A rapid LC-MS/MS assay for detection and monitoring of underivatized branched-chain amino acids in maple syrup urine disease

Hamed Piri-Moghadam, Alan Miller, Debra Pronger, Faye Vicente, Joel Charrow, Shannon Haymond, David C. Lin

Introduction

The branched-chain amino acids (BCAA), valine, alloisoleucine, isoleucine, and leucine, are involved in metabolic pathways that play significant biological roles in the human body [1–3]. In maple syrup urine disease (MSUD) there is a deficiency in branched-chain ketoacid dehydrogenase complex (BCKD), an enzyme needed for metabolism of those amino acids. Specifically, MSUD is the result of deficiency in one of those amino acids. Specifically, MSUD is the result of deficiency in one of those amino acids.

Methods:

Various columns and modes of chromatography were tested. The final optimized method utilized mixed mode chromatography with an Intrafrac column under isocratic condition. Sample preparation utilized the 96-well format. Briefly, extraction solvent containing the internal standard is added to 20 uL of sample, followed by shaking and positive pressure filtering, and the resulting extracted sample is analyzed. The assay was validated based on accepted quality standards (e.g., CLIA and CLSI) for clinical assays.

Results:

The method is linear over a wide range of concentrations, 2.0–1500 µM, with LOD of 0.60 µM and LOQ of 2.0 µM. The precision of the assay was 4–10% across analytes. The method was also validated against reference laboratories via blinded split-sample analysis and demonstrated good agreement with accuracy; 89–95% relative to the external group mean.

Conclusion:

We have developed a method that is accurate, rapid, and reliable for routine clinical testing of patient sample BCAA, which is used in the diagnosis and management of maple syrup urine disease (MSUD). The assay also has desirable characteristics, such as short run time, small sample volume requirement, simple sample preparation without the need for derivatization, and high throughput.

KEYWORDS

Branched-chain amino acid, Mass spectrometry, Quantitation, Maple syrup urine disease, Clinical assay

ARTICLE INFO

Introduction: Quantitation of the isomeric branched-chain amino acids (BCAA; valine, alloisoleucine, isoleucine, leucine) is a challenging task that typically requires derivatization steps or long runtimes if a traditional chromatographic method involving a ninhydrin ion pairing reagent is used.

Methods: The method is linear over a wide range of concentrations, 2.0–1500 µM, with LOD of 0.60 µM and LOQ of 2.0 µM. The precision of the assay was 4–10% across analytes. The method was also validated against reference laboratories via blinded split-sample analysis and demonstrated good agreement with accuracy; 89–95% relative to the external group mean.

Conclusion: We have developed a method that is accurate, rapid, and reliable for routine clinical testing of patient sample BCAA, which is used in the diagnosis and management of maple syrup urine disease (MSUD). The assay also has desirable characteristics, such as short run time, small sample volume requirement, simple sample preparation without the need for derivatization, and high throughput.

Abbreviations: 3NPH, 3-nitrophenylhydrazine; ACN, Acetonitrile; AMR, Analytical measurable range; BCAA, Branched-chain amino acids; BCKD, Branched-chain ketoacid dehydrogenase complex; CAP, The College of American Pathologists; CLIA, The Clinical Laboratory Improvement Amendments; CLSI, The Clinical & Laboratory Standards Institute; CN, Cyanato; CRR, Clinical Reportable Range; ESI, Electrospray ionization; FA, Formic Acid; GC-MS, Gas chromatography-mass spectrometry; HMDB, Human metabolome database; HILIC, Hydrophilic interaction liquid chromatography; IEX, Ion exchange; LC, Liquid chromatography; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; LC-UV, Liquid chromatography-ultra violet; LDT, Laboratory-developed tests; LLE, Liquid-liquid extraction; LOD, Limit of detection; LOQ, Limit of quantitation; MeOH, Methanol; MSUD, Maple syrup urine disease; NM, Nuclear magnetic resonance; PBS-BSA, Phosphate buffered saline with bovine serum albumin; PITC, Phenylisothiocyanate; PTFE, Polytetrafluoroethylene; QC, Quality control; RP, Reverse phase; RPLC, Reverse phase liquid chromatography; S/N, Signal-to-noise ratio; SCX, Strong cation exchange; SPE, Solid phase extraction; SRM, Selected reaction monitoring; UHPLC, Ultra-high-performance liquid chromatography; WAX, Weak anion exchange.

* Corresponding author at: Ann & Robert H. Lurie Children’s Hospital of Chicago, 225 E Chicago Ave, Chicago, IL 60611, USA

E-mail address: dalin@luriechildrens.org (D.C. Lin).

https://doi.org/10.1016/j.jmsacl.2022.04.003
Received 19 October 2021; Received in revised form 19 April 2022; Accepted 24 April 2022
Available online 30 April 2022

2667-145X © 2022 THE AUTHORS. Publishing services by ELSEVIER B.V. on behalf of MSACL. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
H. Piri-Moghadam et al.  

The three catalytic components of the BCKD complex: E1, the branched chain α-keto acid dehydrogenase (EC 1.2.4.4), E2, the dihydrolipoamide branched chain transacylase (EC 2.3.1.168), or E3, the dihydrolipoyl dehydrogenase (EC 1.2.4.4) [4,5]. This leads to the elevation and accumulation of BCAA and ketoacids in body fluids, and contributes to a variety of clinical symptoms, including skeletal muscle, immune system, and central nervous system dysfunctions. MSUD is estimated to affect one in 100,000 to 300,000 births [6]. MSUD can be diagnosed and monitored based on the concentrations of BCAA in blood [7–9]. If MSUD is not diagnosed in the first few days of life, the progression of the disease may result in critical issues including intellectual disability, coma, irreversible brain damage, seizures, and death [10]. If diagnosed early, patients can be managed by monitoring the blood concentrations of BCAA and controlling BCAA intake. By adjusting the composition of the diet and dietary supplements, potential adverse outcomes and risk factors can be prevented or mitigated [7,10]. Therefore, it is critical to develop a reliable and accurate BCAA assay for confirmation of MSUD in newborns, as well as routine monitoring of MSUD patients. As such, mass spectrometry-based targeted metabolomics presents a powerful technique for the diagnosis and monitoring of biochemical genetic diseases and inborn errors of metabolism, such as MSUD. There are several analytical techniques to quantify BCAA in biofluids [9–11]; however, these methods often involve tedious sample processing, long run times, or less than desirable specificity, sensitivity, or accuracy.

Metabolomics is one of the -omics techniques used for discovery and diagnosis of diseases by monitoring small molecules using various platforms [12–17], including NMR [15], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [18,19] and gas chromatography-mass spectrometry (GC–MS) [18]. Like other -omics techniques, it has benefits and shortcomings. Unlike bottom-up proteomics, which requires trypsin digestion, metabolomics extraction of small compounds can be simply achieved by direct application of available extraction techniques, including solid phase extraction (SPE) or liquid–liquid extraction (LLE). On the other hand, the presence of hundreds of thousands of metabolites in biological samples – estimated to be as many as 114,100 based on the human metabolome database (HMDB 4.0) [20] – can pose challenges. For example, there is high potential of false-positive quantitation due to the presence of isomers, isobars, and metabolites with the same molecular weight and fragments. As such, selection of proper chromatographic method and unique MS/MS transitions, simplified sample preparation techniques, and awareness of potential interferences are important in the development of any metabolomics assay.

Another obstacle in metabolomics is related to the lack of a proper chromatography method to retain and separate polar metabolites. Many clinically significant metabolites (e.g., amino acids) are polar, and the use of reverse phase liquid chromatography (RPLC) will result in the elution of many metabolites in the dead volume. For targeted metabolomics, it is essential to tailor the chromatographic method based on the metabolites of interest. Otherwise, co-elution of metabolites will result in inaccurate quantitation and potentially lead to clinical misdiagnosis and improper treatment of patients. Polar metabolites may be derivatized [21] with compounds such as, phenylisothiocyanate (PITC) [22–25], aTRAQ kit [26], and 3-nitrophenyldihydrazine (SNPH) [27] as one solution to decrease the polarity of metabolites and allow better retention on RPLC (e.g., C18) columns. A commonly used method in clinical laboratories for the chromatographic separation of amino acids involves application of a post-column ion-pairing reagent, such as n-hexylamine followed by UV detection and quantitation [28].

Quantitation of BCAA in biological samples showcases the strengths of mass spectrometry-based targeted metabolomics, including high sensitivity, specificity, and wide dynamic range. Circulating amounts of leucine, isoleucine, and valine in healthy people covers a broad range. Reference intervals for each analyte can differ between laboratories depending on the specific partition and analytical technique used, but generally fall around 50–200 µM for leucine, 20–120 µM for isoleucine, and 80–300 µM for valine in the healthy population [29,30]. In MSUD patients, the concentrations of these analytes are often elevated [31], e.g., higher than the upper limit of the reference interval. Detection of abnormal concentrations of alloisoleucine is considered pathognomonic for MSUD. Alloisoleucine is normally “absent” or present at very low concentrations (usually < 2 µM) in healthy individuals [32]. Given its low concentration, accurate quantitation of alloisoleucine is a challenging task due to its co-elution with the leucine and isoleucine isomers. Alloisoleucine and isoleucine are also diastereomers (i.e., stereoisomers that are not enantiomers), which makes it more difficult to achieve baseline resolution in chromatography.

LC-MS/MS-based targeted metabolomics and its application in clinical laboratories continues to be a growing area of interest, particularly for diagnosis of inborn errors of metabolism, such as MSUD. Lack of appropriate validation studies, poor robustness, inter-batch irreproducibility, high cost, and complex sample preparation are some of the possible shortfalls when transitioning a metabolomics assay from the research bench to the clinical laboratory. The current study focuses on the development and validation of an LC-MS/MS-based, targeted metabolomics clinical assay for the diagnosis of MSUD in newborns and monitoring of MSUD patients in a pediatric hospital setting. The LC-MS/MS-based BCAA assay described here was developed after testing various chromatographic modes and columns to identify an appropriate analytical strategy to achieve near-baseline resolution for the BCAA isomers. The assay was validated in accordance with the guidelines and requirements from the Clinical Laboratory Improvement Amendments (CLIA) and Clinical & Laboratory Standards Institute (CLSI) for laboratory-developed tests (LDT) in clinical mass spectrometry laboratories. The strengths of this assay, particularly in comparison to those described in the literature, include 1) short assay run time, 2) small sample volume requirement, 3) simple sample preparation without the need for derivatization, and 4) high assay throughput, all of which are desirable characteristics for a targeted-metabolomics assay to be used in a clinical laboratory. These characteristics, when used along with automated chromatography data processing and reviewing strategy, also makes it possible to provide final results to the healthcare providers within as few as 4 hours after samples are received by the lab. This duration includes time for sample preparation, LC-MS/MS analysis of calibrators, quality controls, and patient samples, as well as data processing and review.

Materials and methods

Chemicals

Individual standards of valine, alloisoleucine, leucine, and isoleucine were purchased from MilliporeSigma (St. Louis, Missouri, USA) for QC preparation. A mixture of amino acid standards (acidic and neutrals) was purchased from MilliporeSigma (St. Louis, Missouri, USA), to prepare calibration curve standards. Individual isotopically labeled internal standards of L-Valine (13C5, 99%; 15N, 99%), L-Leucine (13C5, 99%; 15N, 99%), L-Isoleucine (13C6, 99%; 15N, 99%), and a mixture of internal standards (Metabolomics Amino Acid Mix Standard) were obtained from Cambridge Isotope Labs (Tewksbury, Massachusetts, USA). Phosphate buffered saline with bovine serum albumin (PBS-BSA; PBS with 1% w/v BSA, pH 7.4) was the standard matrix (calibrators, QCs, and blank) and was purchased from MilliporeSigma (St. Louis, Missouri, USA). Optima LC-MS grade methanol, acetonitrile, and ammonium formate were obtained from Fisher Scientific (Fair Lawn, New Jersey, USA). Formic Acid, 1 mL ampule, 99.4%, used as an additive of mobile phase, was purchased from Thermo Fisher (Fair Lawn, New Jersey, USA). Special Reagent Water was obtained from Millipore Integral 5 Purification System. Hydrochloric acid was purchased from MilliporeSigma (St. Louis, Missouri, USA). External proficiency testing samples containing BCAA were purchased from College of American Pathologists (Northfield, Illinois, USA). Control plasma, which contains know concentrations of amino acids, was purchased from Sciei (Framingham, Massachusetts, USA).
**LC-MS/MS method**

Method optimization, development, and validation experiments were performed using ultra-high-performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry with unit mass resolution instruments and Waters Xevo TQ (Milford, Massachusetts, USA). Sample volumes of 5 µL of both standards and extracted analytes were injected into the LC-MS/MS system using an autosampler. Several columns were tested to develop the final chromatographic method. Luna hydrophilic interaction liquid chromatography (HILIC; 150 × 2 mm, 3 µm, 200 Å), Luna NH2 (150 × 2 mm, 3 µm, 100 Å), Luna strong cation exchange (SCX; 150 × 2 mm, 5 µm, 100 Å), Kinetex Polar C18 (50 × 2.1 mm, 2.6 µm, 100 Å), and Kinetex F5 (100 × 2.1 mm, 2.6 µm, 100 Å) were purchased from Phenomenex (Torrance, California, USA). Infinity Lab Poroshell 120 EC-CN (150 × 3 mm, 2.7 µm, 120 Å) and InfinityLab Poroshell 120 Chiral-T (100 × 4.6 mm, 2.7 µm, 120 Å) were obtained from Agilent (Santa Clara, California, USA). Intrada Amino Acid column (150 × 3 mm, 3 µm; pore size is proprietary) was purchased from Imtakt USA (Portland, Oregon, USA). Mobile phase composition, flow rate, and gradient conditions are different for each of the aforementioned columns, and are provided in the [Supplementary Material](#), Sections 2-7. The final optimized method selected for the separation of BCAA utilized the Intrada column and involved isocratic condition with the flow rate of 0.8 mL min⁻¹. Mobile phase composition was MeOH:H₂O (80:20) with 10 mM ammonium formate and 0.25% v/v formic acid. BCAA were completely eluted with a total runtime of 8.5 min. The temperature of the column holder was set at 35 °C. Given that the human plasma samples contain a large number of metabolites, including highly polar metabolites, amino acids with positively charged side chains (e.g., lysine, arginine, and histidine), and other potential residual contam­nants, a column flush was performed at the end of each day using solutions with higher salt and water content to help clear and deep clean the column, as per column manufacturer’s recommendation. Specifically, 100 mM ammonium formate in 100% water was used to flush the column at the end of the day. The column was then flushed with 100% water and switched back to the mobile phase.

Mass spectrometric acquisition was performed in the positive mode of electrospray ionization (ESI). Selected reaction monitoring (SRM) mode was used for quantitative targeted analysis. Transitions of 132.1 → 86 for Leu, Ile, and Allo; 139.1 → 92 for Leu and Ile (13C6-Lys); 118.1 → 72 for valine, and 124.1 → 77 for valine (13C6-Lys) were selected. Mass spectrometric conditions were set as: ESI voltage of + 3 kV; desolvation gas flow of 650 (L/Hr); cone gas flow of 100 (L/Hr); ion source temperature: 150 °C, and desolvation temperature: 350 °C.

**Calibrators and quality control preparation**

The concentration units for calibrators and extraction solvents described in the following sections are volume/volume (v/v), unless otherwise specified. To prepare the standards for the calibration curve, individual standards were weighed and dissolved in a mixture of MeOH: H₂O (70:30) with 1% (v/v) HCl to prepare a stock solution with a concentration of 33000 µM. The individual standards were then mixed in equal volumes to prepare the calibrator and QCs were selected based on historical review (Fig. 1). One line was plotted across undiluted and diluted samples. One line was constructed for each matrix and control, with response on the y-axis and 1000 µM of each analyte divided by area of IS) of different analytes in a given matrix was tested was plotted across undiluted and diluted samples. One line was constructed for each matrix and control, with response on the y-axis and dilution factors on the x-axis. As co-elution of albumin may affect the matrix effect, it should be noted that a centrifuge can be used if a positive pressure extraction device is not available, but it may take up to ~ 10 min longer. Finally, the extract collected on a 96-well plate was run on the LC-MS instrument.

**Chromatography method**

A number of reverse phase (RP), HILIC, and ion exchange (IEX) columns – including polar C18, F5 (polar organic and RP modes), chiral, mixed mode (Intrada), SCX, NH₂ (weak anion exchange, WAX), CN, and HILIC – were tested for their ability to retain and separate BCAA (see [Supplemental Material](#), sections 3-7). The NH₂ column was tested in polar organic mode to ensure compatibility with ESI-MS. The final method chosen used the Intrada column and was based on the isocratic condition of MeOH:H₂O with 0.25% FA and 10 mM ammonium formate.

**Matrix effect**

A parallelism study was used to evaluate the effect of matrix on ionization efficiency of BCAA. Samples used in the parallelism study included 15 clinical plasma samples, as well as control samples, which were prepared by mixing BCAA standards at 400 µM in neat solvent and PBS-BSA. All samples, standards, and QCs were analyzed as described earlier (Fig. 1). Extracts were diluted at 1x (i.e., undiluted), 2x, 5x, 20x, and 50x, and analyzed. To evaluate matrix effect, response (i.e., area of analyte divided by area of IS) of different analytes in a given matrix was plotted across undiluted and diluted samples. One line was constructed for each matrix and control, with response on the y-axis and dilution factors on the x-axis. As co-elution of albumin may affect the measurement of low concentration analytes (33-34), we also evaluated the impact of different albumin removal strategies, including ultrafiltration versus simply diverting to waste during the LC portion of the assay (see [Supplemental Material](#), Section 8). The latter approach was ultimately adopted for the final method in order to simplify the sample.
Assay evaluation

Carryover

Carryover of the method was evaluated two ways: 1) measuring the signal of the blank sample after a high concentration sample (i.e., concentration at the upper limit of the AMR), and 2) running a series of low (L; 4.4 µM) and high (H; 1500 µM) concentration samples in a particular sequence: L1, L2, L3, H1, H2, L4, H3, H4, L5, L6, L7, L8, H5, H6, L9, H7, H8, L10, H9, H10, and L11, and comparing values observed in post-HI LO samples (i.e., low concentration (LO) sample run after a high concentration (HI) sample) versus post-LO LO samples (i.e., LO sample run after a LO sample) for each analyte using t-test.

Limit of detection, limit of quantitation and analytical measurable range (AMR)

The LOD, LOQ, and AMR were obtained by running samples, in four replicates, at 11 concentrations, ranging from 0.5 µM to 1500 µM. The 11 samples were prepared by admixing low (0.5 µM) and high (1500 µM) concentrations of BCAA mixture (Supplementary Material s, Table S1).

Accuracy

Accuracy was evaluated as part of the AMR study and by testing samples of known concentrations, including QCs at three concentrations, three CAP proficiency samples, and one control plasma from Sciex which contains amino acids mixture of known concentration. Accuracy was also assessed based on external lab comparisons (see Inter-lab correlation study).

Clinically reportable range (CRR)

To evaluate the CRR of the assay, PBS-BSA was spiked to produce a CRR level 4 sample at concentrations of 2002.7, 1998.6, 1999.6, and 2001.6 µM for valine, leucine, isoleucine, and alloisoleucine, respectively. The CRR level 4 sample was diluted with PBS-BSA at 1:2 (CRR level 2), 1:5 (CRR level 3), and 1:10 (CRR level 4) dilution factor, and then extracted in 4x technical replicates.

Precision

To evaluate the precision and robustness of the assay, an experiment was performed based on the CLSI EP-05 guideline [35]. Precision study samples were prepared at four concentrations spanning the AMR: 2.0 (4.4 for valine), 100, 750 and 1350 µM. Three technologists on a rotating schedule analyzed the four concentrations of precision samples in duplicates per run, two runs per day (with at least two hours between runs), over the course of 21 days. The precision samples were analyzed with solvent blank, PBS-BSA, and three concentrations of QC to ensure the quality of each run.

Inter-lab correlation study

Accuracy of the assay was also evaluated by comparing results obtained in-house against those measured at external reference laboratories. Briefly, frozen plasma from patients with diagnosed and suspected MSUD were thawed and randomly combined to obtain pooled samples. Pooled samples were aliquoted in cryogenic vials and stored at −20 °C. Labeled correlation samples were placed in storage containers and shipped on dry ice to reference laboratories. Additional aliquots were also prepared for correlation with results obtained using Biochrom 30 amino analyzer (Cambridge, UK), the current method used in the lab, which involves LC-UV and ion pairing with ninhydrine. Plasma samples were deproteinized and stored frozen at −20 °C until they needed to be thawed prior to Biochrom analysis. Forty-nine samples in total were sent out to external labs for comparison. Nine samples did not have in-house measurements via Biochrom analyzer as pooled samples were prepared for send-out comparisons only. Results were not obtained for one of the samples from one of the external laboratories due to transport and labelling issues which made analysis impossible.

Data processing

All chromatographic data were processed using ASCENT (version 3.7; Indigo BioAutomation, Inc., Indiana, USA), a cloud-based mass spectrometry and chromatography data processing software. Briefly, technologists accession the LC-MS/MS run into the ASCENT software and the laboratory information system (LIS) assign samples to a batch. The ASCENT software automatically queries a pre-specified folder on the network and looks for new files generated from the mass spectrometer. Upon completion of the LC-MS/MS analytical run, the raw data file and sample list tables are uploaded by ASCENT, which then
performs the necessary data conversion and analysis based on the chromatogram data, and generates quantitative results for each compound. The results from the analytical run are then available for technologists to review and certify through the ASCENT website. Data from the assay evaluation studies were analyzed using a combination of Microsoft Excel and R (version 4.0.3) in RStudio (version 1.3.1056) [36,37]. Figures were constructed using Excel and R via ggplot2 R package (version 3.3.2) [38]. For the parallelism experiment, linear regression models were fitted for each matrix for comparison. For the external laboratory comparison, the target values (i.e., group mean) were calculated based on the average of values obtained across external labs.

Results

Chromatographic separation of underivatized branched-chain amino acids

Fig. 2A-I shows results of different columns (after optimization) used for separation of leucine, isoleucine, and alloisoleucine. Detailed information of optimization of chromatographic parameters (i.e., solvents, acid, salt, gradient and flow rate) for each column is provided in Supplementary Materials, Sections 3–7. C18 and F5 (Fig. 2A, B and C) columns were not able to retain BCAA, and all analytes of interest eluted within the dead volume. While the CN (Fig. 2D) column retained the BCAA, it did not provide the resolving power required for separating isomers. Although NH2 (Fig. 2E) and HILIC (Fig. 2F) columns showed higher retention factor, they were able to separate structural isomers, but failed to resolve the stereoisomers of isoleucine and alloisoleucine. Although chiral (Fig. 2G) and SCX (Fig. 2H) columns were both capable of partially resolving the three isomers, the isomers overlapped at 30–40% of peak height. As shown in Fig. 2I, the best result was obtained using the Intrada column [39], which uses mixed mode chromatography.

Matrix effect

As shown in Fig. 3, regression lines constructed based on response observed versus dilution factors for each clinical sample generally had slopes near zero (i.e., nearly identical response across dilution factors tested) and were parallel across samples, including those with control samples (i.e., analyte in PBS/BSA or diluent). This suggests that there is neither a significant ion suppression nor enhancement effect, and that the extent of matrix ion suppression or enhancement may be sufficiently similar between the calibrators and test samples. This supports the idea that the use of IS and matrix-matched calibration is able to correct for potential matrix effects. In the different biological matrices tested, if matrix effect is present then we would expect to see: 1) significantly higher response in diluted versus undiluted samples due to ion suppression, and 2) substantially different regression line slopes between clinical and control samples on a response vs. dilution factor plot. Furthermore, since albumin is a major component in the matrix that may co-elute with analytes of interest – particularly those present in low abundance– we optimized the LC gradient and assay run time to reproducibly divert the remaining albumin in samples post-extraction to waste during the first three minutes of the LC isocratic elution. This prevents co-elution of albumin with BCAA and makes robust and accurate quantitation of BCAA possible.

Carryover

We did not observe appreciable carryover effect using either: 1) measuring the signal of the blank sample after a high concentration sample (Supplemental Materials, Fig. S23), or 2) analyzing a series of low and high samples, as the t-tests performed comparing the post LO-LO and the post HI-LO samples concentrations did not show any significant difference for any of the analytes (p > 0.05).

LOD, LOQ, AMR and CRR

LOD and LOQ were obtained based on a signal-to-noise ratio (S/N) cut-off of 3 and 10, respectively (Section 10 of Supplemental Materials). This translates to an LOD of 0.60 µM and an LOQ of 2.0 µM. There are several ways to test the linearity assumption of an assay. One approach is to evaluate linearity by visual inspection (e.g., response vs. concentration or expected vs. measured concentration), which is the least robust approach. Another approach is to examine the fit of the linear regression model based on the R2 value, i.e., how well does the linear regression model “fit”. Yet another approach is to examine and compare straight-line to higher-order polynomial curve fits [40,41]. We utilized a combination of the latter two approaches. Linearity of the calibration curves was obtained over three orders of magnitude, with R2 > 0.99 and intercept < LOQ using weighted calibration (1/x and 1/x2). Table 1 summarizes the performance characteristics of the LC-MS/MS BCAA assay. In this study, linearity was also assessed statistically by testing the model y = x + x2 (where y is the measured concentration and x is the nominal concentration) and evaluating the significance of the x and x2 terms (results not shown). The assay was determined to be linear between concentrations of 2 to 1500 µM for all analytes. Accuracy was also estimated based on the AMR experiment samples which ranged from 80 to 99%, depending on the analyte and the concentration tested (see Supplemental Table S2).

Accuracy of the assay was also evaluated via analysis of reference samples with known concentrations. Table 2 shows acceptable agreement between the BCAA concentrations obtained in-house versus the known concentrations. The CRR of the assay, based on the dilution experiments performed, was determined to be 2–2000 µM for alloisoleucine, leucine, isoleucine, and valine. At 2x dilution, recovery observed was, on average, > 95% for all analytes; alloisoleucine had a 93% recovery rate. The upper limit of the CRR was ultimately set at 2000 µM.

Precision

The overall precision of the assay observed was approximately 4–10%, depending on the analyte and concentration of the sample measured (imprecision was up to ~ 20% at the LOQ, which are generally at concentrations significantly lower than physiological concentrations expected in patient samples). Table 3 summarizes precision results by compound and concentration using Waters Xevo LC-MS/MS. (See section 11 of Supplementary Material for figures, which show the results of the precision study over the course of 21 days for each analyte).

Inter-lab correlation study

Fig. 4a shows acceptable correlation between results obtained using the assay developed and target values calculated based on the average of values obtained by the external reference laboratories. Fig. 4b demonstrates the alloisoleucine concentrations measured by each external reference laboratory and by in-house methods. On average, accuracy ranged from ~ 89–95% when we compared the results obtained in-house versus the target “true” values, which were calculated based on the mean of the external laboratories’ measurements.

Discussion

Chromatographic retention and separation of polar metabolites (i.e., amino acids) is a challenge in clinically-targeted metabolomics. Reverse phase chromatography using C8 or C18 columns [1] results in coelution or poor chromatographic retention and separation. Commonly used systems for the detection of BCAA include the Biochrom 30 amino acid analyzer system (Cambridge, UK), which is based on ion exchange chromatography and post-column derivatization of amino acids using a ninhydrin ion pairing reagent [42,28], followed by UV/VIS photometric detection. While this method was introduced >60 years ago [28], it is
Fig. 2. Chromatography based on various chromatography columns and modes for separation of BCAA. The LC-MS/MS analysis was done on Waters Xevo instrumentation.
Based on our lab’s experience with the Biochrom method, it takes about three hours for separation of 45 amino acids and one hour and forty minutes for BCAA. Long chromatographic separations pose several issues, particularly when results are required in clinically urgent scenarios. To retain and separate the polar metabolites using RPLC in a shorter runtime, several groups [22,24,43] used PITC to derivatize amino acids during sample preparation. However, derivatization has several limitations, including adding extra steps to the protocol, being tedious and time-consuming (including the evaporation/reconstitution step), potential

Fig. 3. Parallelism study to assess matrix effect. Comparison made based on response (y-axis) vs. dilution factor (x-axis) for A) alloisoleucine, B) isoleucine, C) leucine, and D) valine. Sample S00a represents a high concentration standard prepared in PBS:BSA, and S00b represents a high concentration standard prepared in diluent. S01 through S15 represents different clinical samples.

Table 1
Performance characteristics of the LC-MS/MS-based BCAA assay.

| Metabolite       | Internal standard | LOD (µM) | LOQ (µM) | AMR (µM) | CRR (µM) | Weighting | Slope        | Intercept | R²            | Accuracy % (n = 3) | RSD % (n = 3) |
|------------------|-------------------|----------|----------|----------|----------|-----------|--------------|-----------|---------------|------------------|---------------|
| Valine           | Valine (13C5;15 N) | 0.60     | 2.0      | 2.0-1500 | 2.0-2000 | 1/x       | 8.81 e⁻³   | –          | 0.0024        | 0.9993           | 90             | 93            | 94            | 3.2           | 1.1           | 1.5           |
| Leucine          | Leucine (13C6;15 N) | 0.60     | 2.0      | 2.0-1500 | 2.0-2000 | 1/x       | 2.14 e⁻²   | –          | 0.0028        | 0.9996           | 99             | 99            | 98            | 1.6           | 1.0           | 1.5           |
| Isoleucine       | Isoleucine (13C6;15 N) | 0.60     | 2.0      | 2.0-1500 | 2.0-2000 | 1/x       | 1.73 e⁻²   | –          | 0.00097       | 0.9995           | 98             | 97            | 98            | 1.0           | 1.0           | 1.0           |
| Alloisoleucine   | Alloisoleucine (13C6;15 N) | 0.60     | 2.0      | 2.0-1500 | 2.0-2000 | 1/x²      | 1.51 e⁻²   | 0.0075     | 0.9936         | 98              | 80             | 82            | 15            | 1.0           | 1.5           |

Accuracy was calculated as 1-abs(1-(measured concentration/expected concentration)).
Slope and intercept represent those of the calibration curve where y = response, i.e., analyte/internal standard area count ratio, and x = concentration.

L = level. L1 = 25 µM for valine, leucine, and isoleucine, 2 µM for alloisoleucine; L2 = 250 µM for valine, leucine, and isoleucine, 150 µM for alloisoleucine; L3 = 500 µM for valine, leucine, and isoleucine, 300 µM for alloisoleucine. Three concentrations of samples were prepared in PBS:BSA. Also see inter-lab comparison (Fig. 4) for additional accuracy information.

+ clinically significant cut-off chosen in consultation with clinician for testing.

still the method of choice in many clinical laboratories. Based on our lab’s experience with the Biochrom method, it takes about three hours for separation of 45 amino acids and one hour and forty minutes for BCAA. Long chromatographic separations pose several issues, particularly when results are required in clinically urgent scenarios. To retain
Tables

### Table 2
Results of known samples using the developed BCAA assay.

| Sample name | Metabolite     | Target value (µM) | Measured value (µM) |
|-------------|----------------|-------------------|--------------------|
| CAP sample 1 | Valine         | 207               | 216                |
|             | Alloisoleucine | –                 | < 2 µM             |
|             | Leucine        | 138               | 139                |
|             | Isoleucine     | 74                | 72                 |
| CAP sample 2 | Valine         | 203               | 205                |
|             | Alloisoleucine | –                 | < 2 µM             |
|             | Leucine        | 126               | 121                |
|             | Isoleucine     | 68                | 65                 |
| CAP sample 3 | Valine         | 186               | 190                |
|             | Alloisoleucine | –                 | < 2 µM             |
|             | Leucine        | 99                | 97                 |
|             | Isoleucine     | 50                | 55                 |
| Control plasma from Scie | Valine         | 199               | 224                |
|             | Alloisoleucine | –                 | 21                 |
|             | Leucine        | 126               | 129                |
|             | Isoleucine     | 86                | 67                 |

CAP sample target values based on peer-group median. Control plasma amino acid target values were provided by Scie based on aTRAQ reagent assay.

### Table 3
Method precision summary by compound and concentration.

| Compound         | Level | Nominal value (µM) | n | Avg Conc | SD Conc | CV Conc (%) |
|------------------|-------|--------------------|---|----------|---------|-------------|
| Alloisoleucine   | L1    | 2.0                | 82 | 1.70     | 0.26    | 15.3        |
|                  | L2    | 100               | 82 | 101.40   | 3.18    | 3.1         |
|                  | L3    | 750               | 82 | 676.60   | 27.54   | 4.1         |
|                  | L4    | 1350              | 82 | 1193.50  | 35.99   | 3.0         |
| Isoleucine       | L1    | 2.0                | 82 | 1.60     | 0.21    | 13.1        |
|                  | L2    | 100               | 82 | 98.90    | 2.21    | 2.2         |
|                  | L3    | 750               | 82 | 709.10   | 24.22   | 3.4         |
|                  | L4    | 1350              | 82 | 1267.00  | 33.97   | 2.7         |
| Leucine          | L1    | 2.0                | 82 | 1.50     | 0.25    | 16.7        |
|                  | L2    | 100               | 82 | 98.40    | 2.21    | 2.2         |
|                  | L3    | 750               | 82 | 701.30   | 23.43   | 3.3         |
|                  | L4    | 1350              | 82 | 1244.60  | 30.41   | 2.4         |
| Valine           | L1    | 4.0                | 82 | 4.00     | 0.30    | 7.5         |
|                  | L2    | 100               | 82 | 99.20    | 2.27    | 2.3         |
|                  | L3    | 750               | 82 | 706.90   | 25.11   | 3.6         |
|                  | L4    | 1350              | 82 | 1263.10  | 31.66   | 2.5         |

Level = precision sample level (L1 is the lowest, L4 is the highest). Nominal value = based on the standards prepared in-house. n = total # of datapoints used for calculation. Avg Conc = average concentration measured. SD Conc = standard deviation of the concentrations measured. CV Conc = coefficient of variation (CV; in %) observed.

Conclusion

We have developed and validated a clinical LC-MS/MS assay that allows rapid and accurate quantitation of BCAA in plasma samples. As the method can retain and separate polar metabolites – specifically, the branched-chain amino acids – without the need for derivatization, this allows for a straightforward and convenient sample preparation process. The use of extraction solvent that includes the internal standard further simplifies the sample preparation process, while making accurate quantitation possible. Further, the assay requires only a small sample volume (i.e., 20 µL), which makes re-analysis of low-volume samples possible. This may be particularly useful when analyzing samples from Matuszewski et al. [44], typically involve pre- and post-extraction spiking of samples. These approaches are more applicable when dealing with exogenous metabolites, drugs, or when endogenous metabolites are present at very low concentrations. Given endogenous BCAA concentrations in blood samples are relatively high in healthy samples and MSUD patients, a parallelism experiment was used as an alternative method to study the matrix effect [45–48]. Our results suggest that combination of our internal standard selection and sample preparation process minimizes matrix effects, allowing accurate quantitation of BCAA analytes (Figs. 3 and 4).

Another major benefit of the sample preparation method used in this assay is its simplicity. One of the main advantages of using the mixed-mode column is that the initial condition of the mobile phase (polar organic) and the extraction solvent are matched in their composition of organic and water content. This allows direct injection of extract to the LC-MS/MS without the need for extra dilution or solvent evaporation followed by reconstitution. Additionally, since no derivatization is required and internal standards are already incorporated in the extraction solvent, the workflow is further streamlined. Total sample preparation time is generally <30 min per batch of 7 calibrators, 3 quality controls, 2 blank samples, and 12 patient samples from start to finish. This is shorter than the sample preparation time described by other LC-MS/MS methods, which often involve dry down and reconstitution [49,10], or derivatization [50,43]. Furthermore, other BCAA methods described in the literature do not separate and measure alloisoleucine, an analyte critical in the diagnosis and monitoring of MSUD [51].

One clinical consideration for this assay is possible interference of 6-aminocaproic acid. 6-aminocaproic acid is a drug routinely used in cardiovascular surgery. It is a structural isomer of leucine, isoleucine, and alloisoleucine with similar fragments, but different intensities. As such, there is high suspicion that patients being tested for BCAA using this assay might be taking this medication, an alternative method is also available which can also be adapted to avoid the interference by increasing the run from 8.5 to 9.5 min (Supplemental Materials, Fig. S27). At the time of this assay development, we were not aware of any patients who have been put on 6-aminocaproic acid while being tested for BCAA.

This assay has several strengths. One is the minimal sample volume required, which is useful for newborn testing when a limited sample may be available, or if a sample needs to be re-tested for MSUD confirmation. Another strength is the simplicity of the sample preparation. To improve batch-to-batch repeatability and to simplify the method, an internal standard was added to the extraction solvent (as master mix). In the current chromatographic method, polar organic mode (i.e., high content of acetonitrile or methanol as initial gradient) was used. Ensuring the composition of the extract is compatible with the chromatographic method improves peak shapes and avoids tailing or fronting, particularly for early-eluting metabolites. Furthermore, this allows the extract to be directly run in LC-MS/MS without the need for the solvent evaporation and reconstitution step. This shortens the sample preparation time and minimizes variability during sample preparation. Finally, the chromatographic separation is ~10 min per sample, which allows for a rapid analysis-to-result time.

We have developed and validated a clinical LC-MS/MS assay that allows rapid and accurate quantitation of BCAA in plasma samples. As the method can retain and separate polar metabolites – specifically, the branched-chain amino acids – without the need for derivatization, this allows for a straightforward and convenient sample preparation process. The use of extraction solvent that includes the internal standard further simplifies the sample preparation process, while making accurate quantitation possible. Further, the assay requires only a small sample volume (i.e., 20 µL), which makes re-analysis of low-volume samples possible. This may be particularly useful when analyzing samples from health issues (PITC is toxic), and poor batch-to-batch repeatability. In addition, derivatization of amino acids using PITC may not be linear over the range of concentrations for the metabolites. Therefore, a faster, simpler, and safer BCAA method with a high level of reliability and accuracy is desirable. This study showed that the mixed-mode, SCX, and NH2 columns are able to retain polar metabolites. Of the three, the mixed-mode column was best able to separate structural and stereoisomers of BCAA without requiring sample derivatization. Overall, the performance of this assay was determined as acceptable for clinical use. The accuracy, on average, was between 87 and 99% across analytes and AMR concentrations tested (i.e., across low, mid, and high concentrations - see Tables 1, 2, and Supplemental Material Table S2). The precision averaged 4–10% across the AMR (see Table 3 and Supplemental Material Table S2), depending on the analyte of interest. Comparison against external reference laboratories also showed 89–95% accuracy relative to the target group values (see Fig. 4).

Traditional approaches of evaluating matrix effect in quantitative LC-MS/MS analyses of biological fluids, such as those described by

The accuracy, on average, was between 87 and 99% across analytes and AMR concentrations tested (i.e., across low, mid, and high concentrations - see Tables 1, 2, and Supplemental Material Table S2). The precision averaged 4–10% across the AMR (see Table 3 and Supplemental Material Table S2), depending on the analyte of interest. Comparison against external reference laboratories also showed 89–95% accuracy relative to the target group values (see Fig. 4).

Traditional approaches of evaluating matrix effect in quantitative LC-MS/MS analyses of biological fluids, such as those described by
Moreover, this provides the benefits of minimizing the amount of solvent required during sample preparation, and making it easy to adopt a 96-well plate format for high-throughput batch processing. One major limitation of the method is that some of the specifications of the Intrada column, e.g., pore size, are proprietary. Nevertheless, as the field of chromatography and column manufacturing inevitably advances, we believe methods for separating and analyzing metabolites in blood samples will become increasingly easier, and more readily available. Ultimately, the simplicity, speed, and accuracy of the BCAA assay described is one example of a relatively streamlined method that is readily accessible and adoptable by clinical labs.

**Declaration of Competing Interests**

The authors declare that they have no known competing financial interests or personal relationships that could affect the work described in this article.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2022.04.003.
References

[1] M. Sargsyan, A. Trchounian, Development and validation of LC-MS/MS method for determination of branched chain amino acids and α-keto acids in human plasma, Int. J. Mass Spectrom. 453 (2020) 116345.

[2] R.Y. Yang, S.M. Wang, L. Sun, J.M. Liu, H.X. Li, X.F. Sui, M. Wang, H.L. Xiu, S. Wang, Q. He, J. Dong, W.W. Chen, Association of branched chain amino acids with coronary artery disease in a matched pair case-control study, Nutrition, Metabolism and Cardiovascular Diseases 25 (10) (2015) 937–942, https://doi.org/10.1016/j.numecd.2015.06.003.

[3] Y. Zhang, Q. Zhou, R. Yang, C. Hu, Z. Huang, G. Zhang, Q. Liang, R. Gong, X. Zhu, H. Gorga, H. Yuan, C. X. N. Zhang, Z. Yang, L. Sun, Serum branched-chain α-keto acids are associated with leukocyte telomere length and frailty based on residents from Guangxi longevity county, Sci. Rep. 10 (1) (2020) 1–8, https://doi.org/10.1038/s41598-020-58910-5.

[4] T. G Jungo, J.L. Chuang, R.M. Wynn, G. Stylianoudis, M. Korson, D.T. Chuang, A. Drouidotis, Maple syrup urine disease in Cypriot families: identification of three novel mutations and biochemical characterization of the Thr211Met mutation in the Elafin alpha subunit, Genet. Test. Mol. Biomarkers 13 (5) 657–664 (2009), https://doi.org/10.1089/gtm.2009.0065.

[5] K.A. Straus, V.J. Carlson, K. Sortys, M.E. Young, L.E. Bowser, E.G. Puffenberger, K. W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Muelly, D.A. Shellmer, Z. Radcliff, W. Brigatti, K.B. Williams, D.L. Robinson, C. Hendrickson, K. Beiler, C.M. Taylor, B. Haas-Givler, S. Chopko, J. Hailey, E.R. Mue
A. Eshghi, A.J. Pistawka, J. Liu, M. Chen, N.J.T. Sinclair, D.B. Hardie, M. Elliott, L. Chen, R. Newman, Y. Mohammed, C.H. Borchers, Concentration determination of >200 proteins in dried blood spots for biomarker discovery and validation, Mol. Cell. Proteomics 19 (3) (2020) 540–553, https://doi.org/10.1074/mcp.TIR119.001820.

S.A. Carr, S.E. Abbatiello, B.L. Ackermann, C. Borchers, B. Dornon, E.W. Deutsch, R.P. Grant, A.N. Hoofangle, R. Huttenhain, J.M. Koomen, D.C. Liebler, T. Liu, B. MacLean, D.R. Mani, E. Mansfield, H. Neubert, A.G. Paulovich, L. Reiter, O. Vitek, R. Aebersold, L. Andersson, R. Bethoven, E. Boja, J. Botelho, M. Boyne, R.A. Bradshaw, A.L. Burlingame, D. Chan, H. Keshishian, E. Kuhn, C. Kinsinger, J.S. Lee, S.W. Lee, R. Moritz, J. Oses-Prieto, N. Rifai, J. Ritchie, H. Rodriguez, P.R. Srinivas, R.R. Townsend, J. Van Eyk, G. Whiteley, A. Wiita, S. Weintraub, Targeted peptide measurements in biology and medicine: best practices for mass spectrometry-based assay development using a fit-for-purpose approach, Mol. Cell. Proteomics 13 (3) (2014) 907–917, https://doi.org/10.1074/mcp.M113.036095.

F. Poitout, J.L. Colangelo, S. Lavallee, A.D. Aulbach, M.S. Piche, D. Emmulat, M. Ameri, L.I. Boone, Current practices and challenges in method validation, Toxicol. Pathol. 46 (7) (2018) 847–856, https://doi.org/10.1177/0192623318801571.

F. Poitout, J.L. Colangelo, S. Lavallee, A.D. Aulbach, M.S. Piche, D. Emmulat, M. Ameri, L.I. Boone, Current practices and challenges in method validation, Toxicol. Pathol. 46 (7) (2018) 847–856, https://doi.org/10.1177/0192623318801571.

F. Poitout, J.L. Colangelo, S. Lavallee, A.D. Aulbach, M.S. Piche, D. Emmulat, M. Ameri, L.I. Boone, Current practices and challenges in method validation, Toxicol. Pathol. 46 (7) (2018) 847–856, https://doi.org/10.1177/0192623318801571.

Y. Lopez-Hernandez, J.J. Oropesa-Valdez, J.O. Blanco-Sandate, A.S.H. Van Oostdam, J. Zheng, A.C. Guo, V. Lima-Rogel, R. Rajabzadeh, M. Salgado-Bustamante, J. Adrian-Lopez, C.G. Castillo, E.R. Arguelles, J. Monarrez-Espino, R. Mandal, D.S. Wishart, The urinary metabolome of healthy newborns, Metabolites 10 (4) (2020), https://doi.org/10.3390/metabo10040165.

T.T. Le, A. Shafaei, A. Genoni, C. Christophersen, A. Devine, J. Lo, P.L. Wall, M. C. Boyce, Development and validation of a simple LC-MS/MS method for the simultaneous quantitative determination of trimethylamine-N-oxide and branched chain amino acids in human serum, Anal. Bioanal. Chem. 411 (5) (2019) 1019–1028, https://doi.org/10.1007/s00216-018-1522-8.