Comparison of Non-labeling Quantitative Proteomics Techniques Using Ion Trap and QTOF Mass Spectrometers

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Abstract

Introduction: Relative quantitation of proteins within complex samples is an increasingly important aspect of proteomics. Two common non-labeling workflows are often cited: ion trap or QTOF mass spectrometry combined with ion intensity or spectral counting versus comparison of peptide peak height intensities. The purpose of this study was to compare these two commonly utilized quantitative methodologies using a moderately complex mixture of known peptides with spike-ins. Importantly, the comparison was made using a QTOF or ion trap with identical front end components and methods.

A commercially available, standard 48-protein mixture was trypsinized and C18 cleaned and then several proteins spiked in at two- and four-fold molar concentrations. Samples were analyzed using identical HPLC parameters, including a chip cube source on either an Ion Trap (n=3) or QTOF mass spectrometer (n=5). The exact same chip column was used on both instruments, rendering the front end portion of the analyses identical. Ion trap data were quantitated using total peptide precursor ion intensity or peak height intensity in a database searching program. Peptides were analyzed in MS mode on the QTOF and differentially abundant mass features were determined using Genespring MS. Targeted MSMS analyses of differentially regulated features was used to assign protein identifications using the QTOF.

Results: In a relatively rapid (40 minute) single MS/MS run on either instrument, 40 of the 48 proteins are identified with high confidence (high peptide scores and at least 2 peptides per protein) and 4 proteins by a single, high-quality peptide. In a comparison of non-spiked versus 4x-spiked all 5 spike proteins, but no non-spike proteins, were significantly different ($P < 0.05$). Comparisons between other samples (0 vs 2x and 2x vs 4x) showed that the significance of the differences observed appears to be correlated with the number of peptides detected. In general, there appeared to be a correlation between a high number of peptides detected and low standard deviations for ion trap data. Two-fold differences were detected in 4 out of 5 proteins in the ion trap. In order to determine the limitations of quantitating high abundant proteins, we spiked serotransferrin at 1.5x concentration, which was also detected by the ion trap. The results underscore the importance of running technical replicates in either system.

Introduction

Both labeling and non-labeling workflows are currently being utilized in quantitative proteomics and several instruments are available for each workflow. In spite of increasing literature on non-labeling strategies (1-3), there has never been, to our knowledge a direct comparison of the two mass spectrometers typically used for these analyses, namely the ion trap and the QTOF. While the ion trap is generally known for high speed and excellent sensitivity, this comes at a cost of poor mass accuracy and high variance. Conversely, conventional wisdom holds that the QTOF offers high mass accuracy but longer cycle times and poorer sensitivity. The issue of variance/reproducibility of QTOF instruments has recently been challenged with the introduction of the time-to-digital converter to replace the analog-to-digital converter.

In order to directly compare the virtues of the QTOF vs ion trap, it would be necessary to duplicate the front end, HPLC portion of the analyses. This could be challenging with conventional systems since, in order to be identical, the same column and parameters would have to used on both systems. This technical hurdle was overcome in the current study through utilization of an HPLC chip system on both the QTOF and the ion trap whereby the exact same columns could be used on both systems.

Many proof-of-principle studies utilize either a very small set of proteins or a complex biological matrix. In these experiments, we utilized a moderately complex mix of 48 proteins that had been lyophilized and were present in equimolar ratios. Five of the 48 proteins were spiked into the 48-mix digest at 1.5, - 2-, and/or 4-fold molar concentrations.

Table 1: Identity and concentrations of spike-in proteins. These proteins were present in the commercial 48-mix and were digested separately then spiked in to the digested 48-mix proteins.

Protein	Spike (fold)
Serotransferrin	1.5 x
Ubiquitin	2x and 4x
Lactalbumin	2x and 4x
Quinone	2x and 4x
C-reactive protein	2x and 4x

Methods

•Sample Preparation: A standard, lyophilized mix of 48 proteins (Sigma) was resuspended in 8M urea and digested according to common methods using trypsin. Resulting peptide mixtures were cleaned with either C18 tips (Agilent Technologies) or spin columns (Pierce). Five proteins were digested separately and spiked in at 1.5-fold, 2-fold, or 4-fold higher molar concentrations.

•Instrumentation: Peptides were analyzed via LC/MS/MS on an Agilent QTOF (model 6510) or Agilent ETD ion trap (model 6340) mass spectrometer with an HPLC-chip interface. All HPLC components were Agilent 1200. Buffer A of the nanopump was comprised of HPLC grade water and 0.1% formic acid, buffer B was 90% acetonitrile, 10% HPLC water, and 0.1% formic acid. The loading pump utilized 3% acetonitrile, 97% HPLC grade water and 0.1% formic acid. A so-called "long" HPLC chip (Agilent) was used which consisted of a 40 nL enrichment column and a 75 um x 150 mm analytical column combined on a single chip. Gradient and instrument parameters are shown in Tables 2 and 3. A total of 200 fmol of each protein was injected onto the ion trap (n=3) or QTOF (n=5).

•Ion trap data were searched using Spectrum Mill software and both the SwissProt human database and an in-house database containing sequence of the 48 proteins. Protein identifications from ion trap data were subsequently exported into Excel. Data were normalized using either average intensity per run or using the intensity of a single "housekeeping" protein. Averages, standard deviations, and variance (%CV) were determined for each identified protein using either total intensity or total number of spectra for all matched peptides. Data were analyzed using one-way t-tests and fold-change was determined.

•For QTOF experiments, raw data was extracted using Mass Hunter software (Agilent Technologies) with the following parameters: absolute threshold = 5,000, peptide-like isotope abundance profile required, assigned charge states mazimum of 5. Data are then imported as MHD files into GenespringMS (Agilent). Mass and retention time alignments are performed, and features are then filtered in the following order: present in at least 4/5 replicates of each sample, peak intensity variance (CV 15%), analysis of variance (ANOVA) $p < 0.01$, and Fold change >1.5-fold. Final peak lists are used to generate an inclusion list, which is then imported into the QTOF acquisition software and used for targeted MSMS acquisition. Data are validated by cross-referencing the exported mass and retention time list with actual peptide fragmentation (MS/MS) data.

Time (min)	%B
0	3
1	3
50	45
54	45
58	80
65	80
65.1	3
70	3

Table 2: Gradient conditions for analytical (nano) pump on both QTOF and ion trap systems. %B = percent buffer B (90:10 ACN:H2O, 0.1%FA) used.

Table 3: Instrument parameters.

Parameter	QTOF MS only	QTOF MS/MS	Ion trap MS/MS
Voltage	1800	1800	1750
Drying gas	4.0 L/min	4.0 L/min	5.0 L/min
Temperature	300	300	350
m/z range	295-3000	MS: 295-3000 MSMS: 50-1800	300-1800
Precursor ions	N/A	5	4
Active exclusion	N/A	Yes	Yes
Scan rate	1 spectra/ second	MS: 8 spec/sec MSMS: 3 spec/sec	Ultra (26,000 m/z per sec)

Results

•A comparison of total (TIC) and extracted (EIC) ion chromatograms from replicate ion trap and QTOF runs shows that the HPLC portion of the analysis is highly reproducible within an instrument (Figure 1) and nearly identical between instruments (not shown), with a consistent difference in retention time between instruments of 2 minutes. This is in spite of calibrating the HPLC pumps prior to analysis.

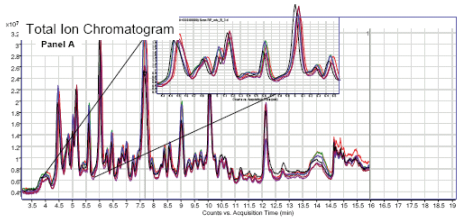


Figure 1: Total Ion Chromatograms (TIC) of 5 replicate QTOF runs overlaid. The inset shows a 2 minutes region magnified.

•A comparison of the total number of proteins identified shows that the ion trap workflow matches 322 distinct peptides matching to 41 proteins with high confidence when 200 fmol of each protein is processed. When quality single peptides are included 44 proteins are identified. When the QTOF is used in untargeted MS/MS mode, 223 distinct peptides are matched to 40 proteins with high confidence. The addition of single high quality peptides results in a total of 41 proteins identified.

•While all methods utilized to analyze ion trap data were at least partially successful, using average intensity per run for normalizing data, followed by the use of total intensity for quantifying data, generated the most accurate determination of differences (Table 4). Regardless which method of normalization was utilized, total intensity was more accurate than spectral counting at determining differences.

		p ≤0.05			FC ≥2 or ≤0.5			p < 0.5 AND FC ≥2 or ≤0.5		
		1 v 2x	2 v 4x	1 v 4x	1 v 2x	2 v 4x	1 v 4x	1 v 2x	2 v 4x	1 v 4x
H TI	Total	7	8	7	5	4	7	-	-	-
	Spike-in	100%	80%	100%	75%	80%	100%	75%	80%	100%
H SC	Total	6	8	6	6	6	7			
	Spike-in	50%	80%	100%	75%	60%	60%	50%	60%	60%
AvInt TI	Total	8	8	12	7	6	7			
	Spike-in	100%	80%	100%	100%	80%	100%	100%	80%	100%
AvInt SC	Total	5	7	6	5	7	5			
	Spike-in	50%	80%	100%	75%	60%	80%	50%	60%	80%

Table 4: Comparison of normalization and analysis methods for ion trap data. Data were normalized using either single housekeeping protein (H) or average intensity per run (AvInt) and analyzed using either total intensity (TI) of all peptides matching to a protein or spectral counting (SC). Total signifies total number of proteins that met that criterion. Spike-in indicates the percentage of five spike-in proteins that met that criterion.

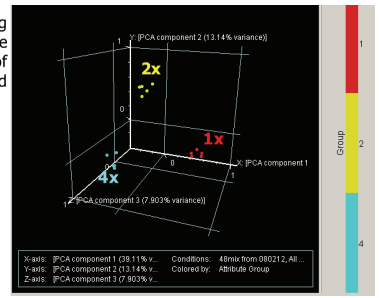
Table 5: Statistical analysis of ion trap data following normalization using AvInt and TI for quantitation. Highlighted boxes indicate significant/accurate differences.

	t-test			Fold Change		
	1v 2x	1 v 4x	2 v 4x	FD 1 v 2	FD 1 v 4	FD 2 v 4
Alpha Lactalbumin	0.01	0.01	0.018	1.42	2.31	1.63
C-reactive protein	0.01	0.003	0.111	3.33	4.24	1.27
Quinone	0.03	0.001	0.002	3.2	7.66	2.39
Ubiquitin	0.00002	0.01	0.014	3.88	9.14	2.36
	1 to 1	*1 to 1.5*	*1 to 1.5*	*1 to 1*	*1 to 1.5*	*1 to 1.5*
Serotransferrin	0.06	0.0003	0.00003	0.91	1.64	1.81

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Total Protein Spectral Intensity	Database Accession #	Protein Name
1	34	25	371.46	29	1.54e+007	39	Serotransferrin
2	10	4	62.11	45	1.07e+007	45	Ubiquitin with Nterm 6 HIS tag
3	5	3	37.89	14	1.18e+007	1	alpha-lactalbumin
4	3	3	34.42	9	2.00e+006	38	Ribosyldihydronicotinamide dehydrogenase [quinone]
5	3	2	24.28	7	2.78e+006	12	C-reactive protein
6	2	1	12.13	5	4.69e+006	35	Peroxiredoxin-1
Totals:	57	38					

Figure 2: Identification of differentially abundant proteins following QTOF mass spectrometry and subsequent GenespringMS analysis. Proteins 1-5 were spiked in at 1.5-, 2-, or 4-fold levels. Protein 6, peroxiredoxin, was correctly identified as being present, but was not a spike protein. Cross-validation with the targeted mass list eliminated peroxiredoxin from this list.

Figure 3: Principle component analysis (PCA) following filtering but prior to ANOVA. PCA is performed both before and after filtering in order to gauge the degree of clustering. Distinct clustering is an indication of good technical reproducibility.



Number of peptides		No. of CVs <20%			
	Proteins	Spectral counting		Total Intensity	
> 16	6	6	100%	6	100%
10 - 15	3	3	100%	3	100%
6 - 9	8	6	75%	6	75%
4 - 5	9	5	56%	5	56%
3	4	2	50%	2	50%
2	10	3	30%	3	30%
<2	4	0	0%	0	0%
Average	44	25	57%	25	57%

Table 6: Variance appears dependent on total number of peptides identified for each protein when the ion trap is used. The proportion of proteins with intensity values differing by <20% CV is less than 60% when fewer than 6 peptides are used to identify a protein. Note that, in the QTOF workflow, filtering results in only peptides with CV's <15% being quantitatively assessed.

Discussion

The most significant differences in terms of workflow are that the QTOF samples a greater number of peptides for use in subsequent quantitative analysis and that statistical analysis is performed before the proteins are identified. Conversely, the ion trap, although faster in MS/MS mode, does not sample as large a number of peptides and statistics is performed only on peptides with MS/MS data. This is apparently reflected in the high error rates when fewer than 6 peptides are used to identify a protein. This is consistent with results by Old, et al (1). Although the QTOF identified a relatively small number of peptides during targeted MS/MS, the high quality of the matches is assured during vigorous filtering and ANOVA prior to targeted MS/MS.

Both instruments identified a large proportion of the proteins (~85%) when a relatively short, 40-minute gradient was used. This is perhaps surprising as QTOFs are generally thought to be less sensitive than ion traps.

In general, both workflows were found to be effective at correctly detecting spike-in proteins when a moderately complex mixture is analyzed. However, the ion trap workflow resulted in inaccurate fold-change determinations and significant p-values for several non-spike proteins. This underscores the importance of performing replicate analyses and using both p-values and fold-change thresholds.

References

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