Clinical Chemistry 00:0
1–12 (2023)

Multiplexing Homocysteine into First-Tier Newborn Screening Mass Spectrometry Assays Using Selective Thiol Derivatization

C. Austin Pickens,[a] Elya Courtney,[a] Samantha L. Isenberg,[a] Carla Cuthbert,[a] and Konstantinos Petritis [a]*

BACKGROUND: Classical homocystinuria (HCU) results from deficient cystathionine β-synthase activity, causing elevated levels of Met and homocysteine (Hcy). Newborn screening (NBS) aims to identify HCU in pre-symptomatic newborns by assessing Met concentrations in first-tier screening. However, unlike Hcy, Met testing leads to a high number of false-positive and -negative results. Therefore, screening for Hcy directly in first-tier screening would be a better biomarker for use in NBS.

METHODS: Dried blood spot (DBS) quality control and residual clinical specimens were used in analyses. Several reducing and maleimide reagents were investigated to aid in quantification of total Hcy (tHcy). The assay which was developed and validated was performed by flow injection analysis–tandem mass spectrometry (FIA-MS/MS).

RESULTS: Interferents of tHcy measurement were identified, so selective derivatization of Hcy was employed. Using N-ethylmaleimide (NEM) to selectively derivatize Hcy allowed interferent-free quantification of tHcy by FIA-MS/MS in first-tier NBS. The combination of tris(2-carboxyethyl)phosphine (TCEP) and NEM yielded significantly less matrix effects compared to dithiothreitol (DTT) and NEM. Analysis of clinical specimens demonstrated that the method could distinguish between HCU-positive, presumptive normal newborns, and newborns receiving total parenteral nutrition.

CONCLUSIONS: Here we present the first known validated method capable of screening tHcy in DBS during FIA-MS/S first-tier NBS.

Introduction

Homocystinuria (HCU) results from the inability to convert homocysteine (Hcy) to cystathionine due to enzyme (1) or vitamin B12 deficiencies (2), causing an elevation of Hcy in blood and urine (3). Classical HCU [OMIM #236200] results from deficient activity in the transsulfuration enzyme cystathionine β-synthase (CBS), a vitamin B_6-dependent enzyme, causing elevated levels of Met and Hcy. The global rate of HCU is 1 in 200 000 to 335,000, and its frequency varies based on geographic location, (1 in 65 000 in Ireland; 1 in 17 800 in Germany; and 1 in 1800 in Qatar (4)). Complications of untreated HCU include ocular issues (i.e., ectopia lentis), skeletal abnormalities, dysfunction and abnormalities of the vascular system (e.g., thromboembolism and vascular occlusions), developmental and intellectual disabilities, failure to thrive, and symptoms similar to Marfan syndrome (1, 5). Decreased morbidity and improved outcomes are associated with early interventions in HCU-positive (HCU+) individuals (6–8), with treatments ranging from vitamin B_6 and/or betaine supplementation to Met-controlled and/or low-protein diets (1, 5). Identification of pre-symptomatic HCU+ individuals is possible through newborn screening (NBS), the routine screening for dozens of inborn errors of metabolism shortly after birth using dried blood spot (DBS) analyses. HCU was added to the US Recommended Uniform Screening Panel (RUSP) in 2006 (9).

Current first-tier NBS methods for HCU utilize Met as a biomarker. In some NBS laboratories, presumptive HCU+ are reflexed to second-tier screening to assess Hcy concentrations. However, Met concentrations are often not elevated enough 24 to 48 h after birth

[a]Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA, United States.
*Address correspondence to this author at: Division of Laboratory Sciences, Centers for Disease Control and Prevention, 4770 Buford Hwy NE, S110-3, Atlanta, GA 30341, United States. E-mail kpetritis@cdc.gov.
Disclaimer: The findings and conclusions in this study are those of the authors and do not necessarily represent the views of the US Department of Health and Human Services, or the US Centers for Disease Control and Prevention. Use of trade names and commercial sources is for identification only and does not constitute endorsement by the US Department of Health and Human Services, or the US Centers for Disease Control and Prevention.
Received August 8, 2022; accepted January 9, 2023.
https://doi.org/10.1093/clinchem/hvad007

to reach cutoff levels in first-tier screening in HCU+ newborns (5). In 2019, the HCU Network America presented at the Advisory Committee on Heritable Disorders in Newborns and Children (ACHDNC), estimating that around 50% of HCU cases are missed using current NBS practices (10). There are numerous case studies in the literature of children and adults identified as HCU+ only after exhibiting clinical symptoms because they received a false-negative NBS result or because HCU screening had not yet been established (10–13). To reduce risk of false negatives, current options for laboratories are limited to lowering Met cutoffs which leads to lower specificity. In order to mitigate for the lower specificity, labs can use the methionine to phenylalanine (Met/Phe) ratio to slightly increase test specificity (14), or to perform second-tier screening for Hcy which leads to a dramatic specificity increase. However, such options can lead to increased false-positive rates, lower throughput, or additional equipment and personnel needs.

In addition to the aforementioned issues regarding Met as a biomarker for HCU, Met is also added to total parenteral nutrition (TPN) formulations administered to premature babies in the intensive care unit, which can artificially elevate amino acid concentrations causing false-positive NBS results. Babies receiving TPN are typically repeat screened within 2 weeks of life and/or after discontinuing TPN. There is no clear rationale in the literature why direct Hcy screening is not included in first-tier NBS assays. One would imagine the reason is related to the complex chemistry of Hcy compared to other NBS biomarkers, since Hcy has a thiol group and is present in both oxidized and reduced forms in the blood. Roughly 1% to 2% of Hcy exists in the blood as free Hcy (fHcy) monomer, with the remaining 98% to 99% of Hcy oxidized as dimers (e.g., Hcy–Hcy dimer [dHcy]) or incorporated into proteins such as albumin (3). To determine the concentration of the total Hcy (tHcy) pool in blood, all Hcy must be reduced to the monomer form. However, given the limitations of using Met as a biomarker of HCU, a paradigm shift towards Hcy as a first-tier biomarker is needed.

Here we present the only flow injection analysis–tandem mass spectrometry (FIA-MS/MS) first-tier NBS method that directly quantifies tHcy from DBS, using tris(2-carboxyethyl)phosphine (TCEP) as a reducing agent and N-ethylmaleimide (NEM) for selective derivatization of Hcy. The method was used to analyze residual newborn specimens, including presumptive normals, those reporting administration of total parenteral nutrition (TPN), and HCU+ specimens, and demonstrated the clinical ability to distinguish HCU+ from the other groups. The ability to screen tHcy during first-tier NBS is a significant step toward reducing HCU false-negative rates, which will enable early identification and intervention to reduce HCU-associated morbidity and mortality.

Materials and Methods

SAMPLE TYPE AND PREPARATION

Detailed information on quality control (QC), linearity materials, and clinical specimens used in analyses; method optimization; extraction methods; solvents and chemicals; and unlabeled biomarkers and isotopically labeled internal standards (ISs) is provided in the online Supplemental Material. Certified values for linearity materials and QCs are presented in online Supplemental Tables 1 and 2. A graphical overview of the sample preparation method is presented in Fig. 1. In all analyses, a 3.2 mm punch was taken from a DBS, which corresponded to approximately 3.1 μL of blood (15).

ACQUISITION, QUANTIFICATION, AND DATA ANALYSIS

Electrospray ionization (ESI) source parameters, peak integrations, quantification methods, biomarker parent and product *m/z*, the IS employed for quantification, and the cone and collision voltages are presented in the Supplemental Material. Ion suppression plots were generated by extracting peak areas of IS using the open-source software Skyline, then visualized using R. All quantified data and peak area data were exported as spreadsheets and imported into R version 4.0.2 (16) for analysis and visualization.

Results

Preliminary data indicated tHcy concentrations were higher than expected in reduced DBS extracts, which led us to hypothesize that interferences were present in the DBS matrix. Online Supplemental Fig. 1, A presents a high-resolution MS (HRMS) parallel reaction monitoring spectrum of the Hcy transition (*m/z* 136 > 90), which indicated numerous isobaric product ions under FIA conditions using nominal mass-resolving MS (i.e., triple quadrupole platforms). Using metabolomic databases, we were unable initially to identify all compounds present in the spectra of Supplemental Fig. 1, A. Using nano-ESI capillary electrophoresis (CE) coupled to HRMS allowed us to determine that the dominant Hcy interferences were, in fact, related to isotopically labeled ISs that were present in the extraction working internal standard solution (WISS) (online Supplemental Fig. 1, B). The most abundant interferent was Met-$^{2}H_3$ (Met-D_3, *m/z* 153.0777) IS, which undergoes in-source fragmentation to a *m/z* 136.0507 fragment ion that dissociates to *m/z* 90.457 (Supplemental Fig. 1, A, B, and C). Since these ISs are used globally in first-tier NBS assays, we explored selective derivatization of thiols

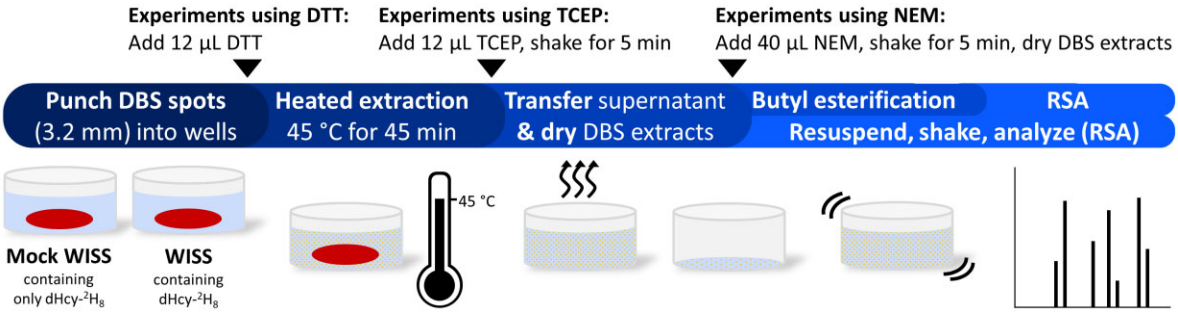

Fig. 1. Sample preparation workflow used to investigate reducing and maleimide derivatizing agents. In all analyses, 3.2 mm DBSs of quality control materials, proficiency materials, and residual clinical specimens were punched into 96-well plates. Arrows (stemming from "Experiments using...") indicate where the first-tier screening method was modified to investigate reducing agents and maleimide derivatizing agents. DBSs were extracted using 100 µL WISS containing ISs for amino acids, acylcarnitines, nucleotides, homocystine (dHcy-$^2H_8$), and other biomarkers, along with formic acid and hydrazine hydrate. A mock WISS containing dHcy-$^2H_8$ along with formic acid and hydrazine hydrate was also used to investigate the presence of interferences from IS. In experiments using DTT, 12 µL of a DTT solution was added to the DBS containing WISS, then incubated for 45 min at 45°C. In experiments using TCEP, the DBS and WISS were incubated for 45 min at 45°C, then TCEP was added to the DBS extract solution and shaken for 5 min at room temperature. Sample extracts were then transferred, dried under nitrogen gas, and the DBS extracts were resuspended in 40 µL of a maleimide solution before being shaken for 5 min at room temperature. Maleimide treated extracts were then dried. Non-butyl ester extracts were resuspended in mobile phase, then analyzed by FIA–MS/MS) In butyl ester-derivatized analyses, DBS extracts were resuspended in acidified butanol, then placed in an oven for 20 min. Butylated DBS extracts were then dried, resuspended in mobile phase, and then analyzed by FIA–MS/MS.

using several reagents to shift Hcy into a separate transition without interferences. Maleimides were most compatible with the typical first-tier NBS assay and biomarkers, and of the maleimides investigated, NEM was most compatible and advantageous for use in the assay (Supplemental Material). Online Supplemental Fig. 2 demonstrates the reaction between Hcy and Hcy-$^2H_4$ (Hcy-D_4) with NEM, to form Hcy-NEM (*m/z* 261.0901) and Hcy-D_4-NEM (*m/z* 265.1155). Both Hcy-NEM and Hcy-D_4-NEM had lower mass range product ions (*m/z* 56.0505 and 60.0755, respectively) and higher mass range product ions (*m/z* 215.0847 and 219.1100, respectively).

Figure 2 demonstrates the impact of Hcy interferences on tHcy quantification, along with the advantages of using NEM to selectively derivatize Hcy. The workflow in Fig. 1 was used to quantify tHcy from low QC samples enriched with dHcy, using either 100 mM dithiothreitol (DTT) or 30 mM TCEP, and either in the absence or presence of 40 mM NEM. DBS extracted with WISS and treated with either DTT or TCEP in the absence of NEM had higher than expected tHcy concentrations due to interferences. On the other hand, DBS samples extracted with a mock WISS containing only dHcy-$^2H_8$ (dHcy-D_8) had tHcy concentrations closer to expected values. DBS extracted with WISS and treated with either DTT and NEM or TCEP and NEM had tHcy concentrations closer to the expected value, successfully demonstrating the mitigation of interferences by selective Hcy derivatization. Both transitions of *m/z* 56/60 and 215/219 pairs were monitored for Hcy-NEM/Hcy-D_4-NEM, with improved precision and sensitivity in the *m/z* 56/60 pair. Therefore, we decided to quantify all subsequent Hcy-NEM data with the *m/z* 56/60 unlabeled/labeled pair, with the option of using the 215/219 pair as qualifying ions if elected by a laboratory. These observations were consistent in both non-butyl ester derivatized data (Fig 2, A) and butyl ester derivatized data (Fig 2, B). Overall, the use of NEM with either TCEP or DTT resulted in roughly 5× more signal when compared to the Hcy-D_4 IS (online Supplemental Table 3).

Since both reducing and thiol derivatizing agents were added to our existing assay, it was important to investigate any deleterious effects of these added reagents on other biomarkers analyzed in the assay. Figure 3 displays the impact of adding reducing agents and reducing agents plus NEM to the existing assay with data normalized to the control in non-butyl ester derivatized data. Online Supplemental Fig. 3 displays the impact of

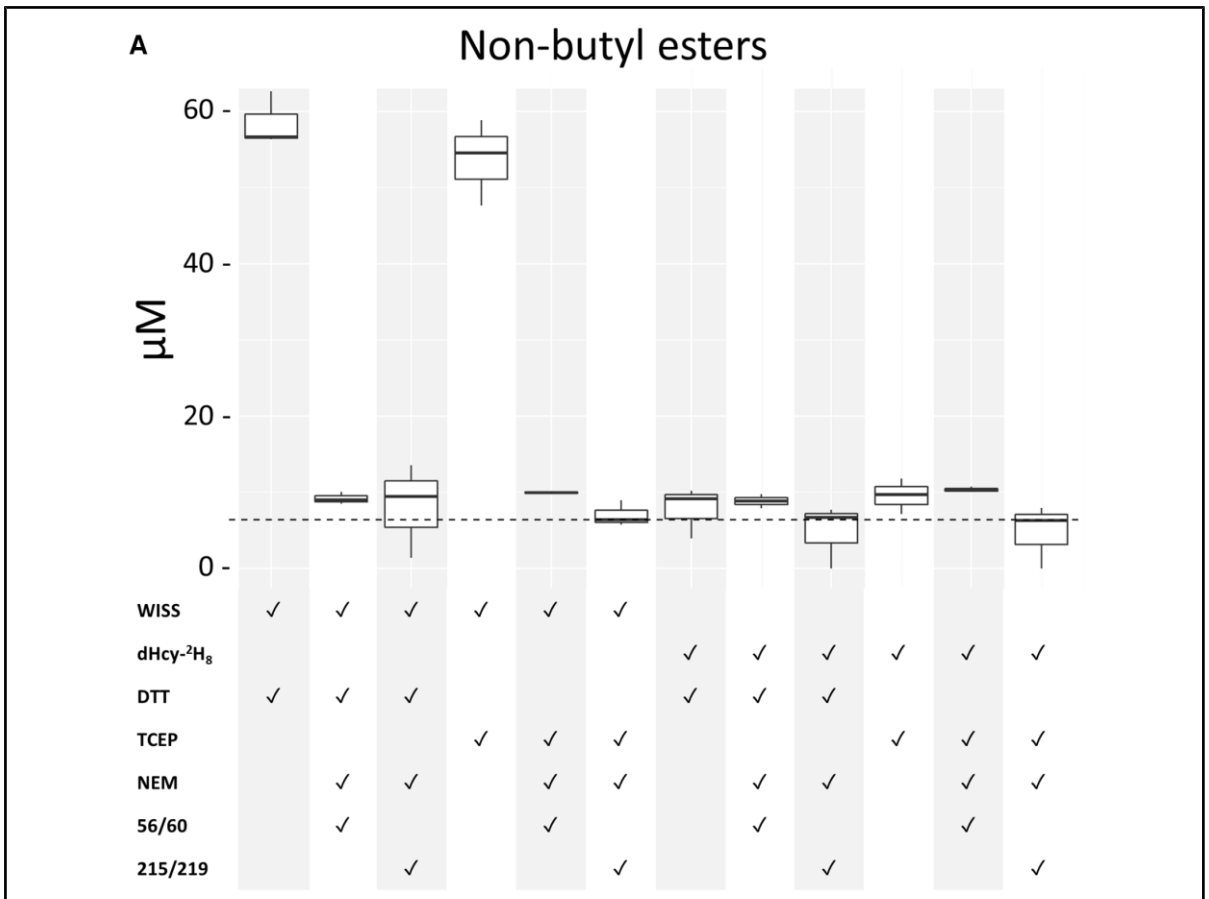

Fig. 2. Hcy and Hcy-NEM concentrations in low QCs in the absence of or treated with reducing agents and NEM. Samples were extracted in either WISS or with a mock WISS containing only isotopically labeled homocystine (dHcy-$^2H_8$). QC DBS extracts were then reduced with either TCEP or DTT to reduce oxidized and protein bound Hcy and yield tHcy in the monomer form. Hcy was either analyzed directly or DBS extracts were treated with NEM to derivatize the Hcy to form Hcy-NEM before analysis. Four additional transitions were monitored for Hcy-NEM (*m/z* 261 > 56 and 261 > 215) and Hcy-$^2H_4$-NEM (*m/z* 265 > 60 and 265 > 219) to ensure equivalency between product ion pairs. The *x*-axis is a matrix of conditions and analysis that each sample (n = 6 per column) was subjected to, and the *y*-axis is the concentration of Hcy or Hcy-NEM. The dashed line indicates the expected concentration. Both (A) and (B) show that WISS-treated DBS extracts had higher than expected Hcy concentrations due to the presence of interferences in the WISS, while DBS extracts treated with mock WISS had Hcy concentrations closer to the expected concentration. Treatment with NEM yielded Hcy concentrations closer to the expected concentration when extracted with WISS and mock WISS.

added reagents to butyl ester-derivatized DBS extracts. Data were visualized as heatmaps due to the large number of biomarkers in the assay. Overall, most of the biomarkers experiencing large percent differences after the addition of reagents were acylcarnitines using surrogate IS such as malonylcarnitine (C3DC) + hydroxybutyrylcarnitine (C4OH), tiglylcarnitine (C5:1), tetradecenoylcarnitine (C14:1), and oleoylcarnitine (C18:1); surrogate IS that were used for these biomarkers are presented in online Supplemental Table 4. The trends were relatively consistent in both low QC (Fig 3, A) and high QC (Fig 3, B) samples. It was expected that TCEP would impact analytes using surrogate ISs since TCEP ionizes in positive mode. Large concentration percent differences with the addition of only DTT were unexpected, since DTT does not ionize in positive mode. DTT was incubated with the DBS and WISS for 45 min because it is slower to react. The water from the DTT solution altered the

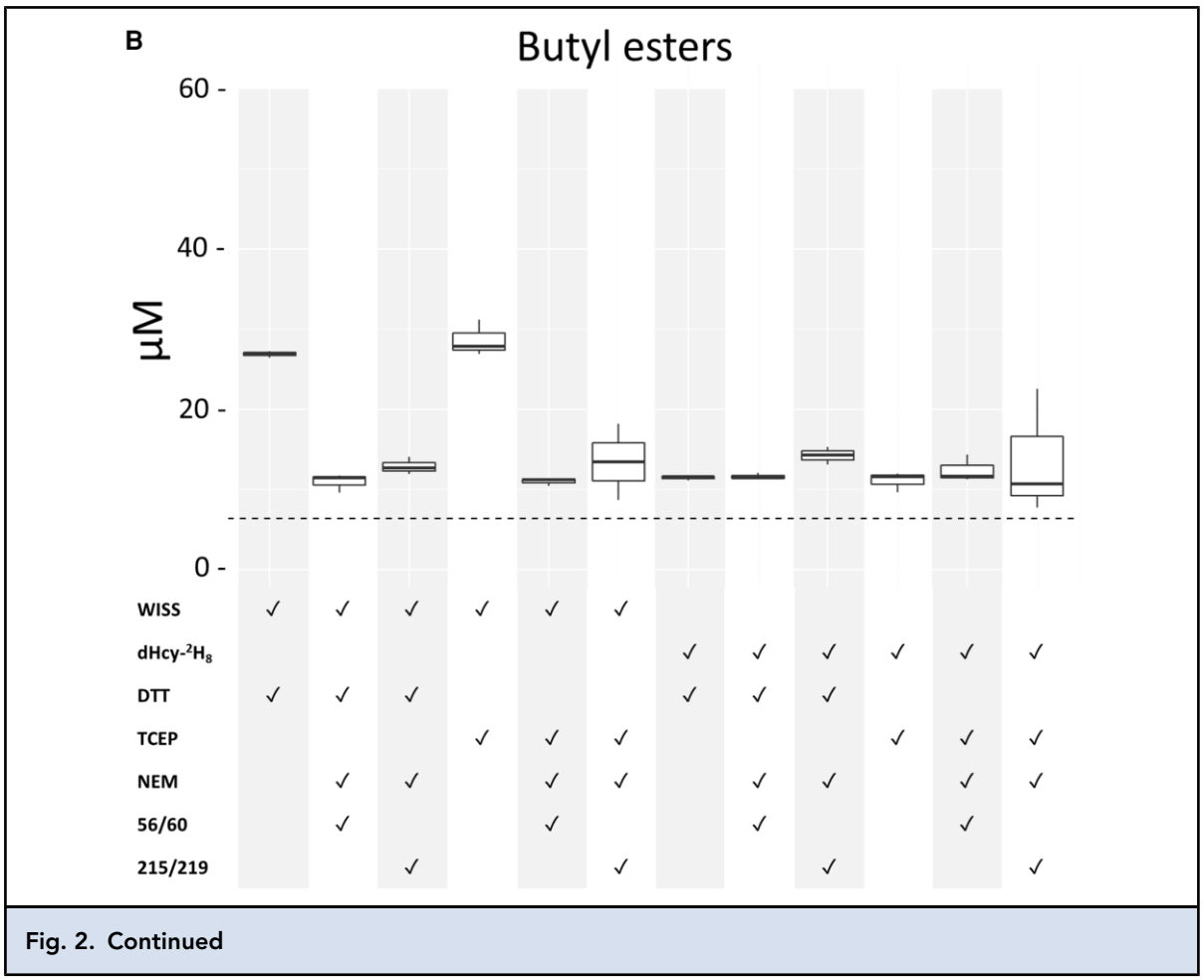

Fig. 2. Continued

aqueous composition of the WISS DBS solution, which likely extracted more salts along with reduced small peptides, contributing to more ion suppression. The addition of NEM with DTT that further exacerbated issues in analytes using surrogates and several other biomarkers was also unexpected. Therefore, we decided to investigate the effects of these added reagents on IS signal.

Figure 4 displays the percentage of matrix effects on ISs in low QC samples treated with either DTT + NEM or TCEP + NEM, and normalized to peak areas from ISs in low QC extracted samples with no reducing agents and NEM. Online Supplemental Fig. 4 displays the impact of the low QC matrix, individually and in combinations with DTT, TCEP, and NEM, that was normalized to peak areas in the neat WISS solution. Interestingly, in Supplemental Fig. 4 we observed that short-chain acylcarnitine IS peak areas decreased with the addition of DTT to the matrix. This led us to hypothesize that short-chain acylcarnitines may react with DTT. Therefore, we took a solution of acetylcarnitine (C2) and DTT and allowed the mixture to react, followed by analysis via HRMS. Online Supplemental Fig. 5 confirms that C2 reacts with DTT to form diacetyl-DTT, likely through acyl chain swapping. It appears that DTT also reacts with other acylcarntines, which can be observed in Supplemental Fig. 4, since the impact of DTT on the percentage of matrix effects seemed to have direct relation with the acylcarnitine chain length (C2-D_3 > C5-D_9 > C12-D_9 > C18-D_3).

In general, we observed significantly greater percentages of matrix effects in samples treated with DTT + NEM in both Fig. 4 and Supplemental Fig. 4, leading us to hypothesize that NEM reacts with DTT to form a strong positively ionizing byproduct. Online Supplemental Fig. 6 displays the theoretical parent and product ion *m/z* of 2 NEM molecules reacting with a single (diNEM+DTT) molecule, since each DTT has 2 thiol groups. This byproduct was confirmed based on parent ion *m/z* and parallel reaction monitoring spectra. A

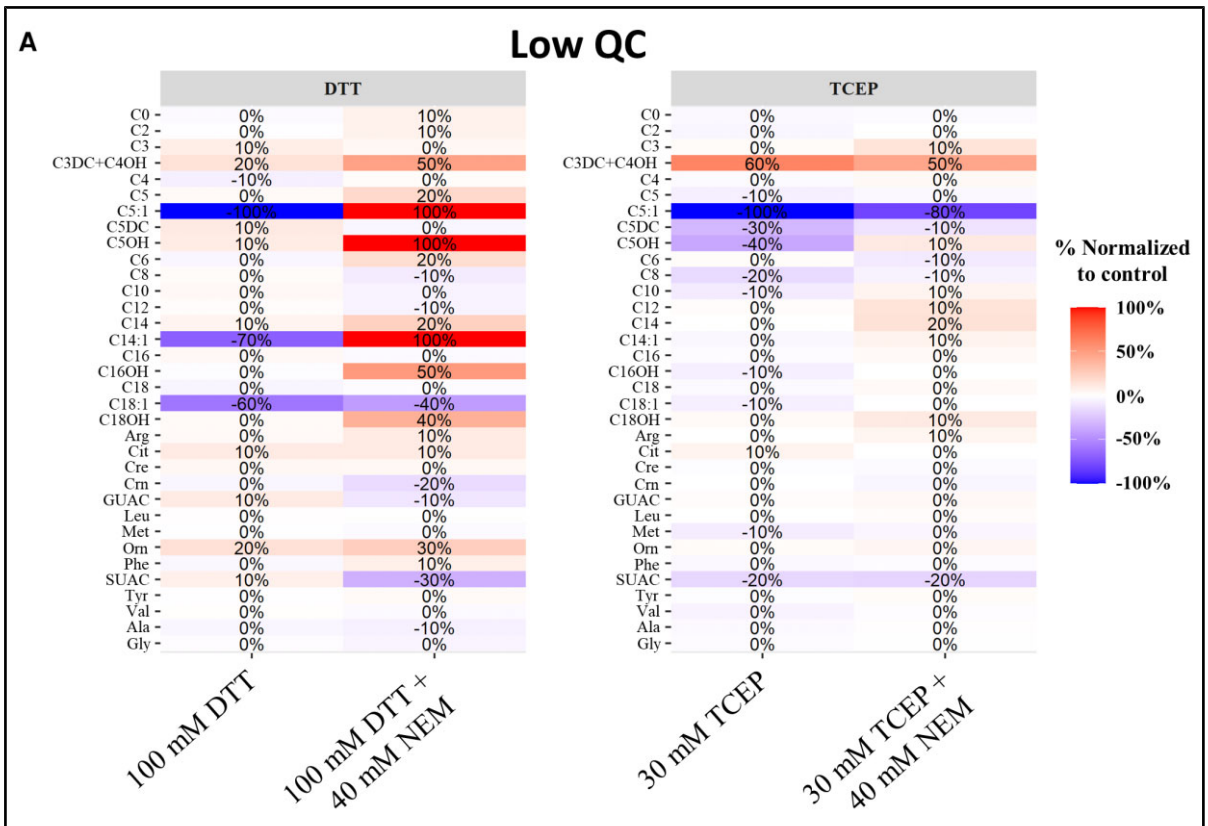

Fig. 3. Impact of adding reducing agents and NEM on existing biomarkers in the assay. The heatmaps indicate the effects of adding reducing agents with and without the presence NEM on biomarker concentrations, in comparison to the method without the addition of reducing agents or NEM. Each group was analyzed in triplicate on a single day. Rounded percentages (e.g., −10%, 0%, 10%) are presented in each of the heatmap cells. The percent normalized to control method was calculated as follows: ((QC treated with either reducing agents or NEM − QC in the absence of reducing agents and NEM)/QC in the absence of reducing agents and NEM × 100).

reaction between TCEP and NEM was first described over 2 decades ago (17), and it was expected that TCEP and TCEP + NEM would cause ion suppression in the assay. TCEP + NEM result in a byproduct that does not have as significant an effect on the matrix as diNEM-DTT (Fig 4, Supplemental Fig. 7). The formation of diNEM-DTT shown in Supplemental Fig. 6, along with the deleterious effects observed in Fig. 3, Fig. 4, Supplemental Fig. 4, and Supplemental Fig. 5, ruled out using DTT alone or in combination with NEM in the multiplexed Hcy first-tier assay. Therefore, we proceeded with TCEP and NEM as reagents for the final method.

Full method validation was performed to assess the performance of the developed method. Results of linearity, precision, limits of detection (LOD), and limits of quantification (LOQ) are presented herein. The method demonstrated excellent linearity for nearly all biomarkers, including Hcy, with $R^2 > 0.99$ (Table 1). The biomarkers with $R^2 < 0.99$ were ornithine (Orn, $R^2 = 0.984$) and C5:1, which uses a surrogate IS and is impacted by the addition of both reducing agents alone and in the presence of reducing agents plus NEM. Online Supplemental Table 5 displays precision results from 3 QC levels analyzed in duplicate on 20 separate days. All biomarkers had residual standard deviations <15% in all QC levels, except for C5:1 (failed low and mid QC), succinylacetone-hydrazone (SUAC, failed low QC), and guanidinoacetic acid (GUAC, failed low and mid QC). Prior to the validation step, the instrument had a repair on the ESI probe which reduced the sensitivity of SUAC in the low QC during validation. Adjusting the probe after validation mitigated our SUAC issue and we did not experience any issues with SUAC in the low QC prior to these repairs. We tested GUAC and the GUAC IS to assess whether TCEP, NEM, or the combination of TCEP + NEM had any

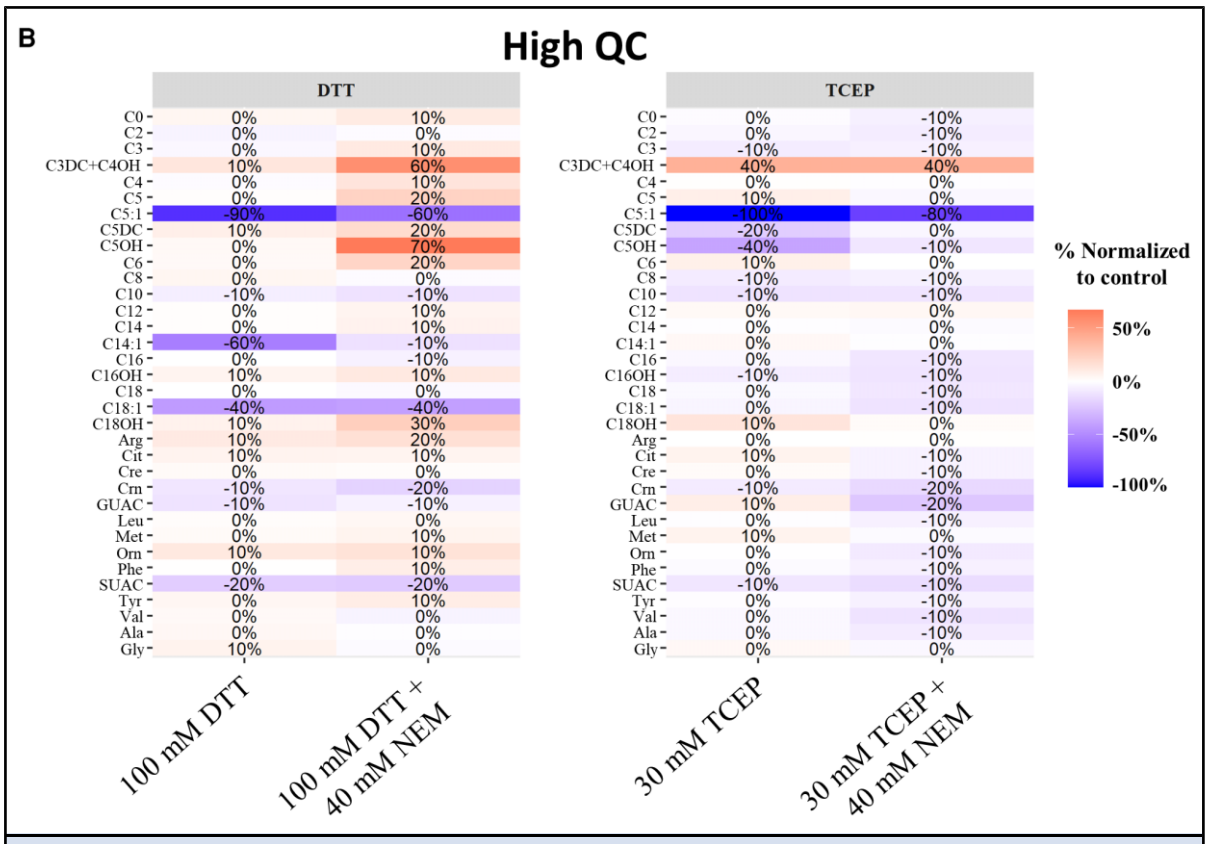

Fig. 3. Continued

reaction, and we observed no reactions or byproducts. LODs and LOQs were comparable to our current method, except for C5:1, with Hcy having an LOQ of 1.67 μM using our method with TCEP and NEM (online Supplemental Table 6).

The next logical step was to test the method on residual clinical NBS specimens to ensure the method could distinguish between presumptive normal (n = 100), those with reported TPN administration (n = 50), and HCU+-confirmed newborns (n = 2). Figure 5 displays a biplot of residual clinical specimens analyzed by the developed multiplexed method with Met concentration on the *x*-axis and tHcy concentration on the *y*-axis. The solid horizontal line represents the average Met cutoff calculated across domestic NBS laboratories in 2021 (n = 43, range 40 to130 μM) and the solid vertical line represents the tHcy concentration at the 1st percentile of 20 newborns with HCU (D. Matern, personal communication, May 2022). Figure 5 clearly demonstrates that the multiplexed method is capable of accurately classifying HCU+ specimens, while distinguishing HCU+ from TPN specimens. As observed in Fig. 5, using only Met as a surrogate biomarker for HCU screening results in high false positives from TPN specimens. Thus, as expected, the multiplexing of tHcy into first-tier screening to identify HCU+ specimens potentially increases the sensitivity and specificity.

Discussion

In our study, we present the first method which has successfully multiplexed direct screening of Hcy into FIA-MS/MS first-tier NBS assays, thus potentially increasing the sensitivity and specificity for HCU screening in first-tier NBS. The developed method extracts all biomarkers that are analyzed in our current assay while also directly quantifying tHcy from DBS. Quantification of tHcy is made possible by converting oxidized Hcy into the reduced tHcy pool with the addition of TCEP and performing selective derivatization of the tHcy pool using NEM. These additional steps using TCEP and NEM add only 20 min to sample preparation compared to our current first-tier screening method. Since the method uses a high organic extraction solution (80/20 acetonitrile/water), the extracts are

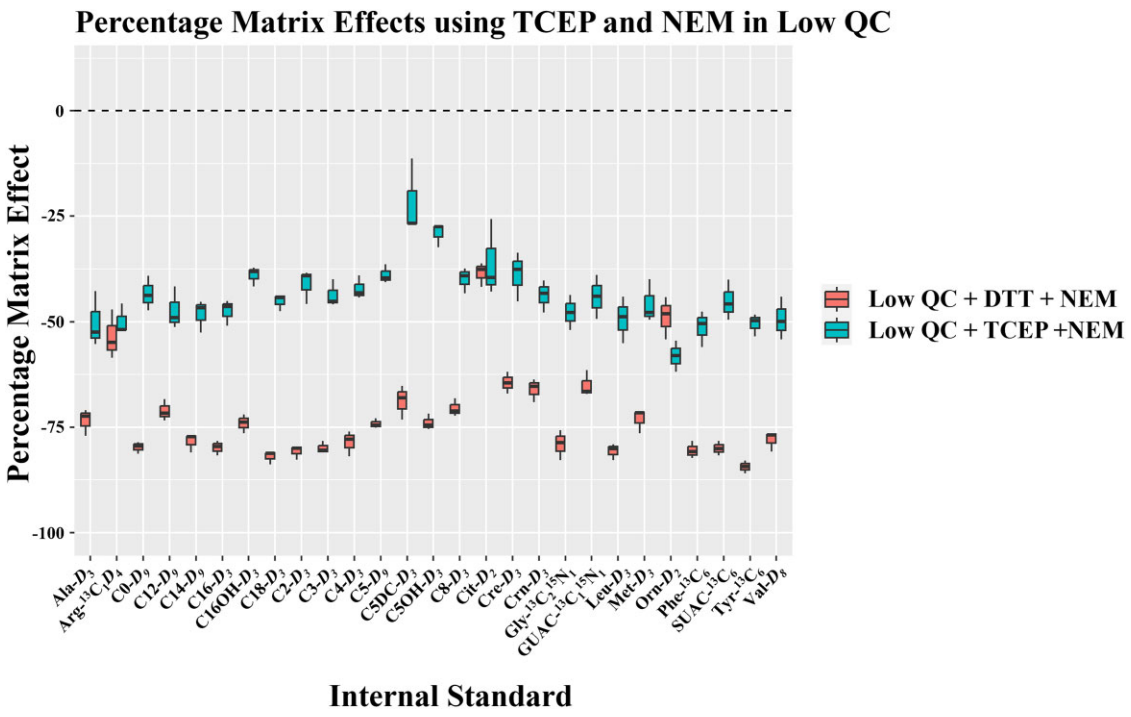

Fig. 4. Comparison of percentage of matrix effect between TCEP and DTT in the presence of NEM. The percentage of matrix effect on ISs was compared between samples consisting of low QC extracts treated with TCEP + NEM or DTT + NEM. Data were acquired FIA–MS/MS. Percentage of matrix effect was calculated using peak area (PA) as follows: ((PA IS from a low QC DBS extract treated with reducing agents and NEM − PA IS from a low QC DBS extract)/PA IS from a low QC DBS extract × 100). Data represent triplicate measurements from NSQAP Lot# 2115 low QC materials.

considerably cleaner compared to second-tier Hcy screening methods that are typically more aqueous (18–20) while maintaining similar recovery (Supplemental Tables 1 and 2). We have analyzed several hundred QC samples per day using the method without noticeable differences in signal, e.g., the method is compatible with a high-throughput environment and would not require source cleaning throughout the day.

The developed method uses NEM dissolved in 50/50 water/methanol, which greatly accelerates drying times of the solution compared to using NEM in 100% water. Additionally, we have preliminary data suggesting that NEM can be added directly to the 50/50 water/acetonitrile resuspension solution, which would further simplify the method to require only 5 min of overhead for TCEP reduction. Notably, the percentage of acid in the resuspension solution may need to be adjusted since the NEM reaction is affected by pH (21). NEM is commonly used in scientific applications, is very low-cost, and widely commercially available. Since DBS and other microsampling devices are of heightened interest in clinical screening (e.g., point-of-care testing), and Hcy is significantly elevated in several other diseases (22–26), our method could be utilized in other clinical applications.

Reduction of oxidized Hcy is a critical step in accurate quantification of tHcy. NBS labs currently use DTT as a reducing agent in second-tier screening assays that quantify Hcy (18–20), likely because it does not ionize in positive mode; however, it was reported to cause abnormal peaks in chromatography when used with DBS (27). Based on results from our study, DTT usage in FIA analysis of Hcy is problematic due to its reaction with short-chain acylcarnitines and reaction with thiol derivatizing agents. Despite TCEP being a strong, positively ionizing reducing agent, there are numerous advantages for its use in NBS. TCEP's reaction is rapid and it can be added at the end of DBS extraction, reducing the time that the DBSs are exposed to higher proportions of water. With 12 µL DTT addition, the extraction solution decreased from 80/20 acetonitrile/water to roughly 70/30 acetonitrile/water during the 45 min heated extraction at 45 °C. TCEP solutions are also stable for longer periods of time compared to DTT (28); in our study we remade TCEP solutions every 2 weeks. We also plan to

Table 1. Linearity data for targeted biomarkers.[a]

Biomarker	*y*-Intercept	Slope	R^2
Alanine (Ala)	0.774	0.937	0.995
Arginine (Arg)	5.002	0.969	0.996
Free Carnitine (C0)	9.181	0.73	0.989
Acetylcarnitine (C2)	3.178	1.196	0.99
Propionylcarnitine (C3)	0.543	0.765	0.989
Malonylcarnitine (C3DC) + hydroxybutyrylcarnitine (C4OH)	0.144	0.4	0.991
Butyrylcarnitine (C4)	0.103	0.927	0.99
Isovalerylcarnitine (C5:0)	0.239	0.831	0.993
Tiglylcarnitine (C5:1)	−0.136	0.3	0.943
Glutarylcarnitine (C5DC)	0.063	1.2	0.995
Hydroxyisovalerylcarnitine (C5OH)	0.318	1.081	0.99
Hexanoylcarnitine (C6)	0.118	0.986	0.994
Octanoylcarnitine (C8)	0.554	0.959	0.989
Decanoylcarnitine (C10:0)	0.374	1.114	0.989
Decenoylcarnitine (C10:1)	0.116	0.163	0.964
Decadienoylcarnitine (C10:2)	0.129	0.367	0.991
Dodecanoylcarnitine (C12:0)	0.184	0.792	0.992
Tetradecanoylcarnitine (C14:0)	0.222	1.034	0.995
Tetradecenoylcarnitine (C14:1)	0.182	0.604	0.993
Palmitoylcarnitine (C16)	0.602	0.817	0.995
Hydroxyhexadecanoylcarnitine (C16OH)	0.093	0.855	0.997
Stearoylcarnitine (C18:0)	0.236	0.898	0.997
Oleoylcarnitine (C18:1)	0.574	0.598	0.994
Hydroxystearoylcarnitine (C18OH)	0.046	0.475	0.996
Citrulline (Cit)	22.955	0.941	0.985
Creatine (Cre)	42.533	0.851	0.984
Creatinine (Crn)	0.507	0.878	0.997
Glycine (Gly)	44.111	0.722	0.993
Guanidoacetic acid (GUAC)	0.525	0.605	0.977
Homocysteine-NEM (Hcy-NEM)	5.156	0.67	0.988
Leucine (Leu)	23.552	0.835	0.997
Methionine (Met)	4.692	0.788	0.99
Ornithine (Orn)	8.94	1.021	0.984
Phenylalanine (Phe)	20.158	0.891	0.996
Succinylacetone-hydrazone (SUAC)	−0.793	0.607	0.998
Tyrosine (Tyr)	2.024	0.901	0.997
Valine (Val)	6.433	0.886	0.998

[a]Linearity data presented were run in triplicate on a single day and compared to certification data acquired by flow injection analysis on triple quadrupole tandem mass spectrometry.

investigate the feasibility of using TCEP linked to magnetic nanoparticles in our method (29), which would allow the post-reaction removal of TCEP from the reduced DBS extracts, drastically reduce ion suppression, and likely reducing the amount of NEM needed in the method since residual TCEP reacts with NEM (online

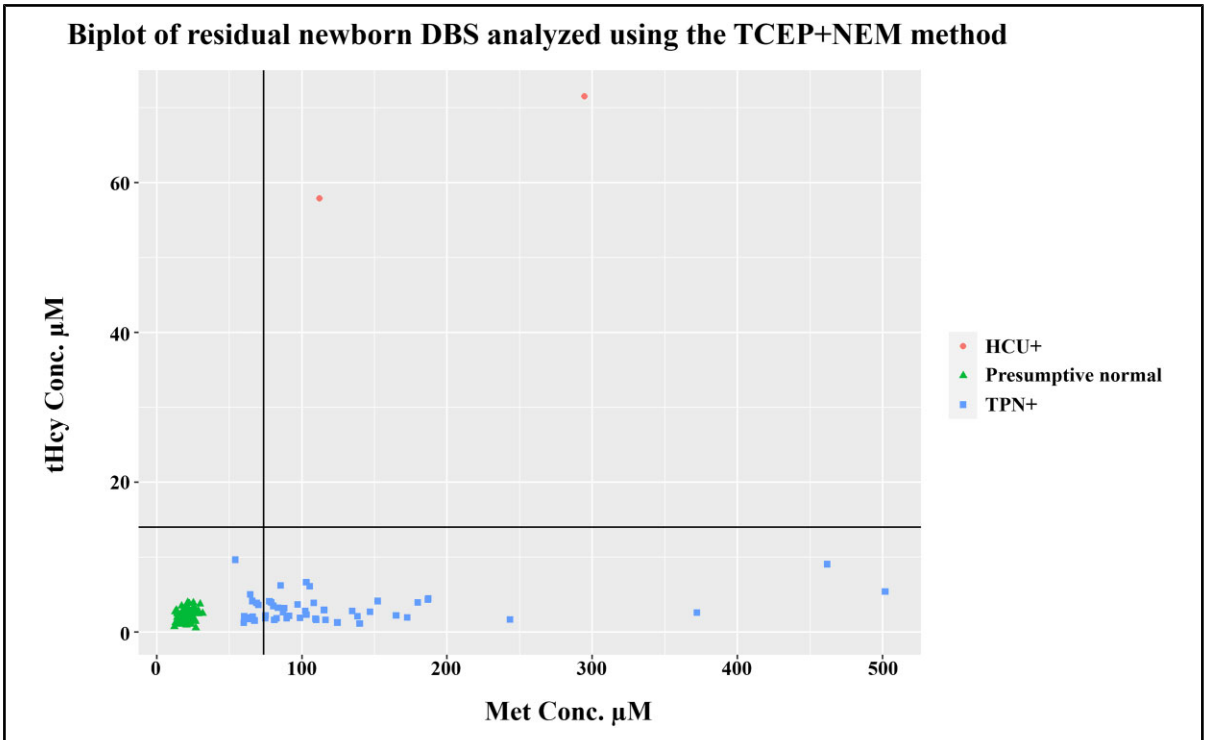

Fig. 5. Analysis of residual clinical newborn DBS using TCEP + NEM method. Residual clinical newborn DBS specimens were analyzed using the method employing TCEP + NEM. tHcy and Met concentrations were plotted, and samples were classified as either presumptive normal, those with reported administration of total parenteral nutrition (TPN+), or homocystinuria positive (HCU+).

Supplemental Fig. 7). Supplemental Fig. 4 indicates that the impact of NEM on the matrix is minimal, with only TCEP, DTT, and TCEP + NEM and DTT + NEM byproducts causing additional ion suppression.

Selective derivatization of Hcy is paramount for the successful quantification of tHcy under FIA conditions using nominal mass-resolving MS such as a triple quadrupole. This is due to interferences including, but not limited to, the Met-D_3 and Leu-D_3 ISs that are commonly used in nearly all commercial NBS assays and NBS laboratory-developed tests. Due to the structural similarity of Met and Hcy, methods employing separation will also encounter similar issues with Met-D_3 as observed in Supplemental Fig. 1, B and a recent publication by our group (30). In addition to NEM shifting the Hcy *m/z* to remove interferences, we also reported Hcy-NEM yielded roughly 5× increased signal intensity compared to Hcy (Supplemental Table 4). It should be noted that the increase in Hcy-NEM signal was in the presence of the TCEP + NEM and DTT + NEM byproducts, so there is likely a higher-fold increase in signal that cannot be observed due to the ion suppression of the byproducts.

Acylcarnitine C5:1, which uses C5:0-D_9 as a surrogate IS, was the worst performing biomarker in the developed method. Based on trends of other biomarkers that use surrogate ISs, we suspect that using a C5:1 IS would mitigate the observed issues. It is also possible in the case of unsaturated acylcarnitines that the redox environment created by reducing agents (31) may damage the double bond; however, we plan to investigate the addition of antioxidants such as butylated hydroxytoluene if we encounter this issue (32). GUAC in lower concentrations also did not perform well in validation and we suspect this may be due to matrix effects and ion suppression between the unlabeled and labeled IS, and we plan to investigate other potential ISs to mitigate this issue. It should be noted that the HRMS parallel reaction monitoring spectrum of pure NEM (*m/z* 126) indicated the presence of a *m/z* 80 product ion (online Supplemental Fig. 8, A), which interferes with the Val-D_8 transition *m/z* 126 > 80 (Supplemental Fig. 8, B). Val concentrations in the enriched QCs did not appear to be impacted by the presence of the NEM interferent with Val-D_8 (Fig 3). Since NEM is consumed by Hcy, we decided to investigate the impact on Val concentration in QCs that were enriched for Hcy. Supplemental Fig. 8, C shows that the concentration of Val in these highly enriched Hcy QCs was comparable in the absence or presence of NEM and TCEP. NBS

laboratories using Val-D_8 could consider a lower conservative cutoff for Val due to the presence of NEM as an interferent. Our group previously reported Val-D_8 has a different optimal collision energy from Val (33), and our group has been investigating Val IS non-deuterated alternatives.

In conclusion, we report the only validated method capable of quantifying tHcy in FIA-MS/MS first-tier NBS assays. Our method's ability to screen Hcy during first-tier NBS has the potential to lead to a paradigm shift towards considering Hcy as a primary screening biomarker, similar to the addition of SUAC to first-tier screening for tyrosinemia type 1 (34). The future directions of this project include refining the method to reduce additional sample prep overhead (e.g., adding NEM to the resuspension solution), adding IS to remove the need for quantifying using surrogate IS (e.g., C5:1, C14:1), and investigating alternative IS for those suspected to have differential ionization (e.g., GUAC) or interferences (e.g., Val-D_8). We will also investigate the use of TCEP linked to magnetic nanoparticles to aid in the removal of TCEP from the DBS extracts, which should reduce ion suppression in the matrix (29). Overall, our method is a significant development towards improving HCU NBS and is expected to reduce false-positive and false-negative rates, in turn decreasing HCU-associated morbidity and mortality.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: HCU, homocystinuria; Hcy, homocysteine; HCU+, homocystinuria positive; NBS, newborn screening; DBS, dried blood spot; tHcy, total homocysteine; FIA-MS/MS, flow injection analysis–tandem mass spectrometry; NEM, N-ethylmaleimide; TCEP, tris(2-carboxyethyl)phosphine; DTT, dithiothreitol; TPN, total parenteral nutrition; IS, isotopically labeled internal standard; HRMS, high-resolution mass spectrometry; WISS, working internal standard solution; Hcy-D_4, Hcy-$^2H_4$; SUAC, succinylacetone; GUAC, guanidinoacetic acid.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved. Nobody who qualifies for authorship has been omitted from the list.*

C. Pickens (Conceptualization-Equal, Data curation-Lead, Formal analysis-Lead, Investigation-Equal, Methodology-Equal, Validation-Lead, Writing—original draft-Lead, Writing—review & editing-Equal), Elya Courtney (Data curation-Supporting, Formal analysis-Supporting, Validation-Supporting, Writing—review & editing-Supporting), Samantha Isenberg (Data curation-Supporting, Formal analysis-Supporting, Methodology-Supporting, Validation-Supporting, Writing—review & editing-Supporting), Carla Cuthbert (Formal analysis-Supporting, Project administration-Equal, Supervision-Equal, Writing—review & editing-Supporting), Konstantinos Petritis (Conceptualization-Equal, Investigation-Equal, Methodology-Equal, Project administration-Equal, Supervision-Lead, Writing—original draft-Supporting, Writing—review & editing-Equal)

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest*:

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: Texas Department of State Health Services Newborn Screening Laboratory provided all the residual newborn screening specimens.
Expert Testimony: None declared.
Patents: This research is covered under provisional patent # 63/333647 (filed on 4/22/2022) (https://www.techtransfer.nih.gov/bundle/tab-3818). Inventors: C.A. Pickens and K. Petritis.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, preparation of manuscript, or final approval of manuscript.

Acknowledgments: The authors thank the Biochemical Mass Spectrometry laboratory (BMSL) and Newborn Screening Quality Assurance Program (NSQAP) lab staff for producing and certifying the QC materials, along with the NSQAP data management team for their data stewardship. The authors would also like to thank the Texas Department of State Health Services Newborn Screening Laboratory for providing the de-identified, residual clinical newborn specimens that were used in this study.

References

1. Sacharow SJ, Picker JD, Levy HL. Homocystinuria caused by cystathionine beta-synthase deficiency. In: Adam MP Mirzaa GM and Pagon RA, editors. Genereviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993–2022.
2. Higginbottom MC, Sweetman L, Nyhan WL. A syndrome of methylmalonic aciduria, homocystinuria, megaloblastic anemia and neurologic abnormalities in a vitamin B12-deficient breast-fed infant of a strict vegetarian. N Engl J Med 1978;299:317–23.
3. Mudd SH, Finkelstein JD, Refsum H, Ueland PM, Malinow MR, Lentz SR, et al. Homocysteine and its disulfide derivatives: a suggested consensus terminology. Arterioscler Thromb Vasc Biol 2000;20:1704–6.
4. National Library of Medicine. Homocystinuria. https://medlineplus.gov/genetics/condition/homocystinuria/#frequency (Accessed May 2022).
5. Levy HL. Early development of newborn screening for HCU and current challenges. Int J Neonatal Screen 2021;7:4.
6. Yamada K, Yokoyama K, Aoki K, Taketani T, Yamaguchi S. Long-term outcomes of adult patients with homocystinuria before and after newborn screening. Int J Neonatal Screen 2020;6:3.
7. Yap S, Naughten E. Homocystinuria due to cystathionine beta-synthase deficiency in Ireland: 25 years' experience of a newborn screened and treated population with reference to clinical outcome and biochemical control. J Inherit Metab Dis 1998;21:738–47.

8. Walter JH, Wraith JE, White FJ, Bridge C, Till J. Strategies for the treatment of cystathionine beta-synthase deficiency: the experience of the Willink Biochemical Genetics Unit over the past 30 years. Eur J Pediatr 1998;157(Suppl 2):S71–6.
9. Watson MS, Mann MY, Lloyd-Puryear MA, Rinaldo P, Howell RR, American College of Medical Genetics Newborn Screening Expert Group. Newborn screening: toward a uniform screening panel and system–executive summary. Pediatrics 2006; 117(Suppl 3):S296–307.
10. Health Resources & Services Administration. Advisory Committee on Heritable Disorders in Newborns and Children, August 1–2, 2019 Meeting. https://www.hrsa.gov/sites/default/files/hrsa/advisory-committees/heritable-disorders/meetings/achdnc-aug-2019-transcript-day1.pdf (Accessed May 2022).
11. Asamoah A, Wei S, Jackson KE, Hersh JH, Levy H. Diagnosis of classic homocystinuria in two boys presenting with acute cerebral venous thrombosis and neurologic dysfunction after normal newborn screening. Int J Neonatal Screen 2021;7:3.
12. Ochoa-Ferraro A, Wanninayake S, Dawson C, Gerrard A, Preece MA, Geberhiwot T. A case series of cerebral venous thrombosis as the first manifestation of homocystinuria. Eur Stroke J 2021;6:420–7.
13. Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE, et al. The natural history of homocystinuria due to cystathionine beta-synthase deficiency. Am J Hum Genet 1985;37:1–31.
14. Alsharhan H, Ahmed AA, Ali NM, Alahmad A, Albash B, Elshafie RM, et al. Early diagnosis of classic homocystinuria in Kuwait through newborn screening: A 6-year experience. Int J Neonatal Screen 2021;7:3.
15. Hall EM, Flores SR, De Jesus VR. Influence of hematocrit and total-spot volume on performance characteristics of dried blood spots for newborn screening. Int J Neonatal Screen 2015;1:69–78.
16. R Core Team. R: A language and environment for statistical computing. Vienna (Austria): R Foundation for Statistical Computing; 2017.
17. Shafer DE, Inman JK, Lees A. Reaction of tris(2-carboxyethyl)phosphine (TCEP) with maleimide and alpha-haloacyl groups: anomalous elution of TCEP by gel filtration. Anal Biochem 2000;282:161–4.
18. Dubland JA, Rakic B, Vallance H, Sinclair G. 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. J Mass Spectrom Adv Clin Lab 2021;20: 1–10.
19. Pajares S, Arranz JA, Ormazabal A, Del Toro M, Garcia-Cazorla A, Navarro-Sastre A, et al. Implementation of second-tier tests in newborn screening for the detection of vitamin B12 related acquired and genetic disorders: results on 258,637 newborns. Orphanet J Rare Dis 2021;16:195.
20. Turgeon CT, Magera MJ, Cuthbert CD, Loken PR, Gavrilov DK, Tortorelli S, et al. Determination of total homocysteine, methylmalonic acid, and 2-methylcitric acid in dried blood spots by tandem mass spectrometry. Clin Chem 2010;56: 1686–95.
21. Russo MST, Napylov A, Paquet A, Vuckovic D. Comparison of N-ethyl maleimide and N-(1-phenylethyl) maleimide for derivatization of biological thiols using liquid chromatography-mass spectrometry. Anal Bioanal Chem 2020;412:1639–52.
22. Wang J, You D, Wang H, Yang Y, Zhang D, Lv J, et al. Association between homocysteine and obesity: a meta-analysis. J Evid Based Med 2021;14:208–17.
23. Lei X, Zeng G, Zhang Y, Li Q, Zhang J, Bai Z, Yang K. Association between homocysteine level and the risk of diabetic retinopathy: a systematic review and meta-analysis. Diabetol Metab Syndr 2018;10:61.
24. Muzurovic E, Kraljevic I, Solak M, Dragnic S, Mikhailidis DP. Homocysteine and diabetes: role in macrovascular and microvascular complications. J Diabetes Complications 2021;35:107834.
25. Xie D, Yuan Y, Guo J, Yang S, Xu X, Wang Q, et al. Hyperhomocysteinemia predicts renal function decline: a prospective study in hypertensive adults. Sci Rep 2015;5: 16268.
26. Hasan T, Arora R, Bansal AK, Bhattacharya R, Sharma GS, Singh LR. Disturbed homocysteine metabolism is associated with cancer. Exp Mol Med 2019;51:1–13.
27. Bartl J, Chrastina P, Krijt J, Hodik J, Peskova K, Kozich V. Simultaneous determination of cystathionine, total homocysteine, and methionine in dried blood spots by liquid chromatography/tandem mass spectrometry and its utility for the management of patients with homocystinuria. Clin Chim Acta 2014; 437:211–7.
28. Getz EB, Xiao M, Chakrabarty T, Cooke R, Selvin PR. A comparison between the sulfhydryl reductants tris(2-carboxyethyl)phosphine and dithiothreitol for use in protein biochemistry. Anal Biochem 1999;273: 73–80.
29. Zwyssig A, Schneider EM, Zeltner M, Rebmann B, Zlateski V, Grass RN, Stark WJ. Protein reduction and dialysis-free work-up through phosphines immobilized on a magnetic support: TCEP-functionalized carbon-coated cobalt nanoparticles. Chemistry 2017;23:8585–9.
30. Pickens CA, Isenberg SL, Cuthbert C, Petritis K. Combining first and second-tier newborn screening in a single assay using high-throughput chip-based capillary electrophoresis coupled to high-resolution mass spectrometry. Clin Chem 2021;67: 1709–20.
31. Lee H, Torres J, Truong L, Chaudhuri R, Mittal A, Johnson ME. Reducing agents affect inhibitory activities of compounds: results from multiple drug targets. Anal Biochem 2012;423:46–53.
32. Pickens CA, Sordillo LM, Zhang C, Fenton JI. Obesity is positively associated with arachidonic acid-derived 5- and 11-hydroxyeicosatetraenoic acid (HETE). Metab Clin Exp 2017;70:177–91.
33. Pickens CA, Petritis K. High resolution mass spectrometry newborn screening applications for quantitative analysis of amino acids and acylcarnitines from dried blood spots. Anal Chim Acta 2020;1120: 85–96.
34. Stinton C, Geppert J, Freeman K, Clarke A, Johnson S, Fraser H, et al. Newborn screening for tyrosinemia type 1 using succinylacetone—a systematic review of test accuracy. Orphanet J Rare Dis 2017; 12:48.