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High resolution mass spectrometry newborn screening applications for quantitative analysis of amino acids and acylcarnitines from dried blood spots

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Abstract

Amino acid and acylcarnitine first-tier newborn screening typically employs derivatized or non-derivatized sample preparation methods followed by FIA coupled to triple quadrupole (TQ) MS/MS. The low resolving power of TQ instruments results in difficulties distinguishing nominal isobaric metabolites, especially those with identical quantifying product ions such as malonylcarnitine (C3DC) and 4-hydroxybutylcarnitine (C4OH). Twenty-eight amino acids and acylcarnitines extracted from dried blood spots (DBS) were analyzed by direct injection (DI)-HRMS on a Q-Exactive Plus across available mass resolving powers in SIM, in PRM at 17,000 full width at half maximum (FWHM), and a developed SIM/PRM hybrid MS method. Most notably, quantitation of C3DC and C4OH was successful by HRMS in non-derivatized samples, thus, potentially eliminating sample derivatization requirements. Quantitation differed between SIM and PRM acquired data for several metabolites, and it was determined these quantitative differences were due to collision energy differences or kinetic isotope effects between the unlabeled metabolites and the corresponding labeled isotopologue internal standards. Overall quantitative data acquired by HRMS were similar to data acquired on TQ MS/MS platform. A proof-of-concept hybrid DI-HRMS and SIM/PRM/FullScan method was developed demonstrating the ability to hybridize targeted newborn screening with metabolomic screening.

GRAPHICAL ABSTRACT

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CRediT authorship contribution statement

C. Austin Pickens: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Writing - original draft.

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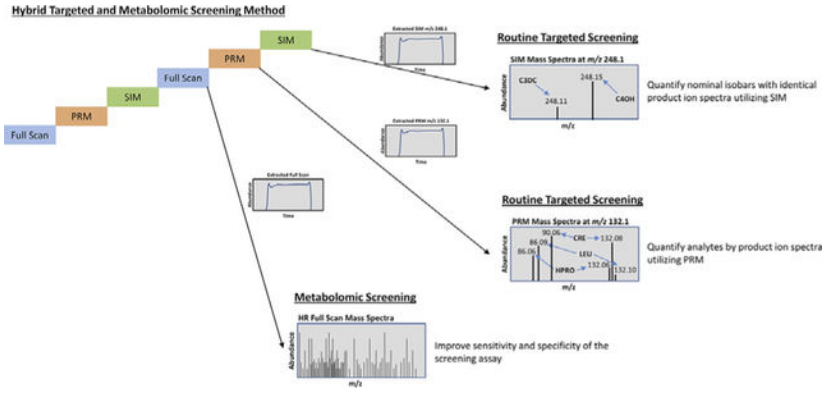
Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2020.04.067.



Keywords

Metabolomics; Newborn screening; High resolution mass spectrometry; Dried blood spot; Metabolic disorders; Amino acids and acylcarnitines

1. Introduction

Newborn screening (NBS) is considered one of the ten great public health achievements in the United States during 2001–2010 [1]. NBS detects asymptomatic newborns at risk for rare diseases such as inborn errors of metabolism (IEM). IEM often result in perturbed levels of amino acids or acylcarnitines, which are used as diagnostic markers for IEM in NBS. Usage of MS/MS in NBS started in the 1990s, and revolutionized NBS by providing greater sensitivity and the ability to screen dozens of disorders in a single multiplexed assay [2–4]. NBS laboratories typically employ first-tier screening for amino acids and acylcarnitines using either derivatized or non-derivatized sample preparation from dried blood spots (DBS). DBS extracts are analyzed by FIA coupled to triple quadrupole (TQ) MS/MS. Although TQ instruments are robust and sensitive, these instruments feature low mass resolving power resulting in difficulties separating nominal isobaric compounds (e.g., <0.1 Da difference in molecular mass, different chemical structure) with identical quantifying product ions. Additional interferences during FIA-TQ-MS/MS analyses include monoisotopic interferences from naturally occurring ^{13}C isotopologues, in-source fragmentation interferences, etc [5–8].

Nominal isobars are especially problematic in NBS between dicarboxyl and hydroxy acylcarnitines such as malonylcarnitine (C3DC, theoretical *m/z* 248.1134) and 3-hydroxybutyrylcarnitine (C4OH, theoretical *m/z* 248.1498), succinylcarnitine (C4DC, theoretical *m/z* 262.1291) and hydroxyisovalerylcarnitine (C5OH, theoretical *m/z* 262.1655), and glutarylcarnitine (C5DC, theoretical *m/z* 276.1447) and hydroxyhexanoylcarnitine (C6OH, theoretical *m/z* 276.1811) [9]. Since the characteristic quantifying product ion of acylcarnitines is *m/z* 85 [9], the aforementioned pairs cannot be distinguished when analyzed non-derivatized by FIA-TQ MS/MS. Issues associated with distinguishing these nominal isobars in first-tier NBS are that elevated levels of each marker indicate a presumptive positive for different disorders. For instance, elevated levels of C3DC are associated with a defect in malonyl-CoA decarboxylase and mutations in the MLYCD

gene [10], while elevated levels of C4OH are associated with a defect in 3-hydroxyacyl-CoA dehydrogenase and mutations in the HADH gene [11].

Indeed butyl ester derivatization of NBS samples improves electrospray ionization (ESI) and distinguishes aforementioned dicarboxylic and hydroxy acylcarnitines when acquired by FIA-TQ MS/MS [9]. Derivatization shifts these metabolite masses by eliminating free carboxylate groups of dicarboxylic acylcarnitines by adding 112 Da and hydroxyl acylcarnitines by adding 56 Da [12]. Issues associated with derivatization are creation of new isobars, in particular, C3DC butyl esters (theoretical *m/z* 360.2386) and hydroxyoctanolycarnitine (C8OH) butyl esters (theoretical *m/z* 360.2750) [13], and glutamic acid butyl esters (theoretical *m/z* 260.1862) and acetylcarnitine (C2) butyl esters (theoretical *m/z* 260.1862). In addition, derivatization elevates free carnitine concentrations due to hydrolysis of the acylcarnitine backbone [14,15] and impacts amino acids concentrations [16,17], thus, adding difficulty comparing data across NBS laboratories [18]. Although chromatographic separation can mitigate several aforementioned limitations [8], due to the high-throughput nature required in universal newborn screening (<2 min sample analysis) FIA is the sample introduction method of choice. Liquid chromatography is instead utilized for second-tier screening on specimens flagged as presumptive positive during first-tier screening, an approach that has greatly reduced false positive rates [19].

High resolution MS (HRMS) allows distinguishing < 0.1 Da differences between metabolites, and hybrid quadrupole HRMS instrumentation provides the ability to select *m/z* for CID while acquiring the HRMS full spectra of product ions. These capabilities appear useful to NBS laboratories since C3DC and C4OH differ by 0.03 Da and could be distinguished by HRMS. Leucine (i.e., transition 132.1 > 86.10) and 4-hydroxyproline (4HPRO, i.e., transition 132.1 > 86.06) are nominal isobars with nominal isobaric quantifying fragments (i.e., transition 132.1 > 86.1) on TQ MS/MS platforms, and both could be distinguished by acquisition of HRMS product ion spectra. Overall HRMS may be viable alternative to overcome limitations experienced by NBS laboratories conducting first-tier screening by FIA-TQ MS/MS. In addition to targeted screening by HRMS, NBS laboratories could also utilize HRMS instrumentation to perform metabolomic screening, which may assist in classification of IEM and further reduce false positive rates [16,20–23]. The objective of this study was to demonstrate the capabilities of DI-HRMS to overcome limitations associated with FIA-TQ MS/MS in NBS, assess NBS analyte quantification across mass resolving powers and develop a MS method to quantify metabolites from non-derivatized DBS sample extracts, and outline a proof-of-concept metabolomic screening workflow that could assist NBS laboratories to increase positive predictive value and reduce false positive rates in first-tier screening.

2. Materials and methods

2.1. Dried blood spot samples and internal standards

Appropriate safety control measures (including engineering, administrative, and personal protective equipment) were used for all procedures based on a site-specific risk assessment that identified physical, health, and procedural hazards. The Centers for Disease Control and Prevention’s (CDC) Newborn Screening Quality Assurance Program (NSQAP) 2018

QC dried blood spot (DBS) cards were analyzed in this study. These DBS cards included a base pool and three linearly enriched versions of the base pool, at varying enrichment levels for each metabolite, and QC production was performed as previously described [24]. Additional information regarding pool preparation and concentrations can be found in the QC Specimen Certification Data reports [25,26] or Supplementary Table A.1. Specific information related to solvents, chemicals, and unlabeled and labeled internal standards (IS) are detailed in Supplementary Information. In brief, the working IS extraction solution was prepared containing fixed concentrations over 20 isotopically labeled IS. The fixed IS concentrations were used as single point calibrators for metabolite quantitation, which is routine in NBS due to lack of commercially available multianalyte DBS-based calibrators. Several metabolites require using IS surrogates since there are no commercially available isotopic labeled IS for some NBS metabolites, and metabolite and corresponding IS used in the study are presented in Supplementary Table A.2.

2.2. Sample preparation for high resolution analysis

Samples analyzed by DI on the HRMS platform were extracted following the protocol as previously described [27]. Dried sample extracts were stored under nitrogen at −20 °C for no longer than three days. Prior to analysis, samples were resuspended in 50:50 acetonitrile:water with 0.1% formic acid, then centrifuged for 7 min at 3500 × G (Rotina 380R, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany) to reduce the amount of DBS fibers toward the sample surface.

2.3. High resolution MS analyses of dried blood spot extracts

HRMS analyses were conducted using a Q-Exactive Plus HRMS (QE+, Thermo Scientific GmbH, Bremen, Germany) utilizing the SIM and parallel reaction monitoring (PRM) MS acquisition methods. Mass accuracy was evaluated daily and calibrated when required. Capillary temperature was set to 210 °C and S-lens RF value was set to 60. Several MS methods were created in order to assess whether analyte quantitation varied across mass resolving powers and comparing SIM to PRM. Mass resolving power is defined as full width at half-maximum. A total of 6 different MS methods were analyzed in this study (Table 1): four methods acquired data using SIM to assess quantitative differences across mass resolving powers, one method acquired data using PRM for comparison of SIM acquired data, and one method hybridized SIM and PRM acquisition functions. A comprehensive list of metabolites, accurate *m/z*, product ions, internal standards, and collision energies (CE) are presented in Supplementary Table A.2. Metabolite CE were optimized from their corresponding IS used in quantification on the QE+.

Sample delivery and ionization were performed using a TriVersa NanoMate ion source (Advion, Ithaca, NY, USA), utilizing chip-based DI in positive ionization mode with 5 μm nominal internal diameter nozzle chips. Sample injection order was randomized on each day of analysis. The TriVersa plate chiller was set to 4 °C, which mitigated acylcarnitine hydrolysis, since at 10 °C there was a linear relationship between acylcarnitine hydrolysis and sample injection order (data not shown). To accommodate the two different MS analysis times outlined in Table 1 (i.e., 1.5 min and 8 min), two method profiles were created on the TriVersa. Both methods applied 1.4 kV, aspirated 8 μL of sample volume 2.4 mm

from the sample well bottom, using 1.0 psi gas pressure, 3 s aspiration delay, and contact closure before starting spray. The 1.5 min analysis time method utilized a sample acquisition delivery time of 1 min 10 s and a 3.0 μL air gap. The MS method for the 8 min analysis utilized a sample acquisition delivery time of 6 min 38 s, a 5.0 μL air gap, and a 20s equalization delay. The contact closure before starting spray and air gaps were used to obtain an adequate number of baseline MS scans before the peak, and the equalization delay was used to obtain an adequate number of baseline MS scans after the peak.

2.4. Sample preparation and analysis by triple quadrupole MS

Samples were extracted following the protocol as previously described [27]. Sample analysis was performed by FIA on a Waters Acquity UPLC system (Milford, MA) with ESI coupled to a Waters Xevo TQD MS/MS system.

2.5. Data analysis and interpretation

Data were acquired on the Q-Exactive Plus using Xcalibur 4.1 (Thermo Scientific) and the ChipMate 8.3.3 software (Advion), and processed using TraceFinder™ 4.1 Clinical (Thermo Scientific). Data were acquired on the TQD MS/MS using MassLynx (Waters) and these data were processed using Neolynx™ (Waters). Quantified data were output into excel format, and analysis and visualization were performed using R version 3.4.3 [28].

3. Results

3.1. Separation of nominal isobaric NBS metabolites by HRMS

Separation of nominal isobaric dicarboxyl and hydroxyl acylcarnitines was achieved using mixed pure standards and extracts from DBS (Fig. 1). Specifically, C3DC *m/z* 248.1131 (theoretical *m/z* 248.1134, mass error 1.2 ppm) and C4OH *m/z* 248.1485 (theoretical *m/z* 248.1498, mass error 5.2 ppm), and C5DC *m/z* 276.1443 (theoretical *m/z* 276.1447, mass error 1.4 ppm) and C6OH *m/z* 279.1793 (theoretical *m/z* 276.1811, mass error 6.5 ppm) were baseline resolved across all mass resolving powers.

3.2. Development of hybridized SIM and PRM HRMS acquisition

The nominal isobaric metabolites displayed in Fig. 1 have identical quantifying product ions and can only be distinguished non-derivatized by FIA MS acquisition in SIM mode. Initially it seemed feasible to conduct sample analysis using SIM at lower mass resolving powers, however, suspected dibutyl phthalate leaching from LC tubing was an isobaric interference with glutarylcarnitine-$^{2}H_{3}$ (C5DC-*d3*) IS at < 70,000 mass resolving power and impacted quantitation in preliminary results (Supplementary Fig. A.1 – A.5). Therefore, a mass resolution of 70,000 was selected for SIM analysis based on the samples, IS, source, and MS employed in this study. Due to the required high-throughput sample acquisition required in first-tier NBS (<2 min data acquisition), the roughly 3.4 Hz scan speed at mass resolving power 70,000 would not provide an adequate number of scans for the 49 *m/z* in the inclusion list. Thus, the hybrid method (Fig. 2) was developed for DI sample introduction to multiplex SIM acquisition for only nominal isobaric NBS metabolites with identical quantifying product ions and corresponding IS, and PRM acquisition for all other *m/z* in the inclusion list. The DI SIM/PRM hybrid method alternates the SIM MS acquisition

function at mass resolving power 70,000 and PRM at mass resolving power 17,000 (Fig. 2). This was possible by alternating included *m/z* to correspond with the alternating SIM and PRM acquisition functions, thus, the hybrid inclusion list was 418 rows. The hybrid method produced a FIA-like chromatogram containing both SIM and PRM acquisition functions, and individual ion traces were extracted and utilized in quantification software.

3.3. SIM vs. PRM vs. hybrid vs. TQ-MS/MS comparison of acylcarnitine and amino acid quantitation

Quantitative acylcarnitine and amino acid data acquired on the QE+ and TQ MS/MS are presented in Figs. 3 and 4. The MS methods labeled 17K, 35K, 70K, 140K, Hybrid, and PRM were acquired on the QE+, and consist of roughly 96 samples acquired across 3 n-ESI chips, except for acylcarnitines tetradecanoylcarnitine (C14:0), tetradecenoylcarnitine (C14:1), palmitoylcarnitine (C16), hydroxyhexadecanoylcarnitine(C16OH), stearoylcarnitine (C18), oleoylcarnitine(C18:1), and hydroxystearoylcarnitine (C18OH). Since the Triversa Nanomate air gap is inconsistent, the spray would often start before baseline scans were acquired of these analytes, in particular, at higher mass resolving powers. Therefore, these peaks were excluded from data presented in Fig. 3. A complete summary of metabolites, IS used in quantitation, quantitative transitions, and CE are outlined in Supplementary Table A.2. The method labeled TQ MS/MS was acquired on the Waters TQD and consists of 40 samples (Figs. 3 and 4). Thus, the variation on the TQ MS/MS platform may appear lower than the data acquired on the QE+. All data displayed are from non-derivatized sample preparation (Figs. 3 and 4), except for C3DC and C4OH data presented from the TQ MS/MS are derivatized, since C3DC and C4OH cannot be distinguished on TQ instruments without derivatization (Fig. 3C and E). Comparison of only MS/MS data acquired by PRM on the QE+ and MS/MS on the TQ platform are presented as bar graphs in Supplementary Fig. A.6.

C3DC and C4OH concentrations were similar comparing non-derivatized data acquired on the QE + to derivatized data acquired on the TQ MS/MS (Fig. 3C and E). A correction factor was applied to C3DC non-derivatized results due to ionization differences between non-derivatized C3DC and the surrogate C5DC-*d*3 IS (Supplementary Information). This difference may be related to differing charge states and poor ionization of non-derivatized dicarboxyl acylcarnitines [9]. C3DC and C5DC data appear variable across SIM resolutions which could be related to the differing charge states or the IS C5DC-*d*3 concomitantly filling in the C-trap with varying amounts of abundant plasticizers and contaminants (Supplementary Fig. A.2.–A.4.), thus, introducing variability. Furthermore, both C4OH and C5OH data acquired by SIM did not experience this issue and both utilize the IS hydroxyisovalerylcarnitine-$^{2}H_{3}$ (C5OH-*d*3).

Overall acylcarnitine concentrations did not vary across MS acquisition method and platform such as propionylcarnitine (C3), C3DC, butyrylcarnitine (C4), C4OH, isovalerylcarnitine (C5), C5DC, C5OH, hexanoylcarnitine (C6), octanoylcarnitine (C8), dodecanoylcarnitine (C12), C14:0, C14:1, C16, C16OH, C18:0, and C18:1 (Fig. 3 B–J and L–R). Interestingly, at mass resolving power 17,000 C16OH (Fig. 3P) data appeared variable, and there was an isobar, *m/z* of 416.3690 (acquired at 140,000 mass resolving

power), which could only be separated at mass resolving powers ≥ 35,000 (data not shown). The analytes C10 and C18OH concentrations appear different across platforms likely due to ionization differences between analyte and surrogate IS, since decanoylcarnitine (C10) uses octanoylcarnitine-$^{2}H_3$ (C8-*d3*) IS and C18OH uses stearoylcarnitine-$^{2}H_3$ (C18-*d3*) IS.

Free carnitine (C0) quantification was overall lower on the QE + compared to the TQ MS/MS platform, and while there were no quantitative differences across mass resolving powers in SIM, data acquired by PRM was on average 27% lower than data acquired by SIM (Fig. 3A). Follow-up experiments were conducted to determine the discrepancy between C0 quantitation on the QE+ (Supplementary Fig. A.7). In brief, equimolar C0 and internal standard free carnitine-$^{2}H_9$ (C0-*d9*) were mixed and data were acquired in full scan (FS) and PRM. On average the ratio of C0/C0-*d9* was 24% lower in PRM compared to data acquired by FS. Initially PRM CE were optimized based on the IS for all compounds. It was later determined that C0-*d9* optimal CE was several volts higher than that of C0, and this higher CE decreased C0 product ion production, which accounted for the lower quantitation observed in PRM. The PRM acquired data for C0 became more similar to the data acquired by SIM after utilizing the percentage abundance differences as a crude correction factor (data not shown). The quantitative differences between platforms may be related to the difference in CE between C0 and C0-*d9* in combination with the different dissociation methods between platforms, the CID used on the TQ MS/MS vs higher-energy collision dissociation (HCD) utilized on the QE+, which can impact fragmentation [29] including acylcarnitines [30].

Quantitative amino acid data acquired on the QE+ and TQ MS/MS platform are presented in Fig. 4. In general, quantification of most amino acids were similar across MS acquisition mode and platform including arginine, citrulline, methionine, and phenylalanine. Interestingly, at mass resolving power 17,000 citrulline (Fig. 4B) data appeared variable and there was an unresolved isobar observed *m/z* 176.0936 (acquired at 140,000 mass resolving power), of citrulline (data not shown). Tyrosine data acquired by PRM appeared lower than data acquired by SIM (Fig. 4H). One would imagine that CE differences between the unlabeled tyrosine and tyrosine-$^{13}C_6$ may be related, since the tyrosine-$^{13}C_6$ is labeled with six ^{13}C in the aromatic ring. However, the IS for phenylalanine is also labeled with six ^{13}C in the aromatic ring and had consistent quantification (Fig. 4F), and both phenylalanine and tyrosine product ions used in quantitation were neutral loss 46 Da which is two carbons away from their aromatic rings. Follow-up experiments were conducted to investigate this discrepancy in tyrosine (Supplementary Fig. A.8). In brief, equimolar tyrosine and tyrosine-$^{13}C_6$ were mixed and data were acquired in FS and PRM. On average, despite having similar CE, the ratio of tyrosine/tyrosine-$^{13}C_6$ was 26% lower in PRM compared to data acquired in FS. Valine data acquired by PRM was higher than data acquired by SIM (Fig. 4I), and this issue was also investigated by follow-up experiments (Supplementary Fig. A.9). In brief, valine-$^{2}H_8$ had slightly higher CE compared to unlabeled valine, which resulted in a 16% increase between valine/valine-$^{2}H_8$ when acquired by PRM compared to FS. The PRM acquired data for tyrosine and valine data became more similar to the data acquired by SIM after utilizing the percentage abundance differences as a crude correction factor (data not shown).

The data for creatine and leucine acquired by MS/MS were similar between the hybrid method and PRM, and across platform comparing the QE + to TQ MS/MS. However, there was enormous variation in data for the analytes creatine and leucine when acquired in SIM mode. It was determined that the signal intensity would drop to roughly 20% throughout the SIM analysis, the issue became exacerbated at higher resolutions, and overall the frequency of signal drops were associated with concentrations of leucine and creatine in affected samples. The mitigation of this issue and follow-up experiments are detailed in Supplementary Fig A.10–A.13. In brief, we optimized the AGC setting of $1e^6$ for low abundance poor-ionizing acylcarnitines (i.e., C3DC). The high AGC setting, in combination with the similar *m/z* of leucine and creatine, and the high concentration of these analytes, may have disrupted the online correction of the eFT, possibly resulting in C-Trap overfilling and saturation leading to a loss of ions. We also observed the *m/z* of unlabeled creatine and leucine in SIM could exceed 10 ppm mass error which was likely associated with ion coalescence [31] as lowering the AGC improved this issue. Since these issues did not affect the labeled IS for leucine and creatine, the frequency of signal drops across the unlabeled SIM trace would decrease peak area yielding lower concentrations and large variation in concentrations. We resolved this issue by selecting a lower AGC value in our SIM acquired data. Concentrations obtained after reanalysis and after excluding samples with signal drops were similar across methods and platform (data not shown).

3.4. Hybridization of targeted screening and metabolomic profiling with high resolution MS

Since hybridizing SIM and PRM MS acquisitions into a single DI method performed similarly to data acquired in each individual MS acquisition, respectively, the next logical step was to multiplex FS MS acquisitions into a hybrid SIM/PRM/FS proof-of-concept method (Fig. 5). The SIM/PRM/FS MS acquisition functions were staggered similar to the hybrid method presented in Fig. 2. The ability to combine crude metabolomic screening with targeted screening in a single analysis harnesses the advantageous capabilities of quadrupole-HRMS instrumentation (e.g., quadrupole time-of-flight, quadrupole ion traps, etc). In the NBS setting, hybrid MS methods could be used to quantify nominal isobaric metabolites with identical quantifying product ions in SIM mode (i.e., C3DC and C4OH), quantify nominal isobaric metabolites with nominal isobaric or differing product ions in a single transition using PRM (i.e., creatine 132.1 > 90.05, leucine isomers 132.1 > 86.09, 4HPRO 132.1 > 86.06), and perform crude metabolomic screening using FS to identify accurate *m/z* or data dependent acquisition to obtain both accurate *m/z* and product ion spectra (Fig. 5). It should be noted that multianalyte SIM/PRM/FS MS acquisition functions can be easily combined with DI or FIA, since unlike liquid chromatography there are no inherent limitations associated with the number of points required to define a chromatographic peak which limits the function duration. DI and FIA peak width is modifiable by users during method development, so MS acquisitions that last several seconds are acceptable. Previous analysis of newborn DBS extracts using a similar HRMS platform under DI conditions identified 278 metabolomic features [16]. While an DI or FIA metabolomics approach used in NBS would greatly underestimate the true number of metabolites in a sample (e.g., isomers, chimeric spectra, contaminants, etc), the complexity of metabolomic data may improve IEM classification and reduce false

positive rates. Alternatively, this workflow could be coupled to liquid chromatography for second-tier screening, to conduct a more comprehensive targeted and untargeted analysis. Chemometric and bioinformatic analysis of DI or FIA metabolomics data could identify candidate metabolomic features for liquid chromatography MS follow-up for structural characterization and identification. After confirmation of candidate metabolite identities, pathway analysis and enrichment could be performed to understand how these metabolites are related to the altered metabolism associated with IEM. NBS laboratories could then investigate the clinical utility of candidate markers by assessing their positive predictive value improvement and reduction in false positive rates, to decide if a metabolite should be utilized as a targeted quantitative marker for IEM screening.

4. Discussion

In our current study we investigate HRMS applications in targeted first-tier NBS assays for amino acids and acylcarnitines. Here we demonstrate utilizing DI-HRMS to overcome several limitations experienced by NBS laboratories when analyzing non-derivatized samples by FIA-TQ MS/MS. In particular, it was possible to distinguish and quantify nominal isobars with identical quantifying product ions, and nominal isobars with nominal isobaric quantifying product ions, through the amalgamation of SIM and PRM MS acquisitions into a single MS method. We report successful quantitation of C3DC and C4OH, and C5DC and C6OH from non-derivatized sample extracts by employing HRMS SIM. The same approach could easily differentiate isobars such as glutamine, a biomarker for ornithine transcarbamylase deficiency and carbamoylphosphatase synthetase deficiency, from lysine. We also observed improved ionization using nanospray sample introduction and lower capillary temperatures for metabolites including the dicarboxyl acylcarnitines C3DC and C5DC (data not shown). One advantage of quadrupole-HRMS instrumentation is the ability to select precursor ions and acquire the HR full spectra produced after dissociation. Utilizing PRM we report distinguishing product ions of leucine isomers (i.e., m/z 86.10) from 4HPRO (i.e., m/z 86.06), and quantifying leucine isomers, 4HPRO, and creatine in a single transition (i.e., m/z 132.1). Additional unique product ions produced during dissociation could be useful as confirmatory and quantifying ions to enhance specificity and sensitivity, but were not used in our study in order to provide a direct comparison to data acquired by a TQ MS/MS platform. Overall nanospray HRMS applications appear advantageous over established TQ-MS/MS approaches, since the targeted PRM acquisition provides exactly the same information newborn screening laboratories are accustomed to, while SIM and FS increase specificity of measurements and introduce metabolomics as an additional screening dimension that has potential to improve specificity and sensitivity of the assay.

There are limited reports detailing HRMS analysis of newborn DBS [16,32,33] that focus on first-tier NBS. Aside from improved sensitivity, higher throughput, and faster scan speeds featured on newer TQ instruments, there have been few modifications to NBS first-tier analysis platforms [34,35]. Typically to overcome limitations with first-tier screening by FIA-TQ MS/MS, NBS laboratories derivatize metabolites with butyl esters or combine metabolites in a single transition for first-tier screening, with LC MS/MS as a follow-up to determine the specific metabolite elevated. For instance, non-derivatized NBS first-tier

assays would quantify C3DC + C4OH (transition 248.1 > 85.0) using butyrylcarnitine-$^{2}H_{3}$ (C4-*d3*) as a surrogate IS. Analyzing these metabolites combined as C3DC + C4OH underestimates the true amount of these metabolites in the sample, due to ionization and CE differences between C3DC, C4OH, and C4-*d3*. For example, we report C3DC and C4OH had quantified concentrations of roughly 1 μM each when acquired by SIM (Fig. 3C and D), while C3DC + C4OH had a concentration of 0.6 μM when acquired by PRM (Supplementary Fig. A.6). The aforementioned issues associated with sample derivatization, IS surrogate selection, varying sample preparation methods, and combined quantification of metabolites contributes to difficulties comparing data across NBS laboratories.

Our group has previously reported that IS surrogate selection can impact quantitative values reported by NBS laboratories [36,37]. In our current study we observed differences comparing quantitative data acquired by SIM and PRM for several analytes. Based on our preliminary investigation (Supplementary Fig. A.7–A.9), these discrepancies appear related to differences in CE or fragmentation efficiency between unlabeled metabolites and their isotopologue IS. These quantitative differences related to isotopologues appear to be compound specific such as the case with phenylalanine and tyrosine, which both utilize six ^{13}C labels in the aromatic ring of their respective IS, and C0 and valine, where both IS are heavily deuterated, but C0 appears lower and valine appears higher in data acquired by PRM compared to SIM. This phenomenon has been previous documented [38] to impact both ^{2}H [39] and ^{13}C [40] labeled molecules, and is likely related to collision energy differences or fragmentation kinetics related to the kinetic isotope effect (KIE) [41]. To our knowledge, KIE has not been investigated with isotopologues commonly employed in NBS, and additional studies should be conducted to better understand how the KIE may impact result comparison across NBS laboratories and determine if correction factors are needed. It should be noted that if the NBS community adopted external calibrator usage for quantitation, instead of relying on single point calibration, that data comparability would greatly improve and mitigate quantitative discrepancies related to different internal standards, instruments, and methods.

In addition to the advantages of using HRMS in targeted NBS, NBS laboratories could also perform metabolomic screening to assist in IEM identification. Previous studies have demonstrated using targeted and untargeted metabolomics data to classify IEM in clinical samples ranging from plasma to DBS [16,20–22]. The disrupted metabolism associated with IEM often results in deficiencies in downstream metabolites and a concomitant toxic accumulation of upstream substrates [42]. Accumulation in substrates often shunts metabolism through alternate biochemical pathways to cope with metabolic stress. For example, defects in phenylalanine hydroxylase associated with classical phenylketonuria (PKU) impact catabolism of phenylalanine, most often resulting in toxic phenylalanine levels which can elevate levels of phenylacetic acid, phenylacetylglutamine, and phenyllactic acid [43,44]. While PKU has been well characterized and studied for over 50 years, NBS diseases with lower incidence rates and those not currently added to the Recommended Uniform Screening Panel (RUSP) may have unique undescribed metabolites that could offer additional clinical utility as diagnostic markers for IEM. In fact, many NBS laboratories are moving away from using single analyte cutoffs, instead utilizing multiple analytes and analyte ratios [45–51], with either in-house developed algorithms [52] or tools such

as the Collaborative Laboratory Integrated Reports (CLIR) [53]. The future utilization of metabolomics appears consistent with current trends in NBS.

The purpose of our study was to investigate HRMS applications in first-tier NBS of amino acids and acylcarnitines, and we acknowledge several limitations. We recognize the generalizability of comparing a single HRMS platform to a single TQ MS/MS platform, and it is possible results could vary across vendors when comparing TQ MS/MS platforms to HRMS platforms. Due to the low abundance of many NBS markers in non-diseased samples, enriched DBS QC materials were used in our analysis. It is possible additional isobaric serum metabolites were not detected, since the enriched DBS QC materials are prepared by mixing red blood cells with charcoal stripped serum [24]. While we identified unresolved isobars of NBS markers at lower mass resolving powers, it is possible there are additional unresolved isobars that could require a mass resolving power >140,000. In addition, a mass resolving power of 70,000 was selected and utilized for SIM acquisition in our hybrid method due to an isobaric contaminant leaching from plastics used in sample prep and analysis. Resolving power requirements for HRMS SIM acquired metabolites may vary based on specific components used in sample preparation workflows, by specific vendor or component lot, and the isotopologue IS employed by a laboratory. While HRMS SIM offers advantages to overcome limitations associated with nominal isobars with identical quantifying product ions under DI and FIA conditions, it is ideal to quantify by MS/MS when possible due to orthogonal confirmation and the large number metabolomic features in the *m/z* range of many NBS markers. Lastly, the proof-of-concept metabolomics workflow outlined in our study would greatly underestimate the true number of metabolites due to DI sample introduction, however, a similar DI-HRMS platform demonstrated metabolomic capabilities from newborn DBS extracts in regards to IEM classification [16].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

4HPRO	4-Hydroxyproline
C0	Free carnitine
C0-*d*9	Free carnitine-$^{2}H_{9}$
C10	Decanoylcarnitine

C12	Dodecanoylcarnitine
C14:0	Tetradecanoylcarnitine
C14:1	Tetradecenoylcarnitine
C16	Palmitoylcarnitine
C16OH	Hydroxyhexadecanoylcarnitine
C18:0	Stearoylcarnitine
C18-*d*3	Stearoylcarnitine-$^{2}H_{3}$
C18:1	Oleoylcarnitine
C18OH	Hydroxystearoylcarnitine
C2	Acetylcarnitine
C3	Propionylcarnitine
C3DC	Malonylcarnitine
C4	Butyrylcarnitine
C4-*d*3	Butyrylcarnitine-$^{2}H_{3}$
C4OH	3-Hydroxybutylcarnitine
C5	Isovalerylcarnitine
C5DC	Glutarylcarnitine
C5DC-*d*3	Glutarylcarnitine-$^{2}H_{3}$
C5OH	Hydroxyisovalerylcarnitine
C5OH-*d*3	Hydroxyisovalerylcarnitine-$^{2}H_{3}$
C6OH	Hydroxyhexanoylcarnitine
C6	Hexanoylcarnitine
C8	Octanoylcarnitine
C8-*d*3	Octanoylcarnitine-$^{2}H_{3}$
C8OH	Hydroxyoctanolycarnitine
CDC	The Centers for Disease Control and Prevention
CE	Collision energy
CLIR	Collaborative laboratory integrated reports
DBS	Dried blood spot FS, Full scan

HCD	Higher-energy collision dissociation
HRMS	High resolution MS
IS	Internal standard
KIE	Kinetic isotope effect
NBS	Newborn screening
NSQAP	Newborn Screening Quality Assurance Program
PKU	Phenylketonuria
PRM	Parallel reaction monitoring
RUSP	Recommended Uniform Screening Panel
TQ	Triple quadrupole MS

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HIGHLIGHTS

- Data acquired by HRMS and triple quadrupole were similar.
- Direct injection HRMS platform overcame newborn screening limitations.
- HRMS allowed combining untargeted metabolomic screening with targeted screening.
- Identified fragmentation differences in unlabeled metabolites and labeled standards.

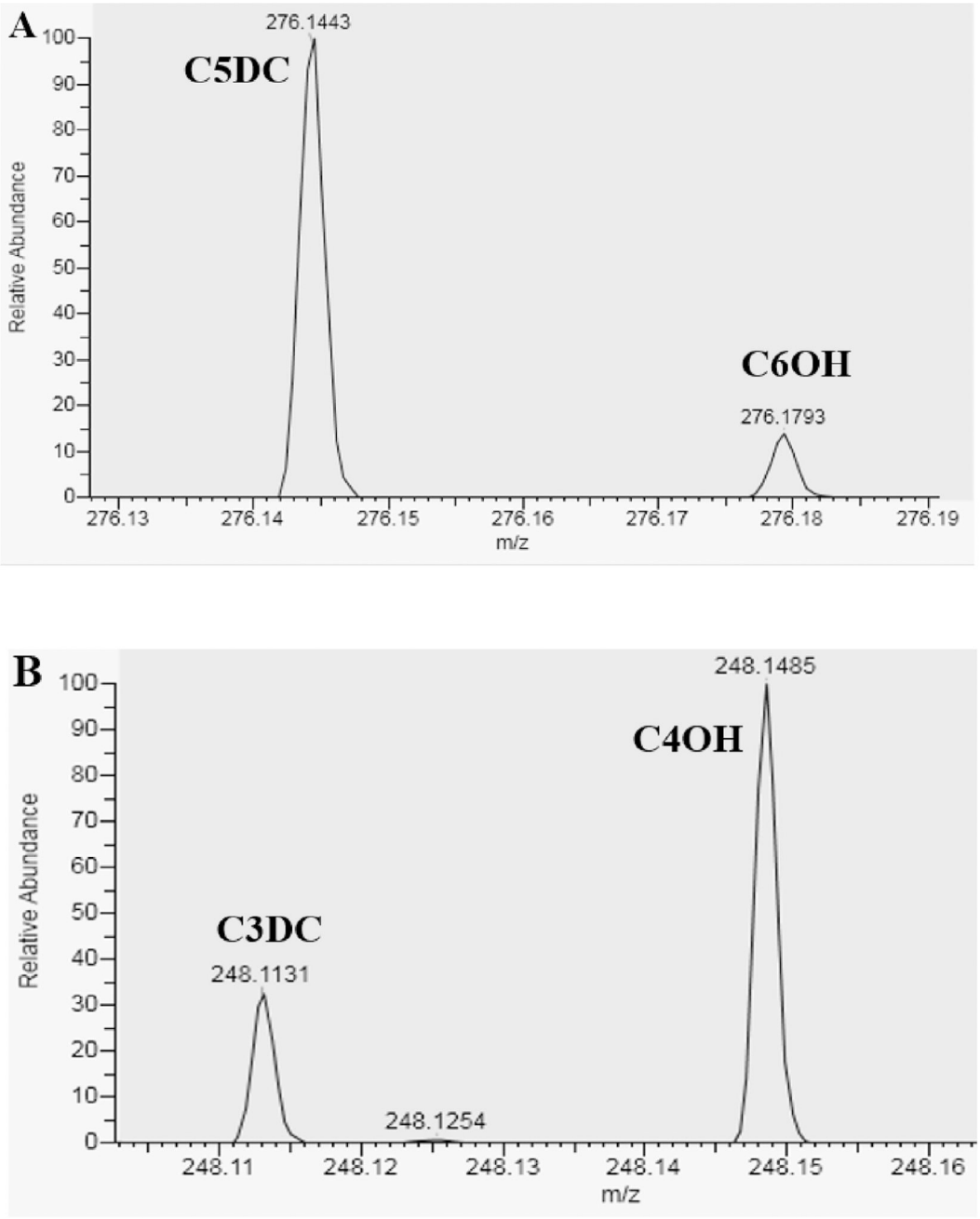


Fig. 1. Separation of nominal isobaric metabolites in newborn screening using high resolution MS.

High resolution separation of nominal isobaric newborn screening metabolites with identical quantifying product ions was performed in SIM at 140,000 mass resolving power. A) gultarylcarnitine (C5DC, theoretical m/z 276.1447, mass error 1.4 ppm) and hydroxyhexanoylcarnitine (C6OH, theoretical m/z 276.1811, mass error 6.5 ppm) and B) malonylcarnitine (C3DC theoretical m/z 248.1134, mass error 1.2 ppm) and hydroxybutyrylcarnitine (C4OH, theoretical m/z 248.1498, mass error 5.2 ppm).

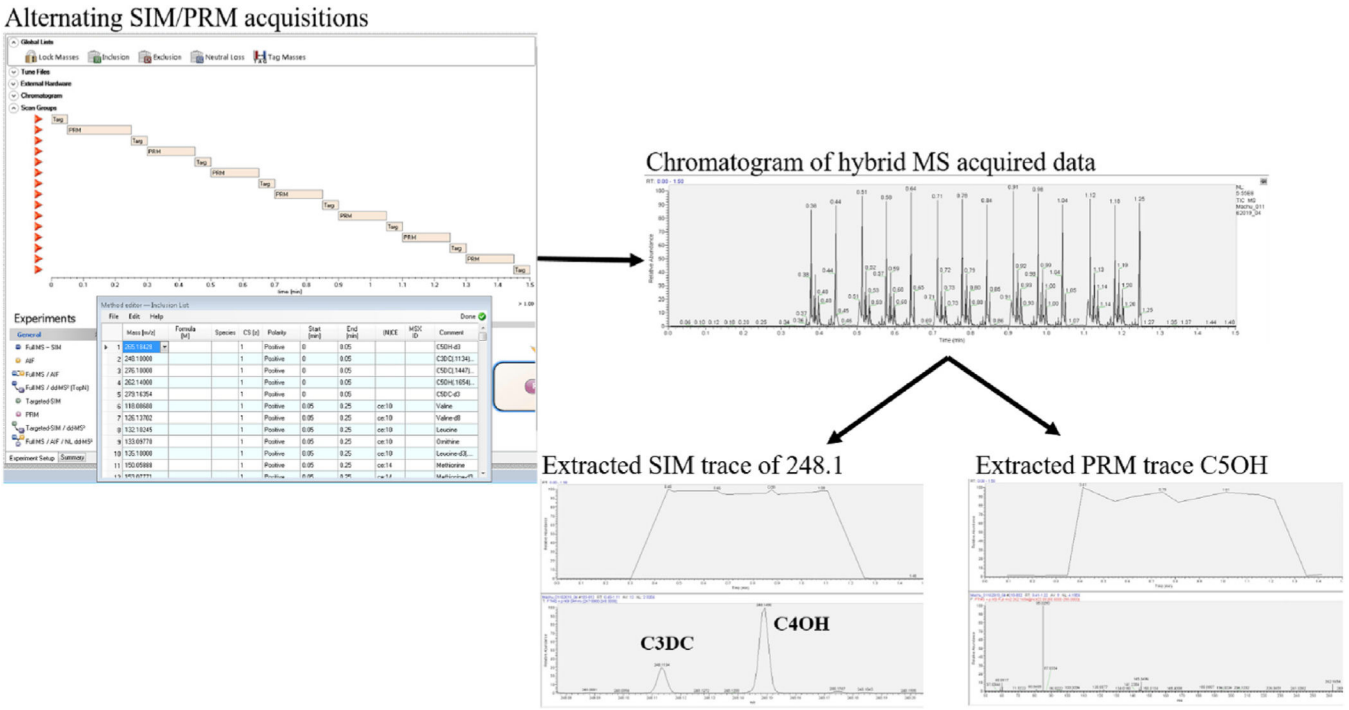
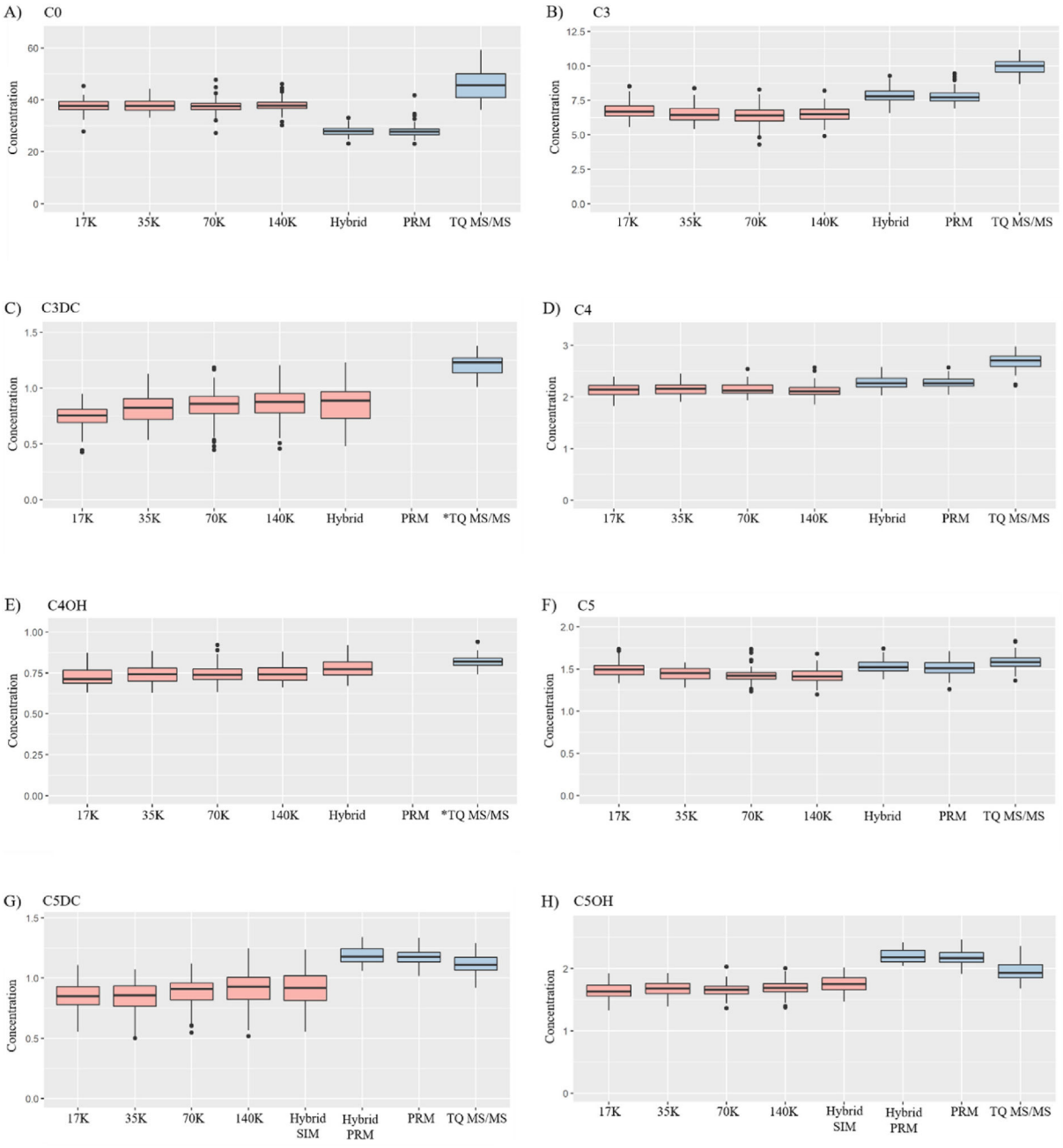


Fig. 2. Quantification of nominal isobaric metabolites through hybridized MS acquisition method.

Routine first-tier newborn screening by FIA MS requires high-throughput methods typically < 2 minute sample acquisition. Therefore, an FIA hybrid SIM and parallel reaction monitoring (PRM) MS method was developed to balance scan speed and sample acquisition time. The method utilizes SIM and a higher mass resolving power for nominal isobars and corresponding internal standards (high selectivity due to accurate mass) and utilizes PRM with a mass resolving power of 17,000 for all other metabolites and internal standards (high selectivity due to orthogonal confirmation with precursor and product ions). The SIM and PRM acquisition functions were alternated over the course of the 1.5 minute method, along with the inclusion list corresponding to the specific time intervals where the MS acquisition methods alternate. The hybrid method yields a chromatogram containing both SIM and PRM MS data. Using TraceFinder 4.1 Clinical these SIM and PRM traces were extracted to individual metabolites and quantified with the corresponding internal standard acquired by either SIM or PRM, respectively. For instance, the m/z 248.1000 in the inclusion list acquired data across SIM functions for malonylcarnitine (C3DC) and 3-hydroxybutarylcarnitine (C4OH), and the m/z 279.1635 for glutarylcarnitine-d3 (C5DC-d3) and m/z 265.1843 3-hydroxyisovalerylcarnitine-d3 (C5OH-d3) were acquired across SIM functions for use as internal standards for C3DC and C4OH, respectively. The hybrid method acquired data for C5DC, C5OH, and corresponding internal standards in both SIM and PRM.



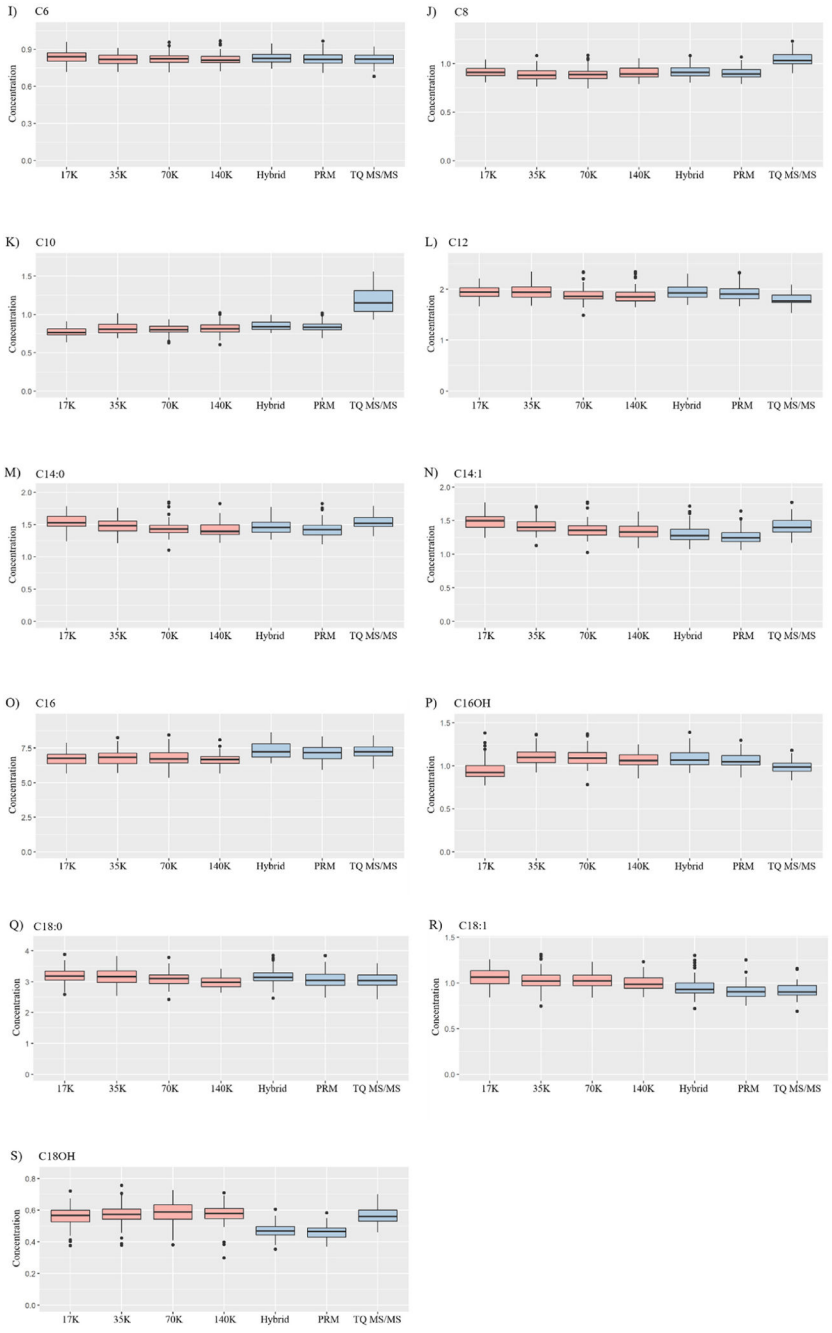


Fig. 3. Comparison of acylcarnitine quantification across MS acquisition method and platform.

Comparison of several acylcarnitines targeted by newborn screening labs. Samples were analyzed by DI-HRMS across MS acquisition methods or FIA-triple quadrupole (TQ)-MS/MS. Each boxplot is colored where red denotes data acquired by SIM, and blue denotes data acquired by fragmentation using parallel reaction monitoring (PRM) or on a TQ MS/MS. SIM and PRM data were acquired on a Q-Exactive Plus. In each plot, the y-axis is the concentration of the acylcarnitine in micromolar (μM) units and the x-axis is the MS acquisition method. The SIM acquisition methods labeled 17K, 35K, 70K, and 140K denote the mass resolving power in thousands (e.g., 17K corresponds mass

resolving power 17,000 in SIM mode). The PRM acquired data used a mass resolving power of 17,000. The hybrid method alternated SIM (70,000), to quantitate nominal isobaric acylcarnitines and corresponding internal standards, and PRM (17,000) for all other acylcarnitines. For C5DC and C5OH, both metabolites were analyzed in the hybrid method by both SIM and PRM, and the acquisition is denoted by Hybrid SIM and Hybrid PRM, respectively. All samples were prepared non-derivatized and compared between platforms and across MS acquisition methods, unless otherwise denoted. Each boxplot acquired on the Q-Exactive Plus represents roughly 96 samples. Data presented from TQ MS/MS platform is 40 samples for non-derivatized and derivatized data, respectively. The * symbol indicates data presented for TQ MS/MS method is from derivatized samples, since C3DC and C4OH are nominal isobars and cannot be distinguished by FIA TQ MS/MS without derivatization. C0, Free Carnitine; C3, Propionylcarnitine; C3DC, Malonylcarnitine; C4, Butyrylcarnitine; C4OH, 3-Hydroxybutyrylcarnitine; C5, Isovalerylcarnitine; C6, Hexanoylcarnitine; C5DC, Glutarylcarnitine; C8, Octanoylcarnitine; C10, Decanoylcarnitine; C12, Dodecanoylcarnitine; C14:0, Tetradecanoylcarnitine; C14:1, Tetradecenoylcarnitine; C16, Palmitoylcarnitine; C16OH, 3-Hydroxyhexadecanoylcarnitine; C18:0, Stearoylcarnitine; C18:1, Oleoylcarnitine; C18OH, 3-Hydroxystearoylcarnitine.

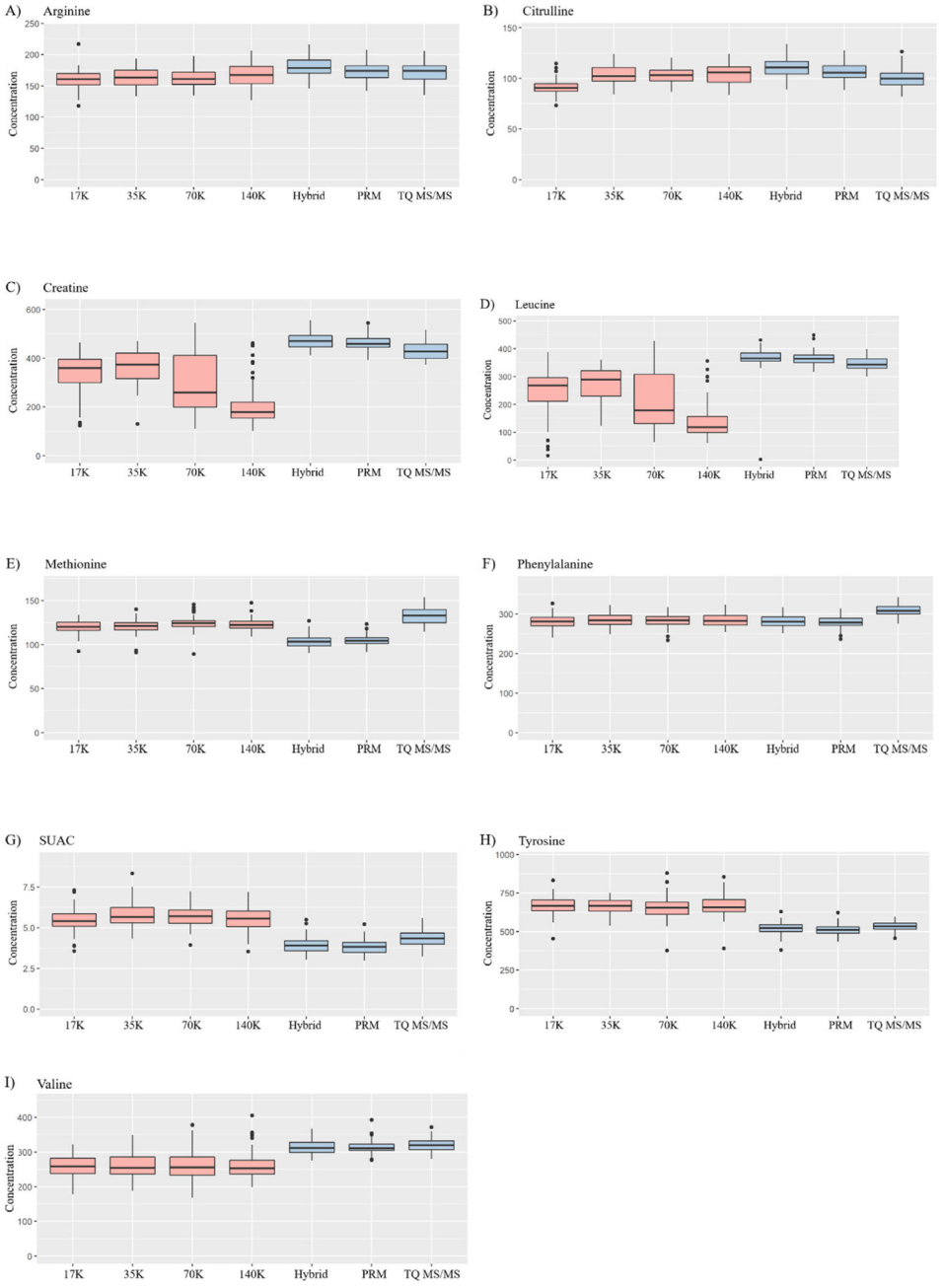


Fig. 4. Comparison of amino acid quantification across MS acquisition method and platform.

Comparison of several amino acids targeted by newborn screening labs. Samples were analyzed by DI-HRMS across MS acquisition methods or FIA-triple quadrupole (TQ)-MS/MS. Each boxplot is colored where red denotes whether the data were acquired by SIM in red, and blue denotes data acquired by fragmentation using parallel reaction monitoring (PRM) or on a TQ MS/MS. SIM and PRM data were acquired on a Q-Exactive Plus. In each plot, the y-axis is the concentration of the metabolite in micromolar (μM) units and the x-axis is the MS acquisition method. The SIM acquisition methods labeled 17K, 35K, 70K, and 140K denote the mass resolving power in thousands (e.g., 17K corresponds mass

resolving power 17,000 in SIM mode). The PRM acquired data used a mass resolving power of 17,000. The hybrid method alternated SIM (70,000), to quantitate nominal isobaric acylcarnitines and corresponding internal standards, and PRM (17,000) for all other amino acids. All samples were prepared non-derivatized and compared between platforms and across MS acquisition methods. Each boxplot acquired on the Q-Exactive Plus represents roughly 96 samples. Data presented from TQ MS/MS platform is 40 non-derivatized samples. Thus, the variation on the TQ MS/MS platform often appears lower than the data acquired on the QE+. Data accounting for the variation in leucine and creatine data are outlined in detail in Supplementary Information.

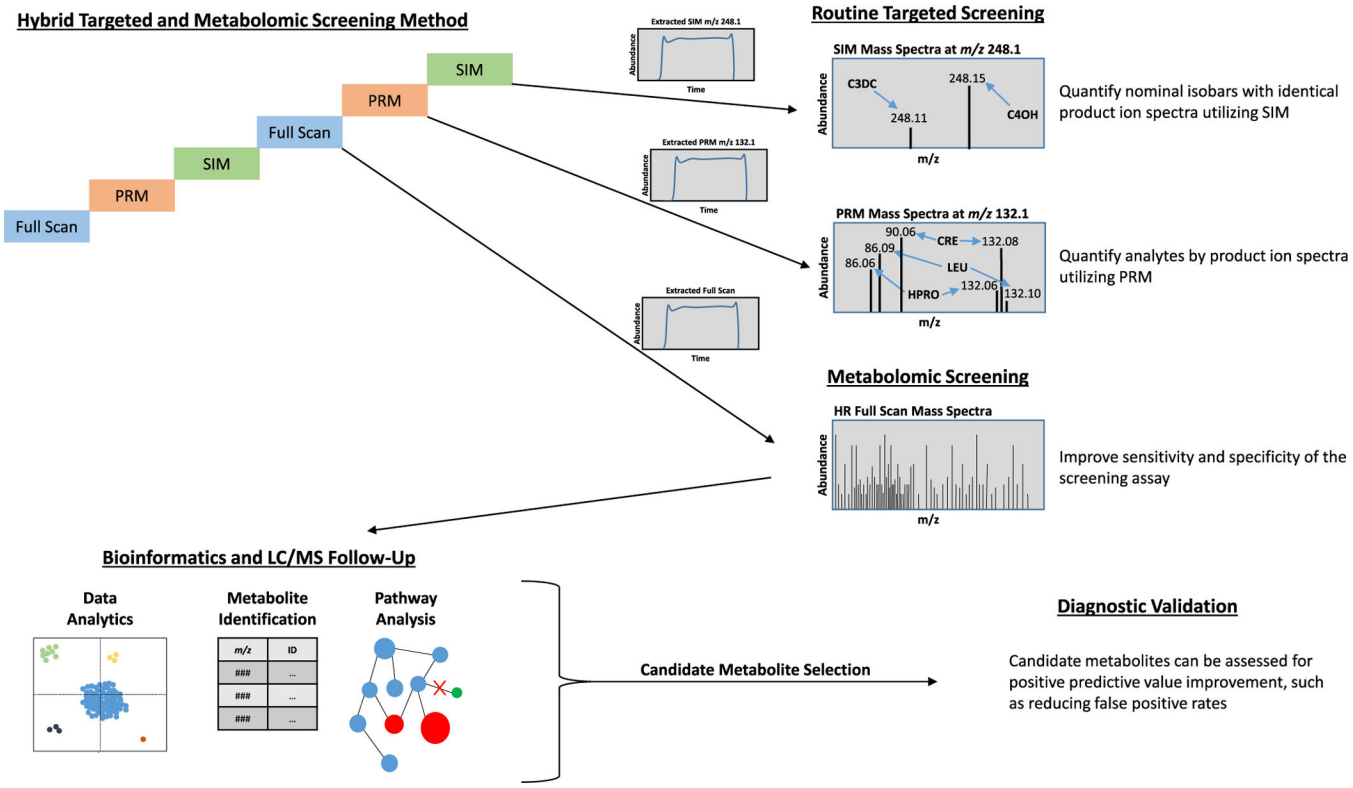


Fig. 5. Hybridization of targeted and non-targeted screening by high resolution MS.

A proof-of-concept workflow to hybridize targeted screening with metabolomic profiling in newborn screening. Since the hybrid SIM/PRM MS acquisition method produced similar results and overcame several limitations in first-tier newborn screening experienced by FIA on triple quadrupole MS/MS platforms, full scan (FS) acquisition was then multiplexed into the HRMS method. This SIM/PRM/FS hybrid method would provide the ability to conduct targeted screening with crude metabolomic screening in a single analysis. C3DC, Malonylcarnitine; C4OH, 3-Hydroxybutyrylcarnitine; LEU, Leucine; CRE, Creatine; HPRO, Hydroxyproline.

Table 1

Overview of MS acquisition methods and parameters.

MS Method Name	MS Acquisition	Mass Resolving Power	Analysis Time	AGC	Maximum IT	Isolation Window
17K	SIM	17,000	8 min	$1e^6$	500 ms	0.4 m/z
35K	SIM	35,000	8 min	$1e^6$	500 ms	0.4 m/z
70K	SIM	70,000	8 min	$1e^6$	500 ms	0.4 m/z
140K	SIM	140,000	8 min	$1e^6$	500 ms	0.4 m/z
PRM	PRM	17,000	1.5 min	$1e^6$	20 ms	0.4 m/z
Hybrid	SIM/PRM	70,000/17,000	1.5 min	$1e^6$	200 ms/20 ms	0.4 m/z

Six MS acquisition methods were compared on the Q-Exactive Plus. Four SIM acquisition methods were utilized, where mass resolving power differed (i.e., 17,000, 35,000, 70,000, and 140,000), but AGC, maximum IT, isolation window, and a sample analysis time of 8 min were constant across the differing mass resolving powers. The long 8 minute method was required to achieve the at least 10 scans for each *m/z* at 140,000 mass resolving power. One method was a full PRM acquisition method, where data was acquired utilizing 17,000 mass resolving power, AGC 1e6, maximum IT 20ms, isolation window 0.4 m/z. The final method was a SIM/PRM hybrid, where SIM data was acquired utilizing 70,000 mass resolving power, AGC 1e6, maximum IT 200ms, isolation window 0.4 m/z, and PRM data was acquired utilizing 17,000 mass resolving power, AGC 1e6, maximum IT 20ms, isolation window 0.4 m/z.