Journal of proteome research

pubs.acs.org/jpr

Article

Skyline for Small Molecules: A Unifying Software Package for Quantitative Metabolomics

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Cite This: *J. Proteome Res.* 2020, 19, 1447−1458

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ABSTRACT: Vendor-independent software tools for quantification of small molecules and metabolites are lacking, especially for targeted analysis workflows. Skyline is a freely available, open-source software tool for targeted quantitative mass spectrometry method development and data processing with a 10 year history supporting six major instrument vendors. Designed initially for proteomics analysis, we describe the expansion of Skyline to data for small molecule analysis, including selected reaction monitoring, high-resolution mass spectrometry, and calibrated quantification. This fundamental expansion of Skyline from a peptide-sequence-centric tool to a molecule-centric tool makes it agnostic to the source of the molecule while retaining Skyline features critical for workflows in both peptide and more general biomolecular research. The data visualization and interrogation features already available in Skyline, such as peak picking, chromatographic alignment, and transition selection, have been adapted to support small molecule data, including metabolomics. Herein, we explain the conceptual workflow for small molecule analysis using Skyline, demonstrate Skyline performance benchmarked against a comparable instrument vendor software tool, and present additional real-world applications. Further, we include step-by-step instructions on using Skyline for small molecule quantitative method development and data analysis on data acquired with a variety of mass spectrometers from multiple instrument vendors.

KEYWORDS: *Skyline, metabolomics, liquid chromatography, tandem mass spectrometry, small molecules, quantitative, targeted, metabolite, data analysis*

INTRODUCTION

Skyline is an open-source quantitative software tool originally implemented for targeted mass spectrometry (MS) proteomics workflows. Since its first public release in 2009, the Skyline software package has proven to be a highly reliable, flexible, and widely used program for targeted selected reaction monitoring (SRM)−MS analysis.[1−3] Subsequently, additional Skyline features were developed to process data-dependent acquisition (DDA) files for quantification using precursor ion extraction from high-resolution MS1 spectra.[4,5] Novel proteomics workflows have been implemented in Skyline as they have emerged and gained acceptance, such as parallel reaction monitoring (PRM)[6,7] and data-independent acquisition (DIA).[8,9] Skyline is currently being used by thousands of researchers worldwide, predominantly for quantitative proteomics applications.[10] While small molecule analysis via mass spectrometry has benefitted greatly from technological advances in mass spectrometry and separation science, there remains a need for data analysis with MS vendor-independent software tools that increase transparency and facilitate the translation of methods between labs and instrument platforms. Instrument manufacturers each have their own method development, data processing, and data analysis solutions. Though vendor software may provide advantages through insights into processing specific instrument data, these proprietary tools limit the transparency of data review by other laboratories that may not have access to the same commercial software and limit translation of methods to other laboratories or even within the same laboratory using other MS platforms. The open-source software tool Skyline provides unique features and is especially known for its comprehensive visualization tools and sophisticated method refinement options such as retention time scheduling and collision energy (CE) optimization,[11] which have been widely adopted in the proteomics field. These features are now also implemented for small molecule workflows such as metabolomics, pharmaceuticals (pharmacokinetics, drug metabolites, drug purity, and toxicology), forensics, food safety, and environmental pollutants.

In the field of open-source software for metabolomics, there are widely utilized packages for nontargeted analysis, such as

Received: September 20, 2019
Published: January 27, 2020

https://dx.doi.org/10.1021/acs.jproteome.9b00640
J. Proteome Res. 2020, 19, 1447−1458

XCMS,[12] MZmine,[13] and MS-DIAL.[14] However, for hypothesis-driven quantification of small molecules, Skyline is one of the few widely applicable open-source tools, others of which include XCMS-MRM[15] and MRMProbs.[16] While Skyline can readily analyze data sets collected with both targeted (SRM and PRM) and nontargeted methods, Skyline differs from traditional nontargeted analysis tools in that it requires as a first step that experiments be defined as a set of target molecules (or "hypotheses") for analysis. Recent developments in Skyline for interrogation of proteomics data-dependent (DDA) and data-independent acquisition (DIA) experiments demonstrate that the number of analytes can be as many as several tens of thousands of peptides.[4,9,17] Nonetheless, specifically for metabolomics, it is important to discriminate between typical nontargeted workflows, which start with feature-finding in MS1 spectra followed by alignment between runs and finally identification, versus the Skyline approach of extracting known targets followed by detection and quantification. Targeted MS data analysis packages typically allow the user to view raw liquid chromatography−mass spectrometry (LC−MS) data in the form of extracted ion chromatograms for the evaluation of separation and peak integration, to adjust automatic peak integration as necessary, often accompanied by approaches utilizing stable isotope dilution or external calibration to quantitatively calculate concentrations of compounds of interest (as applicable). The goal of this manuscript is to describe the extension of the targeted analysis paradigm using Skyline for small molecule workflows and to present a comprehensive metabolomics tool, supporting six major MS instrument vendors, that promises valuable features and excellent analytical performance to the metabolomics community. Additionally, Skyline documents can be readily shared using Panorama via https://panoramaweb.org/, a web-based targeted method and data repository, which can serve as a powerful platform for rapidly translating small molecule methods and results between laboratories.[18,19]

In this report, we demonstrate the utility of Skyline for metabolomics and metabolite quantification and describe experiments we used to examine its ability to process data from several instrument types, including triple quadrupole MS, such as the Xevo TQ-S (Waters) and the 5500 (SCIEX), and also high-resolution mass spectrometers, like the Synapt G2 HDMS (Waters). We first benchmark Skyline for targeted quantitative small molecule analysis using published data from the analysis of clinical samples with a well-established, commercial metabolomics kit (AbsoluteIDQ p180).[20,21] We next characterize a unique strength of Skyline by using it for the development and refinement of a customized assay for the targeted analysis of compounds in the arginine and polyamine pathways, which utilizes ion-pairing reagents and avoids classical derivatization.[22−27] We further pursue an analysis of purine/pyrimidine compounds to evaluate the ability of Skyline to handle positive/negative switching small molecule data in a single project. Finally, we present an example of how to use Skyline to perform the extraction of small molecule targets for quantitative analysis in high-resolution mass spectrometry data.[28] In addition, we have created several small molecule tutorials that provide step-by-step instructions on how to use Skyline for metabolomics analysis, covering topics that include (i) method development and refinement, (ii) calibrated quantification, and (iii) high-resolution metabolomics data analysis. The Supporting Information contains a basic tutorial, "Getting Started with Skyline for Small Molecules", and more detailed tutorials are hosted, updated, and synchronized with new developments on the Skyline website (https://skyline.ms/tutorials.url).

EXPERIMENTAL SECTION

Materials

All solvents used were of LC−MS grade or better; acetonitrile, water, and methanol were purchased from Fisher Scientific (Pittsburg, PA) or Burdick and Jackson (Muskegon, MI). Reagents for metabolite chemistry and quantification kits are described below in the various assay sections.

Biocrates AbsoluteIDQ p180 Analysis

Preparation of serum samples from the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort and analysis of these samples by the Biocrates AbsoluteIDQ p180 targeted metabolomics kit (Biocrates Life Science AG, Innsbruck, Austria) have been described previously by the Alzheimer's Disease Metabolomics Consortium (ADMC).[20,21] The data included in this report presents and discusses the reanalysis of the previously acquired raw mass spectrometric data from a single plate of 76 human serum samples.[20,21] No demographic or clinical data for the samples is included in the current manuscript; this can be obtained with permission from ADNI (adni.loni.usc.edu). Investigators within ADNI provided data but did not participate in analysis or writing of this report. Briefly, amino acids in the p180 kit are derivatized on a filter paper using phenyl isothiocyanate, extracted in buffered methanol, and analyzed by reversed-phase LC−MS/MS using an Acquity ultra performance liquid chromatography (UPLC) coupled to a Xevo TQ-S mass spectrometer (Waters Corporation). In the published data,[20,21] TargetLynx v4.1 was utilized for data analysis. The same curve fit (linear regression with $1/x$ weighting) and quantification parameters were utilized in Skyline-daily version 19.0.9.149 in this manuscript for the purposes of data visualization and processing. The Skyline document for this analysis has been uploaded to Panorama Public at https://panoramaweb.org/SkylineForSmallMolecules.url.

Polyamine Sample Preparation

The following unlabeled standards were obtained in small quantities from Cayman Chemical (Ann Arbor, MI) and Millipore Sigma (St. Louis, MO): agmatine, 5′-deoxy-5′-methylthioadenosine (MTA), S-(5′-adenosyl)-L-methionine chloride (SAM), *S*-adenosylhomocysteine (SAH), spermidine (Spd), *N*1-acetylspermidine (*N*1-AcSpd), *N*8-acetylspermidine (*N*8-AcSpd), *N*1-acetylspermine (*N*1-AcSpm), *N*1- and *N*12-diacetylspermine (DiAcSpm), arginine (Arg), homocysteine (Hcy), γ-aminobutyric acid (GABA), ornithine (Orn), acetylputrescine (AcPut), and spermine (Spm). The stable isotope-labeled internal standards *N*8-AcSpd-d3, *N*1-AcSpm-d3, MTA-d3, DiAcSpm-d6, and GABA-d6 were obtained from Toronto Research Chemicals (Toronto, ON) and CDN Isotopes Inc. (Pointe-Claire, QC). All compounds were dissolved in vendor-recommended solvents to create stock solutions of individual compounds. A 10-point calibration curve of the mix of standards was prepared in the homogenization solvents over a range of 0.01−25 μM.

Cohort 1: urine was collected from thirteen 12 week old CVN mice,[29] following all proper IACUC protocols, for polyamine analysis, and the samples were stored at −80 °C until sample preparation and analysis. The urine samples were

https://dx.doi.org/10.1021/acs.jproteome.9b00640
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removed from the −80 °C freezer and kept over ice during sample preparation. Two aliquots were removed from the original sample: 50 μL used for individual analysis and 10 μL used for the sample pool quality control. For sample and calibration standard preparation, 20 μL of urine or calibration standard in phosphate-buffered saline was removed and diluted with 40 μL of 1:1 MeOH:H_2O containing a mix of internal standards. The samples were then centrifuged at 10 000*g* at 4 °C for 5 min. The supernatant was removed and transferred to a prelabeled LC−MS total recovery vial from Waters (Milford, MA).

Cohort 2: Nineteen brain tissue samples from cognitively normal humans were acquired from the Specimen Repository of the Bryan Alzheimer's Disease Research Center (Duke IRB Protocol 00036140), under exemption based on deidentified samples, and kept at −80 °C until sample preparation and analysis. The samples were removed from the freezer and kept over ice during sample preparation; a variable volume of 67.8:14.2:17.8 MeOH:H_2O:$CHCl_3$ (v/v) spiked with heavy-labeled internal standards was added to each Precellys CK14 tube containing a preweighed brain tissue to normalize each sample to a constant tissue weight per volume of solvent (0.0657 mg/μL). The samples were homogenized using a Bertin Precellys 24 tissue homogenizer (Montigny-le-Bretonneux, France) and cooled to 4 °C with dry ice for three cycles of 10 s each at 28 000*g* and 1 min of rest in between cycles. The samples were centrifuged at 10 000*g* and held at 4 °C for 10 min. From each sample, a 50 μL aliquot of the supernatant was removed and transferred to a prelabeled LC−MS total recovery vial from Waters (Milford, MA).

Polyamine Analysis by LC−MS/MS

Skyline was used to develop and refine a custom method to measure polyamines and acetylated polyamines in urine and brain tissue. The assay used an Acquity UPLC interfaced to a Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation). Electrospray ionization was used with tune parameters: spray voltage 2.8 kV, desolvation gas 1000 L/h, cone voltage 35 V, desolvation temperature 500 °C, and cone gas flow 150 L/h. From each sample, 1 μL was injected into a 1.7 μm Acquity UPLC BEH C18 2.1 × 100 mm^2 column (Waters Corporation Milford, MA). Mobile phase A comprised 0.1% v/v acetic acid (Millipore Sigma, St. Louis, MO) and 0.0125% v/v perfluoroheptanoic acid (PFHA, Millipore Sigma, St. Louis, MO) in water. Mobile phase B comprised 0.1% v/v acetic acid and 0.0125% v/v PFHA in acetonitrile. The separation used a flow rate of 0.5 mL/min and a column temperature of 40 °C. The chromatography program began with 1% mobile phase B and increased to 25% B over the first 1.5 min. The organic phase increased to 40% at 3.0 min, reached 99% at 4.0 min, was held for 0.7 min, and decreased to starting conditions (1% B) at 4.8 min, where it was held for 1.5 min until the end of the 6.3 min analysis. The weak needle wash was composed of mobile phase A, and to minimize carryover the strong needle wash was composed of acetonitrile/methanol/isopropyl alcohol/water/formic acid (50:20:15:15:0.25). Skyline-daily versions 4.2.1.19004 and 4.2.1.19058 were used for LC−SRM−MS data analysis and processing. The Skyline documents for the mouse urine and human brain tissue analyses have been uploaded to Panorama Public at https://panoramaweb.org/SkylineForSmallMolecules.url.

Purines and Pyrimidines by LC−MS/MS

For the purine and pyrimidine analysis, we operated an SCIEX 5500 triple quadrupole LC−MS mass spectrometer fitted with a Turbo V ion source, connected online to an ultrahigh-performance liquid chromatography Agilent 1260 UHPLC system. Analyst v1.6.1 (SCIEX) was used for all SRM data acquisition, the development of the high-performance liquid chromatography (HPLC) method, and the optimization of analyte-specific SRM transitions. Skyline-daily version 4.2.1.19004 was used for LC−SRM−MS data analysis and processing.

For the purine and pyrimidine analysis, urine from five mice was collected from voluntary expulsion, and 20 μL of aliquots was stored at −80 °C until ready for analysis. Urine aliquots were thawed on ice, and 80 μL of methanol was added, containing 2-chloroadenosine (IS) at a final concentration of 2.5 μM to each 20 μL of urine aliquot. The mixture was vortexed vigorously for ∼30 s, and protein precipitation was completed by incubating at −20 °C for 30 min. After this, the samples were vortexed vigorously for ∼30 s and centrifuged at 15 000 rpm for 10 min at 4 °C. An 80 μL aliquot of the supernatant was carefully removed without disturbing the pellets and transferred to an HPLC autosampler vial fitted with inserts; 2 μL was injected per HPLC−SRM−MS analysis.

Synthetic standards for the compounds indicated in Table S1 were obtained from IROA (Mass Spectrometry Metabolite Library of Standards, MSMLS) or Sigma-Aldrich, St. Louis, MO. Stocks of 100 μM concentration were prepared in 80% methanol and stored at −80 °C prior to use. A final standard mixture of all compounds at 5 μM (containing the internal standard/IS, 2-chloroadenosine at 2.5 μM) was prepared prior to analysis and injected at the onset of each biological sample set. The Skyline document for urine purine/pyrimidine analysis has been uploaded to Panorama Public at https://panoramaweb.org/SkylineForSmallMolecules.url.

High-Resolution Diacylglycerol (DAG) Analysis

Demonstration of high-resolution precursor ion metabolomics data analysis utilized a previously published study of the lipidomic response of BT474 breast cancer cells to a fatty acid synthase (FASN) inhibitor, specifically examining the diacylglycerols.[30] Briefly, lipids were extracted from BT474 cell pellets using MeOH/MTBE and then analyzed using a reversed-phase nontargeted lipidomics method, including Acquity UPLC with a CSH C18 2.1 × 10 mm^2 column coupled to a Synapt G2 HDMS mass spectrometer (Waters Corporation). Initial data analysis of this nontargeted lipidomics data set was performed in Progenesis QI (Waters Corporation). Skyline 4.2 was utilized to confirm the differential expression for two diacylglycerol (DAG) species, DAG 30:0 and DAG 32:0. Lockmass correction was enabled and performed within Skyline using leucine-enkephalin $[M + H]^+$ at m/z 556.2771. The Skyline document has been uploaded to Panorama Public at https://panoramaweb.org/SkylineForSmallMolecules.url.

RESULTS

A broad variety of targeted workflows will be presented, featuring some of the most valuable features of Skyline as a novel software tool for small molecules providing convenient, efficient assay development and data processing.

https://dx.doi.org/10.1021/acs.jproteome.9b00640
J. Proteome Res. 2020, 19, 1447−1458

Skyline Small Molecule Quantification: Method Setup and Data Processing

Skyline was originally designed to support quantitative proteomics workflows.[3] Herein, we describe new features that have been implemented to support targeted quantification from both targeted and nontargeted mass spectrometry data acquisition modes for small molecules. A Skyline document for small molecule analytes differs considerably from one for proteomics. Skyline provides a highly flexible environment for getting started with many types of small molecule quantification experiments, so much so that it has been used for proteomics cross-linking experiments.[31] Prior to the release of Skyline for small molecules, multiple groups utilized Skyline and its flexible architecture for peptides to quantify lipids without the tool formally supporting it.[32]

Figure 1 shows a workflow and its key steps for method creation and utilization in Skyline for a small molecule targeted

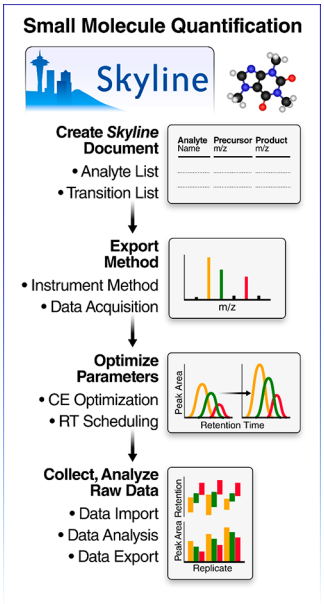

Figure 1. Generalized workflow for small molecule analysis in Skyline. Skyline logo reprinted with permission from Michael MacCoss.

SRM quantification, including method setup, assay refinement, data acquisition, and data processing. To support targeted quantification, the Skyline target tree typically includes the name and at least the m/z and optionally the precursor ion formula of each analyte. The target tree may also include empirical fragment ions (transitions) or transitions from a database or library. Examples of Skyline methods for small molecules typically include a simple list or set of lists of precursor and product ion m/z values for SRM experiments,[33−35] or exact masses for high-resolution precursor ion measurement.[5] In all cases, the molecular formula of a molecule of interest may be provided, allowing the software to derive precise m/z values, as well as isotope distributions for precursor and product ions, though this is not required. Further, Skyline supports a wide variety of chemical adducts such as metal ions and volatile organics (i.e., ammonia and formate) and allows for both positive and negative ion charges. These adducts can be specified in a new Skyline Transition Settings-Filter Tab in Skyline (Figure S1), as well as at the time targets are added. There are also a variety of instrument- and method-specific parameters that can be explicitly defined at the beginning of the workflow, such as collision energy, retention time, cone voltage (Waters), declustering potential (SCIEX), and S-Lens (Thermo). These are available during target addition with the Skyline Edit → Insert → Transition List menu item and may also be set after addition through the Document Grid. The "Getting Started with Skyline for Small Molecules" tutorial in the Supporting Information (S1) covers many of the basics described above and is the best place to start for new practitioners.

The second step in the Skyline-targeted mass spectrometry workflow is to export a method, either in the form of a transition list in a vendor-specific format or as a native instrument method file. As for proteomics methods, exporting to a native instrument method file requires a "template" method file or default method of the correct format for the instrument that will run the method.

After initial method export, usually, a cycle of method optimization will follow, including retention time scheduling, precursor and product ion selection, and collision energy optimization. The optimized method is then exported by Skyline and used to acquire mass spectrometry data on the biological samples, calibrators, and quality controls. The resulting raw data is analyzed by peak integration and calibrated quantification. At this point, Skyline offers extensive visualization options and even the ability to manually adjust integration for data quality control. Finally, peak areas and quantitative metrics may be exported in a tabular report format, using a highly flexible export dialog. A set of small molecule tutorials have been written to describe this workflow in detail and can be accessed in the Supporting Information or on the Skyline website (https://skyline.ms/tutorials.url).

Novel Visualizations in Skyline Aid in Small Molecule Quantification

Like MS vendor software, data analysis starts in Skyline with importing the native instrument files and does not require these files to be converted to an open format. Therefore, after data collection, raw data can be imported directly back into the Skyline document used to export the instrument method for data viewing and further analysis. Skyline is uniquely designed for the user to easily observe retention time, SRM or extracted ion chromatograms, and peak areas for each compound and its heavy-labeled isotope (if present) across numerous injections. A series of screenshots from Skyline are shown in Figure 2 to demonstrate several of the intuitive displays that allow for quick visual inspection of processed data and manual adjustment if necessary. Figure 2A shows the target tree for 5′-methylthioadenosine (MTA), including the precursor and product ions for the native metabolite and d3-MTA stable isotope internal standard. The remaining panels of the figure show an example of how the data may appear if an integration error occurred (left pane, contrived example for file CAL 6) or was correct (right pane). Manual data review is one of the most time-consuming aspects of quantitative mass spectrometry. Figure 2B demonstrates how the replicate retention time view allows the user to quickly recognize an acquisition where an incorrect retention time window was integrated. A simple click on Cal 6 bar in this plot causes Skyline to show the chromatogram view (Figure 2C), where the dashed lines indicate the integration window before and after corrected integration (left and right panes, respectively).

https://dx.doi.org/10.1021/acs.jproteome.9b00640
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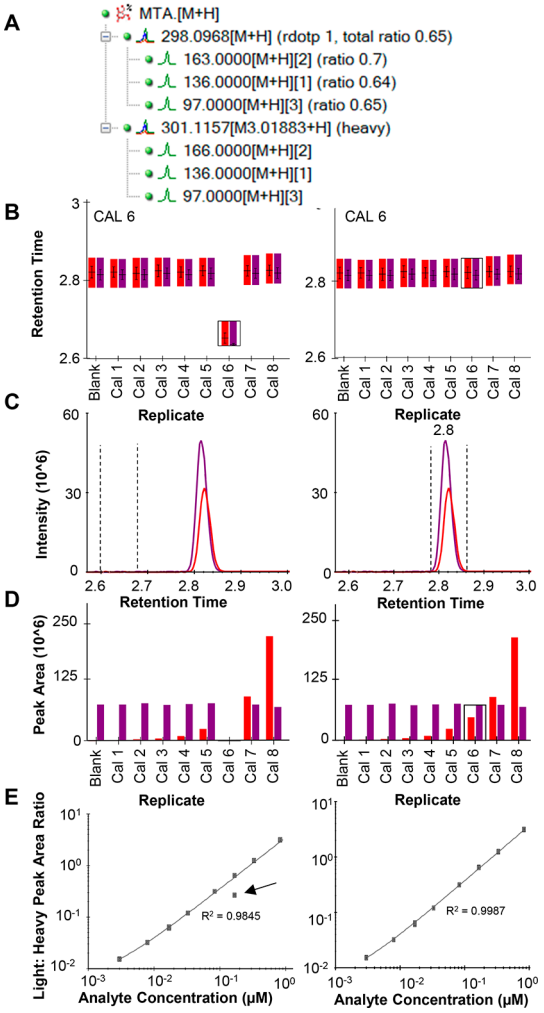

Figure 2. Unique Skyline visualizations are helpful for data interrogation. Left-hand panes demonstrate a hypothetical example of when incorrect retention time is selected for a single acquisition, while right-hand panes demonstrate correct integration. (A) Skyline molecule tree for MTA showing precursor and product ions for the light and heavy isotopes. (B) Retention time replicate display. (C) Chromatogram traces for integrated MTA. The vertical lines indicate where the peak integration was placed. (D) Peak area replicate display. (E) Calibration curve display.

Figure 2D shows the replicate peak area view for the same example data. In calibrated quantification (i.e., using an external calibration curve), it is often useful to visualize the peak intensities of the internal standard for consistency among the samples and standards (shown in purple), as well as the peak intensity of the analyte of interest (shown in red). This view also allows the user to quickly identify an acquisition that may need manual inspection (left pane) and again navigate with a single click to the appropriate chromatogram pane and correct the peak integration as necessary (right pane). Finally, the calibration curve visualization in Skyline (Figure 2E) allows for facile exclusion or reintegration of calibration points that have a high bias. Altogether, these visualizations represent features that can greatly improve accuracy in data analysis by making it easier to locate and adjust or exclude incorrect peak integrations or flag acquisitions that are suspect.

Validation of Skyline as a Tool for Calibrated Quantification

Skyline was recently used in a brief report on quantitative mass spectrometry assays for 25-hydroxy vitamin D and vitamin D binding protein from serum specimens and was found to be equivalent to a vendor tool.[36] While encouraging, this example showed a single small molecule analyte over a limited dynamic range. Therefore, we assessed a much larger data set for this report. The commercial Biocrates p180 analysis kit has been extensively characterized in small molecule analyses, with many publications reporting values obtained from various biological matrices analyzed using the kit, together with the recommended vendor-specific software.[37−39] Typically, the p180 data for the LC−MS/MS portion of the kit is processed in the vendor software of the instrument used for analysis and then imported into Biocrates MetIDQ for reporting. Because the p180 amino acid analysis by LC−MS/MS measures a number of analytes across a reasonably wide dynamic range (0.1−2000 μM) and is highly utilized in the community, we chose it as a test bed for evaluating the quantitative accuracy of Skyline. Serum amino acids from the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort were previously collected using the p180 kit.[20] Herein, we compared the reported concentration values between the two methods of data analysis to benchmark Skyline against TargetLynx (Waters) for this specific assay.

Concentrations were assessed for each of the 19 amino acids included in the Biocrates p180 assay that have corresponding internal standards, using TargetLynx and Skyline, across a set of 76 human serum samples. The raw data that was previously obtained via UPLC analysis for the ADNI cohort was loaded for reanalysis into a Skyline file (version 19.0.9.149) containing the amino acids and the transitions for the light and heavy isotopes where applicable. Peak picking was performed with explicit retention times and integrated automatically using the Skyline peak integration algorithm. Manual adjustments of the peak area integration were only required for a small number of analytes due to differences in explicit retention time listed and the actual retention time, as well as differences in retention time between the light and heavy isotopes of the analyte (some internal standards are deuterated). The concentrations for analytes were calculated using the ratio of light to heavy peak area response and a 7-point calibration curve with $1/x$ weighting covering 10^3 dynamic range; the calibration range for each metabolite varies from 0.1 to 2000 μM, depending on the expected analyte concentration range. Calibration points with greater than ±15% bias above the lower limit of quantification, or outside ±20% bias at the lower limit of quantification, were excluded.

Figure 3A shows a plot of the correlation between the concentrations reported by Skyline and TargetLynx for all 19 amino acids. A linear regression of these points yields a slope of 0.9837 (95% CI 0.9824−0.9850) and an intercept of 0.041 μM (95% CI 0.037−0.045), with an R^2 value of 0.9995. While these numbers indicate a very slight but statistically significant (at 95% confidence) difference from the ideal regression with a slope of 1 and a y-intercept of 0, the correlation coefficient is almost perfectly 1. The very slight difference between Skyline- and TargetLynx-reported absolute concentrations is almost certainly unimportant. Figure 3B displays the calculated accuracy of the concentration values reported via Skyline compared to that of TargetLynx for each individual analyte and sample in the increasing order of average abundance. All of the

https://dx.doi.org/10.1021/acs.jproteome.9b00640
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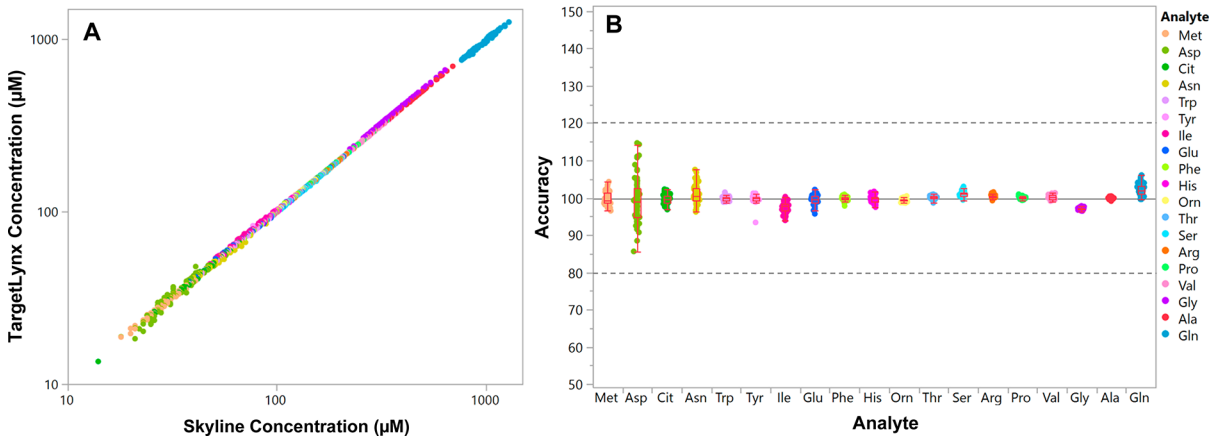

Figure 3. Comparison of clinical serum concentrations obtained for 19 amino acids in the Biocrates p180 assay. (A) Correlation of reanalysis of published data using Skyline results (*x*-axis) versus published concentrations measured in TargetLynx (*y*-axis). (B) Relative accuracy for each analyte comparing Skyline to published concentrations from TargetLynx, sorted low to high analyte concentration (left to right).

compound concentrations reported via Skyline have a mean accuracy within 100 ± 3% accuracy of the published ADNI values, which were calculated with TargetLynx.[20,21] Aspartic acid (Asp), a lower-abundance amino acid, has a notably larger standard deviation than the rest, and this is believed to be due to the software packages using different peak localization and integration approaches. Skyline does not apply boxcar smoothing of the chromatograms prior to peak area integration; the data for Asp suggests that this difference in smoothing use may impact the agreement between the two tools for low S/N analytes. Overall, Skyline performance appears to be nearly identical to the benchmark vendor tool for this particular p180 assay, suggesting that users should feel confident in transitioning existing assays to Skyline or developing new assays that utilize Skyline as the primary quantitative analysis tool.

High-Throughput Method Development Using Skyline: Customized Polyamine Panel

Skyline was used to develop a customized polyamine assay targeting arginine−proline metabolism and its downstream polyamine metabolites. The polyamines and their acetylated forms have important biological implications in cancer and neurology, making their detection and quantification an area of interest.[40−43] In this work, we used Skyline for custom method development and subsequently to analyze LC−MS/MS data generated from human brain and mouse urine samples (also see the workflow, Figure 1). First, a transition list was imported into Skyline including the following molecules for quantitation and analysis: agmatine, hcy, GABA, MTA, spd, *N*1-AcSpd, AcSpm, arg, orn, AcPut, SAH, *N*8-AcSpd, SAM, spm, and DiAcSpm (see Supporting Information Table S2 for precursor and product ion information, collision energy, retention time, and heavy-labeled internal standard used for normalization). Following analysis of these molecules and their corresponding stable isotope-labeled standards, a method was exported with retention time scheduling based on the measured retention times from the prior analysis and a time window setting of 1 min centered on these times. The difference in concurrent transition counts using various (1, 2, 5, and 20 min) retention time scheduling windows is shown in Figure 4A. With a scheduling window (20 min) set for longer than the total chromatographic time, all of the ion transitions will be measured concurrently throughout the entire gradient. As the

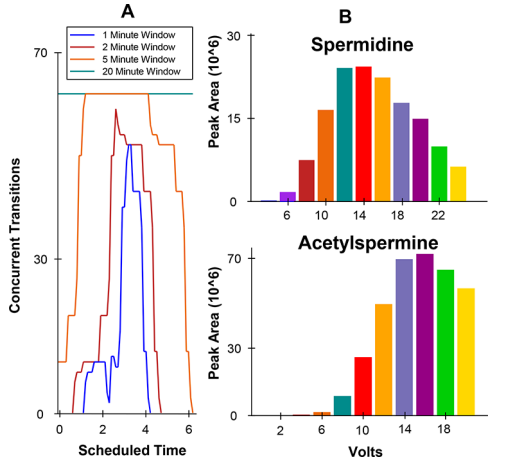

Figure 4. Method development for custom polyamine assay. (A) Comparison of concurrent transition count at different scheduling windows. (B) Utilization of collision energy optimization for two analytes, spermidine (upper) and acetylspermine (lower).

scheduling window narrows, the number of concurrently measured transitions decreases, allowing for a higher duty cycle for each targeted transition that yields a higher number of points across a peak, which in turn leads to improved peak area measurement precision.

In addition to retention time scheduling, Skyline was used for collision energy optimization for all of the compounds in this assay. A method was exported for CE optimization[23,26] with 2 V of increments (steps) for optimization, including five steps above and five below the starting voltage (11 distinct values, spanning a range of 20 V). This workflow is enabled in the Settings → Transition Settings → Prediction tab, as shown in Figure S2. The collision energy for spermidine was optimal in the original method at 14 V, which can be seen as the red bar in Figure 4B; the other collision energies tested from 4 to 24 V resulted in the same (at 12 V) or lower peak areas for the main spermidine transition. Conversely, for acetylspermine, the original collision energy (10 V) was not the optimal energy, and the highest peak area is obtained at 16 V, which would be the optimized CE used for this assay (Figure 4B). A unique feature of Skyline compared to that of other tools is the ability to perform highly multiplexed optimization of collision energy (CE) and other vendor-specific optimization parameters (such

https://dx.doi.org/10.1021/acs.jproteome.9b00640
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as declustering potential) during a chromatographic analysis. Most automated tools perform optimization during analyte infusion, which limits the number of compounds that can be optimized simultaneously and typically involves user intervention. By performing CE optimization in a retention-time-scheduled manner and on the chromatographic time scale, Skyline allows higher multiplexing of analytes for optimization and less user intervention.

Various mobile phase configurations were tested prior to selection of the final mobile phase for the polyamine assay. Included in the testing were 0.025% heptafluorobutyric acid (HFBA) with 0.1% formic acid, 0.025% HFBA with 0.1% acetic acid, 0.0125% perfluoroheptanoic acid (PFHA) with 0.1% formic acid, and 0.0125% PFHA with 0.1% acetic acid. For a majority of the targeted compounds, the combination of 0.0125% PFHA and 0.1% acetic acid provided the largest peak area, as shown in Figure S3. Of the LC−MS/MS methods published for the analysis of underivatized polyamines, many utilize HFBA as an ion-pairing reagent;[23,24,26,27] however, the comparison of HFBA to PFHA revealed that PFHA provides not only better sensitivity but also better peak shape for many compounds.[25] Assessing the peak intensity and peak shape of MTA under four different mobile phase conditions, acetic acid (0.1%) and HFBA (0.025%), formic acid (0.1%) and HFBA (0.025%), formic acid (0.1%) and PFHA (0.0125%), and acetic acid (0.1%) and PFHA (0.0125%), the best peak shape and intensity were obtained with the final mobile phase additive combination (Figure S3B−E). Better peak shape is obtained with acetic acid as seen in Figure S3B,E, and the most intense peak is observed with PFHA combined with acetic acid.

The optimized assay was employed on a series of biological samples, including cognitively normal human brains and mouse urine. Measured GABA is the most abundant compound detected in the human brain using this assay, followed by arginine at ∼800 and ∼400 nmol/g of the human brain, respectively. Also detected in human brains were ornithine, SAM, and SAH along with both *N*1- and *N*8-acetylspermidines and MTA. Acetylputrescine and spermine are not reported in human brains as values are below the limit of detection (LOD) for this assay (see Table 1). In the tested mouse urine, the most abundant compounds quantified with the assay are arginine and ornithine at 4.36 and 3.63 μmol/mmol of creatinine, respectively. Concentrations of SAM and SAH are reported, with levels of SAM in urine being higher than the reported levels of SAH, whereas in the human brain samples the concentrations were comparable. Spermine levels were measured in urine along with several acetylated forms of the polyamines such as *N*1- and *N*8-acetylspermidines and acetylputrescines (see Table 1).

Table 1. Average Concentration of Polyamines in Cognitively Normal Human Brain (n = 19) and CVN Mouse Urine (n = 13), Measured Using the Custom Polyamine Assay and Quantified with Skyline

	brain and urine polyamine concentrations	
	human brain, cognitive normal (nmol/g)	mouse urine, CVN (μmol/mmol creatinine)
GABA	804 ± 432	1.4 ± 1.0
AcPut	<LOD	0.19 ± 0.08
Orn	33 ± 21	3.6 ± 2.3
Arg	401 ± 148	4.4 ± 3.3
*N*1-AcSpd	6.9 ± 5.0	1.2 ± 0.4
*N*8-AcSpd	8.6 ± 4.9	0.9 ± 0.2
Spm	<LOD	1.0 ± 1.5
MTA	1.9 ± 1.2	0.2 ± 0.1
SAH	13.5 ± 4.4	$2.4 \times 10^{-3} \pm 4.4 \times 10^{-3}$
SAM	13.8 ± 8.8	2.2 ± 0.6

Skyline Supports Collection and Analysis of Positive/Negative Switching Ionization

For a specific example to evaluate the positive/negative switching features in Skyline, a purine and pyrimidine analysis was chosen. Purines and pyrimidines serve several important biological roles, such as providing the structural backbone for nucleic acid synthesis (DNA and RNA), acting as precursors to enzyme cofactors (NAD, SAM, etc.), intermediates for energy metabolism (ATP), and a route to excretion of nitrogenous waste (uric acid, allantoin, etc.). They are at the intersection of a variety of metabolic pathways. However, this group of metabolites is structurally heterogeneous and contains compounds that preferentially ionize in either positive or negative mode. To accommodate for this, we used the SCIEX 5500 triple quadrupole mass spectrometer, which offers rapid positive/negative polarity switching and allows for the acquisition of SRM transitions (Q1/Q3) simultaneously under both ionization modes.[44,45] Given the short polarity switching time for this mass spectrometer, 112 transitions could be accommodated with a reasonable total cycle time of ∼1.3 s, with all compounds in the group analyzed in a single LC−SRM−MS acquisition.

To evaluate the ability of Skyline to analyze data from positive and negative ionization modes in a single document, we generated a custom method analyzing purine/pyrimidine compounds on the SCIEX 5500 triple quadrupole mass spectrometer (Figure 5). In Skyline, positively and negatively ionizing compounds can be presented in a combined fashion under the same compound name within the target tree, and the preferred ionization mode for this compound can be indicated by simply including "+1" for positive mode or "−1" for negative mode in the transition list at the precursor (Q1) and/or product ion (Q3) level. In addition, Skyline can indicate the charge state for the Q1 transition (precursor) more specifically, using the IUPAC adduct nomenclature, either as [M + H] or as [M − H], as shown in Figure 5. Skyline imports the data from the LC−SRM−MS acquisitions to allow for simultaneous data processing for all compounds, irrespective of their individual ionization modes. Figure 5 shows the Skyline target tree and representative ion traces for cyclic-GMP, acquired through positive/negative polarity switching of the source. Chromatograms for positive and negative transitions can be viewed individually, together or in a "split chromatogram" view (see Figure 5C). By default, the ionization modes share the same integration time range.

Targeted Analysis of High-Resolution Metabolomics Data

Skyline is not intended as a tool for traditional nontargeted metabolomics workflows. However, an important feature of the software is to perform hypothesis-driven targeted verification and visualization of high-resolution MS data collected for small molecules with nontargeted "discovery" acquisition methods. To demonstrate this, we utilized data from a previously published study that developed fatty acid synthase inhibitors in the context of antitumor activity in a model of HER2$^+$ breast cancer.[28] Alwarawrah et al. reported that profound changes in

https://dx.doi.org/10.1021/acs.jproteome.9b00640
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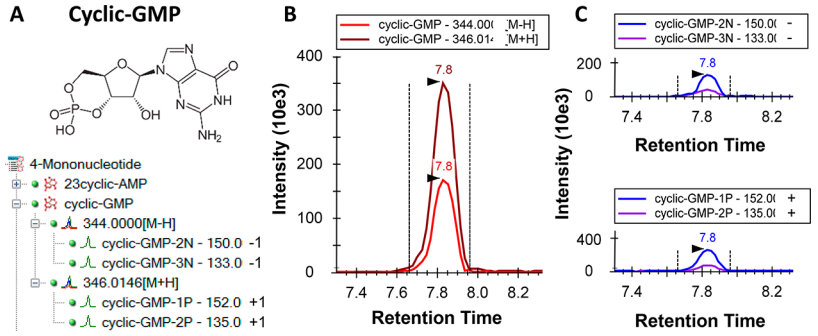

Figure 5. Targeted LC−SRM−MS analysis for purine and pyrimidine analysis using positive/negative switching. (A) Molecular structure of cyclic-GMP and Skyline target tree including molecule annotations and Q1/Q3 transitions, (B) combined ion chromatogram showing data in negative ion mode [M − 1] at *m/z* 344 and in positive ion mode [M + 1] at *m/z* 346, and (C) split graph representation for ion chromatograms in negative ion mode with the Q1/Q3 transition pair 344/(150 and 133) (upper panel) and in positive ion mode with the Q1/Q3 transition pair 346/(152 and 135) (lower panel).

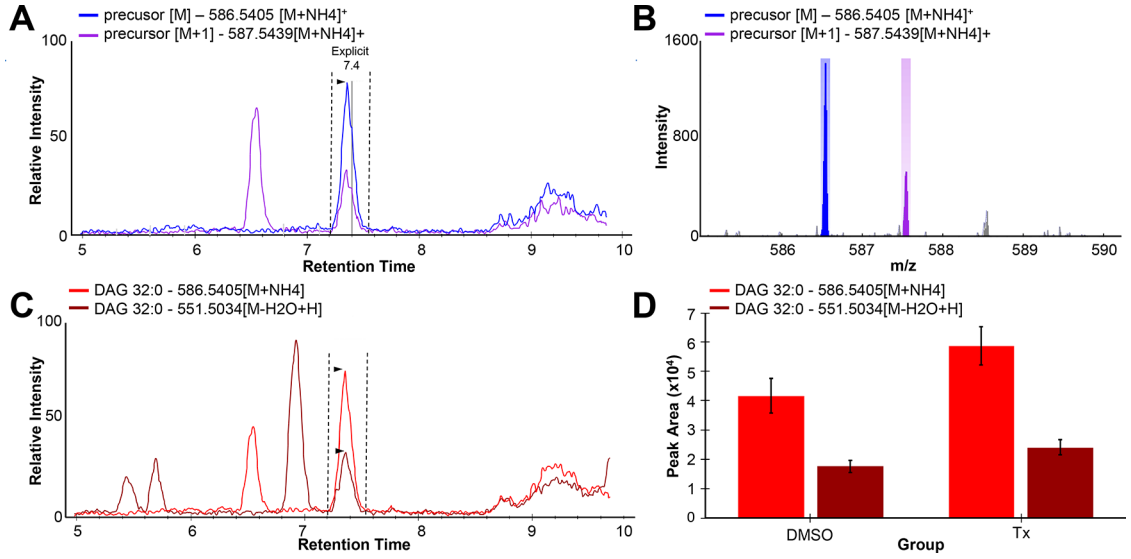

Figure 6. Analysis of high-resolution metabolomics data in Skyline, in particular, for a lipidomics analysis of diacylglycerol 32:0 (DAG 32:0). (A) Extracted ion chromatograms for the M and M + 1 isotopes of DAG 32:0 $[M + NH_4]^+$ adduct, (B) mass spectrum zoomed to show 585−590 *m/z* of the DAG 32:0 $[M + NH_4]^+$ adduct ion viewed in Skyline, (C) extracted ion chromatogram totals of M and M + 1 overlays of DAG 32:0 $[M + NH_4]^+$ and $[M - H_2O + H]^+$ adducts, and (D) grouped comparison peak area view within Skyline of five replicates of each biological condition comparing means and standard deviations of the control and FASN-inhibitor-treated cells.

cellular lipid profiles were observed using a QTOF MS in DDA mode, followed by nontargeted analysis with Progenesis QI software (nonlinear dynamics/Waters).[30] It is worth noting that the Skyline analysis of lipidomics data from a Q Exactive (ThermoFisher Scientific) has previously been demonstrated before Skyline was formally released for small molecules.[32] Here, we focused on the verification of two specific diacylglycerol species, DAG 30:0 and DAG 32:0, from (UPLC)/ESI/MS/MS analysis acquired on a G2 Synapt mass spectrometer (Waters), to demonstrate how the Skyline software can be used to verify accurate mass, isotope distribution, and coelution of ionization adducts and to perform quantitative measurements. Though for the purposes of demonstration we only include a few lipid molecules, there is virtually no limit to the number of metabolites or metabolite features that could be included in such a list for verification, similar to what has previously been demonstrated for MS1 quantification of peptides.[4] Figure S4 shows the transition list and full-scan transition settings used to perform this analysis in Skyline.

As an example, Figure 6A shows the M and M + 1 isotope extracted ion chromatograms for the $[M + NH_4]^+$ adduct of DAG 32:0 from one of the cell line samples. Skyline enables lockmass correction of the high-resolution MS1 diacylglycerol precursor ion data, yielding a −1 ppm relative mass accuracy for this measurement. By clicking on the extracted ion chromatogram, Skyline then accesses the mass spectrum at the clicked retention time from the raw data file and displays the spectrum highlighting the extracted *m/z* ranges (Figure 6B). Additional confidence in the integration of the correct chromatogram peak in lipidomics analysis can be obtained by targeting multiple ionization adducts, similar to targeting multiple charge states for tryptic peptides in proteomics. For diacylglycerols, the two most common ionization states are $[M - H_2O + H]^+$ and $[M + NH_4]^+$. Figure 6C shows that for DAG 32:0 both of these adducts have interference when totaled chromatograms for [M] and [M + 1] are considered independently. Only the peak at 7.4 min, however, shows the coelution of both ionization forms. Finally, the ability of Skyline to define sample groups (here dimethyl sulfoxide and

https://dx.doi.org/10.1021/acs.jproteome.9b00640
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Tx) facilitates visual comparison of the average and standard deviations of treatment groups, for each ionization form (Figure 6D). Taken together, these features are key attributes for Skyline and its use as either the primary quantitative tool or a secondary verification tool in high-resolution metabolomics analysis.

CONCLUSIONS

This study is the first to establish Skyline as a framework for comprehensive quantitative data processing not only for proteomics data but also now fully supporting the analysis of small molecule mass spectrometry data. We have demonstrated great versatility for small molecule workflows and established Skyline as a tool expanding its capabilities for quantitative data processing into the metabolomics field. While all software tools have inherent advantages and disadvantages, we maintain that the primary advantage of Skyline over any other tool is the transparency with which data analysis and interpretation can be shared in the community and in the translation of assays between instruments and laboratories without requiring separate analysis software. Skyline is freely available and actively supported, and the Skyline documents containing the workflows demonstrated here are available in Panorama (www.panoramaweb.org). All Panorama-enabled workflows for peptides, such as automated data import and quality control ("AutoQC"), have also been extended to small molecules.[18,19,46]

Though not explicitly demonstrated herein, the workflows described are also functional in Skyline for Agilent, Bruker, Shimadzu, and Thermo mass spectrometers, in addition to Sciex and Waters used in this report. Additionally, Skyline already supports a number of other workflows utilized in small molecule analysis, including parallel reaction monitoring (PRM), gas chromatography−mass spectrometry (GC−MS), and single-point external calibration. Future work will also implement workflows in Skyline to support fragmentation libraries of small molecules, such as Metlin (http://metlin.scripps.edu/),[47,48] MassBank,[49] the Human Metabolome Database (HMDB, http://www.hmdb.ca/),[50,51] and the National Institute of Standards and Technology Database (NIST https://chemdata.nist.gov/); similar workflows are already available for proteomics experiments.[3,4] Though the expansion of support for GC−MS and all types of ion mobility small molecule data is also ongoing, we would caution that no single tool will be best for all workflows. Especially in the case of latest-generation or vendor-specific workflows, Skyline may lag behind commercial software for a time until support can be implemented. Skyline external tools[52] will also allow for linking small molecule quantitative data sets with other data-processing pipelines.In summary, Skyline has become a versatile and powerful open-source software solution for quantitative mass spectrometry data workflows and can now support both proteomics and metabolomics workflows in a vendor-neutral environment. Combined with the features of Panorama to easily share methods and data sets, Skyline is positioned to drastically improve the transparency and open translation of metabolomics data and methods between laboratories. We believe that Skyline has the ability to gain broad use and acceptance in the small molecule/metabolomics field as it has in quantitative proteomics.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.9b00640.

Ion transitions, collision energy, cell exit potential, declustering potential, and entrance potential for the purine and pyrimidine LC−MS/MS panel (Table S1); on transitions, collision energy values, retention times, and internal standards for targeted compounds in the polyamine custom assay (Table S2); screenshot of the small molecule filter tab in Skyline, allowing the user to define the precursor and fragment ion adducts desired for analysis; additionally, the user can utilize the "ion-type" field to define the analysis of a precursor ("p") or fragment ("f") in a document-wide fashion (Figure S1); collision energy optimization enabled via Settings → Transition Settings → Prediction tab (Figure S2); (A) peak area of targeted compounds with different mobile phase additives; peak shape and intensity for MTA elution with (B) acetic acid (0.1%) and HFBA (0.025%), (C) formic acid (0.1%) and PFHA (0.0125%), (D) and acetic acid (0.1%) and PFHA (0.0125%) (Figure S3); examples of how to set up the transition list (A) and the full-scan parameter settings (B) for the Skyline analysis of high-resolution metabolomics data; specifically, in this case, the transition list allows for analysis of two diacylglycerol lipids, each with two adduct ionization forms (Figure S4); a beginner tutorial called "Getting Started with Skyline for Small Molecules" is included in the Supporting Information; this tutorial, as well as additional tutorials for detailed features of Skyline analysis for small molecules, is available at https://skyline.ms/tutorials.url; also, a short webinar is available at the following link: https://skyline.ms/tutorial_small_molecule.url (PDF)

Accession Codes

All Skyline files are located on the public Panorama Share: https://panoramaweb.org/SkylineForSmallMolecules.url.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

This work was supported by the National Institute of Health: RF1 AG057358 (MPI: Lithgow, Andersen—BS), R56 AG057895 (MPI: Colton, Thompson), R01 AG057895 (MPI: Colton, Thompson), and P41 GM103493, R01 GM103551, P30 AG013280, and R56 AG063885 (to MJM). N.B. was supported by an NIH fellowship grant (T32 AG000266, PI: Campisi).

Notes

The authors declare no competing financial interest.
[∇]Consortium membership can be found at https://sites.duke.edu/adnimetab/team/.

■ ACKNOWLEDGMENTS

Data used in the preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (http://adni.loni.usc.edu) and contributed by the Alzheimer's Disease Metabolomics Consortium (ADMC, https://sites.duke.edu/adnimetab/). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this article. Researchers can apply for ADNI data at http://adni.loni.usc.edu/data-samples/access-data/. Data collection and sharing for this project were funded, in part, by the Alzheimer's Disease Neuroimaging Initiative (ADNI) (National Institutes of Health Grant U01 AG024904) and DOD ADNI (Department of Defense award number W81XWH-12-2-0012). ADNI is funded by the National Institute on Aging, the National Institute of Biomedical Imaging and Bioengineering, and through generous contributions from the following: AbbVie, Alzheimer's Association; Alzheimer's Drug Discovery Foundation; Araclon Biotech; BioClinica, Inc.; Biogen; Bristol-Myers Squibb Company; CereSpir, Inc.; Cogstate; Eisai Inc.; Elan Pharmaceuticals, Inc.; Eli Lilly and Company; EuroImmun; F. Hoffmann-La Roche Ltd. and its affiliated company Genentech, Inc.; Fujirebio; GE Healthcare; IXICO Ltd.; Janssen Alzheimer Immunotherapy Research & Development, LLC.; Johnson & Johnson Pharmaceutical Research & Development LLC.; Lumosity; Lundbeck; Merck & Co., Inc.; Meso Scale Diagnostics, LLC.; NeuroRx Research; Neurotrack Technologies; Novartis Pharmaceuticals Corporation; Pfizer Inc.; Piramal Imaging; Servier; Takeda Pharmaceutical Company; and Transition Therapeutics. The Canadian Institutes of Health Research is providing funds to support ADNI clinical sites in Canada. Private sector contributions are facilitated by the Foundation for the National Institutes of Health (www.fnih.org). The grantee organization is the Northern California Institute for Research and Education, and the study is coordinated by the Alzheimer's Therapeutic Research Institute at the University of Southern California. ADNI data are disseminated by the Laboratory for NeuroImaging at the University of Southern California. Data collection and sharing for this project were funded by the Alzheimer's Disease Metabolomics Consortium (National Institute on Aging R01AG046171, RF1AG051550, and 3U01AG024904-09S4).

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