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The Use of Variable Q1 Isolation Windows Improves Selectivity in LC–SWATH–MS Acquisition

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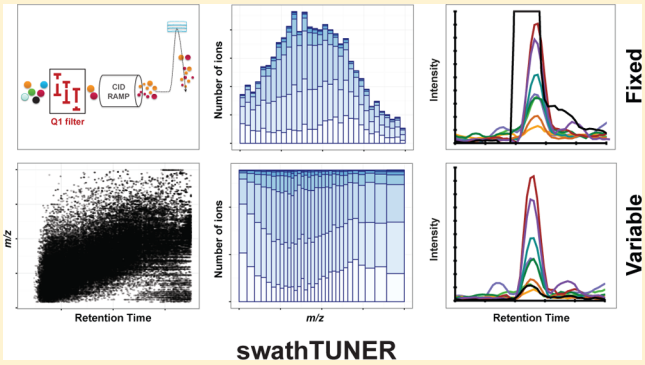
Supporting Information

ABSTRACT: As tryptic peptides and metabolites are not equally distributed along the mass range, the probability of cross fragment ion interference is higher in certain windows when fixed Q1 SWATH windows are applied. We evaluated the benefits of utilizing variable Q1 SWATH windows with regards to selectivity improvement. Variable windows based on equalizing the distribution of either the precursor ion population (PIP) or the total ion current (TIC) within each window were generated by an in-house software, swathTUNER. These two variable Q1 SWATH window strategies outperformed, with respect to quantification and identification, the basic approach using a fixed window width (FIX) for proteomic profiling of human monocyte-derived dendritic cells (MDDCs). Thus, 13.8 and 8.4% additional peptide precursors, which resulted in 13.1 and 10.0% more proteins, were confidently identified by SWATH using the strategy PIP and TIC, respectively, in the MDDC proteomic sample. On the basis of the spectral library purity score, some improvement warranted by variable Q1 windows was also observed, albeit to a lesser extent, in the metabolomic profiling of human urine. We show that the novel concept of "scheduled SWATH" proposed here, which incorporates (i) variable isolation windows and (ii) precursor retention time segmentation further improves both peptide and metabolite identifications.



KEYWORDS: *SWATH, variable Q1 window widths, selectivity, proteomics, metabolomics*

INTRODUCTION

Mass spectrometry (MS) in combination with liquid chromatography (LC) is now widely used as an essential platform to perform global proteomic and metabolomic profiling of complex biological samples.[1−7] For these applications, samples are commonly analyzed following a data-dependent acquisition (DDA, also referred to as information-dependent acquisition) approach. Despite being an efficient strategy to identify and quantify proteins or metabolites, the DDA technique still faces numerous challenges for the analysis of global protein expression or metabolite profiling due to its sampling bias toward high abundant precursor ions, which might hinder the selection of low abundant ones for fragmentation.[8,9] Therefore, a significant fraction of the precursor ions present in a complex mixture may not be identified. Additionally, semirandom on-the-fly selection of precursor ions via DDA leads to the sampling of different subsets of available precursor ions across repeated analyses of the same sample, thereby limiting identification reproducibility.[10] Finally, MS2 quantification can only be performed on a spectral basis, which is not always at the apex of the peak, and the chromatographic resolution is lost.

For comprehensive MS/MS sampling to be achieved, a data-independent acquisition (DIA) approach has emerged. As can be inferred from its name, the acquisition of MS/MS spectra using DIA is independent from the information (i.e., the intensities of the precursor ions) obtained from the previous survey scan. In other words, there is no selection of precursor ions to trigger the acquisition of fragment ion spectra. Instead, either all of the precursors contained in the entire mass range or all of the precursors contained in a number of predefined isolation windows are fragmented together. The former method was utilized by the MS^E technique and performed to acquire both precursor and fragment ion spectra during a single chromatographic run by alternating low and high collision energy (CE) scans.[11] The latter application was pioneered by Venable et al. on a linear ion trap mass spectrometer where the

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sequential isolation and fragmentation of 20 precursor ion windows (10 m/z wide each) were executed to cover m/z 900−1100 mass range.[12] The results demonstrated a dramatic signal-to-noise improvement for quantification using the XICs of fragment ions instead of the XICs of the precursor ions extracted from MS1 spectra, which is the typical approach adopted for DDA data processing.

Despite these advantages, the strategy of using predefined isolation windows was initially not widely adopted due to the limited scan rate of the mass spectrometer and the difficulty to perform efficient peptide identification from the complex MS/MS spectra acquired with low resolving power mass spectrometers. However, with the emergence of high resolution MS, in 2012, Gillet et al. introduced the "SWATH MS" method, which combined a high specificity DIA acquisition method with a novel targeted data extraction and processing algorithm.[13] This DIA approach was applied to a QqTOF system by transmitting all precursor ions within 32 consecutive Q1 isolation windows (25 Da) for fragmentation and recording the convoluted product ion spectra containing all resulting fragment ions. The presence and quantity of the analytes of interest in the injected sample were then determined by analyzing their fragment ion chromatograms derived from the full scan MS/MS spectrum at the defined SWATH window based on the information provided by a previously constructed spectral library (i.e., mass-to-charge ratio of the precursors, the fragment ion signals, and their relative intensities and chromatographic concurrence). The introduction of the SWATH MS (hereafter referred to as SWATH) method demonstrated high sensitivity and reproducibility for proteomics,[13,14] and recently, its application to low molecular weight compound studies has also received considerable interest.[15,16] One of the known advantages of SWATH acquisition resides in the possibility of reprocessing the same data set to obtain previously unidentified features without reacquiring the sample.[13]

On the basis of the initial studies conducted by Gillet et al., the SWATH method generally uses a fixed 25 Da Q1 isolation window width.[17−19] This isolation window width was considered to be equivalent to triple quadrupole SRM selectivity to detect peptide with five or more interference-free transitions when using 10 ppm fragment ion accuracy.[13] In this paper, we compared the use of fixed versus variable Q1 isolation window widths during SWATH acquisition. Specifically, we compared two methods for generating variable windows by considering either (1) the number of precursor ions or (2) the total ion current obtained in each window. A supportive tool, swathTUNER, with a rich graphical user interface was developed in-house to optimize the width of such SWATH windows. The impact of using either of the variable window strategies coupled with liquid chromatographic separation on the qualitative and quantitative analyses of complex proteomic samples obtained from whole cell lysates of human monocyte-derived dendritic cells (MDDCs), and of human urine metabolomic samples, was assessed. The analysis results of proteomic data acquired by these two strategies were also compared with the results obtained by applying variable windows generated by SCIEX SWATH variable window calculator v.1.0.

MATERIALS AND METHODS

Materials

Acetone, triethylammonium bicarbonate (TEAB) (1M, pH 8), sodium deoxycholate (Na-DOC), tris(2-carboxyethyl)-phosphine (TCEP), methylmethanethiosulfonate (MMTS), ammonium formate, ammonium acetate, isopropanol, trifluoroacetic acid (TFA), formic acid (FA), and ammonium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequencing-grade-modified trypsin was obtained from Promega (Madison, WI, USA). Acetonitrile (ACN, LC−MS grade), methanol (MeOH, Chromasolv), and water (LC−MS grade) were purchased from VWR (Radnor, PA, USA). Methanol (HPLC grade) was provided by Fisher Scientific (Leics, United Kingdom).

MDDC Proteomic Sample Preparation

Human monocyte-derived dendritic cells (MDDC) were generated from peripheral blood mononuclear cells after isolation from buffy-coats. Buffy-coats were obtained from anonymous healthy blood donors and were provided by the Blood Transfusion Center of the Hematology Service of the University Hospital of Geneva after approval of our project by the Ethics Committee of the University Hospital of Geneva (ref #0704). MDDC proteomic samples were prepared according to a procedure described in the literature.[20] Briefly, acetone precipitated MDDC proteins were resolubilized in dissolution buffer (0.5 M TEAB, pH 8, and 1% Na-DOC) and then subjected to reduction by 5 mM TCEP at 60 °C for 1 h and alkylation by 10 mM MMTS at room temperature for 10 min. After trypsin digestion with a 1:40 enzyme-to-protein ratio at 37 °C for 16 h, 2 μL of 50% formic acid was added to stop the process. For direct RPLC−MS/MS analysis, an aliquot of the supernatant of the protein digest after centrifugation was diluted to 0.2 μg/μL with 0.1% TFA in water, and then iRT peptides (Biognosys AG, Switzerland) (as retention time anchor peptides) were added at a ratio of 1:50 v/v.

Urine Metabolomic Sample Preparation

Urine samples were collected from ten healthy volunteers and immediately stored at −20 °C. They were centrifuged and pooled on the same day and subsequently stored at −80 °C until analysis. Isotopically labeled internal standards were added before analysis with a final concentration of 1 μg/mL of tryptophane-$^{15}N_2$ and 100 ng/mL benzamide-^{15}N (both provided by Cambridge Isotope Laboratories, Andover MA, USA) and 1 μg/mL of phenylalanine-^{13}C, estrone-$^{13}C_3$, and myristic acid-^{13}C for ESI negative only and 10 ng/mL of testosterone-$^{13}C_3$ for ESI positive only (all provided by Sigma-Aldrich, Buchs, Switzerland). Urine samples were analyzed without further dilution.

Strong Cation Exchange Fractionation for the MDDC Proteomic Samples

The MDDC protein digest was desalted using a Sep-Pak tC18 μElution plate (Waters, MA, USA). The peptide mixture was eluted by ACN/0.1% TFA in water (60:40). Approximately 250 μg of MDDC protein digest was loaded onto a strong cation exchange chromatography (SCX) column (PolySULFOETHYL A, 300 Å, 2.1 mm i.d. x 100 mm, PolyLC, MD, USA) on an UltiMate 3000 micro-LC system (Dionex, Germering, Germany). The separation was performed by a 32 min gradient (0−2 min 0% B; 2−24 min, 0−50% B; 24−29 min, 50−100% B; 29−32 min, 100% B) with mobile phase A (10 mM ammonium formate in 20% v/v acetonitrile, pH 3.0)

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and mobile phase B (500 mM ammonium formate in 20% v/v acetonitrile, pH 3.0) at a flow rate of 150 μL/min. The eluent was collected in a 96-well plate every 2 min. A total of 16 fractions were collected. The first fraction was discarded and the other 15 fractions were dried by a vacuum centrifuge evaporator and resuspended in 100 μL of 0.1% TFA in water for nanoLC−MS/MS analysis using DDA. Before LC−MS/MS analysis, iRT peptides were added at a ratio of 1:50 v/v.

Reversed Phase NanoLC−MS/MS Data Acquisition for the MDDC Proteomic Samples

MDDC proteomic samples were analyzed by a reverse-phase nanoLC−MS/MS system equipped with a NanoLC-Ultra 2D plus system (Eksigent, Dublin, CA, USA) and a TripleTOF 5600 mass spectrometer (SCIEX, Concord, ON, Canada) using a nanoelectrospray ionization source with an uncoated fused silica emitter tip (20 μm inner diameter, 10 μm tip, New Objective, Woburn, MA). Five microliters of sample solution were loaded onto a C18 nano trap column (Acclaim PepMap100, 5 μm, 100 Å, 300 μm i.d. × 5 mm, Dionex, Sunnyvale, CA, USA) and desalted with 0.1% TFA in water at a flow rate of 5 μL/min for 5 min followed by LC separation on a nanoLC column (Acclaim PepMap100, C18, 3 μm, 100 Å, 75 μm i.d. × 15 cm, Dionex, Sunnyvale, CA, USA) using mobile phase A (0.1% FA in water) and mobile phase B (0.1% FA in ACN) at a flow rate of 300 nL/min. The elution gradient was set as follows: 0−2 min, 2% B; 2−5 min, 2−5% B; 5−65 min, 5−35% B; 65−72 min, 35−60% B; 72−73 min, 60−90% B; 73−80 min, 90% B; 80−82 min, 90−2% B; 82−85 min, 2% B.

The mass spectrometer was operated in the positive ion mode, and the spectra were recorded in high-sensitivity mode. After acquisition of every 3 samples, the mass spectrometer was automatically calibrated by injecting 25 fmol ß-galactosidase tryptic digest. For MS analysis in DDA mode (used for building the MDDC spectral library), an MS survey scan with 250 ms accumulation time was performed, followed by MS/MS experiments of the top 10 most intense precursor ions using an accumulation time of 100 ms for each experiment. The total cycle time was 1.3 s. The dynamic exclusion window for MS/MS was set as 15 s. The CE was automatically adjusted by the rolling collision energy function of Analyst TF 1.5.1. For SWATH acquisition, a set of 32 sequential Q1 windows with either a fixed width of 26 Da (containing 1 Da for the window overlap) or variable sizes covering the precursor m/z range of 400−1200 Da (Table S1 in the Supporting Information). The accumulation time for each MS/MS experiment was 100 ms for a total cycle time of 3.3 s including an MS survey scan (100 ms) acquired at the beginning of each cycle. The CE for each window was determined according to the calculation for a charge 2+ ion centered upon the window with a spread of 20. For scheduled SWATH acquisition, the 32 sequential Q1 windows were created based on the precursor ion distribution within the retention time segment of 20−30 min in the LC gradient. All the other parameters were the same as the regular SWATH acquisition.

Reversed Phase HPLC−MS/MS Data Acquisition for the Urine Metabolomic Sample

An UltiMate 3000 RSLC chromatography system (Dionex, Sunnyvale CA, United States) with an Xselect column HSS T3 XP (2.5 μm, 2.1 mm i.d. × 150 mm, Waters, Milford, MA, USA) was used to analyze the urine samples. For positive ionization mode, mobile phase A was 5 mM ammonium formate in water with an adjusted pH of 3.0 by the addition of formic acid, and mobile phase B was methanol. For negative ionization mode, mobile phase C was 5 mM ammonium acetate in water with an adjusted pH of 8.0 by the addition of ammonium hydroxide. The gradient used in positive mode was 0−1 min, 5% B; 1−20 min, 5−95% B; 20−25 min, 95% B; 25−28 min, 5% B. In negative mode, mobile phase C was used instead of mobile phase A. The flow rate was 300 μL/min with the column temperature kept at 40 °C. The injection volume for analysis of the urine samples was 1 μL. Samples were cooled at 6 °C. For SWATH acquisition, a single TOF MS scan followed by 12 MS/MS experiments with fixed or variable Q1 windows (Table S2) with a mass range from m/z 50 to 915 for positive mode and m/z 50 to 932 for negative mode. The cycle time was adapted to 831 ms based on an average LC peak width of 12 s to obtain at least 12 points/peak. A collision energy spread of 50 ± 30 eV was applied. For scheduled SWATH acquisition, 17 individual methods were created for every 1 min time segment in the LC gradient. The LC sync was only applied for the first method from 0 to 1 min and was removed for all subsequent methods to allow MS acquisition using different SWATH methods over the whole LC gradient.

SwathTUNER Software Implementation and Q1 Isolation Window Generation

SwathTUNER is a web application implemented in R (version x64 3.1.1) developed with the *shiny* package for the graphical user interface (GUI) and the *ggplot2* package for generating figures. The input is a text file containing a list of LC−MS peaks (with each peak described by m/z, retention time, and intensity). The parameters can be interactively chosen, including the number of windows and the m/z and retention time ranges. The tool builds the windows according to the following methods: (1) fixed width windows (FIX): the m/z range is divided by the number of windows, and this result is the fixed window size; (2) variable width windows by equalized precursor ion population (PIP): the total number of peaks is divided by the number of windows to determine the fixed number of peaks per window. Window widths are computed from the start of the m/z range to the end. Each window is built by including peaks (from the list sorted by m/z) until the fixed number of peaks per window is reached. The mean m/z value between the last peak included and the next one gives the end of the current window and the start of the next one; (3) variable width windows by equalized precursor total ion current (TIC): the sum of the intensity of all peaks is divided by the number of windows to determine the fixed intensity per window. Window widths are computed from the start of the m/z range to the end. Each window is built by including peaks (from the list sorted by m/z) until the fixed intensity per window is reached. The mean m/z value between the last peak included and the next one gives the end of the current window and the start of the next one. The swathTUNER software tool is available at http://swathtuner.sourceforge.net.

For the experiments presented in this paper, the input file was a list of LC−MS peaks extracted from the TOF MS survey experiment by the tool of Enhance LC/MS Peak-Finding Filter in the PeakView software (v2.0, SCIEX). The peaks were extracted within 6−70 min with an approximate LC peak width of 30 s for proteomic sample and 1−26 min with an LC peak width of 12 s and background subtracted for the urine sample. The generated LC−MS peak list was saved as a text file and imported into swathTUNER. The following software settings were used by swathTUNER for proteomic sample analyses: 32

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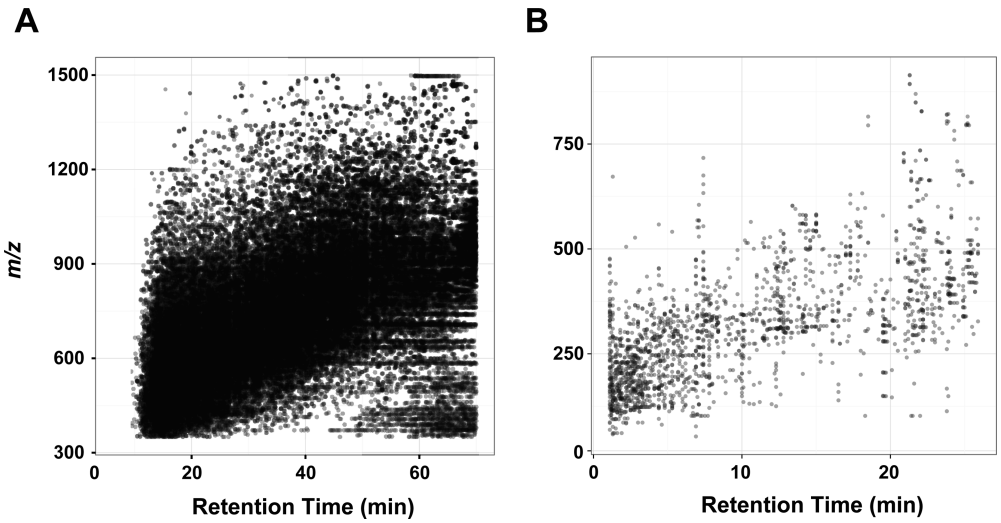

Figure 1. LC−MS features detected in the MS1 acquisition of MDDC tryptic digest (A) and pooled urine metabolomic sample in positive ionization mode (B). Images were taken from the swathTUNER GUI.

windows, m/z range 400−1200, and for urine metabolomic sample analyses: 12 windows, m/z range 50−893 for positive ESI and 50−932 for negative ESI. The resulting tables containing the start and end m/z values of each window were copied (.txt file format) and saved as the files (.wpoa), which were imported into Analyst TF 1.5.1 software for creating the SWATH acquisition method. As an example, a screenshot of the swathTUNER GUI for generating the SWATH Q1 isolation windows for the MDDC proteomic sample is shown in Figure S1.

Data Processing for MDDC Proteomic Samples

Protein Identification for MDDC Spectral Library Construction. The MS raw files acquired by DDA were searched by ProteinPilot software (v4.2, SCIEX) using the Paragon algorithm with Thorough ID search effort and False Discovery Rate (FDR) analysis.[21] The following sample parameters were used: trypsin digestion and cysteine alkylation with MMTS. Data files were searched against a reconstructed database (a combination of UniProt_Human and common contaminants from cRAP and MaxQuant databases, updated on April 12, 2014).

SWATH Data Processing for Peptide Identification and Quantification. Targeted data extraction of SWATH files was performed by Skyline software (v2.5, Seattle, WA).[22] Two DDA spectral libraries were built based on either 1D-LC or 2D-LC separations of the MDDC tryptic digest. Spectral libraries were generated based on the imported ProteinPilot search result (.group file) by using the software package "BiblioSpec" implemented in the Build Library tool from Skyline. A cutoff score equivalent to an FDR (local) analysis of 5% was applied.[23] The same protein sequence database used for ProteinPilot search was set as the background proteome with a maximum missed cleavage of 1 for the tryptic digestion. Only peptides (charge state +2 to +4) with 6−10 fragment ions (charge state: + 1 and +2, types: y and b, m/z range: 350−1500) were selected to perform the subsequent targeted data analysis. XICs of these fragment ions were extracted using the MS/MS filtering function in Skyline with a retention time window of ±3 min centered in the predicted RT (based on the iRT value calculated from the iRT peptides[24]).

Performance Evaluation of SWATH Methods for Proteomics. To compare the performance of different SWATH acquisition methods used for the MDDC proteomic samples, we used three indicators: peptide identification, spectral quality, and peptide peak area variability. Peptide identification is based on the Q-value (cutoff 0.01), which was exported from Skyline as the FDR assessment computed according to the mProphet algorithm using decoy peptides (shuffle sequence).[25] Spectral quality is indicated by the Dot-Product (DotP) value exported from Skyline showing the correlation between the peak areas of the extracted fragment ions in the SWATH spectra and the corresponding intensities in the DDA library spectrum (computed according to the "normalized spectral contrast angle"[26]). Peptide peak area variability is expressed as a coefficient of variation and is calculated based on the summed XIC areas of the SWATH fragment ions for each peptide across the technical replicates.

Data Processing of Urine Metabolomic Samples

Metabolite Identification of SWATH Data Using an Annotated Accurate MS Library. The urine samples were screened for 528 compounds reported in an in-house MS metabolomics library (AMML library).[27] Among these compounds, only 339 showed an LC peak (from TOF MS data) in positive mode and 407 in negative mode at their expected retention time. The following settings were applied to perform the library search: (1) a retention time window of 0.4 min, (2) a signal-to-noise ratio higher than 5 and the intensity higher than 10 counts, (3) a mass accuracy below 10 mmu, and (4) an intensity factor of 1. PeakView 2.2, MasterView 1.1 (SCIEX), and the in-house library were used for automated data processing. Possible retention time shift was investigated through the use of 4−5 isotopic-labeled internal standards, which were added to each urine sample. These standards were well-distributed over the entire LC gradient. The overall shift in retention time for all samples was very small with an average standard deviation less than 0.1 min. Therefore, no alignment was needed, and the shift was covered by the relatively small retention time window of 0.4 min.

Peak Detection of SWATH Features for Comparison of Positive and Negative Ionization Modes. SWATH files corresponding to both positive and negative ionization modes

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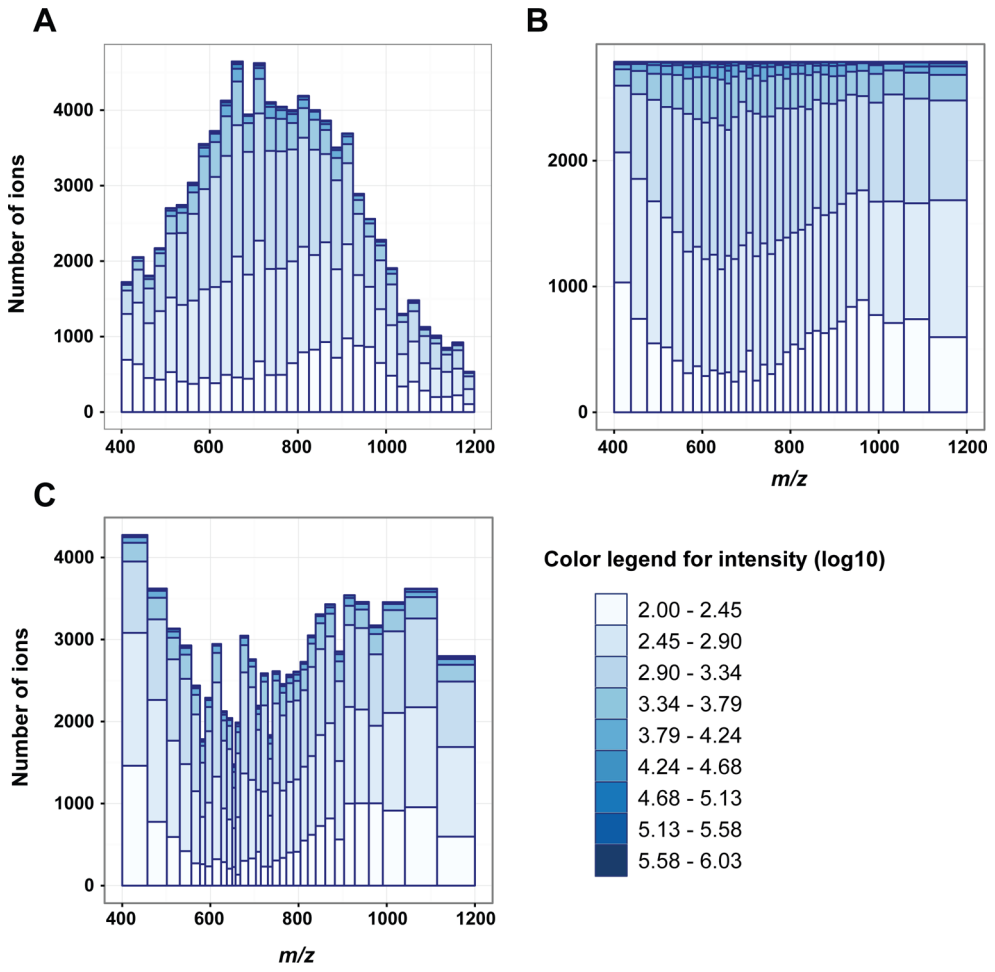

Figure 2. Distribution of the precursor ions detected by MS1 acquisition of the MDDC protein extract digest within the LC retention time range of 6−70 min. Each bin represents a defined mass range (or Q1 isolation window) created by the FIX (A), PIP (B), or TIC (C) approach. The color darkness indicates the total signal intensity. Images were taken from swathTUNER GUI.

were processed by MarkerView (research version 1.2.4.0) to automatically extract the features (LC peaks) of each swath. An in-house C# tool was used to convert the binary files to text files.

Performance Evaluation of SWATH Methods for Metabolomics. The performance of different SWATH acquisition methods for urine samples was evaluated based on metabolite identifications using an MS/MS spectral library search (AMML library, MasterView 1.1) considering the following matching scores: purity score, fit score, and reverse-fit score. The purity score is determined by the number of matched fragment ions (i.e., common fragment ions between the SWATH spectrum and the library spectrum given a mass tolerance). This is similar to the cosine score based on fragment intensities and reflects the compound identification performance. The fit score calculates the fraction of the library fragment ions observed in the SWATH spectrum. This score is used to confirm that the variable isolation window methods do not affect the number of fragment ions belonging to the SWATH spectra compared to the FIX method. The reverse-fit score determines the fraction of fragment ions in the SWATH spectrum observed in the library spectrum. This score evaluates the impact of the variable isolation window strategies on the SWATH spectra.

RESULTS AND DISCUSSION

Generation of the SWATH Q1 Isolation Windows

Liquid chromatography (LC) coupled to mass spectrometry (MS) is a commonly used technique to characterize analytes in proteomic and metabolomic studies. Even though these studies follow similar workflows, they are quite different in terms of analytical strategy. A proteomic sample generally represents a mixture composed of thousands of analytes with comparable chemical diversity (namely peptides in the case of shotgun proteomics), which can be efficiently separated by reversed phase LC before MS analysis. On the contrary, metabolites show a huge diversity of chemical structures and properties and therefore require different types of chromatographic mechanisms (e.g., C18, HILIC). To cover a wide range of metabolites and to cope with acidic and alkaline mobile phases, the same sample is usually analyzed both in positive and negative ionization modes. Figure 1A illustrates the features captured during a nanoLC−MS survey scan of the tryptic digest of proteins derived from monocyte-derived dendritic cells (MDDCs). Figure 1B shows the features detected in the pooled urine samples analyzed by narrow-bore LC−MS.

Although the MS full scan can be used to detect and quantify these features, the identification of analytes relies on their respective MS/MS spectrum, which is typically obtained by a data-dependent acquisition (DDA) approach. Initially devel-

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oped to overcome the limitation of DDA with respect to its sampling bias toward abundant features, data-independent acquisition (DIA) approaches, such as SWATH, generate fragment ions for all the precursor ions across the entire mass range during the same sample analysis run. Because this approach applies wider precursor isolation windows compared to DDA, many more ions are transmitted simultaneously to the collision cell, resulting in the production of multiplexed MS/MS spectra of cofragmented precursor ions. To overcome the resulting loss of selectivity and the impaired precursor identification efficiency, LC separation and mass analyzer resolving power can be improved. For example, Fourier transform (FT) mass spectrometers offer higher resolving power compared to TOF analyzers. However, the resolving power in FT-based instruments is directly proportional to the scanning time. Consequently, due to the faster detection rate afforded by TOF analyzers, the resolving power between an FT-based and a TOF instrument can be considered equivalent when covering the same mass range while acquiring sufficient data points for quantification (elution profile) in typical proteomic applications.[28] In practice, the loss of selectivity cannot be currently overcome by only increasing the resolving power. An alternative approach would be to narrow the Q1 isolation windows, thus reducing the complexity of MS/MS spectra. Nonetheless, using this method, more SWATH events (hereafter swaths) would be required to cover the same precursor ion m/z domain. Each swath would then need to be acquired with a reduced accumulation time to maintain LC peak integrity, thus decreasing the MS/MS spectral quality and overall sensitivity.

Since its introduction, the SWATH approach using 32 isolation windows with fixed width of 25 u has been routinely followed when analyzing a complex proteomic sample. With this window width, a range of m/z 400−1200 can be covered with 100 ms accumulation time per swath for a total cycle time of 3.3 s (3.2 s for stepping through the 32 isolation windows plus 0.1 s for the TOF MS experiment).[13] This is generally sufficient to reconstruct the peptide chromatographic peaks (peak widths are of 30−45 s in our experiment) that allow accurate quantification. Such a window width has been used to subdivide the peptide precursor features observed in Figure 1A into 32 groups of sequential m/z ranges, and the number of precursor ions per window as well as their intensities is illustrated in Figure 2A. In the case of low molecular weight compounds, the precursor mass range is shifted toward lower m/z values. For drug metabolism studies, the Q1 window width is typically 20 u or smaller and can be centered on the m/z value of the desired metabolites.[15] However, the isolation window size is ultimately determined by the LC peak width, the accumulation time needed for each MS/MS experiment, and the precursor m/z domain to be covered. The average peak width of 12 s observed in our experiment for urine sample analyses required an MS cycle time below 1 s. Thus, two acquisition methods with 12 swaths of 71 u or 74 u were devised for the positive and negative ionization modes, covering the precursor domains of m/z 50−893 and m/z 50−932, respectively. The accumulation time for each swath was set to 60 ms because of the relatively large collision energy spread (namely, CE = 50 ± 30 eV). The distribution plots of the precursor features within each swath are presented in Figure S2.

Despite the fact that each window employed by these acquisition methods covers an equivalent m/z interval, respectively, the number of peptide precursors from the MDDC protein digest and the number of metabolite precursors from the urine sample were observed to be unevenly distributed across the entire m/z range (Figure 2 and Figure S2). For the proteomic samples, the density of the precursor ion population tends to get higher near the center of the mass range. This uneven distribution is even more pronounced for the urine samples where a much higher proportion of molecular features was detected over the m/z range of 125−350 in the positive mode and over the m/z range of 175−400 in the negative mode compared to other ranges. When these samples were subjected to SWATH acquisition, the same precursor ion distribution was maintained, and therefore, more ions were directed to the collision cell for the windows with higher ion density, resulting in more complex MS/MS spectra. Therefore, the use of isolation windows of varying widths is required to distribute more evenly the ion population at the precursor level. For this purpose, the swathTUNER software tool was developed to create a series of windows with varied sizes and alleviate the discrepancy of the ion content in each swath. The concept behind it is to equalize the precursor ion population or the total intensity accumulated by the precursor ions among all of the windows. This process is based on the information extracted from MS1 features, such as m/z values, retention time, and peak intensity. Panels B and C in Figure 2 depict the application of both strategies to the MDDC proteomic sample, where isolation windows of a narrower width are assigned to m/z regions of either higher ion density or intensity whereas wider windows are created for less crowded regions. Similar distribution plots for the urine metabolomic samples are depicted in Figure S2.

Recently, SCIEX provided an active Excel spreadsheet template, which is known as SWATH variable window calculator 1.0 (hereafter SWATH calculator) for building SWATH Q1 variable windows. The input precursor list also contains precursor m/z values and peak intensities obtained from the peaks detected in a merged spectrum from all spectra within a user selected retention time window. Unlike swathTUNER, which builds two separate window sets based on either precursor ion count or precursor total ion intensity, SWATH calculator generates one window set by computing the number of precursor ions and taking into account their intensities as a weighting factor. As an example, Figure S3 displays the SWATH isolation window plot generated by this calculator based on the same MS survey scan as in Figure 2A. In this paper, the SWATH method using fixed isolation window width is referred to as FIX, whereas methods using variable isolation windows generated by swathTUNER and by the SWATH calculator are referred to as PIP (equalized precursor ion population), TIC (equalized total ion current), and SCI. Except for the window width, all of the other parameters, such as the duty cycle, the number of windows, and the accumulation time, were consistent for all the SWATH methods, and the same LC method was applied. Therefore, only the effect of precursor isolation window width on peptide/metabolite identification and quantification was evaluated.

Improved Peptide Selectivity

A peptide of interest from a highly complex proteomic sample may still face the challenge of interference from coeluting substances even after extensive chromatographic separation. With the SWATH acquisition method, such interference may arise from the fragment ions generated by coeluting and cofragmented analytes. To account for this issue, the quality of

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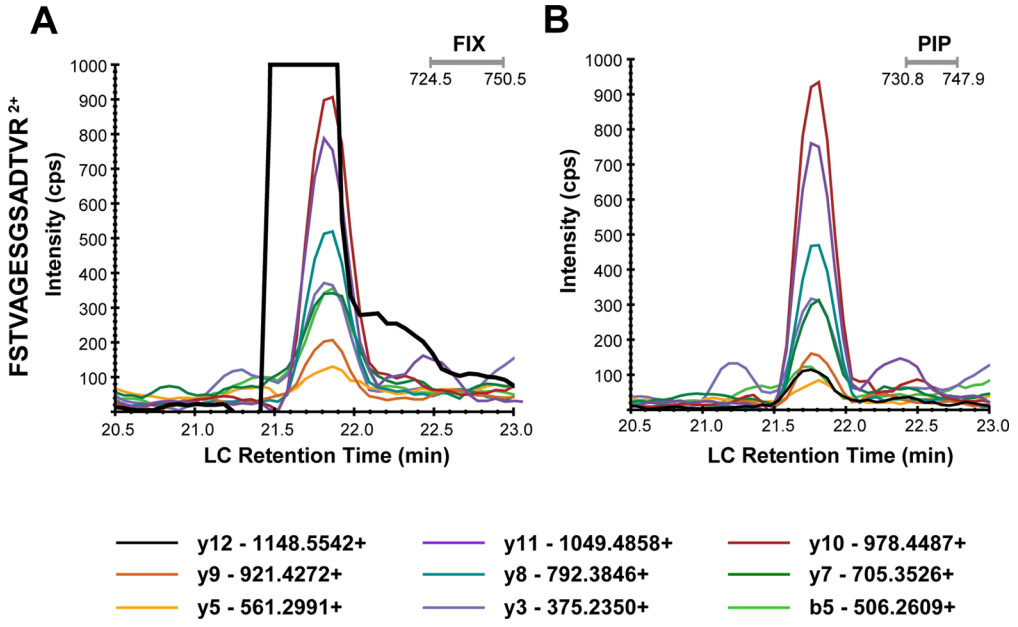

Figure 3. Example of a fragment ion interference removed by the use of a variable isolation window acquisition. XIC traces of the targeted fragment ions for the tryptic peptide FSTVAGESGSADTVR^{+2} (m/z 742.3548) by the FIX (A) and PIP (B) methods. The horizontal gray bars indicate the Q1 isolation window widths.

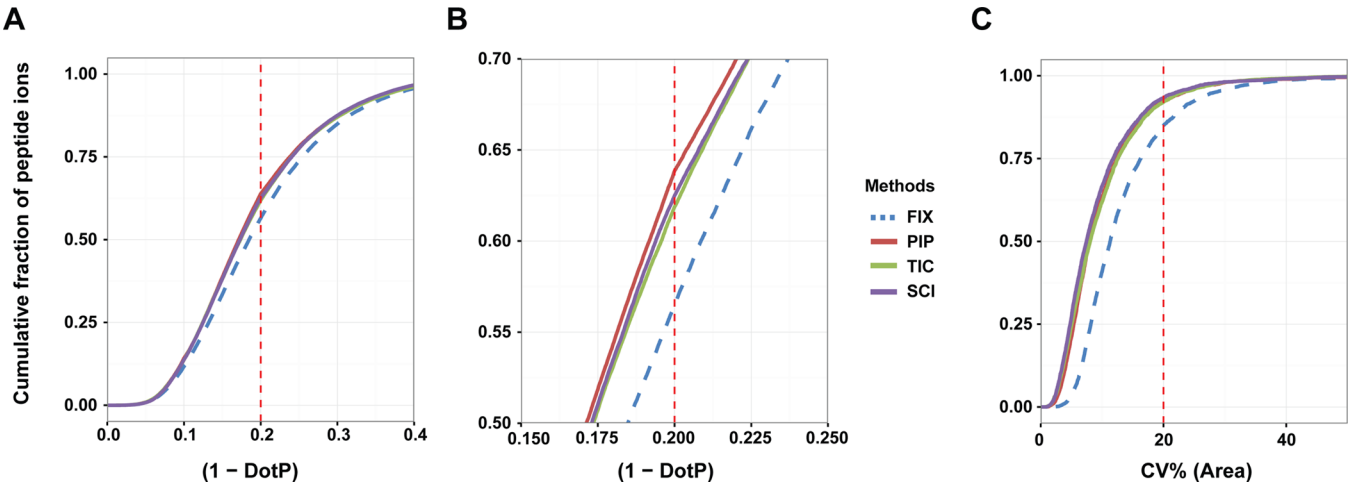

Figure 4. Comparison of the fixed and variable isolation windows methods as a function of 1-DotP value (A) with a partial enlarged view around the DotP value of 0.8 (B), as well as a function of CV% for peptides identified with a Q-value ≤0.01 within five technical replicates (C). The vertical red dashed line indicates a DotP value of 0.8 (A,B) and a CV of 20% (C).

each group of fragment ion XIC traces belonging to a targeted peptide is generally assessed by comparing it with the reference DDA spectrum. Some data processing software, such as Skyline, perform such spectral comparison by calculating a dot-product (DotP)-based score. As an example, Figure 3A shows the XIC traces of nine targeted fragment ions originating from the doubly charged precursor ion of FSTVAGESGSADTVR (m/z 742.3548) (peptide precursor #1). The nine ions were extracted from the MS/MS spectrum generated using the FIX method at the SWATH isolation window of m/z 724.5−750.5. As clearly observed, the XIC trace of the y12$^+$ fragment ion (m/z 1148.5542, black trace) is perturbed by a fragment ion of a cofragmented peptide. As a consequence, the peptide peak group results in an average DotP value of 0.58 (n = 5) corresponding to a low-quality match to the reference library spectrum. By looking more closely at the impaired m/z trace, it is clear that the interfering ion in Figure 3A corresponds to the y10$^+$ fragment ion (m/z 1148.5582) originating from the doubly charged precursor of LGHPDTLNQGEFK (m/z 728.3650, peptide precursor #2). Because SWATH supports the repeated processing of previously acquired data sets, one of the solutions to the presence of interfering fragment ions is to perform post-acquisition data processing to detect and remove impacted XIC traces. Thus, for peptide precursor #1 in Table S3, the y12$^+$ ion can be removed from the targeted fragment list, resulting in the increase from 0.58 to 0.90 for the corresponding spectral library matching DotP score. Nonetheless, when dealing with large-scale data processing, such a post-acquisition strategy is only practically feasible through the use of dedicated tools to remove interfering XIC traces.

A more practical alternative is the adjustment of the Q1 isolation windows of the SWATH acquisition method. This

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provides a relatively simpler strategy to preventively reduce the ion density and therefore directly reduce possible ion interferences by separating the coeluting precursors into distinct fragmentation events, which results in enhanced selectivity. To show the effectiveness of this approach, we analyzed the sample described above with a PIP-based variable isolation windows acquisition method (Figure 3B). Using this variable window width approach, peptide precursor #1 (FSTVAGESGSADTVR, m/z 742.3548) was fragmented within a narrower isolation window (i.e., m/z 730.8−747.9). Conversely, peptide precursor #2 (LGHPDTLNQGEFK, m/z 728.3650) was captured in the immediately preceding swath covering the m/z range of 713.9−731.8. As shown in Figure 3B, the $y10^+$ fragment ion from peptide precursor #2 did not interfere anymore with the XIC trace of the $y12^+$ ion of peptide precursor #1. This in turn resulted in greatly improved selectivity for peptide precursor #1 and gave rise to an average DotP value of 0.93 (Table S3).

To compare the performance of the variable windows methods with the basic fixed-width window approach, the MDDC tryptic digest was analyzed by each SWATH acquisition method, including the SCI-based approach. SWATH data processing was performed by targeting a set of 10,600 peptide precursors found in the spectral library obtained by 2D LC−MS/MS in DDA mode. The DotP value was computed for each of these peptides, and Figure 4A illustrates the resulting cumulative plots. All three variable window width methods outperform the FIX method. This is shown in Figure 4B, where the DotP threshold is set to 0.8, and 56.4% of the precursors are detected with a value higher than or equal to that threshold with the FIX method, whereas 63.8, 62.5, and 61.8% are obtained with the PIP, SCI, and TIC methods, respectively. Surprisingly, the difference between the SWATH methods using variable isolation windows was not significant. Indeed, the precursor ions followed similar distribution patterns based on either their numbers or their intensities, and therefore, the resulting SWATH Q1 windows used across methods were unexpectedly similar. It is worth noting that the SWATH calculator (SCI method) generates the SWATH windows by considering both the precursor ion population density and the total ion current, giving rise to cumulative peptide precursor frequency distributions that appear to be a compromise between those generated by the other two methods.

Improved Peptide Detection Rate

To perform targeted data processing of SWATH data acquired for the MDDC proteomic samples, an initial spectral library containing 3,545 distinct peptide precursors from 723 proteins was built based on the DDA identification results obtained from five LC−MS/MS replicates using only one LC dimension for separation (library #1). The resulting peptide peak groups were evaluated with the mProphet algorithm to estimate the false discovery rate (FDR), and the corresponding results expressed by Q-values are summarized in Figure S4A. Taking advantage of the improved peptide selectivity afforded by each of the variable Q1 window methods, slightly more peptide precursors were confidently identified at an FDR value of 1% (equivalent to Q-value 0.01) as compared to that of the FIX method. Although the increase was subtle (overall <2%), the difference was statistically significant ($p < 0.01$ by t test, data not shown) across all pairwise comparisons between the FIX SWATH method and each of the variable width methods.

For the purpose of increasing the proteome coverage of the MDDC spectral library, we fractionated the protein digests by off-line strong cation exchange chromatography (SCX) prior to nanoLC−MS/MS analysis by DDA. This strategy, commonly known as 2D separation, tripled the number of peptides identified compared to the previous 1D separation experiment. On the basis of the merged identification results from all fractions, a spectral library containing 10,600 distinct peptide precursors with 1,610 associated proteins was built (library #2). This library was then used to perform the targeted analysis of the same SWATH data sets as used above for library #1. The distribution of associated Q-values calculated for each SWATH method is given in Figure S4B, where a more notable improvement is observed by SWATH methods using variably sized windows relative to the FIX method. As shown in Table 1

Table 1. Number of Peptides/Proteins Identified by the Different SWATH Methods[a]

library	ID	FIX	PIP	TIC	SCI
#1	peptides (n = 5)	3405	3476	3474	3456
	CV (%)	0.31	0.23	0.13	0.24
	proteins (n = 5)	708	714	717	713
	CV (%)	0.31	0.24	0.15	0.34
#2	peptides (n = 5)	5714	6503	6193	6259
	CV (%)	2.03	0.57	1.06	1.75
	proteins (n = 5)	980	1108	1077	1072
	CV (%)	2.40	0.58	1.61	0.19

[a]Library #1 was built from the 1D LC−MS/MS data set. Library #2 was built from the 2D LC−MS/MS data set. Peptide identifications were filtered by FDR 1% (Q-value of 0.01).

(library #2), an average number of 5,714 peptide precursors corresponding to 980 proteins was confidently identified by the FIX method that used the constant 25 u isolation window. However, the three SWATH methods using variable Q1 window widths allowed the identification of 789, 479, and 545 additional peptide precursors (consequently, 128, 97, and 92 proteins) for the PIP, TIC, and SCI methods, respectively, representing an increase of 13.8, 8.4, and 9.5% (13.1, 10.0, and 9.5% for proteins). Taking all observations in consideration, we can conclude that the use of variable Q1 isolation window widths improves the identification capacity of the SWATH acquisition method compared with the use of the more common fixed window width. In particular, among the three variable width methods we examined, PIP was associated with the highest peptide identification rate, which is consistent with that presented above. These results suggest that, for a complex sample matrix, the density of precursor ions found within each isolation window has a larger impact on confident peptide identification than ion intensity. Consistently, the largest number of peptide precursors confidently identified by PIP that were not found by FIX were found around the center of the mass range where the highest level of precursor crowding can be typically observed (Figure 5A). On the contrary, because wider window sizes were applied at the edges, the number of confidently identified peptide precursors arising from these regions of the mass range (m/z 400−500 and 1050−1200) was slightly reduced. In addition, we noticed a significant enrichment of low abundance peptide precursors among those that were de novo identified using PIP. Because such peptides are typically very susceptible to interference, this observation suggests that PIP has a higher potential to identify

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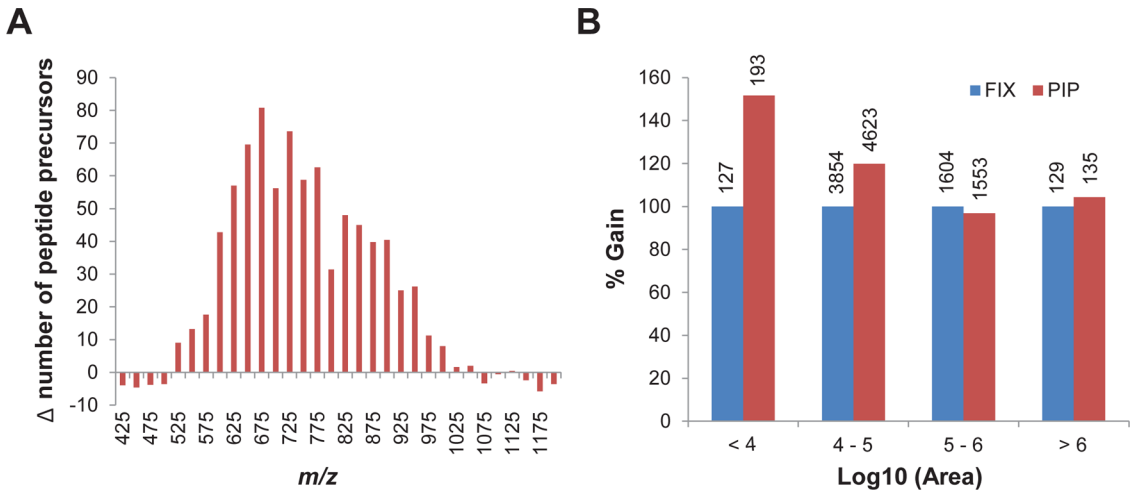

Figure 5. Number of peptide precursors confidently identified (Q-value ≤0.01) within different *m/z* ranges by the PIP method compared to that of the FIX method. (A) Additionally identified peptide precursors with bin size of 25 u representing the isolation window width used by FIX; (B) increase of the peptide precursors identified by PIP against FIX with the number above the bar indicating the absolute number for each method (average number of five technical replicates).

and quantify low abundance biological protein targets than the basic approach (Figure 5B).

Improved Quantitative Reproducibility

One of the major goals for performing proteomic studies is to identify both qualitative and quantitative differences among different sample cohorts (e.g., control vs disease), which potentially leads to the discovery of disease biomarkers. A reproducible method is thus essential to minimize spurious results arising from biological variabilty and potentially undermining the significance of the study. To assess whether the enhanced selectivity provided by the application of variable Q1 isolation windows would also improve assay reproducibility, we performed a comparative study by comparing the quantification results derived from the summed XIC peak areas of the fragment ion peak groups. The coefficient of variation (CV) was calculated for the peptides confidently identified (Q-value ≤0.01) across all four SWATH methods and five LC−MS/MS technical replicates (Figure 4C). Again, the three SWATH methods using variable Q1 window widths performed better than the method using the fixed width and produced a higher number of peptides with higher quantitative reproducibility. In particular, 92.2, 92.1, and 93.3% of the total peptides were found to have a CV value below 20% for the PIP, TIC, and SCI methods, respectively, which are higher than the result obtained by the FIX method (84.9%).

The Use of SWATH Variable Isolation Windows for Metabolomics

The performance of the variable SWATH isolation window approach in metabolomics was evaluated by analyzing a human urine sample. Urine is commonly regarded as one of the most complex metabolomic samples.[29] Samples from ten healthy volunteers were pooled and analyzed without further dilution to increase the number of metabolites present in the sample. The identification of metabolites relies on matching the exact mass, retention time, and MS/MS spectra of the biological sample with reference standards. We used an in-house spectral library containing 528 composite spectra from compounds belonging to the Human Metabolome Database[27] where the CE spread was adapted to the CE ramp applied during the SWATH experiments. Only fragments with structural annotations based on the precursor ion structure were included in the library, as well as the retention time obtained in the same experimental LC conditions. The retention time variation was below 0.1 min for the internal standards added to each sample, allowing the application of a retention time window of 0.4 min. Such small tolerance is useful for metabolite screening where many isomers may be present and good MS/MS matching is not always sufficient for unambiguous identification.

Improved Metabolite Identification

On the basis of the urine sample analysis shown in Figure 1B, three SWATH acquisition methods were created with swathTUNER (Table S2). Panels A and B in Figure 6 show the LC chromatogram obtained in positive ionization and the XIC traces of three representative compounds: (1) pyroglutamic acid (RT = 2.2 min), (2) cyclic AMP (RT = 3.6 min), and (3) 5-hydroxyindoleacetic acid (RT = 6.4 min). For these three compounds, the scores were compared between the fixed and the variable isolation window acquisition methods for three technical replicates. The purity score, which is related to the compound identification, was improved for the PIP and TIC approaches compared to the FIX method for at least two of the selected compounds (Figure 6C). As expected, the Reverse Fit varies similarly to the Purity score, and the Fit score remains the same for all of the SWATH acquisition methods. Finally, Figure 6D illustrates examples of SWATH MS/MS spectrum quality improvement when using the SWATH variable windows for the compound with a low intensity (namely, cyclic AMP), which leads to more confidence in the spectral library search results.

The overall improvement of the purity score for the methods with variable isolation windows is shown in Figure 7A and B by plotting the cumulative fraction of compounds related to their "dissimilarity score" (namely, "1-Purity score"), where a value of 0 reflects a perfect match. The same trend was observed for the Reverse Fit score (Figure S5), indicating an improvement of the MS/MS spectral quality (i.e., with less interfering fragment ions in the SWATH spectra).

In negative ionization mode, no notable improvement was observed for the purity score, especially between 80 and 100%. This is related to the fact that the SWATH spectral library

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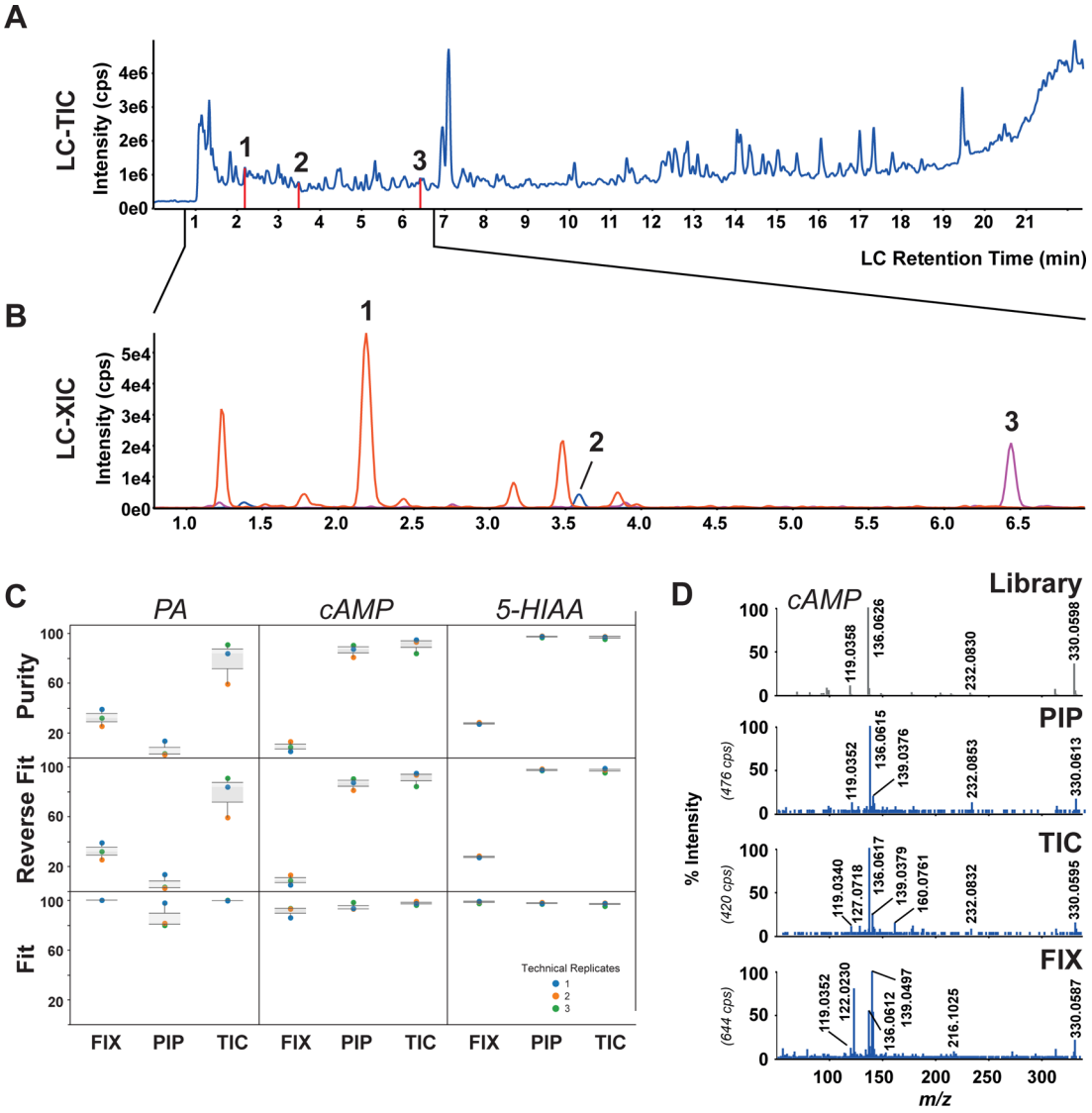

Figure 6. Comparison of fixed and variable isolation window methods for the pooled urine sample (positive ionization). Representative LC TIC chromatogram (A) with extracted traces (B) from (1) pyroglutamic acid (PA, m/z = 130.0499, RT = 2.2 min), (2) cyclic AMP (cAMP, m/z = 330.0598, RT = 3.6 min), and (3) 5-hydroxyindoleacetic acid (5-HIAA, m/z = 192.0655, RT = 6.4 min.). (C) Box plots (n = 3) showing the scores obtained for the FIX, PIP, and TIC acquisition methods along with the three library search scoring algorithms (namely, Purity, Reverse Fit, and Fit). (D) MS/MS spectra of cyclic AMP (compound 2) obtained for the spectral library as well as with the PIP, TIC, and FIX methods.

acquired in negative mode contains less fragment ions per compound than in positive mode (Figure S6A). To further investigate this aspect, we extracted all features within the SWATH spectra obtained by the urine metabolomic samples for both ionization modes. This feature extraction was performed by a peak detection algorithm without using any spectral library. We observed that the overall precursor ion intensity was lower for the negative ionization mode (Figure S6B), and the number of fragment ions detected from the sample was also lower in the negative mode (Figure S6C). As a consequence, the benefits brought by the use of variable isolation window methods are not as significant as in the positive ionization mode for the reduction of interfering ions.

Scheduled SWATH Acquisition

In the experiments presented here, SWATH isolation windows were built based on the assumption that the same precursor ion distribution is maintained across the entire LC elution time. In reality, the precursor distribution pattern differs throughout the LC chromatography as shown for the MDDC digests (RT segments of 10 min, Figure S7) and for the pooled urine samples (RT segments of 1 min, Figure S8). Thus, precursors with smaller m/z values dominated early eluting fractions with a gradual switch to precursors with larger m/z values in the final fractions. Therefore, different sets of variable isolation windows should be generated during the SWATH acquisition (namely, "scheduled SWATH") for each LC retention time segment according to the actual precursor ion distribution. To evaluate whether this "scheduled SWATH" strategy would provide any additional qualitative or quantitative gains, we compared the results obtained from the 20−30 min retention time segment by the PIP method designed for the complete run (see above) with results from the same retention time segment but acquired using a new SWATH method designed specifically for this period. The comparison was performed only for a proof-of-concept purpose. This is because automatic data acquisition using "scheduled SWATH" within a single LC−MS run is not

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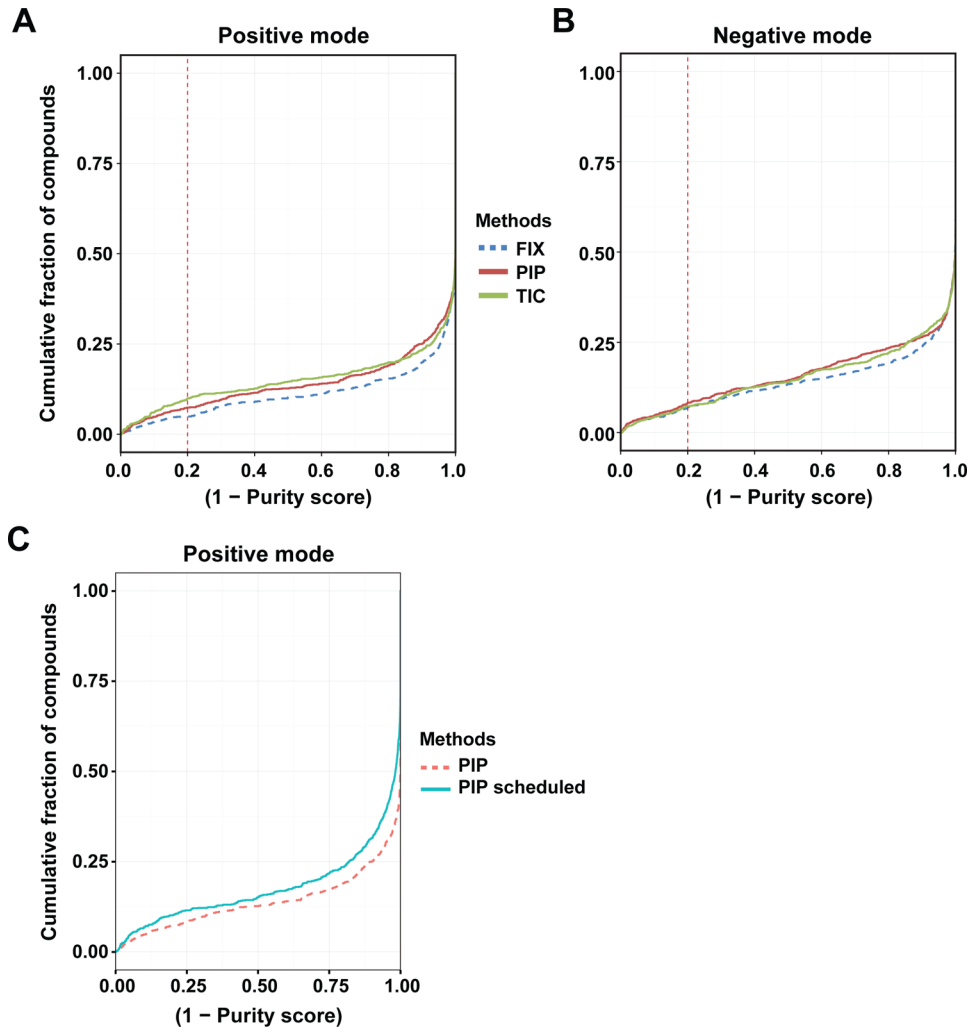

Figure 7. Comparison of the fixed and variable isolation windows methods as a function of 1-Purity score in positive (A) and negative (B) ionization mode. Improvement of the identification rate with the 1 min segments scheduled SWATH acquisition method (C). The vertical red dashed line indicates a Purity score of 80% (A,B).

yet supported by the instrument software. As expected, the results showed an improved overall DotP and Q-values that would lead to more confident identifications for the targeted peptides when using this new method (Figure S9). A similar improvement of the purity scores was observed for the metabolites in the urine sample, where the Q1 windows were designed for each of the retention time segments of one minute (Figure 7C).

CONCLUSIONS

Large-scale accurate identification and differential profiling of proteins and metabolites strongly rely on the ability of performing consistent and sensitive analysis. SWATH is advantageous over DDA methods in terms of both sensitivity and reproducibility. However, the optimal window width of the SWATH method is sample-dependent due to differences in sample matrix and consequent differences in the distribution of precursor ions. By applying narrower Q1 windows for mass regions of higher precursor density or intensity, an additional dimension of analyte separation at the mass level is achieved. This in turn results in improved selectivity of the targeted analytes (i.e., peptides or metabolites) without compromising any other instrument parameters such as the MS cycle time. Consequently, more confident identifications and more reliable quantification results can be obtained with no need for additional sample preparation efforts or sophisticated post data processing. In particular, for proteomic samples, equalizing the number of precursor ions per window was found to be more effective in terms of peptide identification rate than equalizing the precursor ion intensity or using a combination of the two approaches (SCIEX method). However, for samples with fewer detectable LC−MS features, such as pooled human urine, the application of the TIC method appeared to perform slightly better than the other two methods in improving metabolite spectral quality, which is expected to be beneficial for metabolite identification. Finally, swathTUNER combines usability and flexibility for the selection of different SWATH data acquisition strategies to address the requirements of multiple experimental conditions and is expected to be used for several acquisition instruments, including FT-based analysers, where the same principles apply.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00543.

Q1 isolation windows for different SWATH MS acquisition methods used for MDDC proteomic sample and human urine metabolomic sample analyses, DotP value comparisons, swathTUNER GUI screenshot for generating SWATH Q1 isolation windows, SWATH acquisition methods with fixed and variable Q1 isolation windows for the metabolomics urine sample, Q1 isolation windows calculated by the SCIEX SWATH variable window calculator v1.0 for proteomics sample analysis, cumulative fraction of the targeted peptides as a function of the Q-values, cumulative plots for metabolite identification in human urine, comparison of the spectral results obtained by positive and negative ionization modes for the urine metabolites, distribution of the precursors detected in TOF-MS analysis of the MDDC protein extract digest, representative distribution charts of the precursors detected in TOF-MS analysis, and the result comparison of the "scheduled SWATH" and the regular SWATH used for MDDC proteomic sample analysis (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MS, mass spectrometry; LC, liquid chromatography; DDA, data-dependent acquisition; DIA, data-independent acquisition; XICs, extracted ion chromatograms; MDDC, monocyte-derived dendritic cells; TEAB, triethylammonium bicarbonate; TCEP, tris(2-carboxyethyl)phosphine; Na-DOC, sodium deoxycholate; MMTS, methylmethanethiosulfonate; TFA, trifluoroacetic acid; FA, formic acid; ACN, acetonitrile; SCX, strong cation exchange chromatography; FDR, false discovery rate; CV, coefficient of variation; PIP, precursor ion population; TIC, total ion current

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