

## Potential Diagnostic of Branched-Chain Ketoaciduria by HPLC-DAD

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Neste trabalho, um sistema de cromatografia líquida de alta eficiência (HPLC) foi usado para desenvolver e validar um eficiente método para determinar quantitativamente aminoácidos envolvidos na desordem rara conhecida como cetonúria de aminoácidos de cadeia ramificada ou doença do xarope de bordo. As condições analíticas foram desenvolvidas para obter os perfis dos aminoácidos de *L*-valina, *L*-isoleucina e *L*-leucina, sabidamente alterados no plasma sanguíneo dos pacientes. Empregou-se HPLC provido de um detector com arranjo de diodo. Os analitos não possuem grupos cromóforos e, por isso, foram pré-derivatizados com *o*-ftalaldeído (OPA) para tornar possível sua detecção. A validação foi conduzida de acordo com as normas da Agência Nacional de Vigilância Sanitária (ANVISA) (RDC No. 27, de 17 de maio de 2012) e seção de validação de bioanalítica da *United States Food and Drug Administration* (U.S. FDA). Os resultados foram satisfatórios, apresentando alta sensibilidade, boa linearidade, precisão e exatidão, limite de detecção e quantificação, todos parâmetros estabelecidos para métodos bioanalíticos, demonstrando a aplicabilidade e baixo custo do método comparado com outras técnicas como espectrometria de massas. Para os três aminoácidos, *L*-valina, *L*-isoleucina e *L*-leucina, os limites de detecção encontrados foram: 1,61, 1,84 e 1,88 mmol L<sup>-1</sup> e limites de quantificação 4,37, 6,13 e 6,27 mmol L<sup>-1</sup>, respectivamente.

A system of high performance liquid chromatography (HPLC) was used for the development and validation of efficient method for quantitative determination of three aminoacids involved in the inherited metabolic disease Branched-Chain Ketoaciduria (BCK), also called maple syrup urine disease. The analytical conditions were selected in order to obtain baseline separation profiles of the amino acids known to be altered in blood plasma of BCK patients, namely *L*-valine, *L*-isoleucine, and *L*-leucine. Most accurate data were obtained using HPLC/diode detector. As the analytes do not have chromophore groups, they were pre-derivatized with *o*-phthaldialdehyde (OPA), yielding an unsaturated adduct, making thus possible the detection of amino acids. The validation was conducted according to National Health Surveillance Agency (ANVISA) and Guidance for Industry (Bioanalytical Method Validation) United States Food and Drug Administration (U.S. FDA). The results were satisfactory, with high sensitivity, good linearity, precision and accuracy, limit of detection and quantification, all within the established parameters for bioanalytical methods, showing its applicability and low cost compared to other existing techniques such as sequential mass spectrometry. For the three amino acids, *L*-valine, *L*-isoleucine and *L*-leucine, the detection limits (LOD) found were: 1.61, 1.84 and 1.88 mmol L<sup>-1</sup> and the quantification limits (LOQ) 4.37, 6.13 and 6.27 mmol L<sup>-1</sup>, respectively.

**Keywords:** high performance liquid chromatography, maple syrup urine disease, isoleucinose, rare diseases, metabolism, inborn error, branched chain aminoacids,  $\alpha$ -ketoacids

## Introduction

Hereditary metabolic diseases (HMD) or inborn errors of metabolism (IEM) are biochemical disorders genetically determined, involving processes of synthesis, storage or transport of molecules in the organism. In general, the main metabolic diseases associated with amino acid metabolism are attributed to failure of a specific enzyme leading to accumulation or deficiency of intermediates in the corresponding metabolic pathway.<sup>1</sup> In Brazil, the newborn screening public health program contemplates only a few inborn diseases such as phenylketonuria, congenital hypothyroidism, cystic fibrosis and hemoglobinopathies.<sup>2</sup> Other IEM are usually detected too late, when symptoms have been settled and damaged brain is often irreversible.<sup>3</sup> HMD are usually autosomal recessive, and it affects the population of about 1:5,000 live births.<sup>4</sup> HMD is considered individually rare and most often leads to a late diagnosis with serious and irreparable consequences to the central nervous system. Importantly, early intervention in patients with IEM results in favorable clinical outcomes.<sup>5</sup>

Biochemical characteristics and laboratorial diagnosis of Branched-Chain Ketoaciduria (BCK) or maple syrup urine disease (MSUD) is considered a rare metabolic disorder with an incidence of approximately 1:200,000 births. BCK is characterized by a deficiency in the multienzyme complex of the ketoacids dehydrogenases (BCKAD) responsible for the metabolism of branched chain amino acids (BCAAs) *L*-leucine, *L*-isoleucine and *L*-valine. The consequence is the accumulation of BCAAs and its corresponding  $\alpha$ -keto acids (A- $\alpha$ -CCR) in body fluids responsible for their toxicity to the central nervous system (CNS) and the sweet odor that coins the name of the disease - maple syrup urine disease.<sup>6</sup>

Classic BCK, the most common and severe form, manifests between the fourth and seventh day of life with characteristic symptoms such as lethargy and poor milking followed by weight loss and neurological symptoms. Other symptoms include unwillingness to eat, convulsions, hiccups, hypothermia and coma, and ultimately death if the newborn is not treated.<sup>2</sup> Early diagnosis is essential for prognosis, since rapid treatment may prevent neurological deterioration, characterized by reduced density of white matter hypomyelination/demyelination, atrophy and cerebral edema.

Twelve hours after birth, untreated neonates with classic BCK have a maple syrup urine odor and by 12-24 hours, elevated plasma concentrations of the BCAAs. Healthy individuals have reduced plasma concentrations of BCAAs during fasting, since the rate of oxidation of amino acids is higher than the rate of entry of protein in plasma. *L*-leucine

(68-183 mmol L<sup>-1</sup>), *L*-isoleucine (31-105 mmol L<sup>-1</sup>), and *L*-valine (83-300 mmol L<sup>-1</sup>) are the reference values for plasma samples in children under 24 months. Because patients with untreated BCK are not able to oxidize BCAAs, they have increased plasma levels of these amino acids.<sup>7</sup> Laboratory diagnostic tests reveal, in addition to increased blood, plasma or urine level of BCAAs and A- $\alpha$ -CCR, the presence of allo-isoleucine, a non-protein amino acid synthesized *in vivo* from isoleucine. Usually, children under 24 months have undetectable plasma levels of allo-isoleucine. Plasma concentrations of allo-isoleucine greater than 5 mmol L<sup>-1</sup>, allo-isoleucine/isoleucine ratio greater than 0.6, and leucine/alanine ratio above 0.5 (reference values 0.1-0.5) are specific and sensitive markers of BCK. Another important feature is that the increased circulating BCAAs cause a decrease of other neutral amino acids such as tryptophan, tyrosine, methionine, and phenylalanine.<sup>8</sup> The BCK treatment intends to normalize the blood levels of BCAAs by limiting the intake of the three essential amino acids and provide adequate nutrition in order to maintain children growth and development.<sup>7</sup> Current treatment consists of restricting the dietary intake of BCAAs to the absolute minimum amount that is needed for growth.

### Analytical methods for screening and diagnosis of BCK

Qualitative tests are important to provide clues regarding the type of IEM, although not conclusive for diagnostic. Qualitative urinary test with 2,4-dinitrophenylhydrazine (DNPH) can inform the presence of  $\alpha$ -ketoacids in MSUD, but shows elevated *L*-leucine only at concentrations above 700 mmol L<sup>-1</sup>.<sup>9</sup> Amino acid separation techniques applied to biological fluids constitute important tools for diagnosing diseases caused by errors in metabolism. Liquid chromatography has been widely and successfully used in this context because of its high precision, reproducibility and low cost in comparison with other methods. Chromatographic techniques coupled to tandem mass spectrometry (LC-MS/MS) although displaying high resolution, sensitivity and specificity have the disadvantage of high cost, frequently unaffordable for implementation in the public health service.<sup>10-12</sup> On the other hand, high performance liquid chromatography with diode array detection (HPLC-DAD) offers a viable alternative for amino acid analysis due to its lower-cost, good reproducibility, sensitivity and resolution.<sup>13</sup> Here in we develop and validate a lower cost and high reliability HPLC-DAD analytical method for BCAAs determination in blood spot samples collected from normal and BKA patients.

## Experimental

### Chemicals, stock solutions, standards and patient samples

Standards of the aminoacids *L*-valine, *L*-isoleucine and *L*-leucine (Sigma Aldrich, EUA) were used. Ortho-phthaldehyde (OPA), 2-mercaptoethanol and Brij 35 were purchased from (Sigma Aldrich, EUA). The derivatization reagent used was prepared from 5 mg of OPA, 125  $\mu\text{L}$  of methanol (Sigma Aldrich, USA), 1 mL of borate buffer 0.4 mol  $\text{L}^{-1}$  (pH 9.5), 10  $\mu\text{L}$  of 2-mercaptoethanol and 1  $\mu\text{g}$  (10  $\mu\text{L}$ ) Brij 35 obtained from a stock solution containing 0.1 mg  $\text{mL}^{-1}$ . 2-mercaptoethanol was used to prevent OPA-aminoacid adduct to undergo hydrolysis. Brij 35 increases the detector response.

The eluents were prepared with methanol and tetrahydrofuran (HPLC grade) and 10 mmol  $\text{L}^{-1}$  sodium acetate buffer (pH 5.0). All chemicals used were from Sigma Aldrich, EUA. Ten mmol  $\text{L}^{-1}$  aminoacid solutions were prepared in ultrapure water and stored at 4 °C for not more than four months. Water was purified in a Millipore Simplicity equipment and the card used to collect blood was from Schleicher & Schuell 903 (S&S 903) (Germany).

Blood samples of healthy individual were collected with a tube containing EDTA as anticoagulant (VACUETTE™ Brasil). The samples were centrifuged at 1000 rpm for 3 min, and the supernatant (plasma) was removed, diluted 10 times in saline (0.9% NaCl) and filtered through a 0.22  $\mu\text{m}$  Millipore membrane. The samples were kept frozen at -4 °C until use.

### Blood collection card

Blood collection papers contain four circles (diameter, 3.2 mm) to which blood drops were placed side by side until the entire circular areas were filled. Blood samples were reserved for drying at room temperature. After drying, the circles were cut out and the extraction was made with an adapted methodology<sup>14</sup> using water, 1 mmol  $\text{L}^{-1}$  acetic acid and acetonitrile. To optimize the amino acid extraction efficiency, eluent combinations were tested by increasing 10% the concentration of water in acetonitrile, starting from 29% to 89% water and maintaining the acetic acid concentration of 1%.

- i. 29%  $\text{H}_2\text{O}$ ; 1% acetic acid; 70% ACN
- ii. 49%  $\text{H}_2\text{O}$ ; 1% acetic acid; 50% ACN
- iii. 59%  $\text{H}_2\text{O}$ ; 1% acetic acid; 40% ACN
- iv. 69%  $\text{H}_2\text{O}$ ; 1% acetic acid; 30% ACN
- v. 79%  $\text{H}_2\text{O}$ ; 1% acetic acid; 20% ACN
- vi. 89%  $\text{H}_2\text{O}$ ; 1% acetic acid; 10% ACN

The extraction of samples was performed by sonication for 20 min, which was followed by OPA derivatization before chromatographic analyses. This procedure was also employed for analyzing blood samples collected from three BCK patients aged five (two children), and seven years (one child).

### Chromatographic analyses

The HPLC system used comprises: PDA detector, Pump 600, automatic injector Autosampler all Accela (Thermo Scientific, USA), and a Phenosphere 5 microns C18, 80 Å column (Phenomenex, USA) Eluents A was 10 mmol  $\text{L}^{-1}$  sodium acetate buffer (pH 5.0) in methanol and tetrahydrofuran at a ratio of 180:19:1 and eluent B was 100% methanol. A gradient flux of 1 mL  $\text{min}^{-1}$  and an injection volume of 25  $\mu\text{L}$  were used (Table 1). During a total time of 21 min of analysis, the separation column was maintained at 15 °C and absorption at 230 nm used to monitor the analyses.

**Table 1.** HPLC separation of a standards mixture of *L*-Leu, *L*-Ile, and *L*-Val, 1 mmol  $\text{L}^{-1}$ , volume injection: 25  $\mu\text{L}$ . Mobile phase: solvent A: buffer (10 mmol  $\text{L}^{-1}$  sodium acetate pH 5.0, in methanol and tetrahydrofuran at a ratio of 80:19:1) and solvent B: (HPLC grade methanol), with a flow rate of 1.0 mL  $\text{min}^{-1}$ . Excess OPA-containing derivatization solution (5:1, OPA/Sample) was added to the blood extract to assure quantitative conversion of the amino acid to the corresponding OPA adduct

time / min	% A	% B
0	50.0	50.0
15.0	30.0	70.0
20.0	30.0	70.0
21.0	0.0	100
22.0	100	0.0
23.0	0.0	100
24.0	100	0.0
25.0	50.0	50.0
45.0	50.0	50.0

### Method validation

The analytical method validation was conducted according to National Health Surveillance Agency (ANVISA) (RDC No. 27, of May 17, 2012) and the Guidance for Industry (Bioanalytical Method Validation)-FDA. The parameters evaluated were: specificity, linearity, detection limit, quantification limit, accuracy and precision.<sup>15</sup> The study was approved by the institutional (UNIFESP) ethics committee, according to the protocol number 1545/10.

### Specificity

The method specificity was assessed by analysis of plasma samples diluted ten-fold by the addition of standards

*L*-Leu, *L*-Ile and *L*-Val, and by checking interferences and retention time of the analytes.

#### Linearity

Calibration curves were constructed from five dilutions of each biomolecule from the same stock solutions, using the method of chi-square minimization (weighted least squares) and the blank. For the choice of concentrations the maximum limit of detection of aminoacid was observed, therefore the higher concentrations tested were lower than the maximum detection limit of the apparatus.

#### Detection and quantification limits

Detection limits were determined by the formula provided by the ANVISA resolution,<sup>15</sup> which is based on the ratio of three times the baseline noise of the blank calibration curve of each biomolecule. The quantification limits were also determined using the formula given by ANVISA using the calibration curve obtained for each biomolecule.

#### Precision

The precision was determined by testing intra-precision analyses (repeatability) at concentrations of 10, 90 and 180 mmol L<sup>-1</sup> and inter-precision analyses using 90 mmol L<sup>-1</sup> for the three analytes. Precision was expressed as coefficient of variation (CV%), accepted values being below 15%.

#### Accuracy

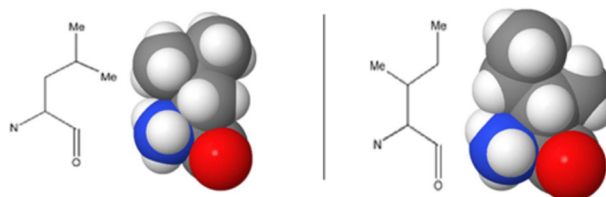
The accuracy was calculated as the percentage of recovery of the amount of *L*-Leu, *L*-Ile, and *L*-Val added to the plasma sample. Fifteen determinations were carried out, which include the linear range of the procedure, i.e., three concentrations (low, medium and high) with five replicates each of 2, 30 and 180 μmol L<sup>-1</sup>. Accuracy was expressed as the ratio between the average concentration determined experimentally and the corresponding theoretical concentration as percentage.

#### Standard stability

The aminoacid standard stabilities were studied within two months storage at 4 °C, and evaluated by the variation of the peak chromatographic areas during validation. The stability of short duration (24 h) was also evaluated.

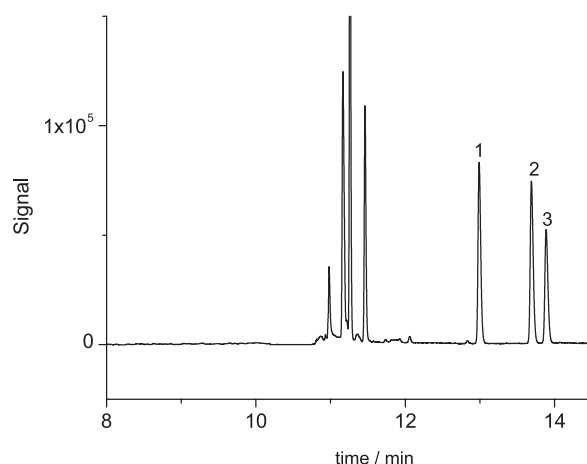
## Results and Discussion

Chromatographic separation of the isomers *L*-Ile and *L*-Leu was afforded by the polarity differences caused by the different positions of methyl groups (Figure 1). Figure 1 shows the isomer spatial representations, *L*-Leu having a larger volume than *L*-Ile, which can be evidenced by the total binding energies of the molecules. *L*-Leu has total energy = 151.149 and *L*-Ile 148.793 kJ mol<sup>-1</sup> (calculated by Avogadro 4.7.2 software). This factor is responsible for the differences in *L*-Ile and *L*-Leu contact surface and contributes to the more effective *L*-Leu retention time in mobile phase and reversed phase chromatographic column.



**Figure 1.** *L*-leucine (a) and *L*-isoleucine (b) spatial representations.

The retention times obtained for the aminoacid standards in the chromatographic conditions described were  $12.73 \pm 0.01$ ,  $15.86 \pm 0.02$  and  $16.73 \pm 0.02$  min for *L*-Val, *L*-Ile and *L*-Leu, respectively (Figure 2).



**Figure 2.** Chromatogram of a standard mixture of *L*-Val (1), *L*-Ile (2) and *L*-Leu (3) at 0.09 mmol L<sup>-1</sup> spiked plasma sample obtained from a healthy adult volunteer. Column Phenosphere 5 μm, C18, 4.1 and 250 mm and 80 Å. Column temperature: 15 °C. Eluent A: sodium acetate buffer 10 mmol L<sup>-1</sup> pH 5.0; methanol and tetrahydrofuran 80:19:1. Eluent B: methanol. Injection volume: 25 μL. DAD λ = 230 nm.

#### Specificity

The aminoacids standard solution were injected independently onto the column in order to compare with blood plasma spiked with the same amino acids and the

results were compared and showed no interference patterns in the respective retention times and peak resolution. Since OPA reacts only with primary amine groups, other compounds found in blood such as bilirubin and lipids do not affect the HPLC traces.<sup>16</sup>

### Linearity

The regression coefficient ( $r^2$ ) of the calibration curve was greater than 0.99 for all three metabolites. The aminoacid concentrations used were 2.0, 10.0, 30.0, 90.0 and 180 mmol L<sup>-1</sup>, and the analyses were performed in quintuplicates. The straight line equation ( $y = bx + a$ ) was determined through the study of linear regression: *L*-Val (1.43E8x – 687950.91), *L*-Leu (1.23E8x – 181002.98), and *L*-Ile (1.52E8x – 149073.31).

### Sensitivity and accuracy

The detection limits (LOD) found for *L*-Val, *L*-Ile, and *L*-Leu, were 1.61, 1.84 and 1.88 mmol L<sup>-1</sup> and the quantification limits (LOQ) 4.37, 6.13 and 6.27 mmol L<sup>-1</sup>, respectively. A HPLC method with UV detection validated by other authors<sup>16</sup> found resulted in 0.43-1.91 mmol L<sup>-1</sup> LOD for all BCAAs. Student's *t* test was used to check deviations from linearity of the calibration curve of the amino acids analyzed. As the tabulated *T* value found is less than the calculated *T* (Table 2), there is a correlation between the data and the calibration curve, with a confidence level of 95%. Therefore, we conclude that this methodology can detect and quantify amino acids levels much above that it is found in blood healthy individuals (Table 2).

**Table 2.** Student's *t* test applied to the amino acids analyzed

Aminoacid	$r^2$	$r$	$n$	$T_c$	$T^a$
<i>L</i> -valine	0.9996	0.9998	5	86.58	2.776
<i>L</i> -isoleucine	0.9992	0.9996	5	61.21	2.776
<i>L</i> -leucine	0.9999	0.9995	5	173.1	2.776

<sup>a</sup>Values found by the Student's *t* distribution table.

The accuracy values for the 3 metabolites are listed in Table 3. All data obtained revealed intra-assay and inter-assay precision values (CV%) falling below 15%.

### Accuracy

Three aminoacid concentrations (2.0, 30 and 180 mmol L<sup>-1</sup>) were analyzed in quintuplicate and the average recovery ranged from 91 to 108%. Another

**Table 3.** Intra-assay and inter-assay precision tests of amino acid analyses

Concentration / (μmol L <sup>-1</sup> )	CV intra-assay / %	CV inter-assay / %
<i>L</i> -valine		
10	6.06	–
90	3.86	4.22
180	9.46	–
<i>L</i> -isoleucine		
10	5.58	–
90	1.83	1.88
180	11.58	–
<i>L</i> -leucine		
10	6.77	–
90	3.83	3.33
180	9.34	–

HPLC method with UV detection tested found similar results: 92-103%.<sup>17</sup> Recently, other authors<sup>17</sup> who used a HPLC method employing a 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate as the derivatizing reagent recovered from 96 to 105% amino acid.

### Stability

No notable stability variations were observed for the three amino acids for either two months of storage at –4 °C or during a short term stability test at room temperature.

### Analyses of BCK patient samples

Untreated BCK carriers, because are not able to oxidize BCAAs, have increased plasma levels of *L*-Leu, 500-5000 (RV reference value: 68-183); *L*-Ile 200-1300 (RV 31-105); and *L*-Val: 500-1800 (RV: 83-300) (μmol L<sup>-1</sup>).<sup>7,18</sup> Increased values were also found in blood spot samples of healthy individuals and BCK samples by Rashed *et al.*<sup>19</sup> and in the present experiments (Table 4). The levels of *L*-Leu and *L*-Ile found here are reportedly compatible with the characteristic diagnostic values. Although patients A and B presented low levels of *L*-Leu, the sum of the two isomers shows compatible values with those of BCK patients. This fact can be attributed to variations in the individual patient's metabolism or can result from undergoing treatment when the blood samples were withdrawn. Accordingly Rashed *et al.*,<sup>19</sup> found similar results for BCK individuals using an electrospray MS/MS method.

Identification and quantification of aminoacids in biological fluids have long been used for studying metabolic disorders. The photodiode array detector coupled to HPLC



**Table 4.** L-Val, L-Ile, and L-Leu concentrations in blood samples of healthy and BCK individuals

Blood spot sample	VAL / ( $\mu\text{mol L}^{-1}$ )	LEU / ( $\mu\text{mol L}^{-1}$ )	ILE / ( $\mu\text{mol L}^{-1}$ )	LEU + ILE / ( $\mu\text{mol L}^{-1}$ )
Healthy individuals <sup>20</sup>	79.9-152.1	ND	ND	70.3-135.7
BCK individuals <sup>20</sup>	28.5-674.0	ND	ND	319.0-3650.0
A	735.3	91.3	453.5	544.8
B	357.9	802.2	239.4	1041.6
C	113.8	164.2	292.6	456.8

ND: not determined; 3 patients (A = girl, 2.2 years old, B = boy, 2.7 years and C = boy 3.6 years).

allows fast, cheap and accurate analysis of amino acids because spans a wide range of the UV-Visible spectrum in a single chromatographic analysis. It makes possible a multivariate analysis with greater accuracy and reduced false positive results. The Brazilian Newborn Screening Public Health program contemplates just a few diseases, thus reflecting the lack of methods and technologies available in Brazilian public hospitals. In this context, the aim of the present study was the development and validation of a HPLC-DAD methodology for simultaneous quantification of the aminoacids that are characteristically altered in BCK: L-Leu, L-Ile, and L-Val. The method developed by this work uses sampling of blood drops, a single extraction phase, OPA derivatization and direct injection onto the HPLC system, which is quite applicable in laboratory scale.

The method is also suitable for routine clinical practice due to its high extraction efficiency, good reproducibility, and simultaneous quantification of three amino acids using a small volume of blood. This analysis is suitable for diagnostic and clinical monitoring of patients on therapeutic treatment. The linearity and the quantification limits obtained are satisfactory to detect individuals with BCK and distinguish them from healthy ones. The methodology performance was ratified through the linearity, accuracy and precision found in the present studies. Aminoacid standards were stable during the interval of time in which they were stocked. Similar blood sample collection and method validation have been employed for other metabolites that characterize several IEM.

The great advantage of this method is that it allows samples to be collected and transported by mail, important for populations that live in areas that do not have access to this type of diagnostic. The Brazilian Newborn Screening Public Health program contemplates just a few diseases, thus reflecting the lack of methods and technologies available in Brazilian public hospitals. In addition, it costs much less and may prevent the treatment of BCK carriers in the adulthood.

## Conclusion

The development of the method described here proved to be consistent with the initial aims, both regarding the

accuracy of results, facility of sample preparation, and interpretation of data. It was specifically designed to quantify BCCAs in BCK but can harness experimental conditions to detect other inherited aminoacid pathologies. The results validation of the bioanalytical method was satisfactory and baseline simultaneous separation of aminoacids. This was confirmed by the sensitivity, linear correlation, recovery, quantification and detection limits way below the levels obtained from healthy donors in the biological matrix. This method can be adopted by continental countries like Brazil, since samples can be mailed to a specialized center and the analyses carried out at a relatively low cost (under \$ 10.00/patient), therefore much cheaper than methods using mass spectrometry.<sup>20</sup>

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## References

1. Saudubray, J. M.; Sedel, F.; Walter, J. H.; *J. Inherit. Metab. Dis.* **2006**, *29*, 261.
2. Nota Informativa-Programa Nacional de Triagem Neonatal Nov/2012 [http://portal.saude.gov.br/portal/arquivos/pdf/2a\\_221112.pdf](http://portal.saude.gov.br/portal/arquivos/pdf/2a_221112.pdf), accessed in October 2013.
3. Centro de Referência em Erros Inatos do Metabolismo (CREIM) São Paulo, 2010. Available in: <http://www.unifesp.br/centros/creim/>, accessed in June 2011.
4. Martins, A. M.; D'almeida, V.; Kyosen, S. O.; Takata, E. T.; Delgado, A. G.; Gonçalves, A. M.; Benetti Filho, C. C.; Martini Filho, D.; Biagini, G.; Pimentel, H.; Abensur, H.; Guimaraes, H. C.; Gomes, J. G.; Sobral Neto, J.; D'almeida, L. O.; Carvalho, L. R.; Harouche, M. B.; Maldonado, M. C.; Nascimento, O. J.; Montoril, P. S.; Bastos, R. V.; *J. Pediatr.* **2009**, *155*, S19.

5. Jardim, L. B.; Prolla, P. A.; *J. Pediatr.* **1996**, *72*, 63.
6. Leonard, J. V.; Morris, A. A. M.; *Acta Paediatr.* **2006**, *95*, 6.
7. Fernandes, J.; Saudubray, M. J.; Berghe, G.; *Inborn Metabolic Diseases: Diagnosis and Treatment*, vol. 1, 5<sup>th</sup> ed., Walter, H. J., ed.; London: Springer, 2006.
8. Oglesbee, D.; Sanders, K. A.; Lacey, J. M.; Magera, M. J.; Casetta, B.; Strauss, K. A.; Tortorelli, S.; Rinaldo, P.; Matern, D.; *Clin. Chem.* **2008**, *54*, 542.
9. Chace, D. H.; Kalas, T. A.; Naylor, E. W.; *Annu. Rev. Genomics Hum. Genet.* **2002**, *3*, 17.
10. Sowell, J.; Pollard, L.; Wood, T.; *J. Sep. Sci.* **2011**, *34*, 631.
11. Kuhara, T.; *J. Chromatogr.* **2001**, *758*, 3.
12. Collins, C. H.; Braga, G. L.; Bonato, P. S.; *Fundamentos de Cromatografia*, 1<sup>st</sup> ed.; Editora Campinas: Campinas, 2006.
13. Raimundo Jr, I. M.; Pasquini, C.; *Quím. Nova*, **1997**, *20*, 83.
14. Zoppa, M.; Gallo, L.; Zachello, F.; Giordano, G.; *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2006**, *831*, 267.
15. Agência Nacional de Vigilância Sanitária (ANVISA); *Guia para Validação de Métodos Analíticos e Bioanalíticos*, RDC No. 27, 2012.
16. Jeong, J. S.; Sim, H. J.; Lee, Y. M.; Yoon, H. R.; Kwon, H. J.; Hong, S. P.; *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2011**, *879*, 2171.
17. Sharma, G.; Attri, S. V.; Behra, B.; Bhisikar, S.; Kumar, P.; Tajeja, M.; Sharda, S.; Singhi, P.; Singhi, S.; *Amino Acids* **2014**, *46*, 1253.
18. Schwahn, B.; Wendel, U.; Schadewalt, P.; Falkenberg, N.; Monch, E.; *Acta Paediatr.* **1998**, *87*, 1245.
19. Rashed, M. S.; Bucknall, M. P.; Little, D.; Awad, A.; Jacob, M.; Alamoudi, M.; Alwattar, M.; Ozand, P. T.; *Clin. Chem.* **1997**, *43*, 1129.
20. Lin, N.; Ye, J.; Qiu, W.; Zhang, H.; Gu, X.; *J. Pediatr. Endocrinol. Metab.* **2013**, *26*, 433.

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