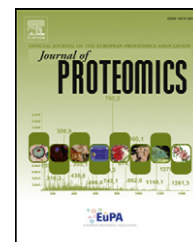


Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jprot

Effects of sample injection amount and time-of-flight mass spectrometric detection dynamic range on metabolome analysis by high-performance chemical isotope labeling LC-MS[☆]

Ruokun Zhou, Liang Li^{*}

Departments of Chemistry, University of Alberta, Edmonton, Alberta, Canada

ARTICLE INFO

Available online 16 August 2014

Keywords:

Dynamic range of detection
Isotope labeling
LC-MS
TOF-MS
Metabolite
Metabolomics

ABSTRACT

The effect of sample injection amount on metabolome analysis in a chemical isotope labeling (CIL) liquid chromatography–mass spectrometry (LC-MS) platform was investigated. The performance of time-of-flight (TOF) mass spectrometers with and without a high-dynamic-range (HD) detection system was compared in the analysis of $^{12}\text{C}_2/^{13}\text{C}_2$ -dansyl labeled human urine samples. An average of 1635 ± 21 ($n = 3$) peak pairs or putative metabolites was detected using the HD-TOF-MS, compared to 1429 ± 37 peak pairs from a conventional or non-HD TOF-MS. In both instruments, signal saturation was observed. However, in the HD-TOF-MS, signal saturation was mainly caused by the ionization process, while in the non-HD TOF-MS, it was caused by the detection process. To extend the MS detection range in the non-HD TOF-MS, an automated switching from using ^{12}C to ^{13}C -natural abundance peaks for peak ratio calculation when the ^{12}C peaks are saturated has been implemented in IsoMS, a software tool for processing CIL LC-MS data. This work illustrates that injecting an optimal sample amount is important to maximize the metabolome coverage while avoiding the sample carryover problem often associated with over-injection. A TOF mass spectrometer with an enhanced detection dynamic range can also significantly increase the number of peak pairs detected.

Biological significance

In chemical isotope labeling (CIL) LC-MS, relative metabolite quantification is done by measuring the peak ratio of a $^{13}\text{C}_2/^{12}\text{C}_2$ -labeled peak pair for a given metabolite present in two comparative samples. The dynamic range of peak ratio measurement does not need to be very large, as only subtle changes of metabolite concentrations are encountered in most metabolomic studies where relative metabolome quantification of different groups of samples is performed. However, the absolute concentrations of different metabolites can be very different, requiring a technique to provide a wide detection dynamic range to allow the detection of as many peak pairs as possible. In this work, we demonstrated that controlling the sample injection amount into LC-MS was critical to achieve the optimal detectability while avoiding sample carry-over problem. In addition, the use of a high-dynamic-range

[☆] This article is part of a Special Issue entitled: Protein dynamics in health and disease. Guest Editors: Pierre Thibault and Anne-Claude Gingras.

^{*} Corresponding author at: Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada. Tel.: +1 780 492 3250; fax: +1 780 492 8231.

E-mail address: Liang.Li@ualberta.ca (L. Li).

TOF system increased the number of peak pairs detected, compared to a conventional TOF system. We also investigated the ionization and detection saturation factors limiting the dynamic range of detection.

This article is part of a Special Issue entitled: Protein dynamics in health and disease. Guest Editors: Pierre Thibault and Anne-Claude Gingras.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Because of the great diversity of chemophysical properties and more than 10^7 -fold concentration differences of metabolites in a metabolomic sample, comprehensive metabolome profiling with an extensive metabolome coverage remains to be a major analytical challenge [1]. While chemophysical properties can be altered by chemical derivatization to achieve some uniformity with the use of a proper labeling reagent (e.g., attaching a hydrophobic aromatic ring to ionic or very hydrophilic metabolites to make them relatively hydrophobic so that they can be retained and separated by reversed phase liquid chromatography) [2,3], the issue of concentration differences is difficult to address. Even after multidimensional fractionation and separation, a resultant fraction, albeit less complex than the original sample, can still contain metabolites with a wide range of concentrations [4]. Thus, analytical techniques offering a wide dynamic range of detection are needed in order to generate a more comprehensive profile of a metabolome.

Chemical isotope labeling (CIL) LC–MS is a technique that can provide improved detection and quantification for metabolomic applications [2,3,5–14]. We have been working on the development of a high-performance CIL LC–MS platform for quantitative and comprehensive submetabolome profiling using rationally designed isotope reagents to improve metabolite separation, detection and quantification simultaneously [2,3,15,16]. In CIL LC–MS, an individual sample is first labeled with a light-chain isotope reagent (e.g., $^{12}\text{C}_2$ -dansyl chloride for labeling amines and phenols) [2], while a pooled sample prepared from all the individual samples is labeled with a heavy-chain isotope reagent (e.g., $^{13}\text{C}_2$ -dansyl chloride). The light-labeled sample is then mixed with the heavy-labeled pooled sample to form a mixture for LC–MS analysis. The labeled metabolites show up in peak pairs in a mass spectrum. The peak intensity ratio of a peak pair is used to determine the relative concentration of the metabolite in a given individual sample vs. the pooled sample. However, due to a limited detection dynamic range of a mass spectrometer, any co-eluting metabolites with different concentrations may not fall within the detection range; some metabolites' concentrations are below the limit of detection while the very high abundance metabolites produce saturated signals. Thus, controlling the sample injection amount is very important in metabolome profiling. It is critical to inject a proper amount of sample into LC–MS in order to maximize the number of metabolites detectable while not causing problems such as signal distortion and sample carryover from one injection to another. In this work, we report a detailed study of the effect of sample injection amount on metabolomic analysis in CIL LC–MS. In addition, a time-of-flight (TOF) mass spectrometer equipped with and without a high-

dynamic-range (HD) detection system was evaluated and compared to determine how the detection dynamic range affects the detectability of peak pairs.

2. Material and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich Canada (Markham, ON, Canada) except those otherwise noted. The synthesis of $^{13}\text{C}_2$ -dansyl chloride has been reported [2]. LC–MS grade of water, methanol and acetonitrile (ACN) were purchased from Thermo Fisher Scientific (Edmonton, AB, Canada).

2.2. Sample preparation

The study of human urine samples was approved by the Ethics Approval Board of the University of Alberta. Human urine sample collection and processing were performed according to a previously reported protocol [16]. Briefly, the second morning urine sample was collected from a healthy volunteer. Within 1 h of urine collection, the urine sample was centrifuged at $2665 \times g$ for 10 min. The supernatant was filtered twice by $0.22 \mu\text{m}$ -pore-size Millipore filter (Millipore Corp., MA) and aliquoted into 0.5 mL vials. The urine sample was stored at -80°C . Dansylation labeling of standards and urine samples was done as described before [16]. In the study of using ^{13}C -natural abundance peaks for peak ratio calculation, 10 mM dansylated methionine (DnsMet) stock solutions was prepared by dansylation of methionine with either $^{12}\text{C}_2$ - or $^{13}\text{C}_2$ -dansyl chloride. $^{12}\text{C}_2$ -DnsMet was mixed with $^{13}\text{C}_2$ -DnsMet in ACN/ H_2O (50/50, v/v) at different concentration ratios. For the urine sample analysis, two 50 μL aliquots of the same urine were taken and labeled separately by $^{13}\text{C}_2$ -dansylation and $^{12}\text{C}_2$ -dansylation. The labeled solutions were mixed to produce a mixture of 1:1 molar ratio of $^{13}\text{C}_2$ - and $^{12}\text{C}_2$ -dansyl labeled urines.

2.3. LC–UV

An LC–UV method previously reported [17] was used to measure the total concentration of labeled metabolites in urine. Briefly, the $^{12}\text{C}_2$ -dansyl labeled urine was injected into LC–UV for absorbance measurement at 338 nm. A calibration curve prepared by LC–UV analyses of varying concentrations of a mixture of 17 dansyl labeled amino acid standards was used to quantify the labeled metabolites in urine. LC–UV was performed using a Waters ACQUITY UPLC system with a photodiode array (PDA) detector. Two microliters of the labeled sample was injected onto a Waters ACQUITY BEH

C18 column (2.1 mm × 5 cm, 1.7 μm particle size, 130 Å pore size) for a fast step-gradient run. Solvent A was 0.1% (v/v) formic acid in 10% (v/v) ACN, and solvent B was 0.1% (v/v) formic acid in ACN. The gradient started with 0% B for 1 min and was increased to 95% within 0.01 min and held at 95% B for 1 min to ensure complete elution of all the labeled metabolites. The gradient was restored to 0% B in 0.5 min and held at this condition for 3.5 min to re-equilibrate the column. The flow rate used was 0.45 mL/min.

2.4. LC-MS

LC-MS analysis was performed using an Agilent 1100 Series Binary LC System (Agilent Palo Alto, CA) connected to a quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker, Billerica, MA). All data were acquired in the positive ion mode. The Q-TOF ion source conditions were: nitrogen nebulizer gas: 1.8 bar, dry gas flow: 8.0 L/min, dry temperature: 230 °C, capillary voltage: 4500 V, end plate offset: 500 V, mass range: m/z up to 1000, spectra rate: 1 Hz, funnel 1: 250 Vpp, funnel 2: 150 Vpp, hexapole: 110 Vpp, quadrupole ion energy: 3.0 eV, and quadrupole low mass: m/z 100. The resolving power of the instrument was typically about 28,000 FWHM at m/z ~500. All chromatographic separations were performed on an Agilent Zorbax Eclipse Plus C18 (2.1 mm × 100 mm, 1.8 μm, New Castle, DE) column. The mobile phase A was 0.1% formic acid in ACN/H₂O (5/95, v/v) and the mobile phase B was 0.1% formic acid in ACN. The 25-min gradient conditions were: 0 min (20% B), 0–3.5 min (20–35% B), 3.5–18 min (45–65% B), 18–21 min (65–95% B), 21–24 min (95–99% B), and 24–28 min (99% B). The column was re-equilibrated with the initial mobile phase conditions for 15 min before injecting the next sample. The flow rate was 180 μL/min and the injection volume was 2.0 μL except in the injection volume study (see Results and discussion).

2.5. Conventional and high-dynamic-range TOF-MS

The Bruker Q-TOF instrument used in this work had two different TOF detection systems. The original instrument was similar to many conventional TOF systems that used an 8-bit digitizer for ion detection. This instrument was then upgraded with a high-dynamic-range (HD) TOF system, which uses a 10-bit digitizer. As a user, one noticeable difference between the two systems is that the upper limit of the ion count increases from $\sim 5 \times 10^6$ counts in the conventional TOF to $\sim 2 \times 10^7$ counts in the HD-TOF.

2.6. Data analysis

LC-MS data were processed using IsoMS [18] a software developed in our laboratory that is freely available from the MyCompoundID.org website. The raw data generated from one or multiple LC-MS runs were automatically processed through peak picking, peak pairing, peak-pair filtering and peak-pair intensity ratio calculation. In peak-pair filtering, only MH^+ ions of the dansyl-labeled metabolites were retained while other forms, including adduct ions from Na^+ , K^+ or NH_4^+ , dimers and other multimers, and in-source fragment ions (usually minus CO_2), were removed. Background and blank peak pairs were also

removed. Thus, a peak pair detected by IsoMS should represent a putative metabolite. Only the high confident, level 1 peak pairs (i.e., all labeled peaks are accompanied with their corresponding ^{13}C -natural abundance peaks) were selected by IsoMS from the LC-MS data. The same peak pairs detected from multiple samples were then aligned using IsoMS to produce a CSV file that contained the metabolite information and peak ratios relative to a control.

2.7. False positive rate determination

To determine the false positive rate (FPR) of peak-pair picking, during the development of IsoMS, all the data from test runs of standards and biofluid samples were manually checked so that the peak pairs picked manually could be compared to those picked by IsoMS. It was found that the FPR was usually less than 5% [18]. However, the manual inspection method is very time consuming and laborious. To improve the efficiency of peak-pair validation, we have developed a novel method of using the intensity ratio to quickly differentiate the false pairs from the correctly picked pairs.

In this method, in a labeled mixture prepared by combining two identical aliquots but labeled by ^{12}C - and ^{13}C -reagents, respectively, the theoretical intensity ratio for each peak pair detected in a mass spectrum should be 1.0. Considering the possible experimental errors and technical variations, the range of the ratio deemed to be acceptable should be 0.67–1.5, i.e.,

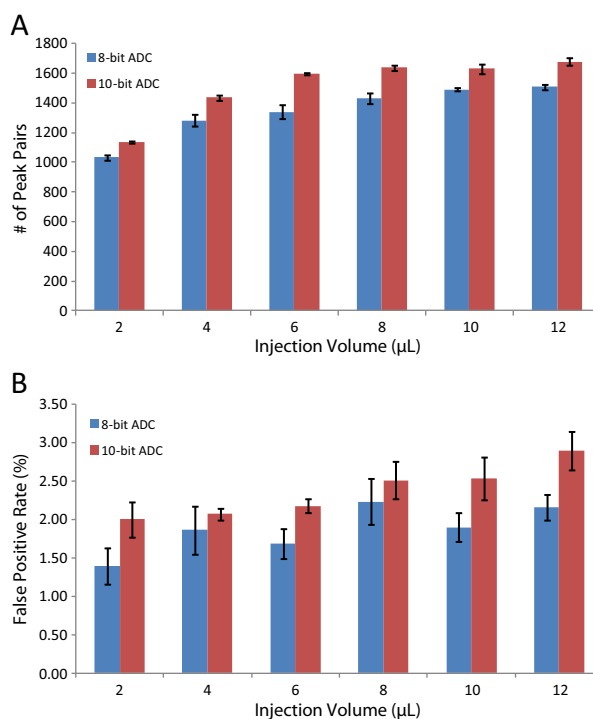


Fig. 1 – (A) Number of peak pairs detected from $^{13}C_2$ / $^{12}C_2$ -dansyl labeled human urine (4.79 mM) as a function of injection volumes. (B) False positive rate (FPR) as a function of injection volumes. The left-bar data sets were obtained using a TOF system equipped with a 8-bit analog-to-digital converter (ADC), while the right-bar data sets were obtained using an upgraded high-dynamic-range (HD) TOF system equipped with a 10-bit ADC.

within the variation of $\pm 50\%$ of the mean (i.e., 1.0). Note that this range is also used as the threshold to analyze the biological variations in real world metabolomic applications (i.e., peak pairs with ratios beyond this range are considered to be caused by biological variations). Any pair with an intensity ratio outside this range when analyzing a 1:1 molar ratio mixture of ^{12}C - and ^{13}C -labeled samples is regarded as a false pair and the number of false pairs can be used to calculate the false positive ratio by using an equation, n/m , where n is the number of peak pairs found outside the range and m is the total number of peak pairs found by IsoMS. From the manual check of many datasets we have generated from $^{13}\text{C}_2$ -/ $^{12}\text{C}_2$ -dansyl labeled biological samples, almost all the peak pairs found within the range of 0.67–1.5 were the correct pairs. In addition, a fraction of peak pairs outside the range were actually correct. Thus, the FPR calculated using this method represents the upper limit; the actual FPR may be lower.

3. Results and discussion

Quantitative metabolome profiling is a key step in applying metabolomics for biological studies and disease biomarker discovery where the metabolomes of two or more groups of samples are compared. To determine the significant

metabolites that cause the separation of the groups, relative concentration differences of individual metabolites in all comparative samples are measured and then compared using statistical tools. Since only relative quantification is needed, differential chemical isotope labeling LC–MS is particularly suitable for quantitative metabolome profiling. The dynamic range for relative quantification of a metabolite present in different samples does not need to be very large. CIL LC–MS can measure a concentration difference of up to 10-fold relative to a pooled sample accurately based on peak ratio analysis of a given peak pair [2,3,16], which is adequate for most metabolomic applications where only small changes (i.e., <10 -fold) are observed; relative concentration changes of >10 -fold among the comparative samples are rare. However, the absolute concentration differences of co-eluting labeled metabolites can be very large. For a given instrument, only a fixed concentration range of analytes can be detected within a mass spectrum. As a consequence, some peak pairs are below the detection limit while the peak pairs from the high abundance metabolites reach the upper limit, resulting in saturated signals. Thus, even though only relative metabolite quantification of a narrow range of fold changes is performed in CIL LC–MS, detecting all the peak pairs of co-eluting labeled metabolites is still very important for comprehensive metabolome analysis.

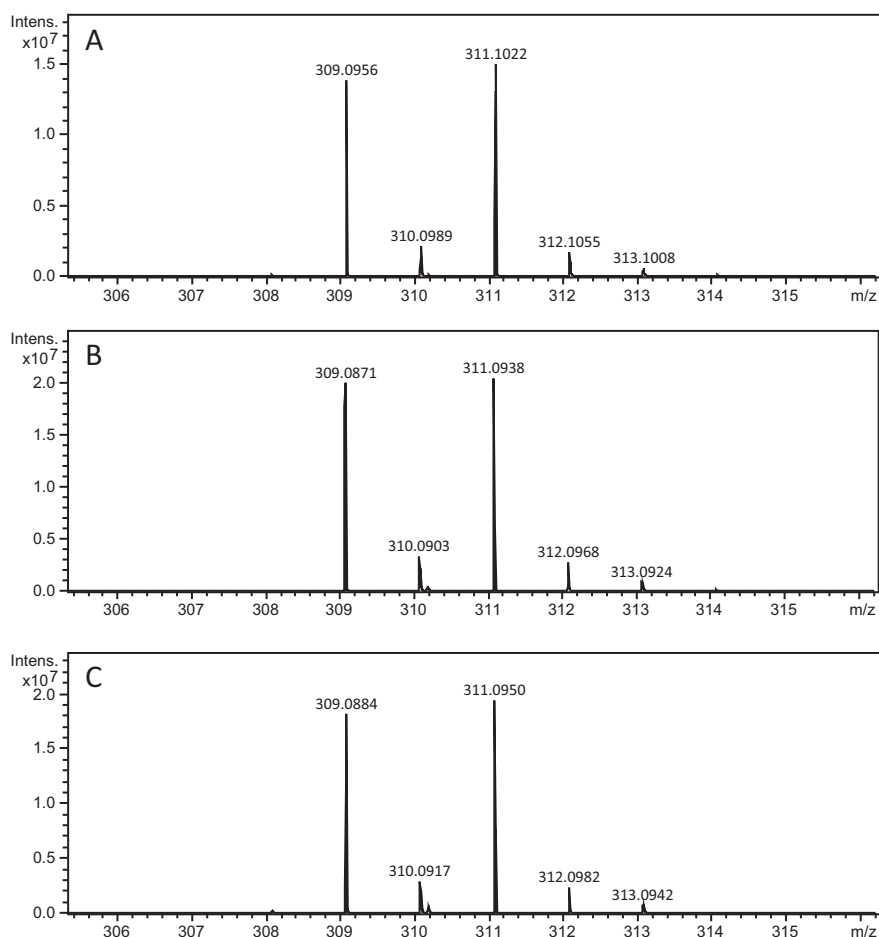


Fig. 2 – Expanded mass spectra of a peak pair obtained from the labeled human urine sample by using the HD-TOF-MS with an injection volume of (A) 2 μL , (B) 8 μL and (C) 12 μL .

There are a number of factors that can affect the overall detection dynamic range. Using a larger size of LC column can accommodate more analytes for separation. The ionization process, such as the electrospray setting, can impose a limit on the number of ions that can be produced. The detection range of a mass spectrometer can also be a limiting factor. Among the experimental parameters associated with the separation, ionization and detection processes, sample injection amount is the only parameter that a user can control after a LC-MS system has been set up. In this work, the performance of two TOF systems on peak pair detectability was evaluated and compared using different amounts of sample injection.

Because of the difficulty of measuring the total concentration of metabolites present in a metabolomic sample, it is often not possible to control the sample injection amount in a conventional LC-MS metabolomic profiling workflow. However, in CIL LC-MS, all the metabolites in a group-based submetabolome are labeled with a rationally designed reagent. For example, dansyl chloride is used for amine/phenol submetabolome analysis. The dansyl group in each labeled metabolite provides an absorption at 338 nm. Thus, a LC-UV method can be used to measure the total concentration of the labeled metabolites in a sample [17]. In this method, a step-gradient LC is used to remove the interference of dansyl-OH

formed from the excess amount of reagents that was quenched by NaOH after labeling [17].

In this work, a mixture of 1:1 molar ratio of $^{13}\text{C}_2$ - and $^{12}\text{C}_2$ -dansyl labeled human urines was prepared and analyzed by LC-MS. The total concentration of the dansyl labeled metabolites in this sample was determined to be 4.79 mM by LC-UV using a calibration curve of 17-dansyl-amino-acid-standards. This corresponded to 20.1 mM of the original urine sample after considering 4.2-fold dilution by the dansyl labeling process. The injection volume of this mixture, reflecting the injection amount, was varied from 2 μL to 12 μL at a 2- μL increment. The number of peak pairs detected at different volumes of sample injection and the corresponding false discovery rate of peak pairs are shown in Fig. 1 for the conventional TOF-MS and HD-TOF-MS detection systems. As Fig. 1A shows, the peak pair number increases as the injection volume increases and the number levels off when the volume is 8 μL or higher. However, the HD system detected more peak pairs compared to the conventional TOF system. For example, in the case of 8 μL injection, an average of 1635 ± 21 ($n = 3$) peak pairs or putative metabolites were detected in the HD-TOF, compared to 1429 ± 37 peak pairs in the conventional TOF, representing an average of 14.4% increase. As Fig. 1B shows, the false positive rates for the HD-TOF are slightly higher, possibly due to higher FPRs among

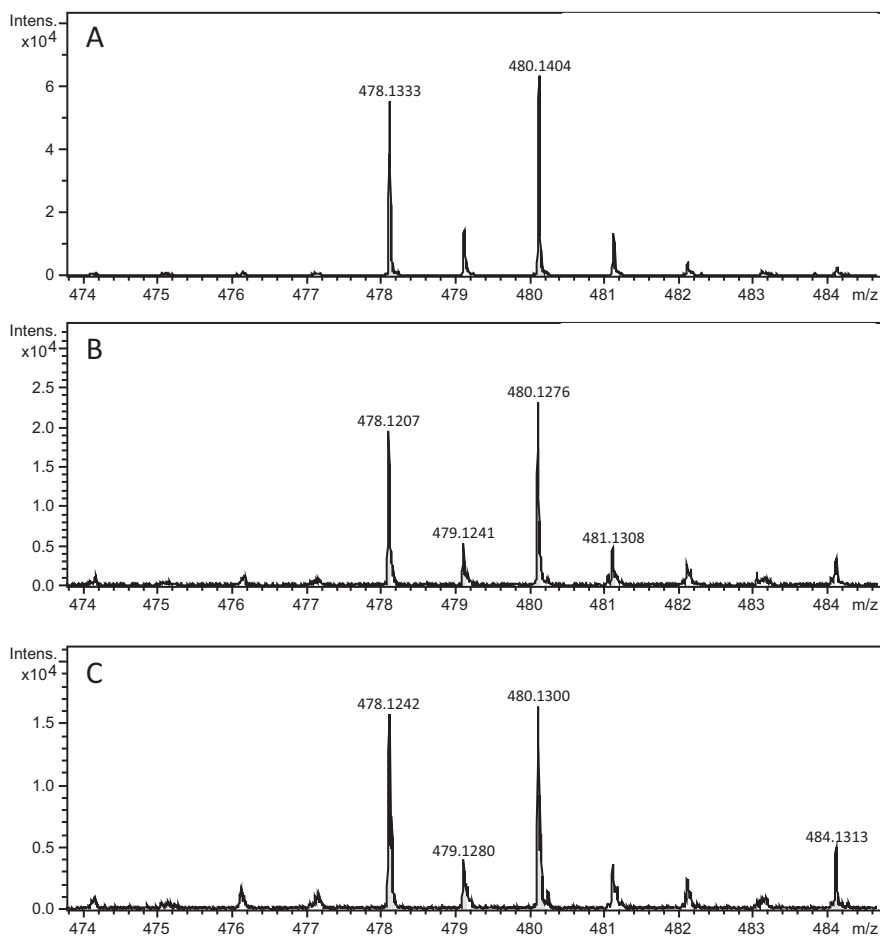


Fig. 3 – Expanded mass spectra of a low-abundance peak pair co-eluting with the peak pair shown in Fig. 2. The spectra were obtained from the labeled human urine sample by using the HD-TOF-MS with an injection volume of (A) 2 μL , (B) 8 μL and (C) 12 μL .

the additional peak pairs detected by the HD-TOF; but all are well below 5%. It should be noted that over-injection can cause problems with sample carry-over. In our setup, injecting the labeled urine sample of up to 12 μL did not cause the carry-over problem after one cycle of washing before the next sample injection. However, we found that injecting more than 12 μL of this 4.79 mM labeled urine sample required extensive column cleaning, which would reduce the sample throughput and potentially reduce the column life time. Moreover, injection of more than 12 μL (up to 24 μL) did not increase the number of peak pairs detected in the HD-TOF (see Supplemental Fig. S2).

To better understand the results shown in Fig. 1 and potentially develop an improved means of expanding the detectability of peak pairs, we examined the datasets obtained more carefully. Fig. 2 shows a series of mass spectra obtained from injecting different volumes of samples in the HD system (i.e., 2, 8 and 12 μL). The four peaks within a peak pair including the ^{13}C -natural abundance peaks increase slightly from 2 to 8 μL injection, and then do not change from 8 to 12 μL injection. This type of behavior suggests that the ionization process for generating these metabolite ions from the 8 and 12 μL injections has been saturated. The ESI process can be saturated when the analyte charges on a droplet surface reach a limit [19]. Any further increase in analyte concentration will not result in an increase in analyte

charge numbers on the surface. Hence the number of gaseous ions produced from the droplet reaches the maximum when a certain concentration limit is reached. This ionization saturation effect does not affect the relative quantification of a differentially labeled metabolite in the mixture. The peak intensity ratio of the peak pair still represents the relative concentration of the metabolite in two comparative samples, although the absolute intensity is no longer proportional to the absolute analyte concentration. However, the intensity of low abundance ions of the co-eluting metabolites cannot be enhanced even when more sample is injected. In fact, some of the co-eluting metabolites may reduce their signals due to increased ion suppression from the high abundance metabolites. This is illustrated in Fig. 3 for a low abundance peak pair (m/z 478/480) co-eluted with the m/z 309/311 peak pairs shown in Fig. 2. In this case, no more ions can be moved to the surface and thus the number of the low abundance metabolite ions cannot be increased by injecting a larger amount of samples. In fact, ion suppression from the high abundance ions is present and apparently increases as the injection volume increases. To overcome the ion suppression effect, collecting the fraction and then subjecting it to an orthogonal mode of separation to remove the high abundance components would be needed. Nevertheless, the peak ratio of this low abundance peak pairs can still be determined. However,

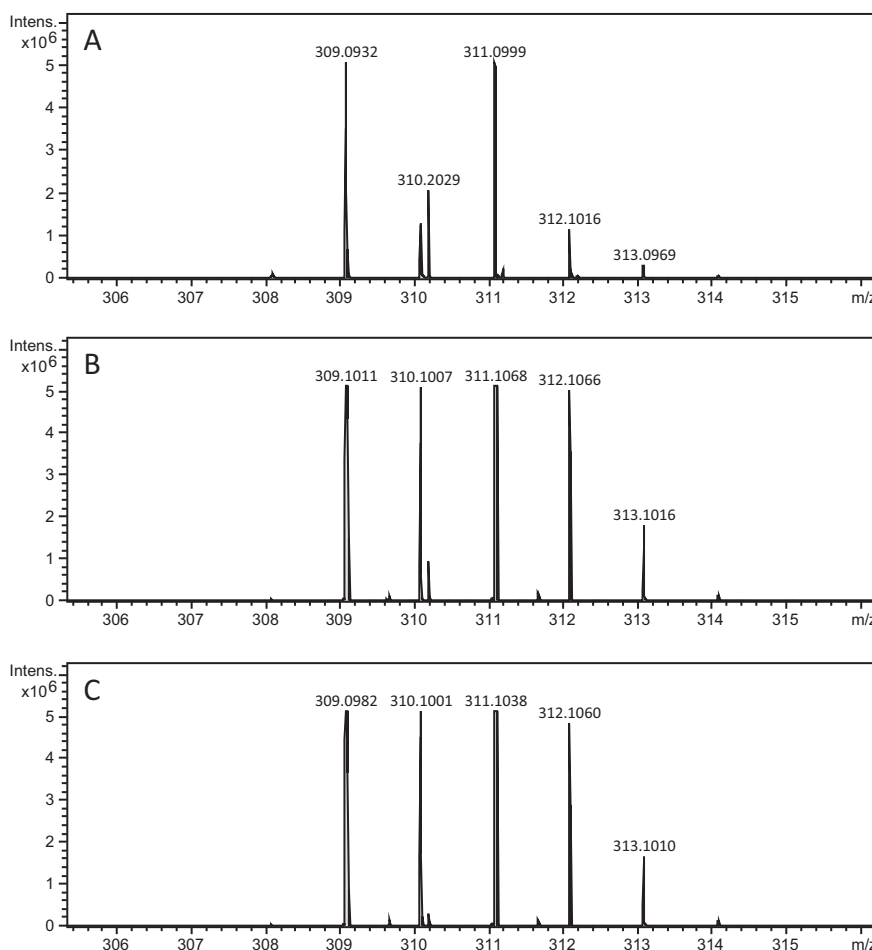


Fig. 4 – Expanded mass spectra of a peak pair obtained from the labeled human urine sample by using the conventional TOF-MS with an injection volume of (A) 2 μL , (B) 8 μL and (C) 12 μL .

many other even lower abundance co-eluting metabolites were likely present and they were not detectable.

Fig. 4 shows a series of mass spectra obtained from injecting different volumes of samples in the non-HD system. The two main peaks within a peak pair (m/z 309/311) become saturated when the injection amount increases from 2 μ L to 8 μ L and their corresponding ^{13}C -natural abundance peaks (m/z 310/312) are also approaching saturation. As Fig. 4A shows, for the 2 μ L injection, the main peaks are already saturated, but the ^{13}C -natural abundance peaks are not. For the 8 or 12 μ L injection (Fig. 4B, C), the main peaks are saturated while the initially unsaturated ^{13}C -natural abundance peaks start to become saturated. The maximum count is 5×10^6 in this non-HD system. With 8 or 12 μ L injection, the number of ions produced from the ionization process exceeds the number of ions that can be counted for by the TOF system. In contrast, the HD system has the maximum count of 2×10^7 . With 8 or 12 μ L injection, the maximum number of ions produced in the ionization process was less than the maximum

counts the HD system can handle. It should be noted that the absolute counts of the two systems are not necessary equivalent to each other; the ion transmission (a larger pump was installed for the HD system) and the electronics are different. In any case, we found that there were rare or no saturated peaks for the ^{13}C -natural abundance peaks in the HD system. Thus, for this high-dynamic-range TOF instrument, detection saturation is not the limiting factor. Instead, ionization saturation is the limiting factor.

The consequence of detection saturation found in the non-HD system is that we reach the upper limit earlier than the HD system and, as a result, we cannot determine the peak ratio on the saturated main peaks. Since many TOF systems currently in use do not use the HD detection, it is desirable to find a way to expand the upper limit for peak pair analysis. We have examined the possibility of using ^{13}C -natural abundance peaks for ratio calculation as a means of expanding the upper limit. The premise was that within a peak pair, the ^{12}C -natural abundance peak may be fully saturated, but the ^{13}C -companion

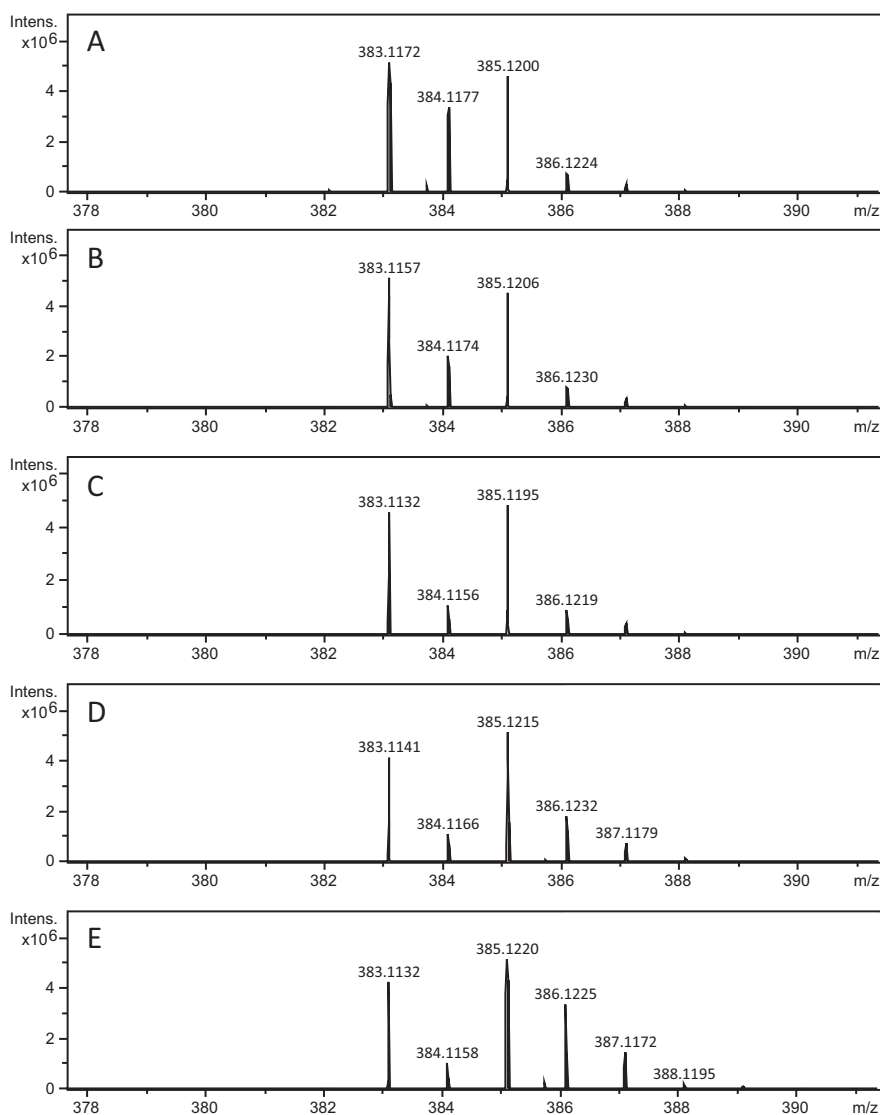


Fig. 5 – Expanded mass spectra of a peak pair obtained by LC-MS analysis of a series of mixtures with varying concentration ratios of $^{12}\text{C}_2$ - and $^{13}\text{C}_2$ -dansyl labeled Met: (A) 4:1, (B) 2:1, (C) 1:1, (D) 1:2, and (E) 1:4.

peak may not be saturated. Because the molecular weights of most of metabolites are below 600 Da, the ^{13}C -natural abundance peak is less than 50% of the ^{12}C peak. In other words, if we use the ^{13}C -natural abundance peaks within a pair for peak ratio measurement, instead of the saturated ^{12}C -natural abundance peaks, we could still perform relative quantification for the peak pair. This could result in an increase in the number of peak pairs quantifiable by CIL LC-MS. We have designed a set of experiments to test this hypothesis.

Fig. 5 shows the expanded mass spectra obtained by LC-MS analysis of a series of mixtures with varying concentration ratios of $^{12}\text{C}_2$ -dansyl labeled Met and $^{13}\text{C}_2$ -dansyl labeled Met (4:1, 2:1, 1:1, 1:2, 1:4). The ^{12}C -natural abundance peaks (m/z 383/385) are saturated, but the ^{13}C -natural abundance peaks (m/z 384/386) are not. Thus, the peak pair ratio determined from the ^{12}C -natural abundance peaks is close to 1 in all the mass spectra shown in Fig. 5 and cannot be used to determine the actual concentration changes. However, the peak pair ratio determined from the ^{13}C -natural abundance peaks follows the concentration changes. The linear regression curve between the measured peak ratio from the ^{13}C -natural abundance peak pair (triplicate runs, $n = 3$) and the expected ratio was found to be linear (see Supplemental

Fig. S1), indicating that even though the ^{12}C -peak is saturated, its corresponding ^{13}C -natural abundance peak can still be used to calculate the peak ratio within a peak pair and thus determine the relative concentration of one sample to the other. Note that the dynamic range for relative quantification is reduced when one or two of the main peaks are saturated, compared to the situation where no peak saturation is present with a peak pair. The closer the ^{13}C -natural abundance peaks to the upper count limit the narrower this range is. In the extreme case where both peaks are saturated (Fig. 3B, C), the peak ratio cannot be used to reflect the relative concentration of a labeled metabolite in two comparative samples.

We have implemented an automated peak selection feature in IsoMS for processing the CIL LC-MS data. This software can be downloaded from the MyCompoundID.org website. IsoMS switches from the use of ^{12}C -natural abundance peak to its ^{13}C -companion peak for peak ratio calculation, when the ^{12}C peak is above the upper limit of the ion counts. By comparing the number of peak pairs obtained with and without using the ^{13}C -natural abundance peak pair for ratio calculation, we found that using the method the number of quantifiable peak pairs could be increased by about 3% for all the injected volumes. Note that the peak pair numbers

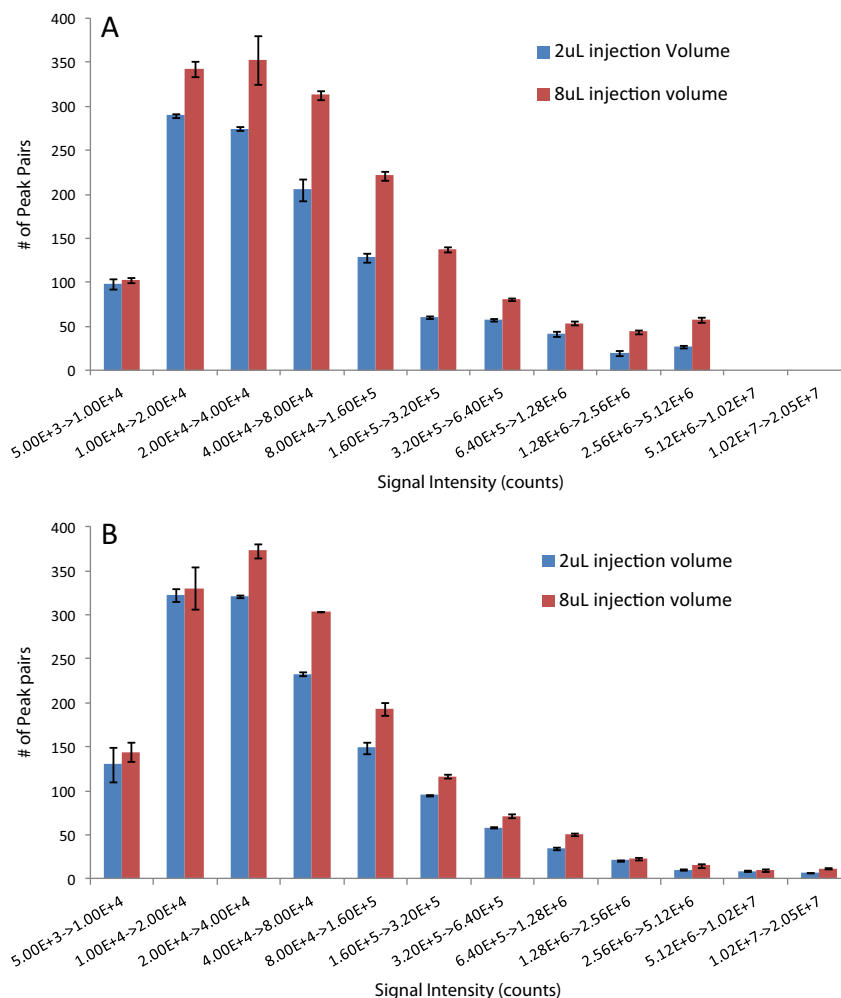


Fig. 6 – Number of peak pairs detected at different ranges of ion counts using (A) the conventional TOF-MS and (B) the HD-TOF-MS.

shown in Fig. 1A were determined using this method. In future work, we will examine the accuracy of peak ratio calculation based on the use of ^{13}C -natural abundance peaks for real samples.

Finally, to gauge the overall detection dynamic range of the two TOF systems for CIL LC–MS, we plotted the number of peak pairs as a function of their absolute intensities (see Fig. 6) from the 2 and 8 μL injections. In both systems, the low limit of ion counts was governed by the chemical noises present in LC–MS analysis of labeled metabolites. Since only the highly confident, level 1 peak pairs (i.e., in each pair, both ^{13}C -natural abundance peaks of the ^{13}C -/ ^{12}C -labeled metabolites are present) were picked, all the main peaks have signal-to-noise ratios of above 40. The effective dynamic range of the non-HD system is from 5×10^3 to 5×10^6 counts, while the dynamic range is from 5×10^3 to 2×10^7 counts for the HD system. To generate the plots shown in Fig. 6, we grouped the peak pairs into 10 bins for the non-HD system and 12 bins for the HD system. Each bin covers a 2-fold ion count range. As Fig. 6 shows, 8 μL injection increases the peak pair numbers in all bins compared to the 2 μL injection except the 1st bin in the non-HD system and the 1st and 2nd bins in the HD system.

Because the dansyl labeled metabolites have more uniform ionization efficiencies than their unlabeled counterparts, the peak-pair intensity distributions shown in Fig. 6 should reflect the distributions of metabolite concentrations in the urine sample. Fig. 6 clearly shows that there are many more low abundance metabolites in urine, compared to the high abundance metabolites. The peak pairs in the 1st bin should be mainly from the very low abundance metabolites. A much smaller number of peak pairs were detected in the 1st bin, compared to the 2nd bin. This is due to the fact that many of the very low abundance metabolites as well as some metabolites with very low ionization efficiency were at the borderline of detection by LC–MS and thus many of them were not detected. Because there are many more lower abundance metabolites than the higher abundance metabolites, improving the detection sensitivity and reducing the background noise of a technique would improve the metabolome coverage significantly. As we have discussed earlier, ionization or detection saturation of high abundance metabolites in a complex sample can be a limiting factor in detecting the lower abundance metabolites. Multidimensional fractionation is required in order to reduce the interference of high abundance metabolites, thereby allowing the injection of an optimal amount of samples that are mainly composed of low abundance metabolites.

4. Conclusions

We report a study of the effects of sample injection amount and TOF–MS detection range on the detectability of metabolites in a high-performance chemical isotope labeling LC–MS platform. In CIL LC–MS, relative metabolite quantification is done by measuring the peak ratio of a $^{13}\text{C}_2$ -/ $^{12}\text{C}_2$ -labeled peak pair for a given metabolite present in two comparative samples. The dynamic range of peak ratio measurement does not need to be very large, as only small changes of metabolite concentrations (<10-fold) are commonly encountered in most metabolomic

studies where relative metabolome quantification of different groups of samples is performed. However, the absolute concentrations of different metabolites can be very different (i.e., from mM to sub nM or $>10^7$ -fold difference), requiring a technique to provide a wide detection dynamic range to allow the detection of as many peak pairs as possible. We have shown that controlling the sample injection amount into LC–MS is critical to achieve the optimal detectability while avoiding sample carry-over problem. In addition, the use of a high-dynamic-range TOF system increased the number of peak pairs detected, compared to a conventional TOF system. In the analysis of the dansyl labeled human urine samples using the LC–MS setup described in this work, ionization saturation was found to be a limiting factor for the upper limit in the HD-TOF–MS, while detection saturation was a limiting factor in the non-HD TOF–MS. When detection saturation was observed, using the ^{13}C -natural abundance peaks for peak ratio calculation, which is done automatically in IsoMS, could increase the number of peak pairs used to calculate the relative concentrations of metabolites. Finally, the intensity distributions of peak pairs detected in human urine showed that there were many more lower abundance metabolites than the higher abundance metabolites. Future work needs to be focused on developing better sample fractionation methods to simplify the complex metabolome samples and developing more sensitive detection systems to further expand the detection dynamic range.

Conflict of interest

There is no conflict of interest.

Acknowledgments

This work was supported by grants from Genome Canada, the Natural Sciences and Engineering Research Council of Canada (NSERC), the Collaborative Health Research Projects of NSERC and Canadian Institutes of Health Research (CIHR), the Canada Research Chairs program and the Alberta Innovates.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2014.08.004>.

REFERENCES

- [1] Patti GJ, Yanes O, Siuzdak G. Innovation: metabolomics: the apogee of the omics trilogy. *Nat Rev Mol Cell Biol* 2012;13: 263–9.
- [2] Guo K, Li L. Differential C-12/C-13-isotope dansylation labeling and fast liquid chromatography/mass spectrometry for absolute and relative quantification of the metabolome. *Anal Chem* 2009;81:3919–32.
- [3] Guo K, Li L. High-performance isotope labeling for profiling carboxylic acid-containing metabolites in biofluids by mass spectrometry. *Anal Chem* 2010;82:8789–93.

- [4] Kuehnbaum NL, Britz-McKibbin P. New advances in separation science for metabolomics: resolving chemical diversity in a post-genomic era. *Chem Rev* 2013;113:2437–68 (Washington, DC, U S).
- [5] Bruheim P, Kvitvang HFN, Villas-Boas SG. Stable isotope coded derivatizing reagents as internal standards in metabolite profiling. *J Chromatogr A* 2013;1296:196–203.
- [6] Wang H, Wang HY, Zhang L, Zhang J, Guo YL. N-Alkylpyridinium isotope quaternization for matrix-assisted laser desorption/ionization Fourier transform mass spectrometric analysis of cholesterol and fatty alcohols in human hair. *Anal Chim Acta* 2011;690:1–9.
- [7] Zhang SJ, You JM, Ning SJ, Song CH, Suo YR. Analysis of estrogenic compounds in environmental and biological samples by liquid chromatography–tandem mass spectrometry with stable isotope-coded ionization-enhancing reagent. *J Chromatogr A* 2013;1280: 84–91.
- [8] Tayyari F, Gowda GAN, Gu HW, Raftery D. N-15-cholamine—a smart isotope tag for combining NMR- and MS-based metabolite profiling. *Anal Chem* 2013;85:8715–21.
- [9] Dai WD, Huang Q, Yin PY, Li J, Zhou J, Kong HW, et al. Comprehensive and highly sensitive urinary steroid hormone profiling method based on stable isotope-labeling liquid chromatography–mass spectrometry. *Anal Chem* 2012; 84:10245–51.
- [10] Mazzotti F, Benabdelkamel H, Di Donna L, Athanassopoulos CM, Napoli A, Sindona G. Light and heavy dansyl reporter groups in food chemistry: amino acid assay in beverages. *J Mass Spectrom* 2012;47:932–9.
- [11] Huang YQ, Liu JQ, Gong HY, Yang J, Li YS, Feng YQ. Use of isotope mass probes for metabolic analysis of the jasmonate biosynthetic pathway. *Analyst* 2011;136:1515–22.
- [12] Armenta JM, Cortes DF, Pisciotto JM, Shuman JL, Blakeslee K, Rasoloson D, et al. Sensitive and rapid method for amino acid quantitation in malaria biological samples using AccQ.Tag ultra performance liquid chromatography–electrospray ionization-MS/MS with multiple reaction monitoring. *Anal Chem* 2010;82:548–58.
- [13] Sun XH, Ouyang Y, Chu JF, Yan J, Yu Y, Li XQ, et al. An in-advance stable isotope labeling strategy for relative analysis of multiple acidic plant hormones in sub-milligram *Arabidopsis thaliana* seedling and a single seed. *J Chromatogr A* 2014;1338:67–76.
- [14] Chokkathukalam A, Kim DH, Barrett MP, Breitling R, Creek DJ. Stable isotope-labeling studies in metabolomics: new insights into structure and dynamics of metabolic networks. *Bioanalysis* 2014;6:511–24.
- [15] Peng J, Li L. Liquid–liquid extraction combined with differential isotope dimethylaminophenacyl labeling for improved metabolomic profiling of organic acids. *Anal Chim Acta* 2013;803:97–105.
- [16] Zhou RK, Guo K, Li L. 5-Diethylamino-naphthalene-1-sulfonyl chloride (DensCl): a novel triplex isotope labeling reagent for quantitative metabolome analysis by liquid chromatography mass spectrometry. *Anal Chem* 2014;85:11532–9.
- [17] Wu Y, Li L. Determination of total concentration of chemically labeled metabolites as a means of metabolome sample normalization and sample loading optimization in mass spectrometry-based metabolomics. *Anal Chem* 2012;84: 10723–31.
- [18] Zhou R, Tseng CL, Huan T, Li L. IsoMS: automated processing of LC–MS data generated by a chemical isotope labeling metabolomics platform. *Anal Chem* 2014;86:4675–9.
- [19] Konermann L, Ahadi E, Rodriguez AD, Vahidi S. Unraveling the mechanism of electrospray ionization. *Anal Chem* 2013;85:2–9.