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Article

Accurate Determination of Amino Acids in Serum Samples by Liquid Chromatography–Tandem Mass Spectrometry Using a Stable Isotope Labeling Strategy

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An accurate and sensitive liquid chromatography–tandem mass spectrometry method was developed for the analysis of amino acids (isoleucine, leucine, valine, tyrosine, phenylalanine and tryptophan) in serum samples using a stable isotope labeling strategy. Amino acid samples and standards were, respectively, derivatized by 10-methyl-acridone-2-sulfonyl chloride (d_0-MASC) and its deuterated counterpart d_3-MASC to form isotopic pairs which co-eluted and were detected by an MS detector at the same time. Accurate internal standard-based quantification was thereby achieved without the use of any internal standard analogy. The labeling reaction of MASC with amino acids is fast, simple and robust. Besides, derivatization increased the molecular weight of amino acids, and therefore they were shifted out of the background noise which was often observed in low mass region. The instrument LODs were in the range of 1.0–2.5 nmol/L. Linearities calculated by comparing theoretical peak area ratios of d_0-/d_3-MASC derivatives with the experimental peak area ratios were excellent with correlation coefficients of >0.995. The proposed method was successfully applied to the analysis of amino acids in serum samples with high sensitivity and accuracy.

Introduction

Amino acids, the primary components of proteins, are very important for biological processes such as protein synthesis and metabolic pathways (1, 2). The imbalance of aromatic amino acids (AAAs, phenylalanine and tyrosine) and branched chain amino acids (BCAAs, leucine, isoleucine and valine) in blood often indicates some disorders (3–6). For example, the metabolic disorders of AAAs are related to many kinds of diseases such as mental disorders, liver disease, kidney disease and immune system diseases (7). The decreased BCAA levels in plasma are often related to patients with liver cirrhosis (3), while liver insufficiency often has great correlations with the BCAA–AAA ratio (3, 5). To obtain better diagnosis and monitoring of these disorders, accurate analysis of these amino acids in blood is of great importance.

Because of the absence of a strong chromophore or fluorophore in most amino acids, there is rare direct ultraviolet (UV) or fluorescence (FL) detection of these compounds (8). Pre- or post-column derivatization of amino acids followed by UV or FL detection became one of the most convenient and widespread analytical approaches to enhance the sensitivity (9, 10). However, due to the complex property of blood samples, interferences were often observed in liquid chromatography analysis. The liquid chromatography–mass spectrometry (LC–MS) method provides a more specific approach, but ionization differences caused by matrix effects may also greatly influence the accuracy. It is desirable to use internal standard to every amino acid of interest. However, it will be quite expensive and not practical to synthesize internal standard to every amino acid. In recent years, a new strategy applying stable isotope labeling has provided an internal standard-based quantification method without the need of using internal standard to every target compound. Samples and standards are simultaneously derivatized with light and heavy isotope-coded labeling reagents, respectively. Then the differentially labeled samples and standards are mixed and analyzed by LC–MS. The isotopic pairs of the labeled analytes coelute within a single run and have identical retention times (Figure 1) (11–14). Matrix effects and ionization differences are overcome by the coelution of analytes and the standards at the same time.

In this article, we report a sensitive and reliable method for the accurate determination of amino acids in serum samples. Differential isotope labeling of amino acids with isotope-coded MASC provided isotopic variants which coeluted on a reversed-phase column. Matrix effects and run-to-run ionization differences which were often encountered in direct LC–MS analysis were greatly reduced by the application of deuterated internal standard generated through derivatization. The hydrophobicity of amino acids was increased through derivatization with MASC, and therefore they can be better retained on the reversed-phase columns. Besides, derivatization increased the molecular weight of amino acids, and therefore they were shifted out of the background noise which was often observed in low mass region. The proposed method was successfully applied to the analysis of amino acids in serum samples.

Experimental

Reagents and chemicals

Analytical standards of amino acids including isoleucine (Ile), leucine (Leu), valine (Val), tyrosine (Tyr), phenylalanine (Phe) and tryptophan (Trp) were all obtained from BDH (UK) with purity higher than 99%. Acetonitrile was of HPLC grade and purchased from Sigma-Aldrich (USA). Pure distilled water was purchased from Watsons (Guangzhou, China). All other reagents used were of HPLC grade or at least of analytical grade. Individual stock solutions of 0.1 mol/L for all compounds were prepared in

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water, and if necessary, 6 mol/L HCl and stored at 4°C in the dark. Standard solutions containing all compounds were mixed and diluted with water, and working solutions of all compounds and calibration concentrations were prepared by appropriate dilution of the stock solutions on the day of analysis.

d_0-MASC or d_3-MASC were synthesized in authors' laboratory according to the method described in our previous work by Zhang *et al.* (15). The derivatizing reagent solution (1.0×10^{-3} mol/L) was prepared by dissolving 3.1 mg MASC in 10 mL of anhydrous acetonitrile. When not in use, all reagent solutions were stored at 4°C.

Extraction of amino acids from serum samples

Serum samples were obtained from Qufu People's Hospital. Serums were collected and stored at 4°C after centrifuging. An aliquot of 200 μL of serum was added into a 5 mL glass centrifuge tube. A 0.8 mL of acetonitrile was then added to precipitate protein. After vortexing for 1 min, samples were centrifuged to remove the precipitation. Then, 100 μL of the serum extraction was added into a 2-mL vial and derivatized by the same procedure described above.

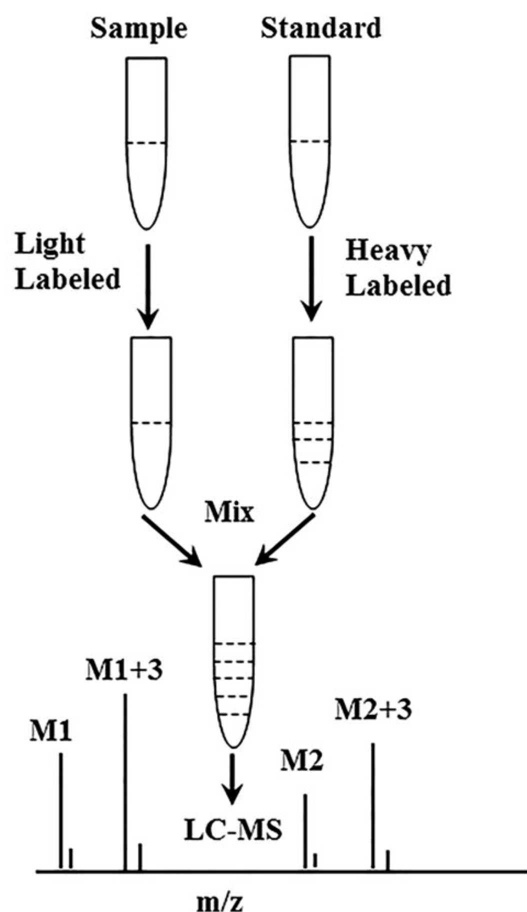

Figure 1. Quantification strategy of stable isotopic labeling.

Derivatization of amino acids

The derivatization of amino acids with MASC proceeded in basic conditions (see Figure 2). To a solution containing an appropriate amount of standard in a 2-mL vial, 50 μL 0.1 M borate buffer (pH 8), 100 μL acetonitrile and 50 μL d_0-MASC or d_3-MASC acetonitrile solution were added. The vial was sealed and vortexed for 1 min, then it was allowed to react at 60°C for 5 min in a water bath. After the reaction was completed, the mixture was cooled to room temperature. A 20 μL 50% (v/v) acetic acid solution was added to adjust pH to <7.0. The derivatized sample solution was then diluted to 500 μL with water–acetonitrile (1:1, v/v).

HPLC–MS-MS analysis

HPLC–MS-MS analysis was carried out by an Agilent 1290 series HPLC system coupled with an Agilent 6460 Triple Quadrupole MS-MS system (Agilent, USA) equipped with an Agilent Jet Stream electrospray ionization (ESI) source. HPLC separation was achieved using a SB C18 column (2.1 × 50 mm, 1.8 μm i.d., Agilent, USA). Eluent A was 0.1% (v/v) formic acid in 5% (v/v) acetonitrile and B was 0.1% (v/v) formic acid in acetonitrile. The flow rate was 0.2 mL/min and the column temperature was kept at 30°C. The elution conditions were as follows: 10–23% B from 0 to 5 min, 23–95% B from 5 to 10 min. The injection volume was 2 μL. The mass spectrometer was operated in a positive ion mode for the monitoring of $[M + H]^+$. The optimal ESI source conditions were as follows: capillary voltage +4.0 kV; nebulizer 40 psi; dry gas 11.0 L/min; dry temperature 300°C; sheath gas temperature 280°C; sheath gas flow 10 L/min. The multiple reaction monitoring (MRM) parameters of the target compounds are listed in Table I.

Method validation

The proposed method was validated in terms of linearity, limit of detection (LOD), limit of quantitation (LOQ) and accuracy. Linearities of d_0-MASC and d_3-MASC derivatives were, respectively, studied by plotting peak areas versus concentrations (10–200 nmol/L). The linearities of the stable isotope labeling method were then evaluated by comparing the theoretical peak area ratios of d_0-/d_3-MASC derivatives with the experimental

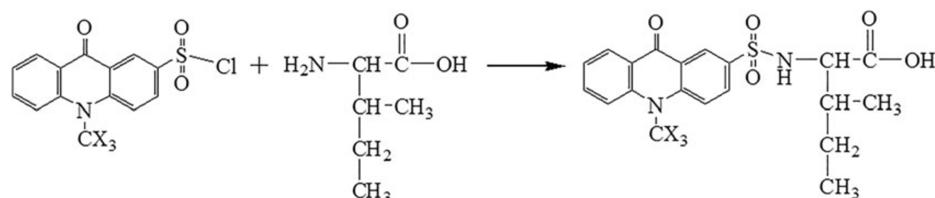

Figure 2. Derivatization scheme of Ile with MASC. X = H or D.

peak area ratios. LODs and LOQs for all target compounds were calculated at a signal-to-noise (S/N) ratio of 3 and 10, respectively. Method accuracy was evaluated by recoveries which were carried out by spiking blank samples with three different concentrations of standard solutions. Intra-day precision was determined by analyzing samples spiked at the same three levels of standards with six replicates, and inter-day precision was determined by running samples with spiked standards at the same levels with three replicates on three different days over a period of 1 week.

Results

Stability of MASC and MASC-AA derivatives

Acetonitrile solution of MASC could be stored at room temperature (25°C) for 1 week without significant decrease in derivatization yields for amino acids compared with those obtained by newly prepared MASC solution. When placed at 4°C, it could be stable for 1 month with peak area deviations of <5% for the derivatized analytes. The stabilities of the corresponding derivatives were also investigated. These solutions were repeatedly analyzed by LC–MS-MS after being placed at room temperature for 0, 4, 8, 12, 24, 48, 72 and 96 h, respectively. The corresponding derivatives were stable with peak area deviations (RSDs) of <4.6%. Therefore, the stability of MASC and MASC derivatives were sufficient for the HPLC analysis.

HPLC separation of amino acids

Ile and Leu are isomers differing only in the iso-butyl and sec-butyl chains. It is difficult to separate the two isomers in a short time, and many studies choose one of them for analysis or use a relative long separation time to fulfill complete separation of them (16, 17). In this study, we found Ile and Leu could be separated by slow gradient elution (25 min). The peak intensities of Ile and Leu were almost the same with peak area deviations of <5%. Since the total amount of BCAA is usually applied in the analysis of disorders, sum areas of Ile and Leu can be accepted for the quantification of the isomers due to the similar mass responses of them. To achieve quick analysis of amino acids, separation of Ile and Leu was not pursued and the analysis time was reduced from 25 to 10 min. Sum areas of Ile and Leu were applied for quantification, and the responses were in good linearity with the concentrations. The same strategy was also applied by other authors (18).

Table I
The MRM Parameters (Agilent 6460)

Compound	Precursor ion		Product ion				Fragmentor (V)	CE[a] (V)
	Light	Heavy	Light		Heavy			
Ile	403.1	406.1	208.1	357.0	211.1	360.0	135	45
Leu	403.1	406.1	208.1	357.0	211.1	360.0	135	45
Val	389.1	392.1	208.1	343.0	211.1	346.0	125	30
Phe	437.1	440.1	208.1	391.1	211.1	394.1	130	30
Tyr	453.1	456.1	209.1	407.1	212.1	410.1	140	40
Trp	476.1	479.1	208.1	430.1	211.1	433.1	135	35

[a]CE for quantitative ions.

ESI–MS-MS analysis of amino acids

Ions common to most d_0-MASC-AA derivatives were at m/z 208.1 and 224.1. These ions were derived from the d_0-MASC moiety. Correspondingly, for the d_3-MASC labeled amino acids, 211.1 and 227.1 were common ions derived from the d_3-MASC moiety. An exception was observed for Tyr, whose representative ion was 209.1, but not 208.1. Its corresponding deuterated counterpart was 212.1. This might be caused by the addition of a H atom into the MASC moiety. Besides ions representing the derivatizing reagent moiety, analyte-specific fragment ions were also observed. $[M-45]^+$ was observed in the product ion spectrum of all the MASC-AA derivatives. For example, the molecular weight of Ile was 403.1, and its fragment ion was 357.1. We propose that this ion is formed by the loss of the carboxyl group from the precursor ion (Figure 3). The precursor and product ions of all MASC-AA derivatives are listed in Table I.

Absolute quantification of the amino acids

LC–MS is popular in absolute quantification due to its high sensitivity and specificity. However, endogenous matrix components may coelute with the analytes of interest and thus affect the reproducibility and accuracy of the method (19–21). The stable isotope labeling method provides a good strategy to overcome matrix effects. Standards and samples were, respectively, labeled by heavy and light versions of derivatizing reagents.

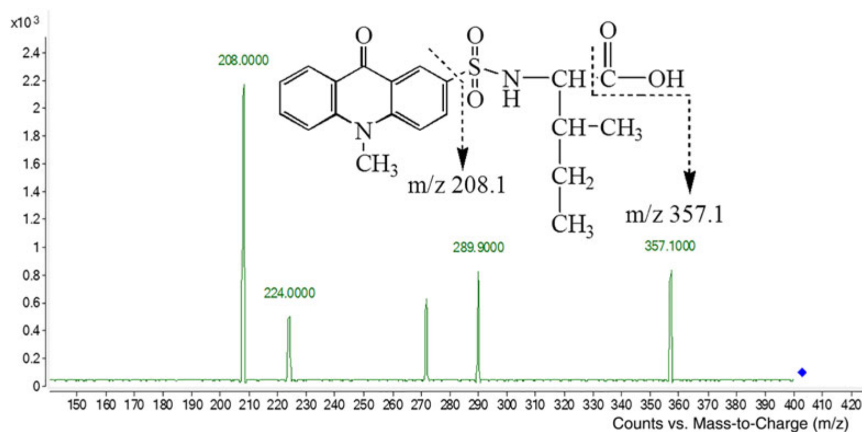

Figure 3. Spectrum of the product ions of Ile. This figure is available in black and white in print and in color at *JCS* online.

After the completion of the labeling reaction, they were then mixed and analyzed by LC–MS. Standards and sample derivatives co-eluted in a single run and experienced the same ionization process. Thus, matrix effects observed for the light-labeled analytes were expected to be identical with the heavy-labeled standards.

The linearities of d_0-MASC and d_3-MASC labeled amino acids were first studied to guarantee the reliability of the stable isotope labeling strategy. The results indicated that both d_0-MASC and d_3-MASC derivatives were in good linearity with correlation coefficients higher than 0.996. d_0- and d_3-labeled amino acid standards were then mixed in ratios of 1:20, 1:10, 1:5, 1:1, 5:1, 10:1 and 20:1 in aqueous solution. The standard mixtures were then injected into the column followed by ESI–MS-MS analysis, and the resulting MRM chromatograms were used for evaluation. As shown in Figure 4, the d_0- and d_3-labeled standards had exactly the same retention times. Linearities calculated by comparing theoretical peak area ratios of d_0-/d_3-MASC derivatives with the experimental peak area ratios were excellent with correlation coefficients of >0.995 (Table II), indicating the robustness of the labeling strategy. Thus, amino acids can be quantitatively and reproducibly analyzed by this strategy.

Method validation

Parameters including LOD, LOQ, precision and trueness were validated according to the procedures described in the 'Experimental' section. As shown in Table II, the method is sensitive with instrument LODs ranging from 1.0 to 2.5 nmol/L, and LOQs ranging from 3.0 to 7.0 nmol/L. According to the usual concentrations of amino acids in serum samples, recoveries were determined by spiking 30, 50 and 100 μmol/L of standards in serum samples. The spiked samples experienced the whole sample pretreatment procedures and light labeled by d_0-MASC, while the standards were heavy labeled by d_3-MASC. Then the spiked samples and standards were mixed and determined by LC–MS-MS. Based on the formula of (measured value – endogenous value)/added value × 100, the recoveries were between 90 and 96% for all amino acids. The results are listed in Table II. The intra-day precisions for the tested samples were in the range of 3.2–6.0%, while the inter-day precisions were between 5.9 and 8.6%.

Application

The proposed method was applied to the analysis of amino acids in the serum of healthy people and hepatitis patients. Serum samples were light labeled by d_0-MASC, while the standards were heavy labeled by d_3-MASC. Then samples and standards were mixed and determined by ESI–MS-MS in MRM mode. The results are listed in Table III. The BCAA/AAA ratios were calculated and compared to study the differences between healthy people and hepatitis patients. As shown in Table III, the BCAA/AAA molar ratios in hepatitis patients were lower than those in healthy people. Although differences between individuals may exist, the molar ratio of BCAA/AAA can be used, to some extent, as an indicator of the damage of liver.

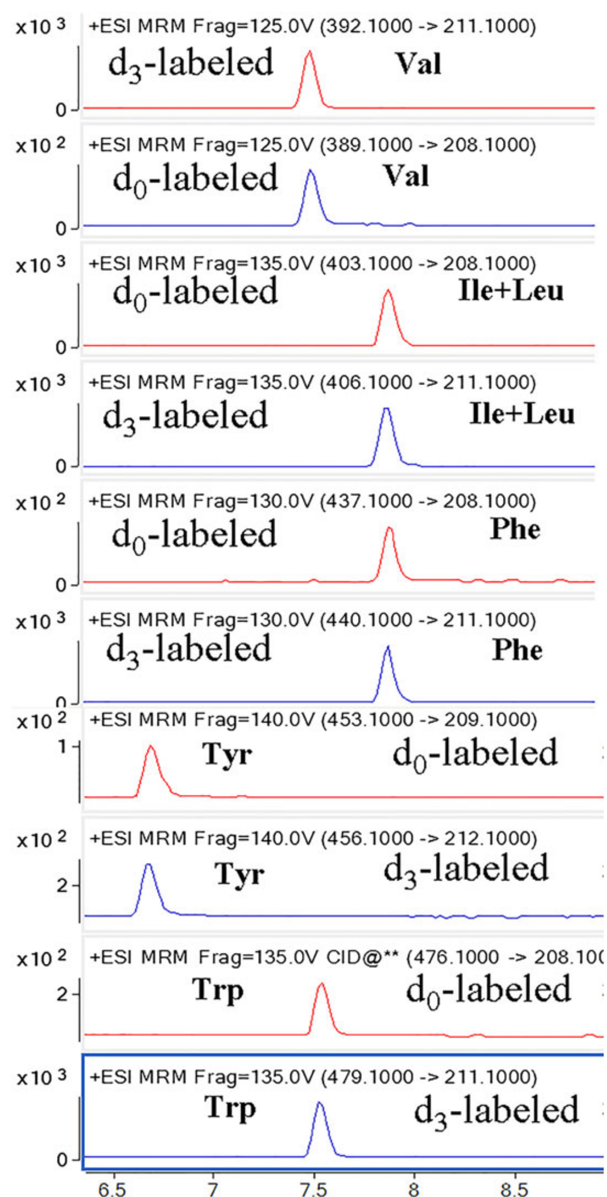

Figure 4. Extracted ion chromatogram of d_0- and d_3-labeled amino acids. This figure is available in black and white in print and in color at *JCS* online.

Table II
Method Linearity, Dynamical Range, LOD and LOQ

Analyte	Linearity	R^2	Dynamical ratio range	LOD (nmol/L)	LOQ (nmol/L)
Ile + Leu	$Y = 0.9661X^a$	0.9996	20:1–1:20	1.0	3.0
Val	$Y = 1.0687X$	0.9952	20:1–1:20	2.0	6.0
Phe	$Y = 1.0287X$	0.9952	20:1–1:20	1.5	4.5
Tyr	$Y = 0.8524X$	0.9978	20:1–1:20	2.5	7.0
Trp	$Y = 0.9523X$	0.9952	20:1–1:20	2.2	6.5

[a]*X*, theoretic concentration ratio; *Y*, experimental mass spectrometric peak intensity ratio.

Discussion

Effect of borate buffer on derivatization

Derivatization of amino acids with MASC proceeded in basic conditions. Sodium bicarbonate buffer and borate buffer were

Table III
Determined Values of Amino Acids in Serum Samples

Samples	Concentration μmol/L					BCAA/AAA[a]
	Ile + Leu	Val	Phe	Tyr	Trp	
Hepatopath 2	112 ± 5.5	300 ± 9.8	111 ± 5.0	83.1 ± 4.0	82.0 ± 3.7	1.49
Hepatopath 2	131 ± 5.8	314 ± 10	112 ± 4.8	76.5 ± 3.9	82.2 ± 3.3	1.64
Health people 1	154 ± 6.2	375 ± 15	106 ± 4.6	37.8 ± 1.6	72.3 ± 2.9	2.45
Health people 2	143 ± 5.7	321 ± 12	105 ± 4.1	44.6 ± 2.3	56.5 ± 2.7	2.25

[a]BCAA/AAA molar ratio.

compared in this study for derivatization. The results indicated that borate buffer was superior to sodium bicarbonate. The effect of pH on the derivatization was then evaluated with borate buffer (0.1 M) in the pH range of 7.5–11.0. Maximum responses of most AA derivatives were obtained in the pH range of 8.0–8.5, while for Tyr, maximum response was obtained at pH 10. Under this condition, the responses of other compounds were decreased by almost 50%. To guarantee the maximum derivatization yields for most amino acids, 0.1 mol/L borate buffer with pH of 8.0 was applied in subsequent derivatization. Under this condition, Tyr mainly existed in its mono-substituted form. This provided great convenience for the quantification.

Effect of MASC concentration on derivatization

The concentration of derivatizing reagent is a vital factor in derivatization. To achieve accurate quantification of the target compound, sufficient reaction of the analytes should be guaranteed. In this study, the effects of MASC concentrations on derivatization were studied in detail. The results indicated that constant MS response was achieved with the addition of a 7-fold molar reagent excess to total molar amino acids. Further increasing the excess of reagent beyond this level had no significant effects on derivatization yields. For the convenience of operation, 1.0×10^{-3} mol/L of MASC was applied in derivatization. This concentration was enough for daily analysis because the contents of amino acids in most serum samples were below this level, and serum samples were usually diluted tens of times before derivatization.

The effect of reaction temperature on derivatization was also evaluated. Derivatization of MASC with amino acids could be finished within 5 min at 60°C. Derivatization yields were constant in the temperature range of 55–70°C. Lower temperature needs longer reaction time, while temperature of higher than 70°C leads to decrease in response. That is probably due to the hydrolysis of the derivatives at high temperature. Thus, 1.0×10^{-3} mol/L of MASC and 60°C were employed for derivatization.

Conclusion

A sensitive and reliable stable isotope labeling method was developed for the analysis of amino acids in serum samples. The labeling reaction of amino acids with MASC can be finished within 5 min under milder conditions. The stable isotope labeling strategy made internal standard-based quantification of amino acids become possible without the need of using expensive internal standard analogy to every analyte of interests. Matrix effects and run-to-run ionization differences, which were frequently observed in LC–MS analysis, were greatly reduced by the coelution of the analytes and standards. The shortcoming of eluting in the low mass region was overcome by derivatization. That was because the molecular weight of the introduced MASC moiety was about two times of those of amino acids. The utility of the method was well validated in the analysis of amino acids in serum samples.

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References

1. Vatanavicharn, N., Ratanarak, P., Liammongkolkul, S., Sathienkijkanchai, A., Wasant, P.; Amino acid disorders detected by quantitative amino acid HPLC analysis in Thailand: an eight-year experience; *Clinica Chimica Acta*, (2012); 413: 1141–1144.
2. Buha, S.M., Panchal, A., Panchal, H., Chambhare, R., Kumar, S., Jain, M., *et al.*; HPLC–FLD for the simultaneous determination of primary and secondary amino acids from complex biological sample by pre-column derivatization; *Journal of Chromatographic Science*, (2011); 49: 118–123.
3. Nishitani, S., Ijichi, C., Takehana, K., Fujitani, S., Sonaka, I.; Pharmacological activities of branched-chain amino acids: specificity of tissue and signal transduction; *Biochemical and Biophysical Research Communications*, (2004); 313: 387–389.
4. Garibotto, G., Sofia, A., Saffioti, S., Bonanni, A., Mannucci, I., Verzola, D.; Amino acid and protein metabolism in the human kidney and in patients with chronic kidney disease; *Clinical Nutrition*, (2010); 29: 424–433.
5. Dejong, C.H.C., van de Poll, M.C.G., Soeters, P.B., Jalan, R., Damink, S.W.M.O.; Aromatic amino acid metabolism during liver failure; *The Journal of Nutrition*, (2007); 137: 1579S–1585S.
6. Schefold, J.C., Zeden, J.P., Fotopoulou, C., von Haehling, S., Pschowski, R., Hasper, D., *et al.*; Increased indoleamine 2,3-dioxygenase (IDO) activity and elevated serum levels of tryptophan catabolites in patients with chronic kidney disease: a possible link between chronic inflammation and uraemic symptoms; *Nephrology Dialysis Transplantation*, (2009); 24: 1901–1908.
7. Li, Y., Tang, A.-G., Mu, S.; HPLC–FLD determination of serum aromatic amino acids: application in chronic kidney disease patients; *Clinica Chimica Acta*, (2011); 412: 1032–1035.
8. Gatti, R., Gioia, M.G., Leoni, A., Andreani, A.; 2,5-Dimethyl-1*H*-pyrrole-3,4-dicarbaldehyde as a precolumn derivatization reagent for HPLC/UV detection of amino acids; *Journal of Pharmaceutical and Biomedical Analysis*, (2010); 53: 207–211.
9. Jámbor, A., Molnár-Perl, I.; Quantitation of amino acids in plasma by high performance liquid chromatography: simultaneous deproteinization

and derivatization with 9-fluorenylmethyloxycarbonyl chloride; *Journal of Chromatography A*, (2009); 1216: 6218–6223.
10. Iwasaki, Y., Nakano, Y., Mochizuki, K., Nomoto, M., Takahashi, Y., Ito, R., *et al.*; A new strategy for ionization enhancement by derivatization for mass spectrometry; *Journal of Chromatography B*, (2011); 879: 1159–1165.
11. Guo, K., Li, L.; Differential ^{12}C-/^{13}C-isotope dansylation labeling and fast liquid chromatography/mass spectrometry for absolute and relative quantification of the metabolome; *Analytical Chemistry*, (2009); 81: 3919–3932.
12. Guo, K., Ji, C., Li, L.; Stable-isotope dimethylation labeling combined with LC-ESI MS for quantification of amine-containing metabolites in biological samples; *Analytical Chemistry*, (2007); 79: 8631–8638.
13. Lamos, S.M., Shortreed, M.R., Frey, B.L., Belshaw, P.J., Smith, L.M.; Relative quantification of carboxylic acid metabolites by liquid chromatography-mass spectrometry using isotopic variants of cholamine; *Analytical Chemistry*, (2007); 79: 5143–5149.
14. Huang, Y.-Q., Liu, J.-Q., Gong, H., Yang, J., Li, Y., Feng, Y.-Q.; Use of isotope mass probes for metabolic analysis of the jasmonate biosynthetic pathway; *The Analyst*, (2011); 136: 1515.
15. Zhang, S., You, J., Ning, S., Song, C., Suo, Y.-R.; Analysis of estrogenic compounds in environmental and biological samples by liquid chromatography–tandem mass spectrometry with stable isotope-coded ionization-enhancing reagent; *Journal of Chromatography A*, (2013); 1280: 84–91.
16. Bosch, L., Alegría, A., Farré, R.; Application of the 6-aminoquinolyl-*N*-hydroxysccinimidyl carbamate (AQC) reagent to the RP-HPLC determination of amino acids in infant foods; *Journal of Chromatography B*, (2006); 831: 176–183.
17. Johnson, D.W.; Free amino acid quantification by LC–MS/MS using derivatization generated isotope-labelled standards; *Journal of Chromatography B*, (2011); 879: 1345–1352.
18. Calderón-Santiago, M., Priego-Capote, F., Galache-Osuna, J.G., Luque de Castro, M.D.; Determination of essential amino acids in human serum by a targeting method based on automated SPE–LC–MS/MS: discrimination between artherosclerotic patients; *Journal of Pharmaceutical and Biomedical Analysis*, (2012); 70: 476–484.
19. Taylor, P.J.; Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry; *Clinical Biochemistry*, (2005); 38: 328–334.
20. Van De Steene, J.C., Mortier, K.A., Lambert, W.E.; Tackling matrix effects during development of a liquid chromatographic–electrospray ionisation tandem mass spectrometric analysis of nine basic pharmaceuticals in aqueous environmental samples; *Journal of Chromatography A*, (2006); 1123: 71–81.
21. Jiang, H., Cao, H., Zhang, Y., Fast, D.M.; Systematic evaluation of supported liquid extraction in reducing matrix effect and improving extraction efficiency in LC–MS/MS based bioanalysis for 10 model pharmaceutical compounds; *Journal of Chromatography B*, (2012); 891–892: 71–80.