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Comparison of Full-Scan, Data-Dependent, and Data-Independent Acquisition Modes in Liquid Chromatography-Mass Spectrometry Based Untargeted Metabolomics

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Abstract

Full-scan, data-dependent acquisition (DDA), and data-independent acquisition (DIA) are the three common data acquisition modes in high resolution mass spectrometry-based untargeted metabolomics. It is an important yet underrated research topic on which acquisition mode is more suitable for a given untargeted metabolomics application. In this work, we compared the three data acquisition techniques using a standard mixture of 134 endogenous metabolites and a human urine sample. Both hydrophilic interaction and reversed-phase liquid chromatographic separation along with positive and negative ionization modes were tested. Both the standard mixture and urine samples generated consistent results. Full-scan mode is able to capture the largest number of metabolic features, followed by DIA and DDA (53.7% and 64.8% respective features fewer on average in urine than full-scan). Comparing the MS^2 spectra in DIA and DDA, spectra quality is higher in DDA with average dot product score 83.1% higher than DIA in Urine(H), and the number of MS^2 spectra (spectra quantity) is larger in DIA (on average 97.8% more than DDA in urine). Moreover, a comparison of relative standard deviation distribution between modes shows consistency in the quantitative precision, with the exception of DDA showing a minor disadvantage (on average 19.8% and 26.8% fewer features in urine with RSD < 5% than full-scan and DIA). In terms of data preprocessing convenience, full-scan and DDA data can be processed by well-established software. In contrast, several bioinformatic issues remain to be addressed in processing DIA data and the development of more effective computational programs is highly demanded.

Introduction

Untargeted metabolomics is emerging as a powerful and attractive technology that has been widely used in many research areas, including, but not limited to, the discovery of disease biomarkers, study of metabolic pathways, monitoring the totality of environmental exposures, and searching for bioactive chemicals that modulate certain disease phenotypes, such as demyelinating disease, diabetes, cancer, etc.[1-5]. Liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) is currently the most prominent analytical platform for untargeted metabolomics, owing to its high throughput, sensitivity and specificity. In LC-HRMS based untargeted metabolomics, LC separates metabolites by their chemical properties to reduce sample complexity, and retention time information of metabolites can be used to assist their identification. Following separation, MS is used to detect metabolic signal intensities that are proportional to metabolite concentrations for quantitative assessments. There are three MS data acquisition modes to record metabolic signals: full-scan, data-dependent acquisition (DDA), and data-independent acquisition (DIA). Conventionally, LC-HRMS based untargeted metabolomics is carried out by first performing LC-full scan MS on each individual sample to obtain accurate mass-to-charge ratio (*m/z*) and relative abundance for all the metabolic features. The acquired metabolic information is then processed using data preprocessing software (*e.g.,* XCMS, MZmine)[6,7] to automatically extract high-confidence metabolic features. Further multivariate and univariate statistical analyses extract significantly altered metabolic features that are pertinent to the given biological question[8]. From there, LC-MS^2 (also called LC-MS/MS or LC-tandem MS) analysis is performed on the pooled sample to collect fragmentation spectra of metabolic features of interest in order to confirm their chemical identities. This conventional approach has been well established but it separates metabolite quantification from structural determination, thus requires extra instrumental analysis time and sample volumes.

To facilitate a more time-efficient approach of data acquisition in untargeted metabolomics, an accelerated and autonomous data acquisition mode, DDA is implemented. In DDA mode, the MS instrument performs MS full-scan immediately followed by MS^2 analysis on a list of precursor ions selected from the full-scan spectrum[9]. In this setting, both quantitative (obtained from the MS full-scan) and structural (obtained the MS^2 spectra) information can be acquired in the same sample analysis, allowing for simultaneous data processing and metabolite identification. Since the selection of precursor ions for MS^2 analysis is intensity dependent, a potential limitation of DDA mode is that metabolic features of interest with low MS abundance may never be selected for fragmentation and will have no MS^2 data associated with them. In addition, since the MS instrument allocates a large portion of its acquisition time for MS^2 spectra generation, the signal intensity for MS^1 features is reduced. This causes a fundamental problem for the detection and quantification of low abundance metabolites in DDA mode.

To address the limitation of DDA in acquiring MS^2 spectra, recent implementation of DIA in metabolomic studies can theoretically generate MS^2 spectra for all precursor ions, therefore capable of detecting and identifying more metabolites that are at lower concentrations[10,11]. In DIA mode, the MS instrument cycles through the precursor ion *m/z* range with a large precursor ion mass width to fragment more than one precursor ion simultaneously. Commonly used DIA methods include all-ion fragmentation (AIF) (*e.g.,* MS^{All}. MS^{E})[12,13], where all precursors are fragmented, and sequential window acquisition of all theoretical fragment-ion spectra (SWATH)[14], where a medium pass window, such as 20 or 25 Da, is used. Theoretically, DIA enables continuous and unbiased acquisition of MS^2 information for all metabolites. Although LC-HRMS in DIA

mode is an attractive metabolomic profiling strategy, the link between precursors and their fragment ions are dissociated due to the complexity of the resulting MS^2 spectra. Thus a great informatics challenge to deconvolute the raw MS2 spectra for subsequent metabolite identification is posed[15].

Given the unique features and limitations of each data acquisition mode, it is rational to believe that the choice of data acquisition mode may affect the quality of metabolomics results. It is also important to note that a common practice in targeted studies is to maximize the detection sensitivity via optimizing sample preparation and LC-MS conditions. However, it is much harder in untargeted analysis to do the abovementioned optimization due to the diverse chemical properties and concentrations of all metabolites. Therefore, it is more practical to select the best data acquisition mode for better analytical performance in untargeted metabolomics. Unfortunately, there is no direct comparison of the three data acquisition modes yet, and the literature of choosing an appropriate acquisition mode for given untargeted metabolomics projects is inadequate. At the current stage, most researchers are more inclined to use the acquisition mode that has already been established in their lab without considering which acquisition method might work better. To address this knowledge gap, we made a comparative study of full-scan, DDA, and DIA AIF modes in LC-HRMS based untargeted metabolomics. The capability of different data acquisition modes was tested using metabolite standard mixture and human urine samples. Complementary LC-MS configurations were assessed, including hydrophilic interaction liquid chromatography (HILIC) and reversed-phase liquid chromatography (RP), as well as electrospray ionization (ESI) in positive and negative modes. Metabolomic data collected in the three data acquisition modes were evaluated from five complementary aspects: (1) metabolic coverage; (2) quantitative precision; (3)

MS^2 spectral quality; (4) MS^2 spectral coverage, the percentage of metabolic features that have MS^2 spectra out of the total metabolic features, and (5) convenience. Our results demonstrate that full-scan provides the best feature detection sensitivity and quantitative precision. In comparison, DDA provides high quality MS^2 spectra and DIA provides better MS^2 spectral coverage.

Experimental Section

Chemicals and solvents. LC-MS grade water (H_2O), acetonitrile (ACN), Methanol (MeOH) and other solvents were purchased from Fisher Scientific (Hampton, New Hampshire). All the metabolite standards and other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri).

Standard solution preparation. The 134 metabolite standards were first prepared individually as stock solutions, each in 1 mL ACN:H_2O (1:1, v:v). To prepare the original standard mixture, all the metabolites were pooled together to reach a final concentration of 8.3 μg/mL. To prepare diluted standard mixture, 10 μL original solution was further diluted 100 times to 8.3 ng/mL. The original standard mixture is indicated as "StdMix(H)" and the diluted standard mixture is indicated as "StdMix(L)". The list of the 134 metabolites is available in **Table S-1**.

Urine sample preparation. One urine sample was collected from a healthy male volunteer. 50 μL urine was mixed with 150 μL LC-MS grade MeOH in a 1.5 mL Eppendorf vial. After vortexing, the solution was spun down at 17,530 g, and the clear urine supernatant was aliquoted to a clean 1.5 mL Eppendorf vial. The clear solution was evaporated using speed-vac, and the dried urine sample was reconstituted in 50 μL ACN:H_2O (1:1, v:v) for LC-HRMS analysis. A diluted urine sample was prepared by a 100 time dilution of a 10 μL aliquot from the reconstituted solution. The reconstituted urine solution before dilution is indicated as "Urine(H)" and the diluted solution is indicated as "Urine(L)".

LC-HRMS analysis. The LC-HRMS analysis was performed on a Bruker Impact II™ Ultra-High Resolution Qq-Time-Of-Flight Mass Spectrometer (UHR-QqTOF-MS)) coupled with an Agilent 1290 Infinity™ II Ultra High-Performance Liquid Chromatography (UHPLC) system. The resolution of this instrument is up to 50,000 (measured at full width at half maximum (FWHM) and at *m/z* 1222 (positive mode) and *m/z* 1334 (negative mode) using TuneMix solution and factory default methods) and the mass accuracy is below 5 ppm. 2 μL sample solutions of each triplicate and solvent blank were injected in sequence onto a Waters ACQUITY UPLC BEH C18 column (130Å, 1.7 μm, 1.0 mm × 100 mm) in RP mode, and a Millipore ZIC-pHILIC column (200Å, 5 μm particle size, 2.1 × 150 mm) in HILIC mode. 2 μL of 150 mM Sodium Formate (NaFA) solution was injected for internal calibration.

We tested the data acquisition modes in RPLC separation coupled with electrospray ionization (ESI) in positive mode and HILIC separation coupled with ESI in negative mode. The combination of RP(+) and HILIC(-) is a well-established method in metabolomics to achieve a global-scale metabolome profiling[16,17]. In RP analysis, mobile phase A was H_2O in 0.1% Formic Acid (FA) and mobile phase B was ACN in 0.1% FA. RP gradient was set as follows: 0 min, 95% A; 8 min, 75% A; 14 min, 30% A; 20 min, 5% A; 23 min, 5% A. Post-gradient equilibrium time was 7 min at 95% A. Flow rate was 0.15 mL/min. In HILIC analysis, mobile phase A was 95% H_2O and 5% ACN with 10 mM Ammonium Acetate at 9.8 pH, and mobile phase B was 5% H_2O and 95% ACN. HILIC gradient was set as follows: 0 min, 5% A; 20 min, 80% A; 25 min, 95% A. Post-gradient equilibrium time was 19 min at 5% A. Flow rate was 0.15 mL/min during the gradient, and 0.2 mL/min during the post-gradient equilibrium. Q-TOF MS was performed in full-scan, DDA, and DIA AIF modes. The detailed MS parameter settings can be found in **Text S-1**.

Data processing. MS-DIAL was used for metabolomic data processing. The computer had an Intel i9-9900k CPU @ 3.60 GHz with 8 cores and 32 GB memory; Windows 10; 64-bit operation system; and 10 processing threads. The parameters were optimized for processing full-scan, DDA and DIA data[18,19]. The detailed parameters can be found in **Text S-2**. After metabolic feature extraction, statistical analysis was performed to calculate the relative standard deviations of metabolic signals in analytical replicates (**Text S-3**). All the raw data has also been uploaded on Metabolights, a public-accessible metabolomics data repository[20]. Researchers can access the raw data via its job ID: MTBLS1572.

Metabolite annotation. For the standard mixture, the metabolites were annotated by matching their accurate mass and retention time to a tandem MS library file created in house using the same instrument and pure chemical standards, thereby containing the accurate mass and retention time for each metabolite in the standard mixture. For urine samples, the metabolites were annotated by MS^2 matching against the publicly available MS spectral database provided by MS-DIAL (MSMS-Public-Pos-VS11 and MSMS-Public-Neg-VS11). The library contains accurate mass and tandem MS information for annotation. The positive mode library includes the tandem MS information for 29,269 metabolites and the negative mode library includes the tandem MS information for 17,810 metabolites. The tandem MS information was collected from Massbank, ReSpec, GNPS, Fiehn lab, CASMI2016, MetaboBase, and RIKEN PlaSMA. The instruments used to generate the MS^2 spectra are Orbitrap and Q-TOF MS. In addition, a MS^2 quality score was calculated for the MS^2 spectral matching by comparing the number of common product ions between the evaluated spectrum and the reference spectrum from MS-DIAL. The score can be represented by a well-

established value of dot product. The detailed explanation of dot product calculation can be found in **Text S-4**.

Results

The schematic workflow for the comprehensive assessment of full-scan, DDA, and DIA AIF is illustrated in **Figure 1.** A standard mixture consisting of 134 endogenous metabolites and a healthy human urine sample (both original concentration and 100-fold dilution) were tested in this study. LC-MS analysis was performed in both RP(+) and HILIC(-), the two most commonly used LC-MS modes for profiling hydrophobic and hydrophilic metabolites, respectively[16,17]. After data collection, the three data acquisition modes were compared in terms of metabolic feature numbers, quantitative precision, MS^2 spectral quality and quantity, as well as convenience.

Analysis of standard mixture. All the metabolites included in the standard mixture and their detectability in different concentrations and acquisition modes are listed in **Table S-1, 2 and 3**, respectively. The retention time and peak intensity of each identified metabolite in each condition are listed in **Table S-4** and **5**. After manual checking of retention time, accurate mass and MS^2 spectra of the metabolite standards, 66, 63, and 66 metabolites were detected in full-scan, DDA, and DIA modes in RP analysis, respectively. In HILIC analysis, 125, 120, and 125 metabolites were detected in full-scan, DDA, and DIA modes (**Table 1**), respectively. The metabolites not extracted by MS-DIAL but recovered by manual checking are also listed in **Table S-7**. From these results, we noticed that the number of confirmed metabolites were consistently slightly higher in full-scan and DIA than in DDA in both RP(+) and HILIC(-) modes.

Metabolite detection at lower sample concentrations. The ability to detect low abundance metabolites is sought after in untargeted metabolomics. To assess the performance of different data acquisition modes in detecting low abundance metabolites, we repeated the analysis of metabolite standard mixture using its 100-fold diluted solution (**Table 1**). Applying the same LC-HRMS

analysis to StdMix(L), we confirmed 44, 23, and 33 metabolites in full-scan, DDA and DIA modes in RP(+) analysis and 57, 43, and 46 metabolites in full-scan, DDA, and DIA modes in HILIC(-) analysis. As expected, with 100-fold dilution, the number of detected metabolites was significantly reduced compared with undiluted original solution (StdMix(H)). Similar to the results obtained using the StdMix(H), full-scan performed the best in terms of the number of detected metabolites. However, compared with StdMix(H) results, DDA had the greatest decrease in the number of identified metabolites (**Table 1**), indicating that DDA is less sensitive in detecting low abundance metabolites than full-scan and DIA. The detailed information about the detected metabolites in StdMix(H) and StdMix(L) are listed in **Table S-1.**

MS^2 spectra quality. In addition to the quantity of detected metabolites, we also compared the quality of MS^2 spectra generated in DDA and DIA modes using the same metabolite standards. The collected MS^2 spectra were compared against the reference MS^2 spectra archived in the MS-DIAL MS^2 spectra library. Dot product was calculated in MS-DIAL to assess the similarity between experimental and reference MS^2 spectra. The detailed matching scores are presented in **Table S-6.** The averaged matching score was 72.8 for DDA results and 46.8 for DIA results. Further t-test analysis showed that there is a significant difference in the MS^2 spectra quality (p-value: 6.4×10^{-17}) between DDA and DIA results, and DDA generates higher quality MS^2 spectra than DIA.

Analysis of urine samples. In addition to the targeted analysis using metabolite standards, we further performed an untargeted analysis on human urine samples in full-scan, DDA, and DIA modes. After the urine metabolomic data was generated, MS-DIAL was chosen as the data

processing software for metabolic feature extraction, alignment, and preliminary metabolite identification. Data processing parameters were manually optimized to achieve the best performance of metabolic feature extraction. Our results showed that the average processing times for three replicate sample files plus one solvent blank file were: 110 seconds for full-scan, 40 seconds for DDA, and 7,620 seconds (127 minutes) for DIA. Although the processing of DIA data can be faster if the MS^1 and MS^2 abundance cut-offs were set higher in MS-DIAL, higher cut-offs may result in the loss of some low abundance metabolic features.

To evaluate the analytical difference between the three data acquisition modes, we first counted the total numbers of detected, MS^1 annotated and MS^2 matched metabolic features (**Figure 2**). To calculate statistical significance among the results of the three data acquisition modes, we also processed the metabolomic raw data individually and therefore have three replicates for each acquisition mode to perform a t-test (**Figure S-1**). Our definitions for these different features are as follows: detected features were the direct output of peak picking software after filtering out low abundance noise, background signals from blank, and different adduct forms; MS^1 annotated features matched *m/z* values with theoretical *m/z* values of at least one metabolite in the MS-DIAL metabolic library; MS^2 matched features have experimental MS^2 spectra matched with reference MS^2 spectra with a minimum of 40% matching fragment ions and 15% similarity of dot product. As shown in **Figure 2 and Figure S-1,** in both RP(+) and HILIC(-) analyses, full-scan and DIA modes were able to cover more metabolic features (statistically significant, p-value < 0.05) than DDA mode regardless of the urine sample concentration. Confidently identified metabolites with dot product > 0.8 from Urine(H) samples in both RP(+) and HILIC(-) modes are listed in **Table S-8**.

Comparison of Quantitative Precision. Besides the number of detected metabolic features, we also investigated the quantitative precision using experimental triplicates of the same urine sample. After feature alignment in MS-DIAL, relative standard deviation (RSD) of the metabolic feature intensities were calculated. As shown in **Figure 3**, although all the three datasets have > 80% metabolic features of RSD < 20%, more metabolic features have excellent precision (RSD < 5%) in full-scan and DIA. This trend is consistent in Urine(H) analyzed in both RP(+) and HILIC(-). The percentages of metabolic features with RSD < 20% are similar between acquisition modes. However, in Urine(L) RP(+) mode, Urine(H) RP(+) mode, Urine(L) HILIC(-) mode, and Urine(H) HILIC(-) mode, the respective percentages of metabolic features with RSD < 5% are 26%, 42%, 37% and 52% in DDA; 37%, 53%, 39%, and 67% in full-scan; and 40%, 72%, 49%, and 57% in DIA. DDA consistently has the smallest percentage of metabolic features with RSD < 5%, independent of sample type, concentration, ionization polarity or chromatographic method. It is also noticed in Urine(L) analysis that metabolic features have broader RSD distribution, and more metabolic features have larger RSD values in comparison to Urine(H) in both RP(+) and HILIC(-) analyses.

Comparison of urine MS^2 spectra. Using human urine samples, we also compared the quantity and quality of MS^2 spectra generated in DDA and DIA modes. The number of metabolic features with MS^2 spectra were 981, 668, 62, and 151 in Urine(H)-RP(+), Urine(H)-HILIC(-), Urine(L)-RP(+), and Urine(L)-HILIC(-) analyses using DDA mode, respectively. On average, 43% of metabolic features have corresponding MS^2 spectra in DDA mode. In comparison, the number of metabolic features with MS^2 spectra were 1381, 1549, 167, and 225 in Urine(H)-RP(+), Urine(H)-

HILIC(-), Urine(L)-RP(+), and Urine(L)-HILIC(-) analyses using DIA mode, respectively. Since all the metabolic MS^2 spectra were deconvoluted mathematically from raw MS^2 data, 100% of the metabolic features have corresponding MS^2 spectra in DIA analysis. Furthermore, by overlapping common metabolic features in DDA and DIA using their retention time and accurate *m/z*, Venn diagrams were prepared to show the shared metabolic features with MS^2 spectra (**Figure 4**). As shown, DIA was able to generate more MS^2 matched features than DDA. However, we noticed that DDA also generated a few unique MS^2 features which were not found in the MS^2 matched features in DIA mode. We manually investigated these unique features and discovered that these are features with low quality peak shapes or low abundance that trigged MS^2 events in DDA mode, and was not picked up in DIA mode. The details can be found in the supplementary information (**Text S-5**).

Besides the comparison of MS^2 spectral numbers, we also evaluated the quality of MS^2 spectra generated from DDA and DIA analyses. The metabolic features generated in Urine(H) by both RP(+) and HILIC(-) analyses were used to compare the MS^2 spectra quality between DDA and DIA. Same as in the metabolite standards analysis, the MS^2 spectra quality of each metabolic feature was assessed by their dot products against reference MS^2 spectra in MS-DIAL. The dot product score distributions of all common metabolic features detected by DDA and DIA are presented in **Figure 5**. Due to the diverse metabolic concentration, the MS^2 spectral quality distribution was similar to those of metabolite standards, and several metabolites had extremely low matching dot product score. A comparison of the dot product score distributions for DDA and DIA results shows that MS^2 spectra generated in DDA have better scores and thus better spectra quality.

293

Discussion

In this work, a comprehensive assessment of full-scan, DDA, and DIA AIF modes in untargeted metabolomics was performed. We tested the modes using standard mixture and human urine samples in original and diluted concentrations and RP(+) and HILIC(-) configurations. Our results demonstrate that each data acquisition mode has its unique features as well as limitations that can significantly affect the metabolomic data quality. **Figure 6** summarizes the performance ranking of full scan, DDA, and DIA in metabolic feature detection, quantitative precision, MS^2 spectral quality and quantity, and convenience. The rankings have three levels represented by three grey pentagon-shape lines in the radar plot (**Figure 6**). The five aspects for comparison are indicated by the five arrow dimensions towards five directions. The further a mode is placed from the center of the plot, the better performance it has in the corresponding dimension. Overall, full-scan is highlighted by its capability to detect the largest number of metabolic features and provide the highest quantification precision. In comparison, DDA is convenient to use, as it streamlines metabolic profiling and metabolite identification, but the number of metabolic features and quantification precision are not as good. Furthermore, between DDA and DIA, the two modes capable of generating MS^2 spectra, DDA provides better quality and DIA provides better quantity. The purpose of this work is to draw the attention of the scientific community to recognize that the choice of data acquisition mode can lead to different metabolomic results. The comprehensive comparison also provides guidance on how to choose the optimal data acquisition mode in order to fully exploit the analytical power of different data acquisition modes in untargeted metabolomics.

In this work, the comparison of the three data acquisition modes was carried out in optimized scan mode parameters using a Bruker Impact II™ UHR-QqTOF-MS. QqTOF-MS is one of the most commonly used HRMS for untargeted metabolomics. Its fast scan speed and short accumulation time allows it to routinely perform full-scan, DDA, and DIA (both AIF and SWATH-type) analyses with high acquisition frequency and good mass resolution. There are three major reasons for the choice of AIF (or MS^{all}) as the DIA method instead of SWATH or SWATH-like workflow for this comparison study. First, AIF is the original DIA method which has been commonly used and widely available for all MS instruments. In comparison, SWATH is only available in several major MS instruments. There remains to be a number of MS instruments that are not yet capable of performing SWATH. Therefore, we decided to compare the more accessible DIA method, which is AIF. More importantly, there are many parameters in SWATH mode that can influence the data acquisition results. These parameters need to be optimized depending on the instrument. However, the optimization has been reported in only a few studies[21], and is a research topic that needs further research. Last but not least, a previous study comparing DDA, SWATH and AIF for drug metabolomics has already shown that SWATH should be better than AIF in terms of MS^2 spectral quality due to smaller isolation window and thus less complex MS^2 spectra[10]. Although we did not include SWATH in our study, it is clear that the implementation and optimization of SWATH in more MS instruments should be addressed in future research[22].

Our results indicate that full-scan mode is able to detect the largest number of metabolic features compared to DDA and DIA. The large difference in feature numbers can be explained by the time the instrument spends on MS^1 data acquisition. Detailed acquisition time information can be found in **Table S-2**. Since in full-scan mode, 100% of the instrument time is spent on MS^1 data

acquisition, more low abundance metabolic features can be captured, as evidenced in both the analysis of metabolite standards and urine samples. Thus, full-scan mode could be more suitable to achieve largest metabolic coverage. In comparison, DDA and DIA is unable to achieve that level of comprehensiveness because part of the instrument time is used for acquiring MS^2 information. Specifically, for the methods used in this study, DDA and DIA respectively spent only approximately 5% and 50% of instrument time on MS^1, comparing to 100% in full-scan mode (**Table S-9**). Therefore, the numbers of detected metabolic features in DDA and DIA modes are much lower than full-scan mode. A detailed illustration of how LC-MS chromatogram is constructed in each acquisition mode is presented in **Figure S-2**. Manual inspection of the DDA data, which contains the fewest number of metabolic features, shows that many of the real metabolic features were not picked up by data processing algorithms because of their poor chromatographic peak shapes and low MS signal intensities. These real metabolic features could potentially be retrieved by our recent effort of integrated feature extraction strategy, which recognizes these low abundance peaks by their valid MS^2 information, rather than their chromatographic peak shapes[23].

Generating precise quantitative information is important for comparative metabolomics, and therefore technical variations should be minimized during sample preparation and data collection[24]. In different data acquisition modes, the quantitative precision is predominantly influenced by peak abundance and peak shape. Our results showed that full-scan is able to have the best quantitative precision, followed by DIA and DDA which generates the least quantitative precision. Although more than 80% of the metabolic features generated in these three data acquisitions have RSD below 20%, which is commonly accepted for LC-MS based metabolomics[25], we noticed that full-

scan mode is able to generate the best quantitative precision. We also manually checked some metabolic features with large RSD values. These features are noticed to have low MS scan numbers, and the peak shapes of these metabolic features are not smooth enough to achieve the most appropriate peak heights. Therefore, the peak shapes of these metabolic features in DDA are not as feasible as full-scan and DIA for peak height determination, and lead to more features with larger RSD. However, the disadvantage of DDA in terms of quantitative precision is not dramatic, so the ranking of DDA is placed between the first and second level in our summary radar plot (**Figure 6**). Comparatively, as a result of the high quantitative precision and presence of structural information DIA mode has been commonly used for targeted quantification in proteomics[26-28] and now starts to be used for targeted quantitative analysis in metabolomics. Recently, there has been an informatics tool developed to automate the quantification process[29].

MS^2 spectra generated in LC-HRMS analysis provides valuable structural information for metabolite annotation via either *de novo* interpretation or matching against reference MS^2 spectra. Besides comparing MS^1 results, we also investigated the quality and quantity of MS^2 spectra generated in DDA and DIA modes. Since metabolic structure elucidation heavily relies on the quality of its MS^2 spectrum, higher spectral quality is favored as it improves the matching accuracy, and leads to a lower chance of false positive identification. Our results showed that DDA mode consistently generates MS^2 spectra with better quality than DIA mode. This result is similar to previous study where DDA and DIA modes were compared for the identification of drug metabolites[10]. It is not difficult to understand the result as in DDA experiment, a narrow precursor ion isolation window (approx. 1 Da) is used to select precursor ions for MS^2. Thus, by isolation of the pure precursor, a "clearer" MS^2 spectra can be achieved. In contrast, in DIA experiment, MS^2

spectra were generated via mathematical deconvolution of a mixture of MS^2 fragments. For co-eluting metabolites, it is often difficult to allocate fragment signals to the right metabolic precursor ions, and better deconvolution algorithms are highly warranted to generate cleaner/higher quality MS^2 spectra in DIA. Although DDA mode outcompetes DIA in terms of MS^2 spectra quality, DIA is able to generate MS^2 spectra for all the metabolic features. To address the limited number of MS^2 spectra generation in DDA analysis, several efforts have been made to improve MS^2 coverage in DDA analysis[30-32].

It is important to note that convenience is a major consideration when carrying out untargeted metabolomics. In terms of metabolic feature extraction, many well-established bioinformatic programs for full-scan and DDA data are available to automatically extract metabolic features and align them across multiple samples[33-35]. In particular, DDA is the most convenient method among all three alternatives, as it can be fully automated from feature extraction to metabolite identification and shortens the full-scan-based workflow from days to hours[9]. More importantly, recent development of spectral similarity analysis (e.g., GNPS) also makes DDA the method of choice to generate large number of MS^2 spectra in each sample for unknown metabolite annotation[36]. In our study, the processing time for DIA-based data is almost 100 times longer than DDA and full-scan-based data under the same parameter settings. In addition, lots of false positive metabolic features were observed in the low signal-to-noise (S/N) range of the DIA results. Although many DIA deconvolution algorithms have been developed in the past few years[37-42], we believe that the further development of software for processing DIA data will promote the increased use of DIA for untargeted metabolomics.

Among the limitations of the present study is the limited choice of LC-MS combinations. We focused on the most commonly used combinations in LC-MS based metabolomics (e.g., RP(+) and HILIC(-)), which is also common practice in other MS method comparison works[10,43-46]. Researchers who want to check their specific LC-MS methods can follow our workflow to perform their comparison. Furthermore, the parameters for LC-MS analysis and data processing have been considered optimized, yet the choice is fairly subjective. We performed parameter optimization via (1) checking recommended settings provided by the data processing program; (2) testing different parameter combinations and manually checking which combination gave the best results; (3) comparing our parameter settings with literature for further optimization. Notably, method optimization via strict Design of Experiments approach of a wide parameter space might generate results with stronger scientific rationale[47]. Nevertheless, our results show that screening through a certain space of the key parameters does not cause significant alterations to the overall trend (**Figure S-3**), suggesting that our method optimization approach is acceptable. Finally, mobile phase pH, column temperature and other factors can affect LC chromatographic separation and thus impact data acquisition performance. These parameters comprise an interesting research topic not addressed in and is beyond the scope of this study.

In summary, this work presents a systematic approach to comparing data acquisition modes in untargeted metabolomics. Our results show that all the three data acquisition modes have distinctive analytical performance in metabolic coverage, quantitative precision, MS^2 spectra quality and quantity, and convenience. Although the underlying difference is simply caused by how instrument time is allocated for MS^1 and MS^2, the consequence is substantial, leading to

different analytical performance. Therefore, the selection of an appropriate data acquisition method should be carefully weighed based on the purpose of the study.

Supporting Information

The Supporting Information is available free of charge.

Text S-1: Detailed MS parameter settings for full-scan, DDA and DIA modes. Text S-2: Detailed parameters for processing full-scan, DDA and DIA data in MS-DIA. Text S-3: Statistical analysis used in this comparative study. Text S-4: Details of dot product calculation. Text S-5: The reasons of DDA having unique MS^2 matched features undetected in DIA. Table S-1: List of metabolites in the standard mixture. Table S-2: Metabolites detected in StdMix(H). Table S-3: Metabolites detected in StdMix(L). Table S-4: Retention time and peak intensity information of metabolites detected in StdMix(H). Table S-5: Retention time and peak intensity information of metabolites detected in StdMix(L). Table S-6: Dot product of the common metabolic feature MS^2 between RP(+) DDA and DIA methods from StdMix(H). Table S-7: Comparison of the statistical results generated using the MS/MS precursor ions and that using manual examination of the chromatogram in the raw data. Table S-8: Table of identified metabolites in Urine(H) samples in both RP(+) and HILIC(-) modes. Table S-9: Detailed acquisition time in each MS mode. Figure S-1**.** Number of statistically significant features in urine analysis. (A) total feature number, (B) MS^1 annotated feature number, (C) MS^2 matched feature number. Figure S-2**.** Illustration of **c**hromatograms in (A) Full-scan; (B) DDA; (C) DIA modes. Figure S-3. Number of total metabolic features as the function of parameter space (A) mass tolerance for constructing extracted ion chromatogram; (B) minimum peak height; (C) algorithm of chromatographic peak reconstruction.

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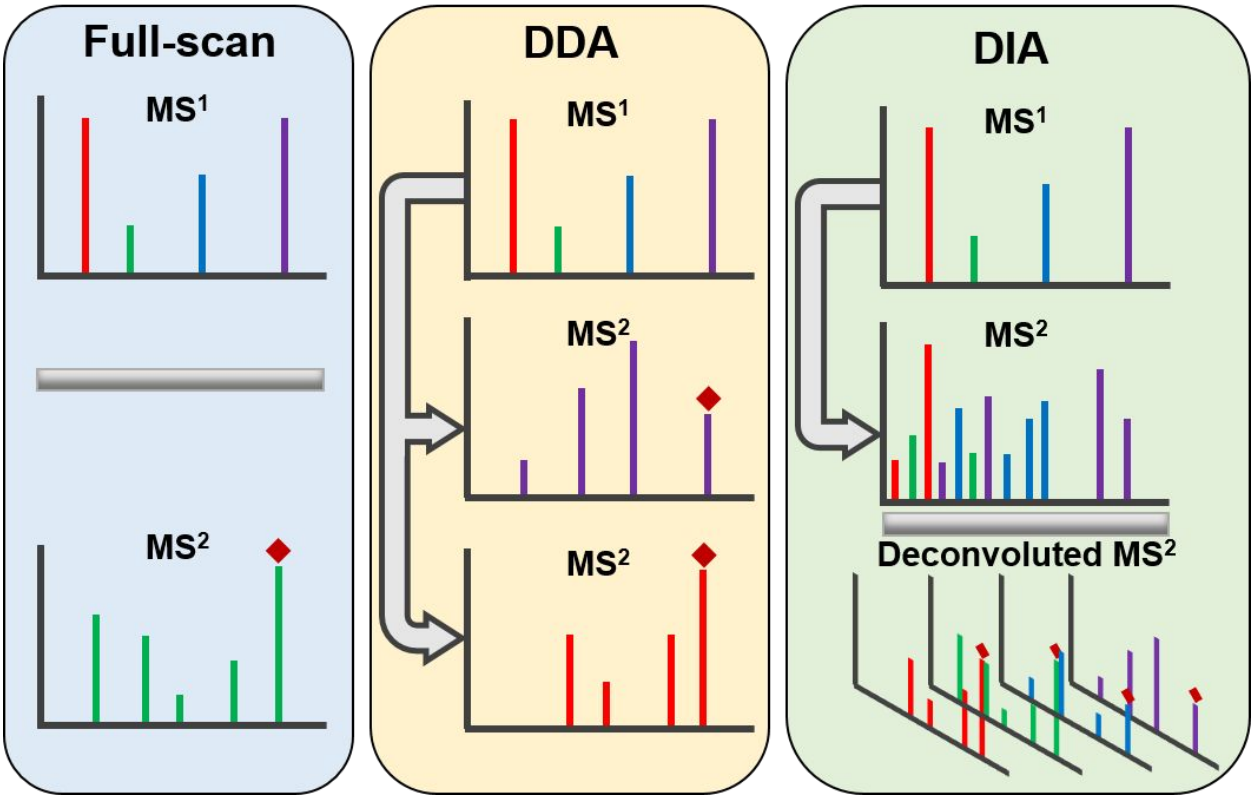
Reference

(1) Patti, G. J.; Yanes, O.; Siuzdak, G. *Nature reviews Molecular cell biology* **2012**, *13*, 263.
(2) Wishart, D. S. *Nature reviews Drug discovery* **2016**, *15*, 473.
(3) Warth, B.; Spangler, S.; Fang, M.; Johnson, C. H.; Forsberg, E. M.; Granados, A.; Martin, R. L.; Domingo-Almenara, X.; Huan, T.; Rinehart, D. *Analytical chemistry* **2017**, *89*, 11505-11513.
(4) Beyer, B. A.; Fang, M.; Sadrian, B.; Montenegro-Burke, J. R.; Plaisted, W. C.; Kok, B. P.; Saez, E.; Kondo, T.; Siuzdak, G.; Lairson, L. L. *Nature chemical biology* **2018**, *14*, 22.
(5) Rinschen, M. M.; Ivanisevic, J.; Giera, M.; Siuzdak, G. *Nature Reviews Molecular Cell Biology* **2019**, 1.
(6) Smith, C. A.; Want, E. J.; O'Maille, G.; Abagyan, R.; Siuzdak, G. *Analytical chemistry* **2006**, *78*, 779-787.
(7) Pluskal, T.; Castillo, S.; Villar-Briones, A.; Orešič, M. *BMC bioinformatics* **2010**, *11*, 395.
(8) Xia, J.; Sinelnikov, I. V.; Han, B.; Wishart, D. S. *Nucleic acids research* **2015**, *43*, W251-W257.
(9) Benton, H. P.; Ivanisevic, J.; Mahieu, N. G.; Kurczy, M. E.; Johnson, C. H.; Franco, L.; Rinehart, D.; Valentine, E.; Gowda, H.; Ubhi, B. K. *Analytical chemistry* **2014**, *87*, 884-891.
(10) Zhu, X.; Chen, Y.; Subramanian, R. *Analytical Chemistry* **2014**, *86*, 1202-1209.
(11) Röst, H. L.; Rosenberger, G.; Navarro, P.; Gillet, L.; Miladinović, S. M.; Schubert, O. T.; Wolski, W.; Collins, B. C.; Malmström, J.; Malmström, L. *Nature biotechnology* **2014**, *32*, 219.
(12) Siegel, D.; Meinema, A. C.; Permentier, H.; Hopfgartner, G. r.; Bischoff, R. *Analytical chemistry* **2014**, *86*, 5089-5100.
(13) Schrimpe-Rutledge, A. C.; Codreanu, S. G.; Sherrod, S. D.; McLean, J. A. *Journal of the American Society for Mass Spectrometry* **2016**, *27*, 1897-1905.
(14) Roemmelt, A. T.; Steuer, A. E.; Poetzsch, M.; Kraemer, T. *Analytical chemistry* **2014**, *86*, 11742-11749.
(15) Nikolskiy, I.; Mahieu, N. G.; Chen, Y.-J.; Tautenhahn, R.; Patti, G. J. *Analytical chemistry* **2013**, *85*, 7713-7719.
(16) Ivanisevic, J.; Zhu, Z.-J.; Plate, L.; Tautenhahn, R.; Chen, S.; O'Brien, P. J.; Johnson, C. H.; Marletta, M. A.; Patti, G. J.; Siuzdak, G. *Analytical chemistry* **2013**, *85*, 6876-6884.
(17) Cajka, T.; Fiehn, O. *Analytical Chemistry* **2016**, *88*, 524-545.
(18) Eliasson, M.; Rännar, S.; Madsen, R.; Donten, M. A.; Marsden-Edwards, E.; Moritz, T.; Shockcor, J. P.; Johansson, E.; Trygg, J. *Analytical Chemistry* **2012**, *84*, 6869-6876.
(19) Libiseller, G.; Dvorzak, M.; Kleb, U.; Gander, E.; Eisenberg, T.; Madeo, F.; Neumann, S.; Trausinger, G.; Sinner, F.; Pieber, T.; Magnes, C. *BMC Bioinformatics* **2015**, *16*, 118.
(20) Kale, N. S.; Haug, K.; Conesa, P.; Jayseelan, K.; Moreno, P.; Rocca-Serra, P.; Nainala, V. C.; Spicer, R. A.; Williams, M.; Li, X. *Current protocols in bioinformatics* **2016**, *53*, 14.13. 11-14.13. 18.
(21) Zhou, J.; Li, Y.; Chen, X.; Zhong, L.; Yin, Y. *Talanta* **2017**, *164*, 128-136.
(22) Wrona, M.; Mauriala, T.; Bateman, K. P.; Mortishire-Smith, R. J.; O'Connor, D. *Rapid Communications in Mass Spectrometry: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry* **2005**, *19*, 2597-2602.
(23) Hu, Y.; Cai, B.; Huan, T. *Analytical Chemistry* **2019**, *91*, 14433-14441.
(24) Huan, T.; Li, L. *Analytical chemistry* **2015**, *87*, 7011-7016.
(25) Shurubor, Y. I.; Paolucci, U.; Krasnikov, B. F.; Matson, W. R.; Kristal, B. S. *Metabolomics* **2005**, *1*, 75-85.
(26) Rosenberger, G.; Koh, C. C.; Guo, T.; Röst, H. L.; Kouvonen, P.; Collins, B. C.; Heusel, M.; Liu, Y.; Caron, E.; Vichalkovski, A. *Scientific data* **2014**, *1*, 140031.
(27) Hu, A.; Noble, W. S.; Wolf-Yadlin, A. *F1000Research* **2016**, *5*.
(28) Doerr, A. *Nature methods* **2014**, *12*, 35.
(29) Li, H.; Cai, Y.; Guo, Y.; Chen, F.; Zhu, Z.-J. *Analytical chemistry* **2016**, *88*, 8757-8764.
(30) Yan, Z.; Yan, R. *Analytical chemistry* **2015**, *87*, 2861-2868.

(31) Mullard, G.; Allwood, J. W.; Weber, R.; Brown, M.; Begley, P.; Hollywood, K. A.; Jones, M.; Unwin, R. D.; Bishop, P. N.; Cooper, G. J. *Metabolomics* **2015**, *11*, 1068-1080.
(32) Calderón-Santiago, M. n.; Priego-Capote, F.; Luque de Castro, M. a. D. *Analytical chemistry* **2014**, *86*, 7558-7565.
(33) Huan, T.; Forsberg, E. M.; Rinehart, D.; Johnson, C. H.; Ivanisevic, J.; Benton, H. P.; Fang, M.; Aisporna, A.; Hilmers, B.; Poole, F. L. *Nature methods* **2017**, *14*, 461.
(34) Li, S.; Park, Y.; Duraisingham, S.; Strobel, F. H.; Khan, N.; Soltow, Q. A.; Jones, D. P.; Pulendran, B. *PLoS computational biology* **2013**, *9*, e1003123.
(35) Pirhaji, L.; Milani, P.; Leidl, M.; Curran, T.; Avila-Pacheco, J.; Clish, C. B.; White, F. M.; Saghatelian, A.; Fraenkel, E. *Nature methods* **2016**, *13*, 770.
(36) Wang, M.; Carver, J. J.; Phelan, V. V.; Sanchez, L. M.; Garg, N.; Peng, Y.; Nguyen, D. D.; Watrous, J.; Kapono, C. A.; Luzzatto-Knaan, T. *Nature biotechnology* **2016**, *34*, 828-837.
(37) Yin, Y.; Wang, R.; Cai, Y.; Wang, Z.; Zhu, Z.-J. *Analytical Chemistry* **2019**, *91*, 11897-11904.
(38) Samanipour, S.; Reid, M. J.; Bæk, K.; Thomas, K. V. *Environmental Science & Technology* **2018**, *52*, 4694-4701.
(39) Nikolskiy, I.; Mahieu, N. G.; Chen, Y., Jr.; Tautenhahn, R.; Patti, G. J. *Analytical Chemistry* **2013**, *85*, 7713-7719.
(40) Bern, M.; Finney, G.; Hoopmann, M. R.; Merrihew, G.; Toth, M. J.; MacCoss, M. J. *Analytical Chemistry* **2010**, *82*, 833-841.
(41) Peckner, R.; Myers, S. A.; Jacome, A. S. V.; Egertson, J. D.; Abelin, J. G.; MacCoss, M. J.; Carr, S. A.; Jaffe, J. D. *Nature Methods* **2018**, *15*, 371-378.
(42) Tsugawa, H.; Cajka, T.; Kind, T.; Ma, Y.; Higgins, B.; Ikeda, K.; Kanazawa, M.; VanderGheynst, J.; Fiehn, O.; Arita, M. *Nature methods* **2015**, *12*, 523.
(43) Canterbury, J. D.; Merrihew, G. E.; MacCoss, M. J.; Goodlett, D. R.; Shaffer, S. A. *Journal of The American Society for Mass Spectrometry* **2014**, *25*, 2048-2059.
(44) Myers, O. D.; Sumner, S. J.; Li, S.; Barnes, S.; Du, X. *Analytical chemistry* **2017**, *89*, 8689-8695.
(45) Koopmans, F.; Ho, J. T.; Smit, A. B.; Li, K. W. *Proteomics* **2018**, *18*, 1700304.
(46) Wu, I. L.; Turnipseed, S. B.; Storey, J. M.; Andersen, W. C.; Madson, M. *Rapid Communications in Mass Spectrometry* **2019**.
(47) Eliasson, M.; Rännar, S.; Madsen, R.; Donten, M. A.; Marsden-Edwards, E.; Moritz, T.; Shockcor, J. P.; Johansson, E.; Trygg, J. *Analytical chemistry* **2012**, *84*, 6869-6876.

TOC graphical abstract



Figures

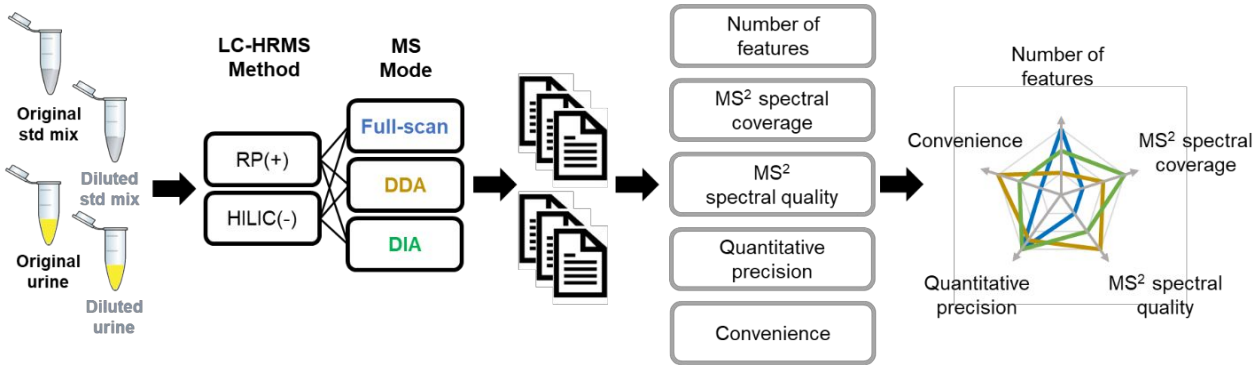


Figure 1. Schematic workflow for the comparison of full-scan, DDA and DIA modes in untargeted metabolomics.

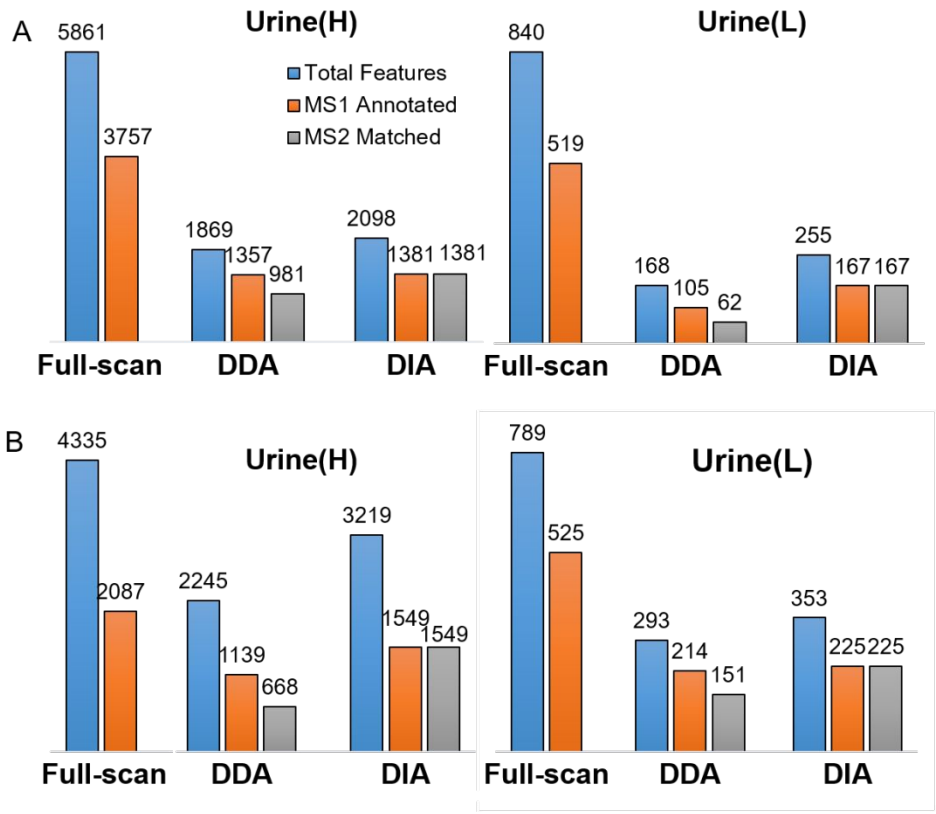


Figure 2. Numbers of total metabolic features detected, MS^1 annotated and MS^2 matched metabolic features in urine samples analyzed by the three data acquisition methods in both (A) RP(+); (B) HILIC(-) analytical configurations.

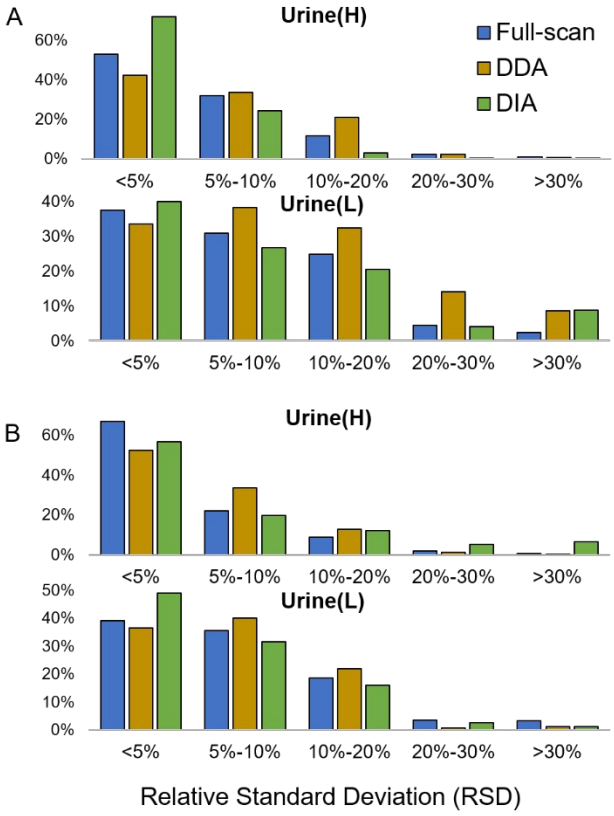


Figure 3. RSD distribution of metabolic feature MS signal intensities in the analysis of (A) RP(+); (B) HILIC(-) analytical configurations.

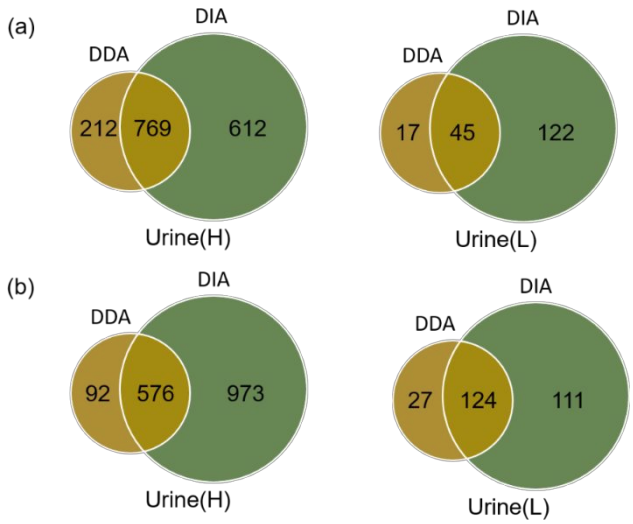


Figure 4. Overlapped MS^2 features detected in DDA and DIA analysis in urine samples. (A) RP(+) analysis; (B) HILIC(-) analytical configurations.

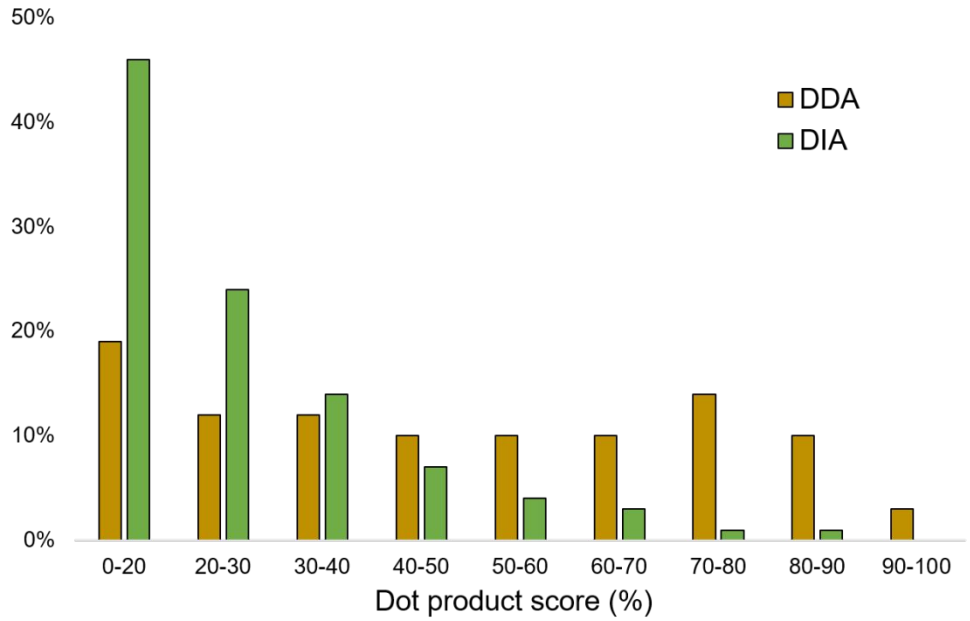


Figure 5. Distribution of spectral similarity scores of DDA vs DIA in RP(+) and HILIC(-) analytical configurations.

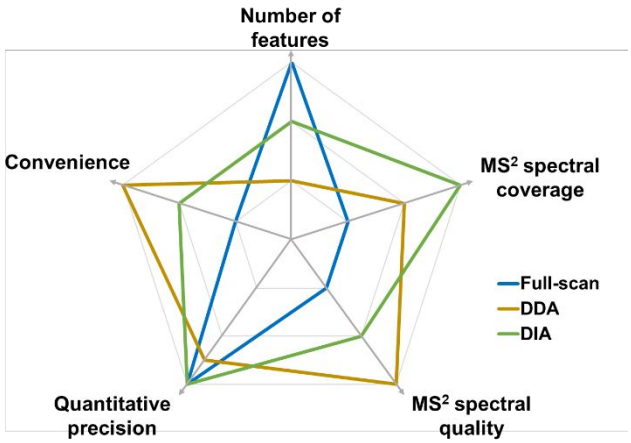


Figure 6. Summary of the assessment of the three data acquisition methods.

Tables

Table 1. Number of confirmed metabolites in the standard mixture analyzed by three data acquisition methods

Identified metabolites in standard mixture		Full-scan		DDA		DIA	
		No.	**%***	**No.**	**%**	**No.**	**%**
StdMix(H)	RP(+)	66		63		66	
	HILIC(-)	125		120		125	
StdMix(L)	RP(+)	44	67	23	37	33	50
	HILIC(-)	57	46	43	36	46	37

*The percentage of detected metabolites in StdMix(L) compared to those in StdMix(H)