A Comparison of nLC-ESI-MS/MS and nLC-MALDI-MS/MS for GeLC-Based Protein Identification and iTRAQ-Based Shotgun Quantitative Proteomics

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The use of nLC-ESI-MS/MS in shotgun proteomics experiments and GeLC-MS/MS analysis is well accepted and routinely available in most proteomics laboratories. However, the same cannot be said for nLC-MALDI MS/MS, which has yet to experience such widespread acceptance, despite the fact that the MALDI technology offers several critical advantages over ESI. As an illustration, in an analysis of moderately complex sample of E. coli proteins, the use MALDI in addition to ESI in GeLC-MS/MS resulted in a 16% average increase in protein identifications, while with more complex samples the number of additional protein identifications increased by an average of 45%. The size of the unique peptides identified by MALDI was, on average, 25% larger than the unique peptides identified by ESI, and they were found to be slightly more hydrophilic. The insensitivity of MALDI to the presence of ionization suppression agents was shown to be a significant advantage, suggesting it be used as a complement to ESI when ion suppression is a possibility. Furthermore, the higher resolution of the TOF/TOF instrument improved the sensitivity, accuracy, and precision of the data over that obtained using only ESI-based iTRAQ experiments using a linear ion trap. Nevertheless, accurate data can be generated with either instrument. These results demonstrate that coupling nanoLC with both ESI and MALDI ionization interfaces improves proteome coverage, reduces the deleterious effects of ionization suppression agents, and improves quantitation, particularly in complex samples.

KEY WORDS: nLC-ESI-MS/MS, nLC-MALDI-MS/MS, protein identification, quantitation, quadrupole linear ion trap, tandem time-of-flight, mass spectrometry.

nano-scale liquid chromatographic tandem mass spectrometry (nLC-MS/MS) is a well-established technique for identification of peptides from complex mixtures, such as protein digests. Even a brief review of the proteomics literature makes it clear that electrospray ionization has become the preferred method for coupling the liquid chromatograph to the mass spectrometer.1–7 With LC-ESI-MS/MS, the chromatographic separation procedure is directly coupled to the MS analysis, which allows high efficiency and increased sample throughput. Additionally, when performed in a data-dependent acquisition mode, it is possible to minimize the collection of redundant data. However, use of LC-ESI-MS/MS also involves tradeoffs between the collection of redundant data and the exclusion of ions of interest from MS/MS experiments. These problems arise because the complexity of most proteomic samples is such that the rate at which peptides elute from the chromatographic column greatly exceeds the maximal scan rate at which mass spectrometers can acquire MS/MS data. Thus, in many LC-ESI-MS/MS experiments the peptides present in the sample are severely under-sampled, resulting in reduced proteome coverage.8,9 For these reasons some recent studies have focused on utilizing a MALDI source as an interface for chromatographic separations10–18 an option that is particularly attractive with the availability of MALDI-TOF/TOF for MS/MS analysis.19

Using an LC MALDI strategy, the collection of MS/MS data can be decoupled from the chromatographic separation, allowing more time to acquire spectra without
missing components as they elute from the HPLC column. Although this approach reduces throughput by introducing an intermediate and semi-automated data analysis step, it allows for a more judicious and rational selection of precursor ions. With MALDI MS, ion chromatogram plots can be generated in silico for each mass detected, and these can be used to minimize collection of redundant data, as well as to ensure the inclusion of isobaric peptides of interest whose elution times differ significantly. MS/MS data can be collected from all peptides at the maxima of their chromatographic responses, increasing sensitivity and the number of components that can be identified from complex mixtures. This type of precursor ion selection would be difficult to implement using ESI methods, where such decisions need to be made on the fly, making it necessary to use dynamic exclusion strategies to reduce redundancy.

Additional benefits associated with LC MALDI include: the ability to archive the sample plate, and to reanalyze the samples using optimized MS/MS parameters after the initial interpretation is complete; its compatibility with a broader selection of chromatographic mobile phases, making it possible to carry out the chromatography under optimized conditions; and its relative insensitivity to interfering compounds in the sample matrix and/or chromatographic eluents. Finally, MALDI and ESI tend to favor the ionization of different sets of peptides. Combining both approaches to analyze the same sample has been shown to improve the number of proteins identified and the confidence of the identifications.

Here we report a comparison of the performance of nLC-ESI-MS/MS, carried out on a 4000 Q Trap linear ion trap (Applied Biosystems, Foster City, CA), and LC-MALDI-MS/MS, carried out on a 4700 MALDI TOF/TOF (Applied Biosystems), in two separate experiments. The first was designed to evaluate the abilities of each TOF (Applied Biosystems), in two separate experiments. MS and LC-MALDI-MS/MS in quantitative applications involving an iTRAQ approach using secreted proteins from the bacterium Thermobifida fusca grown on different carbon sources. However, the protein identification data obtained in the second experiment were also used to extend the findings of the first. The merits, shortcomings, and the complementary nature of each technique are presented and discussed.

**MATERIALS AND METHODS**

**Materials**

All water used in these experiments was deionized using either a Milli-Q ultrapure water system (Milli-pore, Bedford, MA) or a NANOpure system (Barnstead, Dubuque, IA). Sequencing-grade acetonitrile and aceticone were purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium citrate, α-cyano-4-hydroxy cinnamic acid (CHCA, recrystallized prior to use), ammonium bicarbonate, iodoacetamide (IAM), and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO) and were used without further purification. Modified trypsin was obtained from Promega (Madison, WI), and the iTRAQ reagent kit was purchased from Applied Biosystems. The Solka Floc cellulose was obtained from James River Corporation (Berlin, NH). All other chemical reagents, unless otherwise noted, were obtained from Aldrich (Milwaukee, WI).

**Preparation of GeLC Tryptic Digests**

E. coli crude cell extracts were prepared as described previously. The protein concentration was measured by the Bradford assay using BSA as a standard. As indicated in Figure 1, SDS-PAGE analysis was carried out on 20-µg aliquots of this sample in a pre-cast Novex 12% Tris/glycine mini-gel using an Xcell electrophoresis apparatus (Invitrogen, Carlsbad, CA). After completion of the run, the gel was visualized by Colloidal Coomassie Blue stain (Invitrogen). Following visualization of gels, the three highest-molecular-weight bands were cut out, diced into 1- to 2-mm pieces using a scalpel, and placed into 0.5-mL Eppendorf tubes for in-gel digestion by the method of Schevchenko et al., with minor modifications. After digestion, the supernatant was removed, and the gel pieces were washed with 30 µL of 5% formic acid followed by 30 µL of 50% ACN and finally 50 µL of 95% ACN. Each of these extractions was combined with the supernatant and dried in a SpeedVac concentrator (Thermo Savant, Holbrook, NY).

**Preparation of iTRAQ Sample**

A T. fusca YX (strain ER1) starting culture was grown overnight on Hagerdahl minimal medium containing glucose (5 mg/mL) as the sole carbon source. This culture was then used to inoculate cultures of Hagerdahl media containing dried and shredded alfalfa (ALF) (10 mg/mL) or Solka Floc cellulose (SF) (10 mg/mL) as the sole carbon source. These cultures were grown in a water bath at 50°C with shaking at 250 rpm. After 94 h, the supernatant of each culture was harvested by centrifugation, phenylmethanesulfonylfluoride (PMSF) protease inhibitor was added to 0.1 mM, and the supernatants were centrifuged again and stored at −70°C. The protein concentrations, determined by TCA-Lowry protein assays, were 0.45 mg/mL (ALF) and 1.21 mg/mL (SF). Both supernatants showed similar glycosyl hydrolase activity on pretreated
and untreated alfalfa biomass; however, a 1D SDS protein gel showed differences in the types of proteins present in each supernatant.

Samples containing 1 mg of ALF or SF supernatant protein were prepared by adding an equal volume of an 8 M urea solution (run over AG501-xB(D) resin (BioRad) to remove cyanide) combined with 0.5% CHAPS buffer and then centrifuging three times to remove all precipitate. These solutions were concentrated using Biomax 10K NMWL (0.5 mL vol) Ultrafree centrifugal filters (Millipore) by centrifugation at 10,000 g for 10 min at 4°C. A 7 M urea, 2 M thiourea, 4% CHAPS solution was used to wash each sample by dilution and concentration three times. The final protein concentrations were about 5 mg/mL.

Each of the ALF and SF secreted protein samples (50 µg in 17 µL) was denatured by adding 1 µL of 2% SDS and reduced by addition of 2 µL of 50 mM tris-(2-carboxyethyl) phosphine (TCEP). The cysteine residues were blocked using 1 µL of 200 mM methyl methanethiosulphonate (MMTS) as described previously.28,29 After tryptic digestion, samples were dried completely and reconstituted in 50 µL of the dissolution buffer (500 mM triethylammonium bicarbonate). Equal amounts of each sample were aliquoted in duplicate, and each aliquot was labeled with a different iTRAQ tag according to the manufacturer’s instructions (document #4351918A and 4350831C downloaded from http://docs.appliedbiosystems.com/search.taf). The 114 and 115 tags were used to label the peptides in the two identical samples from the SF-grown culture, and the 116 and 117 tags were used for two extracts from the ALF culture. After labeling, the four samples were pooled and subjected to cation-exchange chromatography as described below.

**Strong Cation-Exchange Fractionation**

Strong cation-exchange fractionation (SCX) fractionation was performed using an Applied Biosystems cation-exchange cartridge system. The pooled iTRAQ labeled tryptic peptides samples (~400 µL) were evaporated completely in a SpeedVac concentrator and reconstituted with 1 mL of loading buffer (10 mM potassium phosphate, pH 3.0, 25% ACN). The pH of the sample was adjusted to 3.0 with phosphoric acid prior to cartridge separation. After conditioning of the SCX cartridge with loading buffer, the sample (~100 µg) was loaded and washed with an additional 2 mL of loading buffer. The peptides were eluted in three steps by 1 mL of loading buffer containing 50 mM KCl, 150 mM KCl, and 500 mM KCl, respectively. Desalting of SCX fractions was carried out using solid-phase extraction (SPE) on Sep-Pak Cartridges (Waters, Milford, MA). The SCX fractions were dried at reduced pressure and the sample reconstituted in 0.1% TFA. After washing, the tryptic peptides were eluted in 1 mL of 70% ACN–0.1% TFA. The solvent was removed at reduced pressure and the peptides were reconstituted in 2% ACN–0.5% FA for subsequent nLC analysis.

**Reverse-Phase Separation and Tandem Mass Spectrometry**

All *E. coli* gel band digests were reconstituted in 30 µL of 0.5% FA with 2% ACN prior to mass spectrometry analyses. For initial experiments designed to evaluate the per-
formance of the two LC-MS/MS strategies directly, a single chromatographic system was used. The *E. coli* samples containing peptides from the 1D gel bands were separated on an UltiMate chromatography system equipped with a Switchos, FAMOS autosampler, and Probot spotting robot (Dionex, Sunnyvale, CA). The gel-extracted peptides (6 µL) were injected onto a PepMap C18 trap column (5 µm, 300 µm × 5 mm, Dionex) and then separated on a PepMap C-18 RP nano column (3 µm, 75µm × 15 cm), eluted using a 60-min gradient (for the GeLC) of 5% to 40% ACN in 0.1% formic acid at 350 nL/min. After the chromatographic column, the system was fitted with a flow splitter, which directed 50% of the flow directly to a hybrid triple quadrupole linear ion trap mass spectrometer (4000 Q Trap ABI/MDS Sciex, Framingham, MA) equipped with Micro Ion Spray Head ion source for online analysis, as described below. The remainder of the flow was directed to the Probot spotting robot, where fractions were collected every 20 sec (58.5 nL) on a 576-position OptiTOF sample plate and simultaneously mixed with 0.34 µL of matrix (7 mg/mL CHCA containing 25 fm [Glu] Fibrinopeptide B—Glufib—as internal standard for mass calibration). These samples were then subjected to MALDI MS/MS analysis using a 4700 Proteomics Analyzer equipped with TOF-TOF ion optics (Applied Biosystems, Framingham, MA) and 4000 Explorer version 3.6. The instrument was operated in 1 kV positive ion reflector mode and calibrated with Glufib (Applied Biosystems, Framingham, MA) as an internal calibrant. The laser power was set to 4500 for MS and 5200 for MS/MS with CID off. MS spectra were acquired across the mass range of 850–4000 Da with a minimum S/N filter of 25 for precursor ion selection. MS/MS spectra were acquired for the 15 most abundant precursor ions, with a total accumulation of 2000 laser shots.

The ESI-MS data acquisition was performed using Analyst 1.4.1 software (Applied Biosystems) in the positive ion mode using information-dependent acquisition (IDA) analysis. The nanospray voltage used for all experiments was +2.0 kV, and nitrogen was used as the curtain (value of 10) and collision gas (set to high), with the heated interface at 150°C. The declustering potential was set at 50 eV and Gas1 was 15 psi. In IDA analysis, after each survey scan of the m/z range from 400 to 1550 and an enhanced resolution scan, the three highest-intensity ions with multiple charge states were selected for MS/MS using a rolling collision energy based on the ions’ observed charge states and masses.

For the independently optimized separations used for the analysis of both the SDS-PAGE-separated *E. coli* proteins and the iTRAQ-labeled *T. fusca* proteins, an identical chromatographic program to the one described above was used for the LC-ESI applications, except that the flow rate was decreased to 250 nL/min. However, for the LC-MALDI applications, 0.1% TFA was substituted for the 0.1% FA in addition to the flow-rate change. In other respects, the chromatographic separations were identical. The MS analysis of the independently chromatographed material was carried out as described above for the split samples.

### Data Analysis and Protein Identifications

MS/MS spectra generated from nanoLC/ESI-based IDA analyses for 1D gel samples were interrogated using Mascot 2.1 (Matrix Science, Boston, MA) for searching against the NCBI database (downloaded in August 2006) using the *E. coli* taxonomy. The search parameters allowed for one miscleavage, variable modifications of methionine oxidation, and cysteine carboxamidomethylation, with a peptide tolerance = 1.2 Da and MS/MS tolerance = 0.6 Da. Only significant scores for the peptides defined by a Mascot probability analysis (www.matrixscience.com/help/scoring_help.html#PBM) greater than “identity” were considered to be confidently identified peptides and used for protein identifications. For those protein IDs based on single-peptide hits, the identity score criteria were made more stringent by requiring a confidence interval of ≥99%. For all MS/MS spectra of iTRAQ samples, ProteinPilot software 1.0 (Applied Biosystems, Foster City, CA) with the Paragon search engine was used for interrogation of a *T. fusca* database (downloaded in Nov. 2006 from http://www.ncbi.nlm.nih.gov). The default search settings used for quantitative processing and protein identification were: trypsin cleavage with fixed MMTS modification of cysteine, iTRAQ labeling and variable methionine oxidation. We report only protein identifications with a total ProtScore >1.3, which represents >95% statistical confidence in Protein Pilot. The quantitation of the identified proteins was reported using the 114 and 115 tags as the controls with bias correction. All MS/MS spectra generated from nanoLC/MALDI were submitted for database searching and quantitative analysis using GPS Explorer Software. The search engine used for peptide and protein identification was Mascot version 2.1. The search parameters used for the *E. coli* samples allowed for a single miscleavage and variable modifications of methionine oxidation and cysteine carboxamidomethylation with a mass tolerance of 75 ppm. For the iTRAQ-labeled *T. fusca* samples, the search parameters were adjusted to allow for fixed modifications by MMTS at cysteine residues and iTRAQ labeling, variable oxidation at methionine, and a single miscleavage. The
mass tolerance was set to 75 ppm. Each protein identified was required to have at least one unique peptide identification not shared with any other protein. As above, we report only those proteins with a protein identification confidence interval of $\geq 95\%$.

We recognize the complications introduced by using different search engines for the data obtained by nLC-ESI and nLC-MALDI, but this was necessary because current versions of ProteinPilot with the Paragon search engine (ABI, Foster City, CA) are not compatible with data from the 4000 Series Explorer version 3.6 software utilized by the 4700 mass spectrometer. ProteinPilot was used to analyze the ESI-acquired iTRAQ data because it incorporates algorithms to automatically yield quantitative information and minimize or eliminate false-positive identifications arising from the existence of protein homologs in the database searched. The elimination of false-positive identifications can represent a serious problem in eukaryotic genomes, or when searching very large trans-species databases such as NCBI	extit{nr}. However, in the study reported here, we searched two relatively small prokaryotic genomes in which the process of manually eliminating false positives due to protein homologs, though laborious, is tractable. By carefully eliminating redundant identifications due to homologs through manual inspection of the Mascot results, we believe the data generated by both search engines are comparable. Furthermore, any residual difference in the data sets due to the use of different search engines is relatively small and has little impact on the results.

Physicochemical properties including the grand average of hydropathicity (GRAVY)$^{30}$ index for unique peptides exclusively identified by each technique, were calculated using ProtParam$^{31}$ at http://us.expasy.org/tools/protparam.html.

RESULTS AND DISCUSSION

To compare the performance of nLC-ESI-MS/MS and nLC-MALDI-MS/MS, two common applications were used: GeLC-based protein identifications$^{32}$ and iTRAQ-based shotgun quantitative analysis.$^{28}$ Additionally, two different instrument configurations were used to couple the LC separation to the mass analysis in order to determine the optimal workflow, as shown in Figure 1. A direct nLC split flow with parallel online ESI-MS/MS analysis and spotting for MALDI analysis was compared with the performance of independently optimized nLC for both ESI and MALDI analyses. Since preliminary analyses yielded consistently better results using independently optimized nLC configurations, this was used for the rest of the studies.

Split Flow Analysis of \textit{E. coli} Band 2

Table 1 summarizes the results from an initial experiment in which the peptide separation for \textit{E. coli} band 2 was carried out in a single run. The chromatographic effluent was evenly split for ESI-MS/MS and MALDI-MS/MS. A total of 22 proteins were identified in this band (21 by ESI and 14 by MALDI), of which 8 were detected solely by the online ESI analysis and only a single protein solely by MALDI analysis. Furthermore, the sequence coverage, though variable from protein to protein, was generally greater for the ESI analysis, which was contrary to previous experience and expectation.

It is likely that the relatively poor performance of the LC-MALDI analysis in this instance was due to the use of a chromatographic separation that had been optimized for the LC-ESI application. Since ESI has a much narrower range of compatibility with the various mobile phases that can be used in the chromatographic separation, it was not possible to carry out the ESI analysis using a MALDI-optimized separation protocol.

Independently Optimized Chromatography

The chromatographic separation for each of the two analytical approaches was optimized independently, to take full advantage of the unique properties of each ionization method. Specifically, additional experiments were carried out with \textit{E. coli} band 2, in which 0.1% FA was used as the ion pairing agent for the ESI analysis and 0.1% TFA was used as the ion pairing agent for the MALDI analysis (see Materials and Methods for details). For these experiments, the entire chromatographic flow was directed to the respective ionization source. A total of 29 proteins were identified (Table 2), 20 of which were detected by both approaches, while 6 were unique to the MALDI analysis and 3 were unique to the ESI analysis. The sequence coverage varied between proteins, but was generally higher for the MALDI analysis. The number of proteins identified by ESI using independently optimized chromatography was similar to that resulting from the split flow analysis of band 2, which was not surprising as the split-flow chromatography had already been optimized for ESI analysis. However, the number of proteins identified by the MALDI analysis was considerably larger, suggesting that the optimized chromatography (0.1% TFA) improved the relative performance of the LC-MALDI approach. Furthermore, the average sequence coverage for both techniques was higher than with the split-flow analysis above, which is at least partially due to the use of an optimized chromatographic separation, even for the ESI approach (where the flow rate was changed from 350 nL/min to 250 nL/min).
Figure 2 shows the sequence of a representative protein from this analysis, PLEB_ECOLI. The peptide sequences in green are those that were identified by both techniques, while those in red or blue were identified uniquely by MALDI or ESI, respectively. Sequences given in black were not identified by either approach. This result clearly demonstrates that the sequence coverage substantially increased when the approaches were combined.

To extend these results and to further demonstrate the benefits of using independently optimized chromatographic protocols, two additional E. coli samples were analyzed (bands 1 and 3, see Figure 1). The results of these experiments were combined with those described above and are summarized in Figure 3 and Table 3. A total of 84 proteins were identified from all three bands, of which 53 were identified by both strategies, while 19 and 12 were identified uniquely by ESI and MALDI, respectively. Thus, the additional LC-MALDI analysis increased the number of identified proteins by 16% compared with using LC-ESI analysis alone. While this demonstrates the complementary nature of the two ionization techniques, it should be noted that a second analysis by LC-ESI-MS/MS might also result in the identification of additional peptides.

The reason for the limited increase in proteomic coverage in this instance is likely due to two factors. First, these samples are not particularly complex, as they represent narrow and discreet gel bands and were found to contain only 20–30 proteins each. Second, since all the proteins within each band migrated to the same position in the gel and all three bands were cut from the same section of the gel, they are likely to have similar properties. It is expected that the analysis of a more complex sample containing peptides from a larger number of proteins with different physicochemical properties would reveal a much greater level of complementarity for the two techniques.

### Table 1

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Online NanoLC-ESI-MS/MS</th>
<th>Offline NanoLC-MALDI-MS/MS</th>
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<tr>
<td></td>
<td>Peptide Number</td>
<td>Sequence Coverage %</td>
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<td>ODP2_ECOLI</td>
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<td>38.8</td>
</tr>
<tr>
<td>EFG_ECOLI</td>
<td>20(6)</td>
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Note: parentheses represent unique peptides exclusively identified by each method.
To compare the chemical properties of the peptides exclusively identified by either the MALDI or ESI approach, the masses, pIs, and predicted GRAVY scores for the unique peptides were predicted. As shown in Table 3, the average masses of peptides uniquely identified by MALDI are approximately 25% larger than those by ESI for all three gel band samples. Moreover, the average GRAVY index of the unique peptides by MALDI was more negative than those peptides identified by ESI, suggesting that the MALDI-identified unique peptides are more hydrophilic, in accordance with expectations.20–22

There was no obvious correlation between the ionization technique utilized and the predicted pI of a peptide observed.

Protein Identification in Shotgun Analysis

The shotgun analysis of the iTRAQ-labeled samples from T. fusca was originally carried out to compare the performance of the two analytical strategies in quantitative applications. Nevertheless, they also provide an opportunity to evaluate the relative performance of these two approaches for protein identification in shotgun applications. A single analysis of the three ion-exchange fractions of the iTRAQ-labeled peptides by
nLC-MALDI-MS/MS resulted in the identification of 137 proteins, whereas a single analysis of the same fractions by nLC-ESI-MS/MS identified only 61 proteins. Reanalysis of this sample resulted in little improvement in the protein identification. The relatively poor performance of the ESI approach on this set of samples suggested that the samples contained contaminants that were suppressing peptide ionization. This hypothesis was supported by the observation that a fivefold dilution of the protein sample followed by reanalysis by nLC-ESI-MS/MS resulted in a significant increase (50%) in the number of identified peptides and proteins. A further dilution (1 to 15) showed little further improvement. When the results of all four runs were pooled, a total of 124 proteins were identified by the ESI approach.

The results of this analysis are summarized in Figure 4. The three ion exchange fractions analyzed by both techniques resulted in a total of 180 protein identifications, of which 81 were found by both approaches. The MALDI analysis resulted in 56 unique protein identifications, and the ESI approach produced an additional 43. Thus, in this more complex sample, the addition of an LC-MALDI analysis increased the number of protein identifications by 45% compared with using LC-ESI analysis alone, even when the ESI analysis was repeated four times. Furthermore, it is apparent that the relative insensitivity of MALDI-based MS to the presence of small-molecule impurities represents a significant advantage, particularly in cases where the presence of an ion-suppression agent is likely, or unknown. The large increase in proteomic coverage and the ability of MALDI to accommodate the presence of impurities that would suppress ESI clearly demonstrates the complementary nature of these analytical approaches, particularly in complex samples.

Quantitation with iTRAQ

Quadruplex iTRAQ experiments were performed, containing two technical replicates of each sample. The 114 and 115 tags were used to label the peptides in the two identical samples of SF, and the 116 and 117 tags were used to label the peptides from the two ALF samples. By calculating the iTRAQ ratios between SF- and ALF-derived samples, the precision of these experiments can be determined as a function of analytical approach. Moreover, by calculating the iTRAQ ratios obtained for the technical replicates (114:115 or 116:117), the accuracy of the iTRAQ method can be assessed as a function.
of the analytical approach, since the true value of each ratio must be 1:1. The average of the deviations from this known value for each protein represents a measure of the accuracy of the iTRAQ method for the analytical system used.

Figure 5 shows a plot of the log$_2$(average iTRAQ ratio) for a selected set of 24 proteins identified in the iTRAQ experiments. The ratios determined by LC-MALDI-MS/MS are given in red and those determined by LC-ESI-MS/MS are shown in blue. Error bars are included for each protein, which represent the relative standard deviation (RSD) of four data points for each protein ratio. The error bars associated with the MALDI analysis are consistently smaller than those associated with ESI and the RSD values were 10% and 19%, respectively, when averaged over all the protein ratios. An explanation for this difference is suggested in Figures 6A and B, which show the MS/MS spectra for the same iTRAQ-labeled peptide by MALDI-TOF/TOF and 4000 Q Trap, respectively. The 4700 MALD-TOF/TOF instrument has a much higher mass resolution (Figure 6A) than the linear ion trap used for the LC-ESI experiments and, unlike the

![Venn diagram](image)

**FIGURE 4**

A Venn diagram showing the union and intersection of the two sets of proteins that were identified by independently optimized nLC-ESI-MS/MS and nLC-MALDI-MS/MS of the three SCX fractions of the iTRAQ-labeled peptides.
linear ion trap (Figure 6B), can resolve all the reporter ions to baseline. The partially overlapping peaks that result from the reduced resolution in Figure 6B produce a reduction in the accuracy of the peak integration and, thus, quantitation.

Figure 7 summarizes the comparison of the accuracy of the LC-MALDI-MS/MS and LC-ESI-MS/MS approaches. Although the iTRAQ ratios from the MALDI analysis using the 4700 TOF/TOF had less deviation from the known value than when using the 4000 Q Trap, the accuracy of both methods is certainly acceptable for most proteomic analyses (1.00 ± 0.11 and 1.01 ± 0.15, respectively). The reason for this observation is most likely the high number of protein determinations (79) used to obtain the average ratio. It should also be noted that each of these individual protein determinations is itself an average of the individual peptide determinations. Nevertheless, based on these observations it can be expected that even with a much more limited dataset, reasonably accurate iTRAQ data can be obtained using the 4000 Q Trap.

**CONCLUSION**

The data presented here suggest that nLC-ESI-MS/MS and nLC-MALDI-TOF/TOF are complementary analytical techniques for proteomic analyses. Employing both increases both protein and proteomic coverage over what would be obtained by either alone, particularly for complex samples. Furthermore, the relative insensitivity of MALDI analysis to ionization suppression was found to be a significant advantage, suggesting that the nLC-MALDI approach should be employed whenever the presence of an ionization suppression agent is either suspected, or is unknown.

Additionally, the superior mass resolution of the TOF/TOF instrument improves the precision of quantitative
results obtained in iTRAQ applications and is a consequence of the mass analyzer, rather than the ionization interface. Nevertheless, even when using a relatively low resolution ion trap, such as the 4000 Q-trap, the precision observed for technical replicates is sufficient and much less than the biological variation expected.

Despite the limited mass resolution of the Q trap instrument, the accuracy of the iTRAQ ratios was nearly identical to that obtained with the TOF/TOF. While in this particular case the accuracy observed is due to the large number of replicates used to determine the average values (79), it appears that reasonably accurate results can be obtained, even with fewer replicates.

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