




Improving Harmonization and Standardization of Expanded Newborn Screening Results by Optimization of the Legacy Flow Injection Analysis Tandem Mass Spectrometry Methods and Application of a Standardized Calibration Approach

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BACKGROUND: Newborn screening (NBS) laboratories in the United Kingdom adhere to common protocols based on single analyte cutoff values (COVs); therefore, interlaboratory harmonization is of paramount importance. Interlaboratory variation for screening analytes in UK NBS laboratories ranges from 17% to 59%. While using common stable isotope internal standards has been shown to significantly reduce interlaboratory variation, instrument set-up, sample extraction, and calibration approach are also key factors.

METHODS: Dried blood spot (DBS) extraction processes, instrument set-up, mobile-phase composition, sample introduction technique, and calibration approach of flow injection analysis–tandem mass spectrometry (FIA-MS/MS) methods were optimized. Inter- and intralaboratory variation of methionine, leucine, phenylalanine, tyrosine, isovaleryl-carnitine, glutaryl-carnitine, octanoyl-carnitine, and decanoyl-carnitine were determined pre- and postoptimization, using 3 different calibration approaches.

RESULTS: Optimal recovery of analytes from DBS was achieved with a 35-min extraction time and 80% methanol (150 μ L). Optimized methodology decreased the mean intralaboratory percentage relative SD (%RSD) for the 8 analytes from 20.7% (range 4.1–46.0) to 5.4% (range 3.0–8.5). The alternative calibration approach reduced the mean interlaboratory %RSD for all analytes from 16.8% (range 4.1–25.0) to 7.1% (range

4.1–11.0). Nuclear magnetic resonance analysis of the calibration material highlighted the need for standardization. The purities of isovaleryl-carnitine and glutaryl-carnitine were 85.13% and 69.94% respectively, below the manufacturer's stated values of $\geq 98\%$.

CONCLUSIONS: For NBS programs provided by multiple laboratories using single analyte COVs, harmonization and standardization of results can be achieved by optimizing legacy FIA-MS/MS methods, adopting a common analytical protocol, and using standardized calibration material rather than internal calibration.

Introduction

Flow injection analysis–tandem mass spectrometry (FIA-MS/MS) has the capability to simultaneously detect >50 different compounds in a single dried blood spot (DBS) specimen and has been widely adopted by newborn screening (NBS) laboratories throughout the world.

An issue common to all FIA-MS/MS screening methods is calibration. Two approaches have been adopted, both of which have their advantages and disadvantages. Some laboratories quantify results using stable isotope dilution and a DBS calibration curve, others use stable isotope internal calibration (SIIC) alone. In principle, the former approach benefits from a matrix matched calibration curve and assurance of linearity

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across the defined range of concentrations. However, the practicalities of accurately preparing multiple DBS calibrators in sufficient quantity poses a sizable challenge, and this is before traceability and standardization of the calibrators are considered. The alternative, more practical approach is to rely on SIIC, but the efficacy of this is limited by the purity of the stable isotope internal standard (IS), the accuracy with which they are prepared, and the limitations of what is effectively a single-point calibration.

Although accuracy is an important factor, the clinical utility of a screening test is determined by the evidence base associated with the clinical cutoff values (COVs). For laboratories that use floating COVs, multiple of the median, or Collaborative Laboratory Integrated Reports functionalities (1–3), assay harmonization and standardization are less relevant. However, in England and Wales, laboratories ($n = 14$) must adhere to nationally agreed protocols based on single-analyte COVs: methionine (Met), 45; leucine (Leu), 500; phenylalanine (Phe), 200; tyrosine (Tyr), 240; isovaleryl-carnitine (C5), 1.6; glutaryl-carnitine (C5DC), 0.56; octanoyl-carnitine (C8), 0.4; and decanoyl-carnitine (C10), 0.5 $\mu\text{mol/L}$. Interlaboratory harmonization is therefore critically important, as is standardization to the assay from which the COVs were originally determined. With numerous laboratories relying on in-house methodologies, harmonization and standardization are challenging. Currently, there are no commercially available DBS-based external calibrators.

A recent study demonstrated that interlaboratory variation can be substantially reduced by the use of common stable isotope IS (4), and another study has shown that retrospectively harmonizing screening results to the CDC's Newborn Screening Quality Assurance Program internal quality control (IQC) material reduced interlaboratory variation in a set of proficiency testing samples (5).

The aim of this study was to improve the harmonization and standardization of NBS results by optimizing legacy FIA-MS/MS methods for the measurement of the following screening analytes in DBS; Met, Leu, Phe, Tyr, C5, C5DC, C8, and C10 to implement a standardized approach to calibration. Multicenter studies were undertaken to assess the impact of the following parameters on intra- and interlaboratory variation: DBS analyte extraction procedure, MS/MS set up, mode of sample introduction, mobile phase composition, and calibration approach.

Materials and Methods

CHEMICALS AND REAGENTS

Acetonitrile, methanol, and formic acid were obtained from LGC Standards, HPLC grade ammonium acetate

(>99%) was obtained from Sigma Aldrich, and ultrapure water at 18 $\text{m}\Omega$ was obtained in-house from an Elga water purifier system.

Traceable amino acids standards L-leu, L-meth, L-phen, and L-tyr were obtained from National Metrology Institute of Japan. Each had a certified mass fraction of 0.999 (kg/kg) with expanded uncertainty of 0.002 (kg/kg). Valeryl-L-carnitine was obtained from Larodan Fine Chemicals, and octanoyl-L-carnitine, decanoyl-L-carnitine, and glutaryl-L-carnitine lithium salt were obtained from Sigma Aldrich. The purity of the 4 acylcarnitine species and associated uncertainties at the 95% CI was assigned by quantitative nuclear magnetic resonance (qNMR) prior to use.

L-phe (ring-D5, 98%), L-tyr (ring-D4, 98%), L-met (methyl-D3 98%), and L-leu (5,5,5-D3, 99%) were obtained from CK Isotopes. L-carnitine:HCl, O-isovaleryl (N, N, N trimethyl-D9, 98%), L-carnitine:HCl, O-octanoyl (N methyl-D3, 98%), L-carnitine:HCl, O-decanoyl (N methyl-D3, 98%), and L-glutaryl-carnitine.perchlorate salt (D3, 98%) were obtained from QMX Laboratories. The mixed acylcarnitine stable isotope stock standard solution (50 mL, 1 mmol/L) was prepared in distilled water and then diluted 1:50 in methanol to give final concentration of 20 $\mu\text{mol/L}$. Aliquots (1.0 mL) were dried under nitrogen and stored at -70°C . The mixed amino acid stable isotope stock standard solution (500 mL, 2.5 mmol/L) was prepared in distilled water. Aliquots (4.0 mL) were stored at -70°C . The C5DC stable isotope stock standard solution (50 mL, 1 mmol/L) was prepared in water and then diluted 1:50 in 80% methanol to give a final concentration of 20 μM . Aliquots (1.0 mL) were dried under nitrogen and stored at -70°C .

Extraction eluent, containing the stable isotope IS, was prepared gravimetrically as follows: aliquots of C5DC stable isotope and mixed acylcarnitine stable isotope were reconstituted separately in 80% methanol (4 mL) and quantitatively transferred to a bottle containing the mixed amino acid stable isotope stock standard (4 mL). The vials containing the dried C5DC stock and dried mixed acylcarnitines were rinsed multiple times, and the bottle was made up to volume (2 L) with 80% methanol to give final concentrations of 5.0, 0.04, and 0.04 mmol/L, for the amino acids, acylcarnitines, and C5DC respectively.

STANDARDS AND INTERNAL QUALITY CONTROL

Traceable calibration standards were prepared as follows: individual stock standards were prepared gravimetrically, using a United Kingdom Accreditation Service calibrated balance with weights of the vessel, powder, and diluent recorded. Amino acids stocks were prepared at 2 mg/g in 0.1 mol/L HCl, and acylcarnitines were prepared at 0.5 mg/g in 80% methanol. A second set of individual

stocks was prepared in the same manner by a different analyst. Both sets of stocks were diluted into 80% methanol and mixed gravimetrically to be compared using the FIA-MS/MS method to ensure accurate preparation. All analytes matched concentration to within <2.5%. For preparation of the calibration standards for the study, one set of stocks was diluted into the extraction eluent (80% methanol containing the stable isotope IS) and mixed gravimetrically to accurately produce 5 mixed solutions. Concentration of individual analytes in each standard are summarized in online [Supplemental Table 1](#). IQC material was prepared by enriching blood from healthy volunteers and applying 20 μL onto Whatman 903 filter paper. The DBS IQC material was stored desiccated at -70°C prior to use. In addition, a pooled DBS IQC material was separately prepared for the intralaboratory study by addition of multiple 3.2 mm subpunches to a 96-well plate. Extraction solution (150 μL) was added to each well, and the plate was agitated for 35 min. The resulting solution was removed from each well, pooled, and stored at -20°C prior to use.

ANALYSIS OF DBS SAMPLES, STANDARDS, AND IQC

The routine in-house methods of each laboratory essentially utilized the same methodology (6). A 3.2 mm subpunch was taken from each DBS into a 96-well plate. The equivalent volume of blood in each subpunch was assumed to be 3.1 μL . DBS were extracted by addition of 80% methanol containing stable isotope IS, prior to agitating on a plate shaker for between 20 and 30 min. Sample (10–20 μL) was introduced into the MS/MS using variable flow injection analysis. Mobile phase consisted of 80% methanol containing 0.025% formic acid v/v. The initial flow rate was typically between 200 and 450 $\mu\text{L}/\text{min}$, and after approximately 0.1 min, this was reduced to 10 or 20 $\mu\text{L}/\text{min}$ and then returned to the initial flow rate after approximately 1 min to flush out the ion source electrospray needle and the auto-sampler to ion source peek tubing. Data for quantification were acquired during the period of reduced flow rate.

Typical MS/MS settings were capillary voltage, 3.5 kV; source block, 150°C ; desolvation temperature, 350°C ; and desolvation gas flow, 800 L/h. Cone gas and collision energy were optimized for each analyte. Data were acquired by selective reaction monitoring (SRM) using positive ionization mode (Leu, $132 > 86$; Met, $150 > 104$; C5, $246 > 85$; C5DC, $276 > 85$; Phe, $166 > 120$; C8, $288 > 85$; Tyr, $182 > 136$; C10, $316 > 85$). Quantification of each analyte was made by SIIC. The relative response ratio of each analyte to its respective stable isotope IS was multiplied by the concentration of the appropriate stable isotope IS and then corrected for the nominal volume of blood in the

3.2 mm DBS subpunch: analyte concentration = relative response ratio \times (150/3.1) \times concentration of IS. Analysis was performed on Waters TQD and Xevo TQ-S (Laboratory 1), Waters Xevo TQD (n = 2, Laboratory 2), Sciex API 4500 (n = 2, Laboratory 3), Waters TQD (n = 2, Laboratory 4), Waters Xevo TQD and TQD (Laboratory 5), Sciex API 3200 and a Sciex API 4000 (Laboratory 6), and Waters Xevo TQ-S and Waters TQD (Laboratory 7).

DATA ANALYSIS

Datasets generated by laboratory instrumentation were processed using Microsoft Excel 2016. Statistical analysis of the extraction and interlaboratory study data was carried out using R version 3.6.1 and RStudio version 1.2.5001 (7, 8).

Study Design

OPTIMIZATION OF DRIED BLOOD SPOT SAMPLE

EXTRACTION PROCEDURE

IQC material was prepared from lithium heparin blood collected from healthy volunteers. Five laboratories prepared the IQC at endogenous concentration, and one laboratory enriched the blood to concentrations approximating the COVs. In accordance with a specified protocol, laboratories extracted 2 sets of IQC, each consisting of 3.2 mm subpunches (n = 30) in a 96-well plate. Sample sets were extracted with 80% methanol, Set 1 with 150 μL and Set 2 with 200 μL . Plates were agitated on a plate shaker, and after 5 min, sample eluent from 3 wells was transferred to a separate 96-well plate, effectively stopping the extraction process. This process was repeated at 5-min intervals up to and including 50 min postinitiation of extraction. Sample extracts were analyzed by each of the 6 laboratories using their in-house routine FIA-MS/MS method. The mean measured concentration of each analyte, at each time interval, for each sample set was collated and analyzed to determine the optimum extraction technique.

INTRALABORATORY COMPARISON

The legacy FIA-MS/MS method was set up on 5 instruments from different manufacturers (Waters Quattro Premier XE, Waters Xevo TQ-XS, ABI Sciex 4000 Qtrap, Agilent 6490, and Thermo Vantage). Each instrument was tuned and set up using the nominal SRMs by a single individual in one laboratory. Five replicate injections of pooled IQC material were made on each instrument from the same autosampler vial. The mean and percentage relative SD (%RSD) between replicate injections and instrument was calculated for each analyte. The experiment was repeated with a calibration curve covering the COV after the following method parameters

had been optimized; mobile phase composition, modifier, injection volume, and constant flow injection.

INTERLABORATORY COMPARISON

Interlaboratory variation was determined for the routine in-house legacy FIA-MS/MS screening methods and compared with the optimized FIA-MS/MS method. All 16 laboratories in the UK Newborn Screening Laboratory Network (England, Wales, Scotland, and Northern Ireland) were invited to participate in the study, and 7 laboratories agreed to do so. Each laboratory was supplied with a set of 5 traceable calibration solutions containing stable isotope IS, extraction eluent (80% methanol containing stable isotope IS), and 3 levels of DBS IQC material and instructed to prepare 2 identical 96-well plates as follows. Calibration solutions ($n = 5$) were added to each plate. A 3.2 mm subpunch was taken from routine DBS NBS samples ($n = 20$) and 3 levels of DBS IQC material, the latter being punched in triplicate. Extraction eluent (150 μL) was added to each sample, and the plates were agitated for >30 min prior to analysis of C5, C5DC, C8, C10, Leu, Met, Phe, and Tyr. The procedure was repeated on 2 additional days.

Six of the 7 laboratories also prepared and analyzed identical sets of samples using the optimized FIA-MS/MS method with in-house IS. An aqueous standard was provided to each laboratory and diluted as instructed to prepare a calibration curve. Three alternative calibration approaches were evaluated: SIIC with all laboratories using a common IS, single-point isotope dilution calibration, and multiple point ($n = 5$ plus blank) isotope dilution calibration. The concentration of the d3-C5DC in the common IS was retrospectively corrected against the C5DC in the calibrator, the concentration of which had been determined by qNMR, thus enabling calculation of "C5DC results corrected for d3-C5DC."

STATISTICAL ANALYSIS

For the optimized extraction study, the results were analyzed by fitting nonlinear models to the different combinations of laboratory, analyte, and extraction volume. An exponential model of the following form was used:

$$c = A [1 - \exp(-Bt)],$$

where c is the concentration and t the elapsed time. This model describes a curve that passes through the origin (0 concentration at time 0 is assumed) and increases at a rate defined by B to approach the asymptotic value A . The optimum extraction time was defined as the point at which the concentration reaches 95% of the maximum value A . Rather than attempting to model the extraction process itself in terms of the reaction kinetics and other relevant factors, the aim was to produce fitted curves

that could be used to determine reaction times by interpolation, using a relatively straightforward approach. The resulting set of extraction times was then examined using analysis of variance to determine which, if any, factors were significantly associated with extraction time.

For the interlaboratory study, the results were analyzed using mixed-effects models with maximum likelihood estimation. IS type (common or in-house) and calibration method (single- or multipoint) were treated as fixed effects, while differences between laboratories were included as a random effect.

Results

Results of the extraction study are summarized in Fig. 1 for Met and C5DC. Results for the other analytes are summarized in Supplemental Fig. 1. Optimal extraction of DBS samples was determined by computing the 95th percentile of the set of extraction times obtained from the curve fitting process, which estimates the time within which 95% of samples reach equilibrium. When samples were eluted in 150 μL of 80% methanol, equilibrium of all analytes was reached in 95% of samples within 35 min. When samples were eluted in 200 μL of eluent, equilibrium was achieved within 45 min.

To test the significance of observed differences in time to reach equilibrium, a linear model was applied to the set of individual extraction times, and an analysis of variance was carried out. Differences in extraction time between laboratories were found to be statistically significant, both as a main effect ($P = 0.010$) and as part of the interaction term ($P < 0.0001$). Volume was not significant as a main effect ($P = 0.38$), but any volume effects are also part of the interaction term and thus not separable from the lab effect. In computing the significance levels, laboratory was treated as a random effect. Analyte was found not to be a significant factor and was removed from the analysis.

Structural confirmation and qNMR analysis of the acylcarnitine species showed a mean purity of 85.48% (range 69.94%–93.54%). The fully traceable purities and assigned uncertainty at the 95% CI ($k = 2$) are summarized in Supplemental Table 2.

The optimized mobile phase composition was 10 mmol/L ammonium acetate and 0.1% formic acid in 80% methanol. Sample injection volume was 10 μL . A constant flow loop injection was selected as the mode of sample introduction at a flow rate of 150 $\mu\text{L}/\text{min}$. Acquisition time was 1.2 min.

Prior to optimization of the method, the mean intralaboratory %RSD for the 8 analytes was 20.7% (range 4.1%–46.0%). Postoptimization, the mean intralaboratory %RSD for the 8 analytes was reduced to

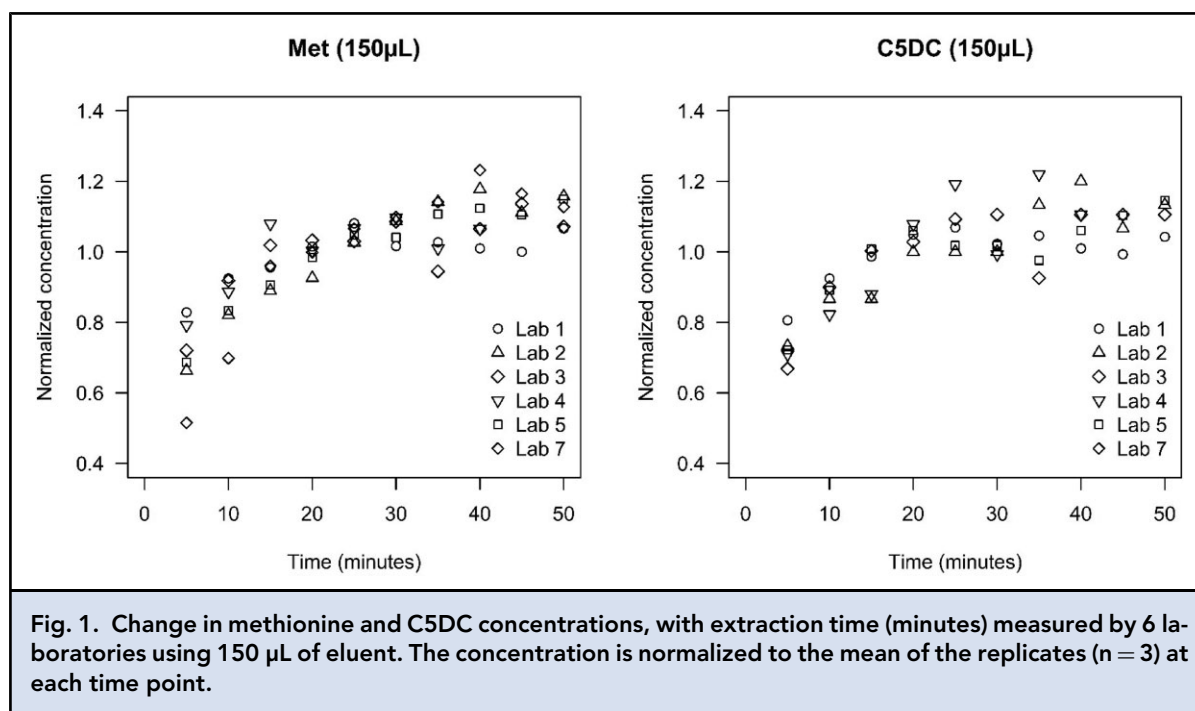


Fig. 1. Change in methionine and C5DC concentrations, with extraction time (minutes) measured by 6 laboratories using 150 μ L of eluent. The concentration is normalized to the mean of the replicates ($n = 3$) at each time point.

5.4% (range 3.0–8.5). Results are summarized in Fig. 2 and Supplemental Table 3A and 3B.

Using the optimized method, the mean interlaboratory %RSD for all analytes was 16.8% (range 4.1%–28.0%) when results were quantified with each laboratory using their in-house IS and SIIC. When results were quantified using a common IS and SIIC, the mean interlaboratory %RSD for all analytes was 11.1% (range 5.0%–24.3%). Using a common IS with single- or multi-point calibration reduced interlaboratory variation, with the mean %RSD for all analytes being 7.0% (range 4.7%–11.4%) and 7.0% (range 4.1%–11.0%), respectively. Results from the 4 different calibration strategies are summarized in Fig. 3 and Supplemental Table 4. The mean concentrations of Phe, C5DC, and “C5DC results corrected for d3-C5DC concentration” are summarized in Fig. 4 for each laboratory, the 3 alternative calibration approaches, and the existing in-house methods.

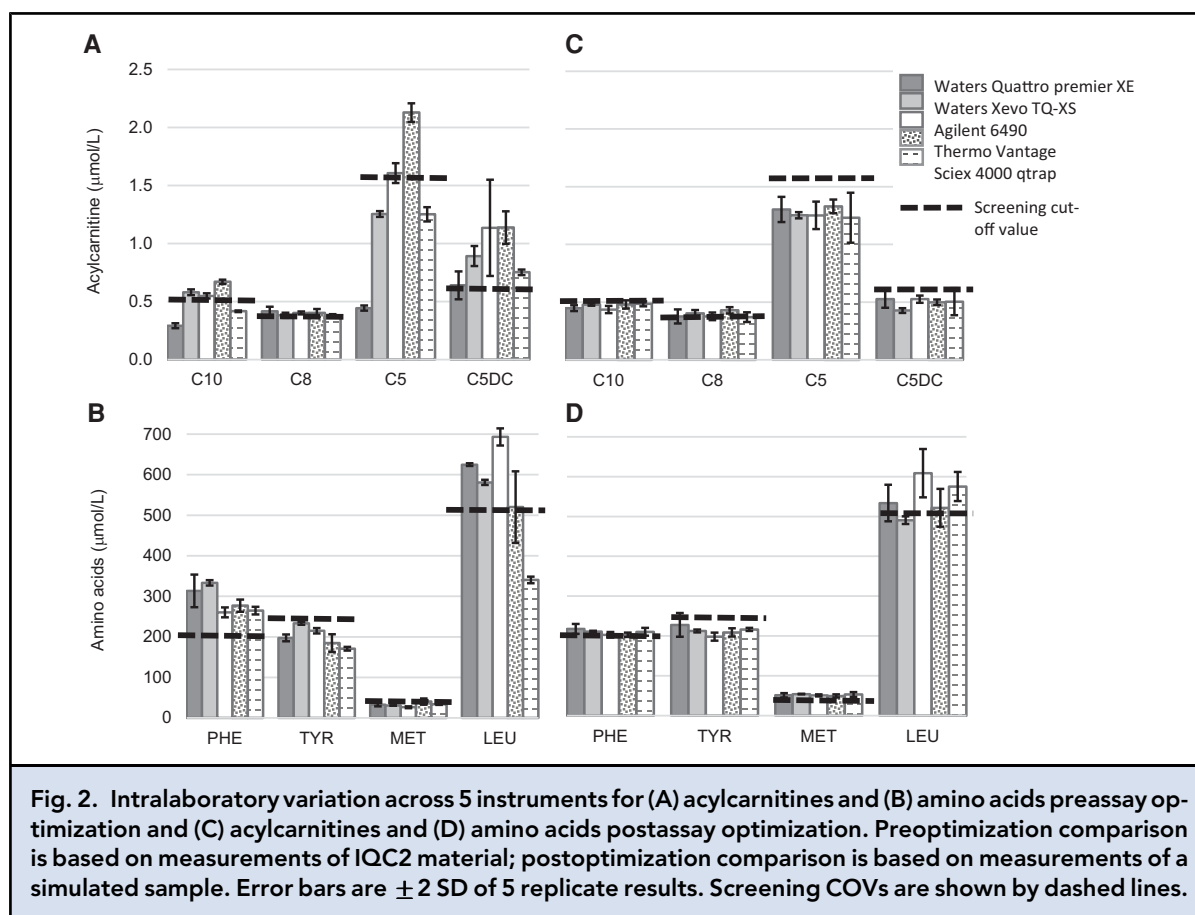
Discussion

Optimization of the extraction procedure demonstrated that the length of time over which the DBS were extracted was the most important factor, with volume of extraction eluent (150 vs 200 μ L) having a lesser effect. Prior to optimization, the extraction time varied from 20 to 30 min between different laboratories. Extrapolating from the fitted curves in the model, this difference

would have contributed a mean under recovery of 3.8% (range 0.1%–13.2%) for 150 μ L of eluent.

Constant flow loop injection eliminates concerns regarding the ability of the liquid chromatography pump to accurately deliver low flow rates (10–20 μ L/min) and optimum peak integrations during processing. More important, prior to optimization, background subtraction was being applied when the flow rate of the solvent was approximately 10 \times greater than that when the analytes of interest eluted, thereby introducing error due to variance in ionization efficiency. However, increasing the amount of matrix entering the source resulted in ion suppression and a loss in sensitivity compared to the cut-flow method, which was found to be consistent across instruments, although not to the same extent. Sensitivity could be increased to some extent by reducing the flow rate, but this had to be balanced against the requirement for an injection-to-injection time no longer than that of the legacy method (1.2 min) due to the workload associated with a typical NBS laboratory.

Methanolic- and acetonitrile-based mobile phase compositions were evaluated (80% and 50%) with and without the presence of modifiers (10 mmol/L ammonium acetate and 0.1% formic acid). The composition of the DBS sample extract in the in-house methods was typically 80% methanol, and matching mobile phase to sample composition gave good results for all analytes. Addition of a modifier gave varying results for the different classes of analytes: 10 mmol/L



ammonium acetate suppressed the signal from the amino acids, with no change to the intensity of the acylcarnitines. The addition of 0.1% formic acid improved the intensity of the acylcarnitines (mean increase 34%, $n = 10$), but did not impact significantly on the amino acids. Suppression of the amino acid signal is useful as often the higher concentration amino acids overload the detector on more sensitive instruments, resulting in users reducing injection volumes or diluting samples, thus creating a challenge for the detection of the acylcarnitines. A combined modifier of 10-mmol/L ammonium acetate and 0.1% formic acid provided the best trade-off between suppression of amino acids and enhancement of the acylcarnitine signal.

The effect of the optimized FIA-MS/MS parameters on harmonization is demonstrated by the significant reduction in intralaboratory variation across 5 different instruments (Supplemental Table 3A and 3B). The postoptimization experiments were performed using a simulated sample (protein-precipitated plasma spiked to achieve analyte concentrations approximating the COV), thus negating differences due to sample extraction. Prior to optimization of the method, it was evident

that both intra- and interinstrument variability were greater than expected (4). From a practical perspective, the intrainstrument variability at the COV was large enough to potentially result in a false-negative or false-positive result (e.g., preoptimization, the %RSD is 46% for C5, 24.3% for Leu, and 22.2% for C5DC (Fig. 2A). The sample matrix differed slightly pre- and postoptimization, a reflection of the practical issues associated with obtaining sufficient volume of a single pool of sample. However, irrespective of the sample matrix used, the variability between instruments was significantly reduced postoptimization, compared to preoptimization. By analyzing an identical sample on all instruments and employing an identical approach to quantification (i.e., SIIC), both sample preparation and calibration effects were effectively excluded from the intralaboratory variation. Each instrument was set up and tuned by a single individual at the same time, excluding operator variability as a contributing factor and indicating that variability reflected instrument differences. It is postulated that these will be differences in mass resolution, SRM set-up (observed transitions vs theoretical), chemical background noise on individual SRM channels,

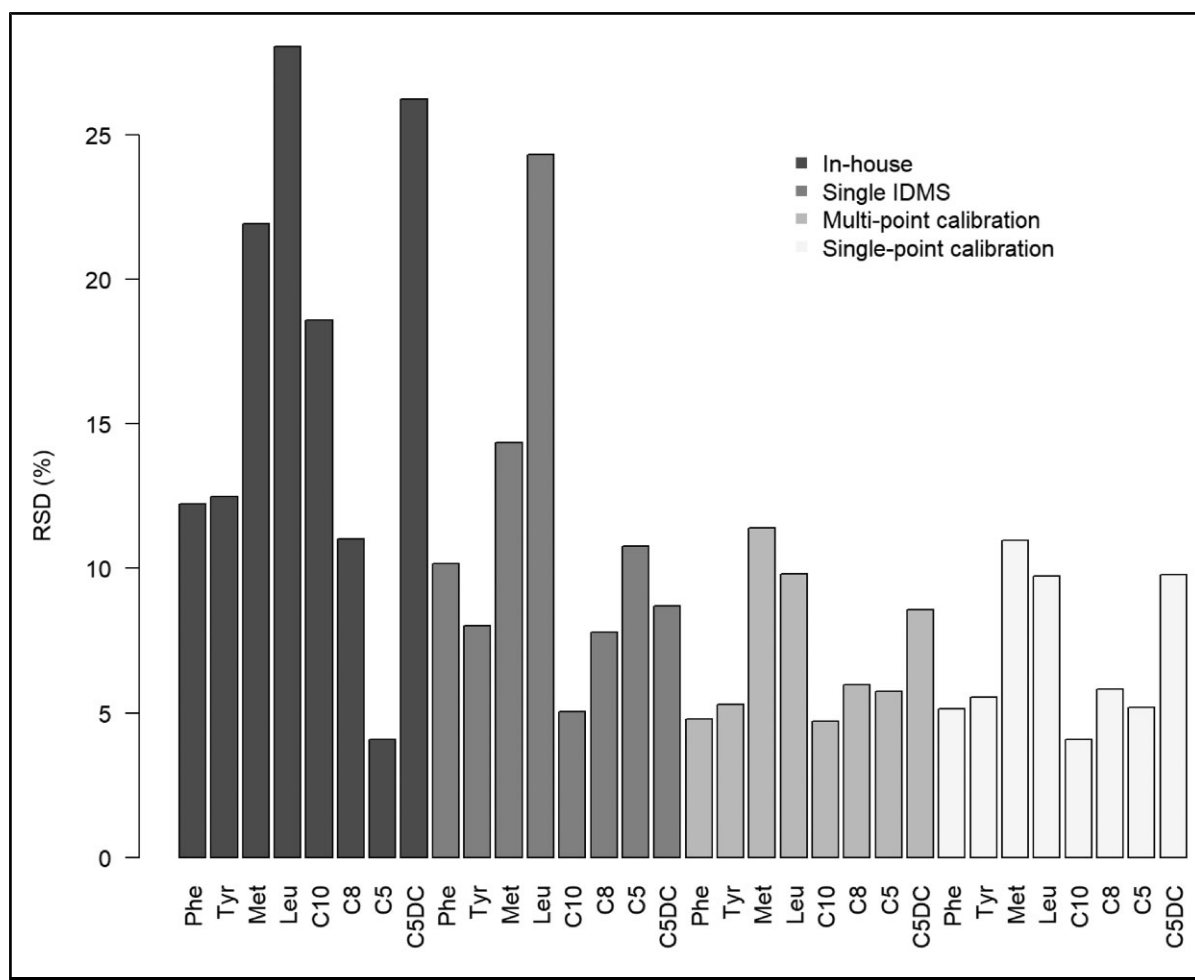


Fig. 3. %RSD of mean results from all laboratories for each analyte by calibration approach.

and the processing of acquired data (continuum vs centroid data processing).

For screening laboratories using common COVs, while both intralaboratory harmonization and comparability to the method used to establish the COVs are of primary importance, the benefits of accuracy, traceability, and access to commutable materials should not be underestimated. The qNMR results for the individual acylcarnitines showed that all acylcarnitines were below the stated $\geq 98\%$ purity, with both C5 and C5DC being reasonably impure at 85.13% and 69.64%, respectively. This highlights the need for laboratories to have access to traceable standards. If laboratories continue to use isotope dilution internal calibration with ISs of questionable purity and/or in-house standards of questionable purity, NBS results will be biased. For example, C5DC had a stated purity of 98% but was only 69.64% pure, contributing a bias of +28.9% to the final result.

The DBS analytes quantified in this study are well-defined small molecular weight molecules, and full

standardization with traceability should be achievable. However, there is a lack of commercially available matrix-matched certified reference material for these analytes in DBS specimens on which to standardize. The accurate preparation of DBS calibrators is challenging owing to the effect of bloodspot size and chromatographic effect of blood distribution on the filter paper, leading to significant analytical biases (9) and maintaining traceability of measurements to the International System of Units when the final sample is a punch from a DBS specimen. This study has shown the use of nonmatrix-matched calibrators (aqueous) combined with stable isotope IS to be a cost-effective way to reduce interlaboratory variation and overcome the issues outlined. It has also demonstrated that accuracy is maintained using a single-point calibrator at the COV. This is advantageous for a high throughput assay and provides a practical solution for a screening test where all results above the COV will be confirmed. As such, the recommended approach is isotope dilution calibration with a single-point aqueous calibrator.

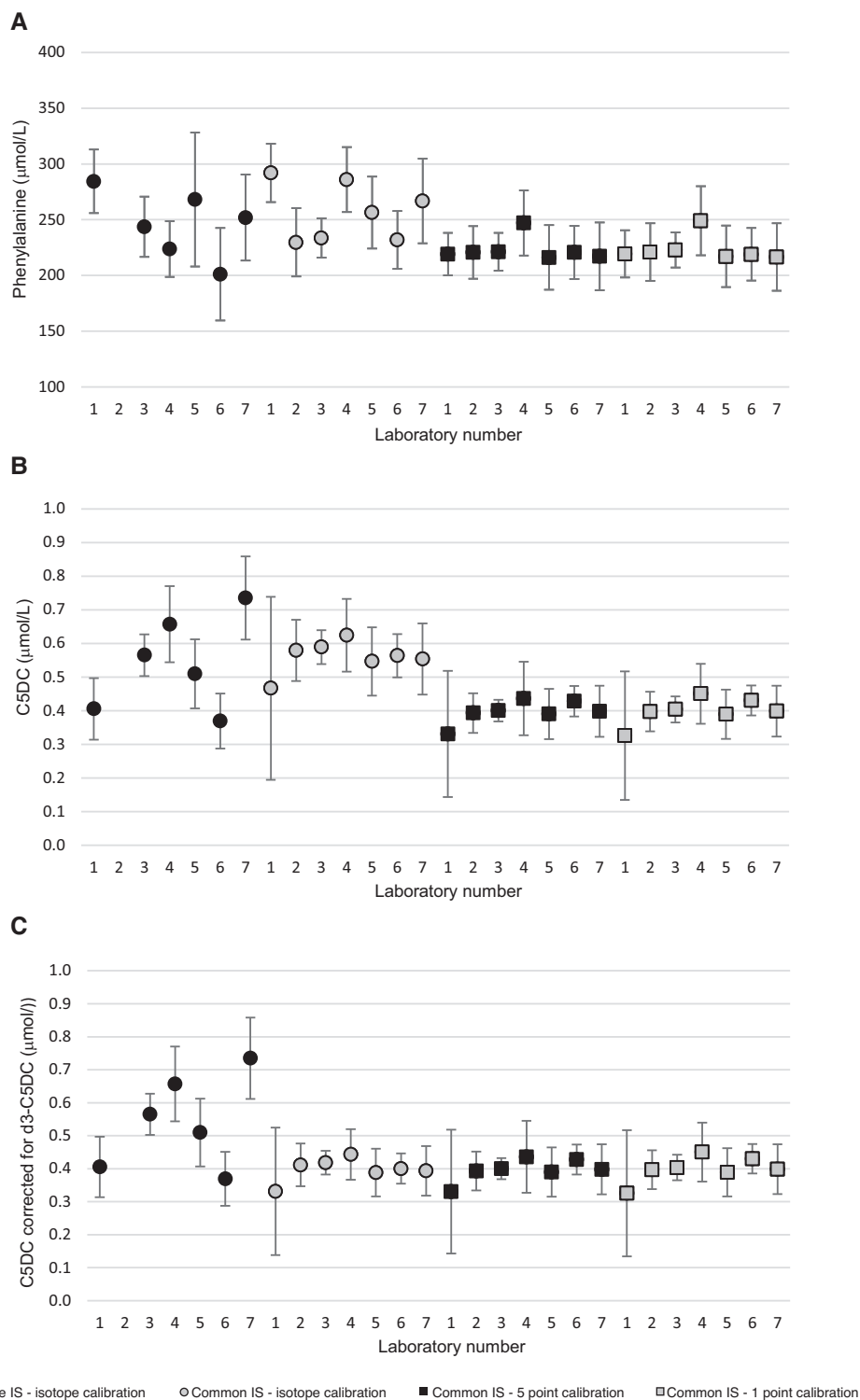


Fig. 4. Impact of the 3 alternative calibration approaches on IQC2 results from each laboratory for (A) Phe, (B) C5DC, and (C) C5DC results corrected for d3-C5DC concentration and comparison with the existing in-house methods. Error bars are ± 2 SD of results. Laboratory 2 did not perform in-house experiments.

This study demonstrates that screening programs can improve both harmonization and standardization of NBS results by optimizing the legacy FIA-MS/MS methods and introducing traceable standards. While this study assessed the analytes utilized in England and Wales, it is expected that the results will be applicable to other analytes commonly included in screening programs worldwide. Furthermore, the findings from this study have important implications in the monitoring of therapy in patients with inherited metabolic disorders as the availability of a traceable calibrator(s) would reduce interlaboratory variation and address concerns raised when utilizing target treatment ranges (10, 11).

The outcomes of this study have led to the national procurement of traceable calibration standards for the UK NBS program. Future work will examine the impact of this calibrator on the COVs currently being utilized.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: FIA-MS/MS, flow injection analysis–tandem mass spectrometry; DBS, dried blood spot; NBS, newborn screening; SIIC, stable isotope internal calibration; IS, internal standard; COV, cutoff value; Met, methionine; Leu, leucine; Phe, phenylalanine; Tyr, tyrosine; C5, isovaleryl-carnitine; C5DC, glutaryl-carnitine; C8, octanoyl-carnitine; C10, decanoyl-carnitine; IQC, internal quality

control; qNMR, quantitative nuclear magnetic resonance; SRM, selective reaction monitoring; %RSD, percentage relative SD.

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