concentrations as the PSA added to denatured serum were digested in 100 mM ammonium bicarbonate buffer instead of serum. Figure 4 shows the linear regression analysis from this experiment along with the corresponding statistics. The slope of the line from this regression is 0.785 (SE = 0.007), while the calculated intercept is −0.006 (SE = 0.006). The limit of detection (LOD) for the digest in buffer was found to be 1.2 mg/L (0.04 $\mu$mol), which is slightly lower than the lowest calibrator at 2.0 mg/L. Additional comparisons were made using the data from the digests of PSA added to buffer and denatured serum. Figure 5 contains a bar graph showing the calculated recoveries from the LC−MS/MS analysis of the denatured serum samples with PSA added that were used to generate the calibration curves shown in Figures 3 and 4. The differences observed for the SE from the two regression analyses suggest that the added complexity of the matrix due to the serum background increases the uncertainty in the mean recovery value as observed from the increase in the 95% confidence interval of the mean for denatured serum samples with PSA added vs buffer samples with PSA added. However, the mean recovery values were not statistically different for both sets of samples above 12 mg/L, which is above the calculated LOD of 1.2 mg/L for samples in buffer and 4.5 mg/L for samples in serum.

**Completion of Digest.** Although trypsin has been used extensively as a tool for generating proteolytic peptides to be identified using tandem mass spectrometry, it has been used primarily in a qualitative way or for relative quantification rather than as a tool for absolute quantification. In order for PC-IDMS to be used as a quantitative methodology the protein being quantified must be reproducibly digested into peptide

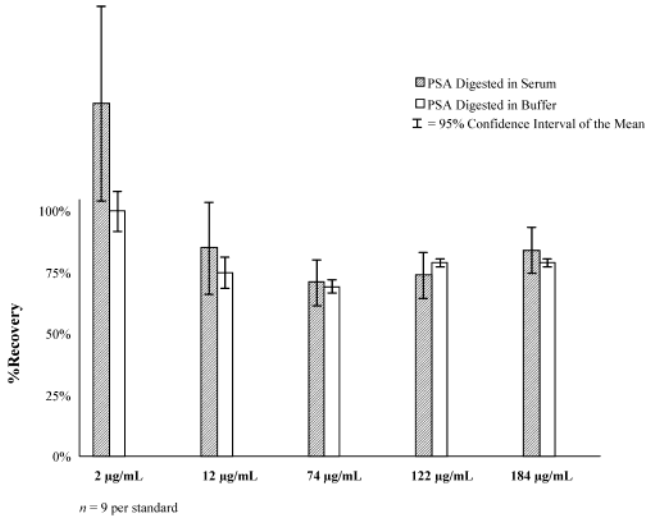


**Figure 5.** Graph compares the calculated recovery values for PSA from digests of PSA added to denatured serum or into buffer using the data from the samples used in the regression analyses in Figures 3 and 4. The results demonstrate that the recovery values for denatured serum samples are very similar to buffer samples for concentrations above 12 mg/L, which is above the LOD for both the digest in denatured serum and in buffer.

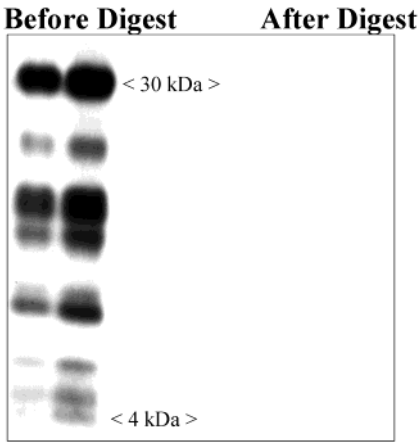


**Figure 6.** Western blot of PSA added to denatured serum before and after digestion with trypsin. The polyclonal antibody recognizes intact PSA at 30 kDa as well as fragments of PSA found in the standard. Fragments of PSA > 4kDa were not detected after digestion using this antibody that has $\mu$g/L sensitivity for PSA and its fragments.

fragments for quantification. To check on the completion of the digestion process, digests of denatured serum samples with PSA added were evaluated by western blot. The extent of digestion was examined by observing the loss of the epitopes recognized by the antibodies used in the Western blot. Figure 6 shows a western blot of PSA added to denatured serum before and after digestion with detection done by using chemiluminescence and film. The absence of any bands after digestion for the same amount of sample loaded indicates that the PSA was digested below the levels of detection for this antibody, which is in the low $\mu$g/L range. Other bands observed in the lanes containing sample before digestion represent fragments of PSA that contain the epitope recognized by the anti-PSA polyclonal antibody. However, the levels of these fragments are not quantitatively represented since Coomassie blue staining

of these samples showed that the greatest intensity was for the band at 30 kDa representing intact PSA (data not shown). These same serum digests were also examined using the immunoassay (ICMA) used to determine the concentration of PSA in the standard material, as described in the Methods. The ICMA did not detect PSA in the serum digests reinforcing the western blot results. Although neither western blot nor ICMA are definitive methods for the detection of residual fragments of PSA that have not been fully cleaved, they do represent orthogonal methodologies that help to support the results from digests of PSA in serum.

**Reduction of Matrix Complexity using 2D-LC.** Although the use of a fast-LC methodology can provide higher throughput when performing LC−MS/MS, there are limitations in the complexity of the sample that can be handled while still providing quantitative results at levels comparable to immunoassay. Using the LC−MS/MS conditions described in the Methods section, the limit of detection (LOD) for the IS and tpPSA peptides in water alone was estimated to be 0.14 $\mu$g/m (0.005 $\mu$mol/L) using a S/N value of 3.3 (data not shown). This concentration is substantially lower than both the LOD from the digests of PSA in buffer [LOD = 1.9 mg/L (0.07 $\mu$mol/L)] and PSA in serum [LOD = 4.5 mg/L (0.16 $\mu$mol/L)]. To evaluate the impact of the background matrix on the separation and ionization processes, denatured serum was digested with trypsin in the absence of PSA and IS peptide then analyzed using the same LC−MS/MS conditions. The results showed no response for either the tryptic fragment of PSA or the IS peptide (data not shown), further supporting the low value observed for the intercept found by LLSRA. Following this control experiment IS and uIS peptide were added to the mixture of tryptic peptides from serum at a concentration of 0.05 $\mu$mol/L then analyzed. The responses of the IS and uIS peptide standards in water were 10 times greater than those observed for IS and uIS added to the digest of the denatured serum control (data not shown). This result suggested that a reduction in the complexity of the sample would most likely increase the sensitivity of the analysis.

Additional experiments were performed in an attempt to alleviate the problem of matrix interference by first using off-line strong cation exchange chromatography (SCX) to reduce the complexity of the digested denatured serum sample with PSA added prior to LC−MS/MS.[24] Fractionation of the SCX effluent was performed using an automated fraction collector with fractions being deposited directly into a 96-well sample plate. Fractions from retention times surrounding the known SCX retention time of the IS peptide were subjected to LC−MS/MS to evaluate the response of IS and tpPSA peptides after digestion. In addition, the enzyme Lys-C was used to cleave the N-terminal peptide IVGGWECEK from PSA since Lys-C only cleaves at lysine residues reducing the number of possible peptides that could be created from the serum protein matrix. Figure 7 illustrates both the before and after base peak ion chromatograms and mass spectra for a digest of PSA added to denatured serum subjected to SCX fractionation. The reduction in complexity of the sample is clearly evident when the LC−MS chromatogram from the digest of denatured serum with PSA added is compared with an LC−MS chromatogram from a fraction off an SCX column containing the IS and tpPSA peptides. The mass spectra from the known retention time for the IS and tpPSA peptides are clearly different when comparing the mass spectrum from the digest of PSA added to denatured serum before SCX vs after SCX. The base peak in the mass

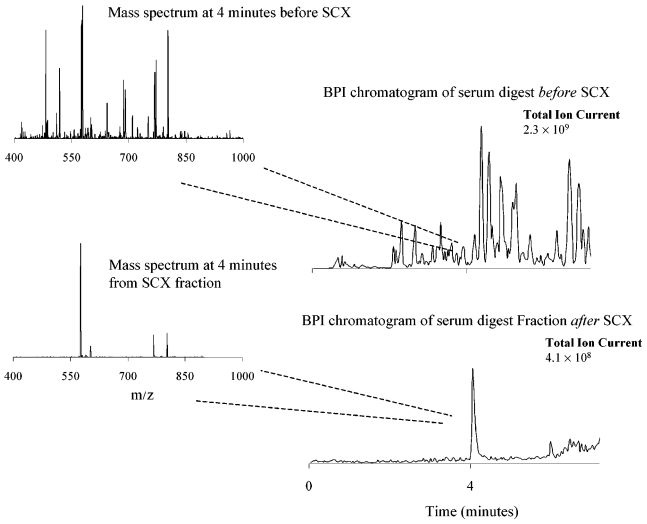


**Figure 7.** Results from the Lys-C digest of PSA added to denatured serum fractionated by strong cation exchange (SCX) and reanalyzed by LC−MS and LC−MS/MS showing base peak ion chromatograms (right side of figure, not normalized) and mass spectra from the retention time for IS and tpPSA peptides (left side of figure, normalized). A comparison of the Base Peak Ion (BPI) chromatograms before SCX (top) vs a fraction off of an SCX column containing the IS and tpPSA peptides (bottom) shows the reduction in the number of components brought about by utilizing SCX. This reduction is further illustrated in the mass spectra shown on the left side of the figure where there are clearly fewer ions detected at the same elution time for the IS and tpPSA peptides in the SCX fraction vs before fractionation. A comparison of the response for the IS peptide before and after SCX showed that there was a 20% increase after SCX fractionation. This result suggests that a reduction in the number of peptides present in a sample by using SCX may allow for a lower limit of detection to be obtained.

spectrum after SCX is a different compound than either the IS or tpPSA peptides showing that there is not complete isolation of the IS and tpPSA peptides. Regardless, the LC−MS/MS responses observed for the IS and the tpPSA were on the order of 20% higher when SCX fractionation was used as opposed to LC−MS/MS responses without SCX fractionation. In addition, there was an increase of 25% in the response from IS and tpPSA peptides using Lys-C vs trypsin coupled with SCX, suggesting that the selective use of a specific enzyme to reduce sample complexity can result in greater response. Although this does not completely negate the loss of signal from the serum matrix, it does show that selective use of an enzyme coupled with off-line SCX can help increase response the response of serum digests analyzed by LC−MS/MS.

## Summary

The results from this study support the findings by Barr et al.[17] that PC-IDMS is a viable technique for the absolute quantification of proteins. Our results also show that PC-IDMS can be used to quantify a biomarker directly from serum with levels of accuracy, precision, and sensitivity that, although less than levels obtained by immunoassay, are worth pursuing as a possible means of standardizing commercially available immunoassays performed in serum. A combination of this antibody independent procedure and a traceable peptide reference standard for PSA would produce a reference mea-

surement system that could measure serum based samples. Such a reference system would be very valuable for reconciling the current diversity of patient test results measured by different immunoassays. A recently approved IFCC reference method for the measurement of glycohemoglobin in the blood of individuals with diabetes has demonstrated that proteolysis coupled with LC−ESI MS can be a useful tool for clinical applications provided there is adequate sensitivity and reproducibility.[25]

This study focused on simple sample processing coupled with reproducible high-flow LC−MS/MS as part of an effort to examine the capacity of PC-IDMS to quantify a protein from a complex matrix such as serum. Denatured serum was used instead of native serum to remove any confounding interference that might be caused by proteins that bind PSA, thus allowing for a thorough examination of the properties of the serum matrix alone. The observations made in this study clearly demonstrate the capacity of background serum matrix to suppress analyte ions using the HPLC system described. The development of reproducible, and sensitive low-flow LC−ESI systems for absolute quantification is currently an area of study in our group, especially for the quantification of proteins from serum. Although lower flows and longer gradients may increase analyte response they also increase the amount of time needed to perform an analysis.

The ability to quantify a protein in the presence of such a broad dynamic range in background protein concentration has not previously been presented for PC-IDMS without prior sample purification. It is worth mentioning that although the levels of PSA added to denatured serum were 1000 times higher than values observed in a clinical setting, they were still $10^4$ times lower than the human serum albumin present in the sample. Our research group continues to build off of previous quantification activities using FT-ICR MS[6,23] by using new techniques designed to enhance sensitivity by further reducing the complexity of the sample after ionization.

**Nonstandard Abbreviations:** LC−MS/MS, liquid chromatography-tandem mass spectrometry; PSA, prostate specific antigen; 2D-LC, two-dimensional liquid chromatography; ESI, electrospray ionization; MALDI, matrix assisted laser desorption ionization; BCR, European Community Bureau of Reference; PC-IDMS, protein cleavage-isotope dilution mass spectrometry; SCX, strong cation exchange; SRM, selected reaction monitoring; FT-ICR MS, Fourier transform-ion cyclotron resonance mass spectrometer; Base Peak, peak with the greatest intensity observed in a mass spectrum.

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