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Performance Metrics for Liquid Chromatography-Tandem Mass Spectrometry Systems in Proteomic Analyses and Evaluation by the CPTAC Network*

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Footnotes

[1]The abbreviations used are: FWHM, full width at half maximum; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MS1, full MS scan; MS2, tandem MS scan; SOP, standard operating procedure.

[2] Certain commercial equipment, instruments, or materials are identified in this document. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the products identified are necessarily the best available for the purpose.

A major unmet need in liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomic analyses is a set of tools for quantitative assessment of system performance and evaluation of technical variability. Here we describe 46 system performance metrics for monitoring chromatographic performance, electrospray source stability, MS1 and MS2 signals, dynamic sampling of ions for MS/MS and peptide identification. Applied to datasets from replicate LC-MS/MS analyses, these metrics display consistent, reasonable responses to controlled perturbations. The metrics typically display variations less than 10% and thus can reveal even subtle differences in performance of system components. Analyses of data from interlaboratory studies conducted under a common standard operating procedure identified outlier data and provided clues to specific causes. Moreover, interlaboratory variation reflected by the metrics indicates which system components vary the most between laboratories. Application of these metrics enables rational, quantitative quality assessment for proteomics and other LC-MS/MS analytical applications.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)[1] provides the most widely used technology platform for proteomic analyses of purified proteins, simple mixtures and complex proteomes. In a typical analysis, protein mixtures are proteolytically digested, the peptide digest is fractionated and the resulting peptide fractions then are analyzed by LC-MS/MS (1, 2). Database searches of the MS/MS spectra yield peptide identifications and, by inference and assembly, protein identifications. Depending on protein sample load and the extent of peptide fractionation employed, LC-MS/MS analytical systems can generate from hundreds to thousands of peptide and protein identifications (3). Many variations of LC-MS/MS analytical platforms have been described and the performance of these systems is influenced by a number of experimental design factors (4).

Comparison of datasets obtained by LC-MS/MS analyses provides a means to evaluate the proteomic basis for biologically significant states or phenotypes. For example, data-dependent LC-MS/MS analyses of tumor and normal tissues enabled unbiased discovery of proteins whose expression is enhanced in cancer (5-7). Comparison of data-dependent LC-MS/MS datasets from phosphotyrosine peptides in drug-responsive and -resistant cell lines identified differentially regulated phosphoprotein signaling networks (8, 9). Similarly, activity-based probes and data-dependent LC-MS/MS analysis were used to identify differentially regulated enzymes in normal and tumor tissues (10). All of these approaches assume that the observed differences reflect differences in the proteomic composition of the samples analyzed, rather than analytical system variability. The validity of this assumption is difficult to assess, because of a lack of objective criteria to assess analytical system performance.

The problem of variability poses three practical questions for analysts employing LC-MS/MS proteomics platforms. First, is the analytical system performing optimally for the reproducible analysis of complex proteomes? Second, can the sources of suboptimal performance and variability be identified and can the impact of changes or improvements be evaluated? Third, can system performance metrics provide documentation to support the assessment of proteomic differences between biologically interesting samples?

Currently, the most commonly used measure of variability in LC-MS/MS proteomic analyses is the number of confident peptide identifications (11-13). Although consistency in numbers of identifications may indicate repeatability, the numbers do not indicate whether system performance is optimal or which components require optimization. One well-characterized source of variability in peptide identifications is the automated sampling of peptide ion signals for acquisition of MS/MS spectra by instrument control software, which results in stochastic sampling of lower abundance peptides (14). Variability certainly also arises from sample preparation methods (e.g., protein extraction and digestion). A largely unexplored source of variability is the performance of the core LC-MS/MS analytical system, which includes the LC system, the MS instrument and system software. The configuration, tuning and operation of these system components govern sample injection, chromatography, electrospray ionization, MS signal detection and sampling for MS/MS analysis. These characteristics all are subject to manipulation by the operator and thus provide means to optimize system performance.

Here we describe the development of 46 metrics for evaluating the performance of LC-MS/MS system components. We have implemented a freely available software pipeline that generates these metrics directly from LC-MS/MS data files. We demonstrate their use

in characterizing sources of variability in proteomic platforms, both for replicate analyses on a single instrument and in the context of large interlaboratory studies conducted by the National Cancer Institute-supported Clinical Proteomics Technologies Assessment for Cancer (CPTAC) network.

Experimental Procedures[2]

Metrics. A list of the 46 metrics described in this report is shown schematically in Figure 1. Short descriptions of the metrics, their assigned reference codes, and the direction indicating improved performance for each can be found in Table 1. While the following section lists the currently used metrics, updates will be described with each release (http://peptide.nist.gov/metrics/). Additionally, all of the data described in this work can be downloaded from the ProteomeCommons.org Tranche network at http://cptac.tranche.proteomecommons.org.

Chromatography. Metrics C-1A and C-1B report the fraction of peptides with repeat identifications either >4 minutes earlier (C-1A) or later (C-1B) than the identification nearest the chromatographic peak maximum. Early identifications indicate bleed (typically non-retained, hydrophilic peptides), whereas later identifications arise from peak tailing of either overloaded peptides or peptides with poor chromatographic behavior. These are reported as fractions of all peptide identifications.

Metric C-2A reports the retention time period over which the middle 50% of the identified peptides eluted and C-2B is the rate of peptide identification during that period. Sample LC-MS chromatograms from analysis of a yeast proteome tryptic digest

on three LTQ instruments are depicted in Figure 2. The longer the time period over which peptides elute, the more time available to acquire MS2 spectra and the greater the number of peptides likely to be sampled and identified. In this work, this period is defined as the time over which the middle 50% of the identified peptides elute, which corresponds to the difference between the end of the first and beginning of the last retention quartiles (also called 'interquartile range') (C-2A). Various measures described later are computed only during this central period (e.g. C-2B, the identification rate in unique peptides/min) to reject early and later uninformative periods of chromatography and correct for differences in absolute start and end times when comparing chromatograms.

Metrics C-3A , C-4A, and C-4B report chromatographic peak widths for identified peptide peaks. Sharper chromatographic peaks generate higher signal intensities and can reduce oversampling, thereby increasing the diversity of peptides identified. Peak widths (full width at half max (FWHM)) were calculated as RT_2-RT_1. Peak width medians (C-3A) and interquartile distances for these values (C-3B) are reported. C-3B is a measure of the distribution of peak widths for all peptides. Smaller values indicate higher degrees of peak uniformity. Peak widths were also calculated on smaller sets of early (decile 1, C-4A) or late (decile 10, C-4B) eluting peptides to measure for peak broadening at the extremes of the gradient.

Peptide elution order can be used to measure elution differences early (hydrophilic) and late (hydrophobic) in the chromatographic gradient. C-6A and C-6B are calculated by first identifying the N(*a*, *b*) peptides in common for a pair of runs, *a* and *b,* and then sorting all peptides in each run by elution time. If we define R1(*a*,*b*) as

the rank of the earliest eluting peptide (rank 1) in run *a* that is also present in run *b* and R1(*b*, *a*) as the equivalent rank of the earliest co-occurring peptide in run *b*, then R1(*a*,*b*)–R1(*b*,*a*) is a measure of the number of extra early eluting peptides in run *a* (C-6A). To make this more robust, we find the maximum of the difference R*n*(*a*, *b*)–R*n*(*b*, *a*) from *n*=1 to *n*= N(*a*, *b*)/10. C-6B is calculated similarly but for the high-ranking, co-occurring peptides. This maximum is divided by the total number of peptides identified in the run, giving the fractional excess (or if negative, deficit) of hydrophobic peptides. For both measures, average differences between all intraseries runs and all interseries runs are reported.

Dynamic Sampling. Metrics DS-1A and DS-1B are measures of peptide ion oversampling, which is controlled by the dynamic exclusion settings in the acquisition software. Ideally, these settings minimize wasteful multiple sampling of a peptide ion by permitting just one MS2 spectrum for a given peptide ion over a chromatographic peak. Ratios of singly to doubly (DS-1A) and doubly to triply (DS-1B) identified peptide ions are reported as measures of oversampling. The ratio of spectrum identifications to peptide ion identifications provides a more general measure of oversampling, but mixes the effects of distorted chromatographic peaks and bleed with effects more directly originating from dynamic sampling.

The numbers of MS (DS-2A) and MS2 (DS-2B) spectra acquired during the middle 50% peptide retention period (C2-A) indicate the effective speed of sampling over the most information rich section of the chromatogram. If, for a given MS spectrum, there is insufficient signal to reach the target threshold, or the dynamic

exclusion settings are not working as expected, the numbers of MS or MS2 spectra may vary substantially between technical replicates.

Metrics DS-3A and DS-3B describe peak sampling. Ideally, chromatographic peaks will be sampled at their maximum intensity. However, current methods make sampling decisions prior to peak maximization and larger chromatographic peaks are often sampled well before reaching the maximum. Because an *m/z* value chosen for MS2 sampling is typically placed on an exclusion list for the theoretical remainder of the peak, it is important that the amount of signal be sufficient to trigger acquisition of an MS2 spectrum with adequate signal-to-nose (S/N) for successful peptide identification. The trade-off is that the threshold value should not be so high as to prevent lower intensity peaks from being sampled. By monitoring the median ratio of the maximum MS1 intensity value over the MS1 intensity at sampling time for all identified peptides (DS-3A), it is possible to look for variability in chromatographic peak sampling. To approximate the sampling ratio for less abundant analytes, ratios for peptides in the bottom 50% by MS1 abundance are calculated separately (DS-3B). The reciprocal values for these metrics, or the percent of the total peak height at sampling, are also useful and perhaps more intuitive. MS1 maximum peak heights were found by linear interpolation from the extracted ion chromatograms (XIC) for each precursor *m/z* using a newly developed method (15-18).

Ion source. Metrics IS-1A and IS-1B are measures of electrospray stability. Short-term electrospray stability is monitored as the median of ratios of MS1 total ion current for each set of adjacent scans, the larger ion current divided by the smaller, over the interquartile time interval C-2A. Electrospray drop-off was monitored by counting

the number of events where the total ion current changed more (IS-1A) or less (IS-1B) than 10-fold in adjacent full MS scans. Values greater than zero for either of these metrics indicate spray tip "sputter."

Instrument setup and tuning, including electrospray optimization, affect distributions of the peptide ions *m/z* values. This is monitored as the median precursor *m/z* (IS-2) of the identified peptide ions. This measure is sensitive to sample loading, with higher concentration of peptides generating higher *m/z* values, presumably at least partly due to the increasing preference for lower charge states with fewer charges available per peptide.

The availability of protons during electrospray ionization, the relative sequence length of peptides and pH can all affect the charge state ratios of the identified peptides. Since doubly charged peptide ions (+2) represent the majority of the identified species from a typical tryptic digest, the ratios of +1/+2 (IS-3A), +3/+2 (IS-3B) and +4/+2 (IS-3C) allow monitoring of the stability of the distributions. Perturbations in these ratios (or in the median precursor *m/z*) either run-to-run or between series may also correlate with decreased overlap of the observed peptide identifications.

MS1 and MS2 Signal and Analysis of Spectra. Reported intensities are the most direct measure of signal strength, although we have found that they can vary substantially between runs and labs for no clear reason. Metrics reporting these values over the C-2A interval are MS1-2B, the median TIC for full MS1 spectra and MS1-3B, the median of maximum MS1 intensities for peptides. MS1-3A is a measure of 'dynamic range', which is taken as the ratio of the 95th/5th percentile of MS1 maximum intensities for identified peptides over C-2A.

The maximum-to-median signal (a measure of S/N) in both MS1 (MS1-2A) and MS2 (MS2-2) spectra has proven to be a more stable measure of signal strength than absolute intensities. However, since these metrics can depend on thresholding and centroiding, they could vary between classes of instruments and data processing systems. For the data generated in this study with Thermo ion trap instruments, this did not appear as a problem and this measure served well for monitoring run-to-run variation. The total base-peak normalized abundance provides a related measure, but can be sensitive to the *m/z* range and background ions. Numbers of reported peaks (MS2-3) provide another highly correlated measure and are also reported. We note the general difficulty of determining whether variations in signal intensity arise from amounts injected or changes in instrument sensitivity. In fact, none of the measures described can reliably distinguish between these two factors.

Median ion injection times for MS1 (MS1-1) and MS2 (MS2-1) spectra are also reported. Short times should be associated with high signal levels or low threshold settings. In many cases the median is also the maximum, so mean ion injection times

are also reported. Reduced ion injection times occur when analyzing higher sample loads, but should be relatively stable between technical replicates.

Since intensity is used to select ions for fragmentation, intensity variation of the same peptide in different runs is a major source of run-to-run variability. A measure of this variability is the median relative deviation of peptide ion intensities in common between two runs. Absolute differences in peptide ion intensity between the two runs must first be corrected. This is done by sorting an array of the ratios of MS1 maximum intensities for peptide ions found in pairs of runs. The quartile values (Qn is the value for the nth quartile) yielding the median relative deviations are calculated using the expression:

$$[(Q2/Q1) + (Q3/Q2)] / 2$$

The average for within series (MS1-4A) and the ratio of within/between series (MS1-4B) are reported.

Another measure of performance is the fraction of identified (scores above threshold) MS2 spectra at different MS1 maximum peptide intensity quartiles (MS2-A-D). These values indicate to what extent intensity variations are sensitive to MS1 signal strength.

An additional class of metrics reports precursor accuracy (i.e., error associated with the identified peptides). All mass error measurements are derived from +2 peptide ions only. Additionally, mass errors > 0.45 m/z were rejected for high-resolution Orbitrap and FT MS instruments, which are largely due to incorrect monoisotopic mass assignments at

acquisition time. MS1-5A reports the median difference between the theoretical precursor m/z and the measured precursor m/z value as reported in the scan header. Reported monoisotopic values were used if available. MS1-5B is the mean of the absolute differences. MS1-5C is the median value of the real differences in ppm and MS1-5D is the interquartile distance for the distribution used to determine MS1-5C.

Peptide identification. Peptide identifications from MS2 spectra, needed for many of the above metrics, were made by matching spectra to those in a reference spectrum library (19). This method, long used in gas chromatography-mass spectrometry (GC/MS), measures the similarity of reference and search spectra. A 'dot product'-based metric measures similarity (20). This function is not only widely used for GC/MS, but has been found to be effective for MS/MS identification and has recently been employed in several peptide spectrum matching search methods (19, 21-23). Spectrum libraries were derived from spectra assigned to peptide ions by sequence search engines (23-25). The yeast library (NIST yeast_consensus_final_true_lib (06/30/08)) contained 79,990 spectra and was derived from a large number of analyses made by many laboratories, including those of the CPTAC network and is available on the web (http://peptide.nist.gov and http://www.peptideatlas.org/speclib/). The chicken egg yolk spectral library (NIST chicken_consensus_final_true_lib (12/05/08)) contained 4,437 spectra and was generated from many dozens of runs at NIST. Both libraries are available from the authors (S.E. Stein) on request. Score thresholds were fixed for all analysis to yield an overall false discovery rate of 1% estimated by searching decoy libraries of unrelated organisms and eliminating

homologous matches. In cases where a spectrum identified more than one peptide, only the peptide identification with the highest score was used.

ReAdW4Mascot2.exe version 2.1 (ConvVer 20081119b), an extension of ReAdW.exe (Patrick Pedroli, Institute for Systems Biology; http://tools.proteomecenter.org/software.php) was used to extract peaklists to mzXML or MGF format . The converter was run using the following arguments for LTQ data: -sep1 -NoPeaks1 -c –MaxPI and for Orbitrap data: -sep1 -NoPeaks1 -c -MaxPI -ChargeMgfOrbi –MonoisoMgfOrbi. The search engine used was SpectraST (version 3.0, TPP v4.0 JETSTREAM rev 2, Build 200807011544) (19). For spectral library searches with SpectraST, enzyme specificity (trypsin), numbers of missed cleavages permitted (</= 2), fixed modifications (none) and variable modifications (carbamidomethyl C, oxidated M, n-terminal Acetyl, Pyro-glu and Pyro-CmC) were determined by the contents of the spectral library. Mass tolerance for precursor ions was 2 *m/z* for LTQ data and 1.0 *m/z* for Orbitrap data. Mass tolerance for fragment ions is not adjustable in SpectraST. SpectraST does not select candidate spectra based on charge. Therefore, charge information in the peaklists is ignored. The cut-off score/expectation value for accepting individual MS/MS spectra was an fval of 0.45. This threshold was based on a global FDR calculation using decoy spectra. FDR was calculated using the formula

$$FDR = FP / (FP + TP)$$

where FP = 2 times the number of false positive matches at or above this score and TP = number of true positive matches at or above this score. Overlapping decoy and target spectra were removed prior to the analysis and FP was scaled according to the ratio of the target and decoy library size. Decoy spectra were actual peptide spectra from an unrelated

organism, and the distribution of random matches between target and decoy libraries was approximately equal.

P-2C, a good overall measure of performance, is defined as the number of distinct identified tryptic peptide sequences, ignoring modifications and charge state. Numbers of unique semi-tryptic peptides (truncated tryptic peptides) were also counted, and the ratio of semitryptics/tryptics is reported (P-3). Since semitryptics can be formed by sample degradation or in the ionization source, higher total peptide values do not necessarily reflect better performance. This metric should be useful for determining how complete a digest is between preparations or for assessing how variable in-source fragmentation is between runs of the same sample. Also reported are the total numbers of identified spectra (P-2A) and the number of identified precursor ions (P-2B). The median score of identified peptides is also reported (P-1). In the case of the default analysis pipeline, this is the median SpectraST (19) f-val for all peptide identifications. A score threshold of 0.45 was applied to all analyses. Relative decreases in the median score can indicate a reduction in MS2 S/N or other problems resulting from a divergence in similarity to consensus library spectra.

A data analysis pipeline for implementation of performance metrics

We have used a newly developed metrics pipeline to calculate of all of the values presented in this paper (Figure 3). The software consists of (1) a data extraction/feature finding algorithm derived from ReAdW.exe (http://sashimi.sourceforge.net/), (2) a peptide identification engine (either SpectraST (19) or OMSSA (24) are currently integrated),

followed by (3) a program which calculates all of the metrics from the extracted data and, (4) a program that generates statistics for the data series.

The pipeline is currently used to analyze Thermo Fisher .RAW files, requiring Thermo Xcalibur™ to be installed. Updates for handling other vendor-specific files are being developed. The entire workflow is driven by a Perl script which is directed to one or more directories full of raw data files producing an output file in tab-delimited-text format. This software is available for download at http://peptide.nist.gov/metrics/.

Egg yolk protein LC-MS/MS studies. Fresh chicken egg yolk was evaporated *in vacuo* and 1 mg samples were stirred in 100 µl of 6 M urea, 0.1 M Tris buffer, pH 8, for 2 hr at room temperature. Cysteines were reduced and alkylated with 1 mmol dithiothreitol (1 hr) followed by addition of 4 mmol iodoacetamide for 1 hr at room temperature. Excess iodoacetamide was reacted with excess dithiothreitol (4 mmol) for 1 hr. The sample was then diluted with water to a total volume of 1 ml, mixed with 20 µg Promega sequencing grade modified trypsin, and stirred at 37 °C for 18 hr. After digestion, the solution was acidified with 20 µl of 50% formic acid. The digest was divided into 3 samples. One was refrigerated for 2 weeks and analyzed (sample 1). A second aliquot was frozen for two weeks, thawed and then analyzed (sample 2). The third was evaporated to dryness *in vacuo* and redissolved in water at the same concentration, frozen for 2 weeks, and then thawed and analyzed (sample 3).

Five technical replicate analyses of each sample were done by LC-MS/MS using a LC-Packings Ultimate 3000 HPLC (Dionex Corp., Sunnyvale, CA) coupled to a Thermo LTQ linear ion trap mass spectrometer (Thermo Scientific, Waltham, MA). Samples (2 µl) were

injected onto a Dionex C_{18} Acclaim PepMap 300 column (300 μm ID, 15 cm long) and eluted with a gradient of water (A)/acetonitrile (B), each containing 0.1 % formic acid as follows: 0 to 40 min – 0% to 50% B, 40 to 45 min – 50% to 95% B, 45 to 48 min – 95% B, 48 to 50 min – 95% to 0% B, 50 to 60 min – 0% B. The flow rate was 4 μl/min. The eluent passed through a 15 μm silica tip (New Objective, Woburn, MA) and was sprayed into the LTQ mass spectrometer. Each MS scan was followed by 8 MS/MS spectra of the 8 most intense peaks, taken in reverse order. A dynamic exclusion time of 20s was used with a list size of 500. The collision energy was set to 35%.

Yeast lysate sample loading studies. An aliquot of the tryptic digest of the CPTAC yeast reference material (see **Supplementary Methods**) was redissolved in 0.1% formic acid in water at a concentration of 1 μg/μl. Solutions at 400 ng/μl, 40 ng/μl, 4 ng/μl, and 400 pg/μl were made by serial dilution and were analyzed by LC-MS/MS using a nanoLC-2D LC pump (Eksigent Technologies, Dublin, CA) coupled to a Thermo LTQ mass spectrometer (Thermo Scientific, Waltham, MA). For each series of LC-MS/MS runs, samples were analyzed back-to-back from low to high concentration. Blank gradient runs were done between each series. Samples were loaded onto an Atlantis dC18 trap column at 4.5 μl/min (Waters Corp., Milford, MA) and eluted onto a 100 μm x 10 cm BioBasic C18 IntegraFrit column (New Objective, Woburn, MA) connected to a 20 μm SilicaTip with a 10 μm tip (New Objective, Woburn, MA). Peptides were separated at 450 nl/min with the following gradient: 2-30.5% B over 90 min., 30.5-90% B over 10 min., 90% B for 2 min., 90-2% B over 3 min., and 2% B for 10 min. (A= 0.1% formic acid in water and B= 0.1% formic acid in acetonitrile). Except for the LC analysis described above, all other instrument settings and

parameters were according to the CPTAC Network Study 6 SOP (see **Supplementary Methods**).

Additional methods. Complete descriptions of the CPTAC yeast protein materials, their preparation and digestion and of the SOPs for CPTAC Network studies 5 and 6 described here are presented in **Supplementary Methods.**

Results

Overview of performance metrics

We developed 46 performance metrics, which map to LC-MS/MS system components as shown in Figure 1. A list annotated with brief descriptions of each metric is provided in Table 1; in the text, we refer to them by category and code. The metrics map to functional system components including the liquid chromatography system, the MS instrument (electrospray source, MS1 and MS2 signal intensities), the MS instrument control system (dynamic sampling) and the data analysis system (peptide identification outputs after database searching).

The metrics were first derived empirically through examination of run-to-run or lab-to-lab differences in datasets. In some cases, characteristics of the datasets suggested relevant metrics. For example, it was noted that on one LC-MS/MS system, early eluting peptides were absent. Based on this observation, an algorithm was written to calculate the median peptide elution rank order differences in the early part of the chromatogram. Further logical examination of variability in the chromatography led to a focus on quantification of the observed variation and thus to the related chromatography metrics. In another example, average precursor *m/z* values for identified peptides were calculated for each run because the data were easily accessible. Differences between instruments for this metric were later attributed to differences in tuning protocols. Variations of this iterative "observe, quantify, evaluate, refine" approach have yielded over 100 metrics, of which 46 are reported here. Further development of performance metrics is a continuing focus of our work (at NIST).

Evaluation of variability in replicate analyses of similar samples

We initially evaluated the behavior of the metrics in replicate analyses of three similar samples on a single LC-MS/MS system. An egg yolk protein extract was digested with trypsin and stored refrigerated for two weeks (preparation A, 6 LC-MS/MS replicates), or frozen for two weeks, thawed and analyzed (preparation B, 6 replicates) or evaporated *in vacuo*, redissolved at the same concentration, stored frozen for two weeks, thawed and analyzed (preparation C, 5 runs). The replicates were each analyzed on a Thermo LTQ linear ion trap mass spectrometer. The median intraseries (replicates within a preparation) and interseries (between preparations) deviations are given in Table 2. The %dev values represent the median absolute difference between all pairs of measurements expressed as a percentage of the median value. The average %dev within replicates for each preparation was less than 2% under these conditions and less than 3% across all runs for the three preparations. As expected, variation was greater on average between preparations A, B and C than between replicate analyses. For example, the variability between preparations in the number of tryptic peptide counts (P-2A) is nearly 3 times greater than the average intraseries variability. This reflects the expected behavior of this metric, in which modest differences between the three preparations should give rise to varying peptide identifications.

The average ratio of interseries to intraseries %dev in Table 2 was 1.29, which indicates that the metrics vary slightly more due to modest sample differences than due to variation between replicate analyses. Thus, the results in Table 2 indicate that the

metrics are stable across analytical replicates and provide estimates of normal ranges for the metrics for typical laboratory handling.

Monitoring system changes in response to controlled operational variation

To evaluate the response of the metrics to controlled experimental variation, we analyzed a tryptic digest of the NCI-CPTAC yeast proteome reference material in triplicate at 10 sample loads ranging from 1.6 to 6,000 ng. Patterns of variation of the metrics with sample load illustrate their response characteristics (Figure 4). Tryptic peptide identification metrics P-2A, P-2B and P-2C all increased with load, but increases were marginal at loads above 100 ng (Figure 4a). This was paralleled by increases in MS1 signal intensity parameters MS1-2B and MS1-3B together with a concomitant drop-off in ion injection time (MS1-1) (Figure 4e), and an increase in median S/N for identified MS1 (MS1-2A) and MS2 spectra (MS2-2) for identified peptides (Figure 4f). These metrics quantitatively illustrate the well-known relationship between higher sample load, greater MS1 and MS2 signal intensities, higher spectral quality and greater numbers of successful database matches. The increased variation in the metric values at lower sample loads is also presumably due to the diminishing concentration of the analytes. The fraction of identified MS2 spectra in each of the 4 MS1 quartiles also increased with sample loading up to 100ng (MS2-4A through MS2-4D) (Figure 4f), as expected. Metrics of ion source performance indicated that, although the median precursor *m/z* for identified peptides (IS-2) increased only slightly with sample load, the number of +1 species identified relative to +2 species (IS-3A) increased sharply at

loads above 160 ng (Figure 4c). This may reflect limited availability of protons at the source at higher analyte concentrations.

Some of the chromatography metrics exhibited striking dependence on sample load (Figure 4b). Most notably, C-2B (peptide identification rate) increased only at lower loading concentrations, whereas the "bleed" metric C-1A increased across the entire range. This latter metric was not accompanied by corresponding changes in peak width metrics (C-3A and C-3B), which suggests that C-1A simply reflects load, rather than loss of column performance. This is consistent with the observation that peptide identifications increased across the load range, albeit more slowly at the highest concentrations.

These data demonstrate that the metrics correctly represent well-understood relationships among system components and demonstrate sensitivity to "real world" variables (e.g., sample load). In this example, the practical inference drawn from the combined metrics is that system performance improves dramatically with incremental sample loading increases below 160 ng, as reflected collectively by identifications, chromatography and MS signal intensities, whereas improvement at higher loads is not as significant. This illustrates the value of the metrics for rational, quantitative optimization of system performance.

System variability in interlaboratory studies

The CPTAC interlaboratory studies (CPTAC studies 5 and 6) involved analyses of a common yeast proteome reference sample on multiple Thermo LTQ and LTQ-Orbitrap instruments in several laboratories. The yeast extract was digested with trypsin and

aliquots were distributed for analysis. Although the participating laboratories used different LC systems and autosamplers with these MS instruments, they also employed a standard operating procedure (SOP), which standardized sample loading, chromatography, MS instrument tuning and dynamic sampling (see Supplementary Material).

As part of Study 6, three replicate analyses of the yeast digest were analyzed on four different LTQ-Orbitrap systems in three different laboratories (Figure 5). Inspection of the peptide identifications summary (Figure 5a) indicates approximately a 40% reduction in the number of peptide identifications by LTQ-Orbitrap @86 compared to the other 3 Orbitraps. Inspection of the other metrics indicates that dynamic oversampling parameters DS-1A and DS-1B were lower for LTQ-Orbitrap@86 than for the others (Figure 5d), indicating excessive repeat sampling of peptide ion signals for MS/MS. This would have the observed effect of lowering peptide identifications. Another major characteristic of underperformance of instrument '@86' is the reduced value for ratios of +3/+2 charge states (IS-3B) and +4/+2 charge states (IS-3C) for identified peptides (Figure 5c). (No +4 peptide ions were identified for this instrument.) This may indicate a shift in the distribution of all peptide ion charge states and may have led to an increased fraction of +1 peptide ions, which were excluded from MS/MS in the Orbitraps. Compliance with SOP dynamic exclusion settings was verified by inspection of the datafile headers. However, further investigation identified inadequate formic acid content in the LC mobile phase as the likely cause, which is consistent with both a decreased proportion of higher charge state ions (IS-3B and IS-3C) and increased oversampling (fall in the value of DS-1A) .

The Study 6 data also identified performance metrics whose variation had little or no impact on peptide identifications, including the MS1 and MS2 signal intensity metrics (Figures 5c and 5e) for the case of 'Orbitrap@86'. Also noteworthy is the modest dip in peptide identifications in the second run for instrument LTQ-OrbitrapP@65 (Figure 5a). This occurred together with evidence of electrospray instability as indicated by 10 fold jumps or drops in MS1 signal intensity (IS-1A and IS-1B, Figure 5c) in adjacent full scans. Manual inspection of the corresponding datafile revealed a periodic "sawtooth" profile for the base peak chromatogram, indicating electrospray instability.

Another noteworthy aspect of the Study 6 data is the high degree of reproducibility of the chromatography metrics across instruments. Although the four Orbitraps used three different combinations of LC and autosampler systems, the SOP specified stationary phase, column measurements, flow rates and injection parameters. Peptide elution periods, peak widths and chromatographic bleed were generally consistent across instruments and replicate analyses, which illustrates the feasibility of SOP-driven studies of LC-MS platforms, even when different hardware components may be employed.

In CPTAC Study 5, the yeast digest was analyzed in six replicates on 3 LTQ and 3 Orbitrap instruments in 5 laboratories. The metric C-6A, which detects differences in the relative numbers of early and late eluting peptides with and between labs, identified large differences within replicates and between labs for 'LTQ2@95' (Figure 6a). These differences were reflected in increased values for chromatographic bleed metrics C-1A and C-1B and decreased peptide identification rate (C-2B) (Figure 6b) and lowered

numbers of peptide identifications (Figure 6c) in the 4th and 5th runs in the series. This enabled identification of wash solvent cross-contamination of the sample injection loop as a probable cause. Correction of the problem partially restored the metrics and peptide identifications to values comparable to the other systems (see 'LTQ2@95-rep', Figure 6).

Performance of metrics across systems and laboratories

Whereas Table 2 described variation in the metrics for a single instrument in replicate analyses, the CPTAC interlaboratory studies provided a means to evaluate variability across multiple laboratories, instruments and analyses. Figure 7A displays average intralab %dev values for the metrics for all 6 instruments (3 LTQs and 3 Orbitraps) in CPTAC Study 5 (6 replicate yeast digest analyses). The metrics have been sorted within categories from lowest to highest and the error bars represent the %dev of the intralab %dev (i.e., they estimate the range of %devs across the labs). The plotted size of the colored bar represents variation within a laboratory; the relative size of the accompanying error bar indicates variation (%dev) of the intralab %dev across laboratories. These values as displayed are not intended to represent interlab variability, which would require comparing measurements between instrument classes directly, but to approximate the variability of the intralab %dev values. For most of the metrics, these values are comparable and average less than 10%. The most highly variable metrics describe MS1 signal intensity, dynamic sampling and chromatographic bleed. Indeed, this latter metric was the most variable both within and between labs, also consistent with the egg yolk studies presented in Table 1.

This comparison can be extended to interlab values for the LTQs and Orbitraps (Figures 7b and 7c). Whereas Figure 6A depicts the variation within labs, Figures 7b and 7c reveal values that are most irregular between labs. The main finding is that interlaboratory variation in peptide identifications and most other performance metrics are comparable between the LTQ and Orbitrap systems, even though there are large differences in the average values for some metrics between them (not shown). Differences in these mass analyzers result in different MS1 signal intensities, but this also results in greater variation in both MS1 and dynamic sampling metrics for the Orbitraps (Figure 7b vs. Figure 7c). This could also be reflected by the fact that one of the Orbitraps outperformed the others in the study by more than 20% in the number of unique tryptic peptide identifications for this Study (data not shown). Nevertheless, variations in MS2 metrics appear comparable for LTQs and Orbitraps.

Figures 7a-c also provide a broad perspective on which analytical techniques pose the greatest challenges to intralaboratory and interlaboratory standardization. For example, the metrics with the highest variation describe peptide chromatography (e.g., C-1A and C-1B), which is subject to more variable influences than any other component of the LC-MS/MS system. Even modest variations in mobile phase composition, gradient delivery, flow control stability, sample contaminants and the composition of previous samples can influence peptide elution. We note that the relative variability of C-1A and C-1B corresponds to typical experience in chromatography, where peak tailing (C-1B) is a more common problem than peak bleed or “fronting” (C-1A) .

Discussion

Troubleshooting poor system performance in LC-MS/MS-based proteomics typically involves a combination of experience, intuition and a highly subjective evaluation of limited data (e.g., "what does the chromatogram look like?"). Although causes of commonly encountered system problems are well-known and can be rationalized in retrospect, the combinatorial possibilities of malfunction among multiple components may preclude a systematic approach to diagnosis. The 46 performance metrics described here enable the implementation of a quantitative, integrated approach to system troubleshooting. These metrics enable diagnosis of poor system performance, such as the ~40% decline in peptide identifications by one Orbitrap compared to others in an interlaboratory study (Figure 5).

However, performance metrics can help diagnose much more subtle, yet important, problems. These metrics provide the first effective means to deal with modest decrements in system performance, which are frequently encountered, yet difficult to diagnose. The metrics are quite sensitive to small changes in performance—most have %dev values of less than 10%. The sensitivity of these metrics enabled detection of electrospray instability as a contributing cause of modestly diminished peptide identification performance in one replicate analysis of a yeast proteome digest in CPTAC Study 6 (LTQ-OrbitrapP@65 in Figure 5).

A relatively large number of metrics (46 presented here) could be considered excessive for purposes of troubleshooting system performance. However, the availability of over 40 metrics does not imply that all metrics are always used together for diagnostic purposes. Indeed, this would probably always be unnecessary. In the

examples we present here, specific system malfunctions are indicated by changes in smaller subsets of metrics. On the other hand, the advantage of the relatively large number of metrics is that they reflect diverse components of the system.

A complete record of system performance should become a critical element of quality control documentation for LC-MS datasets. This requirement for documentation of system performance is important in many applications in proteomics, particularly where analysis and comparison of LC-MS datasets provides the basis for identifying distinct characteristics of biological systems. Apparent differences between phenotypes are detected by comparing datasets from multiple technical replicate analyses of the corresponding samples. A key assumption underlying this approach is that observed differences represent true proteomic differences rather than variability in system performance. This is particularly important when biological differences between samples are relatively modest, as analyses must be able to discern differences comprising a small subset of proteome components. The metrics we describe here could provide an unambiguous basis for quantitatively defining platform stability and could enable idenfication of outlier data that would otherwise confound biological comparisons.

We also have shown here in the context of the CPTAC interlaboratory studies that these metrics display stability in behavior across multiple laboratories and systems. These observations not only further define the utility and normal ranges of the metrics, but provide insights into the aspects of multi-laboratory studies that provide the greatest barriers to platform standardization. However, the implementation of an SOP in CPTAC Study 6 effectively normalized key features of the chromatography (Figure

5c)—a remarkable achievement in view of the use of different LC systems by the participating laboratories. We note that SOPs were employed in the CPTAC studies to enable comparisons under conditions where key system variables were held constant, thus enabling identification of sources of variability in peptide and protein detection. The SOPs represented a balance between performance optimization for peptide detection and practical considerations for interlaboratory studies. They do not represent fully optimized methods and are not intended as prescriptive for the proteomics research community.

This work on performance metrics was done with Thermo LTQ and LTQ-Orbitrap instruments, which are commonly used for LC-MS/MS proteomics and were the principal instruments available in our laboratories (at NIST) and in the participating CPTAC laboratories. Although a few of the metrics we describe (e.g., ion injection times) are not applicable to other instruments, such as quadrupole-time of flight instruments, most of these metrics can be applied to any electrospray LC-MS/MS instrument platform used for proteomics. Extension of the metrics to these other systems will require software to extract data from instrument datafiles and reference datasets to define the behavior of individual metrics in different instrument platforms.

The metrics described here represent a subset of a larger body of measurements explored in our studies of LC-MS systems. Although these metrics were applied to data-dependent LC-MS/MS analyses, subsets of these metrics and variations thereof could be applied similarly to high resolution LC-MS "MS1 profiling" systems or to LC-multiple reaction monitoring-MS on triple quadrupole and quadrupole-ion trap instruments. Indeed, many of the metrics are applicable to the analysis of LC-MS systems for non-

peptide analytes, including metabolites, lipids, carbohydrates and other molecule classes. Implementation of these performance metrics will be facilitated by the distribution of software for extracting the metrics directly from raw data files and by development of graphical user interfaces and integration with standard proteome analysis workflows. We are continuing development and evaluation of new performance metrics and will make available software to facilitate their implementation in the analytical community.

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Table 1. Descriptions of metrics.

Code	Category	Metric Group	Metric	Units	Optimal	Purpose/Use	Description
C-1A	Chromatography	Fraction of Repeat Peptide IDs with Divergent RT	-4 min	fraction	↓	Estimates very early peak broadening	Fraction of all peptides identified at least 4 min. earlier than max MS1 for ID
C-1B	Chromatography	Fraction of Repeat Peptide IDs with Divergent RT	+ 4 min	fraction	↓	Estimates very late peak broadening	Fraction of all peptides identified at least 4 min. later than max MS1 for ID
C-2A	Chromatography	Interquartile Retention Time Period	Period (min)	min.	↑	Longer times indicate better chromatographic separation	Time period over which 50% of peptides were identified.
C-2B	Chromatography	Interquartile Retention Time Period	Pep ID Rate	peps/min.	↑	Higher rates indicate efficient sampling and identification	Rate of peptide identification during C-2A
C-3A	Chromatography	Peak Width at Half Height for IDs	Median Value	s	↓	Sharper peak widths indicate better chromatographic resolution	Median peak widths for all identified unique peptides (s)
C-3B	Chromatography	Peak Width at Half Height for IDs	Interquartile Distance	s	↓	Tighter distributions indicate more peak width uniformity	Measure of the distribution of the peak widths - small values indicate consistency
C-4A	Chromatography	Peak Widths at Half Max over RT deciles for IDs	First Decile	s	↓	Estimates peak widths at the beginning of the gradient	Median peak width for identified peptides in last RT decile (late)
C-4B	Chromatography	Peak Widths at Half Max over RT deciles for IDs	Last Decile	s	↓	Estimates peak widths at the end of the gradient	Median peak width for identified peptides in first RT decile (early)
C-4C	Chromatography	Peak Widths at Half Max over RT deciles for IDs	Median Value	s	↓	Estimates peak widths in the middle of the gradient	Median peak width for identified peptides in median RT decile (middle)

Code	Category	Metric Group	Metric	Units	Optimal	Purpose/Use	Description
C-5A	Chromatography	Average Elution Order Differences*	Between	percent	↓	Estimates peptide elution similarity run-to-run	Average elution rank order difference for identified peptides between series
C-5B	Chromatography	Average Elution Order Differences*	Betw/In	ratio	↓	Estimates peptide elution similarity between series	Ratio of average rank order difference between series to average rank order difference within a series (low values indicate similarity between series)
C-6A	Chromatography	Fraction of Extra Early Eluting Peptides in Row Series (- = Fewer)*	Between	fraction	↓	Used to detect differences in the numbers of early peptides	Estimates relative frequency of early eluting peptides
C-6B	Chromatography	Fraction of Extra Late Eluting Peptides in Row Series (- = Fewer)*	Between	fraction	↓	Used to detect differences in the numbers of late peptides	Estimates relative frequency of late eluting peptides
DS-1A	Dynamic Sampling	Ratios of Peptide Ions IDed by Different Numbers of Spectra	Once/Twice	ratio	↑	Estimates oversampling	Ratio of peptides identified by 1 spectrum divided by number identified by 2 spectra
DS1-B	Dynamic Sampling	Ratios of Peptide Ions IDed by Different Numbers of Spectra	Twice/Thrice	ratio	↑	Estimates oversampling	Ratio of peptides identified by 2 spectra divided by number identified by 3 spectra
DS-2A	Dynamic Sampling	Spectrum Counts	MS1 Scans/Full	count	↓	Fewer MS1 scans indicates more sampling	Number of MS1 scans taken over C-2A
DS-2B	Dynamic Sampling	Spectrum Counts	MS2 Scans	count	↑	More MS2 scans indicates more sampling	Number of MS2 scans taken over C-2A

Code	Category	Metric Group	Metric	Units	Optimal	Purpose/Use	Description
DS-3A	Dynamic Sampling	MS1max/MS1sampled Abundance Ratio IDs	Median All IDs	ratio	↓	Estimates position on peak where sampled for peptides of all abundances	Ratio of MS1 maximum to MS1 value at sampling for median decile of peptides by MS1 maximum intensity (1=sampled at peak maxima)
DS-3B	Dynamic Sampling	MS1max/MS1sampled Abundance Ratio IDs	Med Bottom 1/2	ratio	↓	Estimates position on peak where sampled for least abundant 50% of peptides	Ratio of MS1 maximum to MS1 value at sampling for bottom 50% of peptides by MS1 maximum intensity (1=sampled at peak maxima)
IS-1A	Ion Source	MS1 During Middle (and Early) Peptide Retention Period	MS1 Jumps >10x	count	↓	Flags ESI instability	Number of times where MS1 signal greatly decreased between adjacent scans more than 10-fold (electrospray instability)
IS-1B	Ion Source	MS1 During Middle (and Early) Peptide Retention Period	MS1 Falls >10x	count	↓	Flags ESI instability	Number of times where MS1 signal greatly increased between adjacent scans more than 10-fold (electrospray instability)
IS-2	Ion Source	Precursor m/z for IDs	Median	Th	↓	Higher median m/z can correlate with inefficient or partial ionization	Median m/z value for all identified peptides (unique ions)
IS-3A	Ion Source	IDs by Charge State (Relative to +2)	Charge +1	ratio	↓	High ratios of +1/+2 peptides may indicate inefficient ionization	Number of +1 peptides over +2 peptides
IS-3B	Ion Source	IDs by Charge State (Relative to +2)	Charge +3	ratio	↓	Higher ratios of +3/+2 peptides may preferential	Number of +3 peptides over +2 peptides

Code	Category	Metric Group	Metric	Units	Optimal	Purpose/Use	Description
						favor longer peptides	
IS-3C	Ion Source	IDs by Charge State (Relative to +2)	Charge +4	ratio	↓	Higher ratios of +4/+2 peptides may preferential favor longer peptides	Number of +4 peptides over +2 peptides
MS1-1	MS1 Signal	Ion Injection Times for IDs	MS1 Median	ms	↓	Lower times indicates an abundance of ions	MS1 ion injection time
MS1-2A	MS1 Signal	MS1 During Middle (and Early) Peptide Retention Period	S/N Median	none	↑	Higher MS1 S/N may correlate with higher signal discrimination	Median signal-to-noise value (ratio of maximum to median peak height) for MS1 spectra up to and including C-2A
MS1-2B	MS1 Signal	MS1 During Middle (and Early) Peptide Retention Period	TIC Median	counts/1,000	↑	Estimates the total absolute signal for peptides (may vary significantly between instruments	Median TIC value for identified peptides over same time period as used for MS1-2A
MS1-3A	MS1 Signal	MS1 ID Max	95/5 Pctile	ratio	↑	Estimates the dynamic range of the peptide signals	Ratio of 95th over 5th percentile MS1 maximum intensity values for identified peptides (approximates dynamic range of signal)
MS1-3B	MS1 Signal	MS1 ID Max	Median	counts	↑	Estimates the median MS1 signal for peptides	Median maximum MS1 value for identified peptides
MS1-4A	MS1 Signal	MS1 Intensity Variation for Peptides*	Within Series	percent	↓	Used to monitor relative intensity differences with a series	Average of between series intensity variations for identified peptides

Code	Category	Metric Group	Metric	Units	Optimal	Purpose/Use	Description
MS1-4B	MS1 Signal	MS1 Intensity Variation for Peptides*	Betw/In	ratio	↓	Used to monitor relative intensity differences with a series compared to between series	Ratio of average intensity variation between series to average intensity variation within a series (low values indicate similarity between series)
MS1-5A	MS1 Signal	Precursor m/z - Peptide Ion m/z	Median	Th	↓	Measures the accuracy of the identifications	Median real value of precursor errors
MS1-5B	MS1 Signal	Precursor m/z - Peptide Ion m/z	Mean Absolute	Th	↓	Measures the accuracy of the identifications	Mean of the absolute precursor errors
MS1-5C	MS1 Signal	Precursor m/z - Peptide Ion m/z	ppm Median	ppm	↓	Measures the accuracy of the identifications	Median real value of precursor errors in ppm
MS1-5D	MS1 Signal	Precursor m/z - Peptide Ion m/z	ppm InterQ	ppm	↓	Measures the distribution of the real accuracy measurements	Interquartile distance in ppm of the precursor errors
MS2-1	MS2 Signal	Ion Injection Times for IDs	MS2 Median	ms	↓		MS2 ion injection time
MS2-2	MS2 Signal	MS2 ID S/N	Median	ratio	↑	Higher S/N correlates with increased frequency of peptide identification	Median S/N (ratio of maximum to median peak height) for identified MS2 spectra
MS2-3	MS2 Signal	MS2 ID Peaks	Median	count	↑	Higher peak counts can correlate with more signal	Median number of peaks in an MS2 scan
MS2-4A	MS2 Signal	Fraction of MS2 Identified at Different MS1max	ID Fract Q1	fraction	↑	Higher fractions of identified MS2 spectra	Fraction of total MS2 scans identified in the first quartile

Code	Category	Metric Group	Metric	Units	Optimal	Purpose/Use	Description
		Quartiles				indicate efficiency of detection and sampling	of peptides sorted by MS1 maximum intensity
MS2-4B	MS2 Signal	Fraction of MS2 Identified at Different MS1max Quartiles	ID fract Q2	fraction	↑	Higher fractions of identified MS2 spectra indicate efficiency of detection and sampling	Fraction of total MS2 scans identified in the second quartile of peptides sorted by MS1 maximum intensity
MS2-4C	MS2 Signal	Fraction of MS2 Identified at Different MS1max Quartiles	ID Fract Q3	fraction	↑	Higher fractions of identified MS2 spectra indicate efficiency of detection and sampling	Fraction of total MS2 scans identified in the third quartile of peptides sorted by MS1 maximum intensity
MS2-4D	MS2 Signal	Fraction of MS2 Identified at Different MS1max Quartiles	ID Fract Q4	fraction	↑	Higher fractions of identified MS2 spectra indicate efficiency of detection and sampling	Fraction of total MS2 scans identified in the last quartile of peptides sorted by MS1 maximum intensity
P-1	Peptide Identification	MS2 ID Score	Median	fval	↑	Higher scores correlate with higher S/N and frequency of identification	Median peptide identification score for all peptides - higher scores generally correlate with increased MS2 S/N
P-2A	Peptide Identification	Tryptic Peptide Counts	Identifications	count	↑	Total identifications correlate with high levels of peptide signals - performance	Number of MS2 spectra identifying tryptic peptide ions (total "spectral counts")
P-2B	Peptide Identification	Tryptic Peptide Counts	Ions	count	↑	A good overall performance measure	Number of tryptic peptide ions identified - ions differing by charge state and/or modification state are counted separately

Code	Category	Metric Group	Metric	Units	Optimal	Purpose/Use	Description
P-2C	Peptide Identification	Tryptic Peptide Counts	Peptides	count	↑	A good overall performance measure	Number of unique tryptic peptide sequences identified
P-3	Peptide Identification	Peptide Counts	Semi/Tryp Peptides	ratio		Indicates prevalence of semi tryptic peptides in sample. Increasing ratios may indicate changes in sample or in source	Ratio of semi/full tryptic peptide IDs

* Composite metrics that use values calculated over more than 1 series

Table 2. Variation of metrics in replicate analyses of egg yolk protein digest.

Metric	Avg. value	Avg. intraseries %dev	%Dev of intraseries %dev	Interseries %dev	Ratio avg. intra/inter
C-1A ('bleed' -4 min.)	0.0082	34.88	3.9	42.87	1.23
C-1B ('bleed' +4 min.)	0.1499	4.84	3.98	0.63	0.13
C-2A (IQ pep. RT period)	11.56	2.43	1.4	0.3	0.12
C-2B (peptides/min.)	41.9	2.59	1.21	0.79	0.31
C-3A (med. peak width)	11.34	0.35	1.92	0.14	0.41
C-3B (IQ for peak widths)	2.19	3.04	1.59	3.2	1.05
DS-1A (oversampling - once/twice)	3.31	4.35	2.52	3.11	0.71
DS-1B (oversampling - twice/thrice)	2.51	7.24	1.88	0.25	0.03
DS-2A (MS1 Scans over C-2A)	345.3	1.06	7.63	2.53	2.39
DS-2B (MS2 Scans over C-2A)	1899	2.39	1.45	0.66	0.27
DS-3A (med. MS1max/MS1sampled all IDs)	2.48	2.44	2.49	2.01	0.82
DS-3B (med. MS1max/MS1sampled for bottom 50% by abund.)	1.44	1.45	1.41	1.08	0.75
IS-1A (MS1 >10X jumps)	0	0	0	0	0
IS-1B (MS1 >10X falls)	0	0	0	0	0
IS-2 (med. precursor m/z)	689.61	0.32	2.03	0.52	1.66
IS-3A (ratio IDs +1/+2)	0.263	1.65	1.98	4.98	3.02
IS-3B (ratio IDs +3/+2)	0.393	1.29	1.91	4.18	3.24
IS-3C (ratio IDs +4/+2)	0.105	1.94	1.73	6.46	3.33
MS1-1 (ion injection (ms) for IDs)	5.6	3.17	0	13.86	4.36

Metric	Avg. value	Avg. intraseries %dev	%Dev of intraseries %dev	Interseries %dev	Ratio avg. intra/inter
MS1-2A (S/N)	317.8	1.77	1.42	6.6	3.73
MS1-2B (med. TIC/1e3 over C-2A)	5470.6	1.76	11.98	15.31	8.68
MS1-3A (dynamic range 95th/5th for IDs)	87.2	5.21	1.28	6.16	1.18
MS1-3B (med. MS1 signal for IDs)	66265	2	2.37	13.33	6.66
MS2-1 (Ion injection (ms) for IDs)	100	0	0	0	0
MS2-2 (S/N for IDs)	220.5	1.44	5.15	2.04	1.41
MS2-3 (med. num peaks for IDs)	314.6	1.84	1.61	2.46	1.34
MS2-4A (fract. ID'd Q1)	0.748	1.62	1.26	1.85	1.15
MS2-4B (fract. ID'd Q2)	0.585	1.7	1.15	2.2	1.29
MS2-4C (fract. ID'd Q3)	0.453	1.48	1.28	3.52	2.37
MS2-4D (fract. ID'd Q4)	0.368	3.79	1.54	6.17	1.63
P-1 (med. f-value score for IDs)	0.891	0.24	3.91	0.38	1.54
P-2A (total IDs)	2767.7	1.11	2.6	3.01	2.71
P-2B (unique ion IDs)	1260.5	0.59	1.7	1.6	2.71
P-2C (unique peptide IDs)	859.5	0.55	1.85	0.87	1.59
P-3 (semi/full tryptic peps)	0.13	3.33	1.42	3.28	0.99
MEDIAN VALUES		***1.76***	***1.7***	***2.2***	***1.29***

Figure Legends

Figure 1. Schematic representation of performance metrics mapped to LC-MS/MS system elements.

Figure 2. Illustration of chromatography metric C-2A applied to LC-MS/MS data from three Thermo LTQ systems in analyses of yeast proteome samples in CPTAC Study 5. Time intervals for elution of the middle quartiles of peptide identifications (C-2A) are indicated, as are values for C-2B (peptide identification rate during this interval) and total peptide identifications during the analysis (P-2C). See text for discussion.

Figure 3. Schematic representation of software pipeline to generate metrics. See text for discussion.

Figure 4. Stability and variation of metrics over a range of sample injection amounts. Serial dilutions of a tryptic digest of the CPTAC yeast reference proteome were analyzed in triplicate by LC-MS/MS on an LTQ instrument. Median values for each series were plotted according to the categories in Figure 1. Error bars represent ± the median error. Some values have been scaled as indicated in the panel legends.

Figure 5. Performance metrics for triplicate analyses of a tryptic digest of the CPTAC yeast reference proteome on 4 LTQ-Orbitraps at 3 different sites in CPTAC Study 6. Instruments labeled "@56, @86 and @65O" are LTQ-Orbitraps; the instrument labeled "@65P" is an LTQ-XL-Orbitrap. Panels (**a-f**) display metrics according to category; values for each of the 3 runs is represented by a symbol. Low values for peptide identifications for instrument LTQ-Orbitrap@86 (panel **a**) coincide with low metric values for peptide ion charge states

(metric IS-3B, panel **c**) and dynamic sampling (metric DS-1A, panel **d**) (see text for discussion).

Figure 6. Performance metrics for six replicate analyses of a tryptic digest of the yeast reference proteome on LTQ and Orbitrap instruments in CPTAC Study 5. Instruments labeled "LTQ@73, LTQ@65 and LTQ@95" are LTQ instruments; the instrument labeled "Orbi@56" is a LTQ-Orbitrap; the instrument labeled "Orbi@65" is an LTQ-XL-Orbitrap. Marked variations in the relative number of early and late eluting peptides (panel **a**), chromatography metrics (panel **b**, middle section) and identifications (panel **c**, middle section) for instrument LTQ2@95 led to diagnosis and resolution of the problem (see text for discussion), as indicated by a second set of analyses on this instrument (LTQ2@95-rep). Instrument LTQ@73 is included in both panels as representative of the performance of the other instruments. The Orbitraps were included in the analysis and in panel **a** as a source of diversity from different laboratories and to demonstrate the usefulness of the chromatographic metrics across instrument platforms.

Figure 7. Summary of intralaboratory and interlaboratory variation for metrics for 3 LTQ and 3 LTQ-Orbitrap instruments in 6 replicate analyses of a tryptic digest of the CPTAC yeast reference proteome in CPTAC Study 5. The instruments labeled "LTQ" are all LTQ model instruments; the instruments labeled "Orbis" include two LTQ-Orbitraps and one LTQ-XL-Orbitrap. Metrics are grouped by system category and ranked by code for comparison between panels. Intralaboratory variation in %dev for each metric and variation in %dev are shown in panel **a**. Panels **b** and **c** show interlaboratory variation in metrics for LTQ and Orbitrap instruments, respectively.

Figure 1

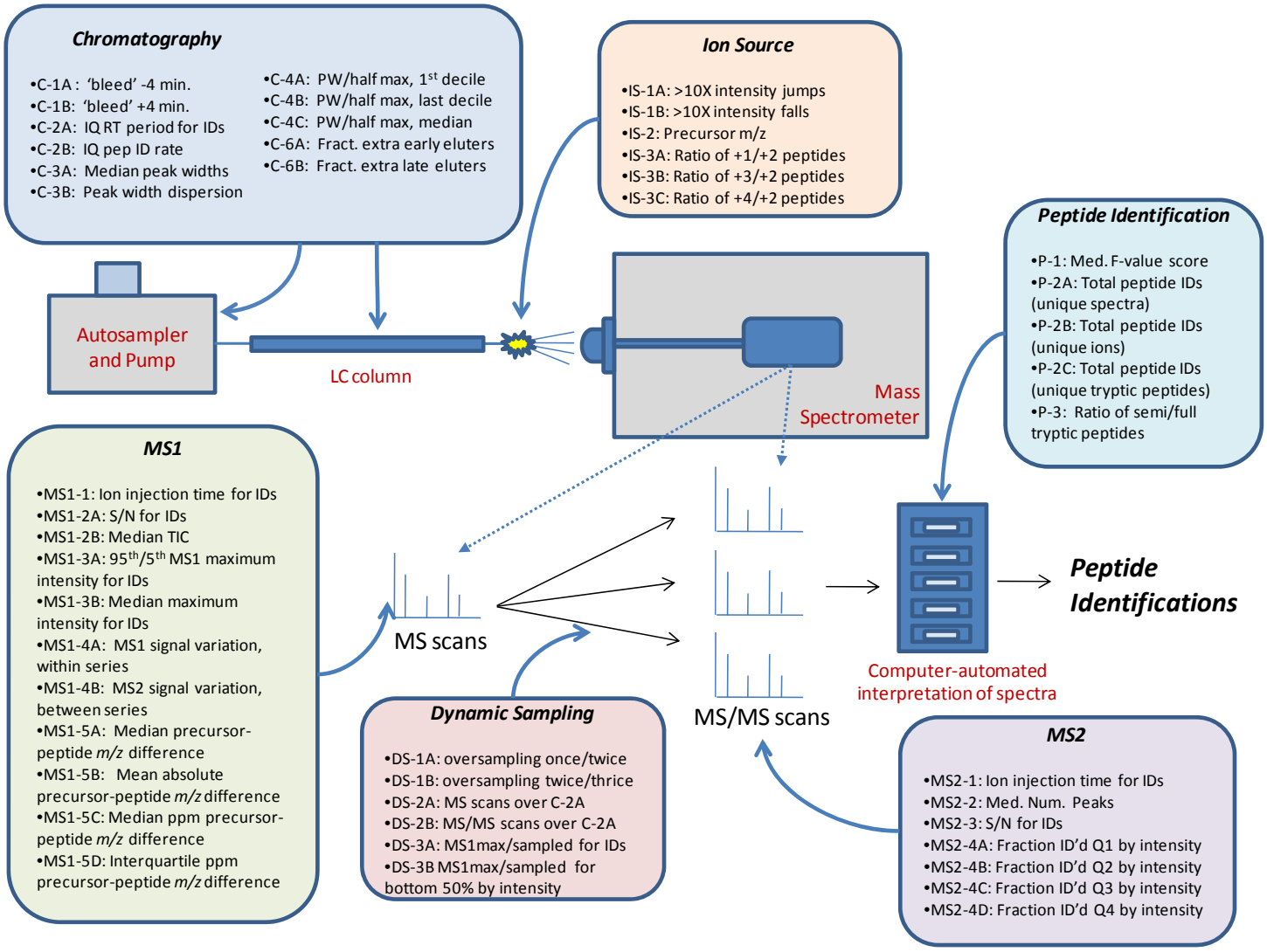
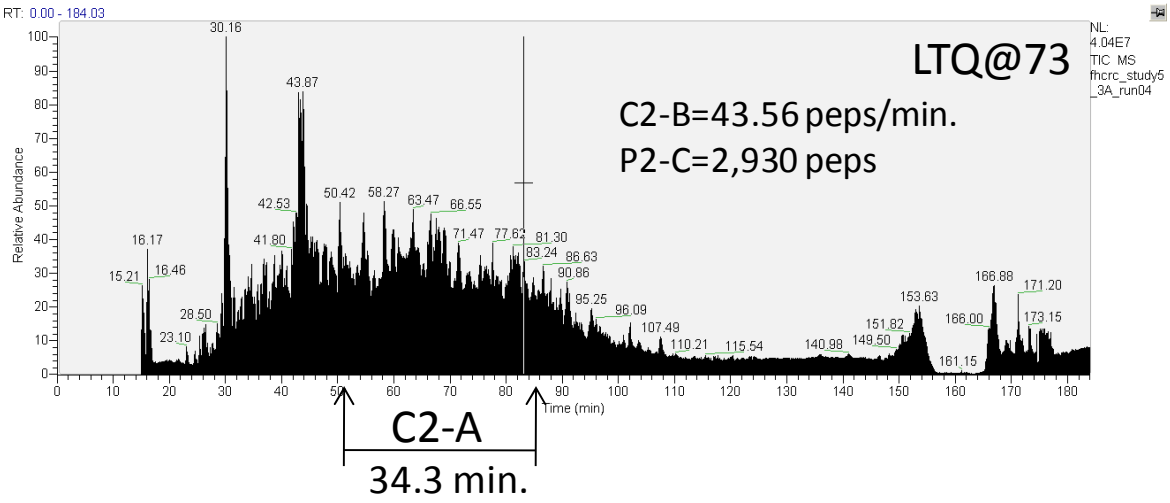
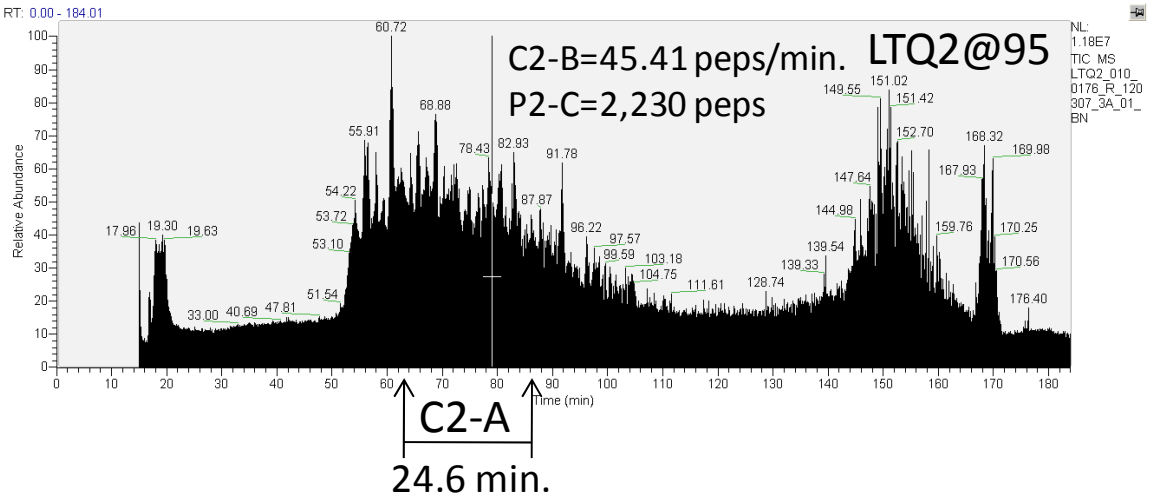


Figure 2





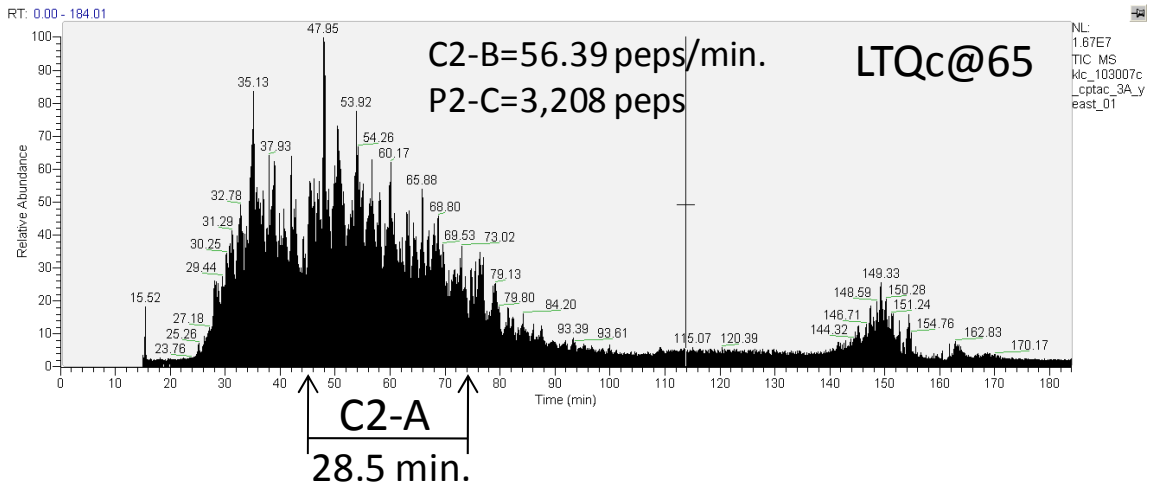


Figure 3

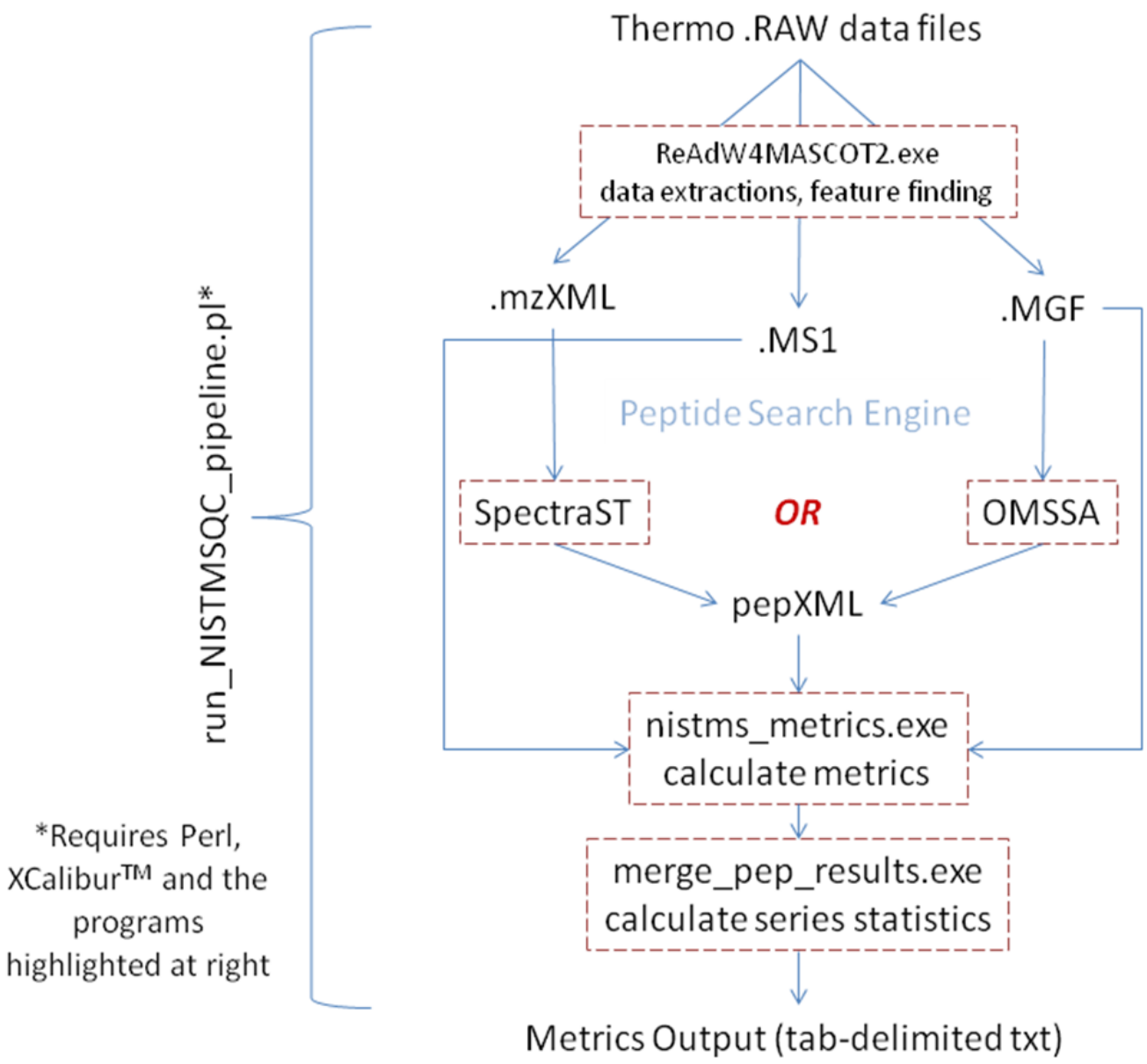


Figure 4

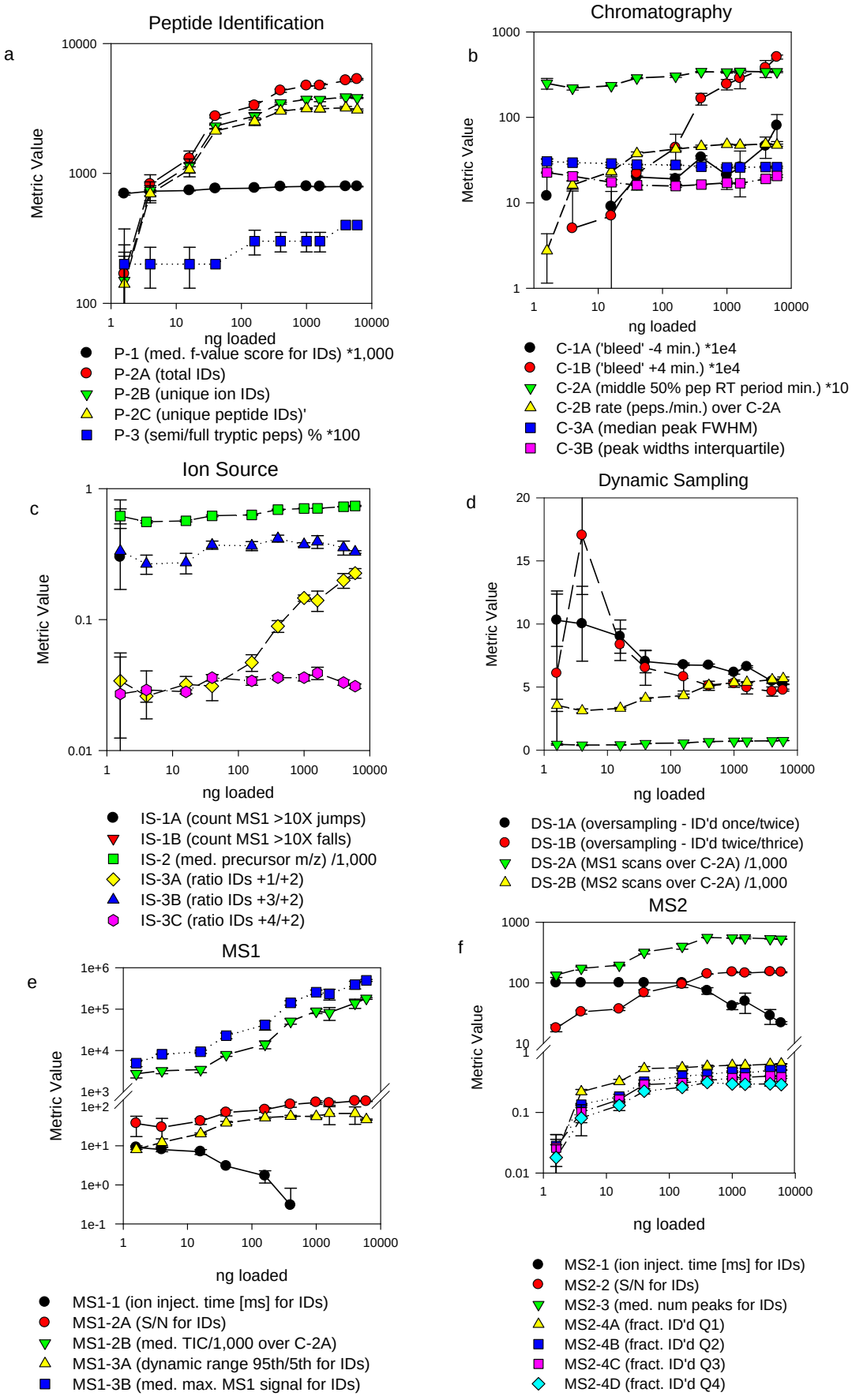


Figure 5

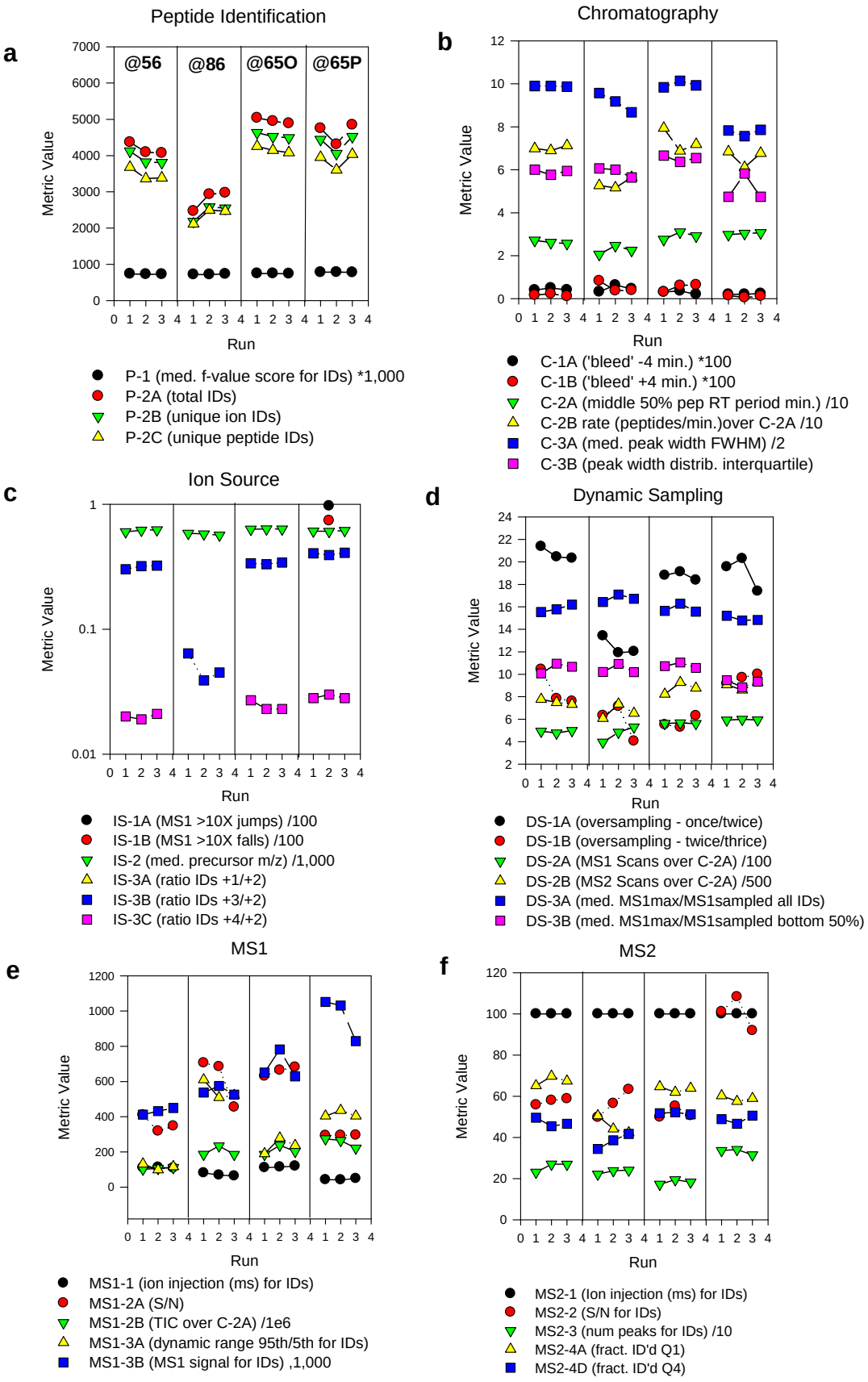


Figure 6

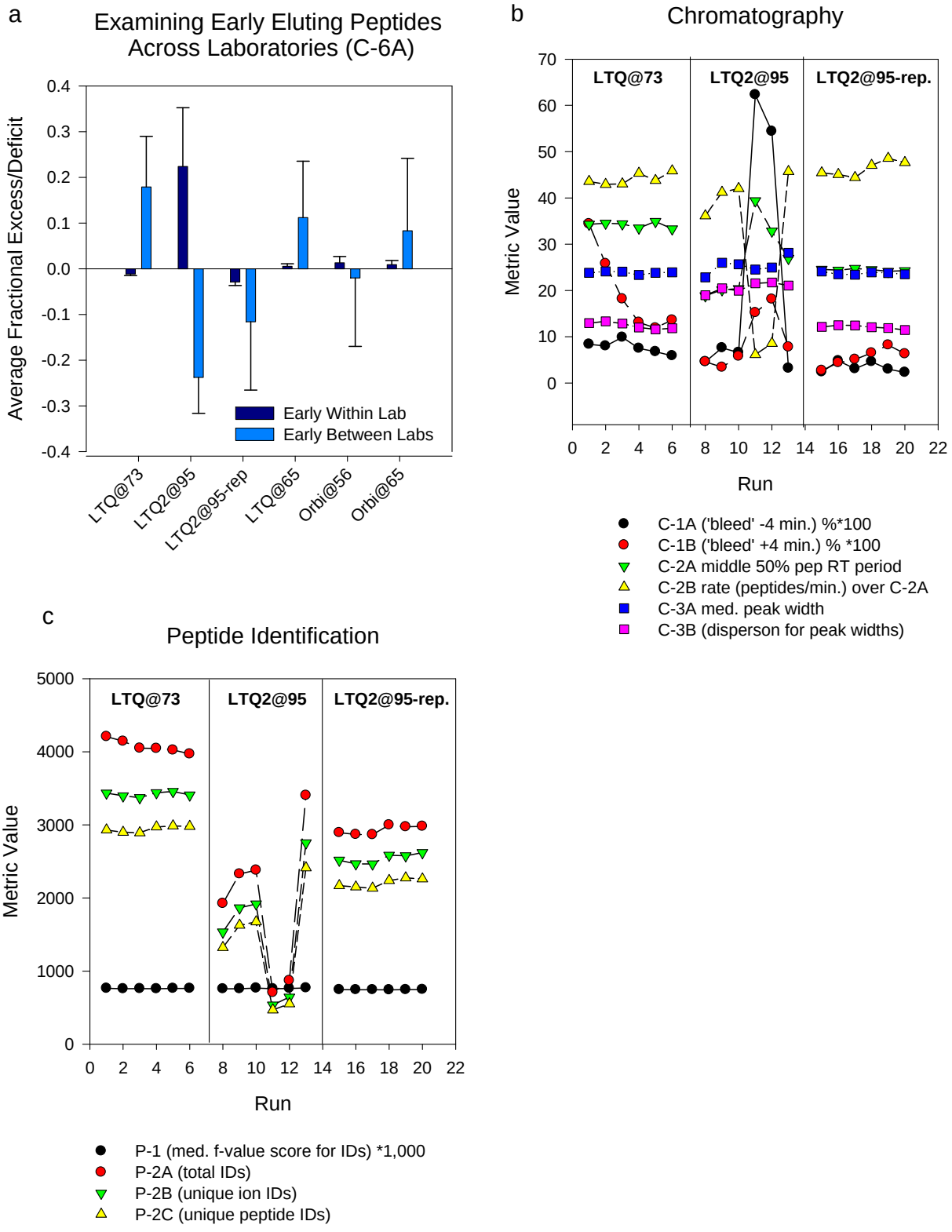


Figure 7