

# Quantification of Proteins and Metabolites by Mass Spectrometry without Isotopic Labeling or Spiked Standards

Weixun Wang,<sup>†</sup> Haihong Zhou,<sup>†</sup> Hua Lin, Sushmita Roy, Thomas A. Shaler, Lander R. Hill, Scott Norton, Praveen Kumar, Markus Anderle, and Christopher H. Becker\*

SurroMed, Inc., 2375 Garcia Avenue, Mountain View, California 94043

**A new method is presented for quantifying proteomic and metabolomic profile data by liquid chromatography–mass spectrometry (LC–MS) with electrospray ionization. This biotechnology provides differential expression measurements and enables the discovery of biological markers (biomarkers). Work presented here uses human serum but is applicable to any fluid or tissue. The approach relies on linearity of signal versus molecular concentration and reproducibility of sample processing. There is no use of isotopic labeling or chemically similar standard materials. Linear standard curves are reported for a variety of compounds introduced into human serum. As a measure of analytical reproducibility for proteome and metabolome sampling, median coefficients of variation of 25.7 and 23.8%, respectively, were determined for ~3400 molecular ions (not counting their numerous isotopes) from 25 independently processed human serum samples, corresponding to a total of 85 000 individual molecular ion measurements.**

This contribution reports on a new liquid chromatography–mass spectrometry (LC–MS) approach to quantitative profiling of large numbers of proteins (proteomics) and small molecules (metabolomics) for the purpose of differential expression measurements and discovery of biomarkers. In this situation, many or most monitored proteins and small molecules are unanticipated at the time of laboratory study, thus eliminating the possibility of prior investigation of relative sensitivity factors (RSFs). Furthermore, methods based on introducing a known amount of a chemically analogous extraneous substance as an internal standard (i.e., “spiking” of a standard reference material) are not practical, whether the analogue is chemically identical and isotopically labeled<sup>1–6</sup> or based on chemical similarity.<sup>7,8</sup>

It is worth noting, however, that quantification using spiking with isotopically labeled compounds (known as the isotope dilution method) has a long history.<sup>1–6</sup> This approach, including quantitative isotopic tracer work, has helped generate many valuable contributions to science, especially in biochemical pathway analysis.

Recently, a quantification approach for proteomics has been devised on the basis of a kind of isotope dilution method in which a specific amino acid in a sample and reference mixture are isotopically labeled and subsequently separated by solid-phase capture, wash, and release—what has become known as the ICAT and related methods (isotope-coded affinity tags).<sup>9–11</sup> Drawbacks of the method include sample processing complexity, material losses, reagent expense, required presence of a specific amino acid, nonspecifically captured peptide background, frequent difficulty in obtaining useful tandem mass spectrometry (MS/MS) fragmentation patterns, and inability to address metabolomics. There is definite reason to explore other approaches, as done in this paper.

Although it has been observed that electrospray ionization (ESI) provides signals that can be linear with concentration,<sup>12–14</sup> historically there has been concern about nonlinearities and ion suppression effects,<sup>15</sup> especially in the circumstance of complex biological matrixes, such as serum. Nevertheless, we have undertaken a broad study to examine whether analyte ion signals can in general reflect concentrations in a linear way, especially for the case of complex matrixes, and thereby form the basis of an analytical method for quantifying proteome and metabolome profile data for differential expression.

\* Corresponding author. Phone: (650) 230-1845. Fax: (650) 230-1960. E-mail: cbecker@surromed.com.

<sup>†</sup> These two authors contributed equally to this work.

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The quantification method reported here relies on the changes in analyte signals directly reflecting their concentrations in one sample relative to another. Samples are not mixed; neither are the samples otherwise manipulated beyond that required for the LC-MS analysis itself. The sample preparation and LC-MS conditions need to be carefully controlled, however, for optimal results.

This quantification technology employs overall spectral intensity normalization by employing signals of molecules that do not change concentration from sample to sample. In this way, a simple correction can be applied for any drift over time in overall LC-MS response or differences in sample concentrations. We have developed a computer application, called MassView software, which among other functions performs normalization by comparing two or more spectra to determine the constant intensity ratio between those unchanging analytes; this forms the basis for identifying the nonchanging concentrations (see below). An alternative would be to spike the samples with some (almost any) exogenous compound(s) and use the intensity of that compound to perform normalization for overall system response; however, we find even this nonmatched spiking is unnecessary and have not used it.

As an initial investigation, we made synthetic mixtures of five proteins at different ratios before enzymatic digestion and observed linear behavior in the signal from digested peptides corresponding to the protein concentrations. Encouraged by the success of this experiment, we spiked various extraneous proteins and small molecules into pooled human serum over a range of concentrations to test this new quantification method. Human blood serum certainly represents a complex biological matrix. Note that the spiking of exogenous compounds in this work was to perform quantification tests and is not part of the methodology. Results for these experiments are presented here.

## EXPERIMENTAL SECTION

**Materials.** Pooled human serum used in the proteome analyses and spiking components (des-asp<sup>1</sup>-angiotensin II, [val<sup>4</sup>]-angiotensin III, vitamin B<sub>12</sub>,  $\alpha$ -endorphine, horse myoglobin, bovine RNase A, BSA, bovine cytochrome *c*, human hemoglobin, and bovine carbonic anhydrase II) were purchased from Sigma-Aldrich (St. Louis, MO). Pooled human serum used in the metabolome studies was a mixture from four anonymous healthy donors collected from Stanford Blood Center (Palo Alto, CA). The handling of these biological materials must be performed in accordance with U.S. Department of Health and Human Services guidelines for level 2 laboratory biosafety, as found in *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed., HHS Publication no. (CDC) 93-8395. Affinity beads for albumin and IgG removal were from ProMetic Biosciences (Cambridge, U.K.). All other general reagents were purchased from either Fisher or VWR Scientific.

**Five-Protein Mixtures.** In all three sets of five protein mixtures, the concentrations of horse myoglobin, bovine RNase A, and BSA were kept constant (0.2 mg/mL) while the concentrations of bovine cytochrome *c*, and human hemoglobin were varied thus: set 1, both 0.2 mg/mL; set 2, bovine cytochrome *c* 1.0 mg/mL, human hemoglobin 0.04 mg/mL; set 3, bovine cytochrome *c* 0.04 mg/mL, human hemoglobin 1.0 mg/mL. All three sets were denatured by 6 M guanidine hydrochloride, reduced by 10 mM

dithioereitol at 37 °C for 4 h, and alkylated with 25 mM iodoacetic acid/NaOH at room temperature for 30 min in dark. The denaturant and reduction-alkylation reagents were removed from the mixtures by buffer exchange against 50 mM (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub> at pH 8.3 using a 5 kDa molecular weight cutoff spin filter (Millipore, Billerica, MA). Modified trypsin (Promega Corp., Madison, WI) of 1% weight equivalence of the proteins was then added to the mixtures with incubation at 37 °C.

**Serum Proteome.** Different amounts of intact horse myoglobin and bovine carbonic anhydrase II were mixed into 20  $\mu$ g of unprocessed human serum protein at amounts from 100 fmol to 100 pmol. Spiked serum was then fractionated into serum proteome and serum metabolome using a 5-kDa molecular weight cutoff spin filter (Millipore Corp., Bedford, MA). Twenty-five microliters of serum proteome was diluted with 25 mM PBS buffer (pH 6.0) before it was applied to affinity beads (Prometic Biosciences, Cambridge, U.K.) for human serum albumin and IgG removal. The albumin- and IgG-depleted serum proteome was denatured, reduced, alkylated, and trypsin-digested following procedures as for the five-protein mixtures.

**Serum Metabolome.** Serum metabolome was separated from the proteome using the 5-kDa cutoff spin-filter procedure mentioned above. Different amounts of an equal-molar compound mixture (des-asp<sup>1</sup>-angiotensin II, [val<sup>4</sup>]-angiotensin III, vitamin B<sub>12</sub>, and  $\alpha$ -endorphine) from 50 fmol to 100 pmol were then spiked into 100  $\mu$ L of metabolome serum fluid. After desalting with a C-18 SPE cartridge (Sep-Pak, Waters Corporation, Milford, MA), the samples were ready for injection into the LC-MS system.

**Nanoflow LC-MS.** A binary HP 1100 series HPLC was directly coupled to a ThermoFinnigan (San Jose, CA) LCQ DECA electrospray ionization (ESI) ion-trap mass spectrometer (using automatic gain control) or Micromass (Manchester, U.K.) LCT ESI-time-of-flight (TOF) mass spectrometer equipped with a nanospray source (New Objective, Woburn, MA). PicoFrit fused-silica capillary columns (5- $\mu$ m BioBasic C<sub>18</sub>, 75  $\mu$ m  $\times$  10 cm, New Objective, Woburn, MA) were run at a flow rate of 300 nL/min after flow splitting. An on-line trapping cartridge (Peptide CapTrap, Michrom Bioresources, Auburn, CA) allowed fast loading onto the capillary column. Injection volume was 20  $\mu$ L for the 20  $\mu$ g of tryptic peptides. Gradient elution of the proteome sample was achieved using 100% solvent A (0.1% formic acid in H<sub>2</sub>O) to 40% solvent B (0.1% formic acid in acetonitrile) over 100 min. Injection volume for the metabolome samples was also 20  $\mu$ L. Separation of the metabolome was performed with a gradient of 10–25% of solvent B in 40 min, followed by 25–90% solvent B in 30 min. The throughput for both proteome and metabolome analysis was 50 samples/week per instrument.

## RESULTS

**Initial Proof-of-Concept Study with a Five-Protein Mixture.** Before applying this direct quantification methodology to complex biological systems, we first applied it to a less complicated model system composed of a five-protein mixture. Three sets of five-protein mixtures were prepared so that the concentrations of three components were kept constant (horse myoglobin, bovine RNase A, bovine serum albumin) while the remaining two (bovine cytochrome *c* and human hemoglobin) varied at ratios of 1/5 and 5 (Figure 1A). After denaturation, reduction, alkylation, and tryptic digestion, the mixtures were analyzed by on-line LC-MS with

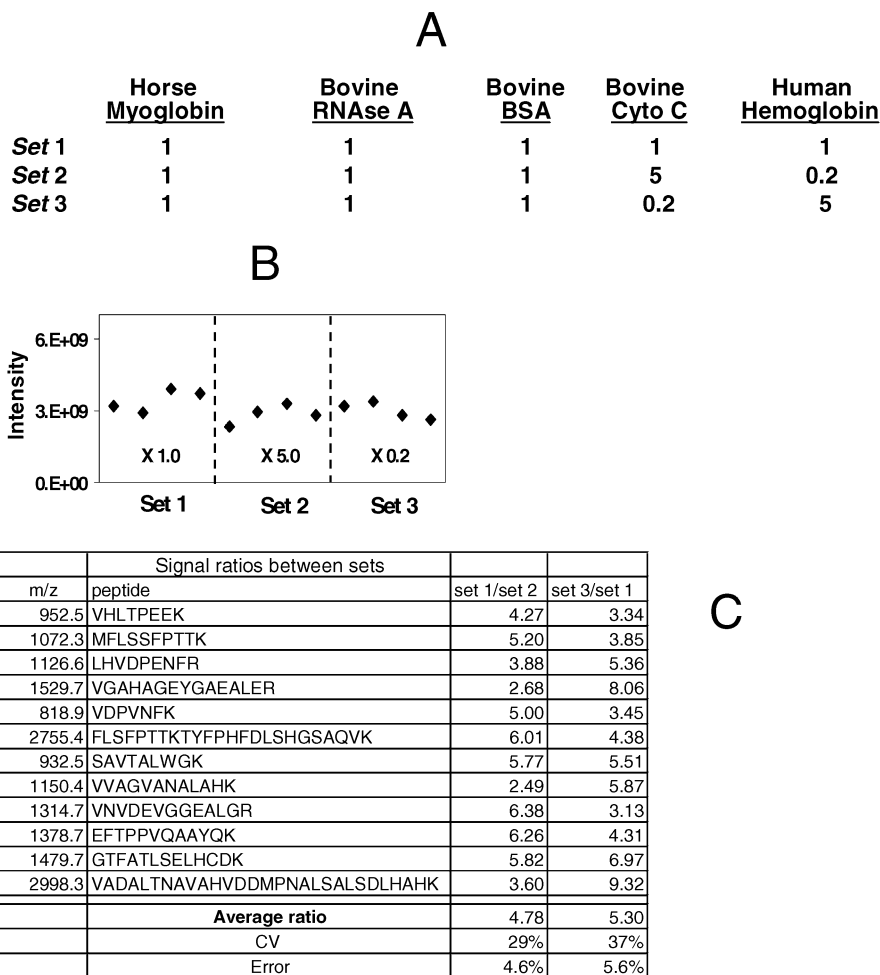


Figure 1. Proof-of-principle quantification study of a simple system composed of a five-protein mixture. (A) Three sets of protein mixtures composed of five proteins each were prepared according to the ratios listed in the table. (B) Normalized intensities of one exemplary hemoglobin peptide for the three sets of mixtures; note scaling factors in the plotting (a flat response shows optimal results). (C) Ratios between different sets of 12 hemoglobin peptides.

the ion-trap mass spectrometer. The sample preparation and analysis were repeated four times for each set of ratios to check reproducibility. Intensities of tryptic peptides from the digested proteins were used to quantify the concentrations between sets. Figure 1B is an example showing how one tryptic peptide from human hemoglobin differentiated the three sets of mixtures. By normalizing against the intensities of the tryptic peptides of proteins whose concentrations were kept constant in all three sets, we observed signal changes for the bovine cytochrome *c* and human hemoglobin that were close to linear with the known relative concentrations with acceptable coefficients of variance (CVs) (Figure 1C).

**Complex Sample: Human Serum Proteome and Metabolome.** We then applied this approach to more complex biological matrixes, including human blood serum. In our experimental design, the high molecular weight (proteome) and low molecular weight (metabolome) fractions are separated before analysis. Both fractions exhibit significant complexity. Figure 2A illustrates a typical LC-MS analysis of a proteome sample from human serum. Figure 2A is a base peak chromatogram of tryptic peptides from the digested proteome. With highly complex samples and complex mass spectra, it became apparent that an automated peak

identification and quantification computer application would be required.

**Data Analysis Software.** The high resolution and high mass accuracy of the TOF system was found to be advantageous for tracking and quantifying large numbers of mass spectral peaks. To track and quantify these peaks, custom software encompassing a variety of functions needed to be designed and built. The various steps in the software are now described.

**Overview.** For every scan (mass spectrum at a given elution time, typically spanning 1 or a few seconds) our software first performs a baseline subtraction, then a smoothing of the data. A method called vectorized peak detection is used to identify valid spectral peaks in the presence of noise sources, notably chemical noise. Next, there is an assignment of an isotopic pattern for the peaks, also known as de-isotoping. An intensity value is then recorded for the molecular ion. Using an arbitrarily chosen reference sample, the LC-MS retention times are adjusted for small variations between samples, and intensities are normalized in a global fashion (one normalization constant for the entire sample). Mass spectral peaks found in multiple samples to correspond to each other become assigned as components of the study. After the final assignment of intensity for each component

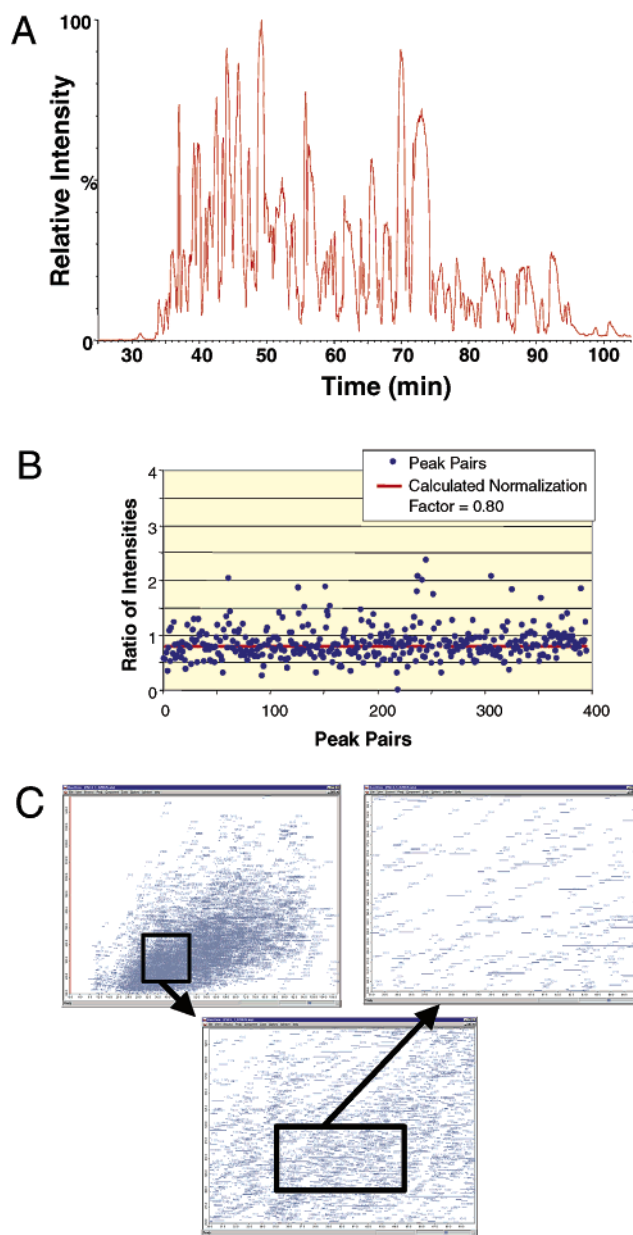


Figure 2. Complexity of the human serum proteome sample. (A) Nanoflow electrospray LC-TOF-MS base-peak intensity profile of 20  $\mu\text{g}$  of digested serum proteome. Peptides are eluted in a gradient from 0 to 40% acetonitrile/0.1% formic acid. (B) Example of normalization; the median is determined for the ratios of intensities for many components between a sample file and a reference file. (C) Two-dimensional view by SurroMed's proprietary MassView software of the LC-MS spectra: the X axis is retention time, and the Y axis is the mass-to-charge ratio. Each plotted element is a separate molecular ion (component).

in each sample, a coefficient of variance is then calculated for each component. In a study comparing groups or pairs of individuals, a statistical analysis such as a two-sided *t*-test is performed to determine the significance of any observed changes between the groups or pairs of individuals.

For the first step, baseline subtraction, a low-order polynomial smoothing filter based on the Savitsky-Golay algorithm is applied using parameters optimized to remove baseline noise and spikes while preserving the signal frequency contained in the peak

shape.<sup>16</sup> This results in a smoothed baseline with well-defined starting and end points for each peak. A set of points belonging to the baseline is then selected, and a low-order polynomial interpolation between these points results in an approximation to the baseline, which is subtracted from the original spectrum.

We then continue with the use of the Savitsky-Golay algorithm for peak smoothing and noise reduction. We select the parameters, the degree of the (high-order) polynomial, and the width of the data channels over which the approximation is to be made, empirically to preserve peak shape and area.<sup>17-19</sup> This two-step approach, using different filter parameters for baseline and signal, proved particularly useful for multifrequency signals (multiple peaks with different peak width).

Peaks pertaining to valid analyte molecular ions are identified by a method called vectorized peak detection.<sup>20</sup> In this method, local maximums are determined in two dimensions, namely the *m/z* dimension and the chromatographic (time) dimension. A local maximum requires an increase in ion counts greater than a settable change (a threshold) over a limited range in *m/z* or time; this is peak sensing. Only those points that pass criteria for the maximums in both dimensions are accepted. This approach allows a flexible threshold definition rather than a constraining global threshold. A primary advantage of the vectorized peak detection algorithm is in a robust elimination of chemical noise while still accepting weak analyte peaks.<sup>20</sup> Acceptance of chemical noise peaks can be eliminated or at least greatly reduced because they do not show local maximums in the chromatographic dimension.

Isotope pattern assignment begins by locating the maximum intensity isotope in a spectral region. The assignment tool is based on a pattern recognition technique called template matching,<sup>21,22</sup> often used in image processing for object recognition. Here, we employ templates derived from an isotopic table to search for patterns of peaks in the original spectra. The isotopic template or pattern is mass-dependent and based on a nominal peptide elemental composition associated with the average occurrence of the various amino acids determined for a large number of proteins.<sup>23</sup> The measure of fit between original data and isotopic pattern template is the cross-correlation (convolution) between the two and is maximized by the isotopic template that matches the one found in the original data. A variety of patterns associated with different charge states (and different masses for the *m/z* region) are systematically examined up to *z* = 5 or 6, and the best fit chosen. Isotopes that are thus determined to belong to a single molecular ion are then removed from the peak list and are no longer considered.

Even though experimental care is exercised to keep the chromatography as constant as possible, small deviations over the ~100-min elution profile unavoidably occur. When spectra are

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dense, as in the current application, minor wobbling or drift in the retention times between samples can limit the number of molecular ions that can be successfully tracked. We have developed a "dynamic time warping" (DTW) approach, which is somewhat analogous to what has been developed in the speech recognition field.<sup>24,25</sup> In this process, one file is compared to a reference file, and for each comparison, a set of peaks in common between the two files are chosen by the software based on proximity of retention time (fairly loosely defined at this stage, usually to  $\pm 5$  min) and  $m/z$  (commonly  $\pm 0.10$  Da). However, it is the corresponding entire mass chromatograms spanning all elution times at these masses that are actually used in the computation (rather than just the peaks themselves). Because the computation is relatively time-consuming, the number of mass chromatograms is limited to  $\sim 200$  by constraints based on intensity of the chosen peaks. These 200 mass chromatograms are then combined to create a matrix, each element being the difference in intensity at a given time and  $m/z$  between the two files, with each row of the matrix originating from the mass chromatogram. Next, a path is found through the matrix that minimizes the intensity differences using dynamic programming techniques.<sup>25</sup> The path provides the warping function to apply to the nonreference file in question. Note that a similar approach has been used in chromatography,<sup>26</sup> but only considered total ion chromatograms (TICs). This work has shown it is essential to consider a more comprehensive data set than just TICs.

The peak intensities are next determined for quantification using the smoothed data by using either the peak area (summed over the elution time of the component, that is, a mass chromatogram) or the maximum peak height in ion counts. Both approaches have been used successfully, and a maximum peak height has been used with the TOF data in this report. Furthermore, the software was written to either sum over all isotopes or use the intensity of the monoisotopic peak; in this work, we have used the monoisotopic peak intensity. Restated, for the TOF data in this work, the maximum value of the smoothed monoisotopic peak in ion counts is assigned as the peak intensity.

To correct for instrument sensitivity drift over time and sample-to-sample concentration variations, next, the data is normalized (see Figure 2B). In the approach used here, normalization is performed by choosing one file as a reference and individually normalizing all other files relative to the reference file, one file at a time. The choice of reference file is generally arbitrary, and results are independent of the file chosen. For each file's normalization, a set of pairs of corresponding peaks from the two peak lists are chosen by the computer software on the basis of close proximity in retention time and  $m/z$ . Unlike the size-constrained list used for dynamic time warping, there is no computational need to constrain the numbers of ratios; consequently, 1000–3000 peak pairs were used. A smaller number are shown in the example in Figure 2B to aid in the visualization. The single normalization constant for each file is taken as the median of the ratios of intensities for all components between the file in question and the reference file. Note that the software does

not select which peaks to use in the comparison. Rather, the median value for the intensity ratio of *all* the spectral peaks between the nonreference and reference files is used as the normalization constant for that file. The median, rather than the average, allows for some strongly changing peaks and is appropriate for comparing like substances, such as the serum from two individuals.

At this point in the software, component building takes place for the study by scanning the de-isotoped peak maximums listed according to their  $m/z$  and retention time values, and if a peak from another sample is within the user-adjustable  $m/z$  and retention time windows, they are considered to represent the same component. For the TOF mass spectrometer, the  $m/z$  window (generally set to  $\pm 0.10$  Da or less) is smaller than for the ion trap, leading to the previously mentioned conclusion, that for highly complex samples and associated dense spectra, a high-resolution instrument is favorable for this profiling. In the component building process, a threshold is user selected (commonly at  $\sim 20$  ion counts). Using a relatively conservative threshold results in a more reliable and robust process. Statistical variation in signal simply due to counting statistics ("shot noise") is not dominant with this threshold setting.

To provide a sense of the spectral density in these serum analyses, Figure 2C shows a two-dimensional view ( $X$  axis, retention time; and  $Y$  axis,  $m/z$ ) of the mass spectral peaks from the separation corresponding to Figure 2A. For the metabolomic fraction, similar results are found revealing large numbers of molecules. Twenty micrograms of tryptic digested serum proteome yielded more than 2700 different molecular ions (components) per file, and the metabolomic fraction from 100  $\mu\text{L}$  of serum yielded 730 different molecular ions that were detected and tracked.

With regard to molecular identification, selected molecules can be targeted for subsequent tandem mass spectrometry (MS/MS) analysis. For peptides, results are searched against protein or DNA expressed sequence tag (EST) databases<sup>27,28</sup> using a commercial program, such as SEQUEST (ThermoFinnigan, Inc.) or Mascot (Matrix Science), or alternatively, de novo sequencing of the MS/MS spectra can be used with a variety of commercial software.

To differentially quantify molecules by this method, however, first the reproducibility of the analytical platform had to be tested and confirmed.

**Reproducibility of Sample Preparation and Nanoflow LC–MS.** Quantification is based on the stability and reproducibility of analyte signals as well as their linear, or near-linear, dependence on concentration. Starting from a human serum pool, a total of 105 individual proteome samples were independently processed and analyzed consecutively by nanoflow electrospray LC–MS over a time period of 13 days (80 on an ion-trap mass spectrometer and 25 on a TOF mass spectrometer). Similarly, a total of 115 serum samples were prepared and analyzed for metabolome analysis over 17 days.

To measure the CVs for larger numbers of molecular components, the MassView software first quantified their intensities relative to a reference sample (in this case, arbitrarily chosen).

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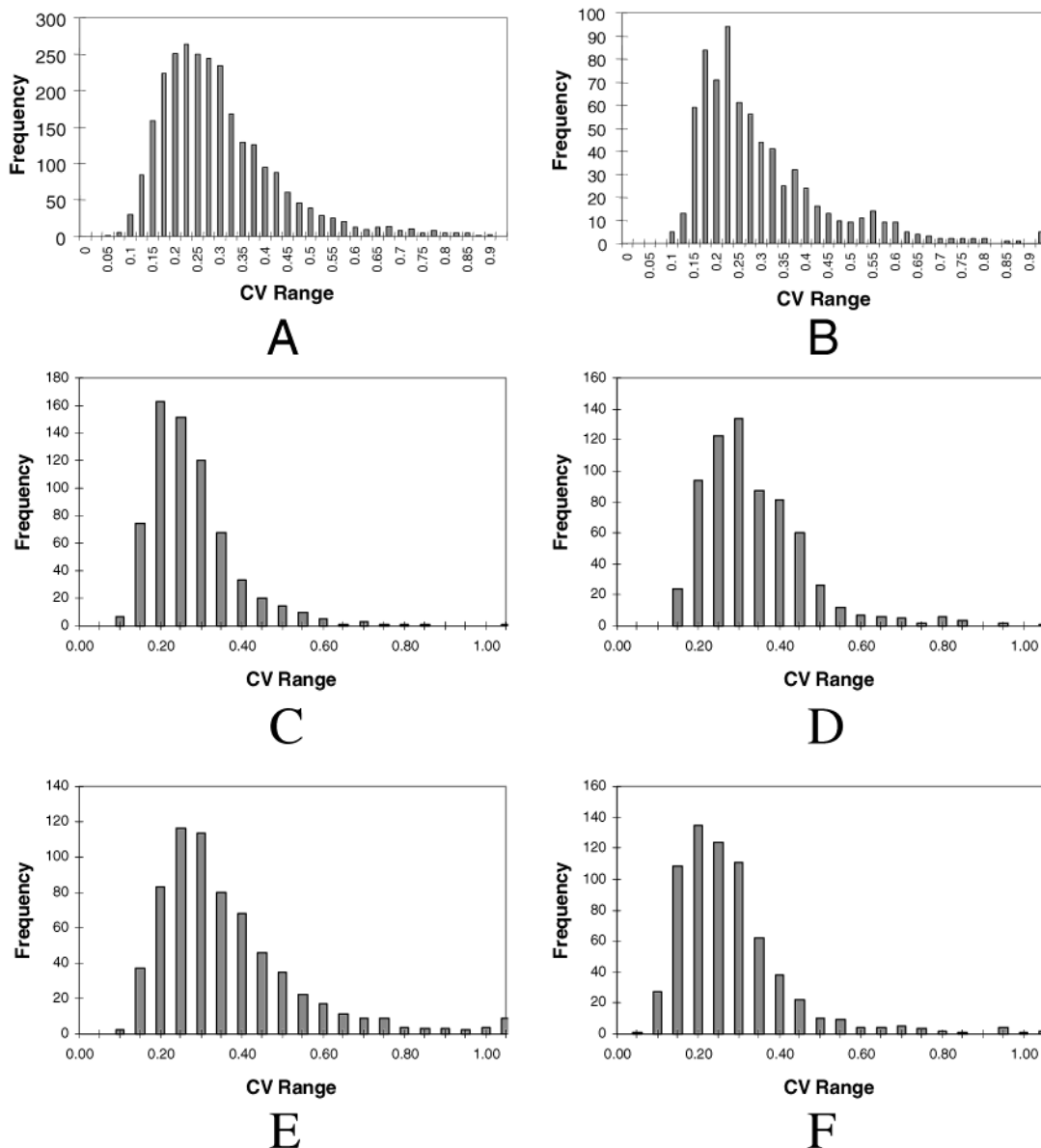


Figure 3. Reproducibility and normalization of serum profiling by nanoflow LC–MS. Parts A and B show coefficient of variance (CV) distributions for proteome and metabolome samples from pooled human serum based on 25 runs each, each sample independently processed. (A) For the serum proteome, the median and average CVs were 25.7 and 29.0%, respectively, with 2700 (de-isotoped) molecular ions analyzed each run. (B) For the serum metabolome, the median and average CVs were 23.8 and 28.5%, with 730 (de-isotoped) molecular ions analyzed each run. These data were obtained from a time-of-flight mass spectrometer. The data for the CV distributions from part A are divided into four quartiles on the basis of the average intensities, each ~670 components, to show how the CV behaves as a function of intensity. Part C is the first quartile (lowest intensities), followed by parts D, E, and F, the second, third, and fourth quartiles, respectively.

From those results, Figure 3A and B displays for proteome and metabolome samples the distribution of CVs of normalized intensities for a total of ~3400 molecular ions from measurements on 25 samples on the TOF instrument, corresponding to a total of ~85 000 individual molecular ion measurements (each molecular ion was de-isotoped as described above). The median and mean CV values were 25.7 and 29.0% for the proteome and 23.8 and 28.5% for the metabolome samples, respectively.

An issue to address is how this method performs for high- and low-intensity molecular ions. Accordingly, the data of Figure 3A were divided into four quartiles on the basis of average intensity for the components. Results are seen in Figure 3C–F, from lowest to highest intensities. It is observed that there is no

large growth in CVs for the lower intensity peaks. In fact, some tightening of the CV distribution is found for the lowest quartile that is due to the influence of the threshold in eliminating some smaller-intensity data. As mentioned previously, it is useful to employ a threshold that is not set too low so that all the signals being tracked have substantial ion counts.

**Quantification: Examples from the Human Serum Proteome.** Two nonhuman proteins (horse myoglobin and bovine carbonic anhydrase II) were spiked in 20  $\mu\text{g}$  of the unprocessed human serum proteome at amounts ranging from 100 fmol to 100 pmol. The spiked serum samples then went through sample processing, including denaturation and digestion with trypsin by standard procedures. The samples were analyzed on ion-trap and

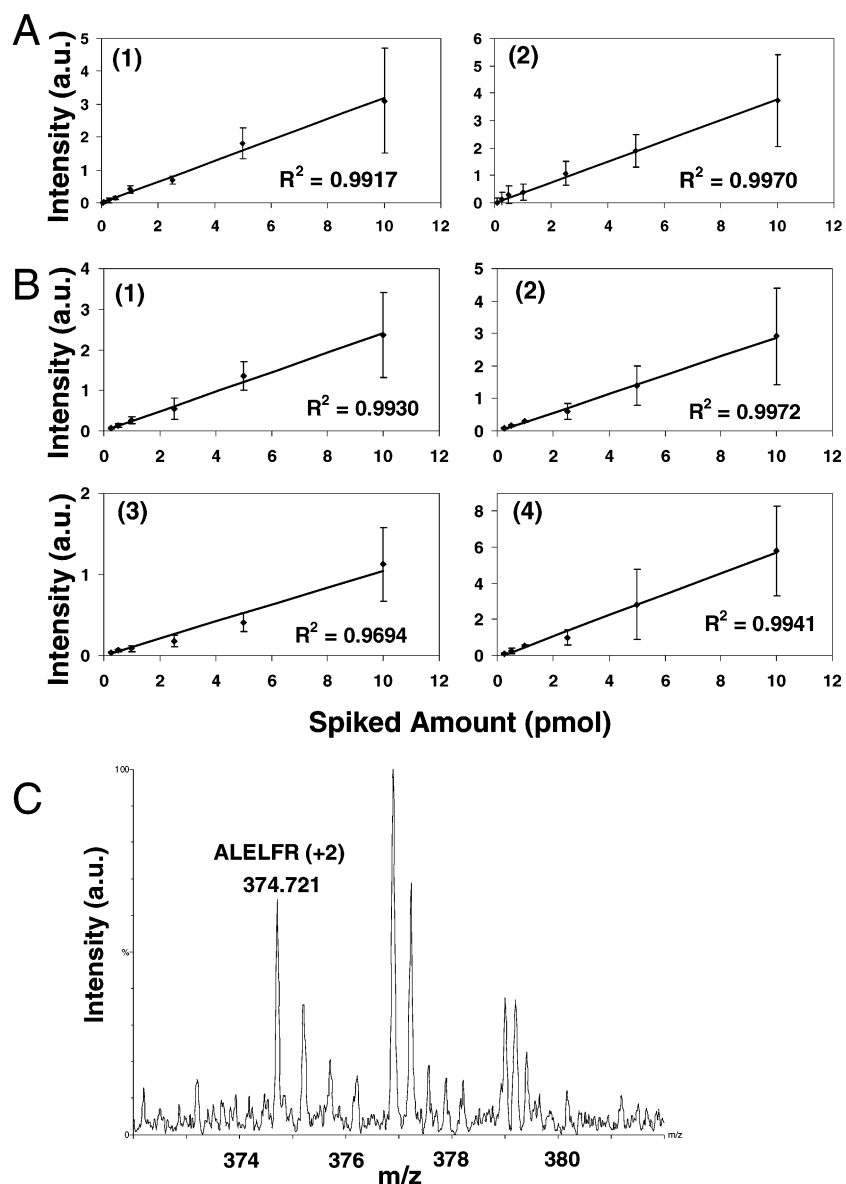


Figure 4. Linear-fit standard curves of normalized peak intensities of peptides from spiked proteins versus spiked amounts. (A) Data collected from an ESI-ion-trap mass spectrometer for horse myoglobin peptides: (1) HGTVVLTALGGILK, (2) GLSDGEWQQVLNVWGK. (B) Data collected from ESI-TOF mass spectrometer: (1) from horse myoglobin, HGTVVLTALGGILK; (2) from horse myoglobin, ALELFR; (3) from bovine carbonic anhydrase, VLDALDSIK; (4) from bovine carbonic anhydrase, AVVQDPALKPLALVYGEATSR. (C) Example of a spectrum obtained from an ESI-TOF mass spectrometer showing one peptide of horse myoglobin (spiked at 100 fmol, the lowest amount for this study) coeluting with other peptides from serum.

time-of-flight mass spectrometers using the same nanoflow electrospray ionization and liquid chromatography arrangement and solvent gradient.

Exemplary standard curves for peptide concentrations versus signal intensity are presented in Figure 4A and B. We observed no exception to linear signal behavior in these experiments. An example of ESI-TOF mass spectral data is shown in Figure 4C for a tryptic peptide from 100 fmol spiked horse myoglobin.

**Quantification: Examples from the Human Serum Metabolome.** Different amounts of an equal-molar test compound mixture from 50 fmol to 100 pmol per component were spiked into 100  $\mu$ L of pooled human serum metabolome prior to sample cleanup by solid-phase extraction using a C18 cartridge. Again, samples were mass-analyzed by both ion-trap and TOF instruments. Figure 5A shows the normalized signal for each compound

as a function of spiked concentration generated on an ion-trap MS. Linear response was observed, although the detection limit observed for  $\alpha$ -endorphine was 1 pmol. With the higher resolution ESI-TOF mass spectrometer, it was easier to go to lower concentrations (see Figure 5B). For example, the signal-to-noise ratio for 50 fmol of spiked angiotensin was above 60 (Figure 5C).

## DISCUSSION

The large-scale studies presented here show utility for differential expression studies with relatively low CVs and significant sensitivity and dynamic range for both the high molecular weight fraction above 5 kDa (proteomics) and low molecular weight fraction (metabolomics) using electrospray ionization. Two types of mass analyzers showed the same behavior. However, the higher resolution (TOF) analyzer allows the avoidance of numerous

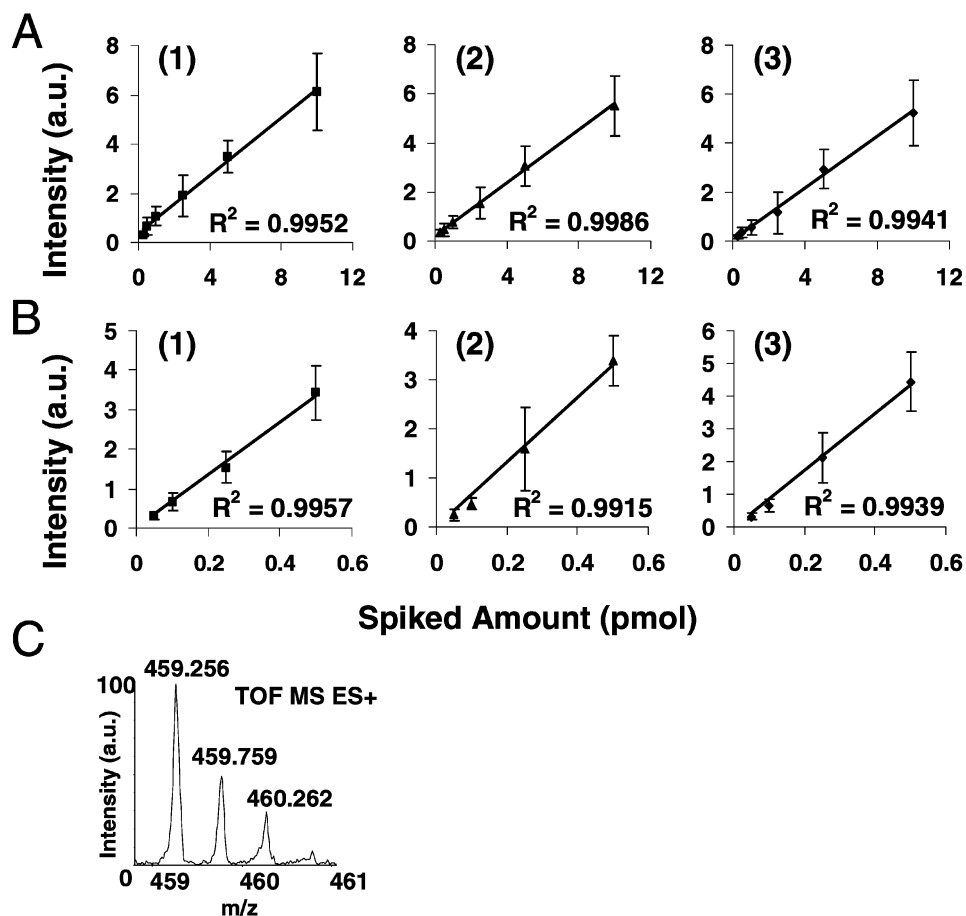


Figure 5. Standard curves for signals from normalized peak intensities of spiked compounds versus the spiked amount in 100  $\mu\text{L}$  serum. These data were collected from (A) an ion-trap mass spectrometer, and (B) an ESI-TOF mass spectrometer. Spiked compounds are (1) vitamin B<sub>12</sub>; (2) [val<sup>4</sup>]angiotensin; and (3) des-asp<sup>1</sup>-angiotensin. (C) Mass spectrum of 50 fmol spiked des-asp<sup>1</sup>-angiotensin obtained from the ESI-TOF mass spectrometer (sum of three scans). Signal-to-noise ratio was >60.

interferences, thus, often effectively increasing the signal-to-noise ratio and compound specificity for low abundance molecules. Direct quantification without isotopic labeling or spiking of matched internal standards offers advantages, such as simple sample processing, applicability to proteome and metabolome samples, and less spectral interferences. Again, note that the spiking of exogenous compounds in this work was to perform quantification tests and is not part of the methodology.

It is imperative that the sample processing and LC-MS platform be highly reproducible, which is feasible by following appropriate standard operating and system maintenance procedures.

The simple five-protein mixture proof-of-principle experiment and the larger experiment using compounds spiked into complex pooled human serum demonstrate that a high degree of linearity is observed for signal versus concentration. This linearity in signal response, together with a high degree of reproducibility reflected in low median and mean CVs, satisfies the fundamental requirements for quantification in differential expression experiments involving unanticipated molecules.

Results for each sample are normalized by multiplication by a single number to account for any long-term drifts in overall LC-MS response. The normalization factor for each sample can be determined by a variety of means. These include finding and using

intensities of compounds, such as housekeeping proteins or creatinine. We have found useful an unbiased normalization procedure based on determining the median of ratios of peak intensities of molecular components in the test sample relative to a reference sample and applying that median ratio as the normalization factor. In addition, it is possible to spike a compound-(s) of nearly any composition into each sample to register overall signal response, although we have not found this necessary.

There were concerns that ion suppression effects would make problematic the direct quantification from mass spectral intensities. We have not observed difficulties, even though we do observe some ion suppression. Signals from the spiked exogenous analytes are lower when the analytes are mixed in the complex serum than when they are in pure form. (Interferences in the complex sample also sometimes restrict the limit of detection, LOD.) Although such ion suppressions were observed, nevertheless, they do not appear to alter the linear response between normalized signal intensities and spiking concentrations. An important observation is that samples of similar general composition are compared in biological studies; thus, similar ion suppression occurs. Furthermore, the significant separation time used ( $\sim 1$  h or more) leads to less ion suppression while having reasonable throughput by avoiding undue complexity of the sample or chromatographic salt fronts at the moment of ionization.



## CONCLUSIONS

The studies described in the present report demonstrate that a quantification method without the need for isotopic labeling or spiking of special chemicals has been established for complex biological samples. These methods are being applied to biomarker discovery in clinical studies, with thousands of proteomic and metabolomic analytes' being profiled across different human and

animal population groups for their quantitative differential expression.

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