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Analysis of 2-methylcitric acid, methylmalonic acid, and total homocysteine in dried blood spots by LC-MS/MS for application in the newborn screening laboratory: A dual derivatization approach

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ABSTRACT

Inborn errors of propionate, cobalamin and methionine metabolism are targets for Newborn Screening (NBS) in most programs world-wide, and are primarily screened by analyzing for propionyl carnitine (C3) and methionine in dried blood spot (DBS) cards using tandem mass spectrometry (MS/MS). Single-tier NBS approaches using C3 and methionine alone lack specificity, which can lead to an increased false-positive rate if conservative cut-offs are applied to minimize the risk of missing cases. Implementation of liquid chromatography tandem mass spectrometry (LC-MS/MS) second-tier testing for 2-methylcitric acid (MCA), methylmalonic acid (MMA), and homocysteine (HCY) from the same DBS card can improve disease screening performance by reducing the false-positive rate and eliminating the need for repeat specimen collection. However, DBS analysis of MCA, MMA, and HCY by LC-MS/MS is challenging due to limited specimen size and analyte characteristics leading to a combination of low MS/MS sensitivity and poor reverse-phase chromatographic retention. Sufficient MS response and analytical performance can be achieved for MCA by amidation using DAABD-AE and by butylation for MMA and HCY. Herein we describe the validation of a second-tier dual derivatization LC-MS/MS approach to detect elevated MCA, MMA, and HCY in DBS cards for NBS. Clinical utility was demonstrated by retrospective analysis of specimens, an interlaboratory method comparison, and assessment of external proficiency samples. Imprecision was <10.8% CV, with analyte recoveries between 90.2 and 109.4%. Workflows and analytical performance characteristics of this second-tier LC-MS/MS approach are amenable to implementation in the NBS laboratory.

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1. Introduction

2-Methylcitric acid (MCA), methylmalonic acid (MMA), and homocysteine (HCY) are clinically important biomarkers for the identification of inherited disorders of propionate, cobalamin (Cbl), and methionine metabolism [1], many of which are recommended for inclusion in newborn screening (NBS) programs [2,3]. These disorders are initially detected in NBS using dried-blood spot (DBS) cards by the presence of elevated propionylcarnitine (C3) concentration or abnormal methionine levels in combination with various analyte ratios [4]. However, C3 elevations are somewhat non-specific, and can be seen in several primary inborn errors of metabolism (e.g., propionic acidemia, methylmalonic acidemia, and several cobalamin disorders), nutritional vitamin B12 deficiency [1], or even secondary to prolonged hyperbilirubinemia [5]. Similarly, methionine is elevated in cystathionine β-synthase

Abbreviations: C3, propionylcarnitine; C2, acetylcarnitine; Cbl, cobalamin; CBS, cystathionine β-synthase; DBS, dried blood spot; DAABD-AE, 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole; DMAP, 4-(dimethylamino)pyridine; DTT, dithiothreitol; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; ESI, electrospray ionization; FA, formic acid; GC, gas chromatography; GPCho's, glycerophosphocholines; HCl, hydrochloric acid; HCY, homocysteine; LC, liquid chromatography; LLOD, lower limit of detection; LLOQ, lower limit of quantitation; MCA, 2-methylcitric acid; Met, methionine; MMA, methylmalonic acid; MPs, mobile phases; MS, mass spectrometry; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; NBS, newborn screening; Phe, phenylalanine; PPV, positive predictive value; QC, quality control; rpm, revolutions per minute; S/N, signal-to-noise.

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deficiency (CBS) and in disorders of the methionine transmethylation cycle (e.g., methionine adenosyltransferase deficiency) [4,6], but also secondary to total parenteral nutrition or liver damage [7,8]. Accordingly, the analysis of C3 and/or methionine alone lacks specificity for primary inborn errors of metabolism that are the target of NBS, and can also suffer from reduced sensitivity as a result of concentration overlap between affected and unaffected neonates [1,4]. The integration of complex algorithms including ratios of C3 to other acylcarnitine species, and ratios of methionine to other amino acids can improve performance, but such algorithms still fail to maintain an acceptable false-positive rate when cut-offs are set low enough to ensure high sensitivity [1,9]. Second-tier testing of more specific biomarkers can, however, improve the positive predictive value (PPV) of a screening program without sacrificing sensitivity.

Increased levels of MCA and/or methylmalonic acid in bodily fluids are more specific biomarkers for identifying, and also differentiating between, C3-related disorders of propionate metabolism, such as propionyl-CoA carboxylase deficiency and methylmalonyl-CoA mutase deficiency or adenosylcobalamin deficiency [4,10–13]. Propionic acidemia is caused by a genetic deficiency in propionyl-CoA carboxylase, a biotin dependent enzyme that catalyzes the carboxylation of propionyl-CoA with bicarbonate to form methylmalonyl-CoA [13]. Abnormal production of MCA is formed via the citrate synthase catalyzed Claisen condensation of accumulated propionyl-CoA with oxaloacetate producing two diastereomers [10,14]. Classical methylmalonic acidemia is caused by a genetic deficiency in methylmalonyl-CoA mutase which, along with the coenzyme adenosylcobalamin, converts L-methylmalonyl-CoA to succinyl-CoA [15]. The absence of MMA in follow-up urine testing is suggestive of propionyl-CoA carboxylase deficiency. Presence of MMA in urine, however, suggests either classical methylmalonic aciduria, adenosylcobalamin deficiency, combined adenosylcobalamin and methylcobalamin deficiency or a nutritional vitamin B12 deficiency [1,10,12,16]. Further assessment of blood homocysteine is able to differentiate classical methylmalonic acidemia and adenosylcobalamin deficiency from combined Cbl disorders and vitamin B12 deficiency [1]. Nutritional vitamin B12 deficiency is usually excluded by further serum vitamin B12 assessment. Molecular testing for Cbl genes is necessary to establish a diagnosis of classical methylmalonic aciduria and Cbl disorders.

Markedly increased HCY is present in classical homocystinuria, caused by genetic deficiency in CBS, which, along with the cofactor pyridoxal 5′-phosphate (vitamin B6), is responsible for the condensation of serine with HCY to form cystathionine [17]. Homocystinuria caused by deficiency in CBS is initially suggested in first-tier NBS by both increased concentration of methionine and the methionine/phenylalanine ratio [17,18]. Hyperhomocysteinemia can also be caused by deficiency in enzymes that are involved with the conversion of methionine to HCY (methylation cycle) resulting in increased methionine first-tier screens [17,18]. Therefore, follow-up testing for abnormal HCY, MMA, and MCA is valuable for improving the diagnosis and differentiation of various disorders associated with propionate, methionine, and Cbl metabolism.

Confirmatory clinical testing for MMA and MCA is typically done by profiling urinary organic acids using gas-chromatography mass spectrometry (GC–MS) [19] or more recently by liquid chromatography-quadrupole time-of-flight MS (LC-QTOF/MS) [20]. Testing for abnormal MMA and MCA levels can also be performed by plasma analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [21]. Follow-up analysis for total HCY is typically done using plasma either by immunoassay, or LC methods with fluorescence or MS detection, and requires the addition of a disulfide reducing agent [21–23]. LC-based metabolomics for first- and second-tier NBS where multiple distinct classes of biologically important molecules are analyzed in a single assay is also gaining interest in the clinical community [24,25], but will require further reports on clinical validation and implementation to reach widespread acceptance. Preferable for NBS, analysis using the original DBS card for second-tier testing eliminates the need for additional neonatal urine or blood collection. Furthermore, it decreases the number of false-positive cases and minimizes unnecessary parental anxiety resulting from an abnormal first-tier screen.

Analysis of DBS MCA, MMA, and HCY in second-tier in NBS programs has been demonstrated to increase PPV [1,18]. However, limited neonatal DBS specimen size, and differences in chemical functionality of these analytes, are inherent challenges to overcome for LC-MS/MS analysis in the NBS laboratory, with few NBS-specific analytical strategies having been reported in the literature [18,26–28]. Of note, MCA is a tricarboxylic acid that displays poor sensitivity using electrospray ionization with LC-MS/MS analysis; to a lesser extent, MMA, a dicarboxylic acid, also has reduced sensitivity when using LC-MS/MS. These challenges can be solved by appropriate sample derivatization. In particular, derivatization of MCA using DAABD-AE, which contains a highly ionizable tertiary amine, can enhance MS sensitivity [26]. HCY has poor chromatographic retention on standard reverse phase LC columns, which can be solved by conversion to a more non-polar molecule through butylation [18]. In our initial investigations, MMA and HCY could not be derivatized using DAABD-AE, and butylation of MCA displayed suboptimal performance. With modification and optimization of previous methods [18,26], herein we describe a dual derivatization LC-MS/MS approach to detecting abnormal MCA, MMA, and HCY levels in neonatal DBS cards for second-tier screening. It is anticipated that this second-tier approach will improve overall screening test performance by both eliminating unnecessary parental anxiety caused by neonatal resampling and narrowing the differential diagnosis window of the screening result.

2. Materials and methods

2.1. Consumables, sample preparation equipment, and stock solutions

MCA [X-4176], MCA-d_3 [D-4162], MMA-d_3 [D-2810], and homocystine-d_8 [D-3030] were from CDN isotopes. MMA [M54058], HCY [44925], *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) [03450], 4-(dimethylamino)pyridine (DMAP) [107700], 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE) [79291], and dithiothreitol (DTT) [D0632] were from Sigma-Aldrich. 3 N HCl in 1-butanol [1-201007-200] was from Regis Technologies, Inc. Formic acid (FA) [A118P-100] was from Fisher Chemical. Methanol [MX0488-1] and acetonitrile [AX0155-1] were from Millipore Sigma. Ultrapure type 1 water was generated using a Purelab Flex 4 system. Whatman 903™ newborn screening cards were from GE Healthcare. Human sodium heparin whole blood was obtained from Tennessee Blood Services. DAABD-AE derivatization of MCA, and butylation of MMA and HCY was performed in polypropylene 96-well deep well plates [76210-524] with silicon microplate sealing mats [76311-636] from VWR. Micro tubes were from Eppendorf [1.5 mL, 022364111] and Sarstedt [0.5 mL, 72.699]. DBS cards were punched using a Panthera Puncher 9 from Perkin Elmer. Other equipment used was as follows: Incu-Mixer MP [H6004] from Benchmark Scientific, microplate evaporator [Evx-192] from Apricot Designs, ultrasonicator (E 300H) from Elma, and an Allegra 6 centrifuge (using rotor PTS-2000) from Beckman Coulter.

Stock solutions of MCA (11.5 mM), MCA-d_3 (8.61 mM), MMA (34.6 mM), MMA-d_3 (17.5 mM), and HCY (29.2 mM) were prepared

in water. A stock solution of homocystine-d_8 (0.861 mM) was prepared in water containing 4% FA. Two intermediate internal standard (IS) solutions were prepared in water, the first containing 200 μM MCA- d_3 and the second containing 50 μM MMA- d_3 and 25 μM homocystine-d_8. All stock and intermediate IS solutions were stored at −80 °C in microcentrifuge tubes. Intermediate IS solutions were further diluted for spiking during DBS sample preparation. Critical reagent solutions of EDC, DMAP, and DAABD-AE were aliquoted in glass vials and stored at −20 °C. An aqueous solution of DTT was prepared from powder on the day of analysis.

2.2. Calibrator and quality control preparation

Whole blood was first adjusted to 55% hematocrit by centrifugation and removal of appropriate amounts of serum. The blood volume of a 3.2 mm DBS punch at 55% hematocrit has been reported to be approximately 3.1 μL [29]. MCA, MMA, and HCY stock solutions were diluted in water to 0.20 mM and 2.0 mM. Calibrators were then prepared by spiking 0.20 and 2.0 mM solutions into whole blood to make final spiked concentrations of 0.5, 1, 2, 5, 10, 15, 25, and 50 μM for MCA and MMA, and 5, 10, 15, 25, and 50 μM for HCY. Similarly, quality control (QC) samples were spiked at 1.5, 8, and 20 μM. Calibrator and QC concentrations were corrected for the presence of endogenous levels of MMA and HCY in blood as indicated in the Results section. One hundred microlitres of standard or QC were then spotted onto Whatman 903™ paper and allowed to dry at ambient temperature overnight. DBS cards were stored at −80 °C.

2.3. Patient samples

DBS samples previously submitted to the British Columbia Newborn Screening Program and residual monitor specimens were utilized in keeping with the residual specimen storage and use policy for method development. Newborn screening DBS cards are usually collected 24–72 h after birth and screening profiles are generally obtained in our laboratory between 3 and 10 days after the sample collection time. Initial normal reference ranges were generated using NBS cards that had been stored at ambient temperature <2 weeks prior to further analysis. Positive first-tier NBS cards and monitor specimens were stored at −80 °C.

2.4. Sample preparation method

For analysis of MCA, single-step DBS extraction and DAABD-AE derivatization was conducted by modification of previously described sample preparation methodologies [9,26,30]. The current workflow allows for automated DBS punching into a 96-well deep well plate format, followed by extraction and derivatization in the same plate without the use of individual sample preparation tubes. Briefly, two 3.2 mm disks of DBS calibrators, QCs, or patient cards were punched into a 96-well deep well plate using a Panthera Puncher 9. To each well was then added, in sequential order, 25 μL of 1.98 μM MCA-d_3 IS in water, 25 μL of 80 mM EDC in water, 25 μL of 80 mM DMAP in acetonitrile, and 50 μL of 1.2 mM DAABD-AE in acetonitrile:water (95:5). The plate was then heated at 60 °C in a Incu-Mixer MP with shaking at 600 rpm. After 60 min 100 μL of water containing 0.1% FA was added to each well. The plate was then shaken at 1000 rpm for 1 min followed by centrifugation at 2800 rpm for 10 min. One hundred and fifty microliters of the supernatant were transferred to a new 96-well deep well plate. The plate was then centrifuged at 2800 rpm for 5 min and 10 μL was analyzed by LC-MS/MS.

For analysis of MMA and HCY, extraction from DBS cards and butylation was conducted based on a prior report with modification [18]. Briefly, two 3.2 mm disks of DBS calibrators, QCs, or patient cards were punched into a 96-well deep well plate. To each well was then added 230 μL of a methanol:water (3.6:1) solution containing MMA-d_3 (2.0 μM) and homocystine-d_8 (1.0 μM) and DTT (54.3 mM). The plate was then heated at 60 °C in a Incu-Mixer MP with shaking at 1000 rpm for 30 min. Subsequently, the plate was centrifuged at 2800 rpm for 10 min. One hundred and eighty microliters of the supernatant were transferred to a new 96-well deep well plate and the solvents removed using a microplate evaporator under a heated stream of air (55 °C) for 30 min. One hundred microlitres of 3 N HCl in 1-butanol were then added to each well and the plate was heated at 60 °C in a heated plate shaker at 1000 rpm for 35 min. The solvents were then removed using a microplate evaporator under a heated stream of air (55 °C) for 20 min. Samples were then reconstituted with 150 μL of methanol:water (1:1). The plate was vortexed at 1000 rpm for 5 min at 60 °C, placed in an ultrasonic water bath at ambient temperature for 5 min, and centrifuged at 2800 rpm for 5 min. Ten microlitres were analyzed by LC-MS/MS.

2.5. LC-MS/MS method

LC was performed using a Waters Acquity UPLC® HSS T3 column (2.1 × 100 mm, 1.8 μm, 100 Å) [Waters part no. 186003539] fitted with a 0.2 μm pre-column filter (2.1 mm) [Waters part no. 289002078] on a Waters Acquity Ultra Performance LC system (fixed loop). The mobile phases (MPs) consisted of ultrapure water containing 0.1% FA (MPA) and methanol also containing 0.1% FA (MPB) at a column temperature of 40 °C. For MCA analysis, a flow rate of 0.4 mL/min was used with the UPLC gradient program based on MPB as follows: 2% from 0 to 2.0 min, 2–60% from 2.0 to 4.0 min, 60–99% from 4.0 to 4.05 min, 99% from 4.05 to 4.50 min, 99–2% from 4.50 to 4.55 min, and 2% from 4.55 to 6.5 min. The elution time for MCA was approximately 3.9 min. For analysis of MMA and HCY, a flow rate of 0.375 mL/min was used with the UPLC gradient program based on MPB as follows: 30% from 0 to 1.2 min, 30–60% from 1.2 to 2.7 min, 60–78% from 2.7 to 2.9 min, 78% from 2.9 to 5.3 min, 78–30% from 5.3 to 5.4 min, and 30% from 5.4 to 7.8 min. The approximate elution times for HCY and MMA were 2.4 min and 4.8 min, respectively. The strong needle wash was methanol:water (90:10 v/v) containing 0.1% FA and the weak needle wash was methanol:water (20:80 v/v). Both the injector needle and 10 μL sample loop were washed with 200 μL of strong needle wash followed by 600 μL of weak needle wash prior to sample injection.

A Waters Xevo TQ MS triple quadrupole mass spectrometer was operated in positive electrospray ionization mode (ESI+). For analysis of MCA, multiple reaction monitoring (MRM) was used to quantify the DAABD-AE derivatives of MCA (499.4 > 151.1 m/z) and MCA-d_3 (502.4 > 151.1 m/z). The MRMs for MCA and MCA-d_3 were previously reported [26]. Instrument settings were optimized in-house, initially by infusion of the derivatized analytes using a syringe pump, and then by LC injection for further fine-tuning of parameters. The compound dependent parameters of cone voltage and collision energy were set to 35 V and 22 eV, respectively. Argon was used as the collision cell gas at a flow rate of 0.17 mL/min. As product ion scans of the precursor 499.4 m/z revealed only one major ion at 151.1 m/z, the same MRM for MCA with the collision energy offset to 19 eV was acquired as a qualifier transition. Source parameters were as follows: capillary voltage of 3.50 kV, source temperature 150 °C, desolvation temperature 400 °C, and desolvation gas flow rate 1000 L/hr. MRM was also used to analyze butyl esters of MMA (quantifier 231.2 > 119 m/z, qualifier 231.2 > 175.1 m/z), MMA-d_3 (234.2 > 122 m/z), HCY (quantifier 192.1 > 56 m/z, qualifier 192.1 > 90 m/z) and HCY-d_4 (quantifier

196.1 > 59 *m*/*z*, qualifier 196.1 > 94 *m*/*z*) with reference to a prior report [18]. Parameters were optimized in-house such that the cone voltage was 15 V for all MMA and HCY MRMs, with the collision energies set to 10 (MMA quantifier), 7 (MMA qualifier), 25 (HCY quantifier), 13 (HCY qualifier) eV respectively. The collision cell gas was set at a flow rate of 0.17 mL/min. Source parameters for MMA and HCY were as follows: capillary voltage of 3.50 kV, source temperature 150 °C, desolvation temperature 550 °C, and desolvation gas flow rate 1000 L/h.

2.6. Data analysis

Data was analyzed using MassLynx software version 4.1 (Waters), Excel (Microsoft), cp-R [31], and Prism version 8 (GraphPad).

2.7. Method validation

Assay validation, as described in the Results section, was conducted with reference to appropriate guidance documents for laboratory developed tests [32–34].

3. Results

3.1. Linearity and imprecision

Linear calibration curves with 1/x weighting were analyzed in duplicate in all analytical runs (average R^2 from 10 runs – MCA R^2 = 0.996; MMA R^2 = 0.998; HCY R^2 = 0.993). The presence of endogenous MMA and HCY was corrected for by calculation of the average calibration slope and *y*-intercept using nominal spiking concentrations from 8 analytical batches followed by determination of the *x*-intercept (endogenous concentration). Calibrator and QC concentrations were then adjusted accordingly by addition of the calculated endogenous concentrations (MMA, 0.61 μM; HCY, 9.43 μM) to the nominal spiking concentrations into blood. Correction for endogenous MCA was not required as no peak was observed in the DBS blank. Assay within-run and between-run imprecision, determined using QC samples, were 4.2–6.8% and 5.3–10.8%, respectively (Table 1).

Imprecision was assessed by analysis of DBS cards prepared from blood spiked at 3 concentrations (1.5, 8.0, 20 μM). Thirty-six replicates across 10 runs.

3.2. Carryover

Carryover was assessed by injecting the highest standard 5 times followed by 2 blank samples. No carryover was observed.

3.3. Recovery

DBS cards were prepared using blood spiked with MCA, MMA, and HCY at three different concentrations (1.5, 8.0, and 20 μM for MCA; 2.5, 8.0, and 20 μM for MMA and HCY). Recovery was then compared against blank DBS cards with addition of aqueous solutions of the analytes equivalent to the blood spiking concentrations at the time of sample preparation. Recoveries were acceptable, ranging between 90.2 and 109.4% (Table 2). Additionally, recoveries from the spiked DBS cards were calculated against the same concentration of analytes without the presence of DBS to evaluate variability in derivatization caused by the presence of matrix. No meaningful differences were observed, with recoveries from these additional experiments ranging between 88.1 and 110.7%.

Recovery was assessed by analysis of DBS cards prepared from blood spiked at 3 concentrations (1.5, 8.0, and 20 μM for MCA; 2.5, 8.0, and 20 μM for MMA and HCY) against blank DBS punches with the addition of aqueous solutions of the analytes into the 96-well plate at the time of extraction (analysis in triplicate).

3.4. Limits of quantitation and detection

Lower limits of quantitation (LLOQ) for MCA, MMA, and HCY were acceptable for NBS. For MCA the LLOQ was set at 0.50 μM (median S/N > 6.3:1; between-run CV 8.7%, n = 10) based on results from spiked DBS cards. The lower limit of detection (LLOD), based on a S/N of 3:1 for MCA, was approximately 0.25 μM (median S/N = 3.4; between-run CV 11.4%, n = 8). Alternative approaches were taken for approximation of the LLOQ for MMA and HCY, as significant endogenous amounts are present and were observable in blank DBS cards prepared from whole blood. For MMA, the LLOQ was set at the endogenous level, which was approximately 0.56 μM (median S/N = 19.2:1; between-run CV 14.7%, n = 10). A practical LLOD for MMA was estimated from the lowest specimen signal observed from 154 unknown neonatal DBS cards to be approximately 0.448 μM (S/N = 6.2:1). For HCY, an acceptable LLOQ below endogenous levels in the blank (approximately 9.43 μM) was estimated by calculation of the concentration percent difference of 1, 4, and 8 DBS punches compared to 2 DBS punches from the same neonatal specimen (n = 5 different neonatal specimens). Dilution concentrations for HCY ranged between 1.32 and 15.4 μM from all specimens with a median percent error of 6.4% (min, max; 0.7%, 18.2%). The lowest observed neonatal specimen (2 DBS punches) from this experiment had a concentration of 2.90 μM (S/N 18.9:1) and was acceptable as a screening LLOQ. From 154 random normal neonatal DBS cards, the lowest HCY concentration observed was 2.04 μM (S/N of 17.6:1) and was set as the LLOD for practical purposes. MRM dwell times were set such that a minimum of 15 data points were collected to define all analyte peaks at the LLOQ.

Anticipating the scenario where results from a second-tier screen might be higher than the calibration ULOQ, an experiment was performed using 1, 2, 3, and 4 DBS punches of the highest calibrator (duplicate for MCA, and triplicate for MMA & HCY). For all analytes, the bias to nominal and the CV were both <10% up to 100 μM MCA, 101 μM MMA, and 119 μM HCY.

Table 1
Imprecision for MCA, MMA, and HCY analysis.

Analyte	Grand mean (μM)	Within-run CV (%)	Between-run CV (%)
MCA	1.55	5.3	8.1
	8.23	5.8	5.3
	20.3	6.8	9.2
MMA	2.11	6.4	7.1
	8.73	4.9	10.5
	20.7	4.2	6.9
HCY	11.0	4.9	7.9
	17.6	5.8	10.8
	29.9	6.1	5.9

Table 2
Recovery of MCA, MMA, and HCY from DBS cards.

Analyte	Expected (μM) in blank DBS + aqueous spike	Measured (μM) in spiked DBS	Recovery (%)
MCA	1.66	1.5	90.2
	8.42	7.63	90.6
	20.9	19.2	91.8
MMA	3.06	3.35	109.4
	8.34	8.92	106.9
	20.2	20.4	100.9
HCY	11.2	11.9	106.8
	15.7	15.9	101.3
	26.9	28.0	104.0

3.5. Matrix effects and interferences

Assessment of ion suppression or enhancement was accomplished by post-column infusion of IS along with the LC injection of neonatal DBS specimens (n = 5) prepared without addition of IS (Supplemental Fig. 1A & B). The elution profile of glycerophosphocholines (GPCho's), lipids known to cause matrix ionization effects, was monitored using the pseudo MRMs 184.1 > 184.1 *m/z* (GPCho's) and 104.1 > 104.1 *m/z* (2-lyso GPCho's) in ESI + mode as previously described [35] (Supplemental Fig. 1C & D). In-source fragmentation of the phosphocholine head group results in a common ion for the mono- and disubstituted GPCho's at 184.1 *m/z*, with 2-lyso GPCho's being further identified by the prominent ion at 104.1 *m/z* [35]. The 184.1 *m/z* and 104.1 *m/z* ions were isolated by both quadrupoles with the cone voltage and collision cell energy parameters optimized in-house to 35 V and 7 eV, respectively. Elution of GPCho's and 2-lyso GPCho's corresponded to areas of ion suppression or enhancement in the LC gradients and were not at the elution times of MCA, MMA, and HCY. LC parameters were developed such that chromatographic resolution was achieved from observable interferences (Fig. 1A–C i). MMA was resolved from the endogenous isomeric metabolite succinic acid, a known interference. The variability in MRM transition ratios was investigated during validation for concentrations above the LLOD in order to provide further diagnostic confidence in elevated second-tier screens (Fig. 1A–C ii).

3.6. Stability studies

Stock solutions of MCA, MMA, and HCY stored at −80 °C were stable for 381 days with deviation from nominal being −3.9%, 1.5%, and 0.25%, respectively (5 replicates). The stability of DBS cards stored at ambient temperature was assessed at 0, 1, 4, 7, 13, 35, and 69 days (Supplemental Fig. 2). MCA, MMA, and HCY were determined to be adequately stable for at least 13 days at ambient temperature. Significant decreases (deviation >10% compared to day 0) in MCA concentration were observed at days 35 and 69. Deviation of MMA and HCY concentration from day 0 values was <10% at all time points up to 69 days. A statistically significant increase (maintaining <10% deviation) in analyte concentration was observed for MMA (20.6 μM) and HCY (29.4 μM) only at 35 days of ambient storage. DBS card freeze–thaw stability for 5-cycles (minimum of 1 hr at ambient temperature on 5 different days with storage at −80 °C) deviated from nominal concentration by <10% for all analytes (Supplemental Table 1). The stability of DBS cards stored at −80 °C was assessed after 449 days and found to be adequate with deviations of <10% from nominal for all analytes, except for HCY having a deviation of −16.9% at a concentration of 10.9 μM (Supplemental Table 2).

Batch performance was acceptable following storage of prepared samples at ambient temperature for 2 h. Reinjection of prepared samples stored in a refrigerated autosampler at 2 and 5 days indicated reproducible data, with the median difference between

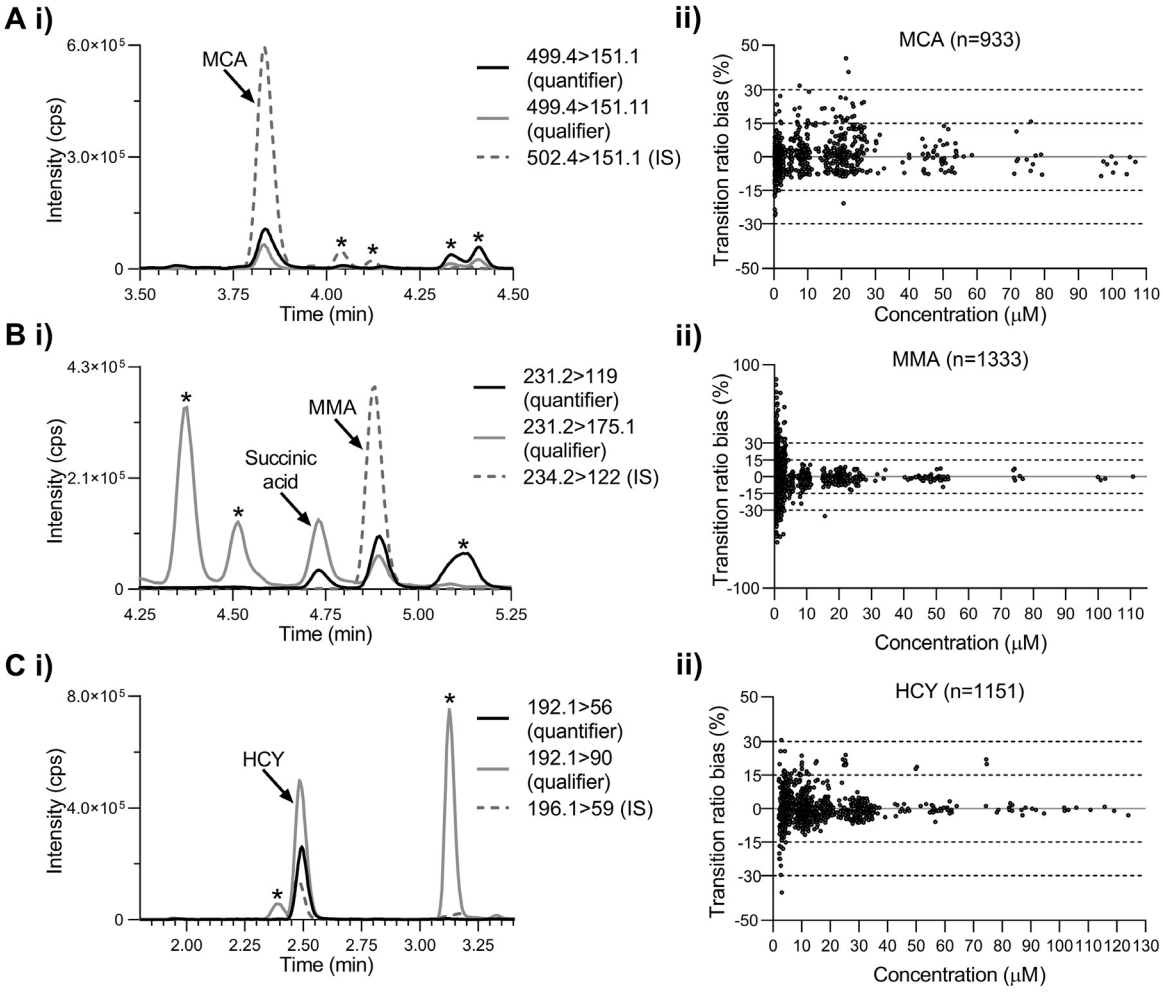

Fig. 1. Chromatographic separation from interferences and transition ratio monitoring. (A–C, i) A low QC sample chromatogram is shown for MCA (1.5 μM), MMA (2.11 μM), and HCY (10.9 μM). Unknown peaks indicated by *. (A–C, ii) MCA, MMA, and HCY quantifier/qualifier transition ratio monitoring is shown for standards, QCs, and specimens during validation.

original and reinjection ranging from −3.5% to 1.8% and Passing Bablok R^2 > 0.995 for all analytes at both reinjection time points compared to the initial injection.

3.7. Evaluation of clinical specimens

As part of the evaluation for clinical screening utility, a retrospective analysis was conducted using residual NBS cards of infants screened negative for all conditions on the screening panel (controls), and available specimen cards from NBS diagnosed patients including both the initial NBS specimen or later specimens collected when on treatment (Table 3). Results of second-tier analysis were able to separate unaffected controls from pathological profiles of patients who screened positive on our current single-tier algorithm. Biochemical patterns clearly differentiated between confirmed cases of propionic acidemia (caused by propionyl-CoA carboxylase deficiency), methylmalonic acidemia (caused by methylmalonyl-CoA mutase deficiency), homocystinuria (caused by CBS deficiency), and cobalamin C deficiency. The proposed two-tier screening work flow integrating these new tests at our site is shown in Supplemental Fig. 3. Representative second-tier LC-MS/MS chromatograms from normal and pathological samples are presented in Fig. 2.

3.8. Interlaboratory method comparison and external proficiency samples

A method comparison was performed for the current derivatized analytical approach against non-derivatized methods from another NBS laboratory (Fig. 3). DBS specimens (n = 37) were analyzed as a blinded comparison, which included samples having normal and elevated levels of MCA, MMA, and HCY. All data points were included in the regression analysis including values below the LLOQ. The LLOQ for MCA, MMA, and HCY from our derivatization approach was set at 0.50, 0.56, and 2.90 μM, respectively, and from the non-derivatized comparison method at 1.0, 1.0, and ~5.5 μM, respectively. Keeping or discarding values below the LLOQ did not change the overall acceptable correlation outcomes (MCA R = 0.9912 vs. R = 0.9863; MMA R = 0.9953 vs. R = 0.9941; HCY R = 0.9613 vs. R = 0.9482). Results below the LLOQ, indicated on the difference plots (Fig. 3A–C ii) by a dashed vertical line, had higher discordance, but the same negative screening result outcome. This is a limitation of this screening assay comparison, as many normal DBS specimen concentrations do fall below the LLOQ. Both assays could detect grossly elevated MCA, MMA, and HCY in DBS adequately for NBS purposes. External proficiency samples, kindly provided by the US Centre for Disease Control Newborn Screening Quality Assurance Program (CDC NQSAP) (courtesy of Konstantinos Petritis), were analyzed (n = 4). Correlation was found to be excellent (Table 4) and our results at all concentration levels were within one standard deviation of the mean determined from all reporting laboratories for the proficiency cycle [36].

4. Discussion

MCA, MMA, and HCY are known analytes useful for the detection and differentiation of disorders of propionate, methionine, and cobalamin metabolism for NBS [1]. These disorders are initially detected by abnormal C3, methionine, and associated ratios in the rapid first-tier screen, but these markers alone lack sufficient clinical specificity and sensitivity. For example, a retrospective review of test performance for our current single-tier algorithm in British Columbia for disorders of propionate metabolism (C3 > 4 μM and C3/C2 > 0.20 and C3/C0 > 0.25) from 2010 to 2019 revealed an acceptable PPV of 45% (13 cases in 29 positive screens). However,

Table 3
Retrospective analysis of NBS specimens and samples collected on treatment.

Sample Type	NBS or treated	C3 (μM)	C3/C2	C3/C0	Met (μM)	Met/Phe	MCA (μM)[a,b]	MMA (μM)[a,b]	HCY (μM)[a,b]
Control[c]	NBS	<4.0	<0.20	<0.25	<70	<1.5	0.140 (0–0.396)	0.702 (0.448–1.06)	3.70 (2.04–6.46)
Propionic academia (2 specimens from 1 case)	NBS	20.7 (16.4, 24.9)	1.03 (0.66, 1.39)	0.89 (0.43, 1.34)	23 (18, 28)	0.5 (0.5, 0.5)	26.5 (13.1, 39.9)	0.527 (0.426, 0.627)	3.29 (2.81, 3.77)
Cystathionine β-synthase deficiency (2 specimens from 1 case)	NBS	1.35 (0.9, 1.8)	0.05 (0.04, 0.06)	0.04 (0.02, 0.06)	565 (108, 1022)	16.3 (2.0, 30.5)	0.006 (0, 0.012)	0.608 (0.551, 0.665)	64.6 (45.8, 83.5)
Methionine adenosyltransferase deficiency (2 specimens from 1 case)	NBS	0.85 (0.7, 1.0)	0.035 (0.03, 0.04)	0.03 (0.02, 0.04)	78 (74, 82)	1.65 (1.6, 1.7)	N.D.	0.815 (0.814, 0.815)	6.95 (6.54, 7.36)
Maternal vitamin B12 deficiency (2 cases)[d]	NBS	7.75 (4.9, 10.6)	0.25 (0.23, 0.27)	0.27 (0.25, 0.28)	23.5 (22, 25)	0.4 (0.4, 0.4)	1.08 (0.117, 2.04)	0.886	4.22
Propionic academia (39 specimens from 6 cases)	Treated						7.78 (2.04–30.2)	0.633 (0.400–1.09)	3.85 (1.40–7.80)
Methylmalonyl-CoA mutase deficiency (10 specimens from 3 cases)	Treated						0.241 (0.044–1.55)	2.48 (1.46–42.4)	3.42 (2.62–5.27)
Cystathionine β-synthase deficiency (1 case)	Treated						0.053	0.580	106
Methionine adenosyltransferase deficiency (1 case)	Treated						N.D.	0.750	27.7
Cobalamin C (10 specimens from 4 cases)	Treated						0.084 (0.058–0.283)	2.41 (1.32–4.12)	16.8 (8.88–30.5)

[a] Data is median (min – max range).
[b] Concentrations are estimates below LLOQ (MCA = 0.5 μM; MMA = 0.56 μM; HCY = 2.90 μM).
[c] n = 122 controls for MCA assay; n = 154 controls for MMA & HCY assay.
[d] Only the MCA assay was performed for one of the cases.

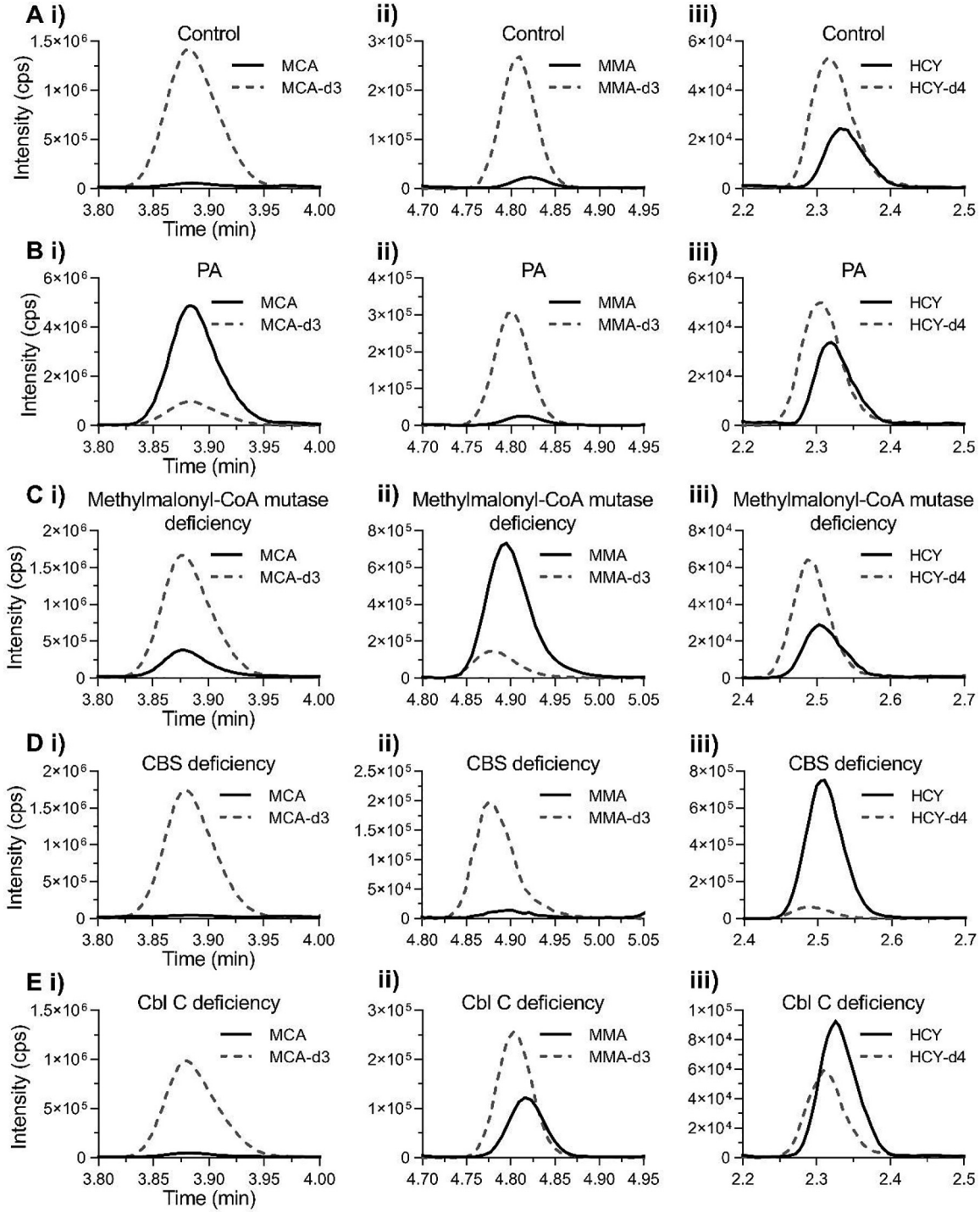

Fig. 2. Representative LC-MS/MS chromatogram profiles from control and confirmed cases. (A, i-iii) Unaffected control NBS specimen, (B, i-iii) Propionic acidemia NBS specimen, (C, i-iii) Methylmalonyl-CoA mutase deficiency specimen from treated patient, (D, i-iii) CBS deficiency NBS specimen, (E, i-iii) Cbl C deficiency specimen from treated patient.

over that time period, two infants with methylmalonyl-CoA mutase deficiency were missed by this algorithm, confirming that sensitivity was being sacrificed to maintain an acceptable PPV. Implementation of second-tier NBS for the more diagnostic markers, MCA, MMA, and HCY, increases overall end-to-end screening performance by allowing for more conservative first-tier cut-off values to reduce the potential for false-negative screens without increasing the false-positive rate. In addition, these second-tier test results narrow the relatively broad differential diagnosis for the initial C3 and/or methionine elevations, allowing for a more rapid and targeted approach to confirm any abnormal screening results.

Analysis of MCA, MMA, and HCY in DBS can be challenging due to the limited neonatal DBS specimen size in combination with inherent differences in the chemical functionality of these molecules. MMA is an achiral di-carboxylic acid, whereas MCA is a tri-carboxylic acid containing a tertiary alcohol group, and is present in the human body as two diastereomers [14]. HCY is an α-amino acid containing a thiol side chain and is present in plasma,

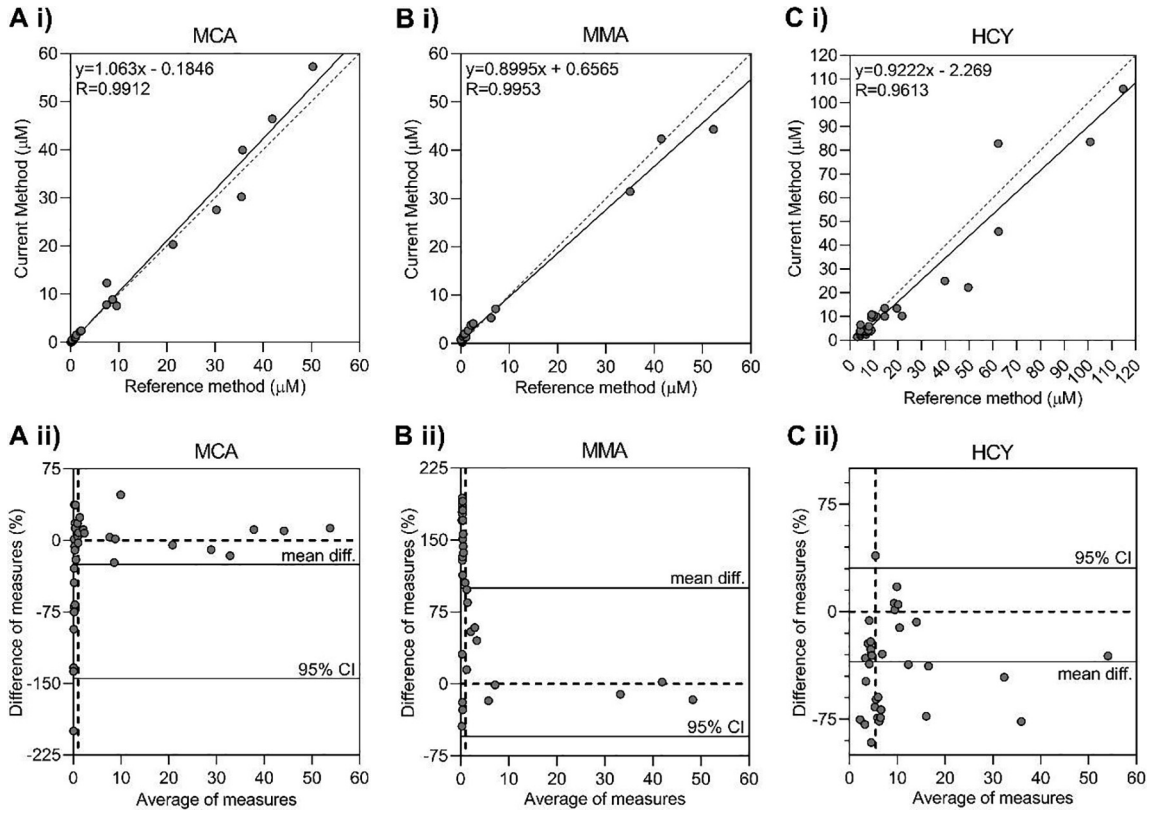

Fig. 3. Interlaboratory method comparison. Analyte concentrations from the current LC-MS/MS method using derivatization were compared against a reference LC-MS/MS method without derivatization in use at another laboratory (n = 37). (A–C, i) Deming regression with dashed line indicating line of identity. (A–C, ii) Difference plots with mean difference and 95% confidence interval (CI) indicated by solid horizontal lines. Dashed vertical lines indicate the higher analyte LLOQ of the two methods being compared (MCA and MMA 1 μM; HCY ~5.5 μM).

Table 4
Results comparison with CDC NQSAP proficiency testing.

Analyte	Nominal spiking concentration (μM)[a]	Group Mean ± total SD[a]	Measured concentration[c] (μM)	R[b]
MCA	1	1.0 ± 0.55	0.667	0.9955
	2.5	2.02 ± 0.67	1.76	
	10	7.73 ± 2.24	6.04	
	25	19.4 ± 5.69	19.4	
MMA	2	2.23 ± 0.74	2.21	0.9989
	5	4.62 ± 1.13	4.38	
	20	18.1 ± 4.52	16.0	
	50	44.0 ± 10.9	43.9	
HCY	0	13.4 ± 7.06	9.72	0.9977
	10	25.4 ± 13.4	17.0	
	50	71.0 ± 38.4	45.5	
	100	130.1 ± 66.7	91.7	

[a] Values from CDC NQSAP 2019 set 1.
[b] Correlation of nominal spiking concentration and measured concentration.
[c] Our method (n = 4).

primarily in the protein-bound disulfide form [37]. Analysis of total HCY requires sample treatment with a disulfide reducing agent, such as DTT, to release protein-bound HCY. Several NBS second-tier approaches to analyzing combinations of MCA, MMA, and HCY in DBS cards by LC-MS/MS have been reported, including analysis of the non-derivatized molecules [27,28], butylation [18], and carbodiimide mediated derivatization with DAABD-AE [26].

Previously published methods were initially investigated with the goal of developing an approach that was amenable to automated DBS punching into a 96-well polypropylene plate for subsequent sample preparation and LC-MS/MS analysis in the NBS laboratory setting. HCY, in its non-derivatized form, was initially observed to have a generally low chromatographic retention on standard reverse phase columns. Acceptable retention for HCY was obtained using hydrophilic interaction chromatography (HILIC), however suboptimal chromatographic performance was observed for MCA and MMA on the same column. Additionally, MCA in its non-derivatized form displayed poor electrospray ionization (ESI) resulting in low sensitivity and variable performance, consistent with a previous report [26]. Using elevated amounts of FA (0.4% vs 0.1%) in the LC MPs increased the sensitivity for MCA, but overall performance remained inadequate for our screening requirements. Butylation is a standard esterification strategy for acylcarnitine and amino acid first-tier analysis in NBS laboratories [38], and is potentially useful for enhancing both LC column retention and ESI+ sensitivity for MCA, MMA, and HCY. For MCA, our

preliminary experiments indicated that butylation was kinetically slow, an observation corroborated by a prior indication [26]. HCY displayed excellent retention on reverse phase columns after butylation and MMA was separable from the isomer succinic acid. Interestingly, the butyl esters were observed to have a binding affinity to 96-well polypropylene plates if the reconstitution solvent was only water, which was reversible on addition of methanol. Peak shape for butylated HCY deteriorated with increased amounts of methanol (or acetonitrile) in the reconstitution solvent; methanol:water (50:50) provided the best balance of results. Butylation in individual glass tubes was not amenable to rapid laboratory workflows at our site.

Derivatization of MCA in DBS using DAABD-AE with modifications to a method based on a previous report [26] was found to provide adequate MS sensitivity and analytical reproducibility. DAABD-AE is a molecule containing an ionizable tertiary amine group designed for enhanced ESI+ detection [39]. Attempts at derivatizing MMA and HCY using DAABD-AE were unsuccessful, presumably a result of steric hinderance and electronic factors stopping the derivatization reaction from proceeding. Overall, butylation of MMA and HCY, and derivatization of MCA using DAABD-AE was deemed the most robust approach using our instrumentation. Our initial investigations had indicated that derivatization improved analytical sensitivity relative to non-derivatized approaches. Similarly, the interlaboratory comparison conducted as part of the validation also suggested lower LLOQs were obtainable by derivatization relative to non-derivatized methods, bearing in mind instrument and sample preparation differences. MPs for our analysis were also optimized such that prepared samples from both MCA and MMA & HCY derivatization approaches could be analyzed on the same LC column. The LC separations avoided the use of ion pairing reagents in the MPs, which differed from previous reports on MCA analysis using DAABD-AE [9,26,30]. The use of ion pairing reagents can be problematic as they can chronically contaminate the LC system, potentially compromising subsequent tests on the same instrument. The approach reported here utilized a standard 96-well plate, an improvement on prior methods that utilized individual sample preparation tubes [18,26].

The technical performance of the method validation was found to be acceptable with imprecision, linearity, storage stability, and limits of quantitation all meeting predefined performance goals. Clinical utility of this methodology for NBS purposes was demonstrated by retrospective analysis of normal and known abnormal specimens, an interlaboratory comparison, and acceptable analytical performance using external proficiency samples. Estimated second-tier normal ranges were comparable to previous NBS reports [18,26,27]. Elevations in diagnostic analyte patterns for affected specimens available at our site were consistent with the known primary defect and would be flagged for further clinical decision making and follow-up based on our established normal ranges from unaffected control samples. Of note, some affected specimens were from patients undergoing treatment, and the concentrations for MMA, MCA or HCY were most likely lower than would be observed at birth, however analyte patterns remained clearly abnormal. As disorders associated with abnormal propionate, methionine, and cobalamin metabolism are rare, second-tier concentration ranges and screening cut-offs for MCA, MMA, and HCY will be periodically assessed and adjusted from longitudinal screening data.

5. Conclusion

We developed a simple and robust LC-MS/MS workflow for the determination of MCA, MMA, and HCY in DBS for the purpose of second-tier neonatal screening. Sample preparation was performed in 96-well plates with automated DBS punching. Both MCA and MMA & HCY assays were performed using the same LC column and the same MPs, which allowed for both tests to be run in series on the same instrument. The application of this second-tier approach in NBS will allow us to establish more conservative first-tier screening cut-offs without increasing the false-positive rate from abnormal C3 and methionine first-tier levels. We anticipate our second-tier screening approach will lessen the number of false-negative screens, and reduce parental anxiety caused by neonatal resampling.

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.

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Appendix A. Supplementary data

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