# Second-Tier Test for Quantification of Alloisoleucine and Branched-Chain Amino Acids in Dried Blood Spots to Improve Newborn Screening for Maple Syrup Urine Disease (MSUD)

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**BACKGROUND:** Newborn screening for maple syrup urine disease (MSUD) relies on finding increased concentrations of the branched-chain amino acids (BCAAs) leucine, isoleucine, and valine by tandem mass spectrometry (MS/MS). D-Alloisoleucine (allo-Ile) is the only pathognomonic marker of MSUD, but it cannot be identified by existing screening methods because it is not differentiated from isobaric amino acids. Furthermore, newborns receiving total parenteral nutrition often have increased concentrations of BCAAs. To improve the specificity of newborn screening for MSUD and to reduce the number of diet-related false-positive results, we developed a LC-MS/MS method for quantifying allo-Ile.

**METHODS:** Allo-Ile and other BCAAs were extracted from a 3/16-inch dried blood spot punch with methanol/$H_2O$, dried under nitrogen, and reconstituted into mobile phase. Quantitative LC-MS/MS analysis of allo-Ile, its isomers, and isotopically labeled internal standards was achieved within 15 min. To determine a reference interval for BCAAs including allo-Ile, we analyzed 541 dried blood spots. We also measured allo-Ile in blinded samples from 16 MSUD patients and 21 controls and compared results to an HPLC method.

**RESULTS:** Intra- and interassay imprecision (mean CVs) for allo-Ile, leucine, isoleucine, and valine ranged from 1.8% to 7.4%, and recovery ranged from 91% to 129%. All 16 MSUD patients were correctly identified.

**CONCLUSIONS:** The LC-MS/MS method can reliably measure allo-Ile in dried blood spots for the diagnosis of MSUD. Applied to newborn screening as a second-tier test, it will reduce false-positive results, which produce family anxiety and increase follow-up costs. The assay also appears suitable for use in monitoring treatment of MSUD patients.



D-Alloisoleucine (allo-Ile)[6] is a pathognomonic marker for maple syrup urine disease (MSUD; OMIM 248600), an autosomal recessively inherited deficiency of the branched-chain ketoacid dehydrogenase complex (BCKDH; EC 1.2.4.4) *(1)*. BCKDH catalyzes the first step of the metabolism of the branched-chain amino acids (BCAAs) isoleucine (Ile), leucine (Leu), and valine (Val), and its deficiency occurs with an estimated incidence of 1:185 000 births worldwide *(2)*. Classic MSUD presents in the neonate with feeding intolerance, failure to thrive, vomiting, lethargy, and a urine odor reminiscent of maple syrup. Left untreated, it progresses to irreversible mental retardation, hyperactivity, failure to thrive, seizures, coma, cerebral edema, and possibly death. Identification of patients before the onset of symptoms is highly desirable, as early initiation of dietary intervention allows for a favorable long-term outcome with reduced mortality *(3)*. Since 1964, MSUD has been added to newborn screening programs by applying a modification of Guthrie's bacterial inhibition assay to detect increased Leu concentrations in dried blood spots *(4, 5)*. With the application of tandem mass spectrometry (MS/MS) and the inclusion of MSUD as a primary screening target by the American College of Medical Genetics *(6)*, MSUD is now included in 45 state screening programs

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[6] Nonstandard abbreviations: Allo-Ile, D-alloisoleucine; MSUD, maple syrup urine disease; BCAA, branched-chain amino acid; MS/MS, tandem mass spectrometry; OH-Pro, hydroxyproline; TPN, total parenteral nutrition.

in the USA (http://genes-r-us.uthscsa.edu/nbsdisorders.htm). Increased concentrations of Leu, Ile, and Val are considered indicative of MSUD; however, abnormal concentrations of these amino acids are often observed in newborns receiving total parenteral nutrition. Furthermore, the currently employed MS/MS determination of BCAA in dried blood spots fails to differentiate between increased concentrations of the isobaric amino acids allo-Ile, Ile, Leu, and hydroxyproline (OH-Pro) *(4)*, the latter of which is increased in the benign biochemical condition of hydroxyprolinemia (OMIM 23700). To improve the specificity of tandem mass spectrometry for the diagnosis of MSUD, we developed an LC-MS/MS method for the unequivocal identification of allo-Ile, Leu, Ile, Val, and OH-Pro and quantification of BCAAs in a single dried blood spot punch.

## Materials and Methods

We purchased allo-Ile, Ile, Leu, Val, and OH-Pro standards, as well as acetonitrile (ACN), formic acid (FA), heptafluorobutyric acid (HFBA), and methanol, from Sigma Chemical Co.; isotopically labeled internal standards for allo-Ile, Leu, and Val from Cambridge Isotope Laboratories; and silent screen filter plates from Fisher Scientific. The filter paper used for sample collection was grade 903 (Whatman).

### SAMPLES

The Mayo Foundation Institutional Review Board approved this study. For the validation of this method, normal control dried blood spot specimens were prepared by spotting whole blood of healthy controls on filter paper or by obtaining anonymized, leftover clinical samples initially submitted to Mayo Clinic's supplemental newborn screening program. We analyzed 560 newborn screening blood spots with normal allo-Ile concentrations to determine a reference interval for allo-Ile, Ile, Leu, and Val. In addition, 37 anonymized and blinded dried blood spots of patients (16 patients with an established diagnosis of MSUD and 21 normal controls) were provided by the Clinic for Special Children in Strasburg, PA, and stored at −80 °C until assayed. High and low BCAA control samples were prepared by spotting blood of healthy individuals that was enriched with all 4 target BCAAs on grade 903 filter paper. Control samples were sealed in plastic bags with desiccant and stored at −20 °C until analyzed.

### STANDARD SOLUTIONS

We made stock solutions by dissolving each standard and internal standard in 0.1 mol/L HCl to a concentration of 1 g/L. From these stock solutions, internal standard working solutions were prepared weekly with 500 mL methanol brought to 1 L with water and diluted to a concentration of 10 μmol/L. Organic mobile phase (mobile phase A) consisted of ACN with 1 mL/L FA and 0.1 mL/L HFBA. Aqueous mobile phase (mobile phase B) consisted of reverse osmosis water with the same concentrations of FA and HFBA.

### SAMPLE PREPARATION

We extracted allo-Ile and other BCAAs from 3/16-inch dried blood spot punches placed in 96-well filter plates with 100 μL water:methanol containing internal standard. After shaking for 30 min at room temperature on an orbital shaker, we collected eluents from filter plates by centrifugation for 2 min at 2200*g* into 96-well round-bottom plates. The water:methanol eluents were evaporated under a steady stream of nitrogen at ambient temperature, and dried specimens were reconstituted into 40 μL of aqueous mobile phase B before transfer to autosampler vials and injection into the LC-MS/MS system.

### INSTRUMENTATION AND ANALYSIS

BCAAs were detected on an LC-ESI-MS/MS (Applied Biosystems SCIEX API 3200) operating in positive multiple reaction monitoring mode at a temperature of 600 °C and a source voltage of 2000 V. We performed chromatography by use of a peripheral Agilent 1100 series LC system with an Applied Biosystems AAA C18 (150 by 4.6 mm, 5 μm) column that was maintained at 50 °C. Sample injection volume was 2 μL. After the application of a specimen to the LC column, a linear gradient was delivered at a rate of 650 μL/min from an initial condition of 5% mobile phase A up to a mixture of 25% mobile phase A and 75% mobile phase B over a course of 7 min. An isocratic wash of 100% mobile phase A was held for 4 min before the column was reequilibrated back to the initial condition within 4 more minutes. Thus, the total analysis time was 15 min including column reequilibration. Parameters for the SCIEX API 3200 instrument are listed in Table 1. The LC-MS/MS system was controlled and the concentration of allo-Ile, Leu, and Val measured by ratios of analyte peak areas to the respective internal standard peak areas of known concentration using Analyst 1.4.2 software running on a desktop PC with the Windows XP operating system. The concentration of Ile was determined by comparison of the Ile peak area to that of the isotopically labeled Leu.

### SAMPLE STABILITY

Preextraction stability was assessed at several interday temperatures and conditions for 3 normal dried blood spots and 3 with BCAAs added at various concentrations to mimic the disease state. Ranges for BCAAs in these dried blood spots were from undetectable to 310 μmol/L for allo-Ile, 48 to 292 μmol/L for Ile, 88 to

**Table 1. MS/MS settings.**

| Analyte | Monitored positive ion transition, *m/z* | DP | FP | CE, eV | CXP |
|---|---|---|---|---|---|
| Val | 118.18/72.2 | 31 | 4 | 17 | 4 |
| Val-$d_8$ | 126.25/80.2 | 31 | 4 | 17 | 4 |
| Allo-Ile/Ile/Leu | 132.10/86.1 | 26 | 6.5 | 13 | 4 |
| Leu-$d_3$ | 135.30/89.2 | 26 | 6.5 | 13 | 4 |
| Allo-Ile-$d_{10}$ | 142.23/96.3 | 26 | 6.5 | 13 | 4 |

DP, declustering potential; FP, focusing potential; CE, collision energy; CXP, collision–cell exit potential.

464 μmol/L for Leu, and 144 to 484 μmol/L for Val. Dried blood spots were analyzed at 0, 1, 3, and 7 days after interim storage at ambient, frozen (−20 °C), refrigerator (4 °C), or warm (37 °C) temperatures or after 2 freeze/thaw cycles.

### ANALYTICAL CHARACTERISTICS

For recovery studies, 3 different healthy control blood samples were used to create dried blood spot specimens with added BCAAs: allo-Ile 5, 20, 40, and 200 μmol/L; Ile 50, 100, 200, and 300 μmol/L; Leu 100, 200, 300, and 500 μmol/L; or Val 100, 200, 300, and 500 μmol/L. Each specimen was analyzed on 3 different days. Recovery was defined as (measured amount − endogenous amount)/added amount.

We compared the LC-MS/MS method with an HPLC assay *(7)* by analyzing 37 blinded specimens in which the concentration of allo-Ile had been measured by the HPLC assay, provided by the laboratory of the Clinic for Special Children. Among the 37 samples, 16 were from patients with an established diagnosis of MSUD and 21 were from others.

### REFERENCE INTERVAL

We measured allo-Ile, Ile, Leu, and Val in 541 control newborn screening dried blood spots and 19 dried blood spots from infants receiving total parenteral nutrition (TPN) over a period of 2 months.

## Results

### SAMPLE STABILITY

With one exception, changes in measured concentrations of BCAAs were <10% at the conditions studied through 7 days and after 2 freeze/thaw cycles (Table 2). In addition, we determined the stability of specimens extracted and prepared from 3 normal and 3 enriched dried blood spots before and after 6 days at −20 °C or 4 °C. Analysis of the prepared specimens yielded concentrations within 9% of expected values (Table 2).

### IMPRECISION AND RECOVERY

Analysis of 10 replicate analyses on 5 specimens within the same day yielded mean (range) CVs of 1.8% (0%–4.8%) for allo-Ile [range of concentrations: undetectable (n = 3) to 282 μmol/L], 5.4% (3.5%–6.4%) for Ile (56–629 μmol/L), 6.0% (5.0–8.1%) for Leu (105–596 μmol/L), and 5.2% (4.2–8.0%) for Val (152–575 μmol/L). We evaluated interassay imprecision using 5 specimens prepared and analyzed over 10 separate days. The mean interassay CVs (range) for the amino acids of interest were 5.6% (0%–14.6%) for

**Table 2. Average analyte stability in dried blood spots and extracts of dried blood spots of normal control (*n* = 3) and samples with added BCAAs (*n* = 3) at various temperatures.**

| | Dried blood spots | | | | | Extracts of dried blood spots | |
|---|---|---|---|---|---|---|---|
| | Ambient (22 °C) | Refrigerated (4 °C) | Frozen (−20 °C) | Incubated (37 °C) | Freeze/thaw | Ambient (22 °C) | Refrigerated (4 °C) |
| Allo-Ile | −4.5 | 15.1 | 9.1 | 1.3 | −3.4 | 3.1 | −8.9 |
| Ile | −2.9 | −4.7 | 1.7 | −0.1 | −0.8 | −3.0 | −1.1 |
| Leu | −7.8 | −4.4 | 3.6 | −3.8 | 0.3 | 2.6 | 2.3 |
| Val | −5.5 | −6.5 | 0.6 | −6.3 | −1.0 | 2.8 | 5.3 |

Results are % change after 7 days (dried blood spots) or 6 days (extracts of dried blood spots) at indicated temperatures or 2 freeze/thaw cycles.

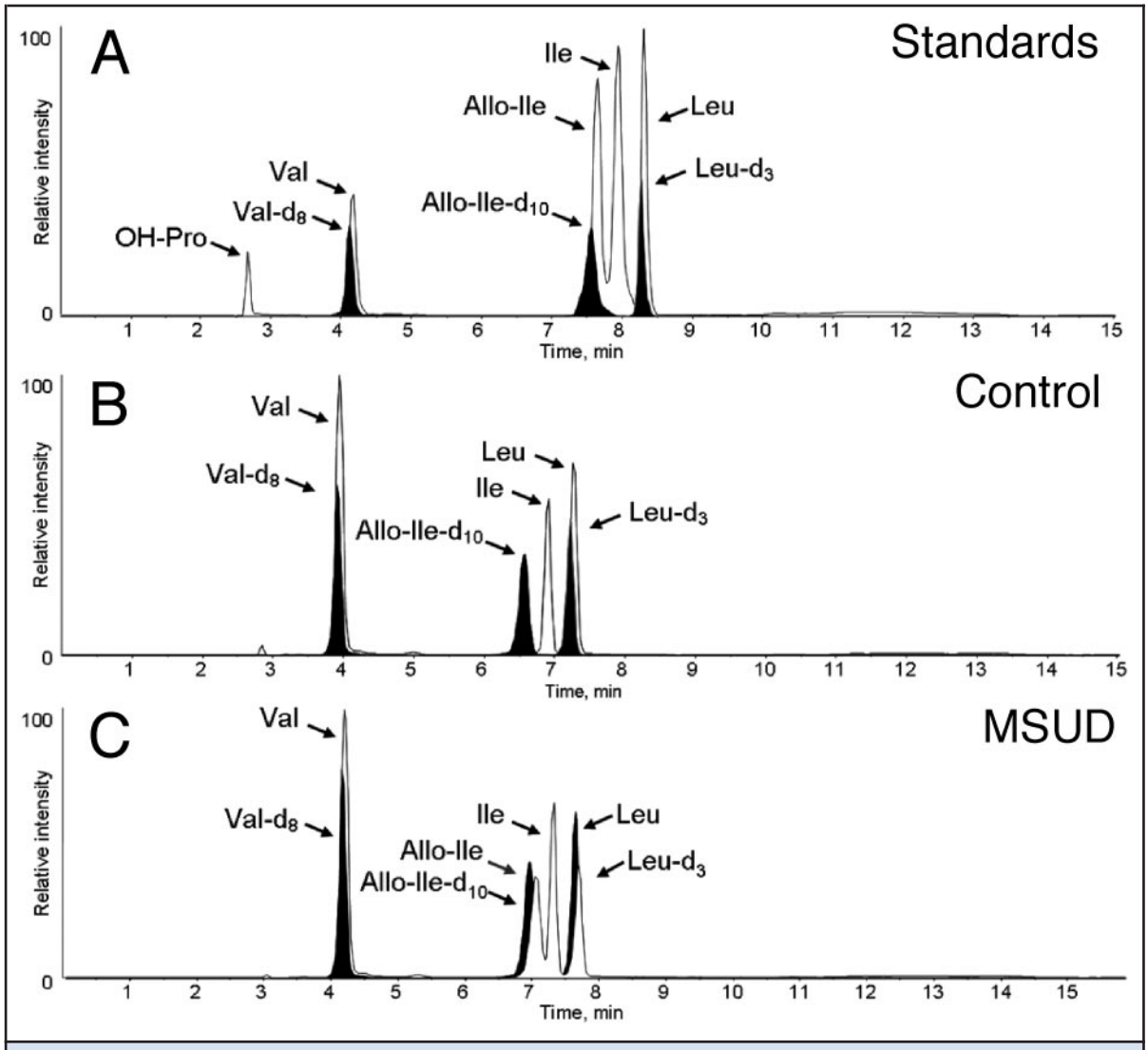


**Fig. 1. Multiple reaction monitoring (MRM) chromatograms of eluents from a sample of pure standards (A), a dried blood spot of a healthy control (B), and a dried blood spot of an MSUD patient (C) showing OH-Pro, Val, allo-Ile, Ile, and Leu as well as deuterated internal standards Val-$d_8$, allo-Ile-$d_{10}$, and Leu-$d_3$ (black peaks).**

allo-Ile [undetectable (n = 3) to 254 μmol/L], 7.4% (3.2%–8.7%) for Ile (55–644 μmol/L), 5.2% (2.6%–7.2%) for Leu (101–610 μmol/L), and 5.0% (3.8%–5.1%) for Val (138–576 μmol/L). The mean (range) recoveries were 132% (100%–218%) for allo-Ile, 112% (100%–125%) for Ile, 100% (90%–110%) for Leu, and 83% (74%–94%) for Val.

### METHOD COMPARISON

As shown in Fig. 2, both the new assay and the HPLC assay *(7)* correctly identified patients with or without MSUD; the average difference of allo-Ile measured was <15%. Further comparison to the HPLC method revealed that the LC-MS/MS method yielded lower concentrations of allo-Ile in most samples, a finding likely related to better positive identification and resolution from potentially interfering substances afforded by the LC-MS/MS approach.

### POPULATION INTERVALS

The mean and the 1st and 99th percentiles for 541 control DBS samples, 19 DBS samples from patients on total parenteral nutrition, and 16 DBS samples of MSUD patients are shown in Table 3 for allo-Ile, Ile, Leu, and Val. All 16 patients with MSUD had concentrations of allo-Ile markedly above the 99th percentile.

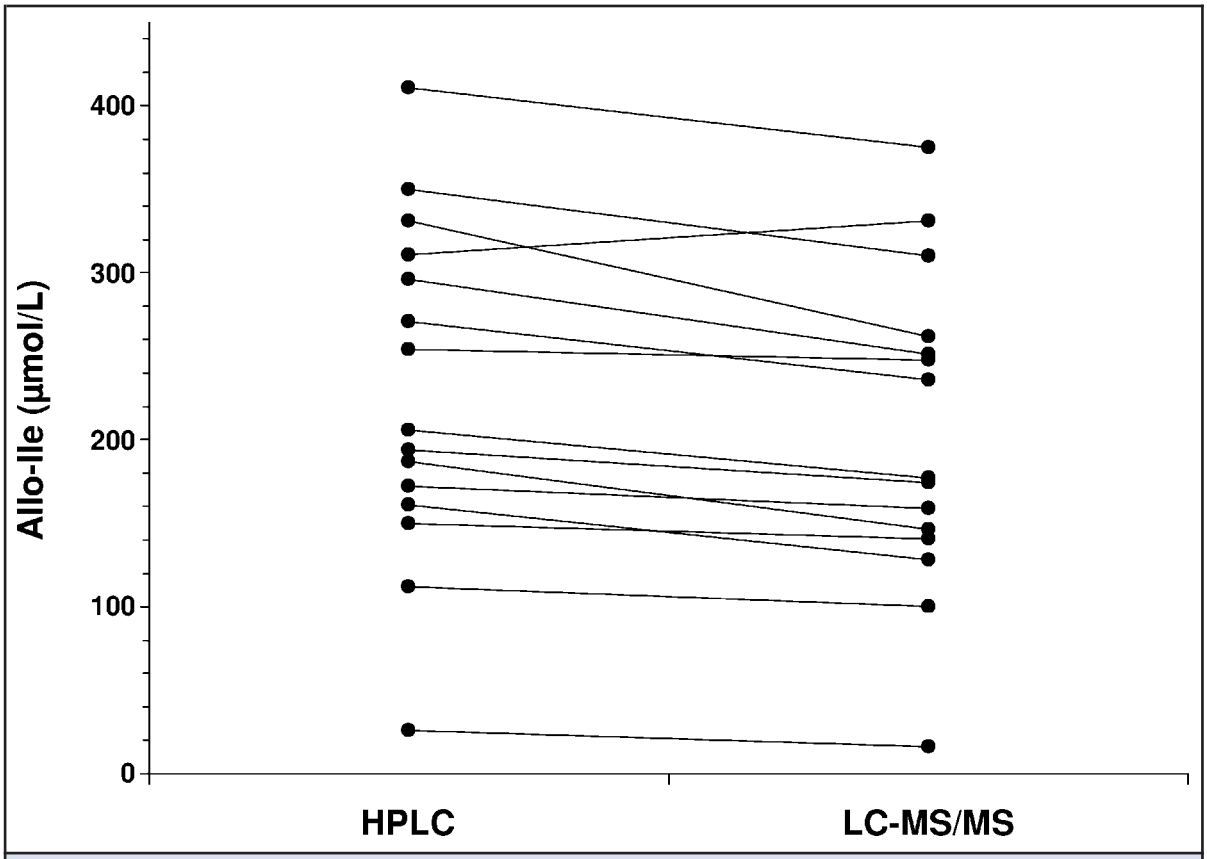


**Fig. 2. Method comparison and clinical validation.**

Allo-Ile was analyzed by LC-MS/MS and HPLC in dried blood spots of 16 patients with an established diagnosis of MSUD and 21 controls. Results were compared to those obtained using an HPLC assay *(7)*. One of the 16 MSUD patient samples was analyzed only by the LC-MS/MS method (allo-Ile: 153 μmol/L) and is therefore not included in the figure. Data for 21 control samples are not shown because all yielded undetectable amounts of allo-Ile by both methods.

## Discussion

The introduction of MS/MS as a newborn screening tool has enabled the expansion of the number of biochemical genetic conditions that can be detected in asymptomatic newborns from a single dried blood spot. BCAA, other amino acids, free carnitine, and acylcarnitines are identified simultaneously in <3 min

**Table 3. Comparison of BCAA concentrations determined by use of the LC-MS/MS method in dried blood spots of healthy control neonates and of babies either receiving total parenteral nutrition or affected with MSUD.**

| | | D-Alloisoleucine, μmol/L | | Leucine, μmol/L | | Isoleucine, μmol/L | | Valine, μmol/L | |
|---|---|---|---|---|---|---|---|---|---|
| | n | Mean (range) | 1st–99th percentile | Mean (range) | 1st–99th percentile | Mean (range) | 1st–99th percentile | Mean (range) | 1st–99th percentile |
| Controls | 541 | ND (ND–1*) | ND–1 | 77 (35–217) | 45–214 | 42 (13–135) | 17–107 | 105 (51–380) | 61–325 |
| TPN | 19 | ND | ND | 316 (74–1444) | 86–1328 | 202 (37–999) | 45–914 | 297 (110–821) | 119–785 |
| MSUD | 16 | 200 (16–375) | 29–368 | 272 (17–600) | 18–596 | 224 (72–563) | 81–528 | 310 (114–598) | 124–567 |

Based on instrument response to the Allo-Ile calibrator at 0.7 μmol/L (signal-to-noise = 8.1), the limit of detection and quantitation was set arbitrarily at 1 μmol/L.
ND, not detected.

after introduction of preferably derivatized blood spot extracts by flow injection into the tandem mass spectrometer (flow injection analysis, FIA). While FIA-MS/MS allows for high-throughput screening, it cannot distinguish isobaric compounds unless an appropriate liquid chromatography step is applied before the mass spectrometric analysis. Allo-Ile, Ile, Leu, and OH-Pro compose one such group of isobaric amino acids. Therefore, infants with hydroxyprolinemia, a clinically benign biochemical trait, or infants with high levels of BCAAs owing to total parenteral nutrition may be subject to unnecessary follow-up to rule out MSUD. To improve the specificity of MS/MS for the detection of MSUD and to spare families the anxiety associated with false-positive screening results *(8)*, we developed a method for the quantification of allo-Ile and other BCAAs from a single dried blood spot. A number of MS/MS-based methods for quantifying amino acids from different sample types, such as plasma *(9, 10)*, serum *(11)*, urine *(10)*, or dried blood spots *(11, 12)*, were recently described. Although an LC-MS/MS method has been reported previously to be suitable for discriminating allo-Ile from Ile and Leu in a mixture of amino acids extracted from dried blood spots *(12)*, neither a comprehensive clinical validation nor a comparison to other methods used for quantifying allo-Ile was included in that report. The method described here allowed for successful identification of infants affected with classic MSUD among a cohort of healthy newborns. In addition, our method can be used to rapidly quantify BCAAs and their isoforms in dried blood spots for follow-up of MSUD patients already being treated. We have also shown through a blinded comparison study that the LC-MS/MS method is comparable to a previously described HPLC assay for quantifying allo-Ile in dried blood spots *(7)*.

The sample preparation procedure described here is amenable to the use of 96-well plates and can be completed in less than 1 h. The analytical time of the LC-MS/MS approach is 15 min from injection of one sample to the next to allow for the chromatographic separation of each BCAA and OH-Pro (Fig. 1). This method is sensitive, requires a small sample size, and is faster than previously published protocols using either an HPLC separation of derivatized amino acids *(7, 13)* or GC-MS *(14)*. This method is also an improvement over traditional methods that apply ion exchange chromatography and postcolumn ninhydrin derivatization, because allo-Ile at near normal concentrations is difficult to distinguish from other nearby eluting ninhydrin-sensitive amines, a complication that may be problematic in some cases *(15)*.

The possibility that sample stability or dried blood spot storage conditions might decrease the concentration of measurable MSUD biomarkers within the small dried blood spot sample was excluded by the short-term stability studies of the target compounds at various temperatures and conditions (Table 2). These findings are supported by a recent study of the long-term stability of amino acids in dried blood spots, which revealed that the measurable concentration of some BCAAs, such as Leu, decreases at a slow rate of 3% per year when the samples are stored at ambient temperature *(16)*. The maximum length of storage at ambient temperature that we tested was 7 days, over which the concentration of all 4 BCAAs on average decreased by only 5.2%, a value well within the imprecision determined for this assay (Table 2).

Some cases of MSUD may escape early detection. Three cases were published whose combined concentration of the isobaric amino acids Leu, Ile, allo-Ile, and OHPro (XLE-OHPro) in the newborn screening samples (344, 325, and 209 μmol/L, respectively) were below the cutoff applied by the performing laboratory *(4, 15)*. The 2 patients described by Bhattacharya et al. *(17)* were affected with the intermediate-severity form of MSUD, which often only reveals abnormal metabolites during metabolic stress. Determination of analyte ratios, such as the XLE-OHPro-to-phenylalanine and Val-to-phenylalanine, can be helpful when BCAAs are only moderately increased *(4)*. The lowest XLE-OHPro concentration determined in the newborn screening sample of a MSUD patient of which we are aware was 229 μmol/L ($n = 111$; 1st percentile of the disease range, 257 μmol/L; see Region 4 Collaborative Project website, www.region4genetics.org). Although the Val concentration (194 μmol/L) was also below the chosen cutoff (200 μmol/L), both the XLE-OHPro-to-phenylalanine ratio (5.2; abnormal >4.5) and the Val-to-phenylalanine ratio (4.4; abnormal >3.0) were informative, and in our laboratory would have prompted further investigation. However, one of the MSUD cases reported by Bhattacharya et al. *(17)* had a XLE-OHPro concentration of only 209 μmol/L and a XLE-OHPro-to-phenylalanine ratio of 3.7. That case would have been missed by our program as well, because our cutoffs for XLE-OHPro had been set at 300 μmol/L and for the ratio to phenylalanine at 4.5. Based on this case report and a comparison of BCAA concentrations in healthy control newborns, babies receiving TPN, and cases with MSUD (Region 4 Collaborative Project, unpublished data), we adjusted the cutoffs for XLE-OHPro to 200 μmol/L, for ratio to phenylalanine to 3.5, and for Val to 140 μmol/L. The cutoff for the Val-to-phenylalanine ratio remained unchanged at 3.0. Following the algorithm depicted in Fig. 3, any newborn screening result exceeding these cutoffs now triggers the second-tier assay for allo-Ile. Review of the last 20 000 newborn screening results obtained by our laboratory suggests that the second-tier test for

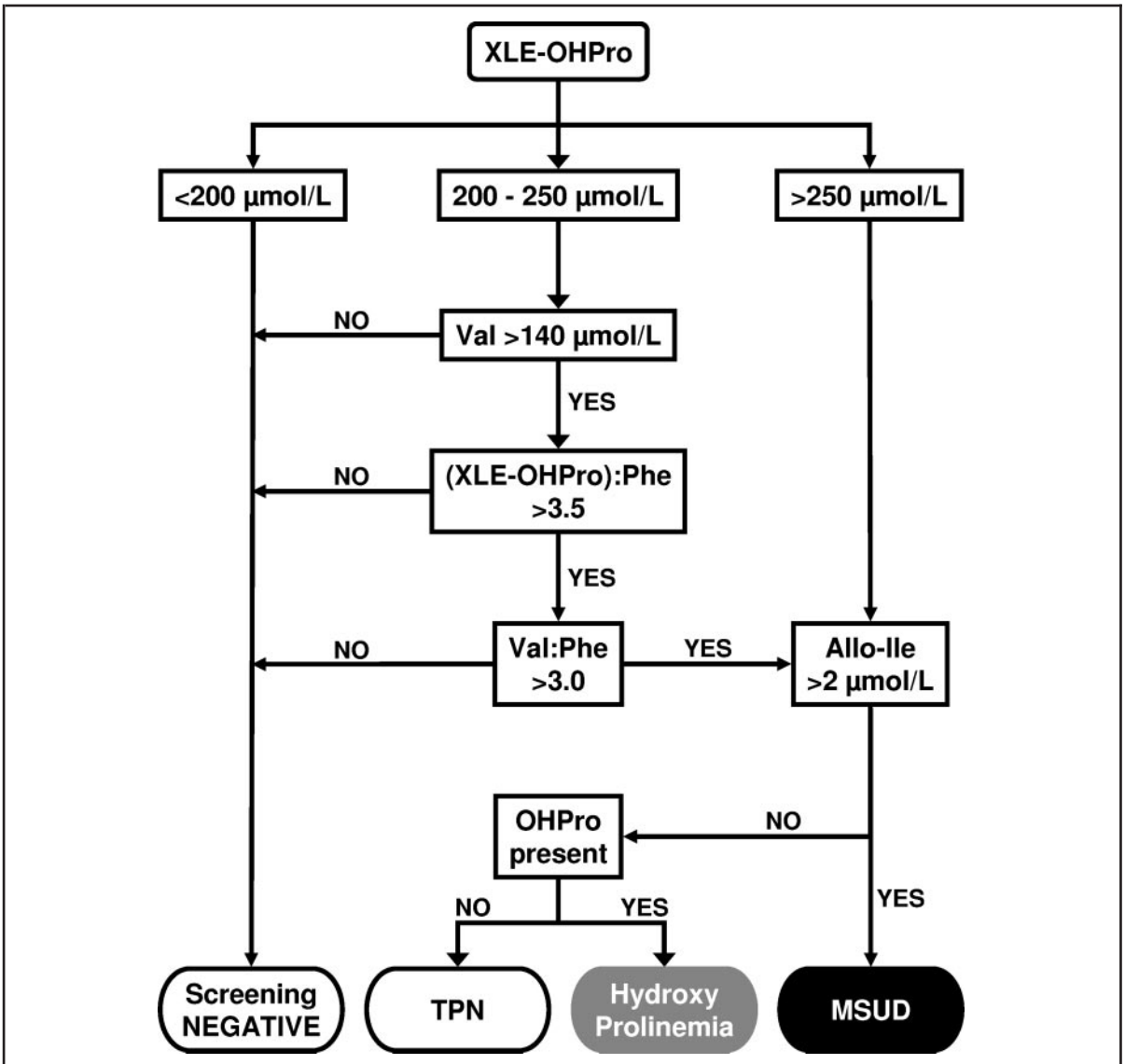


**Fig. 3. Algorithm for newborn screening for MSUD using allo-Ile determination in dried blood spots as a second-tier assay.**

The cutoffs for the isobaric amino acids leucine, isoleucine, D-alloisoleucine, and hydroxyproline (XLE-OHPro) at 200 and 250 $\mu$mol/L correspond to the 98.7th and 99.6th percentiles, respectively, and were established in 20 620 newborn screening samples analyzed in our laboratory between June and August 2007.

allo-Ile will then be performed on 0.45% of submitted newborn screening samples. The cutoff for allo-Ile (2 $\mu$mol/L) was determined by LC-MS/MS analysis of 560 newborn screening samples, which revealed a nearly undetectable allo-Ile concentration regardless of dietary factors such as TPN. Considering that the lowest allo-Ile concentration in the 16 retrospectively studied MSUD cases was 16 $\mu$mol/L, this cutoff value should be clinically adequate to prevent future false-negative results (Table 3).

In summary, we developed a new LC-MS/MS based method for the accurate determination of allo-Ile in newborn screening blood spots. Implementation of this assay as a second-tier newborn screening assay is expected to further reduce the false-positive rate and to increase the positive predictive value of newborn screening for MSUD while avoiding false-negative results. This concept has already proven successful for other critical analytes *(18–20)*. In addition, this assay can aid in the follow-up of treated MSUD patients by

rapid determination of BCAAs including allo-Ile in dried blood spots or plasma.

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