Technical Note

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Quantitative Metabolome Analysis Based on Chromatographic Peak Reconstruction in Chemical Isotope Labeling Liquid Chromatography Mass Spectrometry

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Abstract

Generating precise and accurate quantitative information on metabolomic changes in comparative samples is important for metabolomics research where technical variations in the metabolomic data should be minimized in order to reveal biological changes. We report a method and software program, IsoMS-Quant, for extracting quantitative information from a metabolomic dataset generated by chemical isotope labeling (CIL) liquid chromatography mass spectrometry (LC-MS). Unlike previous work of relying on mass spectral peak ratio of the highest intensity peak pair to measure relative quantity difference of a differentially labeled metabolite, this new program reconstructs the chromatographic peaks of the light- and heavy-labeled metabolite pair and then calculates the ratio of their peak areas to represent the relative concentration difference in two comparative samples. Using chromatographic peaks to perform relative quantification is shown to be more precise and accurate. IsoMS-Quant is integrated with IsoMS for picking peak pairs and Zero-fill for retrieving missing peak pairs in the initial peak pairs table generated by IsoMS to form a complete tool for processing CIL LC-MS data. This program can be freely downloaded from the www.MyCompoundID.org website for non-commercial use.

Introduction

Chemical isotope labeling (CIL) liquid chromatography mass spectrometry (LC-MS) uses differential isotope mass tags to label a metabolite in two comparative samples (e.g., ^{12}C-labeling of an individual sample and ^{13}C-labeling of a pooled sample), followed by mixing and LC-MS analysis. Individual metabolites are detected as peak pairs in mass spectra. The intensity ratio of a peak pair can be used to measure the relative concentration of the same metabolite in two samples. CIL LC-MS can significantly increase the detectability of metabolites by rationally designing the labeling reagents to target a group of metabolites (e.g., all amine-containing metabolites or amine submetabolome) to improve both LC separation and MS sensitivity.[1, 2] It can also overcome the technical problems such as matrix effects, ion suppression and instrument drifts to generate more precise and accurate quantitative results, compared to conventional LC-MS.[3-6] There are a number of new advances reported[3, 5-23] in the area of developing CIL LC-MS for targeted and untargeted metabolomics, particularly for improving labeling chemistries and extending the utility of CIL LC-MS to analyze a broad range of metabolites. However, proper processing of CIL LC-MS data is also critical to maintain high sensitivity (i.e., extracting as many peak pairs as possible from a dataset), high specificity (i.e., keeping low false-positive rate), and high performance quantification (i.e., achieving high precision and accuracy).[24] To this end, we have been involved in developing data processing methods specifically for handling CIL LC-MS data. The software tools related to these methods including IsoMS[24] and Zero-fill[25] are freely available from the www.mycompoundid.org website.

In our data processing workflow, the raw mass spectral data, instead of the chromatographic peak data, are used for metabolite peak detection, peak pairing, peak-pair filtering and peak ratio calculation by IsoMS.[24] This MS-centric approach allows us to detect

more peaks, as many regions of the baseline in a chromatogram still contain mass spectra with low abundance ion peaks. Using a chromatographic peak threshold for peak picking will not detect these peaks. Moreover, it is easier and more reliable to group or remove peaks from the same metabolite using a mass spectrum. This is because the salt/solvent adducts, mono- or hetero-dimers, multimers, common fragment ions (e.g., $-H_2O$ and $-CO_2$) of a molecular ion are present in the same mass spectrum and thus can be readily detected and filtered out. Finally, the Zero-fill program[25] can be used to detect a missing peak pair in a mass spectrum based on the similarity of retention time, accurate mass and ^{13}C-peak intensity (the same amount of ^{13}C-labeled pool is spiked to each sample) to those of the other samples where the peak pair is detected. This algorithm would be difficult to implement using chromatographic peak information.

While processing mass spectral data directly provides some advantages, it is not optimal for extracting quantitative information from the mass spectral peak intensities. Currently, in IsoMS, the peak ratio of a peak pair from a ^{13}C-/^{12}C-labeled metabolite is calculated from a mass spectrum.[24] If the same peak pair shows up in multiple neighbouring scans or spectra, only the highest intensity peak pair is kept. Its peak ratio is calculated and then entered in the metabolite-intensity table. In order to utilize all the peak pairs intensity information, we have now developed a program, IsoMS-Quant, to reconstruct two chromatographic peaks, one for ^{12}C- or light-labeled metabolite and another one for ^{13}C- or heavy-labeled metabolite, for each peak pair shown in the metabolite-intensity table. The area ratio of the two chromatographic peaks measured by the sums of ^{13}C- or ^{12}C-labeled peak intensities is calculated as a measure of relative concentration of the metabolite in light-labeled sample vs. heavy-labeled sample. Using chromatographic peaks for quantification smoothes out signal fluctuations associated with mass spectral peak intensities

in multiple scans, thereby providing better quantification. For targeted metabolite quantification, chromatographic peaks of an analyte are often used. In this report, we describe the IsoMS-Quant program and how it can be used to generate quantitative information in CIL LC-MS. Using examples of urine and serum metabolome analysis, we demonstrate that this program can improve untargeted quantitative metabolome profiling as well as targeted metabolite quantification significantly. The IsoMS-Quant program is freely available at www.mycompoundid.org and this program, along with IsoMS and Zero-fill, forms a complete data processing tool for the CIL LC-MS quantitative metabolomics platform.

Experimental Section

Dansylation Labeling and LC-MS. The labeling reaction (see Supplemental Figure S1 for the reaction scheme) and LC-MS analysis on a Bruker Impact HD QTOF mass spectrometer (Billerica, MA, USA) linked to an Agilent 1100 HPLC system (Palo Alto, CA, USA) and an electrospray ionization source were performed according to a protocol reported previously.[17, 25]

IsoMS-Quant. The IsoMS-Quant program was developed using R, an open source language and environment used in data processing and statistical programming. The user manual is provided in Supplemental Note N1. Supplemental Figure S2 shows the overall workflow for CIL LC-MS data processing. The raw LC-MS data are first processed using a peak-pair picking software, IsoMS. The high-confident level 1 peak pairs (i.e., the pair with two labeled peaks accompanied with their corresponding ^{13}C natural abundance peak) are aligned from multiple LC-MS runs to produce a metabolite-intensity CSV file or table. The Zero-fill program is then used to fill the missing values in the CSV file. The IsoMS-Quant program is applied to the final metabolite-intensity table after the zero-fill process. Although we use the overall workflow

shown in Supplemental Figure S2 to illustrate how IsoMS-Quant is implemented in processing CIL LC-MS data, this program, in principle, should be applicable to other peak-picking software. While it is beyond the scope of this work, comparing different software packages for processing CIL LC-MS data should be valuable from a user's perspective.

During the IsoMS-Quant processing, the program loops through all the available MS-peak-intensity ratios starting from the first sample in the metabolite-intensity table. For each peak ratio, its associated retention time (rt), mz_light, mz_heavy, and ^{13}C-labeled MS-peak intensity (int) are used to locate this peak in the raw MS peak list from the original LC-MS dataset. A matching score is used to find the corresponding ^{13}C-labeled peak that was used to calculate the MS-peak-intensity ratio entered into the metabolite-intensity table. The matching score is defined as:

$$\text{Score} = \left(1 - \frac{\text{rt. diff}}{\text{rt. tol}}\right)/4 + \left(2 - \frac{\text{mz. diff. light} + \text{mz. diff. heavy}}{\text{mz. tol}}\right)/2 + (1 - 2 \times \text{int. diff})/4$$

where

$$\text{rt. diff} = \text{abs}(\text{rt. } {}^{13}C\text{. peak} - \text{rt. rawdata. peak})$$

$$\text{mz. diff. light} = 1\text{E}6 \times \frac{\text{abs}\left(\text{mz. } {}^{12}C\text{. peak} - \text{mz. rawdata. peak} + 2.0067\right)}{\text{mz. } {}^{13}C\text{. peak}}$$

$$\text{mz. diff. heavy} = 1\text{E}6 \times \frac{\text{abs}\left(\text{mz. } {}^{13}C\text{. peak} - \text{mz. rawdata. peak}\right)}{\text{mz. } {}^{13}C\text{. peak}}$$

$$\text{int. diff} = \text{abs}\left(\log\left(\frac{\text{int. } {}^{13}C\text{. peak}}{\text{int. rawdata. peak}}\right)\right)$$

The terms, rt.^{13}C.peak, mz.^{13}C.peak (or mz.^{12}C.peak), and int.^{13}C.peak, refer to retention time, m/z value, and intensity of the labeled peak in the metabolite-intensity table, respectively. The terms, rt.rawdata.peak, mz.rawdata.peak, and int.rawdata.peak, refer to retention time, mz value, and intensity of the labeled peak in the raw MS peak list, respectively. The value, 2.0067,

comes from the mass difference of the two isotope carbons (^{13}C vs. ^{12}C labeling). The default rt tolerance (rt.tol) is 30 s and the default mz tolerance (mz.tol) is 5 ppm. These tolerance values are instrument-dependent and can be adjusted. A different weight (divided by 2 or 4) is assigned to each of the similarity terms in the above score calculation equation. The mz value is deemed to be more important than rt and int and, therefore, given more weight. The MS peak with the maximal matching score is considered to be the correct ^{13}C-labeled peak. Once the ^{13}C-labeled peak is found in the raw peak list data, its corresponding ^{12}C-labeled peak is also identified in the same MS scan based on the mz difference of smaller than mz tolerance (default 5 ppm) from that of the ^{12}C-labeled peak in the metabolite-intensity table.

After both the ^{12}C- and ^{13}C-labeled peaks of a peak pair are identified in an MS scan, peaks in the neighboring MS scans are checked to see if the peak pair is also present. The check procedure stops once either the ^{12}C or ^{13}C peak is not found in a particular MS scan. After this procedure is completed, all the ^{12}C- and ^{13}C-labeled MS-peak intensities in these continuous MS scans over a chromatographic peak are used for chromatographic area calculation. Figure 1 shows how to calculate the chromatographic peak area from the sum of MS peak intensities. In a typical LC-MS experiment, the MS signals are acquired at a constant time interval (e.g., at a spectral acquisition or scan rate of 1 Hz used in this work) and thus the chromatographic peak area can be calculated as the sum of all the segmented areas in trapezoids:

$$\text{Area} = \sum_{1}^{n} Area_i$$

where n is the number of scans where the same peak pair is detected. The area of each trapezoid can be described as:

$$Area_i = \frac{1}{2}(int_k + int_{k+1}) \times time.interval$$

Since the chromatographic peak area ratio of a peak pair is:

$$\text{Ratio} = \frac{Area\ of\ ^{12}C}{Area\ of\ ^{13}C}$$

by substituting the area with MS peak intensity, the ratio becomes:

$$\text{Ratio} = \frac{\sum_1^n int\ \ of\ ^{12}C}{\sum_1^n int\ of\ ^{13}C}$$

Thus the chromatographic peak area ratio of a peak pair can be determined as the sum of all the MS intensity values of the ^{12}C-labeled peaks divided by the sum of all the intensity values of the ^{13}C-labeled peaks.

After IsoMS-Quant completes the ratio calculation, it will compare the new ratio to the original intensity ratio. If the ratio difference is greater than 4-fold, the chromatographic ratio would be rejected; using manual inspections of the ratio results, we found that they belonged to less than 0.5% of the total number of peak pairs found and they were all falsely picked pairs. Otherwise, the new ratio will replace the original intensity ratio in the metabolite-intensity table which can be exported for statistical analysis or other uses.

Results and Discussion

CIL LC-MS is a platform that allows in-depth profiling of chemical-group-based submetabolomes using different labeling reagents targeting different classes of metabolites (e.g.,^{13}C-/^{12}C-dansylation labeling for quantifying amine- and phenol-containing metabolites.[26-29]). The major difference between CIL LC-MS and conventional LC-MS is that in CIL LC-MS all the true metabolites show up in the mass spectra as peak pairs which can be readily differentiated from the singlet peaks originated from background or noise. Thus it is much easier and more reliable to detect the true metabolite peaks. Based on this unique feature of peak pair

detection, we have developed two software modules, IsoMS and Zero-fill, to process CIL LC-MS data to produce a metabolite-intensity table.[24, 25] The peak ratio in the table was calculated from the highest intensity peak pair found in multiple mass spectra. This way of calculation, while it is simple to implement, does not use the intensity ratio information in other neighboring mass spectra. In contrast, IsoMS-Quant utilizes all the mass spectral peak pair information to calculate a peak ratio.

Comparing the performance of using MS peak intensity ratio vs. chromatographic peak area ratio, three cases can be considered. Figure 2A shows an example of good chromatographic peaks where the peak ratios are basically the same: 0.51 from the chromatographic peak area calculation vs. 0.50 from the mass spectral intensity calculation. In this case, the overall mass spectral signals are strong (Figure 2B), representing a high abundance or readily ionizable metabolite found in a ^{12}C-labeled human serum sample mixed with a ^{13}C-labeled pooled sample. However, because of a wide concentration dynamic range of metabolites present in a sample such as human serum, there are many low-intensity peaks detected in LC-MS. For these peaks, the highest intensity peak pair may not be representative of the concentration ratio of the labeled metabolite. Figure 2C shows an example of relatively poor chromatographic peaks for both the ^{12}C- and ^{13}C-labeled mass spectral peaks (Figure 2D). Poor peak shape is likely due to the effects of other co-eluting components or background ions present in a complex sample along with the analyte during the analyte elution; these peaks show up randomly and unpredictably and cannot be mimicked using simple standards. The ratio calculated using the highest mass spectral peak intensities (1.26) does not match well with the ratio determined from the chromatographic peak areas (0.98).

Another case is related to the saturation of MS detection which can lead to distorted peak shapes. Figure 2E shows an example where the MS signals are saturated and the mass spectral peak intensity no longer reflects the real metabolite concentration. IsoMS-Quant addresses this issue by automatically finding the ^{13}C natural isotope peaks of both the ^{12}C- and ^{13}C-labeled metabolite peaks and then using these peaks to reconstruct the chromatographic peaks (Figure 2F) for ratio calculation. Since the ^{13}C natural isotope peak is much lower in intensity, they are less likely to be saturated in MS detection and thus can be used for more accurate quantification. In the Impact QTOF instrument, we rarely observed the saturation of the ^{13}C natural isotope peak; electrospray ionization saturation often occurred before detection saturation. In IsoMS-Quant, a user can enter a threshold above which saturation occurs, depending on the MS instrument used.

The overall performance improvement for quantitative metabolomics can be demonstrated using the results of triplicate analysis of a ^{13}C-/^{12}C-labeled human urine sample. In this experiment, an equal amount of ^{12}C-labeled and ^{13}C-labeled same urine was mixed for analysis and thus the peak ratios for all the metabolite peak pairs should be equal to 1. Figure 3A plots the number of peak pairs detected in multiple neighboring mass spectral scans as a function of the scan number. Out of the 1660 peak pairs detected, only 7 pairs (<1%) were detected in a single mass spectrum. The highest percentage of peak pairs belongs to those detected over 6 to 10 mass spectra or chromatographic peaks of 6 to 10 s. Thus, for most of the peak pairs detected, they appear in multiple scans and integration of peak pair intensities over these scans should improve quantification.

Figure 3B shows a distribution of the number of peak pairs as a function of the peak ratio determined with and without applying IsoMS-Quant. The peak ratio distribution becomes more symmetric after using IsoMS-Quant and there are more peak pairs with peak ratio values close to

1. Figure 3C shows a distribution of the number of peak pairs as a function of the relative standard deviation (RSD) of the peak ratio from the mean from experimental triplicate runs. More peak pairs have their ratios close to the mean after using IsoMS-Quant. Without using IsoMS-Quant, the $^{12}C/^{13}C$ ratios have an averaged RSD of 10.4%, and with IsoMS-Quant, the averaged RSD is reduced to 6.7%. The results shown in Figure 3B,C illustrate that the use of IsoMS-Quant can improve the accuracy and precision for quantitative metabolomics by CIL LC-MS. We note that we have not studied how the retention time precision or mass accuracy of different instruments would affect the degree of improvement achievable by IsoMS-Quant. In our studies, we usually used an LC instrument that can readily provide a retention time precision of better than 30 s and a mass spectrometer that can provide a mass accuracy of better than 5 ppm for CIL LC-MS.

We have used the IsoMS-Quant program for a number of metabolomics research projects and observed improvement in quantitative results that lead to better statistical analysis of the metabolomic data. One example is in a metabolomics study where a set of 109 LC-MS runs of dansyl labeled urine samples collected from 55 bladder cancer patients and 54 controls were processed to search for potential metabolite biomarkers for diagnosis of bladder cancer.[25, 27] The two groups could be readily separated using PLS-DA or volcano plots based on concentration variations of a number of significant metabolites.[25, 27] Supplemental Figure S3 shows a plot of the p-values of three representative significant metabolites obtained before and after applying IsoMS-Quant. The p-values increase by more than 10-fold after using IsoMS-Quant. This level of improvement can be attributed to the fact that IsoMS-Quant generates more precise and accurate peak ratio values, allowing the reduction of intra-group variations and better separation of inter-group differences.

Finally, the use of IsoMS-Quant can also improve the analytical performance of targeted metabolite quantification. For targeted metabolite quantification using CIL LC-MS, a reference sample such as a pooled sample is ^{13}C-labeled, following by spiking ^{12}C-labeled metabolite standards with known concentrations to determine the absolute concentrations of all the metabolites of interest. This reference sample can then be used to quantify the metabolites in an individual sample by measuring the peak ratio of a metabolite peak pair from a mixture of ^{12}C-labeled individual sample and ^{13}C-labeled reference sample. As an example, we performed absolute quantification of 20 metabolites in an individual human serum sample. Standard addition method using ^{12}C-dansyl labeled metabolite standards was used to determine the absolute concentrations of these 20 metabolites in a ^{13}C-labeled pooled sample generated from mixing serums of 100 healthy individuals. An aliquot of the ^{13}C-labeled pooled sample was spiked into a ^{12}C-labeled individual sample in 1:1 volume ratio. The mixture was analyzed by LC-MS. The absolute concentrations of the 20 metabolites in the individual sample were determined by using the peak ratio of a metabolite peak pair and the absolute concentration of the metabolite in the pooled sample. Peak ratio was calculated with and without using IsoMS-Quant.

Table 1 lists the concentrations of 20 metabolites found in the individual serum sample using data processing with and without applying IsoMS-Quant. As Table 1 shows, the percentage of concentration difference for an individual metabolite by the two processing methods or relative error can be up to 32% (for serine) and as high as over 55% (for glycine where mass spectral peaks were saturated); the average difference was 13%. Manual inspection of the concentration data generated from the IsoMS-Quant method indicated that these concentration values were much more reliable, as the chromatographic peak shapes of a peak

pair were well represented. Better precision is also achieved using IsoMS-Quant (mean RSD of 4.2% vs. 6.7% without IsoMS-Quant from triplicate experiments). This example demonstrates that by using IsoMS-Quant better accuracy and precision can be achieved for targeted quantification of metabolites of interest using CIL LC-MS.

Conclusions

We have developed a method of generating quantitative peak ratio data using chromatographic peak areas of a peak pair in CIL LC-MS. A mass spectral peak pair found in the metabolite-intensity table generated by IsoMS and Zero-fill is searched against the raw LC-MS data to find all neighboring mass spectra where the same peak pair is continuously detected. The chromatographic peaks of the light-labeled and heavy-labeled metabolites in the pair are constructed and their peak areas are determined for peak ratio measurement. We implemented this method by developing a software program, IsoMS-Quant, for automatic peak ratio calculation. IsoMS-Quant is demonstrated to provide better precision and accuracy for both untargeted and targeted metabolic profiling work using CIL LC-MS. IsoMS-Quant, along with IsoMS and Zero-fill, forms a complete workflow for rapid processing of raw LC-MS data generated by CIL LC-MS.

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Supporting Information Available

Supplementary materials are available free of charge via the Internet at http://pubs.acs.org.

Table 1. Results of targeted quantification of 20 metabolites in a human serum sample by LC-MS analysis of a mixture of the ^{12}C-labeled sample and the ^{13}C-labeled pooled serum standard with known concentrations of these metabolites.

Name	Absolute conc. from peak area (μM)	Absolute conc. from MS intensity (μM)	Absolute conc. Relative error
Taurine	68 ± 1	69 ± 5	1%
Arginine	364.6 ± 0.3	371 ± 1	2%
Asparagine	10 ± 4	10 ± 4	0%
Glutamine	89 ± 1	96 ± 4	8%
Homoserine	0.98 ± 0.02	0.8 ± 0.1	18%
Serine	428 ± 1	293 ± 6	32%
Aspartic Acid	1289 ± 4	1310 ± 30	2%
Trans-4-Hydroxyl-L-Proline	20.2 ± 0.2	19.1 ± 0.5	5%
Threonine	220.6 ± 0.5	169 ± 3	23%
Aminoadipic acid	2.7 ± 0.2	2.6 ± 0.4	4%
Glycine	411 ± 2	183 ± 4	55%
Glycylproline	0.56 ± 0.04	0.58 ± 0.06	4%
Tryptophan	182 ± 1	206 ± 4	13%
Phenylalanine	330 ± 4	230 ± 19	30%
Isoleucine	128 ± 2	99 ± 1	23%
Lysine	430 ± 8	365 ± 6	15%
4-Hydroxybenzoic acid	0.64 ± 0.01	0.65 ± 0.01	2%
Desaminotyrosine	0.25 ± 0.02	0.24 ± 0.01	4%
Histidine	254 ± 2	211 ± 4	17%
Pyrocatechol	0.0041 ± 0.0005	0.0041 ± 0.0005	0%

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Figure Captions

Figure 1. Schematic of chromatographic peak area calculation from mass spectral intensity values (blue lines).

Figure 2. (A) Extracted ion chromatograms (EICs) of a relatively high abundance or easily ionizable ^{13}C-/^{12}C-labeled peak pair (green: ^{12}C-labeled metabolite; red: ^{13}C-labeled metabolite) found in a mixture of a ^{12}C-labeled individual human serum and a ^{12}C-labeled pooled serum prepared from 100 healthy individuals and (B) the highest intensity mass spectrum of the pair. (C) EICs of a relatively low abundance or not readily ionizable peak pair and (D) the high intensity mass spectrum of the pair. (E) EICs of a saturated peak pair and (F) EICs of the corresponding pair plotted using their ^{13}C natural abundance peaks.

Figure 3. Distributions of the number of peak pairs detected in a 1:1 ^{13}C-/^{12}C-labeled human urine sample as a function of (A) number of neighboring MS scans where a peak pair is detected, (B) peak ratios calculated before and after applying IsoMS-Quant, and (C) relative standard deviations of peak ratios from triplicate experiments (n=3).

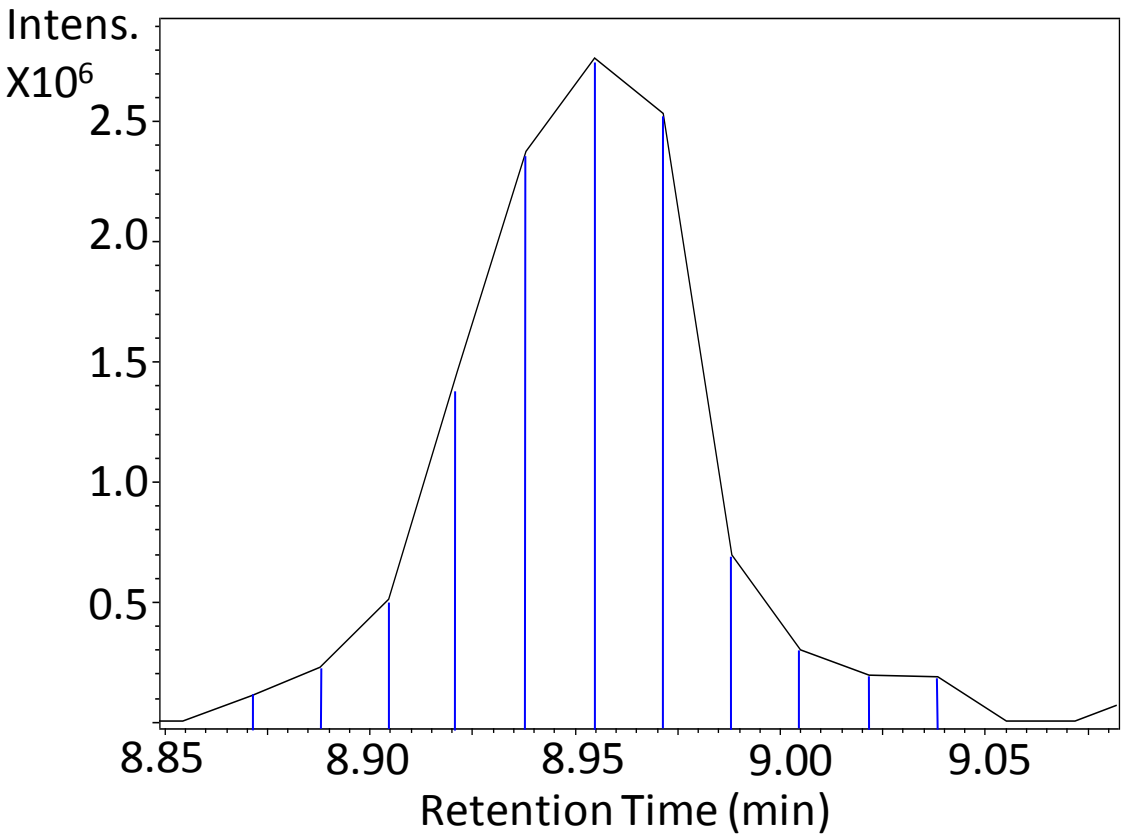
Intens.
X10^6
2.5
2.0
1.5
1.0
0.5
8.85
8.90
8.95
9.00
9.05
Retention Time (min)

Figure 1

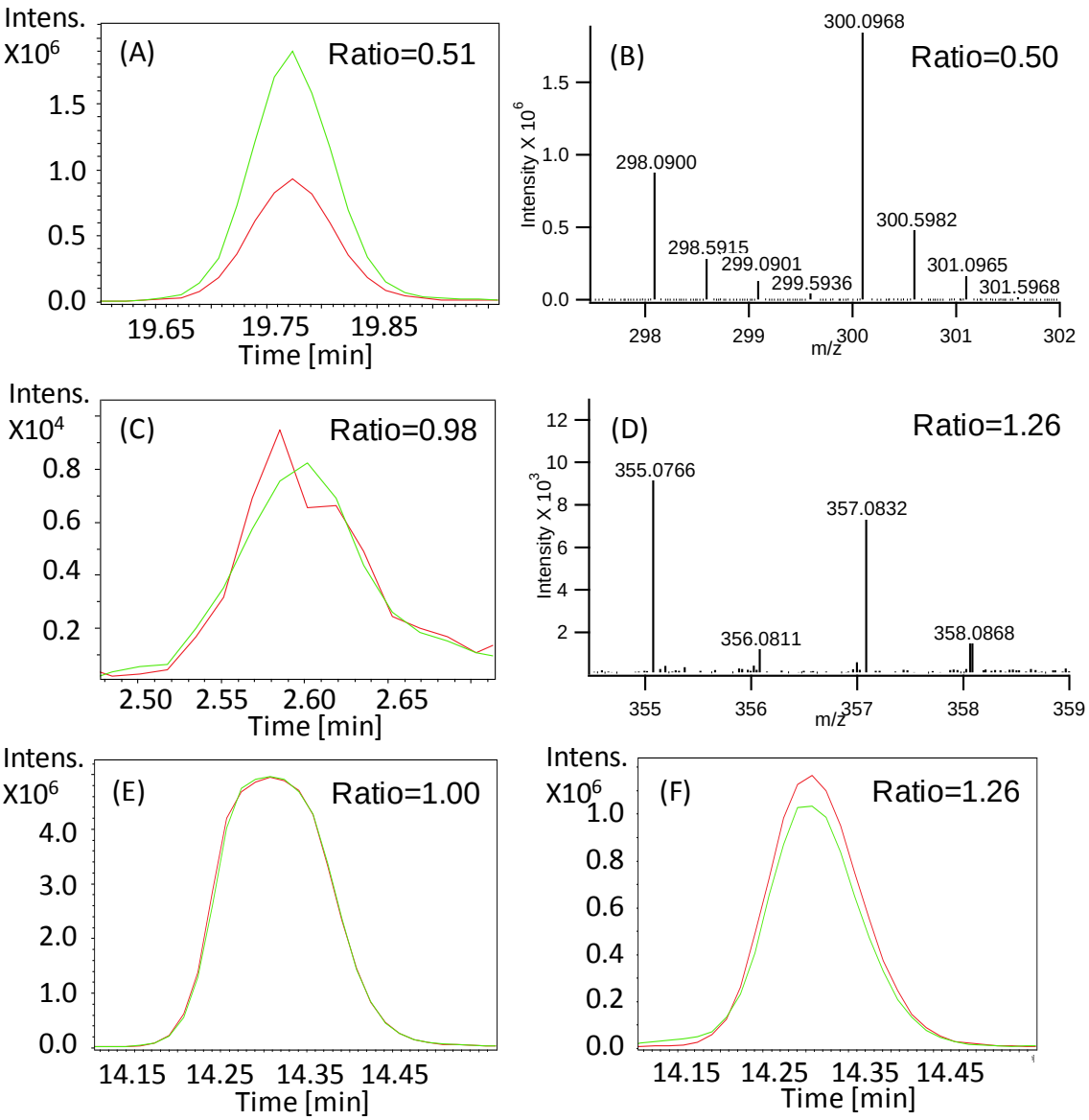
Intens.
X10^6
(A)
Ratio=0.51
Time [min]
(B)
Ratio=0.50
Intensity X 10^6
m/z
300.0968
298.0900
298.5915
299.0901
299.5936
300.5982
301.0965
301.5968
Intens.
X10^4
(C)
Ratio=0.98
Time [min]
(D)
Ratio=1.26
Intensity X 10^3
355.0766
357.0832
356.0811
358.0868
Intens.
X10^6
(E)
Ratio=1.00
Time [min]
Intens.
X10^6
(F)
Ratio=1.26
Time [min]

Figure 2

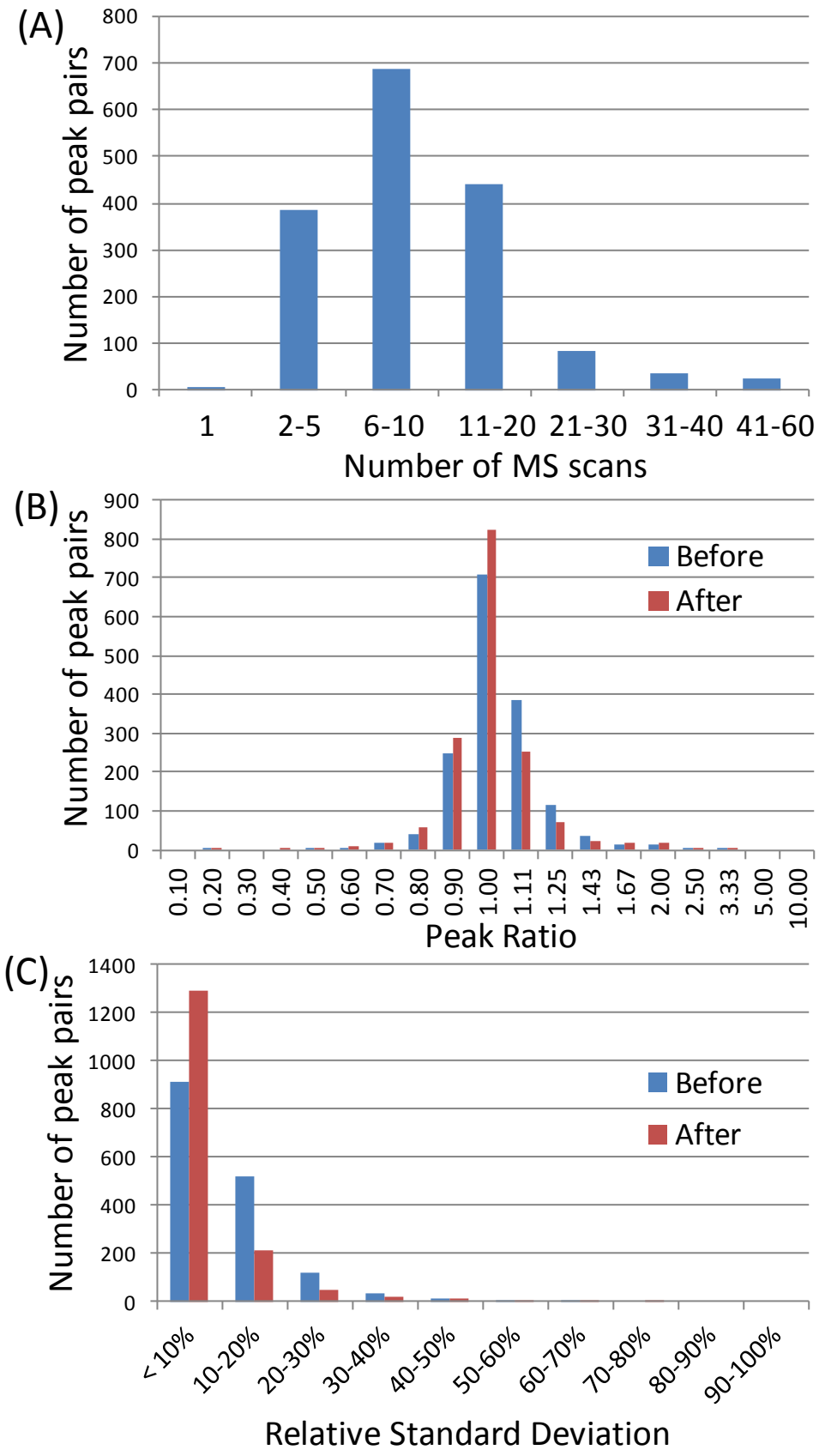
(A)
Number of peak pairs
800
700
600
500
400
300
200
100
0
1
2-5
6-10
11-20
21-30
31-40
41-60
Number of MS scans
(B)
Number of peak pairs
900
800
700
600
500
400
300
200
100
0
Before
After
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.00
1.11
1.25
1.43
1.67
2.00
2.50
3.33
5.00
10.00
Peak Ratio
(C)
Number of peak pairs
1400
1200
1000
800
600
400
200
0
Before
After
<10%
10-20%
20-30%
30-40%
40-50%
50-60%
60-70%
70-80%
80-90%
90-100%
Relative Standard Deviation

Figure 3

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