Journal of proteome research

pubs.acs.org/jpr

Technical Note

Optimizing MS Parameters for Data-Independent Acquisition (DIA) to Enhance Untargeted Metabolomics

Published as part of Journal of Proteome Research special issue "Multiomics in Africa and the Middle East".

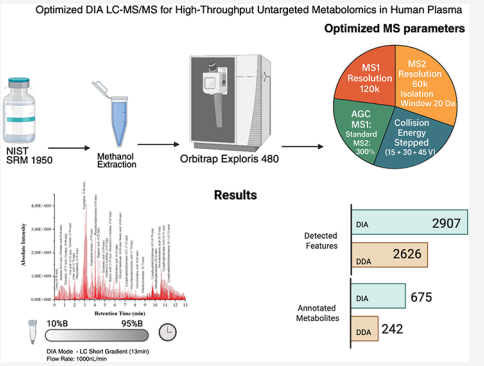
Frederico G. Pinto, Alexander D. Giddey, Rouda S. B. Almarri, Omer S. Alkhnbashi, Timothy J. Garrett, Mohammed J. Uddin, and Nelson C. Soares*

Cite This: J. Proteome Res. 2025, 24, 6311−6319

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Data-Independent Acquisition (DIA) has emerged as a powerful mass spectrometry (MS) strategy for comprehensive metabolomics. This study presents a novel short gradient (13 min) nanosensitive analytical method for human plasma analysis using DIA LC-MS/MS, focusing on in-depth optimization of MS parameters to maximize data quality and metabolite coverage. Key MS parameters, including scan speed, isolation window width, resolution, automatic gain control, and collision energy, were systematically tuned to balance the sensitivity and specificity while minimizing interferences. The optimized method enabled the detection of 2,907 features with 675 annotated compounds, leveraging recent progress in nano-LC-MS/MS for multiomics applications and showcasing the possibility of combining proteomics and metabolomics within a single chromatographic system. Ultimately, a comparison was performed between the data acquired through the DIA and DDA MS approaches in the context of untargeted metabolomics. This optimized analytical method yields more robust and reproducible results, thereby expanding the potential for meaningful discoveries across diverse biological fields.



KEYWORDS: *mass spectrometry, metabolomics, data-independent acquisition, human plasma*

1. INTRODUCTION

Data-Independent Acquisition (DIA) has emerged as a powerful mass spectrometry strategy for metabolomics, enabling comprehensive and reproducible metabolite profiling.[1−3] Unlike Data-Dependent Acquisition (DDA), which selects precursor ions based on intensity, DIA systematically fragments all ions within a defined mass range, ensuring unbiased and high-throughput data collection.[4] This approach enhances metabolite coverage, improves quantitative accuracy, and facilitates retrospective data analysis.[4,5] The ability of DIA to reproducibly capture a complete snapshot of the metabolome makes it particularly valuable for studying complex biological systems,[6] biomarker discovery,[2] and longitudinal studies in metabolomics.[6]

Optimizing DIA is crucial for maximizing its potential in metabolomics, as it directly impacts data quality, metabolite coverage, and quantitative accuracy.[7] Key factors such as scan speed, isolation window width, resolution, automatic gain control (AGC), and fragmentation energy must be carefully tuned to balance sensitivity and specificity while minimizing interferences.[8] Proper optimization enhances reproducibility, quantitative accuracy, and metabolite identification and improves the detection of low-abundance metabolites, making DIA more effective for biomarker discovery and biological interpretation. As metabolomics studies become increasingly complex, continuous advancements in DIA methodologies and data processing strategies are essential for ensuring robust and high-resolution metabolic profiling.[6]

DIA offers broader and more detailed compound coverage compared to DDA, making it especially advantageous in untargeted metabolomics studies.[4] Precise MS parameter adjustments in DIA allow for more comprehensive data capture, supporting biomarker discovery and metabolic pathway studies with greater depth and reliability. Thus, optimizing parameters in DIA-based workflows contributes to more robust and reproducible results, expanding the potential for meaningful discoveries across diverse biological fields.[9]

In this work, we present a novel nanosensitive analytical method using DIA LC-MS/MS for untargeted metabolomics, along with an in-depth discussion of the optimized MS parameters that led to the development of this method.

Received: July 1, 2025
Revised: October 8, 2025
Accepted: November 3, 2025
Published: November 12, 2025

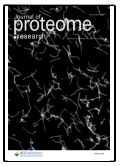


Table 1. DIA LC-MS/MS Parameters Optimized, and Values Tested for Each Parameter[d]

parameter DIA optimization	values tested
Resolution MS^1	60k, **120k**, and 240k
Resolution MS^2 and Isolation Window (IW)	7.5k-5IW, 15k-5IW, 15k-10IW, 30k-10IW, 30k-20IW, 60k-20IW, **60k-60IW**, 120k-60IW, 120k-120IW, and 240k-120IW
Collision Energy (V)	15, 30, 45, 15 + 30, 15 + 45, 30 + 45, and **15 + 30 + 45**
Automatic Gain Control (AGC)−MS^1, %	**Standard**[a], 300, 1000
Automatic Gain Control (AGC)−MS^2, %	Standard[a], **300**, 1000
Maximum Ion Injection (MIT)−MS^1, ms	55, 1000, **Auto**[b]
Maximum Ion Injection (MIT)−MS^2, ms	**Dynamic**[c], Auto, 1000

[a]Standard: The AGC (Automatic Gain Control) standard mode dynamically adjusts ion accumulation time to optimize measurement precision and dynamic range, avoiding detector saturation and improving mass spectral reproducibility. [b]Auto: refers to the maximum duration the mass spectrometer is allowed to accumulate ions during the MS^1 scan (full-scan acquisition of intact precursor ions) before proceeding to the next step, with the instrument automatically adjusting the ion population based on sample complexity and signal intensity. [c]Dynamic: refers to the maximum duration allowed for accumulating ions during MS^2 scans (fragmentation of selected precursor ions), where the instrument dynamically adjusts the actual injection time based on real-time ion abundance and signal intensity. [d]The optimal values for the MS parameters are highlighted in bold.

Although several studies in the literature have utilized DIA for metabolomics, no systematic optimization has been presented and discussed in detail as in this study. Ultimately, a comparison was performed between the data acquired through DIA and DDA MS approaches in the context of untargeted metabolomics.

2. MATERIALS AND METHODS

2.1. Chemicals

HPLC-grade solvents (water, methanol, and formic acid) were purchased from ThermoFisher Scientific (Waltham, MA). The human plasma reference material (SRM 1950) was purchased from the National Institute of Standards and Technology (NIST) (Gaithersburg, MD). All optimization experiments were performed using NIST SRM 1950 (Metabolites in Frozen Human Plasma), a standard reference material extensively characterized for metabolomics applications.[10,11]

2.2. Metabolites Extraction

Metabolites were extracted from NIST SRM 1950 reference human plasma as previously described, with minor modifications.[8] An 800 μL volume of cold methanol was added to 200 μL of plasma in a 1.5 mL Eppendorf tube. The mixture was vortexed for 10 s and incubated for 15 min at 4 °C on a ThermoMixer (Eppendorf Inc., Enfield, CT), prior to centrifugation (18,000g) at 4 °C for 10 min (Centrifuge 5430R, Eppendorf Inc., Enfield, CT). Subsequently, 750 μL of the supernatant was collected and dried using a vacuum concentrator (concentrator RapidVap Vacuum, Labconco, USA). The dried extracts were reconstituted in 100 μL of a water/methanol solution (95:5) containing 0.1% formic acid and transferred to a high-performance liquid chromatography (HPLC) glass vial for analysis.

2.3. LC-MS/MS-Based Metabolomics Analysis

Analyses were conducted using a Vanquish Neo UHPLC coupled to an Orbitrap Exploris 480 mass spectrometer (ThermoFisher Scientific, Waltham, MA) equipped with nanoelectrospray ionization source Thermo Scientific EASY-Spray. A C18 reversed-phase capillary column (75 μm i.d. × 15 cm, 3 μm, 100 Å, ThermoFisher Scientific EASY-Spray) was employed for the chromatographic separation with a Thermo Scientific C18 preconcentrating trap column (300 μm i.d. × 0.5 cm, 3 μm, 100 Å). Mobile phase A was water acidified with 0.1% formic acid. Mobile phase B was 80/20 acetonitrile−water acidified with 0.1% formic acid. The gradient was 1 min 90/10 A/B, 2 min 75/25 A/B, 2 min 40/60 A/B, and 8 min 5/95 A/B. A 2 μL volume was injected for analysis. In untargeted metabolomics, the injected volume is typically optimized to balance sensitivity, chromatographic performance, and column lifetime. Reported values for plasma metabolomics generally range from 1−10 μL, depending on column dimensions and system sensitivity[12,13] (Dettmer et al., 2007; Want et al., 2010; Zhou et al., 2019). In this study, we employed 2 μL, which is consistent with common practice and provided robust and reproducible performance without overloading the column. The flow rate was set to 1000 nL/min. MS spectra were acquired in the range of m/z 50−750. The RF lens was 50%. The ion transfer tube temperature was 275 °C, and a spray voltage was set to 1.9 kV in positive polarity. DIA and DDA modes were used for the fragmentation process. For DIA, the settings optimized for the MS were as follow: MS^1 and MS^2 resolutions, automatic gain control (AGC) and maximum ion injection time (MIT) for MS^1 and MS^2, collision energy, and isolation window for MS^2. We employed a SWATH-type DIA acquisition, in which the full m/z range is segmented into consecutive isolation windows, and all ions within each window are fragmented and recorded. This strategy, originally developed in proteomics[14] and more recently adapted for untargeted metabolomics,[15] enables comprehensive and reproducible fragmentation coverage, thereby improving annotation confidence. Table 1 shows DIA LC-MS/MS parameters optimized and the values tested for each parameter. We have highlighted the optimal performing values in bold (Table 1). The detailed description of the MS parameters used for DDA is given in Table S1. The values for each MS parameter were chosen according to preliminary results. Each experimental condition was analyzed in technical triplicates (n = 3), and data are presented as mean ± standard deviation (SD).

2.4. Data Analysis

For metabolomics, MS-DIAL (v5.4)[16] was used to perform data processing including peak-detection, alignment, gap filling, and compound annotation by using the raw chromatographic files. MS-DIAL parameters were: MS^1 tolerance 0.01 Da, MS^2 tolerance 0.025 Da, retention time tolerance 0.1 min, minimum peak height 5 × 10^3, smoothing method Linear Weighted Moving Average, smoothing level 3 scan, minimum peak width 5 scan, sigma window value 0.5. Metabolite annotation was performed by using strict mass accuracy and spectral similarity criteria. The settings were: MS^1 tolerance = 0.01 Da, MS^2 tolerance = 0.05 Da, and a minimum of 70% fragment match against library spectra. These thresholds fall within the ranges

https://doi.org/10.1021/acs.jproteome.5c00622
J. Proteome Res. 2025, 24, 6311−6319

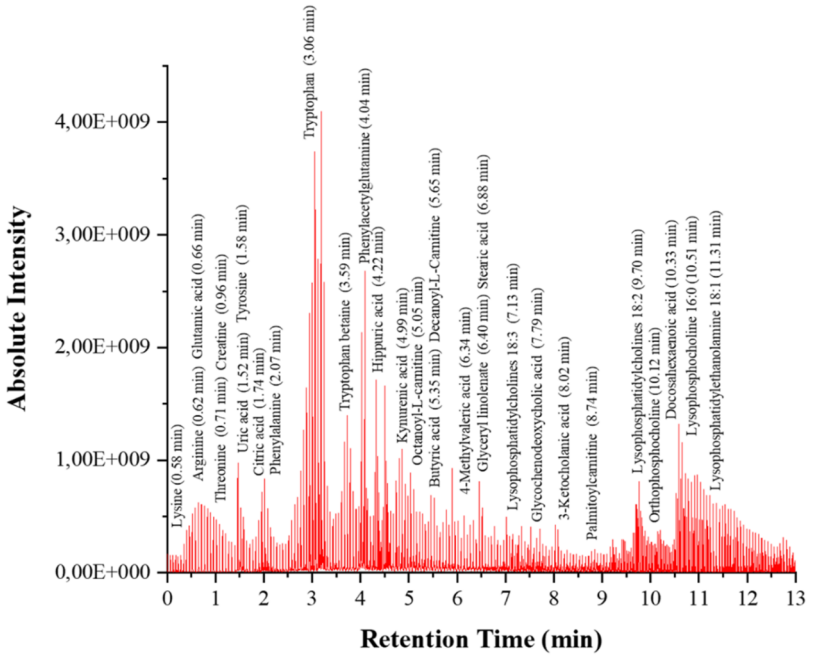


Figure 1. Typical combined total ion chromatogram (TIC), containing both MS^1 and MS^2 signals, displaying the NIST SRM 1950 reference human plasma analysis by DIA LC-MS/MS using the method developed in this work. Several key metabolites or classes are annotated.

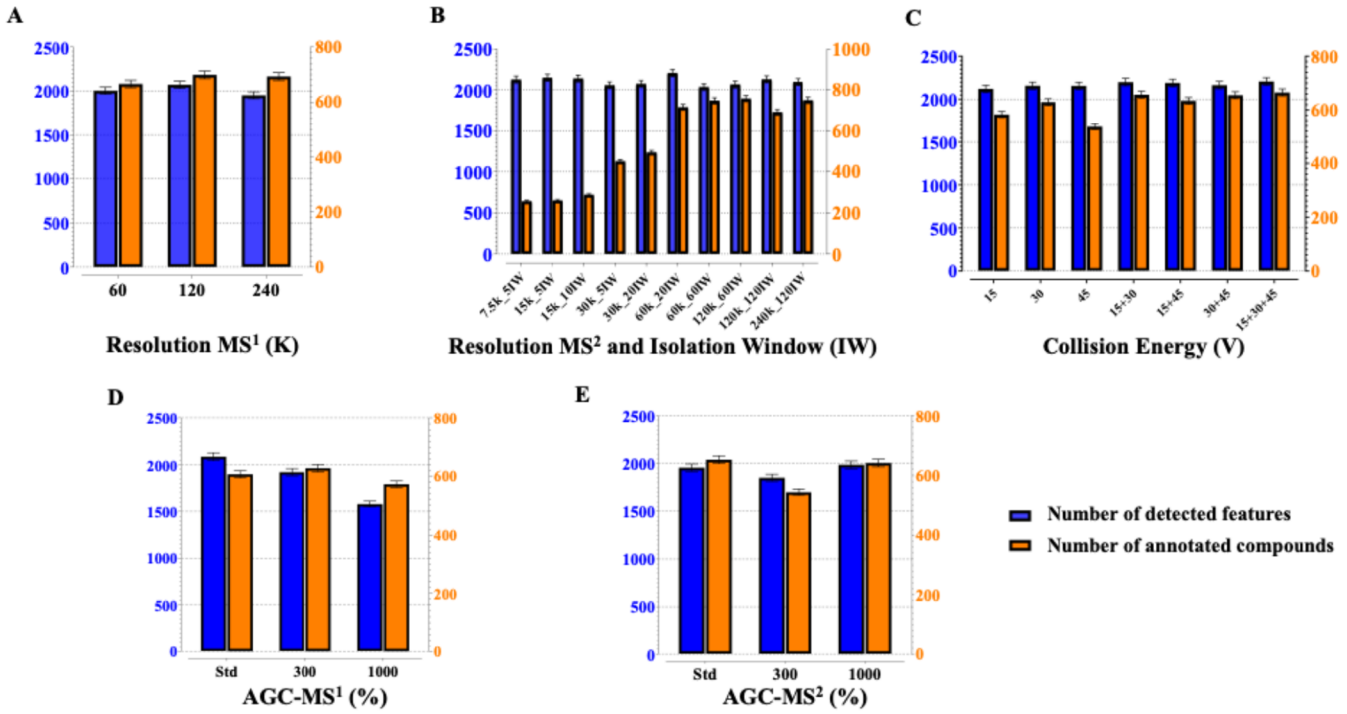


Figure 2. Effect of resolution MS^1 (A), resolution MS^2+isolation window (B), collision energy (C), automatic gain control (AGC) for both MS^1 (D) and MS^2 (E) on the number of detected features and number of annotated compounds for NIST SRM 1950 reference human plasma analysis using DIA LC-MS/MS.

commonly applied in untargeted DIA metabolomics ($MS^1 \leq$ 0.02 Da; MS^2 0.02−0.1 Da; fragment match ≥50−70%) (Tsugawa et al., 2015; Schymanski et al., 2014). The MS^2 spectral library used for compound annotations was the Mass Bank of North America (MoNA) in the msp format. Blank filtering was applied to eliminate background noise. Pseudo-MS/MS spectra were reconstructed using the deconvolution algorithm implemented in MS-DIAL (v. 4.9.2), which applies correlation-based fragment assignment within DIA isolation windows.[17]

3. RESULTS AND DISCUSSION

3.1. DIA LC-MS/MS Analysis

Untargeted metabolomics experiments are designed to detect and quantify as many biomolecules as possible in a sample.[18−20]

https://doi.org/10.1021/acs.jproteome.5c00622
J. Proteome Res. 2025, 24, 6311−6319

In this work, after preliminary tests using different gradients, a nanosensitive short gradient (13 min) method based on DIA LC-MS/MS analysis was developed to maximize coverage of the diverse metabolites using NIST SRM 1950 reference human plasma. Figure 1 shows the total ion current (TIC) chromatogram acquired using the optimized gradient method and MS parameters described in this work. The metabolomics data set identified a total of 2907 features with 675 annotated compounds (Table S2) using the criteria described in Materials and Methods section. While we utilized cutting-edge reference libraries for metabolites and diligently annotated the data set, it is critical to emphasize that our work does not claim exhaustive or definitive identifications.

The method developed in this work leverages recent progress in nano-LC-MS/MS for multiomics applications, which showcased the possibility of combining proteomics and metabolomics within a single chromatographic system. Presently, it is common in the metabolomics field to utilize microliter flow rates or LC solvents with different additives (e.g., 5 mM ammonium formate) or both. Our proposed protocol establishes a flexible and standardized approach for the in-depth characterization of both metabolites and proteins within a unified analytical framework, utilizing the same conditions commonly employed in the proteomics community. By promoting an efficient and reproducible workflow, this approach has the potential to advance multiomics research by maximizing instrument utilization, minimizing solvent requirements for LC separation, and enhancing data consistency between proteomic and metabolomic analyses.

3.2. Optimization of MS Parameters for DIA LC-MS/MS Analysis

In this section are the results on the MS parameters that were studied using DIA LC-MS/MS analysis of NIST SRM 1950 human reference plasma.

Figure 2 shows the number of detected features using DIA acquisition mode and the number of annotated compounds for the following MS parameters investigated in this work at different levels: MS^1 resolution, automatic gain control (AGC) for MS^1 and MS^2, collision energy, and MS^2 resolution + isolation window (IW). Maximum ion injection (MIT) for MS^1 and MS^2 was investigated in this work, but a minimal impact was observed in terms of the number of detected features and annotated compounds. Thus, MIT Auto for MS^1 and Dynamic for MS^2 were used based on previous results in our lab. Throughout the work, the number of detected features refers to those features that have undergone the MS^2 fragmentation process. In this work, metabolite annotation was conducted at MSI level 2 (putatively annotated compounds), based on accurate mass and MS/MS spectral similarity against MS-DIAL libraries.[21] To ensure consistency, annotations identified under one condition were tracked across data sets, and strict similarity thresholds were applied to reduce the likelihood of spurious matches.[16,22] Our evaluation thus focused on the relative effect of acquisition parameters on annotation performance rather than on definitive metabolite identification.

3.3. MS^1 Resolution, MS^2 Resolution + Isolation Window (IW)

Optimizing resolution in mass spectrometry for metabolomics is important to balance sensitivity, selectivity, and speed, ensuring accurate identification of metabolites while maintaining efficient analysis of complex biological samples.[8] Higher resolution can reduce the number of detected features by lengthening scan times,[23] which practically limits the number of points across a chromatographic peak. A minimum of 7 points is needed to define a peak from noise.[24] Thus, it is important to find the right balance between the scanning speed and metabolite coverage. Three different MS1 resolutions were evaluated in this work: 60, 120, and 240k (Figure 2A). The resolution of 120,000 was found to perform best in terms of the number of detected features (2072). As the MS1 resolution increased from 60 to 120k, the number of detected features increased. Increasing MS1 resolution allows the instrument to better distinguish analyte peaks from background and interfering ions, leading to greater sensitivity in detecting features.[25] Thus, it is important to find the right balance between scanning speed and metabolite coverage. Three different MS1 resolutions were evaluated in this work: 60, 120, and 240k (Figure 2A). The resolution of 120k was found to perform best in terms of the number of detected features (2072). As the MS1 resolution increased from 60 to 120k, the number of detected features increased. Increasing MS1 resolution allows the instrument to better distinguish analyte peaks from background and interfering ions, leading to greater sensitivity in detecting features.[25] However, the number of detected features decreased when the resolution increased from 120 to 240k. Although higher resolutions in full scan improve data quality and the ability to distinguish isobaric compounds, it may decrease the number of detected features due to cycle time limitations.[26]

The 120 and 240k resolutions showed a similar ability to annotate compounds (699 and 693 annotated compounds, respectively) with a slightly higher performance than 60k (666 compounds). Increasing resolution for full scan mode in mass spectrometry reduces mass errors, enhancing the ability to distinguish closely related ions by improving mass accuracy.[27]

Optimizing MS^2 resolution and isolation window (IW) is critical for accurate compound identification and structural analysis in mass spectrometry.[4,23,27] High MS^2 resolution improves the ability to resolve closely related fragment ions, leading to better spectral clarity, reducing interference from neighboring ions, and also enhances sensitivity in MS^2 scans. Tuning the IW controls the range of precursor ions present for fragmentation. A narrower IW improves specificity by coisolating fewer precursors, reducing both the complexity of the MS^2 spectra and the required size of the search space when matching potential precursor ions to the spectral library later, while a wider IW reduces the total number of needed scans, allowing faster cycle time for quantitative accuracy and offsetting the disadvantages of slower scan speeds. Striking the right balance between MS^2 resolution and IW ensures precise and efficient data acquisition, maximizing sensitivity without compromising selectivity.[28] The effect of both MS^2 resolution combining with IW was investigated, considering the number of detected features and the number of annotated compounds (Figure 2B). In this work, a minimal impact was observed in our experiments in terms of the number of detected features using the different combinations of MS^2 resolutions and isolation windows tested. The cycle time was maintained at either 3 or 6 s for the different conditions evaluated, values sufficient to not produce large variations in the number of detected features. In terms of the number of annotated compounds, increasing the MS^2 resolution while keeping the isolation window constant led to an increased number of annotated compounds with a significant increase from 15,000 to 30,000 and 30,000 to 60,000. The best performance in terms of the number of annotated compounds was observed when the MS^2 resolution was higher

https://doi.org/10.1021/acs.jproteome.5c00622
J. Proteome Res. 2025, 24, 6311−6319

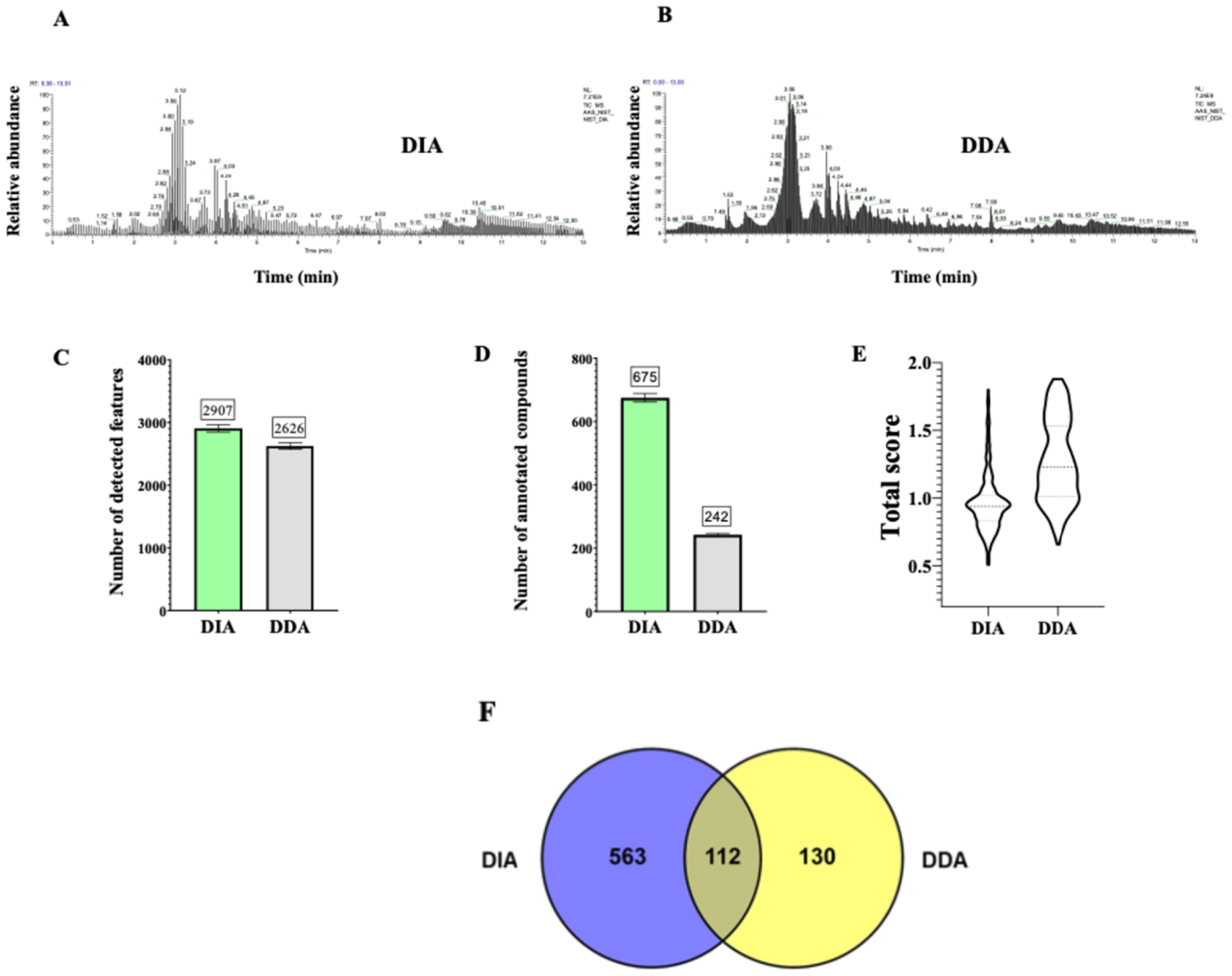


Figure 3. Combined total ion chromatograms (TICs), containing both MS and MS^2 signals, displaying the NIST SRM 1950 reference human plasma analysis using DIA (A) and DDA (B) modes. Effect of different acquisition modes, DIA and DDA, on the number of detected features (C) and on the number of annotated compounds (D) using DIA LC-MS/MS analysis of NIST SRM 1950 reference human plasma; (E) Distribution of spectral similarity scores (total score) for DDA and DIA modes (F) Venn plot representing the number of annotated compounds in each DIA (purple color) and DDA (yellow color) modes, respectively, and their overlap.

than 60,000. Higher resolution in MS^2 improves annotation quality because it enhances the accuracy and clarity of the fragment ion spectra, which are critical for identifying and characterizing compounds.[29] When the MS^2 resolution was constant and the isolation window increased, the number of detected features was minimally impacted while doubling the necessary cycle time; thus, it was determined that a resolution of 60,000 with 20 Da wide isolation width was optimal.

The number of detected features remained relatively stable across acquisition parameters, indicating that feature extraction is robust to parameter variation. In contrast, the number of annotated metabolites varied more substantially, reflecting the dependence of annotation on MS^2 spectral quality and reliable library matching.[16] While resolution and other acquisition parameters strongly influenced annotation outcomes, AGC-MS^2 exerted little effect, likely because ion accumulation was sufficient to generate representative fragmentation patterns.[30] These findings highlight the greater sensitivity of annotation compared to detection and emphasize the importance of optimizing resolution-related parameters in untargeted metabolomics workflows.

3.4. Collision Energy

Collision energy is an important parameter in mass spectrometry to ensure efficient fragmentation of precursor ions, producing informative MS^2 spectra that enable accurate metabolite identification and structural characterization.[31,32] In this work, seven different collision energies were investigated (Figure 2C). The step collision energies showed better performance in terms of the annotated compounds than any single collision energy. Similar results were obtained by Assress et al. 2023[8] for the optimization of MS parameters in data-dependent acquisition for untargeted metabolomics.[8] Stepped collision energy produces better results for untargeted metabolomics because it generates more comprehensive and diverse fragmentation patterns, which are critical for identifying unknown compounds.[33,34] By variation of the collision energy across a range, it ensures that both low-energy and high-energy fragments are captured, allowing the detection of a wider array of diagnostic ions. This increases the likelihood of obtaining structurally

https://doi.org/10.1021/acs.jproteome.5c00622
J. Proteome Res. 2025, 24, 6311−6319

informative fragments, even for compounds with varying fragmentation efficiencies. In contrast, the fixed collision energy may fail to produce sufficient fragmentation for some compounds or miss important ions, limiting the depth of analysis. Stepped collision energy thus enhances coverage and identification accuracy in complex, untargeted workflows.[34] As expected, there is no impact on the number of detected features in different collision energy values.

3.5. Automatic Gain Control (AGC) for MS^1 and MS^2

Optimizing Automatic Gain Control (AGC) for MS^1 and MS^2 in mass spectrometry is essential to regulate ion accumulation, ensuring sufficient ion density for accurate signal detection while preventing overloading, which enhances both sensitivity and data quality in metabolomics analyses.[8,34] In this work, three AGC conditions were tested for MS^1 and MS^2, Standard (Std), 300 and 1000% (Figures 2D2,E). The standard (Std) condition for AGC (Automatic Gain Control) on the MS Thermo Scientific Orbitrap Exploris 480 refers to the default, manufacturer-recommended AGC target setting used for routine operation. Standard (Std) conditions for AGC mean that the system sets the recommended target in an automated fashion. The AGC Std value is commonly used as the starting point for general small molecule workflows. It is considered "standard" because it provides robust performance for most applications without requiring further optimization. The highest performance in terms of the number of detected features was achieved using AGC 1000% for MS^1 and AGC Std for MS^2. In terms of the number of annotated compounds, AGC Std and AGC 1000% showed similar and better performance than AGC 300% for MS^1, but for MS^2, AGC 300% showed the highest performance. The results of the AGC settings for MS^1 and MS^2, which showed great performance, are probably due to the optimum balance between the ion accumulation and scan speed.

3.6. Comparison of Data-Dependent Acquisitions (DDA) and Data-Independent Acquisition (DIA) for LC-MS/MS Analysis

The analytical performances of DDA and DIA modes for LC-MS/MS analysis of the human plasma reference material (SRM 1950) were evaluated in terms of the number of detected features and number of annotated compounds. Figure 3A,B shows the combined total ion chromatograms (TICs), containing both MS^1 and MS^2 signals, displaying the NIST SRM 1950 reference human plasma analysis using DIA and DDA LC-MS/MS, respectively. A greater number of features were detected using DIA (2907) compared to DDA (2626). The higher number of features detected using DIA compared with DDA arises from its systematic fragmentation of all ions within predefined mass windows. This allows DIA to capture a wider range of compounds, including low-abundance ones often missed by DDA, which prioritizes high-intensity ions.[4] Importantly, when analyzing the NIST SRM 1950 reference human plasma via LC-MS/MS, the number of putatively annotated metabolites was significantly higher using DIA (675) than DDA (242), as determined using the criteria outlined in the Materials and Methods section (Figure 3C,D), highlighting the limitations of traditional DDA. To address this limitation, Garrett and colleagues developed a DDA strategy incorporating iterative exclusion.[35] The iterative exclusion process in DDA enhances metabolite coverage by progressively excluding previously fragmented ions across multiple runs, allowing the instrument to target new, less abundant precursors until a plateau of high-quality MS/MS spectra is reached. The higher number of annotated metabolites using DIA compared to DDA for NIST SRM 1950 plasma analysis is due to comprehensive ion coverage of DIA and systematic fragmentation of all ions, capturing low-abundance metabolites often missed by DDA. The ability of DIA to generate more MS^2 spectra, combined with advanced tools for deconvoluting overlapping signals, enhances annotation efficiency despite lower spectral purity.[4] In contrast, DDA without iterative exclusion prioritizes high-intensity ions, limiting its coverage of complex samples like plasma. Figure 3E shows the MS^2 quality score distributions for all annotated metabolites, calculated for the MS^2 spectral matching by comparing the number of common fragment ions between the evaluated DIA and DDA spectra and the reference spectrum from MS-DIAL. Comparing the total score distributions for DDA and DIA, spectra quality generated with DDA had better scores than with DIA acquisition. This result is similar to a previous study where DDA and DIA modes were compared for untargeted metabolomics of urine samples.[4] In metabolomics, DDA generates higher-quality MS^2 spectra than DIA due to its selective fragmentation of the most abundant ions, producing cleaner spectra with fewer coeluting interferences. This results in better spectral matches against reference libraries like MS-DIAL. However, DIA detects more metabolites overall (including low-abundance ones) by fragmenting all ions systematically.

The Venn diagram highlights the complementary strengths of DIA and DDA in metabolomics. DIA uniquely identifies 563 metabolites, leveraging its unbiased fragmentation to detect low-abundance and coeluting compounds often missed by DDA. In contrast, DDA selectively identifies 131 unique metabolites, typically high-abundance ions with robust spectral matches. Both methods share 112 metabolites. The list of unique metabolites detected in each acquisition mode as well as those shared between both modes is provided in Table S3. The metabolites exclusively identified in DIA include creatinine, valine, isoleucine, and butyric acid, etc. Among the compounds uniquely detected in DDA are propionic acid, hypoxanthine, 3-methylhistidine, etc. Examples of metabolites annotated in both acquisition modes include creatine, glutamine, lysine, glutamic acid, histidine, uric acid, arginine, tryptophan, kynurenine, and lysphosphatidylcholine 18:2. The metabolites identified as unique to one acquisition mode (e.g., DIA or DDA) in this study may be detected in the other mode under different sample conditions. For example, low-abundance metabolites exclusive to DIA might become detectable in DDA if their concentrations increase (e.g., due to biological variation, sample enrichment, or improved sensitivity settings). Conversely, high-intensity metabolites unique to DDA could also appear in DIA if interference from coeluting ions is reduced (e.g., via optimized chromatography or narrower isolation windows). This overlap depends on factors like ion suppression, instrument sensitivity, chromatographic resolution, and acquisition parameters, which influence the detectability of compounds across workflows.[4]

Previous studies have addressed DIA optimization in mass spectrometry, focusing on specific acquisition parameters such as the isolation window width, collision energy, or scan speed. For instance, Schulze et al. demonstrated how the choice of precursor isolation windows influences the number and quality of metabolite annotations in untargeted analyses.[36] Similarly, Guzman et al. reported the impact of isolation window size and maximum injection time on sensitivity and proteome coverage,[37] while more recent data-driven frameworks, such as DO-MS, have also provided strategies to optimize precursor window schemes.[38] While these contributions offered valuable insights,

https://doi.org/10.1021/acs.jproteome.5c00622
J. Proteome Res. 2025, 24, 6311−6319

they generally examined isolated factors under limited conditions. In contrast, our study presents a more systematic and integrated evaluation of multiple MS parameters, thereby extending the level of detail and reproducibility beyond what has been previously reported for untargeted metabolomics.

DDA generated cleaner MS/MS spectra that facilitated straightforward spectral library matching; however, its stochastic precursor selection limited the detection of certain metabolites. In contrast, DIA consistently captured additional low-abundance compounds (e.g., butyric acid, creatinine, valine, glycodeoxycholic acid; all with signal intensity $<5 \times 10^6$) that were not observed in DDA runs. These results illustrate the complementary strengths of both approaches, with DDA offering higher spectral clarity and DIA enhancing metabolome coverage in untargeted workflows.

4. CONCLUSION

This study successfully developed and optimized a nanosensitive DIA LC-MS/MS method for comprehensive metabolomics, demonstrating its effectiveness in analyzing complex samples like human plasma. Systematic tuning of MS parameters, including resolution, isolation window, collision energy, and automatic gain control, resulted in enhanced metabolite coverage and annotation efficiency. Optimal results were obtained using MS^1 and MS^2 resolution of 120,000 and 60,000, respectively. The automatic gain control (AGC) target was set to Standard for MS^1 and Custom Normalized 300% for MS^2, and the maximum ion injection time (MIT) was set to Auto for MS^1 and Dynamic with the desired minimum points across peak 5 for MS^2. The stepped collision energy was 15, 30, and 45. The isolation window for MS^2 was 60. The optimized method, leveraging recent advancements in nano-LC-MS/MS for multiomics applications, showcases the possibility of combining proteomics and metabolomics within a single chromatographic system, expanding the potential for meaningful discoveries across diverse biological fields. A comparison between DIA and DDA highlights the strengths of DIA for untargeted metabolomics, offering a robust and reproducible approach for in-depth metabolic profiling.

■ ASSOCIATED CONTENT

Data Availability Statement

The mass spectrometry metabolomics data have been deposited to Metabolomics Workbench (https://www.metabolomicsworkbench.org/) Consortium with the data set identifier datatrack_id:5908 study_id:ST003994.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.5c00622.

Detailed description of the MS parameters applied for the metabolomics based on data-dependent acquisition (DDA) experiments (Table S1) (XLSX)

List of annotated metabolites detected in NIST 1950 reference human plasma matched with MoNA using DIA LC-MS/MS (Table S2) (XLSX)

Lists of annotated metabolites in NIST 1950 reference human plasma matched with MoNA exclusively detected using DIA, DDA, and commonly detected in both DIA/DDA modes by LC-MS/MS (Table S3) (XLSX)

■ AUTHOR INFORMATION

Corresponding Author

Nelson C. Soares − *Center for Applied and Translational Genomics (CATG), Mohammed Bin Rashid University of Medicine and Health Sciences (MBRU), Dubai Health, Dubai 19099, United Arab Emirates (UAE); College of Medicine-Mohammed Bin Rashidand Health Sciences (MBRU), Dubai Health, University of Medicine, Dubai 19099, UAE; Laboratory of Proteomics, Department of Human Genetics, National Institute of Health Doutor Ricardo Jorge (INSA), Lisbon 1649-016, Portugal; Comprehensive Health Research Centre (CHRC), NOVA Medical School, University NOVA of Lisbon, Lisbon 1099-085, Portugal;* orcid.org/0000-0003-2331-8532; Email: nelson.soares@dubaihealth.ae

Authors

Frederico G. Pinto − *Center for Applied and Translational Genomics (CATG), Mohammed Bin Rashid University of Medicine and Health Sciences (MBRU), Dubai Health, Dubai 19099, United Arab Emirates (UAE); Department of Chemistry, Federal University of Viçosa, Viçosa, Gerais 36570-900, Brazil*

Alexander D. Giddey − *Center for Applied and Translational Genomics (CATG), Mohammed Bin Rashid University of Medicine and Health Sciences (MBRU), Dubai Health, Dubai 19099, United Arab Emirates (UAE);* orcid.org/0000-0002-2034-8122

Rouda S. B. Almarri − *Center for Applied and Translational Genomics (CATG), Mohammed Bin Rashid University of Medicine and Health Sciences (MBRU), Dubai Health, Dubai 19099, United Arab Emirates (UAE); Department Applied Biology, College of Sciences, University of Sharjah, Sharjah 27272, UAE*

Omer S. Alkhnbashi − *Center for Applied and Translational Genomics (CATG), Mohammed Bin Rashid University of Medicine and Health Sciences (MBRU), Dubai Health, Dubai 19099, United Arab Emirates (UAE); College of Medicine-Mohammed Bin Rashidand Health Sciences (MBRU), Dubai Health, University of Medicine, Dubai 19099, UAE*

Timothy J. Garrett − *Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, Florida 32608, United States;* orcid.org/0000-0003-1623-009X

Mohammed J. Uddin − *Center for Applied and Translational Genomics (CATG), Mohammed Bin Rashid University of Medicine and Health Sciences (MBRU), Dubai Health, Dubai 19099, United Arab Emirates (UAE); Department Applied Biology, College of Sciences, University of Sharjah, Sharjah 27272, UAE; GenomeArc Inc., Missisauga, Ontario L4Z 1V9, Canada*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jproteome.5c00622

Author Contributions

All authors made a significant contribution to the work reported. F.G.P. contributed to writing—original draft, performed the data analysis and led the investigation and developed the LC-MS methods, prepared and analyzed the samples and data curation; A.D.G. contributed to writing original draft and developed the LC-MS methods, prepared and analyzed the samples and data curation; and N.C.S. contributed to conceptualization, writing original draft, supervision, and performed the data analysis and

https://doi.org/10.1021/acs.jproteome.5c00622
J. Proteome Res. 2025, 24, 6311−6319

led the investigation. All authors contributed to writing, reviewing, and editing the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported by the Center for Applied and Translational Genomics (CATG), Mohammed Bin Rashid University of Medicine and Health Sciences (MBRU), Dubai Health, Dubai, United Arab Emirates. Internal grant MBRU, Dubai Health, College of Medicine grants cycle 2025/2027. Grant Reference No: **MRBU-CM-RG2025-14**, title; *Development of multiomics biomarker test to predict the risk of premature myocardial infarction (MI) in the UAE*. Frederico G. Pinto thanks the National Council for Scientific and Technological Development - CNPq/Brazil (308264/2022-3). TOC graphic created BioRender.com. (Zenati, R., 2025; https://BioRender.com/m3jwauc)

■ ABBREVIATIONS

AGC, automatic gain control; **CVs**, coefficients of variation; **DDA**, data-dependent acquisition; **DIA**, data-independent acquisition; **IW**, isolation window; **LC-MS/MS**, liquid chromatography coupled to tandem mass spectrometry; **MS**, mass spectrometry; **MIT**, maximum ion injection time; **NIST**, National Institute of Standards and Technology; **PSM**, peptide spectrum match; **RT**, retention time; **SPQC**, sample preparation quality control; **TIC**, total ion current; **XIC**, extracted ion chromatogram

■ REFERENCES

(1) Bonner, R.; Hopfgartner, G. SWATH data independent acquisition mass spectrometry for metabolomics. *TrAC, Trends Anal. Chem.* **2019**, *120*, No. 115278.

(2) Wang, R.; Yin, Y.; Zhu, Z.-J. Advancing untargeted metabolomics using data-independent acquisition mass spectrometry technology. *Anal. Bioanal. Chem.* **2019**, *411* (19), 4349−4357.

(3) Amer, B.; Deshpande, R. R.; Bird, S. S. Simultaneous Quantitation and Discovery (SQUAD) Analysis: Combining the Best of Targeted and Untargeted Mass Spectrometry-Based Metabolomics. *Metabolites* **2023**, *13* (5), No. 648.

(4) Guo, J.; Huan, T. Comparison of Full-Scan, Data-Dependent, and Data-Independent Acquisition Modes in Liquid Chromatography−Mass Spectrometry Based Untargeted Metabolomics. *Anal. Chem.* **2020**, *92* (12), 8072−8080.

(5) van der Laan, T.; Boom, I.; Maliepaard, J.; Dubbelman, A.-C.; Harms, A. C.; Hankemeier, T. Data-Independent Acquisition for the Quantification and Identification of Metabolites in Plasma. *Metabolites* **2020**, *10* (12), No. 514.

(6) Ledesma-Escobar, C. A.; Priego-Capote, F.; Calderón-Santiago, M. MetaboMSDIA: A tool for implementing data-independent acquisition in metabolomic-based mass spectrometry analysis. *Anal. Chim. Acta* **2023**, *1266*, No. 341308.

(7) Alka, O.; Shanthamoorthy, P.; Witting, M.; Kleigrewe, K.; Kohlbacher, O.; Röst, H. L. DIAMetAlyzer allows automated false-discovery rate-controlled analysis for data-independent acquisition in metabolomics. *Nat. Commun.* **2022**, *13* (1), No. 1347.

(8) Assress, H. A.; Ferruzzi, M. G.; Lan, R. S. Optimization of Mass Spectrometric Parameters in Data Dependent Acquisition for Untargeted Metabolomics on the Basis of Putative Assignments. *J. Am. Soc. Mass Spectrom.* **2023**, *34* (8), 1621−1631.

(9) Bruderer, R.; Bernhardt, O. M.; Gandhi, T.; Xuan, Y.; Sondermann, J.; Schmidt, M.; Gomez-Varela, D.; Reiter, L. Optimization of Experimental Parameters in Data-Independent Mass Spectrometry Significantly Increases Depth and Reproducibility of Results*. *Mol. Cell. Proteomics* **2017**, *16* (12), 2296−2309.

(10) Phinney, K. W.; Ballihaut, G.; Bedner, M.; Benford, B. S.; Camara, J. E.; Christopher, S. J.; Davis, W. C.; Dodder, N. G.; Eppe, G.; Lang, B. E.; et al. Development of a Standard Reference Material for metabolomics research. *Anal. Chem.* **2013**, *85* (24), 11732−11738.

(11) Simón-Manso, Y.; Lowenthal, M. S.; Kilpatrick, L. E.; Sampson, M. L.; Telu, K. H.; Rudnick, P. A.; Mallard, W. G.; Bearden, D. W.; Schock, T. B.; Tchekhovskoi, D. V.; et al. Metabolite profiling of a NIST Standard Reference Material for human plasma (SRM 1950): GC-MS, LC-MS, NMR, and clinical laboratory analyses, libraries, and web-based resources. *Anal. Chem.* **2013**, *85* (24), 11725−11731.

(12) Dettmer, K.; Aronov, P. A.; Hammock, B. D. Mass spectrometry-based metabolomics. *Mass Spectrom Rev.* **2007**, *26* (1), 51−78.

(13) Want, E. J.; Wilson, I. D.; Gika, H.; Theodoridis, G.; Plumb, R. S.; Shockcor, J.; Holmes, E.; Nicholson, J. K. Global metabolic profiling procedures for urine using UPLC-MS. *Nat. Protoc* **2010**, *5* (6), 1005−1018.

(14) Gillet, L. C.; Navarro, P.; Tate, S.; Röst, H.; Selevsek, N.; Reiter, L.; Bonner, R.; Aebersold, R. Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol. Cell Proteomics* **2012**, *11* (6), No. O111.016717, DOI: 10.1074/mcp.O111.016717.

(15) Zhou, J.; Li, Y.; Chen, X.; Zhong, L.; Yin, Y. Development of data-independent acquisition workflows for metabolomic analysis on a quadrupole-orbitrap platform. *Talanta* **2017**, *164*, 128−136.

(16) Tsugawa, H.; Cajka, T.; Kind, T.; Ma, Y.; Higgins, B.; Ikeda, K.; Kanazawa, M.; VanderGheynst, J.; Fiehn, O.; Arita, M. MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis. *Nat. Methods* **2015**, *12* (6), 523−526.

(17) Tsugawa, H.; Ikeda, K.; Takahashi, M.; Satoh, A.; Mori, Y.; Uchino, H.; Okahashi, N.; Yamada, Y.; Tada, I.; Bonini, P.; et al. A lipidome atlas in MS-DIAL 4. *Nat. Biotechnol.* **2020**, *38* (10), 1159−1163.

(18) Moco, S.; Buescher, J. M. Metabolomics: Going Deeper, Going Broader, Going Further. In *Cell-Wide Identification of Metabolite-Protein Interactions*; Skirycz, A.; Luzarowski, M.; Ewald, J. C., Eds.; Springer US, 2023; pp 155−178.

(19) Wawrzyniak, R.; Ruperez, F. J.; Godzień, J. B. Editorial: Advances and challenges in untargeted metabolomics. *Frontiers in Molecular Biosciences* **2023**, *10*, No. 1097443, DOI: 10.3389/fmolb.2023.1097443.

(20) Gauglitz, J. M.; West, K. A.; Bittremieux, W.; Williams, C. L.; Weldon, K. C.; Panitchpakdi, M.; Di Ottavio, F.; Aceves, C. M.; Brown, E.; Sikora, N. C.; et al. Enhancing untargeted metabolomics using metadata-based source annotation. *Nat. Biotechnol.* **2022**, *40* (12), 1774−1779.

(21) Sumner, L. W.; Amberg, A.; Barrett, D.; Beale, M. H.; Beger, R.; Daykin, C. A.; Fan, T. W.; Fiehn, O.; Goodacre, R.; Griffin, J. L.; et al. Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics* **2007**, *3* (3), 211−221.

(22) Broeckling, C. D.; Afsar, F. A.; Neumann, S.; Ben-Hur, A.; Prenni, J. E. RAMClust: a novel feature clustering method enables spectral-matching-based annotation for metabolomics data. *Anal. Chem.* **2014**, *86* (14), 6812−6817.

(23) Evans, A. M.; Br, B.; Liu, Q.; Mitchell, M. W.; Rj, R.; Dai, H.; Sj, S.; DeHaven, C. D.; Lad, M. High Resolution Mass Spectrometry Improves Data Quantity and Quality as Compared to Unit Mass Resolution Mass Spectrometry in High- Throughput Profiling Metabolomics. *Metabolomics* **2014**, *4*, 1−3.

(24) Malinowski, E. R. Factor analysis in chemistry, (3rd edition). E. R. Malinowski, Wiley, New York, 2002, ISBN 0-471-13479-1, € 117.60. *J. Chemom.* **2002**, *16*, No. 635, DOI: 10.1002/cem.757.

(25) Wang, X.; Shen, S.; Rasam, S. S.; Qu, J. MS1 ion current-based quantitative proteomics: A promising solution for reliable analysis of large biological cohorts. *Mass Spectrom. Rev.* **2019**, *38* (6), 461−482.

(26) Kozhinov, A. N.; Johnson, A.; Nagornov, K. O.; Stadlmeier, M.; Martin, W. L.; Dayon, L.; Corthésy, J.; Wühr, M.; Tsybin, Y. O. Super-

Resolution Mass Spectrometry Enables Rapid, Accurate, and Highly Multiplexed Proteomics at the MS2 Level. *Anal. Chem.* **2023**, *95* (7), 3712−3719.
(27) Kellmann, M.; Muenster, H.; Zomer, P.; Mol, H. Full scan MS in comprehensive qualitative and quantitative residue analysis in food and feed matrices: How much resolving power is required? *J. Am. Soc. Mass Spectrom.* **2009**, *20* (8), 1464−1476.
(28) Li, Z.; Li, Y.; Chen, W.; Cao, Q.; Guo, Y.; Wan, N.; Jiang, X.; Tang, Y. J.; Wang, Q.; Shui, W. Integrating MS1 and MS2 Scans in High-Resolution Parallel Reaction Monitoring Assays for Targeted Metabolite Quantification and Dynamic 13C-Labeling Metabolism Analysis. *Anal. Chem.* **2017**, *89* (1), 877−885.
(29) de Jonge, N. F.; Louwen, J. J. R.; Chekmeneva, E.; Camuzeaux, S.; Vermeir, F. J.; Jansen, R. S.; Huber, F.; van der Hooft, J. J. J. MS2Query: reliable and scalable MS2 mass spectra-based analogue search. *Nat. Commun.* **2023**, *14* (1), No. 1752.
(30) Schrimpe-Rutledge, A. C.; Codreanu, S. G.; Sherrod, S. D.; McLean, J. A. Untargeted Metabolomics Strategies-Challenges and Emerging Directions. *J. Am. Soc. Mass Spectrom.* **2016**, *27* (12), 1897−1905.
(31) Révész, Á.; Hevér, H.; Steckel, A.; Schlosser, G.; Szabó, D.; Vékey, K.; Drahos, L. Collision energies: Optimization strategies for bottom-up proteomics. *Mass Spectrom. Rev.* **2023**, *42* (4), 1261−1299.
(32) Lin, C.; Zhou, X.; Zhang, H.; Fu, Z.; Yang, H.; Zhang, M.; Hu, P. Deciphering and investigating fragment mechanism of quinolones using multi-collision energy mass spectrometry and computational chemistry strategy. *Rapid Commun. Mass Spectrom.* **2023**, *37* (12), No. e9514.
(33) Meybodi, F. Y.; Eslahchi, C. Predicting anti-cancer drug response by finding optimal subset of drugs. *Bioinformatics* **2021**, *37* (23), 4509−4516.
(34) Hilaire, P. B. S.; Rousseau, K.; Seyer, A.; Dechaumet, S.; Damont, A.; Junot, C.; Fenaille, F. Comparative Evaluation of Data Dependent and Data Independent Acquisition Workflows Implemented on an Orbitrap Fusion for Untargeted Metabolomics. *Metabolites* **2020**, *10* (4), No. 158.
(35) Koelmel, J. P.; Kroeger, N. M.; Gill, E. L.; Ulmer, C. Z.; Bowden, J. A.; Patterson, R. E.; Yost, R. A.; Garrett, T. J. Expanding Lipidome Coverage Using LC-MS/MS Data-Dependent Acquisition with Automated Exclusion List Generation. *J. Am. Soc. Mass Spectrom.* **2017**, *28* (5), 908−917.
(36) Schulze, B.; Heffernan, A. L.; Gomez Ramos, M. J.; Thomas, K. V.; Kaserzon, S. L. Influence of extraction windows for data-independent acquisition on feature annotation during suspect screening. *Chemosphere* **2024**, *349*, No. 140697.
(37) Guzman, U. H.; Martinez-Val, A.; Ye, Z.; Damoc, E.; Arrey, T. N.; Pashkova, A.; Renuse, S.; Denisov, E.; Petzoldt, J.; Peterson, A. C.; et al. Ultra-fast label-free quantification and comprehensive proteome coverage with narrow-window data-independent acquisition. *Nat. Biotechnol.* **2024**, *42* (12), 1855−1866.
(38) Wallmann, G.; Leduc, A.; Slavov, N. Data-Driven Optimization of DIA Mass Spectrometry by DO-MS. *J. Proteome Res.* **2023**, *22* (10), 3149−3158.

https://doi.org/10.1021/acs.jproteome.5c00622
J. Proteome Res. 2025, 24, 6311−6319