Developing system suitability criteria and evaluation methods for proteomics experiments

Daniela Tomazela, Jesse D. Canterbury, Andrew Stergachis, Gennifer Merrihew, Brendan MacLean, and Michael J. MacCoss

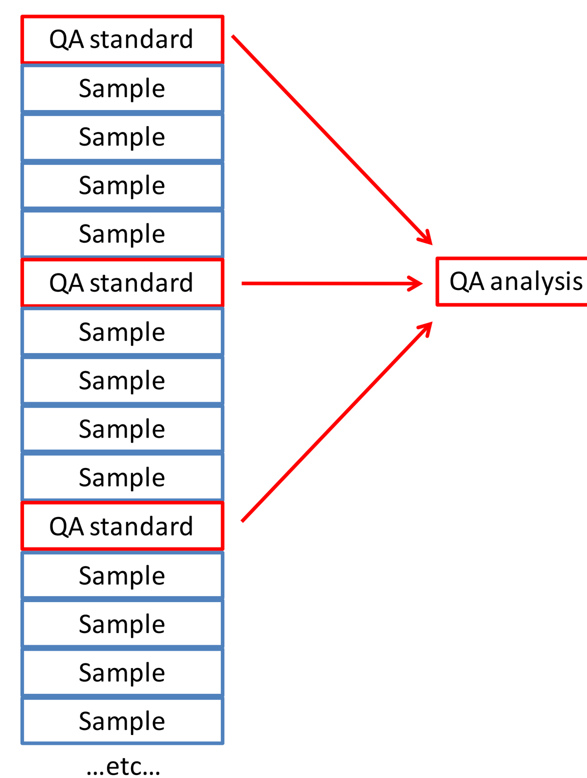
Department of Genome Sciences, University of Washington, Seattle, WA 98195-5065

Introduction & Overview

- One of the biggest challenges facing any proteomics analysis workflow based on LC-MS technology is controlling the quality of liquid chromatography.
- As proteomics technologies begin to be used in clinical settings, it will be useful for researchers in academic laboratories to be able to evaluate the reproducibility of their analyses in a systematic manner.
- Here we describe a method for assessing chromatography quality over the course of long proteomics experiments.
- Our method relies on a making targeted (SRM-style) measurements of retention time and peak area for a set of peptides from a standard mix of 6 proteins from *Bos taurus*. Measurements are made at regular intervals between measurements of laboratory samples.
- Retention time and peak area are measured using Skyline. Deviations of these measurements from the mean are evaluated, with the language of Westgard rules employed to quantify the deviation of individual measurements. Moving calculations of coefficient of variation (CV) and slope/mean are used to determine when a set of measurements is drifting far enough to warrant intervention.

Quality Assessment (QA) sample. A standard mix of typtically digested proteins from *Bos taurus* (Sigma) was used to perform quality assessments during a variety of proteomics experiments.

QA method. Several peptides were selected for monitoring using Skyline (Windows-based software developed in our lab) [1,2]. SRM-style instrument methods were exported from Skyline for different instruments. QA experiments were performed at regular intervals during normal laboratory experiments, as shown at right.

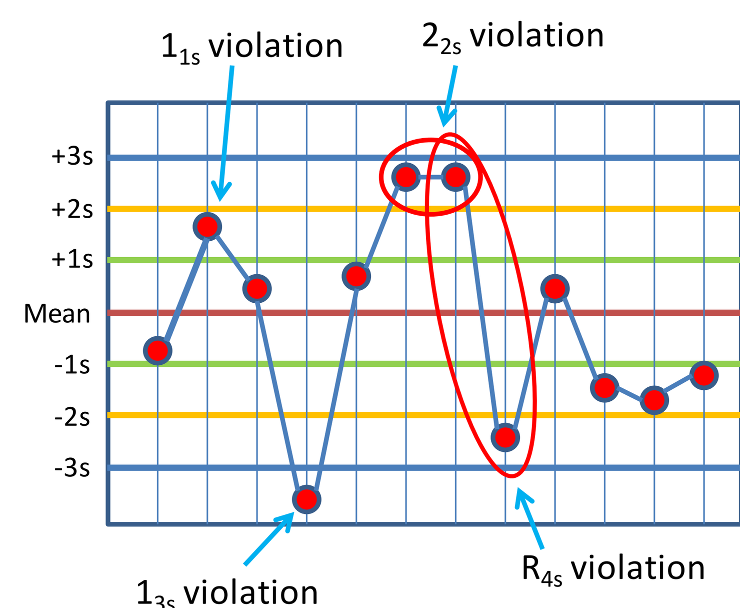


Liquid chromatography. Fused silica liquid chromatography columns of inner diameter 75 µm were packed in-house with C18 reverse-phase material (Phenomenex), and pulled to a ~5 µm diameter tip. Peptides were loaded on to the column and eluted using a linear gradient starting with 95% Buffer A (5% acetonitrile, 0.1% formic acid) and 5% Buffer B (80% acetonitrile, 0.1% formic acid) and progressing to 35% Buffer B over a period 40 min, with a final 5-minute wash of 80% Buffer B, using one of three nanoflow LC systems: Agilent 1100, Eksigent nanoLC, or Waters nanoAcquity. In the case of the Agilent 1100 system, nanoflow was obtained via a split-flow arrangement.

Mass spectrometry. Experiments were performed on three different mass spectrometers: TSQ Vantage, LTQ-FT-Ultra, and LTQ (Thermo Fisher Scientific). In all cases, QA experiments were run in SRM mode. Sample was delivered via an in-house electrospray source. Standard settings were used in all cases except where noted.

Westgard Rules

Westgard rules, named for James Westgard, are simple rules describing when deviations in a given measurement become too large or erratic to be trustworthy (i.e., "out of control"). Deviations of multiple standard deviations or multiple deviations of single standard deviations, among one measurement or a set of measurement, can all serve as useful rules for determining when a given experiment is out of control. Examples are shown below (after [2]).



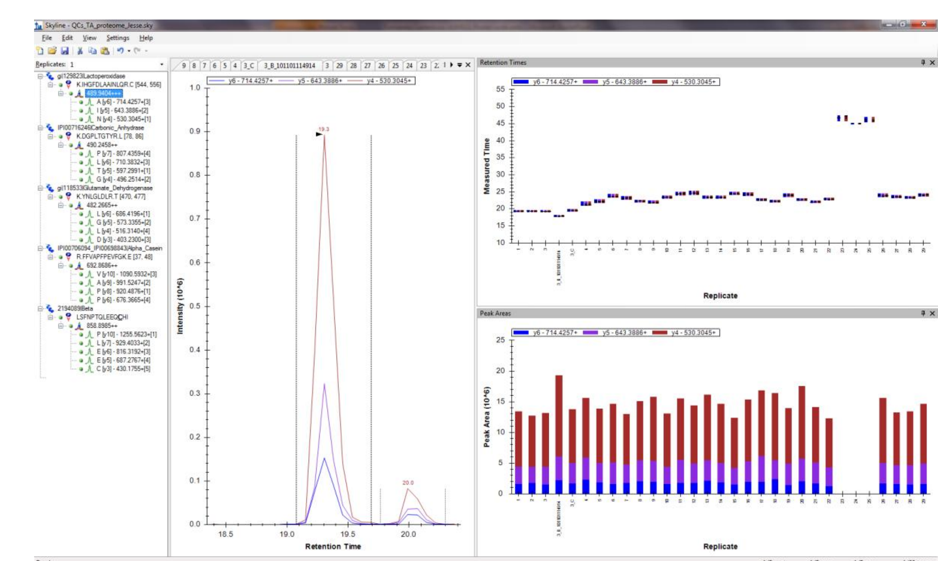
Case 1: Tracheal Aspirates

We have undertaken a study of human infant tracheal aspirate samples as part of research into respiratory distress syndrome (RDS) resulting from hypothesized lack of function of surfactant protein B in affected patients. This study involved collecting data from 150 samples comprised of biological and technical replicates of several cases and controls.

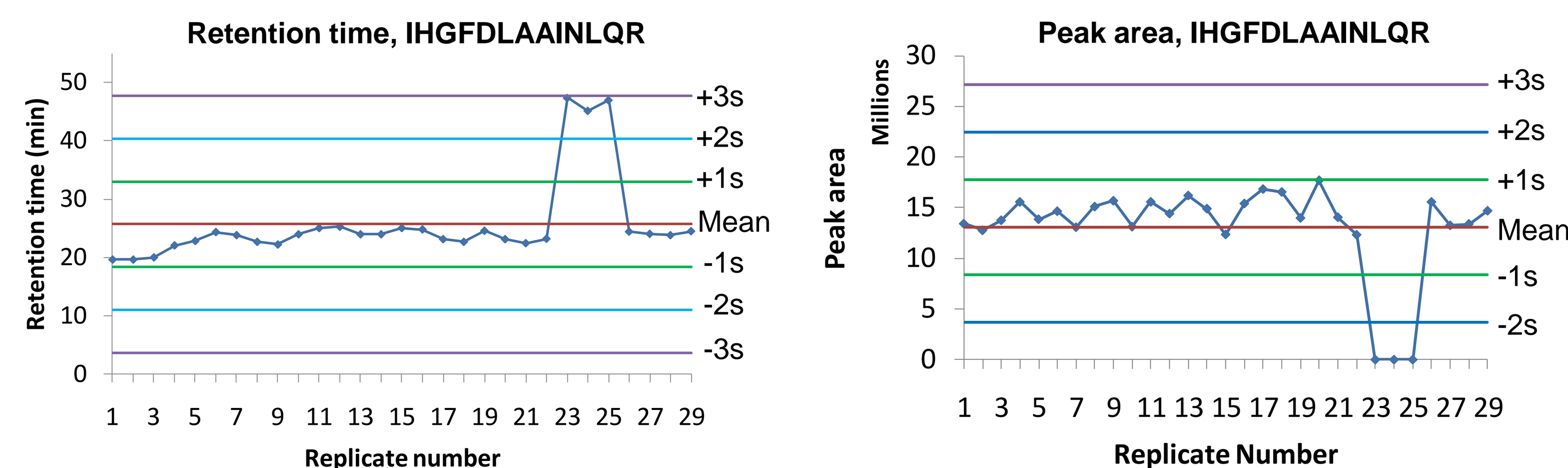
Sample. **Number of QC runs: 29.**

Liquid chromatography. Same conditions as in the Introduction; used Water nanoAcquity UPLC system.

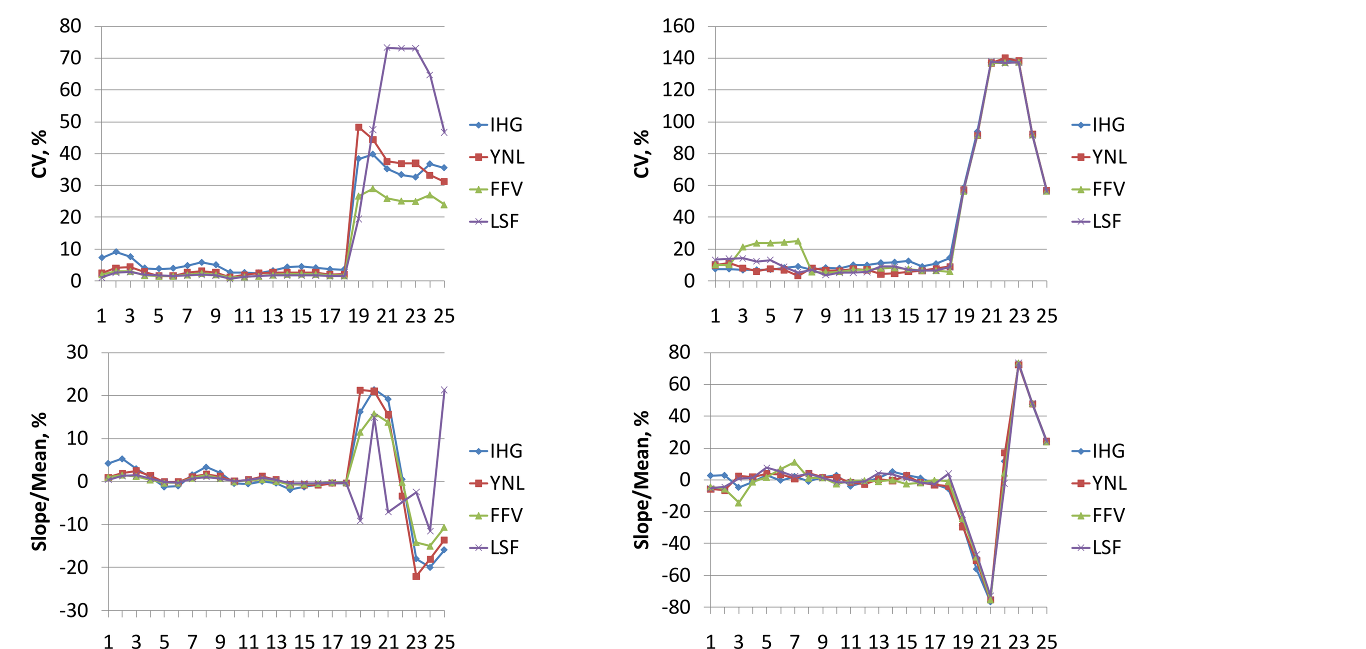
Mass spectrometry. Peptides were mass-analyzed using an LTQ-FT-Ultra mass spectrometer. Peptides from the tracheal aspirate sample were analyzed using a familiar data-dependent scheme. Peptides from the QC sample were analyzed every 5 runs.



Screenshot of Skyline method and analysis. Peptides and transitions are in the far left panel, with a peptide peak displayed in the middle panel. Right two panels show comparison across replicates of retention times (top) and peak areas (bottom) for the highlighted peptide.



Above are shown retention time (left) and peak area (right) data exported into Excel for further analysis. Lines are drawn at 1, 2, and 3 standard deviations from the mean to evaluate application of appropriate Westgard rules. Retention time shows 3 consecutive deviations of +2 standard deviations, while peak areas show 3 consecutive -2 standard deviations (note that peak areas are at zero for those runs).



Above are shown coefficient of variation (CV) (top) and slope/mean (bottom) vs run number for four monitored peptides, computed in a running average of five runs at a time. All graphs show steep increases when the +/-2 standard deviation points come into the calculation. Peak area CVs are more sensitive to variation, since peak area is more variable in general than retention times.

What was the problem? The helium supply to the mass spectrometer was exhausted, which prevented the fragmentation of peptides. Hence no MS/MS spectra could be collected; however MS data was still seen. This problem was solved by reconnecting the helium supply, after which retention times & peak areas normalized, as shown.

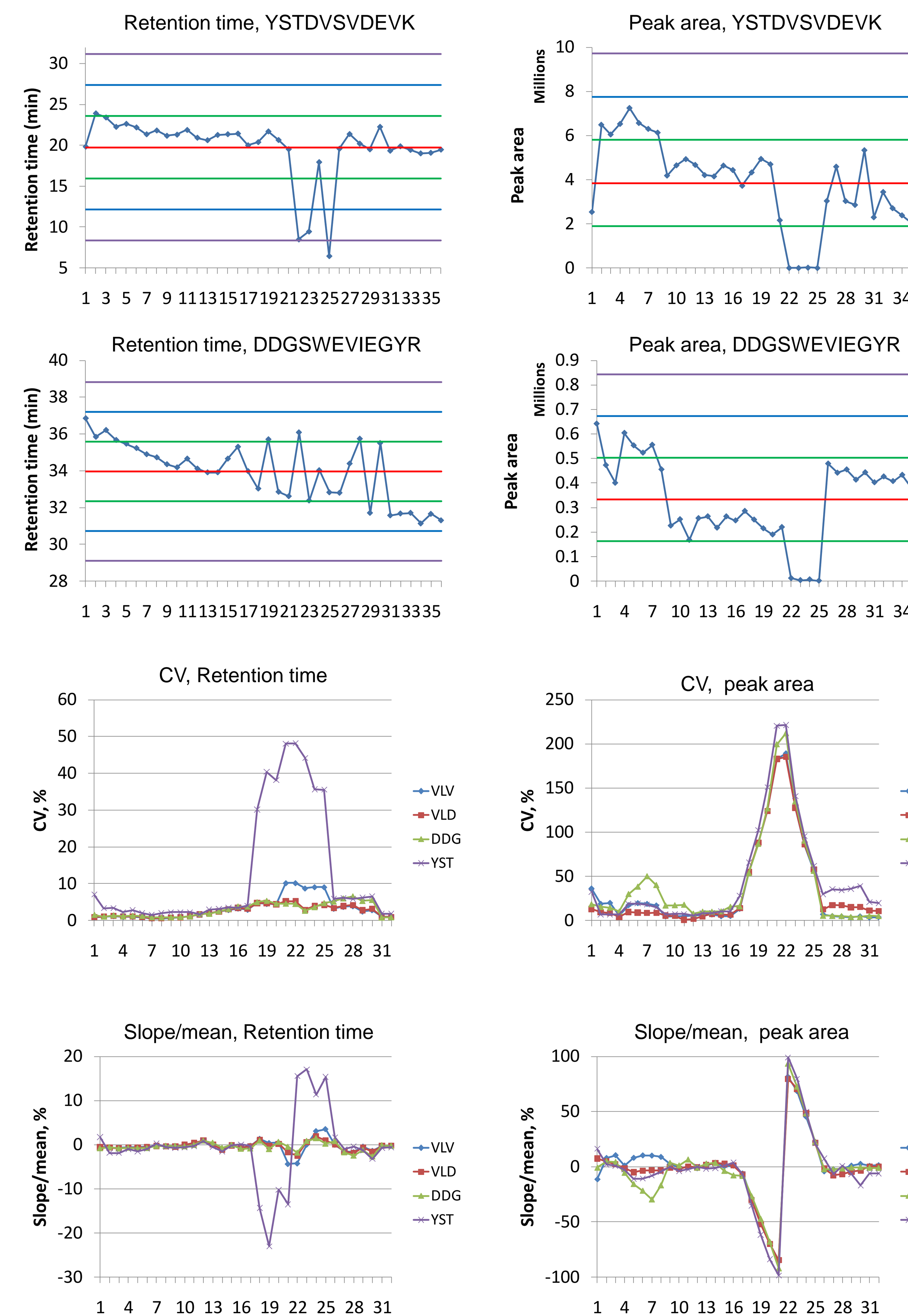
Case 2: System troubleshooting

In this case we were testing laboratory equipment, in particular an aging but functional Agilent 1100 binary pump and autosampler system. The stator on the autosampler injection valve was replaced. Data below are from

Sample. Only the QC sample was used in this study. **Number of QC runs: 36.**

Liquid chromatography. Same conditions as in the Introduction; used Agilent 1100 system.

Mass spectrometry. Peptides were mass-analyzed using an LTQ mass spectrometer. Peptides from the QC sample were analyzed continuously.



These illustrate the importance of monitoring multiple aspects of multiple peptides. Retention times would probably be labeled "in control" for DDG (middle left) but "out of control" for YST (top left), whereas peak areas clearly show zero or near zero for three runs for both peptides (top & middle right). CV graphs of retention times (bottom left) & peak areas (bottom right) show this more clearly, especially the retention time CV graph, where the CV for YST is significantly worse than for the other peptides.

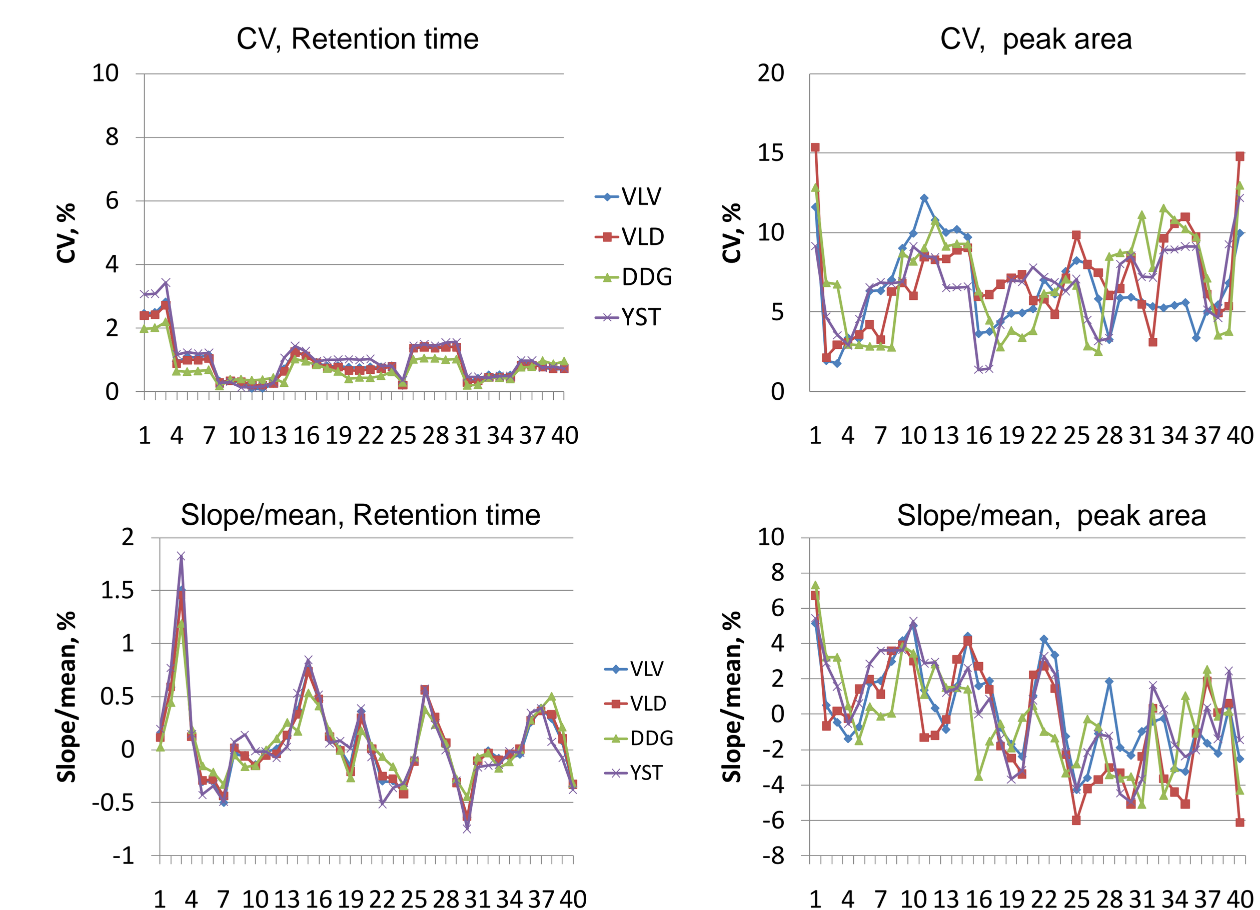
Case 3: In-vitro synthesized transcription factors

We sought to empirically identify 'signature' proteotypic peptides and their fragmentation patterns for 730 human transcription factors and transcriptional regulators. To do this, we generated these proteins *in vitro*, purified full-length forms of the protein, digested these samples with trypsin and monitored all tryptic peptides using a triple-quadrapole mass spectrometer (See WP518 for more details). These 730 protein samples were individually injected and quality-control samples were monitored every 8-12 injections.

Sample. **Number of QC runs: 44.**

Liquid chromatography. Same conditions as in the Introduction; used Agilent 1100 system.

Mass spectrometry. Peptides were mass-analyzed using an LTQ mass spectrometer. Peptides from the QC sample were analyzed continuously.



Here we do not display any raw retention time or peak area data. Based on data displayed in other panels, we can be reasonably sure that CVs below 10% virtually ensure no excursions outside +/- 1s. Slope/mean of retention time data tell a similar story: there are no broad trends in retention time drift, certainly not more than 2%. The more sensitive peak area data show more variation, as in other panels, but overall the CVs are low. While the slope/mean graph does show a broadly positive trend in peak area drift in the first half of injections and broadly negative in the second half, the percent-change is still less than 10%.

Over this large number of sample injections, then, monitored peptides showed good reproducibility. Data collected on experimental samples (not the QA sample) were deemed good and useful.

Conclusions

- Method developed for analyzing a standard set of digested proteins in SRM mode on a variety of instruments, at regular intervals during runs of experimental samples
- Used language of Westgard rules to describe "bad" measurements in terms of numbers of standard deviations away from a mean value
- Explored using running calculations of coefficient of variation (CV) and slope/mean of both retention time and peak area as a way to evaluate system suitability and to assess chromatographic quality on runs in progress.
- Future work will include automating this process so that runs in progress will be stopped when CV and slope/mean deviate from specified limits.

References

[1] Maclean B, Tomazela DM, Abbatiello SE, Zhang S, Whiteaker JR, Paulovich AG, Carr SA, Maccoss MJ., (2010) *Anal Chem.* **82**(24):10116-24.
[2] MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ. (2010), *Bioinformatics* **26** (7): 966-968.
[3] http://www.westgard.com/westgard-rules-and-multirules.htm.

Acknowledgements

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