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1 **Direct liquid chromatography tandem mass spectrometry analysis of amino**
2 **acids in human plasma**

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Abstract

48 Here we describe a new HPLC–MS/MS method using a mixed mode
49 stationary phase and a binary gradient of elution for the rapid separation and
50 quantification of AAs in human plasma without derivatization or ion pairing reagent
51 addition. The sample preparation procedure consists in a single dilution step after
52 protein precipitation with sulfosalicylic acid. The proposed method allows for the
53 unambiguous identification and analysis of 52 AAs and related compounds including
54 the separation of isomers and isobars in an 18 min chromatographic run including the
55 conditioning and the equilibration times. AAs were detected by selective reaction
56 monitoring. Internal calibration was used for the quantification of 37 AAs, including 25
57 using the corresponding isotopically labeled internal standards. External calibration
58 (no internal standard) was used for five additional analytes. Qualitative detection was
59 achieved for the remaining compounds.

60 Validation studies evaluated accuracy, linearity, within- and between-run
61 precision, lower limits of detection and quantification for 37 amino acids present in
62 commonly used quality control samples. For within-run precision CVs averaged 3.8
63 % (n = 30) for all compounds. For between-run precision, CVs averaged 8.6 % for all
64 compounds (n = 20). Correlation with the common standard ion-exchange
65 chromatography with post-column derivatization method was also performed for 32
66 plasma samples. While the proposed method is at least 50 times more sensitive, the
67 data showed good correlation with slopes equal or higher than 0.9 and correlation
68 coefficients mostly higher than 0.90. The method was successfully applied for
69 analysis of plasma samples for detection of inherited disorders of amino acid

70 metabolism.

71 Keywords: Amino acid profiling, human plasma, LC-MS/MS, mixed-mode stationary

72 phases.

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75 **1. Introduction**

76

77 The quantitative analysis of amino acids (AAs) in biological media including plasma,
78 urines, and cerebrospinal fluid (CSF) is one of the earliest chromatographic methods
79 commonly used in clinical laboratories [1, 2]. This analysis is mainly indicated for the
80 diagnosis and the follow-up of inherited metabolic diseases involving amino acids
81 also called congenital disorders of amino acid metabolism [3]. Amino acids patterns
82 are also markers of hepatic, renal, and intestinal functions [3]. Besides, their analysis
83 in plasma is routinely indicated for nutritional assessment in patients under
84 haemodialysis [4, 5] or after bariatric surgery [6].

85 For more than sixty years after its development ion exchange chromatography
86 (IEC) with post-column ninhydrin derivatization coupled to UV-Visible detection had
87 been the conventional standard for amino acids assay [1, 2, 7]. Although robust, this
88 method suffers three main drawbacks: 1- it is very time consuming (2 to 3 hours), 2-
89 it is not always specific due to drug interferences notably in urines, and 3- it requires
90 a dedicated analyser. In addition, some AAs, such as homocitrulline, pipercolic acid,
91 sulfocysteine, argininosuccinic acid, and saccharopine require specific separation
92 methods [3].

93 Recent developments in reversed phase liquid chromatography (RPLC) and
94 hyphenated techniques allowed the implementation of several RPLC methods mainly
95 coupled to tandem mass spectrometry (LC-MS/MS) [8-14]. Gas chromatography
96 coupled to MS has been also proposed for AAs analysis since 2012 [15]. Although
97 more specific than IEC, all these methods require; however, either pre-column
98 derivatization of the AAs present in the sample [7-9, 15] or the use of ion-pairing
99 agents (IPAs) [10, 11] or a combination of both [12], namely in order to enhance the

100 retention and the separation of these highly polar compounds. Pre-column
101 derivatization is costly and time-consuming and it requires controlled conditions to
102 achieve robustness, reliability, and reproducibility. On the other hand, IPAs alter the
103 robustness of the separation in term of reproducibility [16, 17] and their continuous
104 injection into the mass spectrometer irremediably leads to the contamination of the
105 ion source.

106 More recent developments in stationary phases, notably the advent of mixed-
107 mode sorbents [18, 19] may allow the separation of polar compounds without using
108 derivatization or adding IPAs. These silica-based stationary phases are specifically
109 designed for simultaneous separation of pharmaceutical drug substances and
110 counter-ions, as well as mixtures of acidic, basic, and neutral compounds. Providing
111 the adjustment of selectivity by changing mobile phase ionic strength, electrolyte
112 type, pH, and organic solvent, such stationary phases may allow for simultaneous
113 separation of basic, neutral, and acidic analytes, notably the retention of ionic and
114 ionizable analytes such as AAs without using ion-pairing reagents. Hence, we
115 focused on a new mixed-mode stationary phase in order to develop and validate a
116 HPLC-MS/MS method of quantification of AAs in biological samples without
117 derivatization or IPAs addition. The sample preparation procedure consists of a
118 single dilution step after protein precipitation.

119

120 **2. Material and methods**

121

122 2.1. Calibration standards and control samples

123 Calibration standards were prepared by mixing Amino Acids Mixture Standard
124 Solutions, Type B (γ -aminobutyric acid, anserine, arginine, carnosine, ethanolamine,

125 hydroxyl-lysine, histidine, 1-methylhistidine, 3-methylhistidine, lysine, ornithine) and
126 Type ANII (alanine, α -alanine, α -aminoadipic acid, α -aminobutyric acid, α -amino-
127 isobutyric acid, aspartic acid, citrulline, cysteine, cystathionine, glutamic acid, glycine,
128 hydroxyl-proline, isoleucine, leucine, methionine, phenylalanine, phosphor-
129 ethanolamine, phosphoserine, proline, sarcosine, serine, taurine, threonine, tyrosine,
130 valine) both from Wako (Pure Chemical Industries, Osaka, Japan) along with pH 2.8,
131 0.1 M ammonium formate individual solutions prepared from weighed standards (all
132 from Sigma-Aldrich) of L-alloisoleucine, 5-aminolevulinic acid, L-argininosuccinic acid,
133 L-asparagine, L-glutamine, L-homocitrulline L-pipecolic acid, L-saccharopine, S-sulfo-
134 L-cysteine, and L-tryptophan. An individual solution of L-homocystine was prepared
135 separately in 0.1 M HCl. The resulting stock solution was diluted with distilled water to
136 final concentrations of 500 and/or 250, 100, 50 and 10 μ M for all AAS except for
137 sarcosine (1250, 625, 250, 125, and 25 μ M), glutamine (1000, 500, 250, 125, 50, and
138 25 μ M), and L-homocystine (250, 125, 50, and 25 μ M). Hence, a panel of 52 amino
139 acids and related compounds were analyzed (Figure 1 and Supplementary Tables 1
140 and 2).

141 As internal standards we used a Metabolomics Amino Acid Mix Standard
142 purchased from Cambridge Isotopes Laboratories (Tewksbury, Massachusetts, USA)
143 as well as Taurine $^{13}\text{C}_2^{15}\text{N}$, citrulline $^{13}\text{CD}_4$, tryptophan $^{13}\text{C}_{11}^{15}\text{N}_2$, asparagine
144 $^{13}\text{C}_4\text{D}_3^{15}\text{N}_2$, pipecolic acid $^{13}\text{C}_6^{15}\text{N}$, glutamine $^{13}\text{C}_5\text{D}_5^{15}\text{N}$, gamma aminobutyric acid
145 $^{13}\text{C}_4$ all supplied by Eurisotope (Saint-Aubin, France). Ornithine D_6 was from Sigma-
146 Aldrich (St Louis, Missouri, USA). In the end, an internal standard (IS) solution
147 containing twenty-five internal standards was prepared for sample dilution and was
148 used for quantitation. The IS solution was obtained by diluting the Metabolomics
149 Amino Acid Mix Standard and pure solutions of the other amino acid isotopes with

150 the eluent A (pH 2.8, 0.12 M ammonium formate and acetonitrile (98.2/1.2, v/v)). The
151 concentrations of the IS in the initial mixes vary from 1.25 mM (for Cystine, for
152 instance) to 2.5 mM (for arginine, alanine, aspartic acid, histidine, etc.). Thus, after
153 dilution (1/200, v/v) in the eluent A, the final concentrations of the AA isotopes in the
154 IS solution vary from 6.25 μ M to 12.5 μ M according to the considered IS. Internal
155 quality controls, level 1 (L1) and level 2 (L2) batch numbers: 2017.0061 and
156 2017.0062 respectively, used for method validation were obtained from MCA
157 Laboratory (Winterswijk, The Netherlands).

158 2.2. Plasma samples

159 This study using only plasma sample residues was performed according to
160 French Public health regulations (Code de la Santé Publique - Article L1121-3,
161 amended by Law n°2011-2012, December 29, 2011 - Article 5). Blood samples used
162 for this study were received in the routine workload of the laboratory for amino acids
163 assay as part of the follow-up or diagnosis of patients. No other biological
164 investigation was performed than amino acids analysis.

165 2.3. Sample preparation

166 One hundred microliters of each calibrator, quality control, or plasma sample
167 were mixed in a 1.5 mL Eppendorf tube with 100 μ L of a 3 % sulfosalicylic acid
168 solution. The samples were vortex mixed for 30 seconds, refrigerated at 4 °C for 30
169 minutes, and centrifuged at 12,000 RCF for 5 minutes. Fifty microliters of supernatant
170 were added to 450 μ L of internal standard solution and vortex mixed for 30 seconds.
171 Five μ L of the resulting mixture were injected into the LC-MS/MS system.

172 2.4. LC-ESI-MS/MS

173 2.4.1. Liquid chromatography

174 Samples were analyzed on a Thermo Scientific™ UltiMate™ 3000 RS system
175 equipped with an HPG pump, a column oven and a thermostated autosampler. The
176 separation was performed on a 150 × 2.1 mm, 3 μm Thermo Scientific™ Acclaim™
177 Trinity P1 column, protected with a 10 × 2.1mm guard cartridge filled with the same
178 stationary phase at 30 °C. The eluent A is composed of a mixture of pH 2.8, 0.12 M
179 ammonium formate and acetonitrile (98.8/1.2, v/v) and the eluent B is composed of a
180 mixture of pH 6.5, 0.10 M ammonium formate and acetonitrile (80/20, v/v).
181 Ammonium formate and acetonitrile were purchased from Sigma-Aldrich (St Louis,
182 Missouri, USA).

183 2.4.2. MS/MS analysis

184 Compounds were detected on a Thermo Scientific™ TSQ Endura™ triple
185 quadrupole mass spectrometer equipped with a Thermo Scientific™ Easy-Max NG
186 ion source with a heated electrospray ionization probe. Sheath gas was set at 45
187 arbitrary units, auxiliary gas at 15 arbitrary units, and spray voltage at 3500 V for
188 positive ionization and at 2700 V for negative ionization. Vaporizer temperature was
189 set to 370 °C and transfer tube temperature to 270 °C, while source fragmentation
190 was applied at 15 V. Data was acquired in Selected Reaction Monitoring (SRM)
191 mode using a resolution of 0.7 full width at half maximum (FWHM) for both
192 quadrupoles with a 400 ms cycle time.

193 2.5. Ion exchange chromatography

194 For the analysis of AAs in plasma by the IEC conventional method we used
195 the dedicated analyzer “AminoTac JLC-500 V JEOL (Tokyo, Japan)” following a
196 previously described procedure [7].

197 2.6. Method validation

198 The validation of the method was performed according to French Committee
199 of accreditation (Cofrac) guidelines [based on ISO 3534-1 (1993) and ISO 5725-1
200 (1998) guidelines] for bioanalytical methods.

201 The specificity and the selectivity of the method are based on both MS/MS
202 detection and chromatographic resolution. The accuracy of the proposed method is
203 based on the closeness of agreement between the quantity values obtained by
204 measurement with the estimated values (as provided by the Erndim EQA Program)
205 of the analytes contained in the quality controls level 1 (L1) and level 2 (L2) used in
206 this study, which are assumed to be calculated consensus values of the AAs.

207 The limit of quantification (LOQ) for each analyte was determined as the
208 lowest value in the calibration curve giving an average % bias between nominal and
209 back-calculated concentration within $\pm 20\%$ and a % CV below 20% on 10 replicate
210 injections of calibrators.

211 The linearity of the method was checked at least with six levels in the
212 concentration ranges of LOQ to 125, 500, or 5000 μM according to the targeted AA.
213 Six independent determinations were performed at each concentration. Internal
214 calibration was used for 37 compounds, 25 using the corresponding isotopically
215 labeled internal standards. External calibration (no internal standard) was used for
216 five additional analytes. Qualitative detection was achieved for the remaining
217 compounds.

218 Within- and between-run precisions were performed for the 27 compounds
219 that are present in the Internal quality controls, level 1 (L1) and level 2 (L2) plasma
220 control material. For within-run precision, the controls were prepared and analyzed
221 30 times in the same day. For between-run precision, they were prepared 20 times in
222 15 different days.

223 Data was acquired and processed using Thermo Scientific™ TraceFinder™
224 (4.1 software).

225

226 **3. Results and discussion**

227

228 3.1. LC-MS/MS method development

229 3.1.1. Tandem mass spectrometry

230 In order to develop an MS/MS method, we used the product-ions scan (PIS)
231 mode by selecting the $[M+H]^+$ parent ion and scanning in a range from $m/z = 30$ to
232 $M+1$ for each AA. This procedure was performed for collision energy values ranging
233 from 5 to 40 eV.

234 Table 1 summarizes the MS/MS transitions of the studied AAs. As expected,
235 there are several isomers and isobars exhibiting the same precursor ion. Hence their
236 separation must be achieved, notably when their fragmentations produce the same
237 product ions.

238 3.1.2. Selecting the separation conditions

239 Having in common a molecular structure consisting of a side chain R bearing
240 an amino ($-NH_2$) and a carboxylic ($-COOH$) functional groups, the problem with the
241 LC-MS/MS analysis of AAs lies in the large diversity of their R type specific to each
242 amino acid [3]. AAs differ according to R type, locations of functional groups,
243 isoelectric point, and polarity. Therefore, their chromatographic separation with an
244 MS-compatible mobile phase requires an appropriate stationary phase with several
245 possibilities of interactions such as a mixed-mode stationary phase.

246 The first step of method development was column selection according to two
247 selection criteria: resolution of isobaric compounds with an acceptable symmetrical
248 profile for all chromatographic peaks. Preliminary investigations showed that except

249 for the acclaim Trinity P1 stationary phase [19], several bimodal columns (RP-WAX,
250 RP-WCX) and trimodal columns failed to comply with both criteria. Hence, we
251 focused on the Acclaim Trinity P1 stationary phase [19], which exhibits multiple
252 retention mechanisms including anion-exchange, cation-exchange, and reversed-
253 phase. This stationary phase consists of porous silica particles coated with charged
254 nano-polymer beads including an inner-pore area modified with an organic layer that
255 provides both reversed-phase and anion-exchange properties and an outer-pore
256 surface with cation-exchange functionality. Among other similar stationary phases,
257 we chose the Acclaim Trinity P1 because of its strong ion-exchange interactions
258 together with its low RP retention [19]. Indeed, the main objective was to obtain a
259 sufficient retention for the more polar AAs while shortening the retention of less polar
260 ones, in order to obtain a good separation in the shortest possible run-time.

261 In order to separate AAs on the chosen stationary phase we used the simplest
262 MS-compatible mobile phase consisting of a mixture of ammonium formate buffer
263 and acetonitrile. Then we focused on mobile phase composition including pH, and
264 buffer concentration, as well as organic solvent type and concentration, temperature,
265 and flow rate.

266 In order to separate all targeted AAs a gradient elution was necessary, of
267 course because of their large differences in isoelectric points (Ip) and polarities.
268 Hence we chose the simplest procedure consisting of a binary system including an
269 eluent A composed of a mixture of pH 2.8, 0.12 M ammonium formate and
270 acetonitrile (98.2/1.2, v/v) and an eluent B consisting of a mixture of pH 6.5, 0.1 M
271 ammonium formate and acetonitrile (80/20, v/v).

272 Preliminary investigations showed that for such a stationary phase, starting
273 with an acidic pH for eluent A where AAs are mostly protonated is mandatory.

274 Considering the I_p values of most AAs, at pH approaching the mean I_p most polar
275 AAs are not retained, while increasing the pH to obtain the anionic forms of AAs is
276 incompatible with the stationary phase stability. The main objective was to achieve
277 the retention of the more polar AAs while separating isomeric and isobaric ones,
278 notably leucine, isoleucine, alloisoleucine, and hydroxyproline; alanine and
279 sarcosine; and lysine and glutamine. Thus, we first studied the effects of eluent A pH
280 between 2.6 and 3.0 as well as the ammonium formate concentration between 0.05
281 and 0.15 M. Eluent B composition was then developed to achieve the rapid elution of
282 less polar and basic AAs.

283 The optimization of the gradient time, the purging period, the conditioning and
284 the equilibration times were then performed in order to still reach optimum
285 robustness and a run-time as short as possible. Given the specificity of the used
286 selected reaction monitoring (SRM) detection mode, our main objective was to obtain
287 maximum separation efficiency, in order to obtain a sufficient sensitivity, regardless
288 of the resulting resolution except for isomers and isobars.

289 Figure 2 A shows the representative extracted ion current (XIC) chromatogram
290 of the used standard mixture. The concentration and pH of eluent A together with the
291 flow rate and temperature were chosen to achieve sufficient retention and separation
292 efficiency for most polar AAs with optimum robustness, notably with between-assay
293 CVs less than 10 % for retention times.

294 The order of eluting compounds is not only determined by their isoelectric
295 points or molecular weights (Table 1). A further examination of the elution order of
296 some representative AAs (Supplementary Table 1) clearly shows that the side chain
297 plays an unneglectable role. The most polar AAs (hydroxyproline, asparagine,
298 glutamine, serine, threonine) except tyrosine and cysteine elute first followed by three

299 neutral AAs (glycine, alanine, and proline) thus anticipating the elution of acidic AAs
300 (aspartic, glutamic, and alpha-amino-adipic acids). The elution of the remaining
301 neutral AAs (α -amino butyric acid, valine, methionine, leucine, and isoleucine) and
302 citrulline occurs between 2.2 and 3.2 min, while γ -aminobutyric acid elutes at 4.5 min
303 followed by the polar AAs tyrosine and cysteine at 4.5 and 4.9 min and then the
304 neutral AAs phenylalanine and tryptophan at 5.3 and 9.7, respectively, while basic
305 AAs elute between 10.9 and 13.5 min. These results clearly show that the outer-pore
306 cation-exchange mechanism is not the only one involved in the separation. A
307 reversed-phase mechanism is also involved as reflected by the observed retention
308 times of some polar (citrulline, tyrosine, and cysteine) and some neutral AAs (γ -amino
309 butyric acid, phenylalanine, and tryptophan). Also, the need of increasing the
310 concentration of acetonitrile in addition to increasing pH in eluent B in order to
311 accelerate the elution of tryptophan and basic amino acids confirms the existence of
312 the reversed-phase retention mechanism. These results agree with those obtained
313 previously with mixed-mode stationary phases for other compounds [18, 19].

314 Except for critical pairs of isomeric and isobaric AAs, most compounds elute
315 as peaks that were overlapping but unambiguously identifiable by SRM. The best
316 resolution with optimal robustness of critical AAs isomeric pairs sarcosine and
317 alanine, and leucine, isoleucine, allo-isoleucine that are closely eluting is obtained at
318 a pH range between 2.6 and 2.9 and a concentration of ammonium formate between
319 0.05 and 0.15 M. Decreasing pH below 2.7 improves the resolution but at the
320 expense of the stability and column-life. Increasing pH above 2.9 resulted in a
321 decrease of resolution leading to the co-elution of the critical isomer pairs. Similarly,
322 the drop in the concentration of formate buffer below 0.1 M results in the decrease of
323 the resolution accompanied by the alteration of peaks symmetry and a poor

324 reproducibility of retention times. This phenomenon is obviously related to the need
325 for a good buffering effect in order to obtain an appropriate compatibility between
326 eluent A and the solvent of injection.

327 Also, adding a low percentage of at least 1% of a polar solvent such as
328 acetonitrile or methanol in eluent A is necessary in order to obtain a good peaks
329 symmetry. This low percentage of organic solvent probably serves to solvate the
330 hydrophobic sites of the stationary phase resulting in a better accessibility (less
331 overlapping) of these sites of interaction. Methanol may also be used for this purpose
332 but using acetonitrile resulted in a significant reduction of the run-time together with a
333 better sensitivity notably due to the improvement of the peak shapes and the
334 efficiency of the separation.

335 Figure 3 shows representative XIC chromatograms showing the separation of
336 the main circulating AA isomers and other usual isobaric compounds present in
337 plasma samples. The separation of leucine and isoleucine isomers as well as
338 hydroxyproline and 5-aminolevulinic acid all with the precursor ion $[M+H]^+$ at m/z 132
339 (Fig. 3A) is satisfactory achieved ($R_s \geq 1.0$ for leucine isomers). This is the same for
340 alanine, sarcosine, and 3-aminopropanoic acid all exhibiting a parent ion $[M+H]^+$ at
341 m/z 90 (Fig. 3B), as well as for aminobutyric acid isomers with the precursor ion m/z
342 104 (Fig. 3C).

343 3.1.3. Proposed method

344 Considered together these separation developments led us to adopt the
345 following chromatographic conditions for the separation of the targeted AAs:
346 separations are performed on a 150 × 2.1 mm, 3 μm Acclaim™ Trinity P1, protected
347 with a 10 × 2.1 mm guard cartridge filled with the same stationary phase. Eluent A is
348 composed of a mixture of pH 2.8, 0.12 M ammonium formate and acetonitrile

349 (98.8/1.2, v/v) and the eluent B is composed of a mixture of pH 6.5, 0.10 M
350 ammonium formate and acetonitrile (80/20, v/v).

351 The elution of the targeted AAs is performed in 18 min at 30 °C under the
352 following conditions: 100 % A over 5 min, then eluent B is increased to 100 % from 5
353 to 7 min and held constant for 4 min. From 11 to 12 min eluent A is increased to 100
354 % and hold constant during 6 min. The flow rate is set at 0.3 mL/min from 0 to 12
355 min. Then the flow rate is increased to 0.45 mL/min over 0.5 min and held constant
356 for 2 min. Finally, the flow rate is decreased to 0.3 mL/min over 0.5 min and held
357 constant from 15 to 18 min.

358 Figure 2B shows a representative LC–MS/MS chromatogram of a plasma
359 sample obtained with the proposed method. All the targeted AAs are separated in
360 less than 11 min. The proposed stationary phase is able to retain the highly polar
361 AAs as well as acidic AAs even with a starting mobile phase consisting of pH 2.8
362 ammonium formate buffer while achieving the separation of leucine and isoleucine
363 isomers (Fig. 3A).

364 It is worthy to note that the 5-minute isocratic hold of the eluent A widely
365 contributes to the robustness of the method in terms of retention time reproducibility
366 and peak shapes. It can also enable easy method transfer between instruments with
367 differing gradient dwell volume.

368 3.1.4. Optimization of sample preparation

369 The simplest and most appropriate sample preparation conditions were
370 determined by focusing on the nature of the protein precipitating agent, the volume
371 ratio of the precipitating agent, and the dilution level of the resulting supernatant in
372 eluent A in order to minimize matrix interferences while avoiding: 1- altering the
373 robustness of the method notably in terms of retention times reproducibility and

374 separation efficiency, which are dependent on solvent of injection/eluent A
375 compatibility, and 2-the excessive loss of sensitivity due to sample dilution.

376 To sum up, the sample preparation procedure consists of a single dilution
377 (1/10, v/v) of the sample with the IS solution, after protein precipitation with 100 μ L of
378 a 3 % sulfosalicylic acid solution (1/1, v/v).

379 The precipitation of the sample can be achieved with 10 μ L of a 30 %
380 sulfosalicylic acid solution (9/1, v/v) resulting in lower dilution of the initial sample, but
381 this may reduce the precision of the method. In the same way, it is mandatory to
382 dilute the supernatant in the IS solution at least ten times, in order to avoid the
383 robustness alteration of the method as well as an excessive matrix effect.

384 We also used other classical precipitating agents such as methanol but using
385 sulfosalicylic acid resulted in better robustness of the separation together with
386 minimum matrix effect.

387 3.2. LC–MS/MS identification of AAs in plasma samples

388 As shown from the XIC chromatograms (Fig. 2 and 3), retention times, as
389 compared to those of pure standards (Supplementary Fig. 1), and MS/MS transitions
390 allow the unambiguous identification of all targeted AAs. For instance, the MS/MS
391 transitions m/z 132 > 69 and m/z 132 > 43 allow the attribution of the corresponding
392 peaks to isoleucine and leucine, respectively. Also, it is possible to confirm any MS
393 transition from the ESI-MS spectra of the targeted AA by a secondary transition. This
394 applies for the other targeted AAs.

395 3.3. Method validation

396 3.3.1. Specificity

397 The specificity of the method is obviously based on the MS/MS detection
398 mode (3.2. section). In addition, it is well established that the SRM detection method

399 ensures the highest specificity possible for the analysis of AAs and/or any other
400 targeted compound in complex media such as biological ones, providing that the
401 separation of isomers and isobaric compounds is achieved.

402 3.3.2. Accuracy, precision, and linearity

403 In order to accurately quantify the targeted AAs in plasma Internal Standard
404 calibration was used for 37 compounds, 25 using the corresponding isotopically
405 labeled internal standards. External calibration (no internal standard) was used for
406 five additional AAs. Qualitative detection was achieved for the remaining compounds.
407 Details of calibration approach, linearity range, and LOQ for each AA are reported in
408 Table 2 (And supplementary Fig. 2).

409 The obtained results show good linearity over the considered concentration
410 ranges, which allow the determination of most analytes in plasma samples with a
411 single calibration curve for each analyte. The limits of detection (LOD) and
412 quantification (LOQ) of the method were estimated for a signal-to-noise ratio of 3/1
413 and 10/1, respectively. Representative chromatograms at the LOQ are presented in
414 Supplementary Figure 2. Considering the dilution of the sample in the mobile phase
415 and the injected volume, these results show that the proposed method is at least 50
416 times more sensitive than the commonly used IEC methods.

417 For accuracy, within-run, and between-run precision study we used the 27
418 compounds present in the L1 and L2 control samples (Tables 3 and 4, and Product
419 certificate amino acids in the Supplementary material). Calibration was performed in
420 water given that most of the studied compounds are endogenous. The obtained
421 results show good accuracy for the two quality controls in plasma for the 27 studied
422 compounds with recoveries mostly higher than 90 % and lower than 106 % except for

423 a few AAs with low concentration levels close to the LOQ (Table 2), thus confirming
424 the possibility to use this quantitation approach for plasma samples.

425 The use of internal standards corresponding to most of the compounds of
426 biochemical interest corrects for the possible matrix effects that can be observed in
427 the biological medium as compared to the calibrators resulting in a good precision for
428 most compounds (Tables 3 and 4).

429 For the within-run precision, CVs are lower than 7.6 % for both levels except
430 for aspartic acid in L1 control. This is obviously due to the low level of aspartic acid in
431 L1 (12 $\mu\text{mol/L}$), which is very close to the LOQ (10 μM ; Table 2). For the between-run
432 precision, all CVs of L2 control are lower than 11 % while for L1 some CVs can vary
433 from 10.5 to 16.7 %. However, the between-run precision is overall acceptable
434 because of the low concentration levels in the L1 control. This is true even for some
435 AAs without dedicated internal standard such as for cystathionine.

436 3.3.3. Robustness and stability

437 The robustness of the method was evaluated in terms of variation of pH (2.7,
438 2.8, and 2.9 for the Eluent A, and 6.4, 6.5, and 6.6 for the eluent B), salt
439 concentration (0.10, 0.12, and 0.15 M for both eluents A and B), and acetonitrile
440 concentration (1.0, 1.2, and 1.4 % for eluent A, and 18, 20, and 22 % for eluent B) as
441 well as for temperature (29, 30, and 31°C), flow rate (0.28, 0.30, and 0.032 mL/min),
442 isocratic hold (4, 5, and 6 min) and gradient time (1, 2, and 3 min). The acceptance
443 criteria were the reproducibility of retention times (CVs < 10 %, n = 6) along with
444 good shapes and acceptable peak asymmetry (≤ 1.2), and more importantly, a
445 sufficient resolution for leucine isomers ($R_s \geq 1.0$) as well as for sarcosine and
446 alanine ($R_s \geq 1.2$) (Fig. 3).

447 The stability of AAs in plasma samples after protein precipitation with
448 sulfosalicylic acid is well known [2, 11, 12, 14 - 16]. In the case of cysteine,
449 considering the acidic conditions for the protein precipitation, only cystine is present
450 since cysteine is completely oxidized to cystine under these conditions. Cystine is
451 indeed the oxidized dimer form of cysteine (Fig. 1); two moles of cysteine are used
452 for the generation of one mole of cystine.

453 3.3.4. Correlation studies

454 Finally, in order to complete the evaluation of the proposed LC-MS/MS method
455 we compared the results obtained for 32 plasma samples with the proposed method
456 with those obtained with the IEC conventional standard for amino acids assay [8].
457 Table 5 summarizes the obtained results. Apart from a few AAs where the
458 concentration means were very low, mostly lower than 50 μ M, such as for
459 aminobutyric acid, tryptophan, cysteine, asparagine, and hydroxyproline
460 (Supplementary Fig. 3), the correlations between the two methods are rather
461 satisfactory with slopes equal or higher than 0.9 and correlation coefficients mostly
462 higher than 0.90. We were not able to study the correlation for aspartic acid because
463 the concentration levels in the 32 studied plasma samples were below the LOD of the
464 IEC used method.

465 Although the number of plasma samples analysed for correlation purposes is
466 not statistically significant to establish the AAs reference values with the LC-MS/MS
467 method, it is worthy to note that the results obtained by the proposed method
468 (dependent variable) are generally lower by nearly 10 % than those obtained by the
469 IEC method (independent variable). This is probably due to the technological
470 difference between the two methods as well as to the calibration procedures even
471 though we used the same standard solution.

472 3.3.5. Analysis of two known cases of inherited metabolic disorders.

473 The proposed method was applied to the diagnosis of a known case of
474 phenylketonuria [20] detected in neonatal newborn screening in a 20 days female.
475 Classical phenylketonuria is an inherited metabolism disorder characterized by an
476 increase of phenylalanine level in plasma due to a phenylalanine hydroxylase
477 deficiency. The analysis of the amino acids profile by the proposed method allowed
478 to confirm a large increase of phenylalanine concentration in plasma (2270 μM ,
479 Supplementary Table 4 and Supplementary Fig. 4A).

480 The proposed method was also applied to the analysis of the amino acids
481 profile of a suspected case of lysinuric protein intolerance [21], an inherited
482 metabolism disorder due to a defect of renal transporter of dibasic cationic amino
483 acids, in a 21-year-old man. The obtained plasma amino acids profile highlighted an
484 increase of glutamine, and a slight decrease of lysine and cysteine, which lead us to
485 analyse by the same method the amino acids profile in a urine sample of this patient.
486 The obtained data showed an increased urinary excretion of 3 basic amino acids:
487 lysine, ornithine, and arginine (Supplementary Table 4 and supplementary Fig. 4B
488 and C).

489

490 **4. Conclusion**

491

492 A robust HPLC–MS/MS method for the rapid determination of AAs in plasma
493 without derivatization or IPAs addition was validated. The workflow used in this study
494 comprises a simple offline protein precipitation followed by dilution of the sample
495 supernatant with mobile phase containing the internal standards. The proposed

496 method allows the separation of important isomers and the analysis of 52
497 compounds in an 18 min chromatographic run.

498 Accuracy and precision studies confirm the possibility of using aqueous
499 calibrators to quantify at least 32 compounds in plasma. Besides, as long as internal
500 standardization is used, this method is capable of quantifying any amino acid or
501 related compound present in plasma over a wide range of concentrations, ranging
502 from a few tens to a thousand μM .

503 Since the proposed method is necessarily more specific than IEC, it is now
504 necessary to revisit the AAs reference values with the LC-MS/MS method. This work
505 is in progress in our laboratory. Also, additional studies are in progress to adapt the
506 proposed method for other biological matrices.

507

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509

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593 **Table 1.** M/MS transitions. Polarity positive, except for phospho-ethanolamine.
 594 Retention time refers to the center of the retention window. NCP: No calibration
 595 performed.

Compound	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	Collision energy (V)	Internal Standard
Alanine	2.0	90.1	44.4; 90.2	10; 5	Alanine ¹³ C ₃ ¹⁵ N
β-alanine	3.7	90.1	30.7; 72.2	10	Tyrosine ¹³ C ₉ ¹⁵ N ₁
α-aminoadipic acid	2.2	162.1	98.2; 144.1	16; 10	Alanine ¹³ C ₃ ¹⁵ N
α-aminobutyric acid	2.2	104.1	41.5; 58.3	20 ; 10	γ-aminobutyric acid ¹³ C ₄
β-aminoisobutyric acid	3.9	104.1	57.0	20	γ-aminobutyric acid ¹³ C ₄
γ-aminobutyric acid	4.5	104.1	69.2; 87.1	15; 10	γ-aminobutyric acid ¹³ C ₄
5-aminolevulinic acid	4.5	132.1	68.2; 114.1	18; 10	γ-aminobutyric acid ¹³ C ₄
Anserine	11.8	241.0	109.0	20	Lysine ¹³ C ₆ ¹⁵ N ₂
Arginine	13.5	175.1	70.2; 116.1	22; 14	Arginine ¹³ C ₆ ¹⁵ N ₄
Argininosuccinic acid	9.5	291.1	70.2; 116.1	31; 20	Tryptophan ¹³ C ₁₁ ¹⁵ N ₂
Asparagine	1.9	133.2	74.2; 87.1	16; 10	Asparagine ¹³ C ₄ D ₃ ¹⁵ N ₂
Aspartic acid	2.1	134.1	74.2; 88.1	15; 10	Aspartic acid ¹³ C ₄ ¹⁵ N ₁
Carnosine	12.1	227.1	110.1; 210.0	22; 11	Arginine ¹³ C ₆ ¹⁵ N ₄
Citrulline	2.3	176.2	70.3; 159.0	22; 10	Citrulline ¹³ C D ₄
Cystathionine	8.2	223.1	88.1; 134.1	26; 14	Tryptophan ¹³ C ₁₁ ¹⁵ N ₂
Cysteine	4.9	122.1	59.2; 76.2	21; 12	NCP
Cysteine-homocysteine disulfide	7	255.0	134.0	20	NCP
Cystine	6	241.1	74.2; 152	27; 13	Cystine ¹³ C ₆ ¹⁵ N ₂
Ethanolamine	3.8	62.3	44.4; 62.3	10; 5	Tyrosine ¹³ C ₉ ¹⁵ N ₁
Glutamic acid	2.1	148.1	84.1; 130.1	15; 10	Glutamic acid ¹³ C ₅ ¹⁵ N ₁
Glutamine	1.9	147.1	84.1; 130.0	16; 10	Glutamine ¹³ C ₅ D ₅ ¹⁵ N ₂
Glutathione reduced	7.0	308.3	162; 179	16; 12	NCP
Glycine	2.0	76.3	30.5; 76.3	10; 5	Glycine ¹³ C ₂ ¹⁵ N ₁
Histidine	10.9	156.1	83.2; 110.1	23; 13	Histidine ¹³ C ₆ ¹⁵ N ₃
Homocitrulline	3.2	190.0	127.1; 173.0	16; 10	Isoleucine ¹³ C ₆ ¹⁵ N ₁
Homocysteine	3.5	136.1	56.3; 90.1	18; 10	NCP
Homocystine	9.6	269.1	88.2; 136.0	30; 10	Tryptophan ¹³ C ₁₁ ¹⁵ N ₂
Hydroxylysine	10.3	163.0	82.0; 128.0	20; 13	Ornithine D ₆
Hydroxyproline	1.8	132.0	68.2; 86.2	18; 11	Proline ¹³ C ₅ ¹⁵ N ₁
Kynurenine	9.4	209.1	146.0; 192.0	18; 10	Histidine ¹³ C ₆ ¹⁵ N ₃
allo-isoleucine	2.9	132.1	69.2	16	Isoleucine ¹³ C ₆ ¹⁵ N ₁
Isoleucine	2.9	132.1	69.2	16	Isoleucine ¹³ C ₆ ¹⁵ N ₁
Leucine	3.2	132.1	43.4	24	Leucine ¹³ C ₆ ¹⁵ N ₁
Leucine, isoleucine and allo-isoleucine	3.0	132.1	86.2	10	Isoleucine ¹³ C ₆ ¹⁵ N ₁ ; Leucine ¹³ C ₆ ¹⁵ N ₁
Lysine	10.9	147.1	84.2; 130.1	15; 10	Lysine ¹³ C ₆ ¹⁵ N ₂
Methionine	2.7	150.0	104.1; 133.0	10	Methionine ¹³ C ₅ ¹⁵ N ₁

1-Methylhistidine	10.8	170.1	83.2; 124.1	23; 14	Histidine ¹³ C ₆ ¹⁵ N ₃
3-Methylhistidine	10.9	170.1	126.1	12.629	Histidine ¹³ C ₆ ¹⁵ N ₃
Ornithine	10.5	133.2	70.2; 116.1	17; 10	Ornithine D ₆
Phenylalanine	5.3	166.1	103.1; 120.1	28; 11	Phenylalanine ¹³ C ₉ ¹⁵ N ₁
Phosphoethanolamine	1.7	142.0	44.4; 79*	10; 20	Taurine ¹³ C ₂ ¹⁵ N ₁
Phosphoserine	5.0	186.0	70; 8	15	γ-aminobutyric acid ¹³ C ₄
Pipecolic Acid	2.6	130.1	56.3; 84.2	27; 15	Pipecolic Acid ¹³ C ₆ ¹⁵ N ₁
Proline	2.0	116.2	43.5; 70.2	28; 15	Proline ¹³ C ₅ ¹⁵ N ₁
Saccharopine	7.5	277.0	84.2; 213.0	24; 14	Tryptophan ¹³ C ₁₁ ¹⁵ N ₂
Sarcosine	1.8	90.2	44.4; 90.2	10; 5	Alanine ¹³ C ₃ ¹⁵ N
Serine	1.9	106.1	60.3; 88.2	10	Serine ¹³ C ₃ ¹⁵ N ₁
Sulfocysteine	6.3	202.0	74.2; 120.0	23; 11	External calibration
Taurine	1.7	126.1	108.1; 126.1	10; 5	Taurine ¹³ C ₂ ¹⁵ N ₁
Threonine	1.9	120.1	74.2; 102.1	10	Threonine ¹³ C ₄ ¹⁵ N ₁
Tryptophan	9.7	205.0	146.0; 187.9	17; 10	Tryptophan ¹³ C ₁₁ ¹⁵ N ₂
Tyrosine	4.5	182.0	136.0; 164.9	12; 10	Tyrosine ¹³ C ₉ ¹⁵ N ₁
Valine	2.4	118.1	55.4; 72.2	19; 10	Valine ¹³ C ₅ ¹⁵ N ₁

596 * For phospho-ethanolamine the confirmation fragment 79 was acquired in

597 negative mode with a parent ion corresponding to [M-H]⁻ adduct with m/z=140

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612 **Table 2.** Linearity and limit of quantification

Compound	Calibration Type	ISTD	LOQ (μM)	Linearity Range (μM)	Type	Weighting	Origin
Alanine	Internal	Alanine 13C3 15N	20	20-500	Linear	1/X	Ignore
β -Alanine	Internal	Tyrosine 13C9 15N1	2	2-500	Linear	1/X	Ignore
α -Aminoadipic Acid	Internal	Alanine 13C3 15N	1	1-500	Linear	1/X	Ignore
α -Aminobutyric Acid	Internal	γ -Aminobutyric Acid 13C4	5	5-500	Linear	1/X	Ignore
β -Aminoisobutyric Acid	Internal	γ -Aminobutyric Acid 13C4	2	2-500	Linear	1/X	Ignore
γ -Aminobutyric Acid	Internal	γ -Aminobutyric Acid 13C4	2	2-500	Linear	1/X	Ignore
5-Aminolevulinic Acid	Internal	γ -aminobutyric acid 13C4	1	1-500	Linear	Equal	Ignore
Anserine	Internal	Lysine 13C6 15N2	5	5-500	Linear	1/X	Ignore
Arginine	Internal	Arginine 13C6 15N4	2	2-5000	Linear	1/X	Ignore
Argininosuccinic Acid	Internal	Tryptophan 13C11 15N2	1	1-250	Linear	Equal	Ignore
Asparagine	Internal	Asparagine 13C4 D3 15N2	2	2-5000	Linear	1/X	Ignore
Aspartic Acid	Internal	Aspartic Acid 13C4 15N1	2	2-200	Linear	1/X2	Ignore
Carnosine	External	N/A	2	2-500	Linear	1/X ²	Ignore
Citrulline	Internal	Citrulline 13C D4	5	5-200	Linear	1/X	Ignore
Cystathionine	Internal	Phenylalanine 13C9 15N1	1	1-100	Linear	1/X	Ignore
Cysteine		Non applicable, converted to cystine					
Cysteine-Homocysteine Disulfide		No calibration performed for this compound					
Cystine	Internal	Cystine 13C6 15N2	2	2-4000	Linear	1/X	Ignore
Ethanolamine	Internal	Tyrosine 13C9 15N1	2	2-500	Linear	1/X	Ignore
Glutamic Acid	Internal	Glutamic Acid 13C5 15N1	5	5-500	Linear	1/X	Ignore
Glutamine	Internal	Glutamine 13C5 D5 15N2	2	2-1000	Linear	1/X	Ignore
Glutathione Reduced		No calibration performed for this compound					
Glycine	Internal	Glycine 13C2 15N1	50	50-500	Linear	1/X	Force
Histidine	Internal	Histidine 13C6 15N3	5	5-500	Linear	1/X2	Ignore
Homocitrulline	Internal	Isoleucine 13C6 15N1	1	1-250	Linear	Equal	Ignore
Homocysteine		No calibration performed for this compound					
Homocystine	Internal	Tryptophan 13C11 15N2	0.5	0.5-125	Linear	1/X	Ignore
Hydroxylysine	Internal	Tyrosine 13C9 15N1	2	2-500	Linear	1/X	Ignore
Hydroxyproline	Internal	Proline 13C5 15N1	0,5	0.5-500	Linear	1/X	Ignore
kynurenine		No calibration performed for this compound					
Allo-Isoleucine	Internal	Isoleucine 13C6 15N1	5	5-500	Quadratic	Equal	Ignore
Isoleucine	Internal	Isoleucine 13C6 15N1	10	10-500	Linear	1/X	Ignore
Leucine	Internal	Leucine 13C6 15N1	5	5-500	Linear	1/X	Ignore
Lysine	Internal	Lysine 13C6 15N2	5	5-500	Linear	1/X2	Ignore

Methionine	Internal	Methionine 13C5 15N1	5	5-500	Linear	1/X	Ignore
1-Methylhistidine	Internal	Histidine 13C6 15N3	2	2-500	Quadratic	1/X	Ignore
3-Methylhistidine	Internal	Histidine 13C6 15N3	5	5-500	Linear	1/X	Ignore
Ornithine	Internal	Ornithine D6	20	20-500	Quadratic	1/X	Ignore
Phenylalanine	Internal	Phenylalanine 13C9 15N1	0.5	0.5-5000	Linear	1/X2	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 13C6 15N	2	2-500	Linear	1/X2	Ignore
Proline	Internal	Proline 13C5 15N1	5	5-500	Linear	1/X	Ignore
Saccharopine	Internal	Tryptophan 13C11 15N2	1	1-250	Linear	Equal	Ignore
Sarcosine	Internal	Phenylalanine 13C9 15N1	5	5-500	Linear	1/X	Ignore
Serine	Internal	Serine 13C3 15N1	20	20-500	Linear	1/X2	Ignore
Sulfocysteine	External	N/A	5	5-500	Quadratic	1/X	Ignore
Taurine	External	Taurine 13C2 15N	25	25-500	Linear	1/X	Ignore
Threonine	Internal	Threonine 13C4 15N1	10	10-500	Linear	1/X	Ignore
Tryptophan	Internal	Tryptophan 13C11 15N2	5	5-500	Linear	1/X2	Ignore
Tyrosine	Internal	Tyrosine 13C9 15N1	5	5-500	Linear	1/X	Ignore
Valine	Internal	Valine 13C5 15N1	5	5-500	Linear	1/X	Ignore

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615 **Table 3.** Accuracy and within-run precision (n = 30)

Analyte	Control Level 1				Control Level 2			
	Theoretical conc. (μM)	Measured conc. (μM)	Recovery (%)	CV%	Theoretical conc. (μM)	Measured conc. (μM)	Recovery (%)	CV%
Alanine	318.0	309.0	97.2	4.2	925.0	843.4	91.2	4.0
2-aminobutyric acid	31.2	26.6	85.3	6.8	94.7	81.2	85.7	7.6
Arginine	16.6	16.7	99.4	2.9	519.0	490.3	94.5	3.7
Asparagine	107.7	92.7	86.1	3.8	222.0	181.0	81.5	3.4
Aspartic Acid	12.0	14.3	119.2	10.5	99.6	89.6	90	4.7
Citrulline	4.6	5.1	110.9	6.3	415.0	372.1	89.7	3.3
Cystathionine	10.0	7.9	79	4.1	29.6	24.9	84.1	3.7
Cystine	33.0	27.6	83.6	2.8	69.4	52.4	75.5	3.0
Glutamic Acid	107.0	110.8	103.6	3.6	223.0	211.2	94.7	3.7
Glutamine	575.0	577.8	100.5	2.6	1165.0	1100.3	94.4	3.2
Glycine	516.0	523.6	101.5	4.2	1021.0	946.5	92.7	5.1
Histidine	203.0	189.1	93.2	2.0	398.0	343.0	86.2	3.4
Hydroxyproline	48.0	50.4	105	9.6	98.0	126.3	128.9	7.7
Isoleucine	52.1	49.1	94.2	4.2	398.0	393.1	98.8	5.1
Leucine	26.8	25.7	95.9	2.2	890.0	857.7	96.4	4.8
Lysine	271.0	263.3	97.2	2.8	534.0	482.4	90.3	3.6
Methionine	79.9	77.7	97.2	2.6	241.0	222.8	92.4	3.5
Ornithine	159.0	155.2	97.6	2.5	639.0	569.9	89.2	3.0
Phenylalanine	341.0	331.8	97.3	2.0	681.0	626.9	92.1	3.3
Pipecolic Acid	44.6	47.1	105.6	2.1	92.6	90.3	97.5	3.4
Proline	301.0	294.7	97.9	3.3	602.0	556.0	92.4	2.9
Serine	154.0	150.9	98	3.7	463.0	411.7	88.9	5.0
Taurine	213.0	216.8	101.8	3.7	417.0	361.6	86.7	3.2
Threonine	205.0	193.4	94.3	2.7	408.0	396.4	97.2	4.0
Tryptophan	116.0	117.4	101.2	2.7	292.0	291.1	99.7	3.3
Tyrosine	234.0	229.0	97.9	2.2	927.0	877.4	94.6	3.2
Valine	416.0	397.2	95.5	2.8	823.0	761.3	92.5	4.2

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618 **Table 4.** Between-run precision (n = 20)

Analyte	Control Level 1				Control Level 2			
	Theoretical conc. (μM)	Measured conc. (μM)	Recovery (%)	CV%	Theoretical conc. (μM)	Measured conc.(μM)	Recovery (%)	CV%
Alanine	318.0	318.6	100.2	5.9	925.0	938.9	101.5	7.0
2-aminobutyric acid	31.2	28.1	90.1	17.1	94.7	90.9	95.9	8.3
Arginine	16.6	16.7	100.6	10.5	519.0	501.3	96.6	6.8
Asparagine	107.7	99.5	92.3	14.5	222	228.7	103	6.9
Aspartic Acid	12.0	12.0	100.1	14.8	99.6	96.3	96.7	9.6
Citrulline	4.6	4.9	105.5	10.5	415.0	395.3	95.3	5.4
Cystathionine	10.0	8.3	83.0	15.2	29.6	45.5	153.7	6.9
Cystine	32.5	27.4	84.4	7.2	69.4	60.7	87.4	7.2
Glutamic Acid	107.0	104.9	98.0	11.2	223.0	213.3	95.7	9.5
Glutamine	575.0	556.7	96.8	7.6	1165.0	1151.2	98.8	7.7
Glycine	516.0	491.3	95.2	7.8	1021.0	1000.4	98.0	9.1
Histidine	203.0	186.7	92.0	5.8	398.0	381.9	96.0	5.2
Hydroxyproline	48.0	49.9	103.9	12.7	98.0	109.1	111.3	11.4
Isoleucine	52.1	43.5	83.5	6.8	398.0	365.9	91.9	6.2
Leucine	26.8	26.2	97.6	8.8	890.0	825.6	92.8	9.3
Lysine	271.0	253.4	93.5	6.1	534.0	523.0	97.9	6.9
Methionine	79.9	73.8	92.3	5.1	241.0	230.8	95.8	7.0
Ornithine	159.0	142.9	89.9	6.7	693.0	623.9	90.0	10.2
Phenylalanine	341.0	326.2	95.7	7.7	681.0	668.1	98.1	4.8
Pipecolic Acid	44.6	43.8	98.2	16.7	92.6	94.5	102.0	7.0
Proline	301.0	281.8	93.6	6.0	602.0	578.7	96.1	6.1
Serine	154.0	138.9	90.2	6.2	463.0	428.5	92.6	6.2
Taurine	213.0	216.5	101.6	6.0	417.0	433.5	103.9	5.9
Threonine	205.0	192.6	94.0	6.6	408.0	401.6	98.4	6.6
Tryptophan	116.0	116.0	100.0	11.4	292.0	300.8	103.0	11.0
Tyrosine	234.0	219.4	93.7	4.1	927.0	916.8	98.9	5.7
Valine	416.0	386.7	92.9	5.6	823.0	790.3	96.0	5.4

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622 **Table 5.** Correlation study. LC-MS/MS vs IEC for 32 plasma samples

	Slope	Intercept	r ²
Alanine	1.10	-16	0.95
Aminobutyric acid	0.80	1.8	0.87
Arginine	0.90	1.9	0.99
Citrulline	0.90	0.9	0.98
Cystine	0.80	3.0	0.83
Glutamic acid	1.00	0.1	0.96
Glutamine	1.10	-32.0	0.89
Asparagine	0.67	7.3	0.73
Glycine	1.00	1.8	0.98
Histidine	0.90	-1.1	0.97
Hydroxyproline	0.60	3.3	0.87
Isoleucine	0.90	3.0	0.95
Leucine	1.00	-12.1	0.97
Lysine	1.00	0.5	0.94
Méthionine	0.90	-5.0	0.96
Ornithine	1.00	-4.9	0.97
Phénylalanine	1.00	-3.1	0.99
Proline	0.90	4.4	0.97
Sérine	1.10	-17.7	0.86
Taurine	1.10	-0.7	0.92
Thréonine	1.00	2.3	0.94
Tryptophan	0.80	6.4	0.93
Tyrosine	1.00	-2.1	0.98
Valine	0.90	2.6	0.99

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625 **Figure captions**

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627 **Figure 1.** Chemical structures of the analyzed compounds

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629 **Figure 2.** Representative XIC chromatograms: (A) Standard mix; (B) Plasma sample

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631 **Figure 3.** Chromatographic separation of amino acid isomers and isobars: (A) Leucine

632 isomers and other molecules with the precursor ion (m/z 132); (B) Alanine, sarcosine,

633 and other molecules with the precursor ion (m/z 90) C. Aminobutyric acids with the

634 precursor ion (m/z 104).

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