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Direct Amino Acids Analysis in Biological Fluids by Mixed-Mode LC-MS/MS

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ABSTRACT

Amino acids determination in biological fluids at the µmol/l level is of great importance for characterization of metabolism and diagnosis of several pathologies. Usually, amino acids are determined by RPLC-MS/MS after off-line derivatization. This method suffers of major drawbacks: off-line sample preparation, analysis time and high cost per sample.

We propose a new, fast and direct "dilute and shoot" LC-MS/MS method using triple-mode chromatographic separation involving mixed anion exchange, cation exchange and reverse phase interactions.

This new method allows chromatographic resolution of isobaric compounds without adding ion pairing agent and quantification of a large panel of amino acids in plasma for clinical research.

INTRODUCTION

There has been an increasing interest for the analysis of amino acids and other amino-compounds in plasma samples and other biological fluids for clinical research. A large panel of compounds in a single chromatographic run is usually required. This type of analysis has been performed for a long time with HPLC methods based on the post-column derivatization of compounds with ninhydrin (for UV-visible detection) or with o-phtalaldehyde (for fluorescent detection). These approaches require long chromatographic runs and very controlled conditions to achieve a robust method.

Liquid chromatography coupled to mass spectrometry (LC-MS) represents a good option for this kind of analysis. Considering that amino acids are quite polar, a direct reverse phase chromatographic approach is not appropriate because of lack of retention and because separation of some important isomers might be difficult to achieve. Another option would be the use of ion chromatography, but this would require a specific front-end that should be compatible with mass spectrometric detection. For reverse phase approaches, two main options are available: the use of ion pairing reagents or the derivatization of compounds before LC-MS/MS injection. In the first approach, the use of ion pairing agents and their continuous injection onto the MS system could contaminate quickly the ion source; moreover, the robustness of the method could be an issue if the ion pairing is not performed properly, and the method is dependent on the matrix of the sample that might contain a more or less important amount of ionic species in it. As for the second approach, based on derivatization, this adds a step to the sample preparation procedure resulting in a tedious time-consuming, and less robust method.

The objective of this work was to develop and apply a method for the analysis of a panel of 52 amino acids by LC-MS/MS without the use of ion pairing agent and without the need for derivatization of the sample.

MATERIALS AND METHODS

Target Analytes

A panel of 52 amino acids and related compounds were analyzed. The chemical structures of the compounds are presented in Figure 1. Twenty-five internal standards were used for quantitation.

Calibration Standards and Control Samples

Calibration solutions were prepared after appropriate dilution in pure water of HLC 0.1M working solutions. For the working solution preparation, pure solutions of asparagine, glutamine, tryptophan, pipecolic acid, allo-isoleucine, sulfocysteine and homocysteine (Sigma Aldrich) were used as well as two prepared mixtures, Amino Acids Mixture Standard Solution, Type B and Type ANII (Wako Pure Chemical Industries).

An internal standard solution was prepared as well for sample dilution. This solution was obtained by diluting the Metabolomics Amino Acid Mix Standard (Cambridge Isotopes), and pure solutions of taurine $^{13}C_2{}^{15}N$, citrulline $^{13}CD_4$, tryptophan $^{13}C_{11}{}^{15}N_2$, asparagine $^{13}C_4D_3{}^{15}N_2$, pipecolic acid $^{13}C_6{}^{15}N$, glutamine $^{13}C_5D_5{}^{15}N$, gamma aminobutyric acid $^{13}C_4$ (Eurisotop), and ornithine D_6 (Sigma Aldrich), with mobile phase A.

Two quality control levels were used for the analytical validation of the method performed for 27 compounds. The controls consisted in Level 1 (L1) and Level 2 (L2) plasma controls from ERNDIM with the following lot numbers: Lot 2017.0061 and Lot 2017.0062, respectively.

Sample Preparation

One hundred µL of each calibrator, quality control and plasma donor sample were extracted by protein precipitation in a 1.5 mL Eppendorf tube using 10 µL of a 30% solution of sulfosalicylic acid. Precipitated samples were vortex mixed for 30 seconds, refrigerated at 4° C for 30 minutes, and centrifuged at 12000 rpm for 5 minutes. Fifty microliters of supernatant were vortex mixed for 30 seconds with 450 µL of internal standard solution in 100% mobile phase A, and 4µL of this final solution were injected into the LC-MS/MS system.

Liquid Chromatography

A chromatographic method of 18 minutes was used for the analysis of the amino acids using a Thermo Scientific™ UltiMate™ 3000 RS system consisting of an HPG pump, a column oven and an autosampler. The separation was performed on a mixed mode Thermo Scientific™ Acclaim™ Trinity column at 30° C. Mobile phases consisted of ammonium formate in water at pH 2.8 for phase A, and 80% of ammonium formate in water and 20% of acetonitrile for phase B. Chromatographic separation was achieved by gradient elution under the conditions described in Table 1.

Mass Spectrometry

Compounds were detected on a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer equipped with an Thermo Scientific™ EASY-Max NG™ ion source with a heated electrospray ionization probe. Sheath gas was set at 45 arbitrary units, auxiliary gas at 15 arbitrary units, spray voltage at 3500 V for positive ionization and at 2700 V for negative ionization. Vaporizer temperature was fixed at 370° C and transfer tube temperature at 270° C, while source fragmentation was applied at 15V. Data were acquired in Single Reaction Monitoring (SRM) mode using a resolution of 0.7 m/z Full Width at Half Maximum (FWHM) for both quadrupoles with a 400 ms cycle time. The SRM transitions used for this method are presented in Table 2.

Method Evaluation

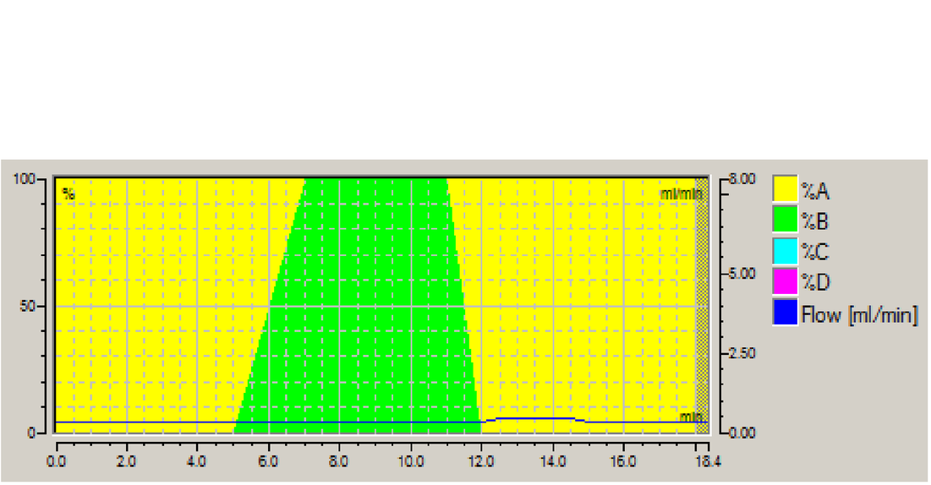
The Limit of Quantification (LOQ) for each analyte was determined as the lowest value in the calibration curve giving an average % bias between nominal and back-calculated concentration within ±20% and a %CV below 20% on 10 replicate injections of calibrators.

Intra and inter-assay precision were performed for the 27 compounds that are present in the AMI-02.1 and AMI02.2 plasma control material from ERNDIM (Lot 2017.0061 and Lot 2017.0062). For intra-assay precision, the controls were prepared and analyzed 30 times in the same day. For inter-assay precision, they were prepared 30 times in different days.

Data Analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software.

Table 1. Gradient profile.



Time (minutes)	Flow rate (mL/min)	%A	%B
0	0.300	100	0
5	0.300	100	0
7	0.300	0	100
11	0.300	0	100
12	0.300	100	0
12.5	0.450	100	0
14.5	0.450	100	0
15	0.300	100	0
18	0.300	100	0

Figure 1. Chemical structures of the analyzed compounds.

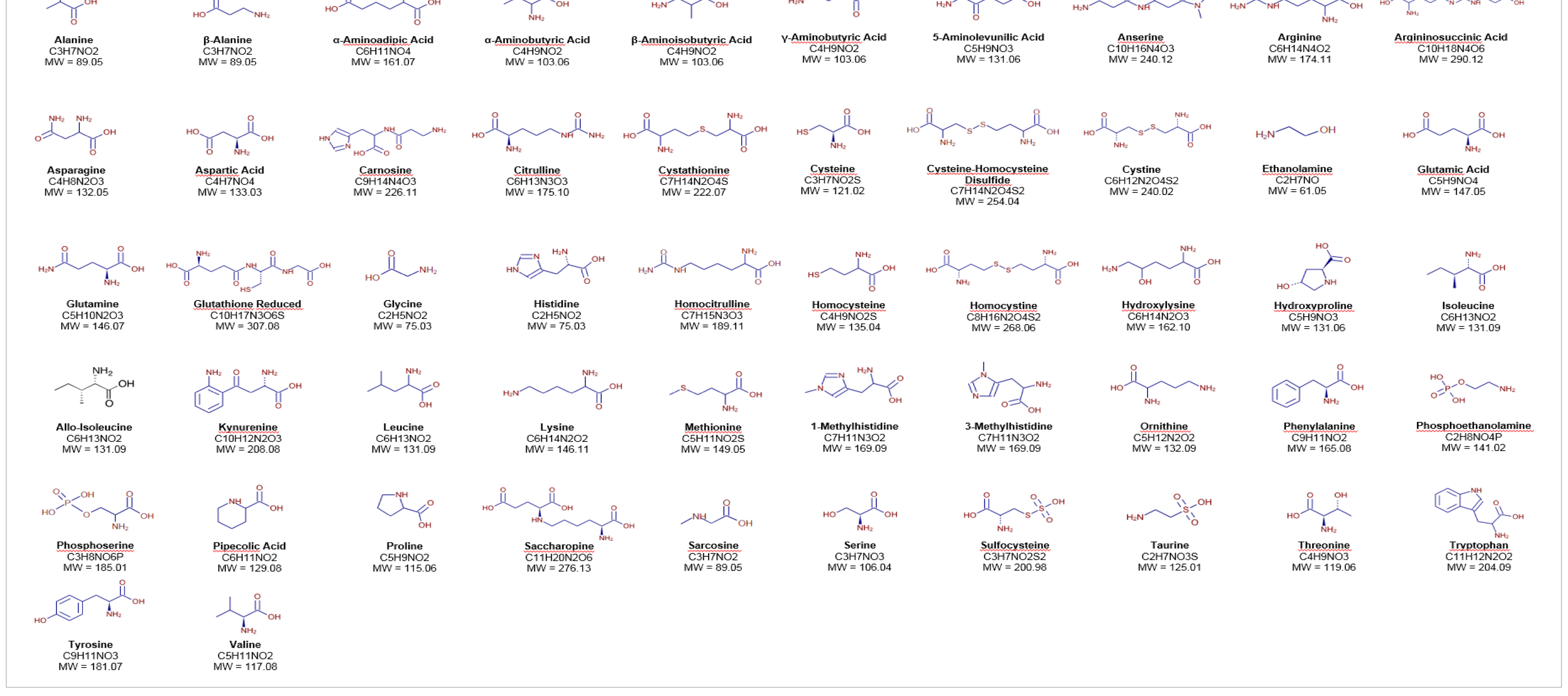


Table 2. SRM transitions.

Compound	Retention Time (min)	RT Window (min)	Polarity	Precursor (m/z)	Product (m/z)	CE (V)	RF Lens (V)	QUAN
Alanine & sarcosine	2.04	4	Positive	90.274	44.44	10.253	56	X
Alanine & sarcosine	2.04	4	Positive	90.274	90.274	5	56	X
Alanine $^{13}C_3$ ^{15}N	2.11	4	Positive	94.243	47.444	10.253	48	X
β-Alanine	3.7	4	Positive	90.252	30.67	10.25	46	
β-Alanine	3.7	4	Positive	90.252	72.21	10	46	X
α-aminoadipic acid	2.2	4	Positive	162.152	98.22	15.764	72	X
α-aminoadipic acid	2.2	4	Positive	162.152	144.11	10.253	72	
α-aminobutyric acid	2.2	4	Positive	104.213	41.5	20.315	47	
α-aminobutyric acid	2.2	4	Positive	104.213	58.33	10.253	47	X
β-aminoisobutyric acid	3.91	4	Positive	104.252	57	20	51	
β- & γ-aminoisobutyric acid	4.05	4	Positive	104.252	86.15	10.253	51	X
γ-aminobutyric acid	4.47	4	Positive	104.252	69.21	14.803	51	
γ-aminobutyric acid	4.47	4	Positive	104.252	87.15	10.253	51	X
γ-Aminobutyric acid $^{13}C_4$	4.47	4	Positive	108.23	91.151	10.253	57	
5-aminolevulinic acid	4.5	4	Positive	132.183	68.26	17.787	67	
5-aminolevulinic acid	4.5	4	Positive	132.183	114.18	10.253	67	X
Anserine	11.82	4	Positive	241	109	20	90	X
Arginine	13.5	3	Positive	175.12	70.26	21.68	83	X
Arginine	13.5	3	Positive	175.12	116.11	13.64	83	
Arginine $^{13}C_6$ $^{15}N_4$	13.5	3	Positive	185.213	75.28	23.601	92	X
Argininosuccinic acid	9.5	4	Positive	291.183	70.29	30.933	151	X
Argininosuccinic acid	9.5	4	Positive	291.183	116.11	19.809	151	
Asparagine	1.87	3	Positive	133.2	74.22	16	60	
Asparagine	1.87	3	Positive	133.2	87.15	10	60	X
Asparagine $^{13}C_4$ D_3 $^{15}N_2$	1.87	3	Positive	142.183	95.222	10	68	X
Aspartic acid	2.12	4	Positive	134.11	74.22	15	65	X
Aspartic acid	2.12	4	Positive	134.11	88.17	10	65	
Aspartic acid $^{13}C_4$ $^{15}N_1$	2.12	4	Positive	139.243	92.222	10.253	59	X
Carnosine	12.13	3	Positive	227.183	110.17	22.4	95	X
Carnosine	12.13	3	Positive	227.183	210.04	11.1	95	
Citrulline	2.33	4	Positive	176.213	70.33	22.337	71	
Citrulline	2.33	4	Positive	176.213	159.04	10.253	71	X
Citrulline ^{13}C D_4	2.33	4	Positive	181.2	164.111	10.253	73	X
Cystathionine	8.2	4	Positive	223.152	88.17	25.573	91	
Cystathionine	8.2	4	Positive	223.152	134.11	13.843	91	X
Cysteine	4.93	6	Positive	122.131	59.28	21.124	48	X
Cysteine	4.93	6	Positive	122.131	76.22	12.225	48	
Cysteine-homocysteine disulfide	7	14	Positive	255	134	20	100	X
Cystine	6	4	Positive	241.1	74.2	27	91	
Cystine	6	4	Positive	241.1	152	13	91	X
Cystine $^{13}C_6$ $^{15}N_2$	6	4	Positive	249.091	156	13.893	98	
Ethanolamine	3.77	4	Positive	62.365	44.44	10.253	51	
Ethanolamine	3.77	4	Positive	62.365	62.37	5	51	X
Glutamic acid	2.08	4	Positive	148.1	84.15	14.955	62	X
Glutamic acid	2.08	4	Positive	148.1	130.11	10.25	62	
Glutamic acid $^{13}C_5$ $^{15}N_1$	2.08	4	Positive	154.183	136.111	10.253	68	X
Glutamine	1.91	3	Positive	147.12	84.15	16.371	64	
Glutamine	1.91	3	Positive	147.12	130.04	10.253	64	X
Glutamine $^{13}C_5$ D_5 $^{15}N_2$	1.91	3	Positive	159.191	141.04	10	71	X
Glutathione reduced	7	14	Positive	308.335	162	16.219	110	
Glutathione reduced	7	14	Positive	308.335	179	11.871	110	X
Glycine	2.04	4	Positive	76.3	30.5	10	46	
Glycine	2.04	4	Positive	76.3	76.3	5	46	X
Glycine $^{13}C_2$ $^{15}N_1$	2.04	4	Positive	79.274	32.556	10.253	41	
Glycine $^{13}C_2$ $^{15}N_1$	2.04	4	Positive	79.274	79.274	5	41	X
Histidine	10.89	4	Positive	156.13	83.22	23.298	73	
Histidine	10.89	4	Positive	156.13	110.1	13.034	73	X
Histidine $^{13}C_6$ $^{15}N_3$	10.89	4	Positive	165.183	118.111	14.601	81	X
Homocitruline	3.2	4	Positive	190.098	127.11	15.562	75	
Homocitruline	3.2	4	Positive	190.098	173.06	10.253	75	X
Homocysteine	3.5	4	Positive	136.13	56.33	17.584	61	
Homocysteine	3.5	4	Positive	136.13	90.17	10.253	61	X
Homocystine	9.57	4	Positive	269.152	88.22	30.326	83	
Homocystine	9.57	4	Positive	269.152	136.04	10.253	83	X
Hydroxylysine	10.31	4	Positive	163	82.	20	65	
Hydroxylysine	10.31	4	Positive	163	128	13	65	X
Hydroxyproline	1.77	3	Positive	132.04	68.28	18.191	65	
Hydroxyproline	1.77	3	Positive	132.04	86.22	11.163	65	X
Kynurenine	9.4	4	Positive	209.183	146.04	18.494	82	
Kynurenine	9.4	4	Positive	209.183	192.06	10.253	82	X
Isoleucine & allo-isoleucine	2.91	5	Positive	132.18	69.26	16.017	55	X
Isoleucine $^{13}C_6$ $^{15}N_1$	3.01	4	Positive	139.243	74.262	17.685	59	X
Isoleucine $^{13}C_6{}^{15}N_1$ & Leucine $^{13}C_6{}^{15}N_1$	3.01	5	Positive	139.243	92.222	10.253	59	X
Leucine	3.15	4	Positive	132.18	43.44	24.157	55	
Leucine, isoleucine & allo-isoleucine	3.01	5	Positive	132.18	86.22	10.253	55	X
Leucine $^{13}C_6$ $^{15}N_1$	3.01	4	Positive	139.243	46.44	25	59	
Lysine	10.89	4	Positive	147.17	84.22	15.916	64	X
Lysine	10.89	4	Positive	147.17	130.11	10.25	64	
Lysine $^{13}C_6$ $^{15}N_2$	10.89	4	Positive	155.213	137.111	10.253	69	X
Methionine	2.66	4	Positive	150.098	104.1	10.253	61	
Methionine	2.66	4	Positive	150.098	133.07	10.253	61	X
Methionine $^{13}C_5$ $^{15}N_1$	2.66	4	Positive	156.152	138.058	10.253	65	X
1- and 3-Methylhistidine	10.8	4	Positive	170.12	109.17	15.258	75	
1-Methylhistidine	10.75	4	Positive	170.12	83.22	22.539	75	
1-Methylhistidine	10.75	4	Positive	170.12	124.11	14.096	75	X
3-Methylhistidine	10.92	4	Positive	170.12	126.1	12.629	75	X
Ornithine	10.48	4	Positive	133.243	70.26	16.978	62	X
Ornithine	10.48	4	Positive	133.243	116.11	10.253	62	
Ornithine D_6	10.48	4	Positive	139.274	76.262	18.242	73	X
Phenylalanine	5.34	4	Positive	166.12	103.17	27.747	62	
Phenylalanine	5.34	4	Positive	166.12	120.17	11.365	62	X
Phenylalanine $^{13}C_9$ $^{15}N_1$	5.34	4	Positive	176.183	129.111	13.135	75	X
Phosphoethanolamine	1.71	3	Positive	142.07	44.44	10.253	53	X
Phosphoethanolamine	1.71	3	Negative	140.00	79	20	53	
Phosphoserine	5.04	6	Positive	186	70	15	60	
Phosphoserine	5.04	6	Positive	186	88	15	60	X
Pipecolic Acid	2.58	4	Positive	130.16	56.33	27.393	67	
Pipecolic Acid	2.58	4	Positive	130.16	84.22	15.006	67	X
Pipecolic Acid $^{13}C_6$ $^{15}N_1$	2.58	4	Positive	137.222	90.222	16.067	76	X
Proline	2.02	4	Positive	116.243	43.5	28.404	67	
Proline	2.02	4	Positive	116.243	70.26	15.006	67	X
Proline $^{13}C_5$ $^{15}N_1$	2.02	4	Positive	122.213	75.28	15.511	66	X
Saccharopine	7.5	4	Positive	277.077	84.21	23.854	105	X
Saccharopine	7.5	4	Positive	277.077	213.06	13.388	105	
Serine	1.87	3	Positive	106.191	60.33	10.253	47	X
Serine	1.87	3	Positive	106.191	88.22	10.25	47	
Serine $^{13}C_3$ $^{15}N_1$	1.87	3	Positive	110.213	63.333	10.253	52	X
Sulfocysteine	6.25	4	Positive	202.091	74.22	22.539	79	
Sulfocysteine	6.25	4	Positive	202.091	120.06	11.213	79	X
Taurine	1.65	3	Positive	126.152	108.11	10.253	82	
Taurine	1.65	3	Positive	126.152	126.15	5	82	X
Taurine $^{13}C_2$ $^{15}N_1$	1.65	3	Positive	129.13	111.04	10	81	X
Taurine $^{13}C_2$ $^{15}N_1$	1.65	3	Positive	129.13	129.13	5	81	
Threonine	1.87	3	Positive	120.16	74.28	10.253	58	X
Threonine	1.87	3	Positive	120.16	102.1	10.253	58	
Threonine $^{13}C_4$ $^{15}N_1$	1.87	3	Positive	125.213	78.222	10.253	58	X
Tryptophan	9.71	4	Positive	205.078	146.06	17.129	71	
Tryptophan	9.71	4	Positive	205.078	187.99	10.253	71	X
Tryptophan $^{13}C_{11}$ $^{15}N_2$	9.71	4	Positive	218.191	200.058	10.253	79	X
Tyrosine	4.54	4	Positive	182.078	136.04	12.427	73	X
Tyrosine	4.54	4	Positive	182.078	164.99	10.253	73	
Tyrosine $^{13}C_9$ $^{15}N_1$	4.54	4	Positive	192.183	174.04	10.253	77	X
Valine	2.36	4	Positive	118.17	55.44	19.35	45	
Valine	2.36	4	Positive	118.17	72.26	10.253	45	X
Valine $^{13}C_5$ $^{15}N_1$	2.36	4	Positive	124.243	77.28	10.253	56	X

RESULTS

Internal calibration was used for 37 compounds, 25 using the corresponding isotopically labelled internal standards. External calibration (no internal standard) was used for 5 additional analytes. Qualitative detection was achieved for the remaining compounds. Details of calibration approach, linearity range and LOQ for each analyte are reported in Table 3.

In the case of cysteine, considering the acidic conditions for the protein precipitation, only cystine is present since cysteine is completely oxidized to cystine under these conditions. Since cystine is the oxidized dimer form of cysteine, two mol of cysteine are used for the generation of one mol of cystine. Representative chromatograms at the LOQ are presented in Figure 2.

The results obtained for intra-assay accuracy and precision are presented in Table 4, and the same results for inter-assay study are presented in Table 5. Representative chromatograms for the 27 compounds in the L1 and L2 control samples are reported in Figure 3.

Calibration was performed in water in view of the fact that the studied compounds are primarily endogenous compounds. The obtained results show good accuracy for the two quality controls in plasma for the 27 studied compounds, which confirms the suitability of this quantitation approach for plasma samples. The use of internal standards corresponding to most of the compounds in the panel correct the eventual matrix effects that can be observed in the biological matrix as compared to the calibrators. For the intra-day study, the highest bias is observed for cystathionine in L1. This is probably due to matrix effects, because this compound doesn't have its own internal standard. For both intra and inter-day studies, we also observed a significant %CV for aspartic acid in L1 control. This is due to the low level of aspartic acid in L1 (12µmol/L), which is very close to the LOQ (10µmol/L).

Table 3. Calibration data.

Compound	Calibration Type	Internal Standard	Linearity Range (µmol/L)	Type	Weight	Origin
Alanine	Internal	Alanine $^{13}C_3$ $^{15}N_1$	20-500	Linear	1/X	Ignore
α-aminobutyric acid	Internal	γ-Aminobutyric ac. $^{13}C_4$	10-100	Linear	1/X	Ignore
Arginine	Internal	Arginine $^{13}C_6$ $^{15}N_4$	5-500	Linear	1/X	Ignore
Asparagine	Internal	Asparagine $^{13}C_4$ D_3 $^{15}N_2$	24-600	Linear	1/X	Ignore
Aspartic Acid	Internal	Aspartic Acid $^{13}C_4$ $^{15}N_1$	10-200	Linear	$1/X^2$	Ignore
Citrulline	Internal	Citrulline ^{13}C D_4	5-200	Linear	1/X	Ignore
Cystathionine	Internal	Phenylalanine $^{13}C_9$ $^{15}N_1$	2.5-100	Linear	1/X	Ignore
Cystine	Internal	Cystine $^{13}C_6$ $^{15}N_2$	10-500	Linear	1/X	Ignore
Glutamic Acid	Internal	Glutamic Acid $^{13}C_5$ $^{15}N_1$	5-500	Linear	1/X	Ignore
Glutamine	Internal	Glutamine $^{13}C_5$ D_5 $^{15}N_2$	10-500	Linear	1/X	Ignore
Glycine	Internal	Glycine $^{13}C_2$ $^{15}N_1$	50-500	Linear	1/X	Force
Histidine	Internal	Histidine $^{13}C_6$ $^{15}N_3$	5-500	Linear	$1/X^2$	Ignore
Hydroxyproline	Internal	Proline $^{13}C_5$ $^{15}N_1$	10-500	Linear	1/X	Ignore
Isoleucine	Internal	Isoleucine $^{13}C_6$ $^{15}N_1$	10-500	Linear	1/X	Ignore
Leucine	Internal	Leucine $^{13}C_6$ $^{15}N_1$	5-500	Linear	1/X	Ignore
Lysine	Internal	Lysine $^{13}C_6$ $^{15}N_2$	5-500	Linear	$1/X^2$	Ignore
Methionine	Internal	Methionine $^{13}C_5$ $^{15}N_1$	5-500	Linear	1/X	Ignore
Ornithine	Internal	Ornithine D_6	20-500	Quad.	1/X	Ignore
Phenylalanine	Internal	Phenylalanine $^{13}C_9$ $^{15}N_1$	2-500	Linear	$1/X^2$	Ignore
Pipecolic Acid	Internal	Pipecolic Acid $^{13}C_6$ $^{15}N_1$	2-500	Linear	$1/X^2$	Ignore
Proline	Internal	Proline $^{13}C_5$ $^{15}N_1$	5-500	Linear	1/X	Ignore
Serine	Internal	Serine $^{13}C_3$ $^{15}N_1$	20-500	Linear	$1/X^2$	Ignore
Taurine	Internal	Taurine $^{13}C_2$ $^{15}N_1$	25-500	Linear	1/X	Ignore
Threonine	Internal	Threonine $^{13}C_4$ $^{15}N_1$	10-500	Linear	1/X	Ignore
Tryptophan	Internal	Tryptophan $^{13}C_{11}$ $^{15}N_2$	5-500	Linear	$1/X^2$	Ignore
Tyrosine	Internal	Tyrosine $^{13}C_9$ $^{15}N_1$	5-500	Linear	1/X	Ignore
Valine	Internal	Valine $^{13}C_5$ $^{15}N_1$	5-500	Linear	1/X	Ignore

Compound	Calibration Type	Internal Standard	Linearity Range (µmol/L)	Type	Weight	Origin
β-alanine	Internal	Tyrosine $^{13}C_9$ $^{15}N_1$	10-500	Linear	1/x	Ignore
α-aminoadipic acid	Internal	Alanine $^{13}C_3$ $^{15}N_1$	5-250	Linear	1/x	Ignore
β-aminoisobutyric acid	Internal	GABA $^{13}C_4$	5-500	Linear	1/x	Ignore
γ-aminobutyric acid	Internal	GABA $^{13}C_4$	2-500	Linear	1/x	Ignore
5-aminolevunilic acid	No[1]					
Anserine	Internal	Lysine $^{13}C_6$ $^{15}N_2$	5-500	Linear	1/x	Ignore
Argininosuccinic acid	No[1]					
Carnosine	External	N/A	2-500	Linear	$1/x^2$	Ignore
Cysteine	N/A[2]					
Cysteine-homocysteine disulf.	No[1]					
Ethanolamine	Internal	Tyrosine $^{13}C_9$ $^{15}N_1$	2-500	Linear	1/x	Ignore
Glutathione Reduced	No[1]					
Homocitrulline	No[1]					
Homocysteine	No[1]					
Homocystine	No[1]					
Hydroxylysine	Internal	Tyrosine $^{13}C_9$ $^{15}N_1$	10-500	Linear	1/x	Ignore
Allo-isoleucine	Internal	Isoleucine $^{13}C_6$ $^{15}N_1$	5-500	Quad.	Equal	Ignore
Kynurenine	No[1]					
1-Methylhistidine	Internal	Histidine $^{13}C_6$ $^{15}N_3$	2-500	Quad.	1/x	Ignore
3-Methylhistidine	Internal	Histidine $^{13}C_6$ $^{15}N_3$	5-500	Linear	1/x	Ignore
Phosphoethanolamine	External	N/A	10-250	Linear	Equal	Ignore
Phosphoserine	External	N/A	10-250	Linear	Equal	Ignore
Saccharopine	No[1]					
Sarcosine	Internal	Phenylalanine $^{13}C_9$ $^{15}N_1$	12.5-1250	Linear	1/x	Ignore
Sulfocysteine	External	N/A	5-500	Quad.	1/x	Ignore

Figure 2. Representative chromatograms at the LOQ.

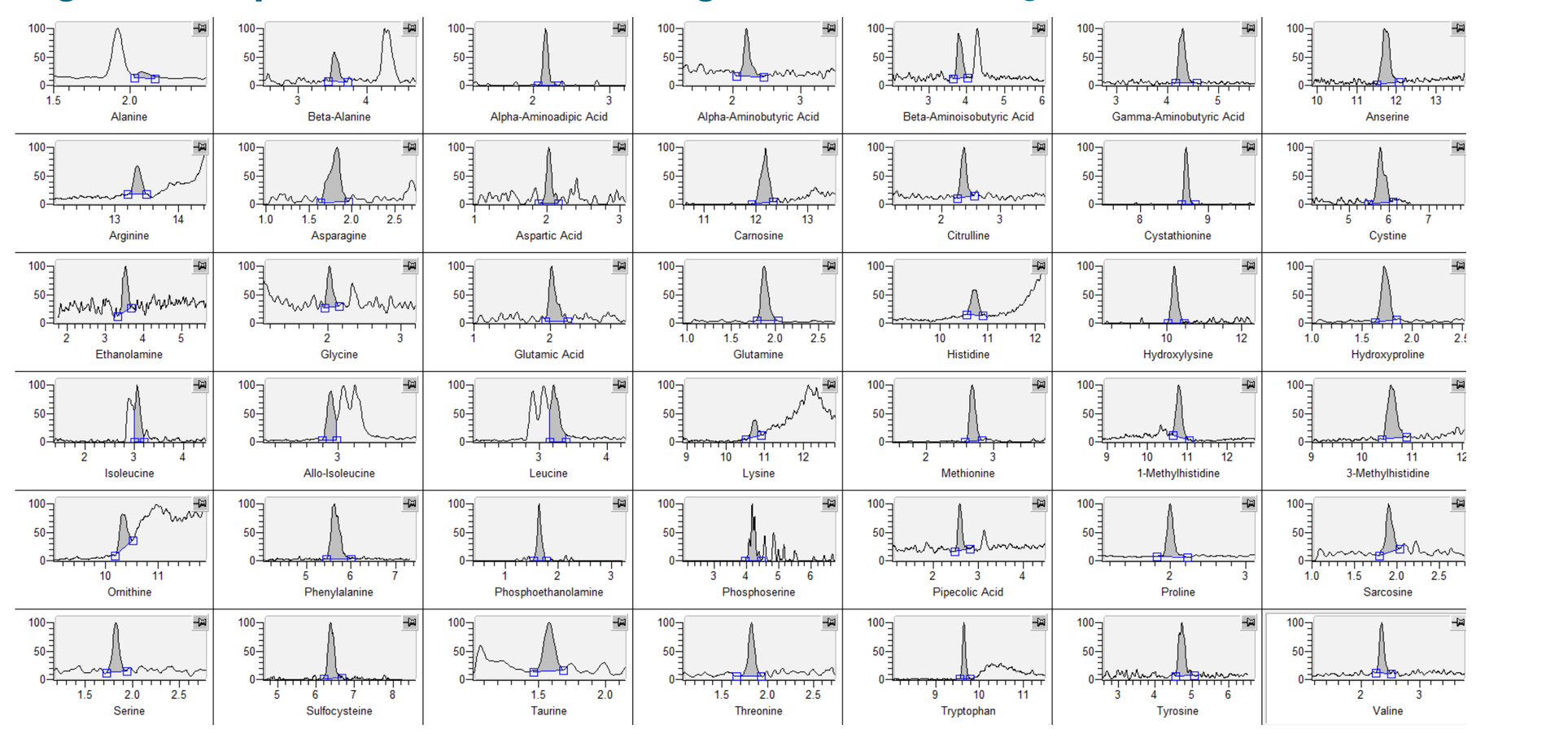


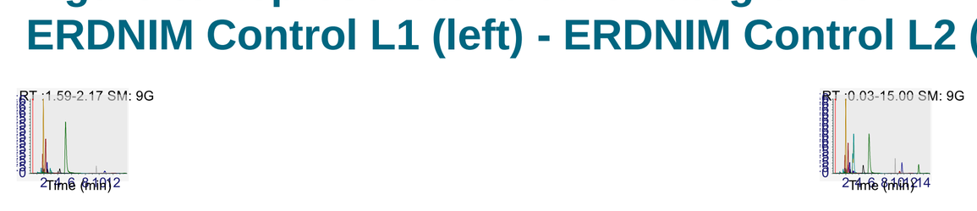
Table 4. Intra-assay accuracy and precision.

Compound	ERDNIM Control L1				ERDNIM Control L2			
	Theoretical concentration (µmol/L)	Average concentration (µmol/L)	Bias (%)	CV (%)	Theoretical concentration (µmol/L)	Average concentration (µmol/L)	Bias (%)	CV (%)
Alanine	318	296.99	-6.6	10.8	925	1077.96	16.5	8
α-aminobutyric acid	31.2	25.69	-17.6	7.5	94.7	82.1	-13.3	4.2
Arginine	16.59	15.63	-5.8	4.7	519	511.6	-1.4	1.3
Asparagine	107.7	86.83	-19.4	8.1	222	188.58	-15.1	5.7
Aspartic acid	12	10.34	-13.8	43.1	99.6	103.57	4	8.9
Citrulline	4.63	4.86	4.9	15	415	422.3	1.8	1.8
Cystathionine	9.95	7.78	-21.8	4.9	29.6	24.88	-15.9	4.8
Cystine	32.5	26.91	-17.2	6	69.4	62.69	-9.7	16.7
Glutamic Acid	107	102.9	-3.8	5.4	223	201.98	-9.4	8.4
Glutamine	575	603.97	5	2.6	1165	1238.26	6.3	3.5
Glycine	516	478.69	-7.2	7.2	1021	934.59	-8.5	8.8
Histidine	203	187.42	-7.7	3.1	398	395.04	-0.7	1.5
Hydroxyproline	48	45.93	-4.3	4.3	98	105.74	7.9	5.1
Isoleucine	52.1	44.39	-14.8	6.6	398	386.13	-3	3.4
Leucine	26.8	25.02	-6.7	5.4	890	802.54	-9.8	2.6
Lysine	271	244.56	-9.8	5.6	534	522.32	-2.2	2.8
Methionine	79.9	74.93	-6.2	4.4	241	243.52	1	3.3
Ornithine	159	150.83	-5.1	7.6	639	609.69	-4.6	6.1
Phenylalanine	341	334.96	-1.8	1.7	681	693.24	1.8	0.8
Pipecolic acid	44.6	47.45	6.4	1.8	92.6	99.18	7.1	1.3
Proline	301	291.83	-3	1.9	602	607.65	0.9	2
Serine	154	157.44	2.2	8.2	463	479.55	3.6	5.2
Taurine	213	231.42	8.6	5.9	417	475.29	14	7.6
Threonine	205	205.02	0	6.5	408	386.23	-5.3	3.1
Tryptophan	116	112.08	-3.4	6	292	289.24	-0.9	1.3
Tyrosine	234	221.76	-5.2	1.6	927	952.51	2.8	1.6
Valine	416	394.71	-5.1	2.2	823	815.09	-1	2.2

Table 5. Inter-assay accuracy and precision.

Compound	ERDNIM Control L1				ERDNIM Control L2			
	Theoretical concentration (µmol/L)	Average concentration (µmol/L)	Bias (%)	CV (%)	Theoretical concentration (µmol/L)	Average concentration (µmol/L)	Bias (%)	CV (%)
Alanine	31.2	28	-10.3	8.8	82.1	85.95	4.7	8.3
α-aminobutyric acid	318	330.23	3.8	14.8	1077.96	1066.44	-1.1	18.6
Arginine	16.59	17.29	4.2	9.2	511.6	524.26	2.5	6.7
Asparagine	107.7	88.31	-18	9.4	188.58	193.97	2.9	8.4
Aspartic acid	12	11.64	-3	37	103.57	109.19	5.4	11.6
Citrulline	4.63	4.68	1	20.6	422.3	425.32	0.7	5.4
Cystathionine	9.95	8.09	-18.7	5.5	24.88	25.65	3.1	6.5
Cystine	32.5	27.65	-14.9	6.6	62.69	61.42	-2	5.4
Glutamic acid	107	101.27	-5.4	8.2	201.98	229.48	13.6	14.1
Glutamine	575	609.32	6	7.4	1238.26	1254.61	1.3	6.8
Glycine	516	496.94	-3.7	13.5	934.59	946.25	1.2	13.3
Histidine	203	195.94	-3.5	5.4	395.04	411.28	4.1	8.2
Hydroxyproline	48	49.67	3.5	5.4	105.74	110.99	5	5.6
Isoleucine	52.1	47.45	-8.9	7.8	386.13	386.76	0.2	7
Leucine	26.8	24.78	-7.5	8.7	802.54	811.21	1.1	4.8
Lysine	271	256.41	-5.4	6.4	522.32	536.71	2.8	9.1
Methionine	79.9	77.56	-2.9	4.7	243.52	248.37	2	9.5
Ornithine	159	152.38	-4.2	11.4	609.69	574.51	-5.8	7.8
Phenylalanine	341	339.74	-0.4	2.9	693.24	692.82	-0.1	4.5
Pipecolic Acid	44.6	48.51	8.8	5.2	99.18	100.96	1.8	5.8
Proline	301	300.35	-0.2	4.5	607.65	619.23	1.9	5.7
Serine	154	141.25	-8.3	11.4	479.55	449.18	-6.3	7.9
Taurine	213	232.07	9	13.3	475.29	492.47	3.6	6.9
Threonine	205	196.88	-4	6.8	386.23	406.89	5.3	5.7
Tryptophan	116	113.81	-1.9	6.2	289.24	303.24	4.8	7.8
Tyrosine	234	224.33	-4.1	3.7	952.51	955.86	0.4	5.3
Valine	416	408.91	-1.7	3.7	815.09	826.08	1.3	4.6

Figure 3. Representative chromatograms.
ERDNIM Control L1 (left) - ERDNIM Control L2 (right).



CONCLUSIONS

An LC-MS/MS method for the clinical research analysis of amino acids and related compounds in plasma was developed and tested on a TSQ Endura triple quadrupole mass spectrometer. The approach includes a simple offline sample extraction by protein precipitation followed by dilution with mobile phase. The use of a specialty column and controlled pH conditions allowed for the separation of important isomers and allowed the analysis of 52 compounds in a 18 minutes chromatographic run.

Accuracy and precision studies have confirmed the possibility to use aqueous calibrators to quantify at least 27 compounds in plasma. For the remaining compounds, further work on the effects of plasma matrix and other matrices is ongoing.

TRADEMARKS/LICENSING

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