Published in final edited form as:

Rapid Commun Mass Spectrom. 2008 October ; 22(20): 3137–3146. doi:10.1002/rcm.3719.

A comparison of relative quantification with isobaric tags on a subset of the murine hepatic proteome using electrospray ionization quadrupole time-of-flight and matrix-assisted laser desorption/ionization tandem time-of-flight

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Abstract

The use of isobaric tagging for relative and absolute quantification (iTRAQ) has increased dramatically over the past few years. Many factors can affect the accuracy of quantification. Some of these include the number of biological/technical replicates, sample complexity, instrumentation, method of peptide/protein identification and the statistical techniques used for data analysis. It has been observed that the low collision energies normally used in electrospray ionization quadrupole time-of-flight (ESI QTOF) can result in low iTRAQ reporter ion abundances. We used two-way analysis of variance (ANOVA) to compare the iTRAQ ratios that were generated on an ESI QTOF and a matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI TOF/TOF). It appears that iTRAQ analyses performed on an ESI QTOF without any special modifications to instrumental parameters produce essentially the same protein ratios as those obtained on a MALDI TOF/TOF.

The ability to detect protein expression changes is vital for understanding the underlying biology of cellular processes at different times or in different states. For example, a single low dose of the organochlorine pesticide chlordane (CD) has been shown to alter cholesterol homeostasis in mice.^{1,2} Although membrane transport proteins like ATP-binding cassette protein G8 and scavenger receptor class B type I were not affected, the possibility of alterations in soluble cholesterol-binding proteins is currently under investigation.³ A great deal of effort has been expended in the development of a variety of stable isotope methods for the relative quantification of changes in protein expression.⁴ Stable isotopes or isotopically labeled amino acids can be added to media and incorporated into proteins during growth,^{5,6} but this method is not easily adapted for use outside of the cell culture arena. Chemical labeling methods are required for tissue analysis and many have been developed that react with a variety of functional groups in proteins and peptides.^{4,7} These techniques can be mass spectrometry (MS) or tandem mass spectrometry (MS/MS) based depending on the properties of the label itself. Isotope coded affinity tag (ICAT) technology is MS-based and was first developed by Gygi *et al.*⁸ It uses light and heavy cysteine-specific tags bound to a biotin moiety so the cysteine-containing peptides can be enriched using affinity chromatography. Not all proteins contain cysteine, however, and with the early versions of

the ICAT reagents, the different masses of the light and heavy tags resulted in different chromatographic performance. Additionally, only two tags (light and heavy) were available so multiplexing (running more than two samples at the same time) was impossible. The iTRAQ methodology was developed by Ross *et al.*⁹ and commercialized by Applied Biosystems, Inc. It was designed to overcome some of the liabilities of the ICAT-labeling methodology, and its use has increased dramatically over the past few years. The isobaric tagging molecule consists of a charged *N*-methylpiperazine-based reporter group, a carbonyl balance group and an *N*-hydroxysuccinimide (NHS) peptide reactive group. The masses of the reporter and balance groups sum to 145.1 *m/z*, and this is kept constant through use of ¹³C, ¹⁵N and ¹⁸O atoms. Originally, only four different reporter groups were available, *m/z* 114.1, 115.1, 116.1 and 117.1, but the current version is available with eight reporters, which allows the comparison of up to eight states or time points. The NHS moiety reacts with primary amines, and labeling is conventionally performed after trypsin digestion. Consequently, the *N*-terminal and any lysines present in the peptide are labeled. Since the tags are isobaric, the peptides from different experimental groups containing different labels elute at the same time and appear in MS spectra at the same *m/z*, significantly simplifying the spectra. *N*-Methylpiperazine is a basic moiety, which accepts protons readily and enhances the overall ionization efficiency of the peptides.⁹ iTRAQ is MS/MS-based in the sense that the charged reporter ions are released during collision-induced dissociation (CID) with the carbonyl undergoing a neutral loss. Relative quantification is achieved using either the peak area or intensity of the reporter ions. Successful use of this technique has been demonstrated in many organisms and tissues.⁹⁻¹⁴

There are numerous factors that can affect the accuracy of iTRAQ quantification. Some of these include the number of biological/technical replicates, sample complexity, instrumentation, method of peptide/protein identification and the statistical techniques used for data analysis. It has been observed that the low collision energies normally used in ESI QTOF can result in low iTRAQ reporter ion abundances.¹⁵ The question naturally arises whether or not iTRAQ data generated with ESI and MALDI sources can be directly compared. We had the opportunity to compare iTRAQ ratios generated on an ESI QTOF and MALDI TOF/TOF from a relatively small data set that was generated as part of a larger experiment looking at the effect of acute, low dose chlordecone on soluble cholesterol-binding proteins. It is not our intention to discuss the biological aspects of the study in this paper; those interested in the biological results should see chapter 2 of Scheri.¹⁶ In the study presented here, we address the question of whether or not iTRAQ data obtained by different sources yield similar results by using multifactor analysis of variance (ANOVA) to compare the iTRAQ ratios that were generated on an ESI QTOF and MALDI TOF/TOF from a modest set of samples.

EXPERIMENTAL

Materials

Chlordecone (CD) was purchased from Chem Service (West Chester, PA, USA). Purity (99%) was confirmed by gas chromatography/electron ionization mass spectrometry. Radiolabeled cholesterol ([4-¹⁴C]cholesterol, 55 mCi/mmol) was obtained from Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Bicinchoninic acid (BCA) protein assay reagents were purchased from Pierce Biotechnology Inc. (Rockford, IL, USA). iTRAQ reagents were purchased from Applied Biosystems (ABI, Foster City, CA, USA). Burdick and Jackson solvents (VWR, West Chester, PA, USA) were used for all high-performance liquid chromatography (HPLC). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

Animals (cholesterol-binding protein enrichment)

Previous dose response and time course experiments^{1,2} provided optimal doses and sampling times for CD pretreatment and the exogenously administered lipid bolus containing radiolabeled cholesterol ($[^{14}\text{C}]\text{CH}$). Male C57BL/6 mice (6–7 weeks old) were purchased from Simenson Laboratory (Gilroy, CA, USA). They were randomly assigned to one of three groups; (1) Control, (2) Low dose (5 mg/kg) CD, or (3) High dose (15 mg/kg) CD. They were individually housed in a temperature ($22\pm 1^\circ\text{C}$) and light-controlled (12 h light/12 h dark daily) facility with free access to water and fed AIN93 diet (Dyets, Inc., Bethlehem, PA, USA) *ad libitum*. Seven days were allowed for acclimatization before treatments were initiated. Corn oil was used as a vehicle (5 mL/kg). Mice received a single dose of either corn oil or CD (5 or 15 mg/kg) via intraperitoneal (IP) injection. After 72 h a lipid bolus of corn oil (5 mL/kg) containing 10 mg/kg CH (2 $\mu\text{Ci}/\text{mouse}$) was administered IP. After injection the mice continued to be housed individually and were allowed free access to water. They were killed 16 h later by carbon dioxide anesthesia and exsanguination. Livers were removed for analysis. The procedures for animal use were approved by the Oregon State University Institutional Animal Care and Use Committee (IACUC).¹⁷

Animals (iTRAQ experiment)

Male C57BL/6 mice (6–7 weeks old) were purchased from Simenson Laboratory (Gilroy, CA, USA). They were randomly assigned to one of two groups; 1) Control or 2) High dose (15 mg/kg) CD. They were individually housed in a temperature ($22\pm 1^\circ\text{C}$) and light-controlled (12 h light/12 h dark daily) facility with free access to water and fed AIN93 diet (Dyets, Inc.) *ad libitum*. Seven days were allowed for acclimatization before treatments were initiated. Corn oil was used as a vehicle (5 mL/kg). Mice received a single dose of either corn oil or CD (15 mg/kg) via intraperitoneal (IP) injection. After 72 h they were fasted for 4 h and killed by carbon dioxide anesthesia and exsanguination. Liver, gall bladder and blood were harvested. Here again, the procedures for animal use were approved by the Oregon State University Institutional Animal Care and Use Committee (IACUC).

Tissue preparation

Liver was homogenized with a Polytron PT 3000 PT (Brinkmann Instruments, Westbury, CT, USA) in buffer consisting of 0.01 M potassium phosphate, pH 7.4, 0.15 M potassium chloride (KCl), 1.0 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM butylated hydroxytoluene (BHT) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). All centrifugation was performed at 4°C . The homogenate was first centrifuged at 1200 g for 30 min. The pellet produced consisted of plasma membrane/cellular debris. The supernatant was then centrifuged at 12 000 g for 30 min to eliminate mitochondria. The supernatant from this run was then centrifuged at 100 000 g for 90 min in a Ti 70 rotor. The resulting supernatant and pellet were the cytosolic and microsomal fractions, respectively. Microsomes were resuspended in 0.1 M potassium phosphate, pH 7.25, 1.0 mM EDTA, 30% glycerol, 20 μM BHT, 1.0 mM dithiothreitol (DTT) and 0.1 mM PMSF. The BCA assay was used to determine protein content.

Radioactivity quantification assessed fraction enrichment of cholesterol-binding proteins

Subcellular fractions were prepared as described in the previous section, but, in this experiment, the mice were injected with $[^{14}\text{C}]\text{CH}$. Fractionation of $[^{14}\text{C}]\text{CH}$ was applied in order to evaluate the concentration of cholesterol-binding proteins. 50 mL/L of 30% streptomycin sulfate was added to cold hepatic cytosol and gently shaken overnight at 4°C . The resulting precipitate was dissolved in 8.0 M urea, 10 mM potassium phosphate, pH 7.4, and dialyzed overnight in 1.0 M urea, 10 mM potassium phosphate, pH 7.4. Aliquots of the

streptomycin-precipitated cytosolic fractions (supernatant and solubilized precipitate) were added to 10 mL of Hionic-Fluor (Perkin-Elmer, Boston, MA, USA). The samples were mixed and then placed in the dark overnight to allow the chemiluminescence to decay. Samples were counted in a Beckman LS7500 liquid scintillation counter. The BCA assay was used to determine protein concentrations.

Fluorescent spectroscopy

The fluorescent analog NBD cholesterol (22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholesterol-3 β -ol) was purchased from Invitrogen/Molecular Probes (Carlsbad, CA, USA). Experiments were performed on a Perkin Elmer LS50 luminescence spectrometer (Waltham, MA, USA).

Tissue preparation for iTRAQ experiments

Cholesterol-enriched fractions were prepared by streptomycin precipitation as outlined above. A BCA assay was used to determine protein concentrations. 100 μ g samples of the streptomycin precipitate fraction were precipitated with six volumes of ice-cold acetone and labeled with iTRAQ reagents as detailed below.

iTRAQ labeling

Following the manufacturer's protocol, samples were labeled with iTRAQ reagents according to the scheme shown in Fig. 1. Briefly, 100 μ g samples were dissolved in 20 μ L of 0.5 M tetraethyl ammonium bicarbonate (TEAB), denatured with 0.1% sodium dodecyl sulfate (SDS) and reduced with 5 mM tris-(2-carboxyethyl) phosphine (TCEP). After incubation for 1 h at 60°C, cysteines were blocked with 10 mM methyl methane thiosulfonate (MMTS) in isopropanol, and the samples were incubated at room temperature for 10 min. Sequencing grade trypsin (Promega, Madison, WI, USA) was added in a trypsin/protein ratio of 1:20, and the samples were incubated at 37°C for 16 h. The samples were vacuum concentrated, resolubilized in 30 μ L TEAB and labeled with 70 μ L iTRAQ reagent in isopropanol. Control samples were labeled with iTRAQ 114.1 or 116.1, while CD-treated samples (15 mg/kg) were labeled with iTRAQ 115.1 or 117.1 and incubated at room temperature for 2 h. One control mouse and one treated mouse comprised one biological sample. Four samples, i.e., a total of eight mice, were prepared. Control and CD-treated samples were randomly combined and vacuum concentrated to ~30 μ L. Samples were cleaned up by running on strong cation-exchange (SCX) spin columns (The Nest Group, Inc., Southboro, MA, USA) and eluted with four salt fractions; 75 mM, 150 mM, 350 mM and 750 mM KCl in 25% acetonitrile, 10 mM potassium phosphate, pH 3.0. The eluted fractions were vacuum concentrated to ~30 μ L, and 300 μ L of 0.1% trifluoroacetic acid (TFA) was added. Samples were desalted on C-18 spin columns (The Nest Group), eluted with 80% acetonitrile, 0.1% TFA and vacuum concentrated to dryness. Samples were resuspended in 20 μ L 0.1% TFA before performing nano-HPLC/MS.

HPLC/ESI-MS/MS

Nano-HPLC/ESI-MS/MS was performed on a nanoAcquity Ultra-Performance LC system (Waters, Milford, MA, USA) coupled to a quadrupole orthogonal time-of-flight mass spectrometer (QTOF Ultima Global, Micromass/Waters, Manchester, UK). Samples of 4 μ L were injected. Peptides were trapped and washed on a Symmetry C18 column (5 μ m, 180 μ m $\times$ 20 mm, Waters) using 2% acetonitrile in 0.1% formic acid at a flow rate of 4 μ L/min for 6 min. Peptides were separated using an ethylene bridged hybrid (BEH) column (1.7 μ m, 75 μ m $\times$ 150 mm, Waters) with a binary gradient consisting of 2% acetonitrile in 0.1% formic acid (solvent A) and acetonitrile in 0.1% formic acid (solvent B) over 70 min at a flow rate of 260 nL/min.

The ion source was operated with a spray voltage of 2.2 kV in the positive ion mode. Data-dependent analysis (DDA) MS/MS mode was utilized with a 0.6 s survey scan and a 2.4 s MS/MS scan on the base peak in the MS survey scan. Previously selected m/z values were excluded from DDA for 60 s. The MS/MS collision energy ranged from 25–65 eV and was based on the precursor charge state selected by the quadrupole. No effort was made to optimize the transmission of low m/z ions through the instrument, i.e., the instrument was run in its standard operating mode. Calibration was performed with [Glu¹]-fibrinopeptide B (MH⁺ 1570.6774 Da, monoisotopic) fragment ions. Lock mass correction was also performed using [Glu¹]-fibrinopeptide B ([M+2H]²⁺ 785.8426 Th) every 30 s for one scan. Peak list (pk1) files were generated using Protein Lynx Global Server (PLGS, Waters, Manchester, UK).

HPLC/MALDI-MS/MS

Peptide separation was performed on a nanoAcquity Ultra Performance LC system (Waters) coupled to a Probot (Dionex/LC Packings, Amsterdam, The Netherlands) MALDI spotting device. Samples of 2 μ L were injected. Peptides were trapped and washed on a Symmetry C18 column (5 μ m, 180 μ m $\times$ 20 mm, Waters) using 0.1% TFA at a flow rate of 4 μ L/min for 6 min. Peptides were separated using an Atlantis C18 column (3 μ m, 75 μ m $\times$ 150 mm, Waters) with a binary gradient consisting of 0.1% TFA (solvent A) and acetonitrile in 0.1% TFA (solvent B) over 60 min at a flow rate of 300 nL/min.

Column effluent was mixed in a 1:4 ratio with MALDI matrix (3 mg/mL α -cyano-4-hydroxycinnamic acid in water/acetonitrile/TFA/ammonium phosphate dibasic (6 mg/mL), 37.6:56.4:0.1:6, v/v/v/v) via a tee-junction. Fractions (20 s/spot, 144 spots/plate) were spotted onto an Opti-TOF MALDI plate system (ABI) using stainless steel plates.

MALDI plates were analyzed on an ABI 4700 Proteomics Analyzer equipped with a 200 Hz Nd:YAG laser operating at a wavelength of 355 nm. Data were acquired in the MALDI reflector mode over a mass range of 800–4000 m/z using five spots of ABI calibration mixture as an external standard. ABI calibration mixture consists of the following peptides (monoisotopic mass of the singly protonated ion is given in parenthesis in Da); [Glu¹]-fibrinopeptide B (1570.6774), adrenocorticotrophic hormone fragment 18-39 (2465.1989), angiotensin I (1296.6853) and [des-Arg¹]-bradykinin (904.6774). Mass spectra were obtained from each spot using 1000 laser shots per spectrum. Peaks with a signal/noise (S/N) ratio greater than 30 were selected for MS/MS. Up to ten MS/MS spectra could be obtained from each spot. MS/MS spectra were acquired with 1500 laser shots, starting with the least intense peak and ending with the most intense peak. Constant laser fluence was used and kept low enough to prevent signal saturation. Ions formed in the source were accelerated to 8 keV. Precursors were selected using timed ion selection (resolving power=100 FWHM) and then decelerated in the collision cell to 1 keV for collision-induced dissociation (CID). Air was used as the collision gas at a pressure of about 5.5×10^{-7} Torr. Fragment ions were accelerated to 14 keV before entering the reflector.

Data processing and database searching

MALDI-MS/MS data were processed with GPS Explorer¹⁸ to create Mascot searchable files. MS/MS peak list files for the four salt fractions, which constituted one biological sample pair (control and treated), were combined using Mascot Daemon¹⁹ and searched against the mammalian Swiss-Prot database (V55.0) using the Mascot¹⁹ search engine. β -Methylthiolation [methylthiocysteine] was set as a fixed peptide modification. Variable peptide modifications included methionine (M) oxidation and iTRAQ labeling of the N-terminal, lysine (K) and tyrosine (Y). One missed cleavage site was selected with trypsin/P as the digestion enzyme. ESI-MS/MS precursor and fragment ion mass tolerances were set

to ± 0.1 and ± 0.12 Da, respectively; MALDI-MS/MS precursor and fragment ion mass tolerances were set to ± 0.2 and ± 0.2 Da, respectively.

iTRAQ ratios were generated in Mascot and median normalized. Tyrosine-labeled data was excluded from the iTRAQ ratio calculations. Data sets were further analyzed with Scaffold.²⁰ Only peptides with a probability of 80% or greater as determined by Scaffold were utilized. In the case of duplicate peptides or different charge states, the iTRAQ ratio with the highest Mascot score was used. A minimum of two peptides was required for protein identification.

Statistical methods

An iTRAQ sample consisted of 50 μ g of protein from a control animal pooled with 50 μ g of protein from a CD-treated animal. One animal pair was used for each iTRAQ sample, and four pairs in all were analyzed. Each animal pair was analyzed twice on each instrument to generate technical replicates A and B (Fig. 2(A)). The iTRAQ ratios from the A and B replicates were averaged for every protein with two or more peptides detected in each replicate. If two or more peptides of a protein were detected in only one replicate, that iTRAQ ratio was used in subsequent calculations. Mean iTRAQ protein ratios were generated for each biological pair on the basis of each instrument alone and the average ratios of pairs from both instruments; these mean ratios were then averaged across the biological sample pairs (Fig. 2(B)).

Statistical analyses were conducted with either StatGraphics²¹ or S-Plus.²² Normality of data was assessed based on standardized kurtosis, standardized skewness and homogeneity of variance. Data for iTRAQ ratios was analyzed in logarithm (natural), ln, space. Means and standard deviations are geometric. Student's t-test was used to assess whether the mean iTRAQ ratio for a particular protein was different than 1 (0 in ln space), both within a biological sample pair and across the four biological sample pairs. Two-way ANOVA was used to compare the effect of the factors sample (pair) and instrument (ESI QTOF, MALDI TOF/TOF) on the dependent variable, the ln(iTRAQ ratio). In all cases, a 95% confidence level was the criterion for significance. Grubbs' Test (assumes normality) was applied for outlier identification.

RESULTS AND DISCUSSION

NBD CH showed little change (<10%) in emission characteristics (500–600 nm) over a range of streptomycin concentrations ranging from 0.05–15 μ M in methanol (data not shown). This indicated little interaction between cholesterol and streptomycin in this solvent.

Mass spectrometry has a limited dynamic range, and the cytosolic proteome is complex. Enrichment of the fraction of interest was explored as a means to simplify matters and make the mass spectrometry more tenable. Original enrichment experiments were performed with [¹⁴C]CH as described in the Experimental section. Streptomycin precipitation of cytosol proved to be effective in concentrating the label while at the same time decreasing the amount of protein present (Fig. 3). Clearly the label is enriched in the precipitate (Fig. 3(A)), most of the protein remains in the supernatant (Fig. 3(B)), thus reducing the number of proteins that need to be detected by mass spectrometry.

Approximately 75 proteins were detected with the ESI QTOF and 40 with the MALDI TOF/TOF under the conditions described in the Experimental section (data not shown). The observed difference between the two techniques was probably due to the differences in HPLC methodology. The overall relatively low number of proteins detected probably

resulted from the limited number (4) of isocratic KCl elutions from the SCX spin columns. The proteins detected by each technique and the overlap observed is shown in Fig. 4. All of the proteins detected in the MALDI TOF/TOF were detected in the ESI QTOF.

Wiese *et al.*¹⁵ showed that higher collision energies were needed for efficient liberation of iTRAQ reporter ions from lysine residues on multiply charged peptides. Peptide VFIEDVSK was chosen to illustrate this effect (Fig. 5). Their group also reported a loss of peptide sequence information under the same conditions. They reported good iTRAQ quantification with singly charged peptides and MALDI MS/MS analysis. Figure 5 shows the product ion mass spectra of the iTRAQ-labeled peptide VFIEDVSK generated via ESI (Fig. 5(A)) and MALDI (Fig. 5(B)). The higher collision energy of the MALDI instrument resulted in the 115.1 m/z reporter iTRAQ ion being the base peak. The lower collision energy of the ESI instrument coupled with the fact that the transmission of low m/z ions was not optimized through the instrument resulted in iTRAQ reporter ion intensities well below the base peak (in this case the y_2 ion). Both spectra show extensive b and y ion ladders that indicated efficient fragmentation. Intact iTRAQ ions resulting from the cleavage of the amide bond between the label and the primary amine group without neutral loss of the carbonyl moiety were observed at m/z 145.1.

The question of whether or not iTRAQ data generated using multiply charged peptides and lower collision energies (ESI QTOF) would be comparable to that of singly charged peptides and higher collision energies (MALDI TOF/TOF)¹⁵ was addressed in the present study by performing two-way ANOVA on proteins that were detected in all four biological sample pairs and with both instruments. Specifically, ANOVA was used to determine the extent to which factors sample and instrument affected the $\ln(\text{iTRAQ ratio})$. Proteins detected in all four animal pairs and with both instruments are listed in the tables shown in Figs. 6(A) and 7(A). Figure 6 is comprised of data generated from the QTOF, while, in Fig. 7, data from the MALDI TOF/TOF is shown. The mean iTRAQ ratios and standard deviations generated from both instruments are tabulated in Figs. 6(A) and 7(A). Mean iTRAQ ratios and the 95% confidence intervals are plotted in Figs. 6(B) and 7(B), respectively. The mean and standard deviation (SD) of the iTRAQ ratio for each protein in each pair are geometric and were generated from the two technical replicates generated for each pair (Fig. 2(A)). The SDs could clearly be improved with more technical replicates, however, cost considerations precluded generation of larger data sets for this study. Figure 8(A) shows the mean iTRAQ values and the 95% confidence intervals after the QTOF and TOF/TOF data were combined and then averaged across all four pairs. This data is plotted in Fig. 8(B). Myosin 9 was the only protein with an observed mean iTRAQ ratio significantly different than 1. Its possible role in vesicular intracellular transport²⁴ will be addressed in future work.¹⁶

Volcano plots are commonly used in the microarray arena to compare the statistical significance with the magnitude of the change in expression level. Figure 9 shows a volcano plot for the data in Fig. 8. For comparison a volcano plot of all the proteins detected by both instruments in three or more biological samples is shown in Fig. 10. In these plots the ordinate shows the $-\log_{10}$ of the p -value for the t-test of whether or not the iTRAQ ratio is different than 1. Higher values represent smaller p -values. The magnitude of the change of the iTRAQ ratio is plotted on the abscissa as the $\log_2$ of the mean iTRAQ ratio. Proteins that are significantly different than 1 are labeled. Overall, only four proteins showed a mean iTRAQ ratio significantly different than 1.

Table 1 shows the results of performing ANOVA on the tabulated data presented in Figs. 6 and 7; the p -values associated with each factor for each protein are given at the 95% confidence level. Clearly, the sample has a significant effect on the $\ln(\text{iTRAQ})$ ratio for

more than half of the proteins. This could be due to the biological variability inherent in the samples themselves. In contrast, the *p*-values for the instrument factor are greater than 0.05 in all cases, indicating that the instruments had no significant effect on the ln(iTRAQ ratio).

It appears, therefore, that iTRAQ analyses performed on an ESI QTOF without any special modifications of the instrumental parameters produce essentially the same protein ratios as obtained on a MALDI TOF/TOF. Although this observation needs to be tested with a larger data set produced with other instruments, it indicates that it should be possible in general to compare iTRAQ data obtained from these two types of instruments.

Acknowledgments

This work was supported in part by the Environmental Health Sciences Center (2P30ES000210), the Oregon Agricultural Experiment Station (ORE00871), and grant number T32 ES007060 from the National Institutes of Health.

Contract/grant sponsor: Environmental Health Sciences Center; contract/grant number: 2P30ES000210.

Contract/grant sponsor: The Oregon Agricultural Experiment Station; contract/grant number: ORE00871.

Contract/grant sponsor: National Institutes of Health; contract/grant number: T32 ES007060.

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Figure 1.

Flow chart showing the sequence of iTRAQ labeling and MS analysis. A total of four animal pairs were analyzed.

Figure 2.

Flow chart showing how iTRAQ samples are analyzed: (A) breakdown for one biological sample and (B) three procedures used for averaging the iTRAQ protein ratios over all four biological samples.

Figure 3.

Enrichment of $[^{14}\text{C}]\text{cholesterol}$ and decrease in protein observed in the supernatant and streptomycin precipitate of hepatic cytosol: (A) cholesterol equivalents/mg protein versus chlordecone treatment and streptomycin fraction ($n=3$ pooled samples, bar heights=mean of three liquid scintillation runs) $\pm$ SD and (B) cytosolic protein recovery versus chlordecone treatment and streptomycin fraction $n=3$ pooled samples; one analysis was done for each pooled sample therefore no error bars are included.

Figure 4.
Number of proteins detected by ESI-QTOF and MALDI TOF/TOF.

Figure 5.

Mass spectra of the iTRAQ-labeled peptide VFIEDVSK: (A) spectrum generated on the ESI TOF from the doubly charged precursor and (B) spectrum generated on the MALDI TOF/TOF. The fragment ions are labeled according to the nomenclature of Beermann.²³ The insets in the spectra show enlargements of the reporter ion region.

Figure 6. Proteins detected with the ESI QTOF in all four animal pairs. Means and standard deviations (SDs) are geometric and were generated from the two technical replicates run for each pair. (A) Mean iTRAQ ratios and SDs. (B) Plots of mean iTRAQ ratios and 95% confidence intervals.

Figure 7. Proteins detected with the MALDI TOF/TOF in all four animal pairs. Means and standard deviations (SDs) are geometric and were generated from the two technical replicates run for each pair. (A) Mean iTRAQ ratios and SDs. (B) Plots of the mean iTRAQ ratios (bold triangles) and 95% confidence intervals.

Figure 8.

Mean iTRAQ values and the 95% confidence intervals after the instrument data were combined and then averaged across all four pairs. (A) Calculated values. Ratios significantly different than one are shown in bold font and (n) is the number of sample pairs used in the calculation of the geometric mean. (B) Plots of the data including the mean iTRAQ ratios (bold triangles) and the associated confidence levels.

Figure 9. Volcano plot of data shown in Fig. 8. Myosion 9 was the only protein observed to have a mean iTRAQ ratio significantly different than 1.

Figure 10.

Volcano plot of all detected proteins from the combined QTOF and TOF/TOF data present in at least three of the biological samples. The four proteins that exhibited mean iTRAQ ratios significantly different than 1 are labeled.

p-values associated with the factors sample and instrument at the 95% confidence level generated by ANOVA on the data shown in Figs. 6 and 7. The asterisks indicate *p*-values less than 0.050

Table 1

ANOVA (Multifactor)			
NCBI Accession Number	Protein	<i>p</i> -Value (Sample)	<i>p</i> -Value (Instrument)
P60710	Actin, cytoplasmic 1	0.011*	0.162
P00329	Alcohol dehydrogenase 1	0.022*	0.261
Q91Y97	Fructose-bisphosphate aldolase B	0.073	0.060
Q61176	Arginase-1	0.049*	0.166
P16460	Argininosuccinate synthase	0.030*	0.747
O35490	Betaine-homocysteine S-methyltransferase 1	0.025*	0.953
P12710	Fatty acid-binding protein, liver	0.199	0.715
P16858	Glyceraldehyde-3-phosphate dehydrogenase	0.195	0.629
P30115	Glutathione S-transferase A3	0.120	0.550
P10649	Glutathione S-transferase Mu 1	0.152	0.443
P19157	Glutathione S-transferase P 1	0.009*	0.157
P01942	Hemoglobin subunit alpha	0.006*	0.994
Q8VDD5	Myosin-9	0.019*	0.905
P35700	Peroxioredoxin-1	0.863	0.707
P08228	Superoxide dismutase [Cu-Zn]	0.239	0.950
P68369	Tubulin alpha-1A chain	0.006*	0.556
P68372	Tubulin beta chain	0.003*	0.355