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Validation of plasma amino acid profile using UHPLC-mass spectrometer (*QDa*) as a screening method in a metabolic disorders reference centre: performance and accreditation concerns.

Bruno C^{1,2,6}, Veyrat-Durebex C^{1,2,6}, LumbuLukuntonda C H^{1,6}, Andres CR^{1,2,6}, Moreau C⁷, Bendavid C⁷, C.Homedan⁸, Labarthe F^{3,4,6}, Tardieu M^{3,6}, Bigot A^{5,6}, Maillot F^{2,5,6}, Benz-de Bretagne I^{1,2,6}, Blasco H^{1,2,6}

1-Laboratoire de Biochimie et Biologie Moléculaire, CHRU de Tours, Tours, France

2-Unité INSERM U1253, équipe “neurogénomique et physiopathologie neuronale”, Université de Tours, Tours, France

3-Service de Médecine pédiatrique, CHRU de Tours, Tours, France

4-UMR *INSERM* U 1069 - Nutrition, Croissance et Cancer (N2C), Université de Tours, Tours, France

5-Service de Médecine Interne, CHRU de Tours, Tours, France

6- Centre de référence des maladies héréditaires de métabolisme – filière G2M

7- Laboratoire de biochimie-toxicologie, Hôpital Pontchaillou CHU Rennes, France, Université de Rennes, Inserm, Inra, Institut NuMeCan, CHU Rennes, France

8-Laboratoire de biochimie et génétique, Centre hospitalier universitaire, Angers, France.

***Corresponding Author :** Dr Clément Bruno

Laboratoire de Biochimie et Biologie Moléculaire

Hôpital Bretonneau, Bâtiment B2A

2 Boulevard Tonnelé

37044 TOURS.

Tel +33 2 47 47 72 36

Fax : +33 2 47 47 86 13

Email : clement.bruno@univ-tours.fr

Introduction

Amino acid (AA) analysis in plasma is essential for diagnosis and monitoring of inborn errors of metabolism (IEM). The efficacy of patient management is governed by the rapidity of AA profile availability, along with the robustness of the method. French quality guidelines and progress made in analytical techniques have led biologists to develop AA profile exploration via mass spectrometry (MS).

Objectives

The aim of this study was to validate an analytical method with a single quadrupole mass spectrometer (MS) and to suggest reference values in regard to French quality and IEM society recommendations.

Design and methods

Plasma samples from patients were deproteinised and derivatised with AccqTag™ reagent. Analysis was performed by reverse-phase chromatography coupled to QDA detector. We evaluated accuracy, intra-days and inter-days precision and limit of quantification by the β -expectation tolerance interval method for 27 AA. Method comparison was performed with the standard method (ion exchange chromatography, IEC) on Jeol Aminotac® and to tandem MS. Reference values were established on AA concentrations of the cohort of patients who had no IEM.

Results

Our method allowed the separations of almost all amino acids with a total run time of 12minutes. Separation of isoleucine and alloisoleucine was incomplete ($R = 0.55$) but without impact on biological interpretation. Precision, accuracy and quantification were satisfactory (intra-days coefficient of variation (CV) was <5%, inter-days precision CV <10% and accuracy <15%). The limits of quantification were validated between 1 and 900 $\mu\text{mol/L}$. Results were comparable between the new method and IEC.

Conclusion

Ultimately, we validated a rapid method on plasma for quantifying 27 amino acids that can be used in routine practice, according to French quality laboratories and SFEIM (French Society of Inborn Error of Metabolism) recommendations. Furthermore, estimated reference values were similar to those found in published studies focusing on other methods. Despite a lower specificity compared to tandem MS, the simplicity and rapidity of our method are the main advantage of this technique and place it as a major tool in IEM diagnosis and management.

1. Introduction

Inborn errors of metabolism (IEM) are rare diseases, mostly expressed during first months of life and characterised by non-specific clinical signs that can lead to irreversible damage¹. Patient prognosis will depend on the time of therapeutic initiation, inherent to the reliability of the diagnosis, mainly based on clinical and biological criteria². Furthermore, diseases decompensations may lead to severe outcomes. In all these cases, early treatment is crucial to improve patient support. Measurement of amino acid (AA) concentrations in biological fluids is one of the most crucial explorations to establish diagnosis of AA metabolism abnormalities, urea cycle disorders and other metabolic disorders, but also to monitor therapeutics and diet strategies. Until recently, the gold standard in AA analysis was ion exchange chromatography with ninhydrin post-column derivatisation (IEC). Despite some significant advantages such as repeatability³ and broad dynamic range, this technique is characterised by a long analytical time and the use of toxic reagents for detection. Tandem mass spectrometry is also used³⁻⁹, but this is an expensive technology. A recent method developed with a single quadrupole on plant extracts showed satisfying results concerning AA resolution and quantification¹⁰. Currently, the French Society of Inborn Errors of Metabolism (SFEIM) recommends providing results to clinicians within 48 hours in cases of suspected metabolic disorders. Moreover, the French obligation to follow the international Norm ISO 15189 for any biological analysis requires internal quality controls (IQC) to be used daily to assess the reliability of results, combined with the regular use of standards to calibrate the analytical method¹¹. We suggest that the method presented here and initially developed for nutrition concerns¹² is of particular interest to fulfil the criteria for quality standards and SFEIM recommendations with acceptable sensitivity and robustness¹³. As this technique has not been initially developed for health concerns, a validation based on ISO 15189 norm and SFEIM recommendations was necessary to use it in clinical practice.

In this context, the objective of this study was to validate a rapid and sensitive method for AA analysis based on a method involving ultra-high performance liquid chromatography coupled to mass

spectrometry (LC-MS) with derivatisation, according to ISO 15189 recommendations and finally to propose adapted reference values for AA plasma concentrations.

2. Design and methods

2.1 Reagents

Analytical grade AccQ-Tag kit containing acetonitrile, borate buffer and AQC reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) were purchased from Waters (Millford, MA, USA). Sodium hydroxide 2M, hydrochloric acid 0.1M and sulfosalicylic acid 10% solutions were prepared by NaOH powder, hydrochloric acid 37% and sulfosalicylic acid dihydrate powder from Sigma Aldrich (St Louis, MO, USA). ULC-MS grade water, acetonitrile and formic acid were purchased from Biosolve (Dieuze, France). As internal standard, we used the MSK-A2 commercial mix from Cambridge Isotopes Laboratories (Tewksbury, MA, USA). The standard solution was prepared for each use, by two AA mixes (A6282 and A6407) obtained from Sigma-Aldrich (St Louis, MO, USA), along with a homemade solution composed of alloisoleucine and glutamine from Sigma-Aldrich (St Louis, MO, USA) to obtain a 1 mmol/L concentration for each of the 24 AAs, with the exception of 500 $\mu\text{mol/L}$ for cystine. A6282 and A6407 were composed respectively of basic AAs (arginine, homocystine, lysine and ornithine) and neutral and acidic AAs (alanine, asparagine, aspartic acid, citrulline, cystine, glutamic acid, glycine, hydroxyproline, isoleucine, leucine, methionine, phenylalanine, proline, serine, taurine, threonine, tyrosine and valine). Six calibration standards were then prepared by serial dilution. Two lyophilised quality control (QC) plasma levels were obtained from Recipe (Munich, Germany) (Table 1). Finally, precipitation reagent was prepared by adding 10 μL of internal standard solution to 990 μL of sulfosalicylic acid 10%.

2.2 Samples

The validation method involved a performance evaluation step and a comparison step. For performance evaluation, we prepared 6 concentrations: 10 μM , 25 μM , 100 μM , 300 μM , 600 μM and 900 μM for each AA (respectively 5 μM , 12.5 μM , 50 μM , 150 μM , 300 μM and 450 μM for

cystine). To compare the new method with an existing tandem MS technique, we collected patients' samples from daily analysis. Blood samples were collected in heparinised tubes and transported to the laboratory within 1 hour in a refrigerated container. The tubes were then centrifuged at 3000 g for 10 minutes at 5 ± 3 °C. Plasma was decanted and then frozen at -20 °C until analysis.

2.3 Sample preparation

First, 100 μ L of precipitation reagent were added to 50 μ L of patient's plasma. The mix was then vortexed before one minute to optimise protein precipitation and centrifuged for 10 minutes at 3000 g at 5 ± 3 °C. Ten microliters of supernatant were transferred to an empty MS grade glass vial. Seventy microliters of borate buffer and 20 μ L of derivatisation reagent were added and the sample was incubated for 10 minutes at 55 °C.

2.4 LC-single quadrupole mass spectrometer

Samples were analysed on a Waters UHPLC system consisting of a UHPLC Quaternary Solvent Manager (QSM), a Flow through Needle manager injector (FTN), a column manager oven (CM) and a single quadrupole mass detector (QDA). Separation was performed on a Cortecs UPLC C18 (1.6 μ m, 2.1 x 150 mm) column maintained at 55 °C during the analysis. The volume injected on the column was 2 μ L. Gradient elution was performed with constant flow rate (0.5 mL/min) using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. Gradient started at 99% A and 1% B during 1 minute. After, mobile phase B increases linearly (curve 6) to 13% in 1 minute. Then mobile phase B increases to 15% in 4.5 minutes and finally to 95% in 2 minutes. This condition is held during 1 minute and then brought back instantaneously to the start condition, which is held during 2.5 minutes.

2.5 Q-Orbitrap

Samples were prepared following 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization as described in "2.3 Sample preparation". Samples were then analysed on a Thermo UHPLC system

Ultimate 3000 coupled to a high resolution Q-Orbitrap tandem mass spectrometer. Separation was performed on a Cortecs UPLC C18 (2.7 μm , 3.0 x 150 mm) column maintained at 55 °C during the analysis.

2.6 Triple quadrupole analysis

Samples were prepared following 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization as described in "2.3 Sample preparation" purposed AB Sciex. Samples were then analysed on a HPLC 1260 Infinity II (Shimadzu) coupled to a high resolution triple quadrupole 4500 tandem mass spectrometer. Separation was performed on an AB Sciex C18 column.

2.7 Validation methodology

2.7.1 Specificity

Validation was conducted according to the guidelines of the International Council for Harmonisation (ICH)¹⁴ and ISO15189 recommendations¹⁵. First, the specificity of the method was tested by injection of a standard AA solution. All mass channel acquisitions were overlaid. The absence of supplementary peaks and resolution (when necessary) were manually checked. Resolution (R) was calculated as the retention time difference divided by the mean of peak width and should be superior or equal to 1.5.

2.7.2 Limit of detection

Lower and higher limits of quantification were determined as the lower and higher concentrations studied with sufficient precision, accuracy and uncertainty relative to scientific society recommendations¹⁶ (RICOS; last update in 2014). Pipecolic acid was absent from calibration standards. As pipecolic acid is lower than 5 $\mu\text{mol/L}$ in physiological state¹⁷, we only established lower limit of detection analysing four levels (0.5 $\mu\text{mol/L}$, 2.5 $\mu\text{mol/L}$, 5 $\mu\text{mol/L}$ and 10 $\mu\text{mol/L}$) each day during 6 days to assess precision and accuracy. Lower limit of detection was established as the lower concentration with precision and bias below 20%.

2.7.3 Protocol of validation

Intra-days and inter-days precision, accuracy, uncertainty and measurement range were assessed by the β -expectation tolerance interval method using Neolicy® software as previously described¹⁸. Briefly, each validation level was prepared every day for 8 days. Each sample was injected in triplicate. Precisions are reported as coefficient of variation (%) and uncertainty and accuracy as deviation of the theoretical value (%). Regarding the 3 following amino acids not systematically quantified: homocitrulline, sulfocysteine, and arginosuccinic acid, each level was analysed during 6 days to assess inter-days precision and accuracy.

The β -expectation tolerance interval must be included in the specification range to assess the compliance between our methods and recommendations. The confidence interval was calculated with accuracy and inter-day variability. It contained 80% of predicted measurements for each value. The method is declared conform if β -expectation tolerance interval is included in recommendations range. Accuracy was also evaluated by quality external evaluation (QEE). QEEs are samples analysed by numerous labs. The results are compared by an independent body. Results are then interpreted according to biases and Z-score (number of standard deviations) to consensus value. Results are considered to be conform if the bias is less than 20% and if the Z-score is between -2 and 2. Eight QEEs purchased from ERNDIM were analysed and compared to other labs. In this case, samples were analysed by 292 labs using diverse methods, including LC-MSMS and IEC as well.

2.7.4 Contamination

Inter-sample contamination was evaluated by analysing a high-concentration sample (750 μ M, with the exception of 375 μ M for cystine) 3 times consecutively (H1, H2, H3), followed by a low-concentration sample (50 μ M, with the exception of 25 μ M for cystine) (L1, L2, L3). The sequence was repeated 5 times. Then, contamination index was calculated as $(\text{mean}(L3-L1))/(\text{mean}(H-L))$. Contamination indices were judged acceptable when inferior to 1%.

2.7.5 Comparison

We collected two independent sets of plasma samples from patients explored for diagnostic or metabolic disease monitoring. We compared AA concentrations measured by LC-MS to those measured by IEC (Jeol® Aminotac, as previously published³) on 38 plasma samples. Furthermore, we compared AA concentrations of 20 other plasma samples measured by LC-MS to those measured by LC-MSMS method performed by the Biochemistry laboratory of the University Hospital of Rennes (France) and by LC-MSMS method in process of accreditation performed by the Biochemistry laboratory of the University Hospital of Angers (France). We analysed the correlation of sample concentrations for each AA between the studied method (LC-MS) and the IEC or LC-MSMS technique using a Bland and Altman plot. Findings were interpreted after comparison with acceptable total error recommended by RICOS. Pipecolic acid was compared only with triple quadrupole methodology with 2.5 and 5 $\mu\text{mol/L}$ spiked samples.

2.8 Matrix effect

Ten different plasma samples collected from patients explored in routine practice were spiked with different solutions containing 10 $\mu\text{mol/L}$, 25 $\mu\text{mol/L}$, 50 $\mu\text{mol/L}$ and 100 $\mu\text{mol/L}$ of each amino acid and were then analysed. Mean recovery was calculated for most of the amino acids. Recovery acceptability was determined as accuracy limits recommended by RICOS.

2.9 Determination of reference values

We collected all AA profiles obtained over a one-year period in our Hospital and we excluded each patient known to have a disease or a treatment that could modify AA balance. Finally, 238 results were then divided into classes according to age. It's known that AA concentrations tend to decrease during the first year of life, increasing in some cases between 4 and 6 and after 13-15 years¹⁹. Accordingly, our results were divided in four classes (0-1 year, 1-4 years, 4-13 years and >13 years). Finally, reference values were determined as the range containing 95% of AA values in selected samples, according to age class.

3. Results

3.1 Chromatographic profile

The analytical method allowed AA separation within 6 minutes out of a total run time of 12 minutes including column equilibration time. All 25 targeted AAs were detected at different masses, resulting in a single peak separated on different acquisition channels. Some exceptions were observed for isobaric AAs (leucine/isoleucine/alloisoleucine and alanine/beta-alanine/sarcosine) as shown in Figure 1. The resolutions were satisfactory however ($R > 1.55$), with the exception of alloisoleucine that was not well-separated from isoleucine ($R = 0.55$) and leucine ($R = 1.2$).

3.2 Method performance

Accuracy and precision performances were acceptable according to scientific recommendations (Table 2). Intra-days and inter-days precision ranges were respectively between 0.78 and 3.34% and between 3.63 and 12.39%. Accuracy was estimated between 1.86 and 12.85% (in absolute value). For almost all amino acids, these criteria respect RICOS acceptability criteria. Only glutamine had an average bias of 11.82%, whereas RICOS recommended a maximum of 9.45%. Furthermore, the accuracy profile obtained showed stability of the precision and accuracy throughout the measurement range, confirming the satisfactory biases and variability of the method. Confidence ranges were always included in specification ranges (Figure 2). The lower and higher limits of quantification of the method were 1.0 $\mu\text{mol/L}$ and 900 $\mu\text{mol/L}$ for all amino acids including homocitrulline, homocystine and arginosuccinic acid (half for cystine), except pipercolic acid. Precisions obtained on 1 $\mu\text{mol/L}$ sample were estimated between 3.15 and 16.72% and accuracy between -0.8 and 24.8%, that are considered as acceptable regarding to RICOS values (Table 2). We observed no significant contamination with all contamination indices $< 0.25\%$ (Table 2). QEE results were considered as compliant for almost all AAs (Table 3). Only asparagine, hydroxyproline and taurine were respectively 35%, 29% and 32% overestimated, while citrulline was 34% underestimated. Regarding pipercolic acid, lower limit of detection was estimated at 2.5 $\mu\text{mol/L}$.

3.3 Recovery and matrix effect

All recoveries were between 80% and 120% for each amino acid. Higher matrix effects were found for phenylalanine (91%) and for aspartic acid (119%). In addition, each bias measured on these samples was lower to uncertainty acceptability. Moreover, matrix effect was stable between different samples (CV between 0.78% (for alanine) and 10.38% (for aspartic acids)). Finally, we observed no significant impact of plasma matrix effect on the amino acids analysis with this method.

3.4 Comparison with other methods

We obtained a good agreement between the LC-MS and IEC methods. Correlation coefficients (R^2) were from 0.70 to 0.99 and Bland and Altman analysis confirmed the absence of discordance between both methods (Figure 3). On the other hand, cystine, hydroxyproline and aspartic acid could not be compared due to the fact that too many samples were below the lower limit of quantification for the IEC method. Correlation coefficients between LC-MS and LC-MSMS Q-Orbitrap were between 0.74 and 0.99. Bland and Altman analysis showed a mean bias of 3.87 %, with and only five amino acids (arginine, aspartic acid, cystine, hydroxyproline and taurine) showing a bias >20% (Figure 4). Correlation coefficients between LC-MS and LC-MSMS triple quadrupole were between 0.74 and 0.99. Bland and Altman analysis showed a mean bias of -2.49 % with only glutamic amino acids showing a bias >20% (Figure 5). However, these biases were lower than accuracy limits recommended by RICOS (Table 2) and don't have any impact on biological interpretation. Furthermore, 95% of measured concentrations were included in the acceptable total error range, except aspartic acid for comparison with Q-Orbitrap method (Figure 4) and no discordance were observed with the triple quadrupole method, except aspartic acid (Figure 5). Unfortunately, sulfocysteine, arginosuccinic acid, homocitrulline, homocystine were not included in these method comparisons as the concentrations of patients samples were under the lower limit of detection. Concerning pipercolic acid detection, no false positive and no false negative have been found.

3.5 Reference values

The calculated reference values are shown in Table 4. Some AA concentrations decreased during the first 4 years (taurine, hydroxyproline, and proline) by contrast to branched-chain AA levels that increased during the first year of life. Finally, AA levels were similar between the 4-13 years and >13 years age groups, with the exception of alanine (increasing) and cystine (decreasing).

4. Discussion

In this study, we validated an accurate and robust method using LC-MS for the quantification of 27 amino acids in plasma for routine diagnosis and monitoring of metabolic disorders. We also demonstrated that this method was able to detect pipercolic acid from 2.5 $\mu\text{mol/L}$. SFEIM considers pipercolic acid as an important complementary amino acid for IEM diagnosis. Consequently, all samples in which pipercolic acid is detected are sent to another lab for specific quantification.

4.1 Need for a change of analytical method

As previously described, underivatized AA methods have benefits but also drawbacks. IEC is an efficient reverse-phase chromatography technique, but involves a processing time (almost 2 hours) that may be incompatible with emergency situations. Over the past years, mass spectrometry (MS) analysis has emerged as an alternative detector for small molecules, including AAs^{3-5,8-10,20,21}. Accordingly, different strategies have been tested, but no standard method has been defined and recognized by the various IEM societies or by the consortium of biologists. LC-MS has already shown potential in biological fluids (drug monitoring²², steroids²³). Conversely, hydrophilic interaction liquid chromatography (HILIC) allows faster analysis (15-20 minutes²⁴) but with low separation between leucine, isoleucine and alloisoleucine isobars²⁰. Currently, derivatization would appear to be the best compromise between analysis time, chromatographic resolution and cost²⁴.

4.2 High technical performance of LC MS (QDA)

Taking into account both the limits of the previous gold standard method (IEC) and advances in mass spectrometry technologies, we decided to change our analytical method: the new one, based on pre-

column derivatisation *via* aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), quickly leads to stable products²¹, followed by detection via tandem mass spectrometry after UHPLC separation. This method showed low variability and excellent accuracy. Intra-days and inter-days precision values were comparable or sometimes even lower than the variability found in other studies quantifying AA by liquid chromatography coupled to mass spectrometry^{5,7}. Lower and upper limits of quantification were in line with clinical needs for all AAs. Even though diagnosis is not based on a single amino acid, and often revealed by high AA concentrations, quantification down to 1 $\mu\text{mol/L}$ can be important to help in some diagnoses and to initiate treatment earlier²⁵. For example, in the case of MTHFR deficiency, methionine levels are decreased at birth and can be at almost 1 $\mu\text{mol/L}$ ²⁶. Similarly plasma citrulline (Cit) and arginine (Arg) concentrations may also be lower than 1 $\mu\text{mol/L}$ in ornithine transcarbamylase (OTC) deficiency²⁷. Accordingly, some studies have estimated that high sensitivity (lower limit of quantification between 1.3 and 5.3 $\mu\text{mol/L}$ ^{21,28}) may be of interest for some AAs. A dilution protocol will be required for AA concentrations that could be higher than the upper limit of quantification, at least for phenylalanine, glutamine and leucine, that can be higher than 1000 μM respectively in phenylketonuria²⁹, urea cycle disorders³⁰ and Maple Syrup Urine Disease (MSUD)³¹.

We found a good correlation of the concentrations measured by mass spectrometry and ion exchange chromatography with ninhydrin post-column derivatisation^{3,20}. Even though the QEE results were not satisfactory for four AAs, the difference can be explained by the variability of the analytical method used by the different laboratories (IEC, LCMSMS, LCMS, etc.), as shown in table 4. Moreover, *Casado et al* previously showed a poor level of correlation between LCMSMS and IEC for hydroxyproline, aspartic acid and citrulline, that could explain these differences between laboratories³². However, comparison between LC-MS and LC-MSMS showed mean bias lower than 20% except for aspartic acid, arginine, hydroxyproline, cystine and taurine. Furthermore, even for these five amino acids, the uncertainty met RICOS recommendations. In most of the cases, this deviation has no significant impact on the biological interpretation. However, we have to be careful with these amino-acids and those that have low concentrations. We must be aware of the lower

specificity of single MS compared to tandem MS, and we highly recommend the re-analysis of samples in case of inconsistency between clinical and biological data. Moreover, recovery was stable between samples and linearity was conserved between different spiked solutions. We can conclude that matrix effect doesn't have any significant effect on plasma amino acids analysis. In addition, as we have estimated new reference values biological interpretation is not impacted. Finally, we noted the absence of contamination, thus completing the validation of the new technique. However, IEM diagnosis can be also provided based on amino acids analysis in urine and in CSF. In both matrixes, some amino acid concentration may be found lower to $1\mu\text{mol/L}$. Even if simple quadrupole mass spectrometry is in agreement with all recommendations concerning plasmatic amino acids analysis, it should be evaluated for CSF and urine samples to evaluate its place in metabolism disorders investigations.

4.3 Main advantages of UHPLC-mass spectrometry (QDA) in clinical practice

Sample preparation has the advantage of being simple and rapid (no more than 30 minutes). Finally, the total analysis time of only 12 minutes, compatible with regular calibration with multiple standards and daily control analysis as imposed by the French quality committee and ISO15189 standard¹¹ is in line with metabolic emergency and allows the analysis of numerous samples per day as required. One of the most common limitations of MS techniques for AA exploration is the separation of the branched-chain AAs leucine and isoleucine. This was a crucial validation point, especially to diagnose and follow patients suffering from Maple Syrup Urine Disease (MSUD), characterised by a deficiency in branched-chain alpha-keto acid dehydrogenase required to metabolise branched-chain AAs (BCAAs). A recent study has shown that early diagnosis, combined with a restrictive diet and close leucine monitoring, have a significant effect on neurological evolution³¹. Furthermore, the treatment of many metabolic disorders is based on protein intake control and plasmatic BCAAs are crucial parameters to evaluate nutrition status. For this, BCAA separation and specificity is an advantage over other techniques that quantify the sum of both. As

described in urea cycle disorders³³ or phenylketonuria³⁴, the specific diet (low protein diet) requires regular and strict monitoring of BCAA (and other essential AAs) to avoid deficiencies and at the same time to allow the correct growth of children³⁵.

4.4 Main limits of the study

The proposed method for metabolic disease screening based on simple quadrupole mass spectrometry has shown satisfying results, regarding to SFEIM organization and RICOS requirements. The proposed single quadrupole analysis should be associated, as previously described to a triple quadrupole analysis to validate unexpected results and to exclude occasional interferences, notably for low concentrated plasma amino acids. The clinical interpretation of amino acids analysis is made from the interpretation of a global profile, but for some amino acids that we cannot quantitate at low levels, we send the sample to another laboratory for accurate quantification. As for the incomplete chromatographic separation of alloisoleucine and isoleucine, we assume that alloisoleucine levels are usually relatively high and recognizable compared to isoleucine in the affected patients. Finally, we have compared 2 series of 20 samples between our method and other MS methods and the results were correct. We could test false positives and negatives in more samples than is required for accreditation purposes and we could regularly plan this kind of cross validation, . The comparison of methods presented on a large number of samples and the satisfying results of external quality controls ensure the reliability of the technique. Finally, as CSF samples have not been evaluated in this study, and because amino acids concentrations are lower than in plasma samples, we recommend to use a validated method on tandem MS.

4.5 Reference values

As shown in Table 5, *Nakamura et al*³⁶ described similar reference values in various Asian populations. In this study, the AA concentrations of Japanese, Korean and Chinese populations

according to gender, age and body mass index were compared. Only glutamine was significantly different ($p=0.0013$). This study, however, was conducted exclusively on an Asian population. By contrast, reference values seem to be different between the studies of *Casado et al*³² and *Filee et al*⁶, respectively conducted on Spanish and Belgian populations (Table 5). We suggest that this difference is a combination of the variability of local analytical methods and of population variations. Furthermore, AA concentrations evolve according to age³⁷. Even though numerous studies have grouped reference values into different age classes, there is no consensus concerning their limits^{6,32,38}. Reference values are difficult to estimate because of the difficulty obtaining non-pathological samples in hospitals. Furthermore, AA concentrations are governed by daily nutrition, activity³⁹ and associated diseases⁴⁰. Finally, concentrations appear to differ according to analytical method (IEC, LCMSMS, LCMS, etc.) and population tested (ethnic group, age, etc.). Thus, we recommend estimating new reference values for each validated method and checking the extent of the discrepancy. Our calculated reference values were comparable to those previously published in LCMSMS methods^{6,32,36,38,41} whatever the age class. We also confirmed the evolution of AA concentrations over the first years of life.

5. Conclusion

In this study, we validated an LCMS method to quantify 27 amino acids in 12 minutes according to French quality committee criteria. This method allows the separation of all amino acids (except leucine, isoleucine and alloisoleucine) with a satisfactory degree of precision and accuracy, allowing their quantification between 1.0 and 900 $\mu\text{mol/L}$. Even if separation of isoleucine, allosisoleucine and leucine was incomplete, our method is able to quantify these three amino acids, without impact on biological interpretation. Pipecolic acid detection was established at 2.5 $\mu\text{mol/L}$ without testing quantification performances. Finally, we estimated our reference values on samples from patients without known metabolic disorders. This method also allows daily use for diagnosis and monitoring of metabolic diseases according to minimum requirements and plays a crucial role in IEM screening despite the need of triple quadrupole in some contexts.

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	Level 1	Level 2
Hydroxy-proline	7.30 - 10.9	44.6 - 66.9
Asparagine	32.2 - 48.3	148 - 222
Arginine	23.2 - 34.6	205 - 307
Taurine	35.6 - 53.4	161 - 218
Serine	80.4 - 121	247 - 334
Glutamine	359 - 538	756 - 1134
Glycine	165 - 230	533 - 800
Aspartic acid	7.17 - 13.3	83.9 - 126
Glutamic acid	83.4 - 125	166 - 249
Citrulline	19.3 - 28.9	102 - 138
Threonine	96.3 - 130	178 - 241
Alanine	351 - 475	589 - 796
Proline	185 - 278	392 - 530
Ornithine	118 - 160	284 - 384
Lysine	136 - 204	216 - 324
Tyrosine	52.2 - 78.4	170 - 230
Methionine	23.3 - 34.9	57.0 - 77.1
Valine	203 - 275	368 - 498
Isoleucine	70.2 - 95	111 - 150
Alloisoleucine	13.3 - 24.7	149 - 223
Leucine	119 - 178	232 - 349
Phenylalanine	67.2 - 90.9	358 - 484

Table 1: Amino acids reference values for QC plasma samples

Compound	Intra-days precision (%)		Inter-days precision (%)		Accuracy (%)		Uncertainty (%)		Recovery (%)	Contamination (%)
	RICOS	Mean	RICOS	Mean	RICOS	Mean	RICOS	Mean		
Hydroxy-proline	19.50	3.10	25.90	6.86	24.90	4.41	76.70	15.15	102.1	0.08
Asparagine	6.90	2.07	9.20	8.52	11.40	10.84	29.80	20.46	94.4	0.24
Arginine	10.90	3.34	14.50	5.71	14.70	3.49	43.70	12.45	98.5	0.01
Taurine	17.30	3.11	22.95	6.92	20.10	4.45	66.00	15.37	105.2	0.07
Serine	7.20	2.33	9.60	4.73	16.80	7.15	36.00	10.79	99.5	0.01
Glutamine	6.80	1.98	9.10	4.14	9.45	11.82	27.65	9.91	99.4	0.07
Glycine	6.70	1.98	8.85	3.88	15.75	5.84	33.45	9.07	101.6	0.10
Aspartic acid	17.60	1.55	23.40	4.43	23.70	5.02	70.50	10.00	109.1	0.07
Glutamic acid	15.47	2.09	23.20	4.05	23.12	5.34	69.52	9.09	97.8	0.01
Citrulline	12.00	3.34	16.00	5.71	18.30	3.49	50.30	12.45	97.5	0.08
Threonine	10.10	2.31	13.40	4.52	14.10	4.33	40.90	9.96	99.7	0.05
Alanine	8.30	2.39	11.00	4.34	21.60	7.26	43.60	10.21	100.5	0.11
Proline	9.60	2.01	12.80	4.10	39.60	3.33	65.20	8.95	100.3	0.11
Ornithine	10.40	2.43	13.80	5.75	21.80	12.85	49.40	13.76	98.3	0.02
Cystine	21.60	2.41	28.70	5.31	23.10	1.86	80.50	11.43	99.8	0.12
Lysine	6.80	1.66	8.60	4.52	15.00	5.99	32.20	10.17	101.7	0.02
Tyrosine	5.90	0.94	7.90	3.67	23.25	2.84	39.05	8.04	98.4	0.01
Methionine	8.30	0.88	11.00	3.76	17.30	3.93	39.30	8.33	98.3	0.03
Valine	6.00	0.78	8.00	3.74	15.60	3.50	31.60	8.25	99.3	0.10
Homocystine	4.15	1.69	8.30	5.83	8.63	5.00	25.23	13.65	-	0.02
Isoleucine	8.70	1.17	11.60	3.70	18.00	3.02	41.20	8.32	100.7	0.00
Alloisoleucine	10.00*	1.53	15.00*	5.63	20.00*	3.07	50.00*	12.39	-	0.03
Leucine	8.30	0.83	11.10	3.68	17.40	3.11	39.60	8.08	100.0	0.02
Phenylalanine	5.40	0.78	7.10	3.67	15.60	3.08	29.80	8.07	99.1	0.04
Homocitrulline	10.00*	-	15.00*	3.63	20.00*	-5.38	50.00*	-	-	-
Sulfocysteine	10.00*	-	15.00*	12.39	20.00*	-2.52	50.00*	-	-	-
Arginosuccinic acid	10.00*	-	15.00*	10.60	20.00*	1.07	50.00*	-	-	-

Table 2: Method performances for the quantitative analysis of 24 amino acids

*: Absence of recommendations from RICOS. These limits are based on FDA recommendations for chromatographic methods

Analyte	Accuracy		
	(mean)		(deviation)
	Our Lab	All labs	%
Alanine	732	723	+1.24
Arginine	297	276	+7.61
Asparagine	78.9	64.7	+21.95
Aspartic Acid	52.2	55.9	-6.62
Citrulline	367	512	-28.32
Cystine	51.2	55.2	-7.25
Glutamic acid	118	116	+1.72
Glutamine	801	778	+2.96
Glycine	563	556	+1.26
Histidine	173	169	+2.37
Hydroxy-proline	59.2	44.5	+33.03
Isoleucine	377	353	+6.80
Leucine	336	324	+3.70
Lysine	217	214	+1.40
Methionine	318	307	+3.58
Ornithine	294	259	+13.51
Phenylalanine	369	341	+8.21
Proline	382	366	+4.37
Serine	206	198	+4.04
Taurine	262	202	+29.70
Threonine	199	200	-0.50
Tyrosine	251	239	+5.02
Valine	312	301	+3.65

Table 3: Mean results of QEE and deviation (in percentage) relative to results from all participating labs (n>200)

	Necker hospital									Our study			
	0-1 month	1-6 months	6-12 months	1-2 years	2-4 years	4-7 years	7-10 years	10-13 years	>13 years	0-1 year	1-4 years	4-13 years	>13 years
	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$
Ala	184-380	166-514	157-481	174-374	151-407	134-502	148-412	167-517	221-481	115.5-335.2	108.1-447.3	84.9-468.6	144.5-473.9
Arg	35-91	32-128	41-117	34-106	38-110	47-123	29-129	29-129	40-124	33.6-102	21.5-110.7	19.6-103.3	17.4-114.9
Asn	28-72	24-80	24-76	19-71	25-69	19-71	29-65	36-60	24-72	25.0-69.6	23.9-81.4	32.6-78.3	29.1-87.8
Asp	0-29	0-29	0-33	0-25	2-14	0-24	0-24	0-11	0-20	1.8-30.5	1.0-33.9	1.0-12.9	1.0-15.0
Cit	4-36	13-41	18-38	14-42	7-43	16-40	10-42	18-42	13-41	7.2-35.1	3.7-39.4	5.2-38.2	3.4-54.9
Cys	39-127	46-118	40-124	45-113	40-112	48-120	56-112	53-113	43-131	14.9-54.3	13.8-49.7	22.9-53.2	3.46-54.9
Gln	256-760	311-675	323-675	362-606	315-619	334-666	368-692	331-715	338-658	272.6-753.5	262.4-674.8	399.6-682.8	384.5-698.4
Glu	0-319	9-193	2-174	19-123	4-156	2-118	3-115	0-124	0-129	15.8-251.3	15.9-167.9	9.5-76.5	10.2-95.1
Gly	138-342	141-305	157-333	160-264	142-302	149-301	134-314	147-319	125-349	156.9-334.5	125.6-287.9	131.1-317.9	121.8-341.1
His	46-106	110-282	35-127	52-104	53-101	48-112	61-109	55-109	61-109	34.7-140.6	41.7-98.4	49.3-100.8	38.1-113.2
Hcys										<1	<1	<1	<1
Hyp	16-60	0-73	0-43	0-29	0-30	0-36	0-36	0-28	0-37	17.3-30.5	5.9-35.9	2.5-37.0	2.6-35.8
Ile	20-100	39-107	30-86	31-79	37-85	37-89	47-87	40-96	42-90	32.8-102.9	28.5-91.2	31.8-91.3	25.9-107.6
Leu	43-187	82-182	57-161	59-151	67-175	68-168	86-182	79-179	87-175	62.5-180.6	60.5-158.2	60.5-168.1	74-183.5
Lys	106-246	53-117	90-262	85-241	85-241	113-269	108-264	140-224	120-268	93.8-313.9	67.6-241.0	61.4-231.3	72.6-252.3
Met	17-41	17-45	13-37	17-29	12-32	11-43	14-38	17-37	14-42	15.2-38.9	8.6-29.7	11.3-28.6	12.8-41.3
Om	22-154	39-119	22-114	24-92	23-91	31-107	18-114	20-132	25-125	34.2-122.9	15.8-85.4	16.5-98.3	22.0-111.1
Phe	35-75	40-76	32-80	36-64	35-67	33-77	38-78	26-86	32-76	30.0-77.6	35.6-64.1	33.2-71.1	32.2-85.2
Pro	107-275	83-291	91-279	93-233	90-238	61-285	92-240	45-345	115-263	90.5-257.3	56.6-231.9	60.5-336.2	93.7-306.5
Ser	79-191	84-192	68-240	87-183	68-200	80-192	85-189	83-166	83-195	35.2-289.4	48.0-225.8	65.8-204.0	46.1-165.5
Tau	0-240	0-200	11-151	4-148	26-114	13-149	12-176	11-139	0-159	83.5-135.8	33.1-119.5	16.3-122.1	21.5-98.1
Thr	50-206	65-213	36-204	63-131	43-159	66-170	56-176	42-226	47-247	34.4-207.2	29.1-181.3	51.1-192.9	63.2-224.3
Tyr	25-133	38-122	35-87	39-79	35-91	38-94	30-102	36-104	37-89	35.5-118.2	31-84.5	33.7-94.5	29.7-90.1
Val	93-301	147-303	139-303	133-293	133-349	158-310	179-327	167-335	178-318	108.8-300.3	143.7-255.9	133.7-294.7	95.1-301.6

Table 4: Amino acids age-related reference values in plasma in French population. Necker values were estimated by Necker hospital (Paris) by IEC

	Filee et al 2014 ⁶			Casado et al 2018 ³²			Nakamura et al 2016 ³⁶			Akiyama et al, 2014 ³⁸		M.Z Peng et al2019 ⁴¹			
	0-2 years	2-12 years	>= 12 years	0-1 month	2 months-2years	>2 years	Japanese	Korean	Chinese	0-3 years	>3 years	0 - 1year	1 - 3 years	3 - 6 years	6 - 15 years
	μmol/L	μmol/L	μmol/L	μmol/L	μmol/L	μmol/L	μmol/L	μmol/L	μmol/L	μmol/L	μmol/L	μmol/L	μmol/L	μmol/L	μmol/L
Ala	122-426	111-518	153-592	190-337	167-439		250.1-595.6	241.7-671.3	196.2-681.5	236-484	246-506	221.8 - 550	156.9 - 475.1	167.3 - 475.6	191.6 - 522.9
Arg	14.4-164	18.4-102	11.2-128	47-122			53.6-138.6	69.5-135.8	59.6-151.2	49.0-100.8	44.4-99.8	30.6 - 115.7	14.7 - 119	29 - 113.7	23.5 - 120.1
Asn	27.1-123	28.4-102	22.7-115	31-120			38.0-65.1	34.2-71.5	33.9-71.8	33.0-68.4	36.4-59.6	32.6 - 71.3	24.2 - 71.3	29.8 - 76.7	28 - 79.9
Asp	6.1-69.4	4.6-60.3	4.4-65	2-20						2.1-8.5	0.8-6.4	3.5 - 11	2.8 - 12.9	2.2 - 12.7	3 - 7.4
Cit	7.9-33.6	8.4-40.7	13.5-63.3	8-40			17.5-41.7	19.6-46.5	16.5-42.9			14.7 - 40	13.6 - 32.8	17.4 - 41.2	15.1 - 34.5
Cys	6.4-124	0.9-39.1	7.3-58.5	15-59						26.7-51.7	37.9-57.9				
Gln	336-691	300-688	296-884	326-750	325-676	330-632	474.8-743.8	402.4-706.4	437.7-700.0	450.1-644.7	423.3-572.5	287.7 - 588.5	331.5 - 565.5	360.2 - 566.3	294.3 - 625.6
Glu	38-353	35-288	41.9-236	5-80			10.1-50.3	8.3-64.9	11.7-86.7	48.4-103.4	32.8-68.2	39.8 - 134.2	36.4 - 97.4	36.8 - 95.6	31.5 - 113.1
Gly	113-458	164.7-402	94.9-463	109-293			95.3-397.7	119.1-381.0	166.1-326.0	153.6-281.6	216.8-406.0	110.5 - 271.7	92.7 - 307.4	136.9 - 252.4	90.1 - 286.4
His	35-117	34.2-114	35.4-136.2	45-104			65.5-106.0	63.9-99.2	68.1-98.0	57.0-82.4	59.1-93.5	60 - 116.3	61 - 116.6	59.6 - 108.9	50.1 - 116.5
Hyp	10.8-76.2	8.7-31.9	4.8-34.6	12-99		5-40						17.8 - 48.1	12.1 - 37.4	12.1 - 44	8.7 - 51.2
Ile	30.3-129	24.7-94.7	28.3-167.8	32-90			38.0-115.3	33.6-104.2	42.8-96.7	50.6-89.8	48.7-92.7	39.8 - 149.9	39.8 - 115.4	37.4 - 163.9	50.8 - 154.5
Leu	44.9-198	44.5-158	61.2-203.8	57-155			70.1-206.0	67.7-169.3	85.2-177.1	90.9-148.6	95.9-163.1	65.7 - 250.7	75.9 - 196.9	70.7 - 248.9	89 - 257.5
Lys	64.5-375	75.5-228	105-253	67-252	112-240		113.0-255.2	131.8-249.7	102.6-276.7	97.6-199.0	130.6-216.8	105.2 - 271.5	71.8 - 242.1	117.6 - 272.2	111.5 - 213.6
Met	9.4-67.6	9.3-31	9.6-44.4	12-37			18.5-44.5	18.5-40.6	13.6-47.3	15.7-32.3	16.7-33.5	13.4 - 59.6	9.5 - 50.9	11.1 - 51.8	10.7 - 36.1
Orn	19.9-181	23.4-165	42.8-186	30-175		30-109	17.8-83.3	26.6-79.7	24.2-83.5			41.3 - 136.4	38.1 - 117.7	41 - 135.5	59.1 - 156.4
Phe	25.5-131	27.7-95.8	23.5-104	40-70			45.5-89.5	40.0-78.2	46.3-88.8	34.7-67.5	42.2-70.4	40.1 - 94.7	47.2 - 80.8	47 - 95.2	48.2 - 88.9
Pro	74.9-276	89.2-286	85.8-327.8	90-355			67.1-271.2	97.2-272.7	73.2-348.2	140.3-272.9	107.9-302.1	109.1 - 283.7	74.5 - 304.7	99 - 331.1	80.1 - 358.1
Ser	62-253	61.8-230	53.7-216	92-197			62.1-179.1	82.4-166.5	80.3-161.1	120.2-177.8	125.2-180.4	101.3 - 206.4	77.6 - 172.5	94.7 - 217.7	93.3 - 212.6
Tau	34.8-309	34.9-266	30.3-223	30-150											
Thr	41.7-252	51-167	58.3-206	58-292		78-197	81.3-235.5	74.0-238.6	62.2-192.2	73.3-183.5	87.9-153.5	79 - 233.2	46.7 - 164.2	74.5 - 211.7	53.4 - 195.3
Tyr	24-154	27.3-92.1	34-101	41-206	39-87		38.3-116.9	31.6-90.9	40.5-107.2	43.2-81.8	55.4-98.0	45.4 - 136.9	37.9 - 104.9	45.2 - 113.5	52.3 - 125.9
Val	72.6-277	94.8-261	99.2-329	102-294			134.3-368.1	118.3-312.0	156.4-282.5	155.2-272.2	189.6-298.4	107.7 - 353.3	140 - 360.3	115.8 - 376.3	152.1 - 362

Table 5: Amino acids-age-related reference values in plasma

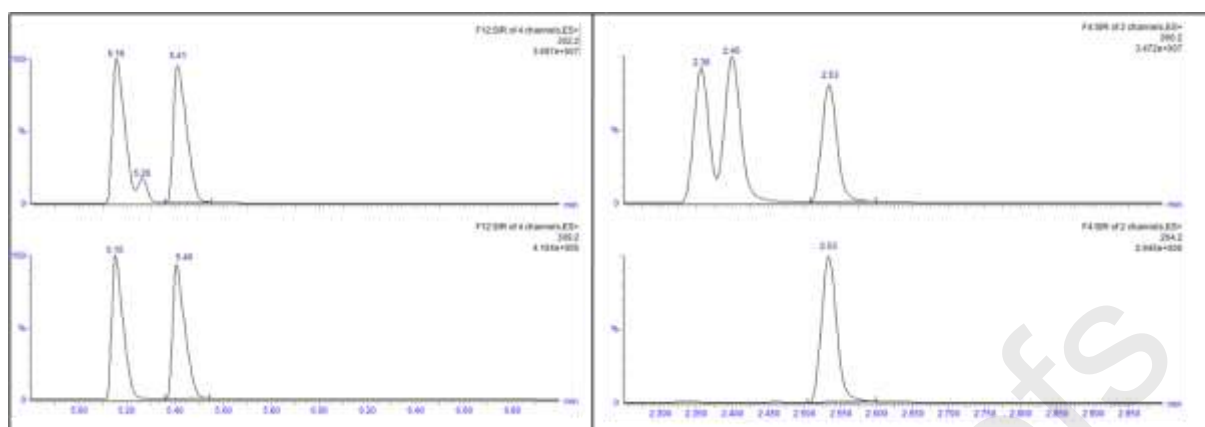


Figure 1: Chromatograms obtained by SIR (Single Ion Reaction) analysis targeted on 260.2, 264.2, 302.2, and 309.2 m/z . Isoleucine, alloisoleucine and leucine are detected respectively at 5.16, 5.26 and 5.41 minutes on channel 302.2. $^{13}C_3$, ^{15}N , isoleucine and $^{13}C_3$, ^{15}N , leucine (internal standards) are detected respectively at 5.15 and 5.40 minutes on channel 309.2. Sarcosine, beta-alanine and alanine are detected respectively at 2.36, 2.40 and 2.53 minutes on channel 260.2. $^{13}C_3$, ^{15}N , alanine (internal standard) is detected at 2.53 minutes on channel 264.2.

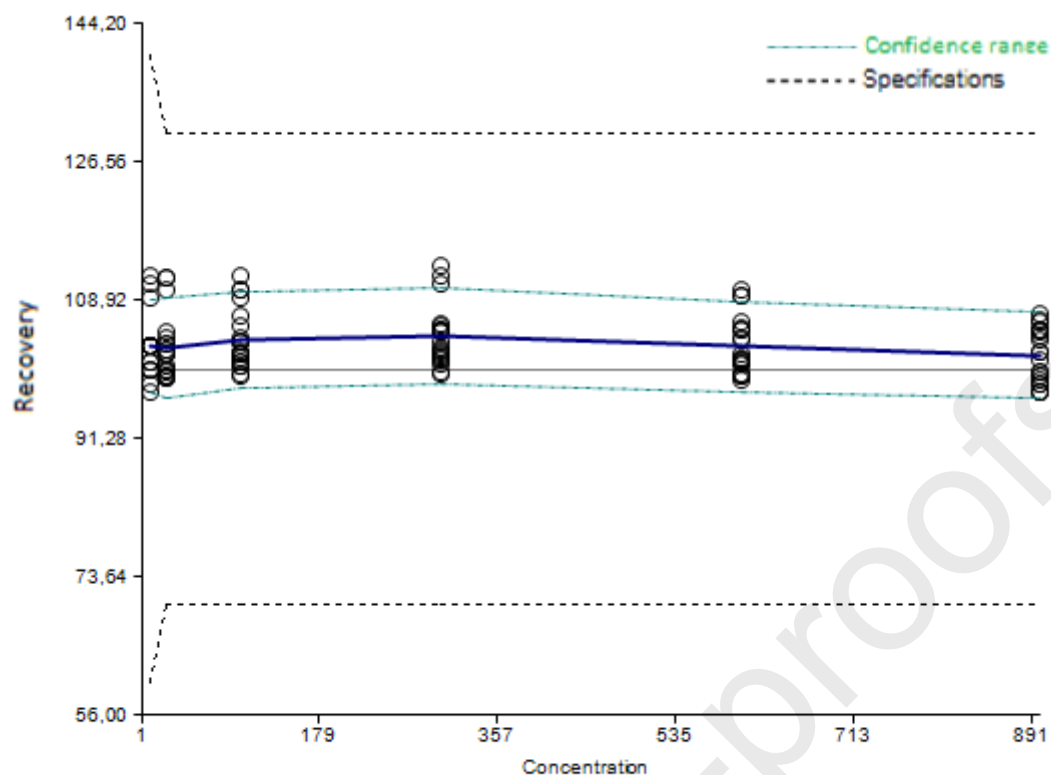


Figure 2: Example of the β -expectation tolerance interval of an AA (i.e. isoleucine). Isoleucine was analysed by UPLC-MS between $10\mu\text{mol/L}$ and $900\mu\text{mol/L}$. Black and continuous line represents the target and black discontinuous line the range of acceptance. Each circle represents a measurement, the blue line is the mean of each measurement and the green dotted line is the β -expectation tolerance interval. The β -expectation tolerance interval is included in specifications (black dotted line). The measured mean bias was 3.63% with an inter-days precision of 4.44%. β -expectation tolerance interval of all AA are present in supplemental data.

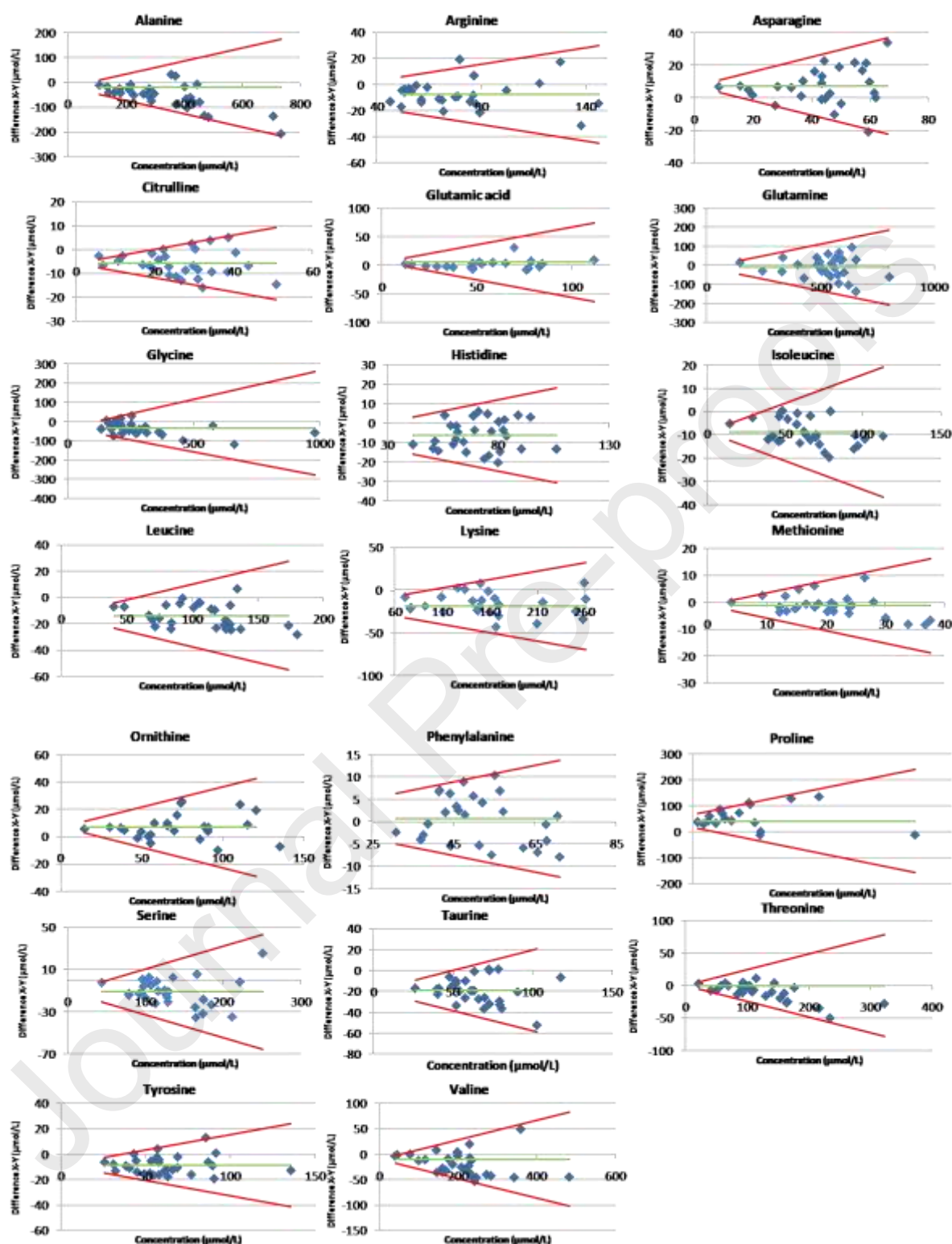


Figure 3: Bland and Altman comparison between LC-MS (method X) results and IEC method (method Y) results obtained on 20 plasma samples. Red lines represent the acceptance range, based on RICOS uncertainty recommendations

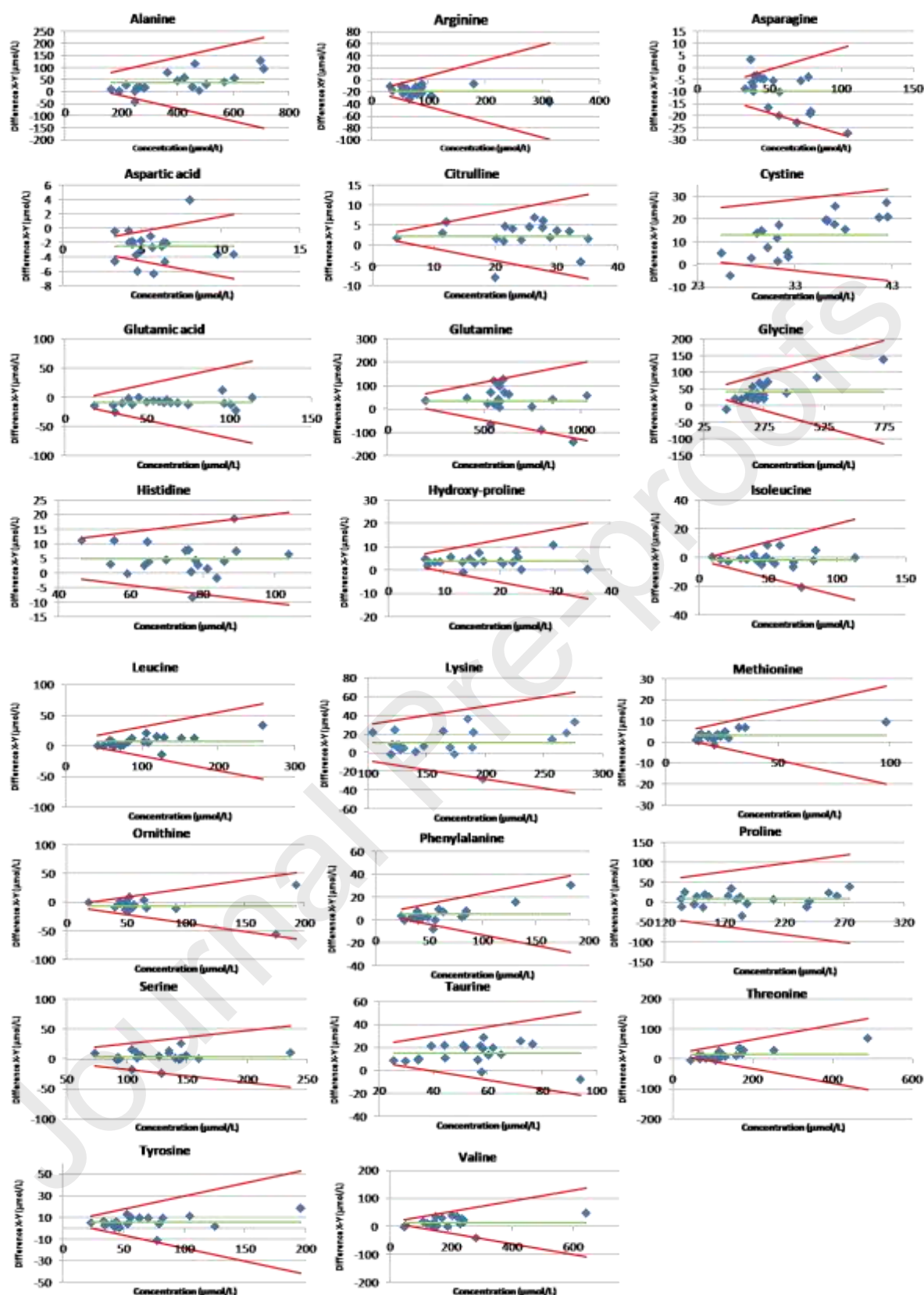


Figure 4: Bland and Altman comparison between LC-MS (method X) results and Q-Orbitrap method (method Y) results obtained on 20 plasma samples. Red lines represent the acceptance range, based on RICOS uncertainty recommendations

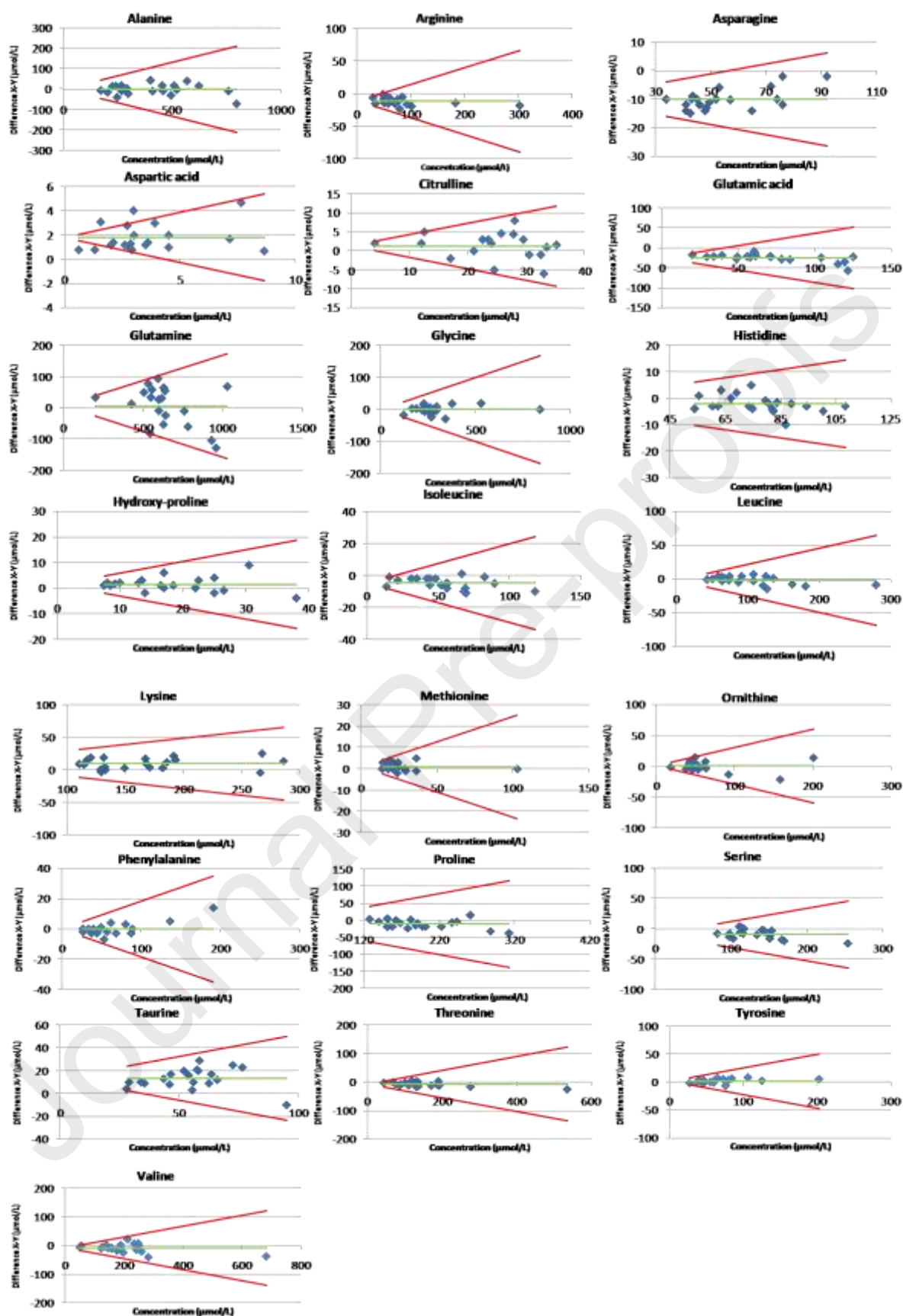


Figure 5: Bland and Altman comparison between LC-MS (method X) results and triple quadrupole method (method Y) results obtained on 20 plasma samples. Red lines represent the acceptance range, based on RICOS uncertainty recommendations