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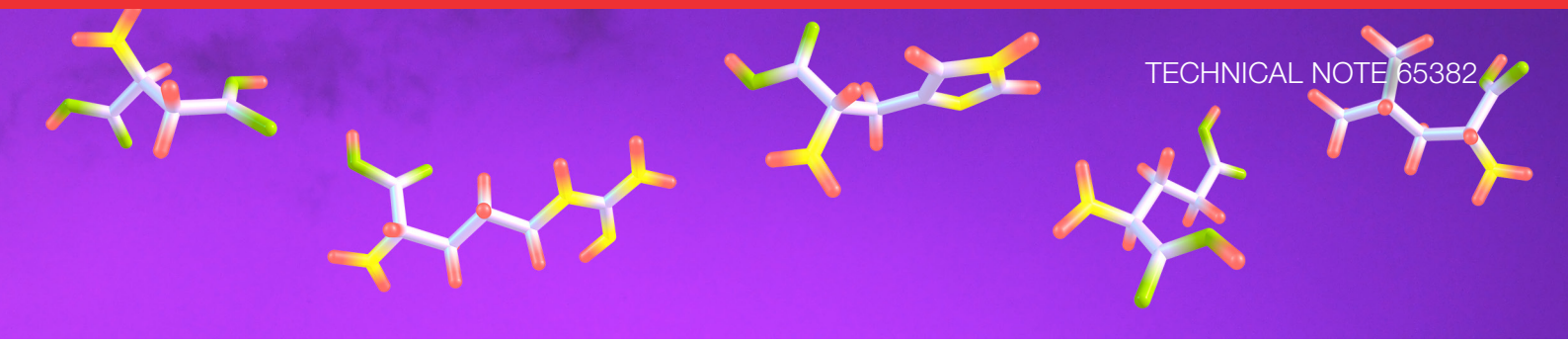

TECHNICAL NOTE 65382

Direct quantification of amino acids in plasma by liquid chromatography-tandem mass spectrometry for clinical research

Authors

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Keywords

Amino acids, human plasma, mass spectrometry, TSQ Endura

Application benefits

- Large panel of amino acids in a single run
- Single dilution step after protein precipitation for sample preparation
- Chromatographic resolution of isomers

Goal

Development and implementation of an analytical method for the quantification of 52 amino acids in plasma with a simple chromatographic approach without derivatization or ion pairing reagent addition.

Introduction

There is increasing interest in the rapid analysis of amino acids and other amino compounds in plasma samples and other biological fluids for clinical research. Typically, the expectation is to analyze a large panel of compounds in a single chromatographic run without adding ion pairing reagents and/or tedious sample preparation steps. Until now, this type of analysis has been performed with liquid chromatography (LC) methods based on post-column derivatization with ninhydrin (for UV-visible detection) or with pre-column derivatization with *o*-phtalaldehyde (for fluorescent detection). While these derivatization workflows are quite popular, they are time-consuming and require controlled conditions to achieve desired robustness, reliability, and reproducibility.

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LC coupled to mass spectrometry (MS) represents a good option for this kind of analysis. Considering that amino acids are quite polar, a direct reversed-phase chromatographic approach is not appropriate because of lack of retention and because separation of some important isomers cannot be achieved. In this regard, another option to reversed-phase chromatography would be to use ion chromatography; however, implementation of the ion chromatography–MS technology requires a specific front-end that is compatible with MS. For reversed-phase approaches, typically, two main options are available: (i) use of ion pairing reagents or (ii) derivatization of compounds before LC-MS/MS injection. In (i), the use of ion pairing agents alters the robustness of the method at the level of the separation and their continuous injection to the MS irremediably leads to the contamination of the ion source. For (ii), 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 an ion pairing reagent-free method for the analysis of a panel of 52 amino acids by LC-MS/MS without the need for derivatization of the sample.

Experimental

Target analytes

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

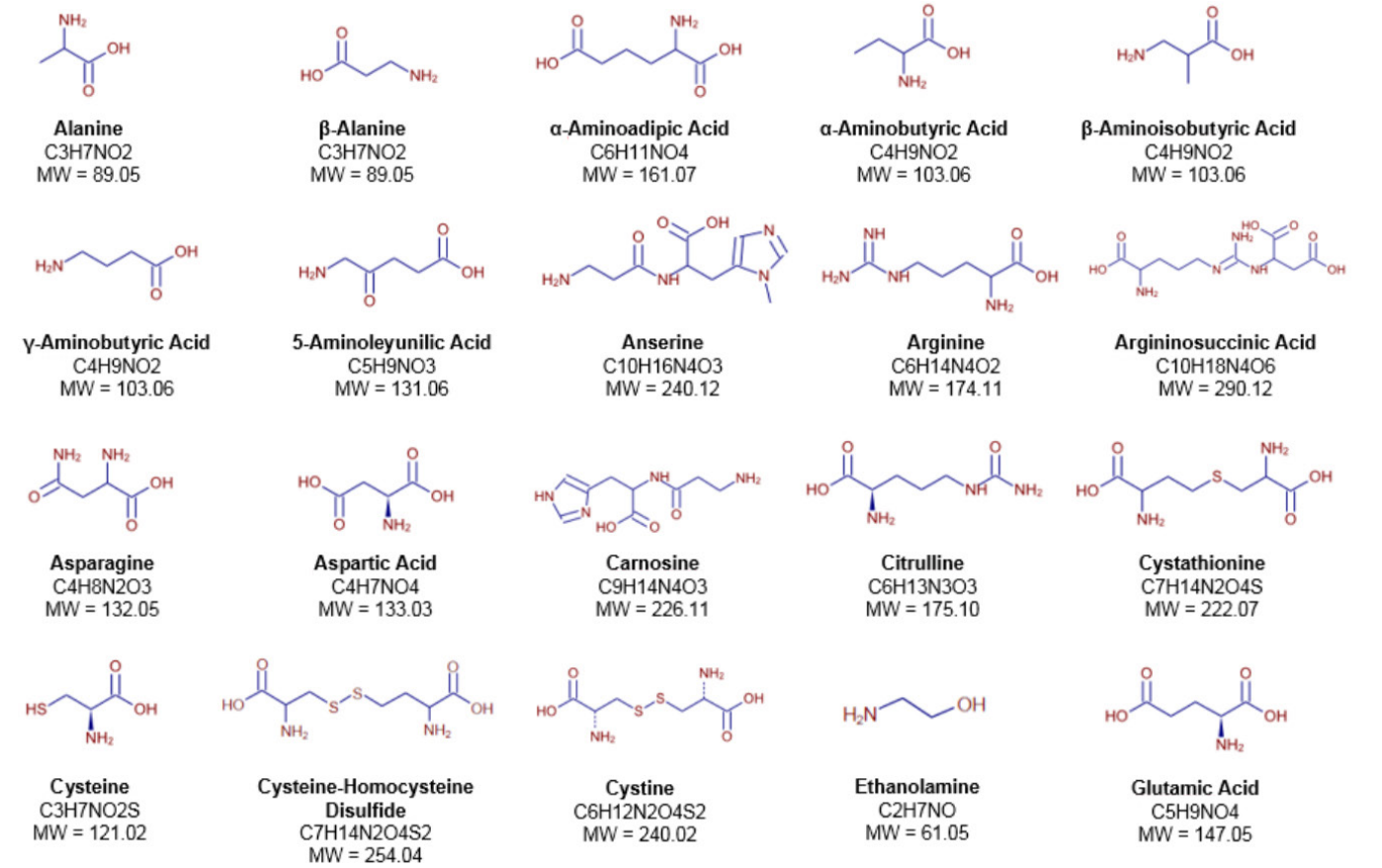

Figure 1A. Chemical structures of the analyzed compounds

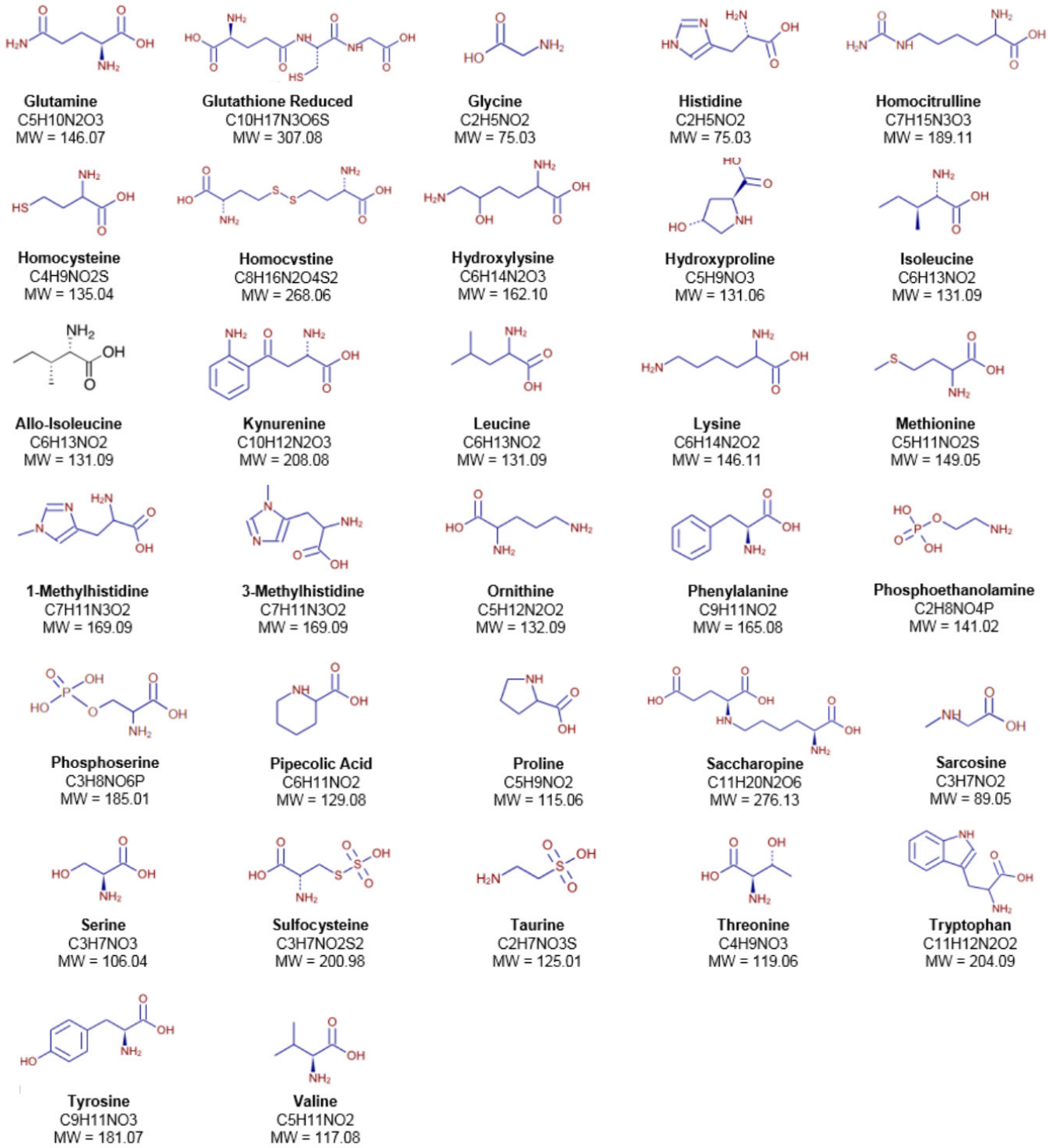

Figure 1B. Chemical structures of the analyzed compounds

Calibration standards and control samples

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

An internal standard solution was also prepared for sample dilution. This solution was obtained by diluting the Metabolomics Amino Acid Mix Standard (Cambridge Isotope Laboratories, MA, USA) 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, France), and ornithine D_6 (Sigma-Aldrich, France), with mobile phase A.

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

Sample preparation

One hundred microliters of each calibrator, quality control, and plasma donor sample were mixed with 10 µL of 30% sulfosalicylic acid in a 1.5 mL Eppendorf tube. After vortexing for 30 seconds, the mixture is refrigerated at 4 °C for 30 minutes, and centrifuged at 12,000 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 4 µL 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™ 3000RS system consisting of an HPG pump, a column oven, and an autosampler. The separation was performed on a Thermo Scientific™ Acclaim™ Trinity mixed mode column at 30 °C. Mobile phases consisted of ammonium formate in water at pH 2.8 for phase A and a mixture of ammonium formate in water and acetonitrile (80/20 v/v) for phase B. Chromatographic separation was achieved by gradient elution under the conditions described in Table 1.

Table 1. Gradient

Time (min)	Flow Rate (mL/min)	%A	%B
0	0.3	100	0
5	0.3	100	0
7	0.3	0	100
11	0.3	0	100
12	0.3	100	0
12.5	0.45	100	0
14.5	0.45	100	0
15	0.3	100	0
18	0.3	100	0

Mass spectrometry

Compounds were detected on a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer equipped with a 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, and spray voltage at 3500 V for positive ionization and at 2700 V for negative ionization. Vaporizer temperature was set to 370 °C and transfer tube temperature to 270 °C, while source fragmentation was applied at 15 V. Data was acquired in Selected Reaction Monitoring (SRM) mode using a resolution of 0.7 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 was performed for the 27 compounds that are present in the AMI-02.1 and AMI-02.2 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 was acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software.

Table 2 (Part 1). SRM transitions

Compound	Retention Time (min)	RT Window (min)	Polarity	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)	RF Lens (V)	Quan
Alanine and sarcosine	2.0	4	Positive	90.3	44.4	10	56	X
Alanine and sarcosine	2.0	4	Positive	90.3	90.3	5	56	X
Alanine $^{13}C_3$ ^{15}N	2.1	4	Positive	94.2	47.4	10	48	X
β-alanine	3.7	4	Positive	90.3	30.7	10	46	
β-alanine	3.7	4	Positive	90.3	72.2	10	46	X
α-aminoadipic acid	2.2	4	Positive	162.2	98.2	16	72	X
α-aminoadipic acid	2.2	4	Positive	162.2	144.1	10	72	
α-aminobutyric acid	2.2	4	Positive	104.2	41.5	20	47	
α-aminobutyric acid	2.2	4	Positive	104.2	58.3	10	47	X
β-aminoisobutyric acid	3.9	4	Positive	104.3	57.0	20	51	
β-aminoisobutyric acid and γ-aminobutyric acid	4.1	4	Positive	104.3	86.2	10	51	X
γ-aminobutyric acid	4.5	4	Positive	104.3	69.2	15	51	
γ-aminobutyric acid	4.5	4	Positive	104.3	87.2	10	51	X
γ-aminobutyric acid $^{13}C_4$	4.5	4	Positive	108.2	91.2	10	57	
5-Aminolevunilic acid	4.5	4	Positive	132.2	68.3	18	67	
5-Aminolevunilic acid	4.5	4	Positive	132.2	114.2	10	67	X
Anserine	11.8	4	Positive	241.0	109.0	20	90	X
Arginine	13.5	3	Positive	175.1	70.3	22	83	X
Arginine	13.5	3	Positive	175.1	116.1	14	83	
Arginine $^{13}C_6$ $^{15}N_4$	13.5	3	Positive	185.2	75.3	24	92	X
Argininosuccinic acid	9.5	4	Positive	291.2	70.3	31	151	X
Argininosuccinic acid	9.5	4	Positive	291.2	116.1	20	151	
Asparagine	1.9	3	Positive	133.2	74.2	16	60	
Asparagine	1.9	3	Positive	133.2	87.2	10	60	X
Asparagine $^{13}C_4$ D_3 $^{15}N_2$	1.9	3	Positive	142.2	95.2	10	68	X
Aspartic acid	2.1	4	Positive	134.1	74.2	15	65	X
Aspartic acid	2.1	4	Positive	134.1	88.2	10	65	
Aspartic acid $^{13}C_4$ $^{15}N_1$	2.1	4	Positive	139.2	92.2	10	59	X
Carnosine	12.1	3	Positive	227.2	110.2	22	95	X
Carnosine	12.1	3	Positive	227.2	210.0	11	95	
Citrulline	2.3	4	Positive	176.2	70.3	22	71	
Citrulline	2.3	4	Positive	176.2	159.0	10	71	X
Citrulline ^{13}C D_4	2.3	4	Positive	181.2	164.1	10	73	X
Cystathionine	8.2	4	Positive	223.2	88.2	26	91	
Cystathionine	8.2	4	Positive	223.2	134.1	14	91	X
Cysteine	4.9	6	Positive	122.1	59.3	21	48	X
Cysteine	4.9	6	Positive	122.1	76.2	12	48	
Cysteine-homocysteine disulfide	7.0	14	Positive	255.0	134.0	20	100	X
Cystine	6.0	4	Positive	241.1	74.2	27	91	
Cystine	6.0	4	Positive	241.1	152.0	13	91	X

Table 2 (Part 2). SRM transitions

Compound	Retention Time (min)	RT Window (min)	Polarity	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)	RF Lens (V)	Quan
Cystine $^{13}C_6$ $^{15}N_2$	6.0	4	Positive	249.1	156.0	14	98	
Ethanolamine	3.8	4	Positive	62.4	44.4	10	51	
Ethanolamine	3.8	4	Positive	62.4	62.4	5	51	X
Glutamic acid	2.1	4	Positive	148.1	84.2	15	62	X
Glutamic acid	2.1	4	Positive	148.1	130.1	10	62	
Glutamic acid $^{13}C_5$ $^{15}N_1$	2.1	4	Positive	154.2	136.1	10	68	X
Glutamine	1.9	3	Positive	147.1	84.2	16	64	
Glutamine	1.9	3	Positive	147.1	130.0	10	64	X
Glutamine $^{13}C_5$ D_5 $^{15}N_2$	1.9	3	Positive	159.2	141.0	10	71	X
Glutathione reduced	7.0	14	Positive	308.3	162.0	16	110	
Glutathione reduced	7.0	14	Positive	308.3	179.0	12	110	X
Glycine	2.0	4	Positive	76.3	30.5	10	46	
Glycine	2.0	4	Positive	76.3	76.3	5	46	X
Glycine $^{13}C_2$ $^{15}N_1$	2.0	4	Positive	79.3	32.6	10	41	
Glycine $^{13}C_2$ $^{15}N_1$	2.0	4	Positive	79.3	79.3	5	41	X
Histidine	10.9	4	Positive	156.1	83.2	23	73	
Histidine	10.9	4	Positive	156.1	110.1	13	73	X
Histidine $^{13}C_6$ $^{15}N_3$	10.9	4	Positive	165.2	118.1	15	81	X
Homocitruline	3.2	4	Positive	190.1	127.1	16	75	
Homocitruline	3.2	4	Positive	190.1	173.1	10	75	X
Homocysteine	3.5	4	Positive	136.1	56.3	18	61	
Homocysteine	3.5	4	Positive	136.1	90.2	10	61	X
Homocystine	9.6	4	Positive	269.2	88.2	30	83	
Homocystine	9.6	4	Positive	269.2	136.0	10	83	X
Hydroxylysine	10.3	4	Positive	163.0	82.0	20	65	
Hydroxylysine	10.3	4	Positive	163.0	128.0	13	65	X
Hydroxyproline	1.8	3	Positive	132.0	68.3	18	65	
Hydroxyproline	1.8	3	Positive	132.0	86.2	11	65	X
Kynurenine	9.4	4	Positive	209.2	146.0	18	82	
Kynurenine	9.4	4	Positive	209.2	192.1	10	82	X
Isoleucine and allo-isoleucine	2.9	5	Positive	132.2	69.3	16	55	X
Isoleucine ^{13}C6 $^{15}N_1$	3.0	4	Positive	139.2	74.3	18	59	X
Isoleucine ^{13}C6 $^{15}N_1$ and leucine $^{13}C_6$ $^{15}N_1$	3.0	5	Positive	139.2	92.2	10	59	X
Leucine	3.2	4	Positive	132.2	43.4	24	55	
Leucine and isoleucine and allo-isoleucine	3.0	5	Positive	132.2	86.2	10	55	X
Leucine $^{13}C_6$ $^{15}N_1$	3.0	4	Positive	139.2	46.4	25	59	
Lysine	10.9	4	Positive	147.2	84.2	16	64	X
Lysine	10.9	4	Positive	147.2	130.1	10	64	
Lysine $^{13}C_6$ $^{15}N_2$	10.9	4	Positive	155.2	137.1	10	69	X
Methionine	2.7	4	Positive	150.1	104.1	10	61	
Methionine	2.7	4	Positive	150.1	133.1	10	61	X
Methionine $^{13}C_5$ $^{15}N_1$	2.7	4	Positive	156.2	138.1	10	65	X

Table 2 (Part 3). SRM transitions

Compound	Retention Time (min)	RT Window (min)	Polarity	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)	RF Lens (V)	Quan
1- and 3-Methylhistidine	10.8	4	Positive	170.1	109.2	15	75	
1-Methylhistidine	10.8	4	Positive	170.1	83.2	23	75	
1-Methylhistidine	10.8	4	Positive	170.1	124.1	14	75	X
3-Methylhistidine	10.9	4	Positive	170.1	126.1	13	75	X
Ornithine	10.5	4	Positive	133.2	70.3	17	62	X
Ornithine	10.5	4	Positive	133.2	116.1	10	62	
Ornithine D_6	10.5	4	Positive	139.3	76.3	18	73	X
Phenylalanine	5.3	4	Positive	166.1	103.2	28	62	
Phenylalanine	5.3	4	Positive	166.1	120.2	11	62	X
Phenylalanine$^{13}C_9$ $^{15}N_1$	5.3	4	Positive	176.2	129.1	13	75	X
Phenylalanine	5.3	4	Positive	166.1	120.2	11	62	X
Phenylalanine $^{13}C_9$ $^{15}N_1$	5.3	4	Positive	176.2	129.1	13	75	X
Phosphoethanolamine	1.7	3	Positive	142.1	44.4	10	53	X
Phosphoethanolamine	1.7	3	Negative	140.0	79.0	20	53	
Phosphoserine	5.0	6	Positive	186.0	70.0	15	60	
Phosphoserine	5.0	6	Positive	186.0	88.0	15	60	X
Pipecolic Acid	2.6	4	Positive	130.2	56.3	27	67	
Pipecolic Acid	2.6	4	Positive	130.2	84.2	15	67	X
Pipecolic Acid $^{13}C_6$ ^{15}N	2.6	4	Positive	137.2	90.2	16	76	X
Proline	2.0	4	Positive	116.2	43.5	28	67	
Proline	2.0	4	Positive	116.2	70.3	15	67	X
Proline $^{13}C_5$ $^{15}N_1$	2.0	4	Positive	122.2	75.3	16	66	X
Saccharopine	7.5	4	Positive	277.1	84.2	24	105	X
Saccharopine	7.5	4	Positive	277.1	213.1	13	105	
Serine	1.9	3	Positive	106.2	60.3	10	47	X
Serine	1.9	3	Positive	106.2	88.2	10	47	
Serine $^{13}C_3$ $^{15}N_1$	1.9	3	Positive	110.2	63.3	10	52	X
Sulfocysteine	6.3	4	Positive	202.1	74.2	23	79	
Sulfocysteine	6.3	4	Positive	202.1	120.1	11	79	X
Taurine	1.7	3	Positive	126.2	108.1	10	82	
Taurine	1.7	3	Positive	126.2	126.2	5	82	X
Taurine $^{13}C_2$ ^{15}N	1.7	3	Positive	129.1	111.0	10	81	X
Taurine $^{13}C_2$ ^{15}N	1.7	3	Positive	129.1	129.1	5	81	
Threonine	1.9	3	Positive	120.2	74.3	10	58	X
Threonine	1.9	3	Positive	120.2	102.1	10	58	
Threonine $^{13}C_4$ $^{15}N_1$	1.9	3	Positive	125.2	78.2	10	58	X
Tryptophan	9.7	4	Positive	205.1	146.1	17	71	
Tryptophan	9.7	4	Positive	205.1	188.0	10	71	X
Tryptophan $^{13}C_{11}$ $^{15}N_2$	9.7	4	Positive	218.2	200.1	10	79	X
Tyrosine	4.5	4	Positive	182.1	136.0	12	73	X
Tyrosine	4.5	4	Positive	182.1	165.0	10	73	
Tyrosine $^{13}C_9$ $^{15}N_1$	4.5	4	Positive	192.2	174.0	10	77	X
Valine	2.4	4	Positive	118.2	55.4	19	45	
Valine	2.4	4	Positive	118.2	72.3	10	45	X
Valine $^{13}C_5$ $^{15}N_1$	2.4	4	Positive	124.2	77.3	10	56	X

Results and discussion

Internal calibration was used for 37 compounds, 25 using the corresponding isotopically labeled internal standards. External calibration (no internal standard) was used for five 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. Cystine is indeed the oxidized dimer form of cysteine; two moles of cysteine are used for the generation of one mole of cystine. Representative chromatograms at the LOQ are presented in Figure 2.

Table 3 (Part 1). Calibration approach, linearity range, and LOQ for each analyte

Compound	Calibration Type	ISTD	LOQ (µmol/L)	Linearity Range (µmol/L)	Type	Weighting	Origin
Alanine	Internal	Alanine $^{13}C_3$ ^{15}N	20	20–500	Linear	1/X	Ignore
β-Alanine	Internal	Tyrosine $^{13}C_9$ $^{15}N_1$	10	10–500	Linear	1/X	Ignore
α-Aminoadipic Acid	Internal	Alanine $^{13}C_3$ ^{15}N	5	5–250	Linear	1/X	Ignore
α-Aminobutyric Acid	Internal	γ-Aminobutyric Acid $^{13}C_4$	10	10–100	Linear	1/X	Ignore
β-Aminoisobutyric Acid	Internal	GABA $^{13}C_4$	5	5–500	Linear	1/X	Ignore
γ-Aminobutyric Acid	Internal	GABA $^{13}C_4$	2	2–500	Linear	1/X	Ignore
5-Aminolevunilic Acid	No calibration performed for this compound						
Anserine	Internal	Lysine $^{13}C_6$ $^{15}N_2$	5	5–500	Linear	1/X	Ignore
Arginine	Internal	Arginine $^{13}C_6$ $^{15}N_4$	5	5–500	Linear	1/X	Ignore
Argininosuccinic Acid	No calibration performed for this compound						
Asparagine	Internal	Asparagine $^{13}C_4$ D_3 $^{15}N_2$	24	24–600	Linear	1/X	Ignore
Aspartic Acid	Internal	Aspartic Acid $^{13}C_4$ $^{15}N_1$	10	10–200	Linear	$1/X^2$	Ignore
Carnosine	External	N/A	2	2–500	Linear	$1/X^2$	Ignore
Citrulline	Internal	Citrulline ^{13}C D_4	5	5–200	Linear	1/X	Ignore
Cystathionine	Internal	Phenylalanine $^{13}C_9$ $^{15}N_1$	2.5	2.5–100	Linear	1/X	Ignore
Cysteine	Non applicable, converted to cystine						
Cysteine-Homocysteine Disulfide	No calibration performed for this compound						
Cystine	Internal	Cystine $^{13}C_6$ $^{15}N_2$	10	10–500	Linear	1/X	Ignore
Ethanolamine	Internal	Tyrosine $^{13}C_9$ $^{15}N_1$	2	2–500	Linear	1/X	Ignore
Glutamic Acid	Internal	Glutamic Acid $^{13}C_5$ $^{15}N_1$	5	5–500	Linear	1/X	Ignore
Glutamine	Internal	Glutamine $^{13}C_5$ D_5 $^{15}N_2$	10	10–500	Linear	1/X	Ignore
Glutathione Reduced	No calibration performed for this compound						
Glycine	Internal	Glycine $^{13}C_2$ $^{15}N_1$	50	50–500	Linear	1/X	Force
Histidine	Internal	Histidine $^{13}C_6$ $^{15}N_3$	5	5–500	Linear	$1/X^2$	Ignore
Homocitrulline	No calibration performed for this compound						
Homocysteine	No calibration performed for this compound						
Homocystine	No calibration performed for this compound						
Hydroxylysine	Internal	Tyrosine $^{13}C_9$ $^{15}N_1$	10	10–500	Linear	1/X	Ignore
Hydroxyproline	Internal	Proline $^{13}C_5$ $^{15}N_1$	10	10-500	Linear	1/X	Ignore
Kynurenine	No calibration performed for this compound						
Allo-Isoleucine	Internal	Isoleucine $^{13}C_6$ $^{15}N_1$	5	5–500	Quadratic	Equal	Ignore
Isoleucine	Internal	Isoleucine $^{13}C_6$ $^{15}N_1$	10	10–500	Linear	1/X	Ignore
Leucine	Internal	Leucine $^{13}C_6$ $^{15}N_1$	5	5–500	Linear	1/X	Ignore
Lysine	Internal	Lysine $^{13}C_6$ $^{15}N_2$	5	5–500	Linear	$1/X^2$	Ignore
Methionine	Internal	Methionine $^{13}C_5$ $^{15}N_1$	5	5-500	Linear	1/X	Ignore
1-Methylhistidine	Internal	Histidine $^{13}C_6$ $^{15}N_3$	2	2–500	Quadratic	1/X	Ignore
3-Methylhistidine	Internal	Histidine $^{13}C_6$ $^{15}N_3$	5	5–500	Linear	1/X	Ignore
Ornithine	Internal	Ornithine D_6	20	20–500	Quadratic	1/X	Ignore

Table 3 (Part 2). Calibration approach, linearity range, and LOQ for each analyte

Compound	Calibration Type	ISTD	LOQ (µmol/L)	Linearity Range (µmol/L)	Type	Weighting	Origin
Phenylalanine	Internal	Phenylalanine $^{13}C_9$ $^{15}N_1$	2	2–500	Linear	$1/X^2$	Ignore
Phosphoethanolamine	External	N/A	10	10–250	Linear	Equal	Ignore
Phosphoserine	External	N/A	10	10–250	Linear	Equal	Ignore
Pipecolic Acid	Internal	Pipecolic Acid $^{13}C_6$ ^{15}N	2	2–500	Linear	$1/X^2$	Ignore
Proline	Internal	Proline $^{13}C_5$ $^{15}N_1$	5	5–500	Linear	1/X	Ignore
Saccharopine	No calibration performed for this compound						
Sarcosine	Internal	Phenylalanine $^{13}C_9$ $^{15}N_1$	12.5	12.5–1250	Linear	1/X	Ignore
Serine	Internal	Serine $^{13}C_3$ $^{15}N_1$	20	20–500	Linear	$1/X^2$	Ignore
Sulfocysteine	External	N/A	5	5–500	Quadratic	1/X	Ignore
Taurine	External	Taurine $^{13}C_2$ ^{15}N	25	25–500	Linear	1/X	Ignore
Threonine	Internal	Threonine $^{13}C_4$ $^{15}N_1$	10	10–500	Linear	1/X	Ignore
Tryptophan	Internal	Tryptophan $^{13}C_{11}$ $^{15}N_2$	5	5–500	Linear	$1/X^2$	Ignore
Tyrosine	Internal	Tyrosine $^{13}C_9$ $^{15}N_1$	5	5–500	Linear	1/X	Ignore
Valine	Internal	Valine $^{13}C_5$ $^{15}N_1$	5	5–500	Linear	1/X	Ignore

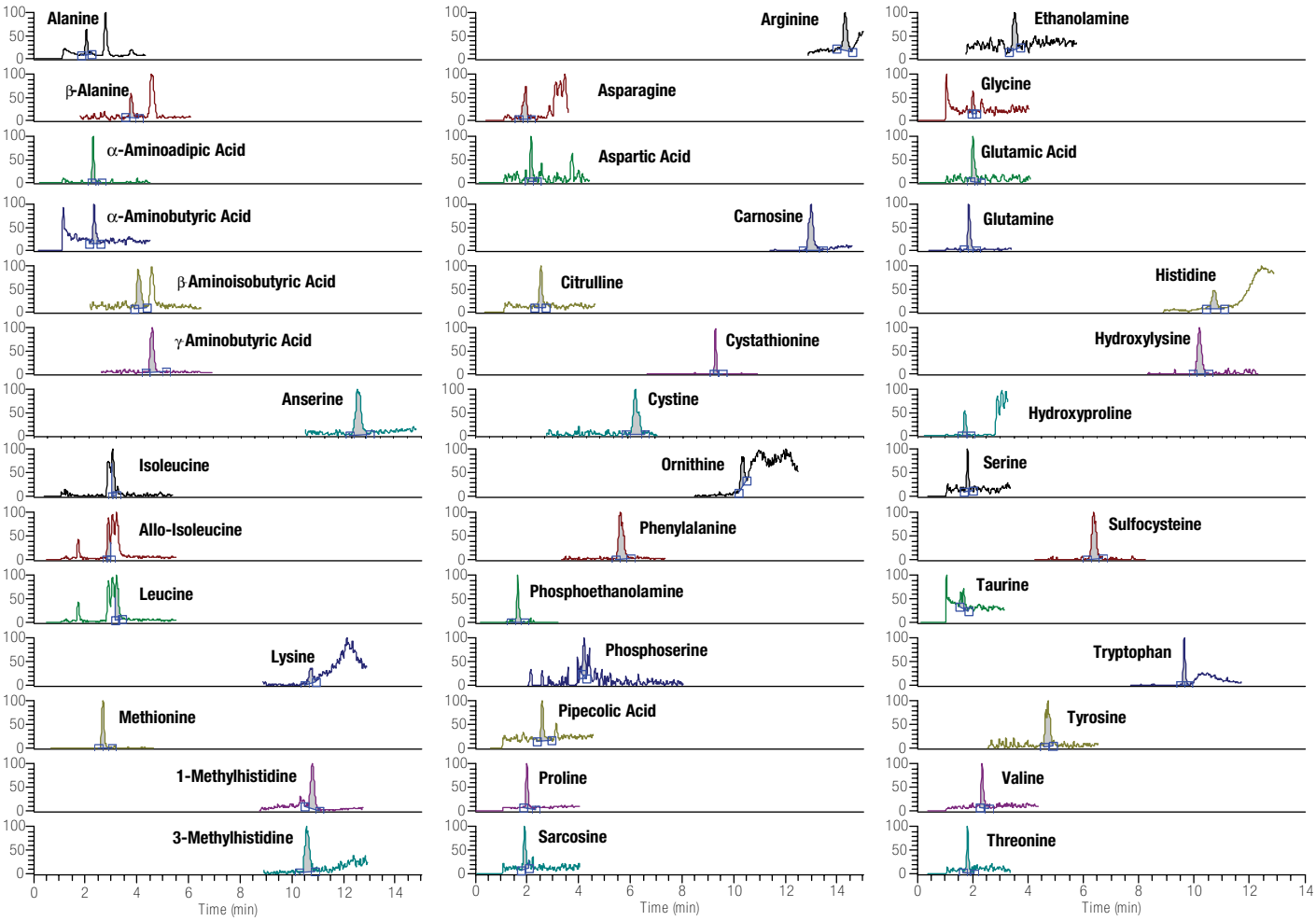

Figure 2. Representative chromatograms at the LOQ

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 and Figure 4, respectively. Calibration was performed in water considering that most of the studied compounds are endogenous. The obtained results show good accuracy for the two quality controls in plasma for the 27 studied compounds, which confirms the possibility to use this quantitation approach for plasma samples. The use of internal standards corresponding to most of the compounds in the panel corrects for the possible 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, which can be attributed to matrix effects, as cystathionine did not have a dedicated internal standard. For both intra- and inter-day studies, we also observed an important %CV for aspartic acid in L1 control. This is obviously due to the low level of aspartic acid in L1 (12 µmol/L), which is very close to the LOQ (10 µmol/L).

Table 4. Intra-assay accuracy and precision

	Control L1				Control L2			
Compound	**Theoretical Conc. (µmol/L)**	**Average Conc. (µmol/L)**	**Bias (%)**	**CV (%)**	**Theoretical Conc. (µmol/L)**	**Average Conc. (µmol/L)**	**Bias (%)**	**CV (%)**
Alanine	318	297	-6.6	10.8	925	1078	16.5	8.0
α-Aminobutyric Acid	31.2	26	-17.6	7.5	94.7	82	-13.3	4.2
Arginine	16.59	16	-5.8	4.7	519	512	-1.4	1.3
Asparagine	107.7	87	-19.4	8.1	222	189	-15.1	5.7
Aspartic Acid	12	10	-13.8	43.1	99.6	104	4.0	8.9
Citrulline	4.63	5	4.9	15.0	415	422	1.8	1.8
Cystathionine	9.95	8	-21.8	4.9	29.6	25	-15.9	4.8
Cystine	32.5	27	-17.2	6.0	69.4	63	-9.7	16.7
Glutamic Acid	107	103	-3.8	5.4	223	202	-9.4	8.4
Glutamine	575	604	5.0	2.6	1165	1238	6.3	3.5
Glycine	516	479	-7.2	7.2	1021	935	-8.5	8.8
Histidine	203	187	-7.7	3.1	398	395	-0.7	1.5
Hydroxyproline	48	46	-4.3	4.3	98	106	7.9	5.1
Isoleucine	52.1	44	-14.8	6.6	398	386	-3.0	3.4
Leucine	26.8	25	-6.7	5.4	890	803	-9.8	2.6
Lysine	271	245	-9.8	5.6	534	522	-2.2	2.8
Methionine	79.9	75	-6.2	4.4	241	244	1.0	3.3
Ornithine	159	151	-5.1	7.6	639	610	-4.6	6.1
Phenylalanine	341	335	-1.8	1.7	681	693	1.8	0.8
Pipecolic Acid	44.6	47	6.4	1.8	92.6	99	7.1	1.3
Proline	301	292	-3.0	1.9	602	608	0.9	2.0
Serine	154	157	2.2	8.2	463	480	3.6	5.2
Taurine	213	231	8.6	5.9	417	475	14.0	7.6
Threonine	205	205	0.0	6.5	408	386	-5.3	3.1
Tryptophan	116	112	-3.4	6.0	292	289	-0.9	1.3
Tyrosine	234	222	-5.2	1.6	927	953	2.8	1.6
Valine	416	395	-5.1	2.2	823	815	-1.0	2.2

Table 5. Inter-assay accuracy and precision

	Control L1				Control L2			
Compound	Theoretical Conc. (µmol/L)	Average Conc. (µmol/L)	Bias (%)	CV (%)	Theoretical Conc. (µmol/L)	Average Conc. (µmol/L)	Bias (%)	CV (%)
Alanine	318	330	3.8	14.8	925	1066	15.3	18.6
α-Aminobutyric Acid	31.2	28	-10.3	8.8	94.7	86	-9.2	8.3
Arginine	16.59	17	4.2	9.2	519	524	1.0	6.7
Asparagine	107.7	88	-18.0	9.4	222	194	-12.6	8.4
Aspartic Acid	12	12	-3.0	37.0	99.6	109	9.6	11.6
Citrulline	4.63	5	1.0	20.6	415	425	2.5	5.4
Cystathionine	9.95	8	-18.7	5.5	29.6	26	-13.4	6.5
Cystine	32.5	28	-14.9	6.6	69.4	61	-11.5	5.4
Glutamic Acid	107	101	-5.4	8.2	223	229	2.9	14.1
Glutamine	575	609	6.0	7.4	1165	1255	7.7	6.8
Glycine	516	497	-3.7	13.5	1021	946	-7.3	13.3
Histidine	203	196	-3.5	5.4	398	411	3.3	8.2
Hydroxyproline	48	50	3.5	5.4	98	111	13.3	5.6
Isoleucine	52.1	47	-8.9	7.8	398	387	-2.8	7.0
Leucine	26.8	25	-7.5	8.7	890	811	-8.9	4.8
Lysine	271	256	-5.4	6.4	534	537	0.5	9.1
Methionine	79.9	78	-2.9	4.7	241	248	3.1	9.5
Ornithine	159	152	-4.2	11.4	639	575	-10.1	7.8
Phenylalanine	341	340	-0.4	2.9	681	693	1.7	4.5
Pipecolic Acid	44.6	49	8.8	5.2	92.6	101	9.0	5.8
Proline	301	300	-0.2	4.5	602	619	2.9	5.7
Serine	154	141	-8.3	11.4	463	449	-3.0	7.9
Taurine	213	232	9.0	13.3	417	492	18.1	6.9
Threonine	205	197	-4.0	6.8	408	407	-0.3	5.7
Tryptophan	116	114	-1.9	6.2	292	303	3.8	7.8
Tyrosine	234	224	-4.1	3.7	927	956	3.1	5.3
Valine	416	409	-1.7	3.7	823	826	0.4	4.6

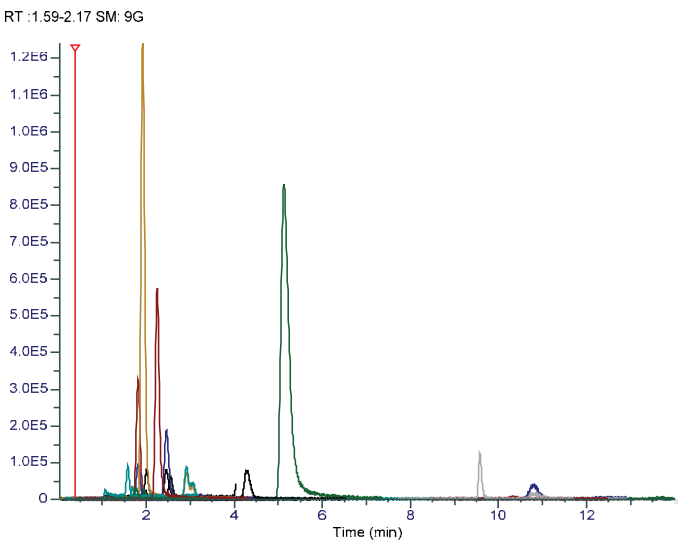

Figure 3. Representative chromatograms for L1 Erdnim control

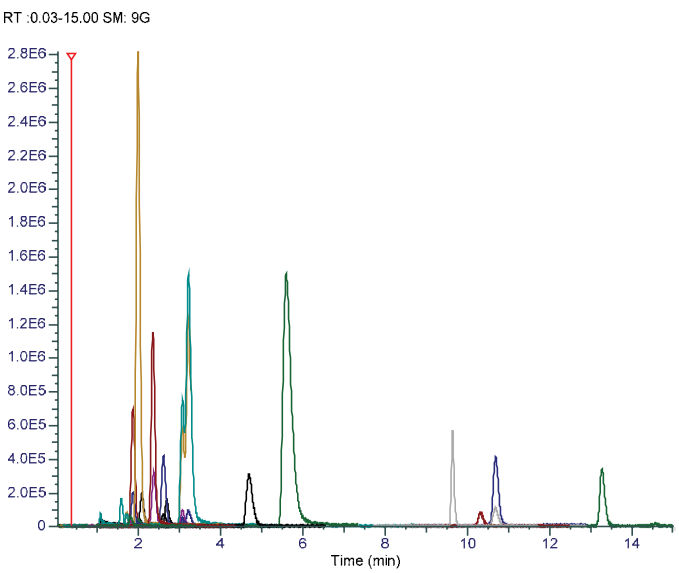

Figure 4. Representative chromatograms for L2 Erdnim control

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Conclusions

A robust, reliable, reproducible 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 workflow used in this study comprises a simple offline protein precipitation followed by dilution of the sample supernatant with mobile phase. The use of a specialty column and controlled pH conditions allowed for the separation of important isomers and allowed the efficient analysis of 52 compounds in an 18-minute chromatographic run.

Accuracy and precision studies confirm the possibility to use aqueous calibrators to quantify at least 27 compounds in plasma. For the remaining compounds, additional studies are in progress to determine the effects of plasma and other matrices.

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