A clinically validated method to separate and quantify underivatized acylcarnitines and carnitine metabolic intermediates using mixed-mode chromatography with tandem mass spectrometry

Carolina Luna[a], Chandler Griffin[a], and Marcus J. Miller[a*]

[a]Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN 46202

*Correspondence should be addressed to:

Marcus J. Miller PhD, FACMG

Department of Medical and Molecular Genetics

Indiana University School of Medicine

975 W. Walnut St., IB-344C

Indianapolis, IN 46202

(317) 278-1140

majamill@IU.edu

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ABSTRACT:

Acylcarnitines are intermediate metabolites of the mitochondria that serve as biomarkers for inherited disorders of fatty acid oxidation and amino acid metabolism. The prevailing clinical method used to quantify acylcarnitines involves flow-injection tandem mass spectrometry, an approach with a number of limitations; foremost the inability to separate and therefore distinguish key isobaric acylcarnitine species. To address these issues, we report a clinically validated liquid chromatography tandem mass spectrometry method to quantify acylcarnitines, free carnitine, and carnitine metabolic intermediates in human plasma. Importantly, this method resolves clinically relevant isobaric and isomeric acylcarnitine species in a single 22 minute analysis without the use of ion pairing or derivatization reagents. This unique combination of features is not achievable by existing acylcarnitine methods and is made possible by the use of a novel mixed-mode chromatographic separation. Further clinical validation studies demonstrate excellent limits of quantification, linearity, accuracy, and inter-assay precision for analyses of 38 different calibrated analytes. An additional 28 analytes are semi-quantitatively analyzed using surrogate calibrators. The study of residual patient specimens confirms the clinical utility of this method and suggests expanded applicability to the diagnosis of peroxisomal disorders. In summary, we report a clinically validated acylcarnitine method that utilizes a novel mixed-mode chromatographic separation to provide a number of advantages in terms of specificity, accuracy, sample preparation time, and clinical utility.

Key Words:

Isomeric separation of acylcarnitines, mixed-mode chromatography, peroxisomal dicarboxylic acylcarnitine biomarker, acylcarnitine profile analysis; carnitine metabolism

1. Introduction

Plasma acylcarnitine profile analysis is one of the most commonly ordered tests in a clinical biochemical genetics laboratory and is considered part of a comprehensive metabolic evaluation. The detection of acylcarnitine abnormalities can provide important diagnostic clues for numerous inherited disorders impacting fatty acid oxidation and amino acid metabolism [1]. In the clinical setting, acylcarnitine testing is typically completed using a method called flow injection tandem mass spectrometry (FI-MS/MS). In this approach, a prepared specimen is slowly infused into a tandem mass spectrometer without chromatographic separation and 30-60 endogenous acylcarnitines are detected by either multiple reaction monitoring or precursor ion scanning using the carnitine product ion, m/z = 85 [2]. FI-MS/MS methods are also employed by newborn screening laboratories to monitor a subset of clinically relevant acylcarnitine biomarkers in blood [3].

Despite its widespread use, FI-MS/MS has a number of well-documented limitations [4]. Most importantly, FI-MS/MS relies almost exclusively on a tandem mass spectrometer with collision-induced disassociation to provide analytical specificity. Therefore, it is incapable of separating and unambiguously identifying isobaric compounds that are prevalent amongst the large family of endogenous acylcarnitines. Examples include methylmalonyl-carnitine (C4:DC) and 3-hydroxy-isovalerylcarnitine (C5:OH), which are both detected by the MS/MS transition 262 > 85 in underivatized methods, or structural isomers such as valerylcarnitine, (C5), isovalerylcarnitine (C5), 2-methylbutyrylcarnitine (C5), and pivaloylcarnitine (C5), which are all detected by the MS/MS transition 246 > 85 in underivatized methods. Additional limitations of FI-MS/MS analysis include inaccuracy due to an inability to effectively calibrate most analytes studied and the requirement for derivatization under heated conditions leading to hydrolysis of acylcarnitine esters thus falsely inflating the pool of free carnitine and reducing the levels of acylcarnitines [5]. A solution addressing many problems

inherent to FI-MS/MS involves the use of liquid chromatography tandem mass spectrometry (LC-MS/MS).

Numerous LC-MS/MS acylcarnitine methods have been reported and most rely on liquid chromatography separations using either reversed-phase [6-10] or hydrophilic interaction liquid chromatography (HILIC) chemistries [11-13]. While these methods provide clear advantages over FI-MS/MS analysis, they too have a number of challenges [14]. Reversed-phase columns offer poor retention of polar compounds such as free carnitine or malonylcarnitine (C3:DC). To overcome this limitation, specimen derivatization and/or ion pairing mobile phase additives are often used [6-10]. Derivatization can be time consuming and a source of error within the workflow. Ion pairing agents are highly retentive chemicals that can contaminate LC-MS/MS equipment and cause interference in ESI negative applications. In HILIC methods, polar compounds are well retained and samples can be quickly prepared without derivatization in solutions with high organic solvent content thereby increasing MS signal for many compounds. The main disadvantage of this approach is the inability to separate isomeric acylcarnitine species. Collectively, these aforementioned limitations may explain why LC-MS/MS methods have not been widely adopted for acylcarnitine quantification in a clinical setting.

Mixed-mode chromatography is an emerging technique that shows great promise in the LC-MS/MS analysis of small isomeric analytes [15]. A mixed-mode chromatographic column combines two or more retention mechanisms (e.g., reversed-phase and cation-exchange) to separate compounds. The inclusion of mass spectrometry compatible ion-exchange properties improves isomeric selectivity enabling the successful application of mixed-mode chromatographic separations in the analysis of complex metabolite mixtures [16, 17]. Importantly, mixed-mode chromatography can be completed using sample preparations and mass spectrometry compatible solvents similar to those used in HILIC methods (i.e., without ion pairing agents) thereby improving sensitivity and assay robustness.

In the following, we report the development of a LC-MS/MS method that applies a unique mixed-mode chromatographic approach to analyze acylcarnitines, key isomeric species, and carnitine metabolic precursors. We further chronicle the performance metrics of this method in clinical validation studies and demonstrate the clinical utility of this method through the analysis of numerous residual specimens from patients with inherited metabolic diseases.

2. Materials and methods

2.1. Reagents

Optima LC-MS grade water, formic acid, methanol, and acetonitrile (Fisher Scientific) and bio ultra-grade 10 M ammonium formate (Sigma Millipore) were used to prepare solutions. Fatty acid free bovine serum albumin (BSA) 30% in saline (Sigma Millipore) was chosen as the surrogate matrix following verification studies demonstrating low acylcarnitine contamination in this material (data not shown). High purity powder stocks were purchased for 38 analytes and 32 isotopic internal standards and each were individually resuspended in either water or methanol to a final concentration of 1 mg/ml (Table S1). The isotopic internal standard mixture (isotope mix) was prepared by combining all isotope stocks and diluting with 100% methanol to a final concentration of 0.5 µM for all analytes with the exceptions of deoxycarnitine-d9, trimethylamine N-oxide-d9, and trimethyllysine-d9, which were made to 5 µM, and carnitine-d9, which was made to 20 µM. Isotope mix was stored at -20 °C prior to use and remade every six months from lyophilized stock mixtures. Injection solution was prepared by mixing 1.5 mL of formic acid in 500 mL of acetonitrile.

2.2. Calibrator and control preparation

Individual acylcarnitine stock concentrations and purity were verified using the approaches described below and combined to form calibrator mix “Cal_X”, which was dried to completion under nitrogen and stored at -20 °C. The remaining non-acylcarnitine calibrator materials were combined to form aqueous

calibrator “Cal_Y”, which was stored at -20 °C. Immediately prior to use, Cal_X was resuspended and combined with Cal_Y to form the final calibrator working solution. The calibrator working solution was diluted in 90% methanol to make a six-point standard curve (Table S2). Standard curve specimens were prepared by mixing 40 µL of diluted calibrator working solution with 40 µL of isotope mix, 20 µL of BSA, and 580 µL of injection solution. A calibration curve was prepared and analyzed at the beginning of each run. Control samples were prepared using pure analyte stock material to make a combined solution in methanol in which analyte concentrations approached the upper limit of our calibration range. Sub-aliquots were taken from this mixture for use as high control specimens. This mixture was also diluted 1 to 25 in methanol and used to prepare low control sub-aliquots. All control materials were dried to completion under nitrogen and stored at -20 °C. Prior to use high and low control samples were resuspended in 20 µL of water and 20 µL of BSA and then processed identically to patient specimens. Low and high control specimens were analyzed at the beginning and end of each run.

2.3. Sample preparation

Samples were prepared by combining 40 µL of plasma + 40 µL of isotope mix + 600 µL of injection solution, vortex mixing, and centrifuging at top speed (21,130 x g) for 2 minutes on a bench top microcentrifuge. Clarified lysate was transferred to a glass auto-sampler vial for LC-MS/MS analysis. Patient specimens were obtained from residual stocks of heparinized plasma in our clinical testing laboratory. Sample collection procedures were approved by the Indiana University Institutional Review Board (Protocol #1804038720).

2.4. LC-MS/MS analysis

Liquid chromatography was performed using an Aquity i-class UPLC system (Waters) equipped with a Scherzo SS-C18 (100 mm X 3 mm, particle size = 3 µm; Imtakt) maintained at 35 °C. Mobile phase A consisted of 10% acetonitrile, 0.3% formic acid, 15 mM ammonium formate and mobile phase B consisted of 90% acetonitrile, 0.3% formic acid, 20 mM ammonium formate. Five microliters of

specimen were injected into the LC-MS/MS system and chromatographic separation was achieved at a flow rate 0.5 mL/min using a gradient of mobile phase A and B (see Table S3 and Fig. S1 for inlet method parameters). Mass spectrometry analysis was completed by scheduled reaction monitoring (SRM) on a Xevo TQS micro (Waters) in ESI positive mode using the following tune parameters: capillary voltage= 0.4 kV, desolvation temperature 650 °C, cone gas = 20 L/hr, desolvation gas = 750 L/hr (see Table S4-S6 for MS method details). For calibrated analytes, tune parameters were optimized by infusing solutions of pure compounds. TargetLynx software (Waters) was used for peak integration and analyte concentration calculations. Calibration response values were fit to a linear model (1/X) that included the origin.

2.5. Free and total carnitine quantification

Acylcarnitine stock concentrations were verified using free and total carnitine analysis as previously proposed be Minkler et al. [7]. A 90 µL aliquot of a dilution of each calibrator stock solution was mixed with 30 µL 75 µM L-carnitine-d9 (Cambridge Isotope) to make the "starting mixture". For total carnitine analysis, 60 µL of starting mixture was combined with 15 µL of 1 M potassium hydroxide and heated at 65 °C for 15 minutes to hydrolyze acyl-carnitine ester bonds. Samples were next mixed with 15 µL of 1 M hydrochloric acid, centrifuged to remove insoluble precipitate, and supernatant was diluted in methanol prior to LC-MS/MS analysis. For free carnitine analysis an aliquot of starting mixture was deproteinated using methanol, centrifuged to remove insoluble precipitate, and analyzed by LC-MS/MS. Carnitine was quantified using our laboratory's clinically validated LC-MS/MS free and total carnitine analysis that is benchmarked semiannually to CAP proficiency testing specimens. Each stock solution was analyzed in triplicate and acylcarnitine stock concentrations were calculated by subtracting the average free carnitine concentration from the average total carnitine concentration.

2.6. Method validation

The limit of quantification was determined by studying a dilution series of calibrator material and identifying the lowest point that met the following criteria, (i) fit to the linear model (residual < 20%), (ii) signal to noise > 10 and (iii) raw peak area >= 1,000. Carry-over was measured by monitoring the signal in a blank specimen analyzed immediately following the analysis of the highest standard curve point. Intra-assay imprecision was determined by analyzing low (std2) and high (std4) concentration calibration materials five times each within the same batch. Inter-assay imprecision was determined by analyzing aliquots of low and high control samples in ten independent batches run on ten different days. Imprecision is reported in terms of the coefficient of variation (CV). The linear range of detection was determined by analysis of calibration materials and finding the range in which all points had a residual < 20%, and the correlation coefficient (r^2) was ≥ 0.98.

Accuracy was studied by spiking five different heparinized plasma samples each with four different concentrations of pure analyte stocks to achieve a concentration increase that spanned the calibration range. Unspiked versions of each specimen were also analyzed. Accuracy was determined by plotting all observed concentrations against the expected concentration and calculating the slope of the linear model and Pearson's correlation (r). In addition, bias was calculated by determining the overall average percent difference between observed and expected values.

3. Results

3.1. Mixed-mode chromatographic method development

The primary goal of this study was to develop a simple LC-MS/MS method to separate isobaric acylcarnitine species without using ion pairing agents or specimen derivitization. To quickly screen for a potential chromatographic solution, we prepared test samples containing mixtures of isobaric acylcarnitines (C3:DC/C4:OH, C4s, C5s, C5:1s, C4:DCs/C5:OH, and C5:DCs). We analyzed these samples using three different mixed-mode columns [Intrada amino acid column (Imtakt), Newcrom AH (SEILC),

and Scherzo ss-c18 (Imtakt)] and for each column we tested multiple column dimensions and mobile phase conditions including varying concentrations of ammonium formate, formic acid, methanol, and acetonitrile. Method development proceeded in a stepwise manner using simple isocratic inlet methods to test one variable at a time; see for example preliminary studies on the impact of ammonium formate concentrations on analyte retention (Fig. S2). All columns tested provided some level of isobaric separation but the Scherzo ss-c18 column provided the most comprehensive overall analyte resolution and was therefore used for the remainder of this study (Fig. 1A-F). For comparison see Fig. S3 and S4 for representative results from the study of the Intrada amino acid (Imtakt) and Newcrom AH (SEILC) columns.

We next expanded our LC-MS/MS method to cover a full acylcarnitine profile spanning from small hydrophilic malonylcarnitine (C3:DC) to large hydrophobic octadecanoylcarnitine (C18; Fig. 2). To properly retain and separate small polar analytes while allowing timely elution of large hydrophobic long-chain acylcarnitines we found that precise concentrations of both ammonium formate and organic solvent were necessary. Ammonium formate more strongly influenced the elution of small polar compounds (e.g., C3:DC) whereas acetonitrile had a greater impact on the elution of large hydrophobic compounds (e.g., C18; Fig. S5). Table 1 shows the retention times, paired isotopic internal standards, and MRM transitions for calibrated analytes in our fully developed LC-MS/MS method. Notably, the void time for this method was empirically determined to be 0.9 min; all analytes therefore had a K prime > 1. In addition to the classic acylcarnitine species studied by traditional methods, we were able to retain and analyze small polar analytes related to carnitine biosynthesis (carnitine, deoxycarnitine, and trimethyllysine) and gut microbial metabolism of carnitine (trimethylamine N-oxide).

3.2. Calibrator creation and verification

Calibration is fundamental to accurate quantification but unfortunately a comprehensive commercial calibration mixture does not exist for acylcarnitine studies. We therefore prepared our own calibrator mix from individual commercial acylcarnitine stocks (n =38; Table S1). Each stock was individually verified by two methods (1) LC-MS/MS acylcarnitine analysis and (2) free and total carnitine analysis (Table S7). The results from study 1 confirmed appropriate calibrator stock contents and detected interference from contaminating acylcarnitines; for example, propionyl-carnitine contamination accounted for 5.54% of overall acylcarnitine in our methylmalonyl-carnitine stock. Study 2, the measurement of free and total carnitine, verified each stock's true concentration and detected free carnitine contamination that can arise in acylcarnitine stock materials due to degradation or improper preparation. We found high levels of free carnitine (>10% of total carnitine) in a subset of our acylcarnitine stocks including all three stocks prepared for a prior study and stored in methanol at 4 °C for > 1 year (Table S1). Following these observations, all liquid stock materials were stored at -80 °C and under these conditions we have seen long term stability of all analytes (>6 months).

Using the collective results from all verification studies, stocks were then combined and diluted to create our calibration stock mixture. Also included in our method are 28 analytes for which pure calibration material was not available or was prohibitively expensive. For these analytes we adopted the approach used in FI-MS/MS methods and applied surrogate calibration using the most chemically similar calibrated compound (Table S6).

3.3. Assay validation

Table 2 summarizes key results from clinical validation studies. These include assays establishing the limit of quantification (LOQ), carryover, linear range of detection, reproducibility (inter-assay imprecision), and accuracy. Validation studies were completed over the course of multiple months using two different manufacturer's lots of analytic columns. Throughout, inter-assay imprecision remained

within the acceptable range, with all analytes achieving a coefficient of variation < 15% at high concentrations and <20% at low concentrations (Table 2). Retention times and isomeric selectivity also remained consistent (Table 1).

Many additional studies were completed to test for factors that could impact the accuracy or reproducibility of the assay. We searched for unrecognized contamination and/or interference by individually analyzing: (i) the calibration mix for isotopic interference, (ii) the isotopic internal standard mix for analyte interference, and (iii) multiple un-spiked matrix specimens for isotopic interference. These studies failed to detect significant levels of interference with the exception of the previously discussed calibrator contamination (Table S7). Similarly, multiple commercial sources of bovine serum albumin were tested for acylcarnitine contamination prior to selecting our surrogate matrix. Stability was monitored and all analytes were found to remain essentially unchanged in plasma following three freeze/thaw cycles, when stored for at least one week at -20 °C, or when prepared and left on the LC-MS/MS autosampler at 4 °C for at least three days. To study matrix effects (e.g., signal suppression or enhancement) we compared isotopic internal standard signals (n= 30) in multiple different plasma samples to isotopic internal standard signals in specimens prepared with water in place of matrix. Surprisingly, the inclusion of plasma substantially improved the signal for succinylcarnitine (+248%), methylmalonycarnitine (+521%) and trimethyllysine (+2,379%). but for all other analytes, minimal matrix effects were noted (average signal change in water vs matrix = 1.9%, range = -32.1% to 13.7%). Finally, calibrator recovery was studied by analyzing replicates of calibrator mixtures before and after complete nitrogen dry down and resuspension. On average acylcarnitine concentrations in the calibration mix were -2.24% lower following dry-down and resuspension (range -5.5% to 4.0%) but these findings failed to achieve statistical significance (homoscedastic t-test >> 0.05 for all analytes).

3.4. Clinical utility

To explore clinical utility we applied our method to the analyses of numerous residual patient specimens and were able to detect, at a very granular level, known disease related abnormalities. For example, the disease associated isomeric acylcarnitine elevations of methylmalonylcarnitine (C4:DC), 3-methylcrotonylcarnitine (C5:1), and 3-methylglutarylcarnitine (C6:DC) were correctly resolved in patients with methylmalonic acidemia (OMIM 251000), 3-methylcrotonyl-CoA carboxylase deficiency (3MCC; OMIM 609010), and 3-hydroxy-3-methylglutaryl-CoA lyase deficiency (OMIM 246450), respectively (Fig. 3A-C). Interestingly, a second unknown C5:1 peak was detected in all 3MCC cases (n= 5). This compound is not present in our pure 3-methylcrotonylcarnitine stocks nor has it been detected in a sample from an unaffected individual to date by our laboratory.

Important pathognomonic acylcarnitine abnormalities could also be detected for numerous uncalibrated analytes. For these biomarkers we could gain confidence in the analyte identity due to three observations: (i) MS transition matching, (ii) appropriate retention time (e.g., the retention time of CX:DC < CX:OH < CX:1 < CX; Fig. 4A), and (iii) segregation with disease. For example, in samples from patients with peroxisomal biogenesis disorders due to *PEX1* loss of function (OMIM 214100) we detected the abnormal accumulation of long chain (C16:DC, C18:1:DC, and C18:DC) and very long chain (C20:DC, C22:DC) dicarboxylic acylcarnitines (Fig. 4B and C). Prior studies have identified long chain dicarboxylic acylcarnitines as potential biomarkers for peroxisomal biogenesis disorders [18].

4. Discussion

In the future, it is likely the field of clinical acylcarnitine testing will continue to move away from FI-MS/MS methods in favor of the selectivity, sensitivity and accuracy afforded by LC-MS/MS methods. To expedite this transition, simple, robust, and cost-effective LC-MS/MS methods are needed. Additional studies are also needed to help make sense of LC-MS/MS acylcarnitine data, i.e. to more fully define reference intervals and disease related abnormalities. Surprisingly, there remain multiple examples of

inherited metabolic diseases that are unequivocally associated with a plasma acylcarnitine abnormality but due to a failure to resolve isomeric species, the identity of the disease-related acylcarnitine remains ambiguous [1].

Although advances have been made in recent years, the commercial availability of pure acylcarnitine materials remains a significant limitation in the development of LC-MS/MS acylcarnitine analyses. One clinically important example involves the monounsaturated acylcarnitines (e.g., C10:1 and C14:1). Only the trans-2 forms of monounsaturated acylcarnitines are currently commercially available but these are not the predominate monounsaturated species in either normal human plasma or in clinically affected individuals thus making them suboptimal calibration materials (Fig. 5A and B). For example, in patients with medium chain acyl-CoA dehydrogenase deficiency (OMIM 201450) the C10:1 elevation is driven by accumulation of cis-4-decenoate not trans-2 decenoate [19]. The expanded availability of more comprehensive acylcarnitine reference material will ultimately improve our ability to calibrate and therefore quantify disease related acylcarnitine abnormalities.

5. Conclusion

The preceding manuscript describes a clinically validated LC-MS/MS acylcarnitine assay that can generate a full acylcarnitine profile including the unambiguous detection of isomeric species, carnitine metabolic precursors, and very long chain dicarboxylic acylcarnitines. Importantly, this assay uses a simple sample preparation with a single analytic column and common MS-compatible solvents. These unique characteristics are not currently available by other methods and are made possible by the application of a novel mixed-mode liquid chromatography separation. In conclusion, the simplicity and comprehensive nature of this method make it an attractive alternative for laboratories considering moving beyond flow-injection assays.

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356 **Table 1** LC-MS/MS parameters for calibrated analytes

Analyte	Class	Transition	Retention time [a]	Internal standard	Transition (internal standard)
malonyl	C3:DC	248 >85	2.019 ± 0.038	C3:DC_malonyl-d3	251 >85
carnitine	C0	162 >85	2.604 ± 0.028	C0_carnitine-d9	171 >85
deoxycarnitine	other	146 >86.8	3.034 ± 0.035	deoxycarnitine-d9	155 >87
methylmalonyl	C4:DC	262 >85	3.062 ± 0.060	C4:DC_methylmalonyl-d3	265 >85
succinyl	C4:DC	262 >85	3.379 ± 0.067	C4:DC_succinyl-d3	265 >85
acetyl	C2	204 >85	3.540 ± 0.057	C2_acetyl-d3	207 >85
trimethylamine-N-oxide	other	76 >59	3.673 ± 0.046	TMAO-d9	85 >68.1
glutaryl	C5:DC	276 >85	4.091 ± 0.084	C5:DC_glutaryl-d3	279 >85
3-hydroxyisovaleryl	C5:OH	262 >85	4.602 ± 0.087	C5:OH_3-hydroxyisovaleryl-d3	265 >85
adipoyl	C6:DC	290 >85	5.303 ± 0.099	C6:DC_adipoyl-d3	293 >85
propionyl	C3	218 >85	5.378 ± 0.084	C3_propionyl-d3	221 >85
3-methylglutaryl	C6:DC	290 >85	5.731 ± 0.117	C6:DC_adipoyl-d3	293 >85
isobutyryl	C4	232 >85	7.340 ± 0.094	C4_isobutyryl-d3	235 >85
butyryl	C4	232 >85	7.666 ± 0.093	C4_butyryl-d3	235 >85
suberoyl	C8:DC	318 >85	8.295 ± 0.091	C8:DC_suberoyl-d3	321 >85
tiglyl	C5:1	244 >85	8.761 ± 0.092	C5:1_tiglyl-d3	247 >85
3-methylcrotonyl	C5:1	244 >85	8.907 ± 0.088	C5:1_3-methylcrotonyl-d3	247 >85
trimethyllysine	other	189 >83.7	9.336 ± 0.125	Trimethyllysine-d9	198 >84
2-methylbutyryl	C5	246 >85	9.383 ± 0.094	C5_2-methylbutyryl-d3	249 >85
isovaleryl	C5	246 >85	9.644 ± 0.088	C5_isovaleryl-d9	249 >85
valeryl	C5	246 >85	9.934 ± 0.088	C5_valeryl-d3	249 >85
sebacoyl	C10:DC	346 >85	11.062 ± 0.068	C10:DC_sebacoyl-d3	349 >85
hexanoyl	C6	260 >85	11.642 ± 0.039	C6_hexanoyl-d3	263 >85
heptanoyl	C7	274 >85	12.307 ± 0.021	C8_octanoyl-d3	291 >85
trans-2-octenoyl	C8:1	286 >85	12.527 ± 0.016	C8_octanoyl-d3	291 >85
3-hydroxydecanoyl	C10:OH	332 >85	12.637 ± 0.057	C8_octanoyl-d3	291 >85
octanoyl	C8	288 >85	12.732 ± 0.014	C8_octanoyl-d3	291 >85
trans-2-decenoyl	C10:1	314 >85	13.145 ± 0.165	C10_decanoyl-d3	319 >85
decanoyl	C10	316 >85	13.476 ± 0.012	C10_decanoyl-d3	319 >85
trans-2-dodecenoyl	C12:1	342 >85	13.42 ± 0.015	C12_dodecanoyl-d3	347 >85
dodecanoyl	C12	344 >85	14.129 ± 0.016	C12_dodecanoyl-d3	347 >85
trans-2-tetradecenoyl	C14:1	370 >85	14.825 ± 0.024	C14_tetradecanoyl-d3	375 >85
tetradecanoyl	C14	372 >85	15.117 ± 0.030	C14_tetradecanoyl-d3	375 >85
trans-2-hexadecenoyl	C16:1	398 >85	16.230 ± 0.047	C16_hexacecanoyl-d3	403 >85
3-hydroxyhexadecanoyl	C16:OH	416 >85	15.778 ± 0.152	C16:OH_3-hydroxyhexadecanoyl-d3	419 >85
cis-9-octadecenoyl	C18:1	426 >85	16.688 ± 0.051	C18:1_octadecenoyl-d3	429 >85
hexacecanoyl	C16	400 >85	16.690 ± 0.057	C16_hexacecanoyl-d3	403 >85
octadecanoyl	C18	428 >85	19.231 ± 0.099	C18_octadecanoyl-d3	431 >85

357 [a] +/- 2 standard deviations

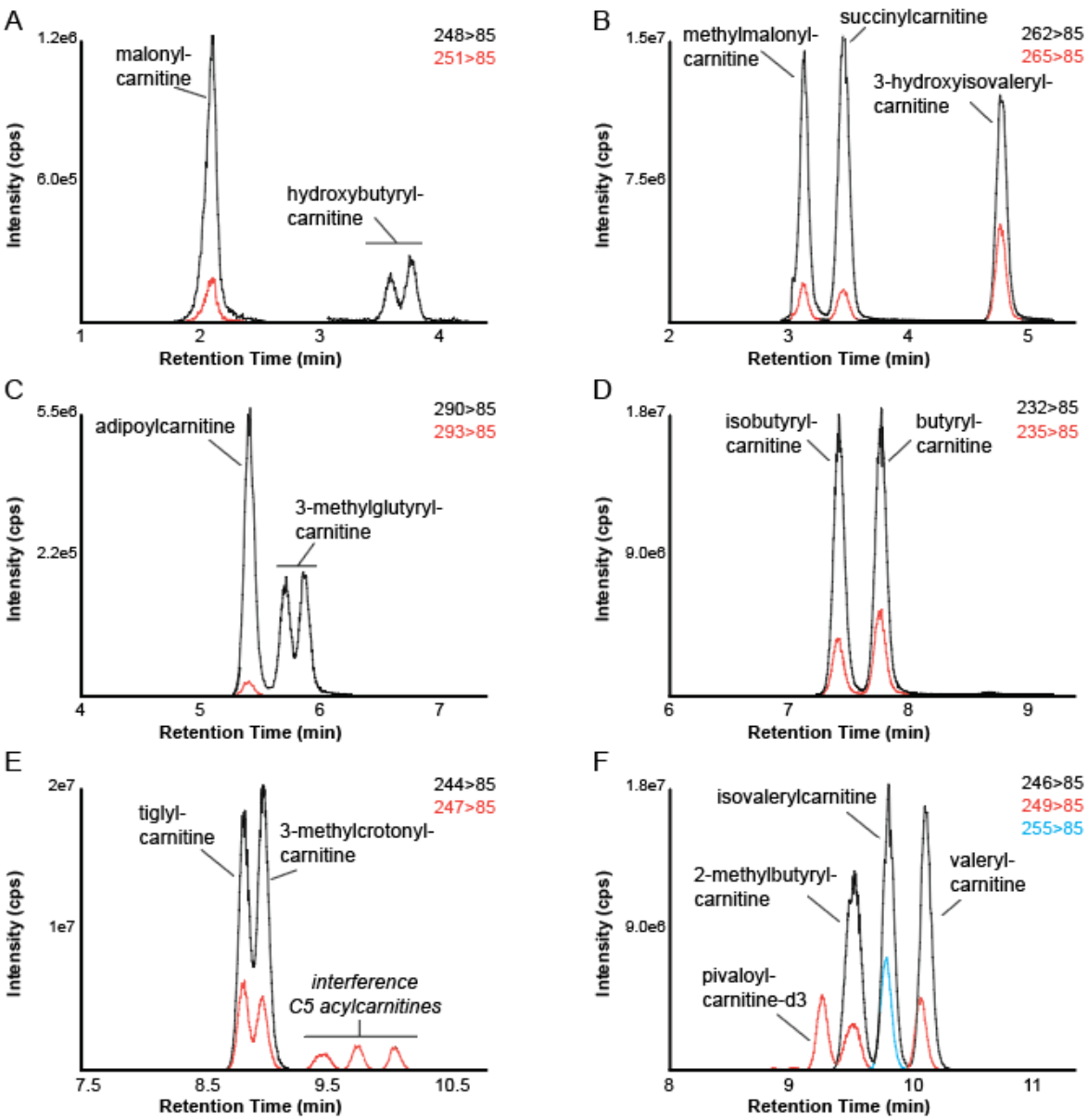
358

Table 2 Summary of key performance metrics

					Inter-assay imprecision		Spike accuracy		
Analyte	**Class**	**Limit of quantification**	**Carryover**	**Linear range**	**Low control[a]**	**High control[a]**	**Bias**	**Slope**	**r**
malonyl	C3:DC	0.05	0.00%	0.05 - 20	0.228 (13.2%)	5.35 (11.7%)	3.4%	1.058	0.999
carnitine	C0	0.094	0.00%	0.75 - 300	3.056 (8.5%)	79.093 (6.9%)	0.7%	1.010	0.998
deoxycarnitine	other	0.047	0.00%	0.75 - 300	3.051 (7.9%)	79.365 (6.7%)	6.1%	1.060	0.999
methylmalonyl	C4:DC	0.006	0.00%	0.05 - 20	0.212 (12.1%)	5.136 (11.9%)	4.2%	1.063	0.999
succinyl	C4:DC	0.003	0.00%	0.05 - 20	0.213 (11.5%)	5.281 (10%)	7.3%	1.053	0.999
acetyl	C2	0.019	0.00%	0.3 - 120	1.302 (7.9%)	32.561 (8.8%)	0.8%	1.016	0.999
trimethylamine-N-oxide	other	0.031	0.00%	0.5 - 200	2.1 (8.0%)	53.159 (5.8%)	2.8%	1.037	0.999
glutaryl	C5:DC	0.003	0.00%	0.05 - 20	0.212 (9.4%)	5.23 (7.9%)	3.4%	1.053	0.998
3-hydroxyisovaleryl	C5:OH	0.003	0.00%	0.025 - 10	0.105 (8.3%)	2.638 (6.0%)	1.1%	1.013	0.999
adipoyl	C6:DC	0.006	0.00%	0.025 - 10	0.108 (10.7%)	2.669 (7.9%)	7.2%	1.089	0.999
propionyl	C3	0.008	0.00%	0.125 - 50	0.537 (8.7%)	13.567 (6.6%)	4.7%	1.061	0.998
3-methylglutaryl	C6:DC	0.006	0.00%	0.025 - 10	0.099 (10.1%)	2.594 (8.7%)	-2.1%	1.002	0.999
isobutyryl	C4	0.002	0.00%	0.025 - 10	0.107 (8.2%)	2.643 (6.0%)	1.1%	1.031	0.998
butyryl	C4	0.006	0.00%	0.025 - 10	0.107 (8.7%)	2.628 (6.6%)	0.4%	1.014	0.999
suberoyl	C8:DC	0.006	0.00%	0.025 - 10	0.103 (9.1%)	2.626 (8.4%)	2.5%	1.031	0.999
tiglyl	C5:1	0.002	0.00%	0.025 - 10	0.105 (8.5%)	2.628 (6.7%)	1.0%	1.008	0.999
3-methylcrotonyl	C5:1	0.002	0.00%	0.025 - 10	0.104 (8.6%)	2.637 (6.3%)	3.8%	1.052	0.999
trimethyllysine	other	0.375	0.00%	0.375 - 50	0.473 (14.9%)	13.541 (9.6%)	-2.7%	0.974	0.996
2-methylbutyryl	C5	0.002	0.00%	0.025 - 10	0.104 (8.1%)	2.636 (6.2%)	2.4%	1.024	0.998
isovaleryl	C5	0.006	0.00%	0.025 - 10	0.108 (7.2%)	2.637 (6.1%)	2.1%	1.035	0.999
valeryl	C5	0.002	0.00%	0.025 - 10	0.106 (8.0%)	2.641 (6.5%)	3.4%	1.049	0.999
sebacoyl	C10:DC	0.003	0.00%	0.025 - 10	0.107 (8.6%)	2.654 (7.8%)	4.2%	1.045	0.999
hexanoyl	C6	0.002	0.00%	0.025 - 10	0.105 (7.9%)	2.643 (6.7%)	1.9%	1.027	0.998
heptanoyl	C7	0.008	0.01%	0.125 - 10	0.562 (8.5%)	12.921 (12.1%)	4.8%	1.002	0.998
trans-2-octenoyl	C8:1	0.003	0.01%	0.025 - 10	0.105 (7.7%)	2.599 (6.0%)	2.4%	1.038	0.998
3-hydroxydecanoyl	C10:OH	0.002	0.00%	0.0125 - 5	0.053 (7.9%)	1.261 (7.1%)	3.0%	1.061	0.996
octanoyl	C8	0.002	0.00%	0.025 - 10	0.108 (7.9%)	2.682 (6.3%)	1.9%	1.033	0.998
trans-2-decenoyl	C10:1	0.002	0.01%	0.025 - 10	0.103 (8.6%)	2.668 (7.4%)	5.1%	1.085	1.000
decanoyl	C10	0.003	0.01%	0.025 - 10	0.107 (8.8%)	2.737 (8.3%)	4.7%	1.069	0.999
trans-2-dodecenoyl	C12:1	0.002	0.01%	0.025 - 10	0.103 (9.9%)	2.666 (7.7%)	7.9%	1.110	0.996
dodecanoyl	C12	0.002	0.01%	0.025 - 10	0.105 (9.5%)	2.676 (7.9%)	4.3%	1.045	0.999
trans-2-tetradecenoyl	C14:1	0.002	0.02%	0.025 - 10	0.109 (11.5%)	2.742 (11.2%)	2.8%	1.038	0.994
tetradecanoyl	C14	0.002	0.02%	0.025 - 10	0.109 (11.1%)	2.684 (10.2%)	6.5%	1.071	0.999
trans-2-hexadecenoyl	C16:1	0.003	0.03%	0.025 - 10	0.101 (14.2%)	2.685 (11.5%)	1.5%	1.057	0.999
3-hydroxyhexadecanoyl	C16:OH	0.003	0.00%	0.0125 - 5	0.054 (11.1%)	1.352 (10.6%)	6.4%	1.088	0.999
cis-9-octadecenoyl	C18:1	0.002	0.05%	0.025 - 10	0.115 (15.4%)	2.65 (14.2%)	3.3%	1.033	0.999
hexacecanoyl	C16	0.002	0.04%	0.025 - 10	0.116 (12.5%)	2.757 (12.6%)	2.5%	1.034	0.999
octadecanoyl	C18	0.003	0.07%	0.025 - 10	0.133 (17.8%)	3.014 (14.3%)	8.6%	1.137	0.998

All concentrations are listed in micromoles/L

[a] Average concentration (percent coefficient of variation)

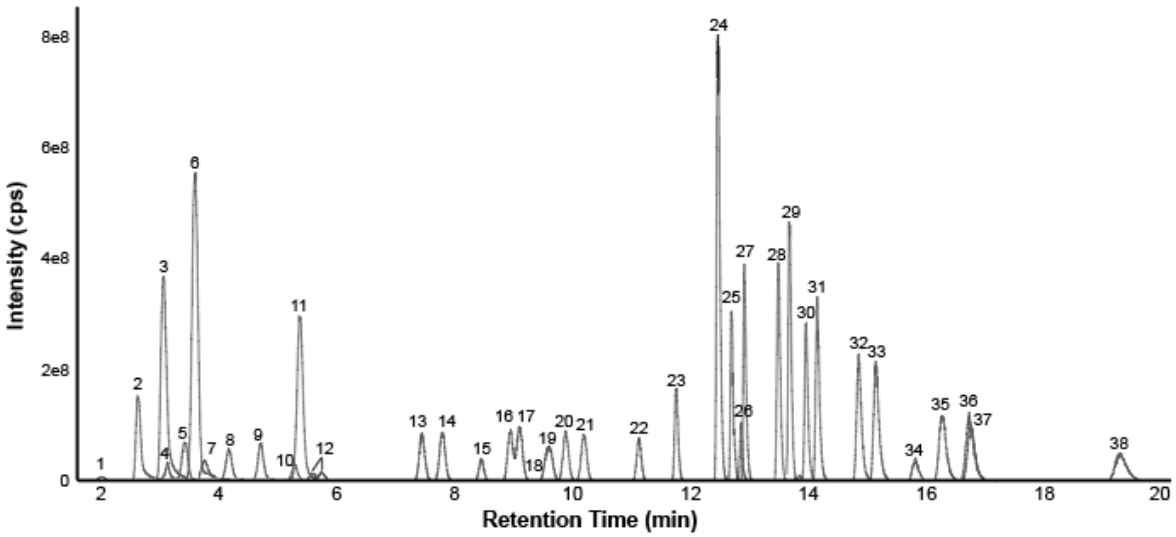


363

364 **Fig. 1.** Chromatographic resolution of clinically relevant isobaric and isomeric acylcarnitine species: (A)

365 C3:DC and C4:OH, (B) C4:DCs and C5:OH, (C) C6:DCs, (D) C4s, (E) C5:1s, (F) C5s. Shown are analyses of

366 standard 5 with acylcarnitines in black and paired isotopic internal standards in red or blue.



367

368 **Fig. 2.** Full chromatogram of acylcarnitines included in the calibrator: (1) malonyl-L-, (2) L-carnitine, (3)
369 (3-carboxypropyl) trimethyl-ammonium chloride, (4) methylmalonyl-L-, (5) o-succinyl-L-, (6) acetyl-L-, (7)
370 trimethylamine N-oxide, (8) glutaryl-L-, (9) 3-hydroxyisovaleryl-L-, (10) adipoyl-L-, (11) propionyl-L-, (12)
371 3-methylglutaryl-L-, (13) isobutyryl-, (14) butyryl-, (15) suberoyl-L-, (16) tiglyl-L-, (17) 3-methylcrotonyl-
372 L-, (18) Nε,Nε,Nε-trimethyllysine, (19) 2-methylbutyrl-L-, (20) isovaleryl-L-, (21) valeryl-L-, (22) sebacoyl-
373 L-, (23) hexanoyl-L-, (24) heptanoyl-L-, (25) trans-2-octenoyl-L-, (26) 3R-3-hydroxydecanoyl-L-, (27)
374 octanoyl-L-, (28) trans-2-decenoyl-L-, (29) decanoyl-L-, (30) trans-2-dodecenoyl-L-, (31) lauroyl-L-, (32)
375 trans-2-tetradecenoyl-L-, (33) myristoyl-L-, (34) trans-2-hexadecenoyl-L-, (35) 3R-3-
376 hydroxyhexadecanoyl-L-, (36) oleoyl-L-, (37) palmitoyl-L-, (38) stearoyl-L-carnitine.

377

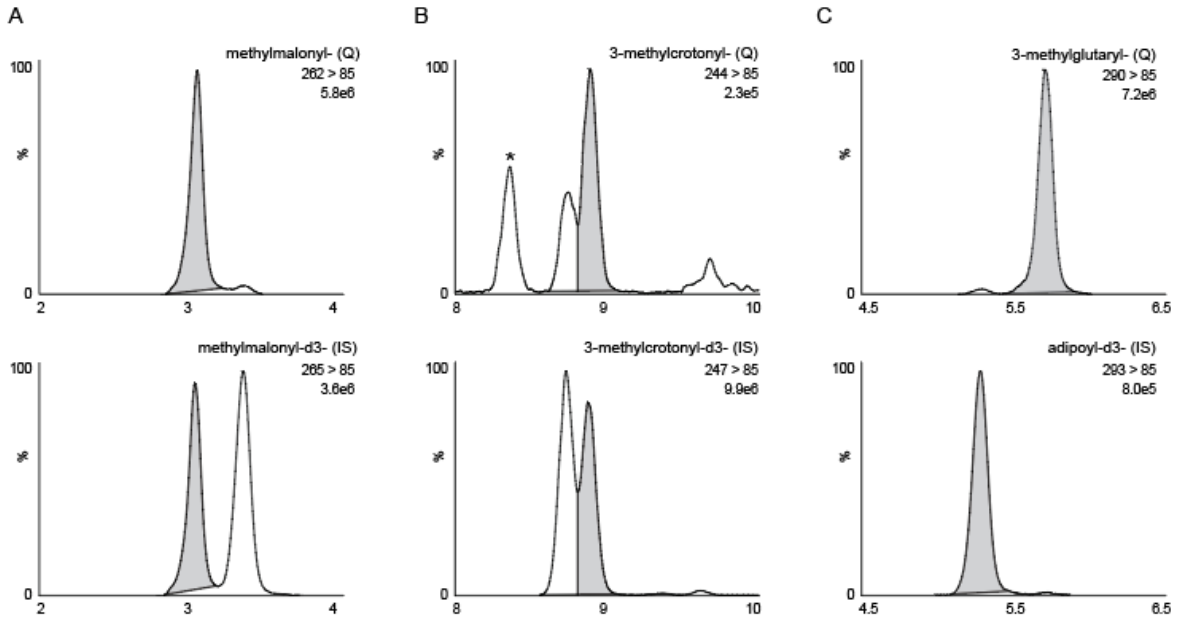
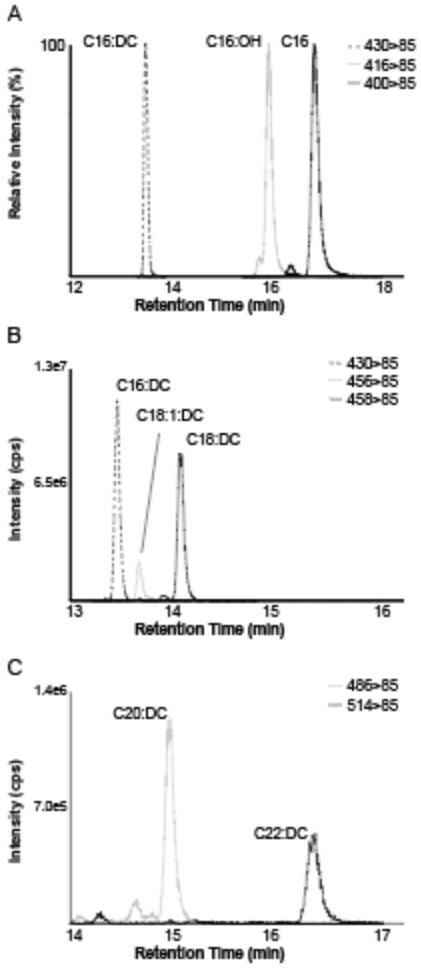
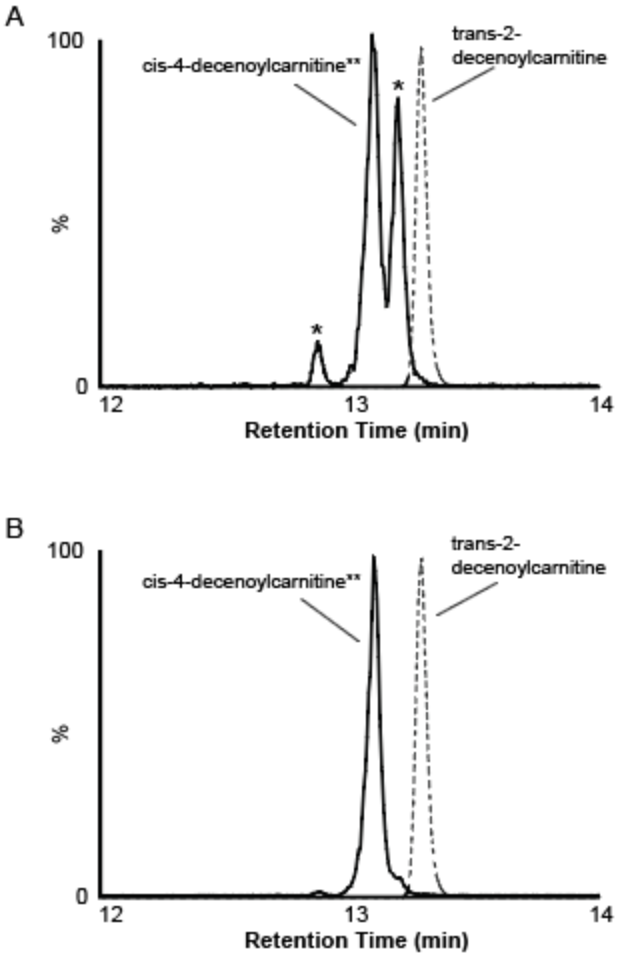


Fig. 3. Clinical application of isomeric separation. (A) methylmalonylcarnitine quantification (0.843 micromoles/L) in a patient with methylmalonic acidemia. (B) 3-methylcrotonylcarnitine quantification (0.006 micromoles/L) in a patient with 3-methylcrotonyl-CoA carboxylase deficiency. (C) 3-methylglutarylcarnitine quantification (2.614 micromoles/L) in a patient with HMG-CoA lyase deficiency. Panels show peak integrations for the quantified analyte (upper) and the isotopic internal standard (lower). *indicates an unknown biomarker for 3-methylcrotonyl-CoA carboxylase deficiency.



386

387 **Fig. 4.** Plasma long chain dicarboxylic acylcarnitine abnormalities in peroxisomal disorders. (A) Analysis
388 of pure C16 reference materials demonstrates the relationship between retention time and the
389 presence of a hydroxyl or dicarboxyl group(s). Detection of (B) long chain and (C) very long dicarboxylic
390 acylcarnitine species in the plasma of a patient with a peroxisomal biogenesis disorder due to *PEX1* loss
391 of function (OMIM 214100).



392

393 **Fig. 5.** Unsaturated acylcarnitine peak complexity and calibration challenges. Representative plasma
394 C10:1 acylcarnitine chromatographic profiles generated using transition 314 > 85, are shown for (A) a
395 typical unaffected individual and (B) a patient with medium chain acyl-CoA dehydrogenase deficiency
396 (C10:1 = 0.181 micromoles/L). A dotted chromatogram overlay shows the retention time of trans-2-
397 decenoylcarnitine, the C10:1 acylcarnitine species used to calibrate our assay. *indicates unknown
398 analytes. **The cis-4-decenoylcarnitine peak identity is assumed based on the literature but it is not
399 confirmed in these specimens.