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Quality Control, Preparation, and Protein Stability Issues for Blood Serum and Plasma Used In Biomarker Discovery and Proteomic Profiling Assays

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Proteomic Profiling of Serum

There is an increasing emphasis in clinical and translational research on the discovery and development of biomarkers that are indicative of a disease state. While biomarkers are not exclusively proteins, the emergence of new mass spectrometry platforms combined with the human genome databases has rejuvenated the search for biomarker proteins, especially in readily available body fluids such as blood. There is currently a tremendous need for an improved ability to "mine" the full depth of the proteome in a high throughput manner. To advance clinical proteomics, methodologies are needed that can accommodate higher throughput while facilitating the ability to observe large numbers of protein events. Currently, many systems that couple robotic handling of samples in the front-end to a matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer are being evaluated for clinical utility. In terms of discriminating accuracy, the most successful of these has been a modification of the MALDI-TOF termed Surface Enhanced Laser Desorption/Ionization-TOF (SELDI). The advantages of these systems are that they can serve as powerful discovery tools leading to protein identification, yet they can also develop protein expression profiles or patterns reflective of multiple biomarkers that can be used to distinguish one group from another, i.e., cancer versus non-cancer or for prognostic purposes.[1–7]

Besides the instrumentation and methodologies related to these mass spectrometry applications, the quality and quantity of the clinical samples to be tested are equally important. Blood serum has emerged as the most common sample used in biomarker discovery and protein profiling, because: it is easily obtained; a large proportion of blood clotting factors are removed; and it remains a rich source of molecules that may indicate systemic function. Blood plasma, which contains all of the clotting factors, can also be utilized for profiling. However, plasma collection relies on various anticoagulants that can affect the chemistry of the interaction with the affinity surface of the MALDI or SELDI platform. Concerns about sample integrity have emerged in bioprocessing including issues related to blood sample collection, processing, and storage. Although these issues are by no means unique to the development of proteomic assays, the scope of profiling a clinical sample's entire proteome raises new concerns about ensuring the integrity of all proteins within the sample.

Our research group was the first to demonstrate in a peer-reviewed study that SELDI profiling patterns could distinguish cancer from non-cancer cases.[1] In particular, we showed that SELDI profiling coupled to a classification algorithm allowed for automated diagnostic assay for prostate cancer.[2] This paper along with the results in ovarian cancer released earlier this year were significant steps toward realization that this process could function as a diagnostic clinical tool.[3] Multi-center analytical and clinical validation studies are currently ongoing for SELDI protein profiling in the early detection of prostate cancers. While these protocols were being developed, many issues regarding blood sample integrity were addressed, especially regarding the use of archived specimens banked over

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a 15-year period. In this paper, we will describe the quality control and automation issues faced in developing reproducible proteomic profiles of clinical serum samples, evaluations of differences in serum and plasma protein profiles, and effects on protein stability during processing and freeze/thaw cycles.

Automated Sample Processing and Reproducibility

Technological developments in high-throughput mass spectroscopy using SELDI-TOF (Ciphergen Biosystems, Inc.) have made it possible to compare proteomics expression patterns of clinical specimens on a large scale. As with any new technology, acceptable levels of reproducibility needed to be established, with sample preparation being the most critical step in the production of reproducible spectra. We have optimized the SELDI system for high-throughput assay robotics, and previous work in our laboratory has determined that mass accuracy of the SELDI generated spectra is highly reproducible with a coefficient of variations (CV) of 0.05 percent. Normalized intensity values for individual peaks are below a 20% CV for samples prepared robotically, which is well within acceptable limits for typical clinical assays. Using the serum processing steps described below, we have found that manual processing of large serum samples can be much more variable, especially as the number of samples and individuals involved in a particular experiment increase.

A modified Beckman automated robotic handling system (BioMek 2000) is used with a 96-well format Ciphergen BioProcessor to concurrently process, dilute, and load serum samples on 12, 8-spot ProteinChips. The only step that is not performed robotically is the addition of the raw serum to a 96-well plate at the beginning of the assay. We routinely use volumes that are five percent larger than the necessary assay volume to ensure that the robot has enough material to perform the processing. This compensates for any potential errors from pipetting by a technician and/or pipette calibration problems. For processing, serum samples (20µl) are mixed with 30µL of 8M urea containing 1% CHAPS in phosphate-buffered saline (PBS). This is performed in a 96-well plate and incubated for 10 minutes at 4° C on a MicroMix shaker (DPC, Randolf, NJ). A volume of 100 µL of 1M urea/PBS containing 0.125% CHAPS is then added to each sample and mixed. A final dilution (1:5) is made in PBS and the diluted samples are then applied to the corresponding well of a BioProcessor containing IMAC3 chips (Ciphergen Biosystems) previously activated with $CuSO_4$ (IMAC-Cu). The bioprocessor is then sealed and agitated on the MicroMix shaker for 30 minutes. The excess serum mixture is discarded, and the chips are washed two times with PBS, followed by two washes with HPLC water (all robotically). The chips are then air dried, and stored until matrix addition and SELDI analysis. Matrix addition is also performed robotically by the addition of 2 x 1µl solution of 12.5 mg/ml sinapinic acid in acetonitrile/TFA.

Using this procedure, we can process four BioProcessors or 48 ProteinChips per day, which represents 384 samples. Routinely, each clinical sample is run in triplicate, thus 128 specimens per day can be efficiently prepared. Once chip processing is complete, the BioProcessor top is removed and the chips are allowed to air dry. To ensure that each chip is treated in the same way, especially when a large number of samples are processed, chips are allowed to dry overnight when feasible. The matrix solution is applied the following morning prior to profiling analysis. We have observed that removing residual water from the chips also improves spectra quality and reproducibility. In a final step, a Proteinchip AutoLoader accessory from Ciphergen directs the automated data collection from up to 24 protein chips at a time.

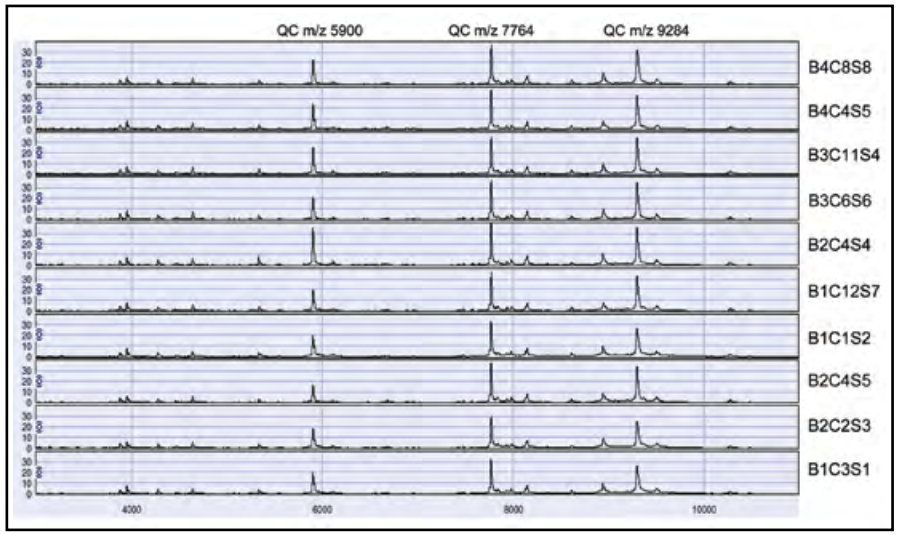

Figure 1. QC Serum Spectra from Multiple Bioprocessors. Mass profiles (3,000 – 11,000 m/z) from ten representative serum QC spectra spotted across 44 IMAC-Cu protein chips on four BioProcessors. For each spectra, the BioProcessor number (B#; 1–4), chip number within each BioProcessor (C#; 1–12), and spot position (1–8; S#) are indicated.

Quality Control Serum

After an effective biomarker or diagnostic pattern has been discovered, the portability of an assay must be clarified. Variability associated with sample processing and data collection protocols must be addressed when different laboratories or instruments attempt to repeat and use the developed assay. In order to facilitate this, we collected a large volume quality control, and pooled human serum samples for SELDI analysis. This quality control (QC) sample consists of pooled serum obtained from 360

healthy individuals, 197 women and 163 men, resulting in almost two liters of serum. Serum from each individual was collected by venipuncture into a 10cc SST vacutainer tube. Blood was allowed to clot at room temperature for 30 minutes, and the tubes were centrifuged at 3,000 rpm for 10 minutes. Each individual serum sample was then decanted and pooled into a 3L beaker on ice. The pooled serum was separated into 0.4 ml aliquots and stored at -80° C. This volume amount is important to ensure the capability of maintaining an instrument's continuous performance and allows consistent evaluation and comparisons of protocols over the long term. Routine calibration and QC serum processing should be performed on a weekly basis to monitor instrument performance as well as the integrity of processing protocols.

We use the SELDI data obtained from the QC sample as a benchmark for the integrity of robotic processing, instrument standardization, and intra-experimental validation. Instrument standardization is accomplished by adjusting the laser intensity, detector voltage, and detector sensitivity so that three consistently present protein peaks (m/z 5,900, 7,764, 9,284 ± 0.2%) in sera are displayed to designated criteria. Specifically, the signal to noise ratios are required to be ≥40 for m/z 5,900, and ≥80 for m/z 7,764 and 9,284. Examples of these QC profiles from a large serum cohort study are shown in Figure 1. These 10 QC spectra are from a serum profiling experiment that required 44 IMAC-Cu protein chips, with each chip having a QC sample loaded on one of the eight possible spots per chip. The ten spectra are reflective of chips from four different BioProcessors, as well as each spot position as indicated in the spectra. Using the highlighted three m/z values, the reproducibility of these spectra are useful for both intraexperimental variation for each chip used in an experiment, as well as a benchmark for comparison with previous, or future, profiling experiments. Appropriate quality control samples can be developed for any body fluid used for SELDI experimentation.

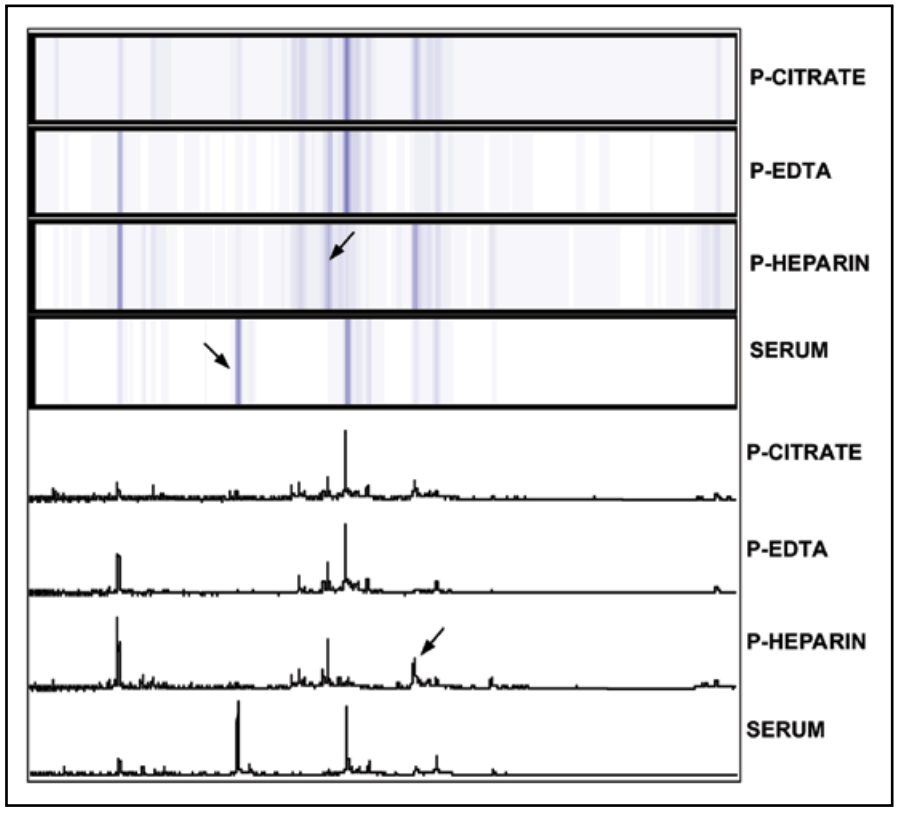

Figure 2. Comparison of Plasma and Serum Profiles on IMAC-Ni Protein Chips. Plasma (heparin, EDTA, or citrate anti-coagulants) and serum samples from the same individual were robotically processed on IMAC3-Ni^{2+} protein chips. Representative gel and trace views generated by SELDI are shown for the 2,500 to 15,000 m/z ranges. Clear differences between different types of plasma and sera can be seen on each chip type (arrows).

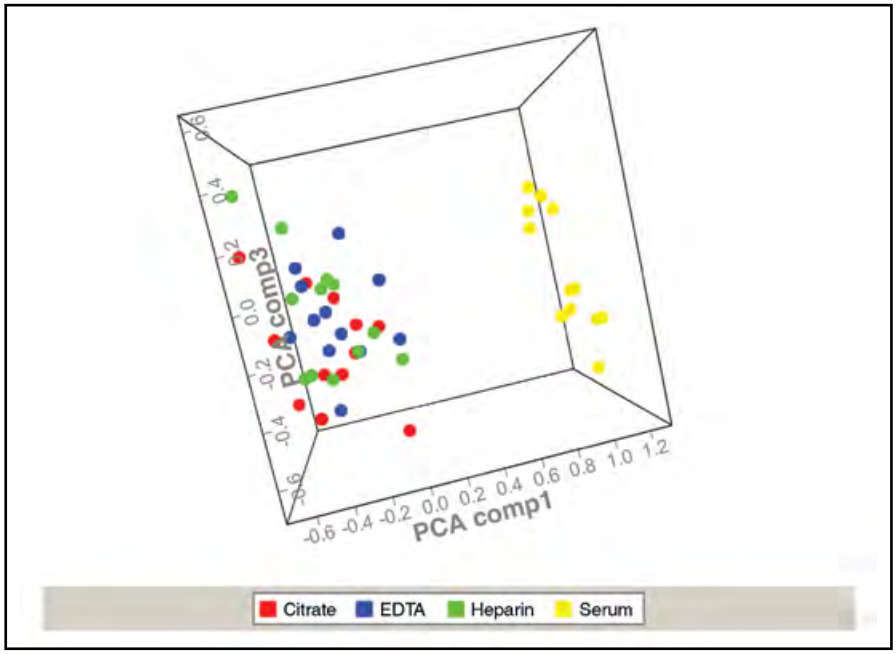

Figure 3. Principle Component Analysis of Differences in Plasma and Serum Protein Profiles. Plasma (heparin, EDTA or citrate anti-coagulants) and serum samples from five individuals (in duplicate) were robotically processed on IMAC3-Ni^{2+} protein chips. The three most significant peak features for each set of spectra were used to generate the three dimensional representations for each group. Yellow - Serum; Red - Citrate; Green - Heparin; Blue - EDTA.

Serum Versus Plasma Protein Profiles

When analyzed by SELDI, serum and plasma samples from the same individuals demonstrate a number of proteins or peptides that are unique to the sample type and anticoagulant used. For example, we have found that plasma collected with heparin or citrate is more suitable for use on strong anionic or weak cationic ProteinChips, and that it yields profiles that are distinct from serum applied to the same chip surface. Representative profiles on IMAC-Ni chips for serum and three plasma preparations (citrate, heparin, EDTA) from the same individual are shown in Figure 2. Figure 3 shows a principle component analysis of the resulting protein peak features for the four sample collection conditions from five individuals. Each color represents a sample processing condition, and reflects the three most important peak features in each individual spectra that distinguishes one group from another. It is apparent from these evaluations that when comparing differences in larger sample cohorts, it is imperative that all samples are prepared the same way, especially for plasma. Comparisons of clinical plasma samples prepared with different anticoagulants should be avoided.

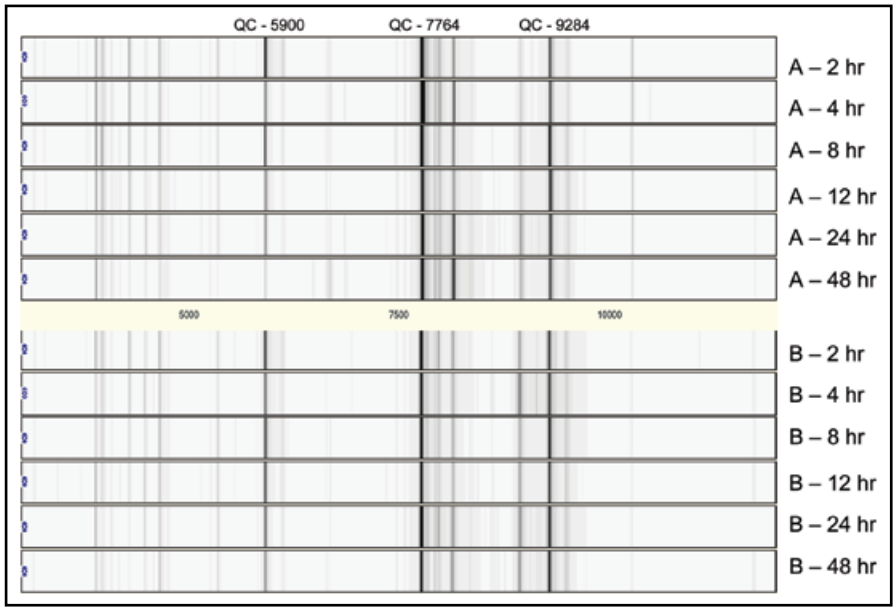

Figure 4. Time Course of Serum Protein Stability in Clotted Samples at Room Temperature. Mass profiles (3,000 – 11,000 m/z) in gel view on IMAC-Cu protein chips of clotted serum derived from two individuals (A and B) For 2, 4, 8, 12, 24, or 48 hrs. The three QC peaks at m/z 5,900, 7,764, and 9,284 are also indicated.

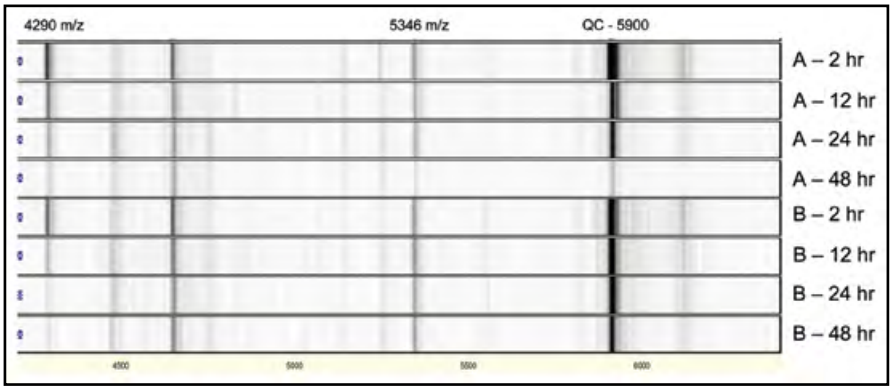

Figure 5. Low-Mass Serum Protein Stability at Room Temperature. Mass profiles in gel view (4,200 – 6,500 m/z) on IMAC-Cu protein chips of clotted serum derived from two individuals (A and B) at 2, 12, 24, or 48 hrs are shown.

Effect of Room Temperature and Time on Processing and Stability

In general, we have used the serum sample preparation procedure described in the previous QC section. However, for obtaining samples from clinical settings, this time frame (30 min) may not be realistic and will be highly variable at different sites. Therefore, we evaluated the effects of time and temperature on the stability of low-mass serum protein profiles. Ten vacutainer tubes of serum were drawn from each of three volunteers. These tubes were left on the benchtop at room temperature for different time points (2, 4, 8, 12, 24, and 48 hours) prior to centrifugation of the clotted material. The resulting serum samples were frozen at -80° C, and then aliquots were later thawed and robotically loaded on IMAC-Cu chips for SELDI analysis. As shown in Figure 4, profiles for each time point from two individuals indicate differences in relative intensities of low mass proteins between 12 to 24 hours, with very noticeable changes in proteins <7,000 m/z after 48 hours. This lower mass range from 4,200 – 6,500 m/z is further highlighted in Figure 5 for the later time points. This data further illustrates the rapid decline in the intensity of multiple proteins (m/z 4,290, 5,346, 6,120) in this mass range after 12 hours. Although limited in scope by the number of individuals examined, this data suggests that clotted serum vacutainers can be left on the benchtop for at least eight hours without significant loss of proteomic features. Beyond this time point, stability becomes variable and will likely differ from sample to sample. Further follow-up studies are ongoing that will evaluate a greater number of individuals on the time-line for stability of clotted samples stored in the refrigerator prior to freezing.

Effect of Freezing and Thawing

It has been widely accepted and assumed that there are a finite number of freeze/thaw cycles for a given sample. As a rule, we generally do not use a serum sample for proteomic analysis that has been thawed more than four

times. Currently, careful separation of the serum samples into multiple 0.3–0.4 ml aliquots minimizes these concerns in our biorepository, however, this is frequently not the case with most archived serum specimens. To evaluate how many freeze/thaw cycles can be done before loss or alteration of protein peak features occurs in the low mass SELDI spectra, four serum specimens (0.4 ml) were thawed in a water bath at 37° C, then snap frozen in liquid nitrogen for five cycles. After each thaw, a 50 ul aliquot was removed for subsequent loading on IMAC-Cu chips. As shown in Figure 6, for one of these representative samples it is clear that the spectra is stable through at least five thaws. Direct comparison of the intensities and resolution of the peaks in these five spectra did not detect any major differences. While this evaluation does examine the physiochemical aspects of the freeze/thaw process, it does not account for how long a sample may have been stored prior to thawing, or the variable time intervals between freezing/thawing. However, it does suggest that samples thawed less than four times retain most of their proteomic integrity and can be used for proteomic analyses. In designing experimental profiling protocols, we have found that the number of freeze/thaw cycles (when known) can be controlled to ensure that one disease group does not contain a large proportion of samples with high freeze/thaw rates relative to another cohort with low freeze/thaw numbers.

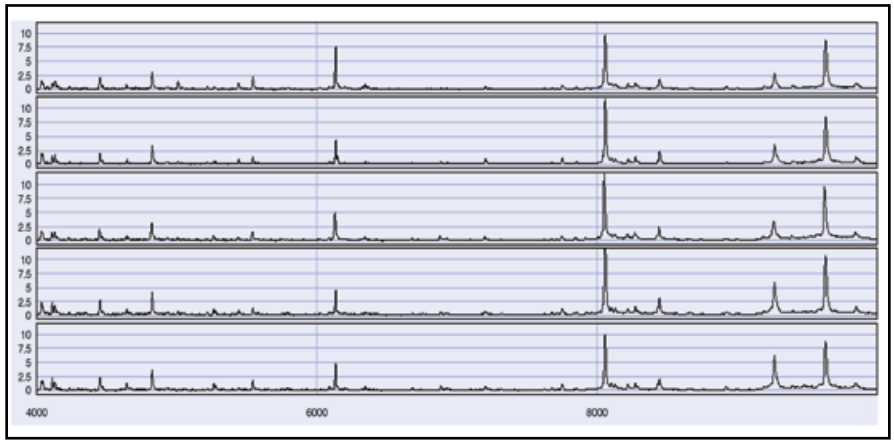

Figure 6. Multiple Freeze-Thaw Cycles for Serum Protein Stability. Mass profiles (4,000 – 10,000 m/z) in peak trace view on IMAC-Cu protein chips from serum frozen and thawed for five cycles.

Figure 7. Effect of Hemolysis on Serum Protein Profiles. Normal and hemolyzed serum were loaded on an IMAC-Cu surface for SELDI analysis (peak and gel views, 4,000 – 17,000 m/z). The α-globin and β-globin chains of hemoglobin are indicated at 15.1 and 15.8 m/z respectively, and the doubly charged ions at 7.5 and 7.9 m/z are also indicated (red arrows).

Effect of Hemolysis

Within large sets of archived serum samples, it is common to have hemolytic serum samples that appear pink to red in color due to red blood cells rupturing during the processing steps. With regard to using these samples in proteomic profiling assays versus normal non-hemolytic serum samples, we have empirically concluded that these samples should be excluded. A proteomic profile comparison of a normal serum sample and an aliquot of the same sample spiked with 1 µl of disrupted red blood cells extracted from the pellet is shown in Figure 7. On an IMAC-Cu surface, the α-globin and β-globin chains of hemoglobin are prominent in the hemolytic sample at 15.1 and 15.8 m/z respectively. Doubly charged ions of these species can also be detected in the same spectra at 7.5 and 7.9 m/z (indicated by the arrows). Also illustrated in this hemolytic spectra is the diminished intensity of other serum proteins bound to the IMAC-Cu surface when compared to the non-hemolytic sample. Besides adding spurious peaks derived from hemoglobin subunits, the high concentration of these same subunits compete for, or alter, the affinity of other serum proteins for binding to the chip surface. This is an unacceptable condition when comparing or distinguishing multiple serum specimens reflective of a given disease state.

Summary

Understanding the stability and integrity of blood sample components will remain a critically important consideration in this age of emerging "omic" technologies, especially proteomics. Using standard laboratory handling conditions, the stability of the low-mass proteins present in serum is clear. There appears to be at least an eight hour window of protein stability in clotted serum at room temperature, and multiple freeze thaws are feasible. These are not new observations in the context of past uses of archived blood samples, but these were necessary studies in the context of new proteomic technologies that can potentially examine the entire proteome of a tissue or fluid.[8,9]

No matter how many improvements arise in the evolution of proteomic, immunologic, and tissue array technologies, standardized sample collection and processing procedures will remain important considerations for new diagnostic and biomarker assay developments.

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