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# 1 **Analysis of free amino acids with unified chromatography-mass** 2 **spectrometry – Application to food supplements**

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4

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9

## 10 **Abstract**

11 Amino acids are most often analyzed in reversed-phase liquid chromatography after a  
12 derivatization procedure to render them sufficiently hydrophobic and detectable with UV or  
13 fluorimetric detection. Simpler methods should be possible to avoid additional chemical  
14 reactions. We present an improved method to analyze free amino acids with unified  
15 chromatography, that is to say with a wide elution gradient starting with supercritical fluid  
16 chromatography (SFC) conditions (high percentage of carbon dioxide) and ending with high-  
17 performance liquid chromatography (HPLC) conditions (100% co-solvent). The mobile phase  
18 composition was carefully adjusted to permit the elution of 21 natural amino acids (among  
19 which 19 proteinogenic) with very good peak shapes from a zwitterionic cinchona-based  
20 stationary phase (Chiralpak ZWIX(+)). Chiral separation was not desired. The mobile phase  
21 finally selected comprised carbon dioxide and a co-solvent (methanol containing 2% water  
22 and 20 mM methanesulfonic acid), ranging from 10 to 100% in 7 minutes followed by 3  
23 minutes re-equilibration at 25°C. A reversed pressure gradient (15 to 11 MPa) and a  
24 reversed flow rate gradient (3 to 1 mL/min) were applied to avoid reaching the upper  
25 pressure limit of the pumping system (40 MPa) and to favor high chromatographic efficiency  
26 at every stage of the elution gradient. Detection was achieved with electrospray ionization-  
27 mass spectrometry (ESI(+)-MS). The method is then fast and straightforward as no  
28 derivatization step is necessary, and all isobaric species were chromatographically resolved.  
29 To demonstrate the applicability of the method, it was applied to the quantitation of amino  
30 acids in food supplements commonly consumed by sportsmen, containing taurine (a  
31 common natural amino acid) or branched-chain amino acids (BCAA), namely valine, and the  
32 isobaric leucine and isoleucine. A standard addition method was examined for sensitivity,  
33 linearity, repeatability and intermediate precision.

34

35 **Keywords:** dietary supplements; native amino acids; supercritical fluid chromatography;  
36 unified chromatography; zwitterionic stationary phases

## 37 1. Introduction

38  
39 Supercritical Fluid Chromatography (SFC) was described for the first time during the  
40 1960s [1]. After a long period of moderate interest, in the years 2000s, SFC was mainly used  
41 for chiral separations, and especially for preparative-scale separations [2–4]. In the last  
42 decade, SFC has known a renewed interest, due to the improved instruments now available.  
43 These new instruments have yielded a significant increase in achiral applications of SFC [5].  
44 The coupling to mass spectrometry has also led to the development of some application  
45 fields that were previously little explored in SFC, like bioanalysis and complex samples of  
46 natural products [6–8]. Supercritical CO<sub>2</sub>, the major fluid used in SFC, is a rather non-polar  
47 solvent, with elution strength close to hexane. To elute more polar molecules, it is therefore  
48 necessary to add a polar organic co-solvent, to increase the polarity of this mobile phase. An  
49 additive, and even in some cases small amounts of water are added to analyse highly polar  
50 compounds. The effects of additives are complex and varied, and not yet fully understood.  
51 However, it is known that, at the usual concentrations employed (typically, arbitrary  
52 concentration of about 0.1-0.5% in the co-solvent) they have a small effect on mobile phase  
53 polarity [9], can have a significant effect on the mobile phase apparent pH [9,10] and adsorb  
54 on the surface of the stationary phase, thereby changing the retention of analytes [11]. In  
55 addition, the elution of ionic or ionisable species is especially favoured by ionic or ionisable  
56 additives that may form ion pairs with the analytes. When using an ionic stationary phase,  
57 the ionic mobile phase additive may act as a displacer [12].

58 While SFC mostly employs solvent proportions up to 50%, enhanced-fluidity liquid  
59 chromatography (EFLC) [13] is a separation method described as an intermediate technique  
60 between SFC and HPLC: it uses co-solvent percentages greater than 50%, up to 100% co-  
61 solvent [6,14]. More recently, following the proposal of Bamba and co-workers [14], wide  
62 elution gradients starting in SFC conditions, going through EFLC conditions and ending with  
63 HPLC conditions have shown interesting possibilities to achieve the elution of a wide range  
64 of analytes, from the least polar to the most polar [6], in a single experiment that can be  
65 named “unified chromatography” (UC).

66 Free (or native) amino acids possess two ionizable functions, one acidic and one basic,  
67 thus they can be zwitterionic (deprotonated acid function, protonated basic function) in  
68 certain mobile phase compositions. Typically, this should be the case in neutral liquid mobile  
69 phases and in SFC mobile phases containing carbon dioxide and an alcoholic co-solvent.  
70 The simultaneous presence of a negative and positive charge renders them quite polar and  
71 difficult to retain in reversed-phase high-performance liquid chromatography (RP-HPLC). RP-  
72 HPLC analysis of amino acids is thus most often done after derivatization to render them  
73 more hydrophobic and more detectable with UV or fluorimetric detection [15]. The first liquid-

74 phase separation of underivatized amino acids on a starch column was demonstrated by  
75 Synge in 1944 [16], while the first enantioseparation was proposed by Davankov and  
76 Rogozhin in 1971 [17]. Free amino acids are now mainly analyzed in liquid-phase  
77 chromatography using ion-exchange chromatography with cation- or anion-exchange  
78 stationary phases, or with ion-pairing RP-HPLC, and detected with evaporative light  
79 scattering detection (ELSD) [18] or mass spectrometry (MS) [19]. More recently, hydrophilic  
80 interaction liquid chromatography (HILIC) also proved to be efficient [20], especially as  
81 hyphenation to MS is favoured by the high concentration of organic solvent required for  
82 HILIC retention. When enantioresolution is desired, zwitterionic chiral selectors [21,22],  
83 macrocyclic antibiotics [23] and polysaccharide phases [24] may all be used. Further details  
84 on this topic can be found in a recent review [25]. A small number of papers report SFC  
85 methods to analyze free amino acids as their zwitterionic character renders them rather  
86 difficult to elute with usual SFC mobile phases. The first such experiment was reported in  
87 1992 by Camel et al. [26]. No enantioresolution was desired, but the method achieved the  
88 elution of several proteinogenic amino acids. However, the mobile phase used was  
89 incompatible with mass spectrometric detection. A second study focused on the  
90 enantioseparation of the three aromatic amino acids using UV detection [27]. Another one  
91 was focused on optimizing mass spectrometric detection for a small group of proteinogenic  
92 amino acids [28]. Finally, another paper reported enantioresolution of a larger selection of  
93 proteinogenic amino acids but the most basic ones were not reported [29]. Recently, we have  
94 demonstrated that the elution of all proteinogenic amino acids was possible with unified  
95 chromatography and a carefully selected mobile phase composition, while enantioresolution  
96 was achieved for most of them on a zwitterionic cinchona-based chiral selector (Chiralpak  
97 ZWIX(+)) stationary phase [30]. However, peak shapes were not totally satisfying for all  
98 amino acids, particularly the most basic ones. Building on this first attempt, in the present  
99 paper we mostly aimed at a rapid elution of all free amino acids, with improved peak shapes  
100 to hope for better sensitivity. Enantioresolution was not desired in this instance. Food  
101 supplements were selected as an interesting sample application, as many of them contain  
102 natural amino acids.

103 Food supplements, also called dietary supplements, are “foodstuffs the purpose of which  
104 is to supplement the normal diet and which are concentrated sources of nutrients or other  
105 substances with a nutritional or physiological effect, alone or in combination, marketed in  
106 dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms,  
107 sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of  
108 liquids and powders designed to be taken in measured small unit quantities” [31]. By  
109 nutrients, the legislation means vitamins and minerals. Global sales of food supplements are  
110 growing rapidly, especially those based on plant extracts, because they are considered safer

111 and healthier than synthetic drugs. They are also thought to be free of side effects. These  
112 increasing sales attest to the great popularity of food supplements. However, this market is  
113 vulnerable, with the possibility that food supplements containing adulterants are sold [31,32].  
114 Validated methods to achieve an efficient quality control of such samples are thus highly  
115 desirable.

116 In this paper, we present the development and partial validation of a fast method to  
117 quantify free amino acids in food supplements with unified chromatography hyphenated to  
118 electrospray ionization – mass spectrometry. Limits of detection and quantitation, linearity  
119 and precision are examined and compared to previously reported methods.

120

## 121 **2. Material and methods**

122

### 123 2.1 Chemicals and solvents

124 The aim of this study was to develop and validate (in part) an efficient method to quantify L-  
125 amino acids in food supplements. Food supplements analyzed were tablets of Amino Acids  
126 Complex (AAC), and capsules of Branched-Chain Amino Acids (BCAA) and Taurine and L-  
127 Theanine complex (TTC) from VITALL +<sup>®</sup> (Etik & Bio, France). Standards of L-amino acids  
128 used for method validation were all purchased from Sigma-Aldrich (Merck, France) and are  
129 described in Table 1. L-Theanine was not available and only examined in the TTC sample. L-  
130 Tryptophan, which was present in no sample, was used as internal standard (IS). Ultra-pure  
131 water was supplied by an Elga Purelab Flex station from Veolia (Antony, France). The HPLC  
132 gradient grade methanol (MeOH) used as the mobile phase co-solvent was supplied by VWR  
133 (Fontenay-sous-Bois, France). Methanesulfonic acid (MSA) was purchased from Janssen  
134 Chimica (Geel, Belgium). Hydrochloric acid 37% was supplied by Fisher Chemicals and Air  
135 Liquide (Paris, France) provided CO<sub>2</sub> with a purity ≥ 99.7%.

136

#### 137 *Samples and standards preparation for **external calibration method**:*

138 Food supplements tablets were dissolved in 20 mL of water with 0.1 M HCl during 1 hour  
139 minimum. Then, the mixture was handily agitated and filtered through 0.45 μM syringe filters.  
140 Samples were diluted by five in MeOH before injection. L-Tryptophan was chosen as internal  
141 standard and was added in stock solutions and food supplement samples at 2000 ppm.  
142 Seven mixtures of stock solutions were prepared (see Table 2), to avoid isobaric compounds  
143 in each group. From each of these mixtures, diluted solutions were prepared to build a  
144 calibration curve with six points, and with concentrations in accordance with the expected  
145 concentrations in the samples prepared from food supplements.

146

#### 147 *Samples and standards preparation for **standard addition method**:*

148 As significant matrix effects were observed with external calibration (even considering the  
149 internal standard), a standard addition method was preferred. Three successive additions  
150 were done: 100  $\mu$ L of food supplement solution (in 0.1M HCl in water) were put in each vial,  
151 a standard solution containing Taurine (for the TTC sample) or Valine, Leucine and  
152 Isoleucine (for the BCAA sample) was added in increasing amount (from 25 to 625 ppm) and  
153 MeOH was used as diluent to obtain the same final volume of 1 mL.

154

## 155 2.2 Instrumentation

156 The analytical system used for method development was an ACQUITY Ultra Performance  
157 Convergence Chromatography™ (UPC2®) from Waters Corporation (Milford, MA, USA). It  
158 was equipped with a binary solvent delivery pump, compatible with mobile phase flow rates  
159 up to 4 mL/min and pressures up to 414 bar. The system had an autosampler including a  
160 partial loop volume injection system. A backpressure regulator and 2-position column oven  
161 compatible with 150 mm length columns were also present. Two detectors were available: a  
162 photodiode-array (PDA) detector and an ACQUITY QDa® single-quadrupole mass  
163 spectrometric (MS) detector with electrospray ionization source (ESI). An isocratic solvent  
164 manager was used as a make-up pump and was positioned before the mass detector. The  
165 main flow stream was split after the introduction of make-up fluid: a portion of the column  
166 flow went to the backpressure regulator and another (unknown) portion went to the mass  
167 detector. Empower®3 software was used for system control, data acquisition and data  
168 retreatment. Because methanesulfonic acid (MSA) was employed in the mobile phase, it was  
169 necessary to modify the exhaust valve on the QDa system. It is worth mentioning that MSA  
170 may damage some mass spectrometric systems (corrosion of certain parts) and care must  
171 be taken when employing this acid.

172

## 173 2.3 Analytical method

174 All experiments were carried out on Chiralpak ZWIX(+), (150 x 3.0 mm; 3  $\mu$ m) from Chiral  
175 Technologies (Illkirch, France). This column was previously demonstrated to be most  
176 effective in the retention and enantioresolution of amino acids in unified chromatography [30].  
177 The co-solvent was MeOH containing 2% water and 20 mM methanesulfonic acid. Initial  
178 conditions were as developed in our previous works [30]: flow rate was set at 0.5 mL/min,  
179 oven temperature at 25°C and back-pressure at 15 MPa. The elution was done with a  
180 gradient from 10% to 100% co-solvent for 22 minutes. The elution gradient was then  
181 improved in order to achieve a faster and most efficient analytical method as further  
182 described in results section. In the optimized method, the elution gradient was faster (7  
183 minutes instead of 22). The oven temperature was still 25°C but a reversed pressure  
184 gradient (15 down to 11 MPa) and a reversed flow rate gradient (3 down to 1 mL/min) were

185 adopted to minimize analysis time and optimize efficiency along the elution gradient as is  
186 further discussed in the results section.

187  
188 The injection volume of each standard and sample solution was 2  $\mu$ L. As most amino acids  
189 have no chromophore, chromatograms were recorded with UV at 210 nm and with ESI-MS  
190 detection (positive ionization mode) and ion extraction from Total Ion Chromatogram (TIC).  
191 The make-up solvent to favor mass spectrometric detection and avoid analyte precipitation  
192 upon CO<sub>2</sub> decompression was MeOH containing 2% water and 1% formic acid at 0.3  
193 mL/min. ESI-MS parameters were set as follows: 600°C for the probe temperature, 0.8 kV for  
194 the capillary voltage and 15 V for the cone voltage. The mass range was set from 75 Da to  
195 250 Da.

## 196 197 2.4 Method validation

198 A partial method validation was carried out on the dietary supplement samples with standard  
199 addition method and with the optimized elution gradient. For this purpose, the limits of  
200 detection and quantification of each amino acid, repeatability, linearity and intermediate  
201 precision of the method were estimated, according to ICH guidelines although these are not  
202 pharmaceutical products [33]. To demonstrate the interest of the method, only the  
203 quantification of Valine, Leucine and Isoleucine in BCAA sample and Taurine in the TTC  
204 sample were validated.

### 205 2.4.1 Limit of detection and limit of quantification

206 The ESI-MS LOD and LOQ of 20 amino acids (all amino acids from Table 1 apart from L-  
207 Theanine) was assessed with standard solutions (see Table 3). Nine stock solutions of 5000  
208 ppm were prepared. Successive dilutions were performed until the LOD of each amino acid  
209 was observed. LOD is considered as 3 times the baseline noise level, while LOQ is 10 times  
210 the noise level.

### 211 212 2.4.2 Linearity

213 To determine the linearity of the analysis using external calibration or standard addition  
214 method, the solutions of different concentrations of amino acids shown in Table 2 were  
215 injected. An internal standard (Tryptophan) was added in all solutions at the same  
216 concentration. Peak areas at each concentration level were recorded and a plot of amino  
217 acid peak area divided by internal standard peak area versus amino acid concentration  
218 divided by internal standard concentration was done.

### 219 220 2.4.3 Repeatability

221 To assess repeatability, three concentration levels of doped BCAA sample were examined.  
222 Each solution was analyzed three times.

223 Relative standard deviation (RSD) was calculated as below:

$$224 \text{ (\% RSD)} = \frac{\text{Standard deviation of AAA over ISA}}{\text{Mean of AAA over ISA}} \times 100$$

225  
226 AAA and ISA are respectively the amino acid peak area and the internal standard peak area.  
227 In dietary supplements, RSD values inferior to 10% are considered acceptable [34].

#### 228 229 2.4.4 Precision

230 The ability to reach the same value, for each sample analysis, in the same analytical  
231 conditions but with different technicians or during different days, was examined. Firstly, at  
232 each concentration level, six solutions were prepared and each of them was analyzed three  
233 times. This served to determine intra-day precision. Secondly, the previous experiments (six  
234 solutions analyzed three times) were reproduced during 4 days and relative standard  
235 deviation served to determine inter-day or intermediate precision. In this case, the RSD value  
236 should be less than 15% [34].

### 237 238 3. Results and discussion

#### 239 240 3.1 Development of an optimized method to elute all amino acids

241  
242 Previously, we had developed a method to elute and resolve the enantiomers of  
243 proteinogenic amino acids [30]. Chiralpak ZWIX(+) and Chiralpak ZWIX(-) stationary phases  
244 had been selected for their recognized ability to resolve enantiomeric pairs of amino acids in  
245 the liquid phase. Even without the need for enantioresolution, these stationary phases would  
246 be excellent options for amino acids due to their multimodal interactions, allowing for the  
247 retention and separation of a wide range of amino acids carrying hydrophobic, polar non-  
248 ionic, acidic or basic side-chains. In this first stage of method development, a wide elution  
249 gradient was employed (10 to 100% co-solvent in CO<sub>2</sub>) [30], and three different mobile  
250 phase additives had been tested: one base (isopropylamine), one acid (trifluoroacetic acid)  
251 and one salt (ammonium formate). Ammonium formate had proven to be the most effective  
252 in eluting all free proteinogenic amino acids and resolving a majority of enantiomeric pairs  
253 (16 out of 19). Some improvement was however wanted as (i) peak shapes for the basic  
254 analytes (Histidine, Lysine and Arginine) were still imperfect; (ii) the isomeric compounds  
255 Leucine and Isoleucine remained co-eluted and (iii) the method was still rather long.



256 Looking for some improvement in all these points, we explored other mobile phase  
257 compositions with less common additives. Methanesulfonic acid (MSA), a strong acid with  
258 aqueous  $pK_a = -1.9$ , appeared as an interesting alternative. MSA is rather UV transparent  
259 and fully compatible with MS detection, making it an interesting option. MSA employed in  
260 reversed-phase HPLC is known to be an excellent ion pairing agent for basic drugs, favoring  
261 their elution with excellent peak shapes [35]. In hydrophilic interaction liquid chromatography  
262 (HILIC) mode, MSA was shown to provide different selectivity and generally improved peak  
263 shapes when compared to trifluoroacetic acid [36]. In normal-phase HPLC, sulfonic acids  
264 were shown to be beneficial to the enantioresolution of free amino acids [24]. Along with the  
265 slightly less acidic ethanesulfonic acid (ESA), MSA was also employed several times in SFC  
266 to achieve the enantioresolution of acidic or basic analytes on polysaccharide stationary  
267 phases [37,38]. When compared to other acidic additives (with higher or lower  $pK_a$  values) or  
268 to basic additives, ESA and MSA both provided improved enantioresolution due to improved  
269 enantioselectivity together with improved efficiency. The benefits were mostly attributed to (i)  
270 the changes in ionization state of the analytes, impacting their interaction with the stationary  
271 phase and (ii) the formation of ion pairs between the additive and basic analytes, impacting  
272 their solubility and interaction with stationary phase. Indeed, in the carbon dioxide – methanol  
273 mobile phase, MSA should be fully deprotonated in methanesulfonate. It should also acidify  
274 the mobile phase significantly (apparent pH around 1-2 [9]), thus the amine functions of  
275 amino acids would probably be in their cationic form while acidic functions would be in their  
276 neutral form. This way, methansulfonate should form ion pairs with cationic amino acids. On  
277 another hand, the stationary phase sulfonic group should remain unaffected by the presence  
278 of MSA, while the quinuclidine group should be fully protonated.

279 In the present case, the use of MSA was not so favorable to enantioresolution of free amino  
280 acid pairs on the zwitterionic stationary phase: only 12 amino acids (Asn, Asp, Cys, Gln, Glu,  
281 Ile, Leu, Phe, Pro, Ser, Tyr, Val) out of 19 could be resolved, with the notable case of  
282 aspartic acid that was well resolved with MSA while it had not been resolved with any of the  
283 additives previously tested. As enantioresolution was not an objective in the present paper,  
284 no further attention was paid to the unresolved pairs. However, MSA proved to be an  
285 excellent alternative to achieve elution of all free amino acids with good peak shapes,  
286 supporting the theory of ion-pair formation. The formation of an ion pair should reduce the  
287 overall polarity of the analyte, thereby reducing retention through the combined effects of  
288 improved mobile phase solubility and reduced interactions with the stationary phase.

289 Whenever the protonated amino group of the amino acid analytes was taking a significant  
290 part in the enantiorecognition process, its masking by the MSA anion would however be  
291 unfavorable to enantioseparation. The MSA anion may also adsorb on the stationary phase

292 cation, thereby shielding it from the analyte. In other words, MSA somewhat reduced  
293 enantioselective binding to the stationary phase.

294 Our initial method was rather long, with a slow elution gradient from 10 to 100% co-solvent in  
295 CO<sub>2</sub> and low flow rate (0.5 mL/min) to avoid reaching the upper pressure limit of the pumping  
296 system at the end of the elution gradient, in liquid mobile phase. We then proceeded to  
297 accelerate the method in progressively increasing the slope of the elution gradient and the  
298 flow rate. To avoid the pressure limitations, a reversed pressure gradient was however  
299 designed, starting at 15 MPa and ending at 11 MPa back-pressure, together with a reversed  
300 flow rate gradient, starting at 3 mL/min and ending at 1 mL/min (Figure 1). This needed to be  
301 optimized step by step, in a trial-and-error process, because it is difficult to predict the inlet  
302 pressure resulting from all operating parameters in a gradient program in SFC.

303 The flow rate reversed gradient also had another motive. It was shown in previous works  
304 from Desfontaine et al. [6] that the optimal flow rate (based on Van Deemter curves) should  
305 vary significantly along a wide elution gradient such as the one employed here: while the  
306 analytes eluting at the beginning of the gradient would reach optimal chromatographic  
307 efficiency at a fast flow rate (as is normally observed in supercritical fluids), the analytes  
308 eluting at the end of the gradient would reach optimal chromatographic efficiency at lower  
309 flow rates (as should be the case in liquids). Having made similar observations in this  
310 chromatographic system, rather than selecting an intermediate flow rate that would provide a  
311 compromise efficiency, we reasoned that the reversed flow rate gradient should be the best  
312 option to favor both early-eluting and late-eluting analytes.

313 The final method was a 7-minutes gradient with 3 minutes re-equilibration, thus cycle-time  
314 was 10 minutes (Figure 1). With this method, all free amino acids analyzed (19 proteinogenic  
315 amino acids and Taurine) eluted from the column with good peak shapes, as appears in  
316 Figure 2.

317 Figure 2 is divided into three parts to see (a) the amino acids with hydrophobic side chains  
318 (Alanine, Valine, Leucine, Isoleucine, Methionine, Phenylalanine, Tyrosine and Tryptophan);  
319 (b) the amino acids with polar side chain and special cases (Serine, Proline, Threonine,  
320 Taurine, Asparagine, Glutamine, Glycine) and (c) the amino acids with acidic or basic side  
321 chains (Aspartic acid, Glutamic acid; Arginine, Histidine and Lysine).

322 In the first group (Figure 2a), retention was mostly in accordance with increasing  
323 hydrophobicity. However, the isomeric Leucine and Isoleucine were very well resolved, as is  
324 more clearly visible from Figure 3b. In these accelerated conditions, the enantioresolution  
325 was lost, but in the longer gradient program, it was possible to separate all four isobaric  
326 compounds with the two enantiomers of Leucine being separated from the two enantiomers  
327 of Isoleucine (Figure 3a). The other isobaric pair formed by Lysine and Glutamine was also  
328 chromatographically resolved with the optimized method, thus the UC-ESI-MS method

329 permits the full identification of all the L-amino acids examined, based on analysis time and  
330 MS single-ion recording trace.

331

332 We then proceeded to apply this optimized method to samples of food supplements  
333 containing natural L-amino acids.

334

### 335 3.2 Quantification of amino acids in food supplements

336

337 Three different samples were examined. The first one contained 17 proteinogenic L-amino  
338 acids (see Table 1) (Amino Acid Complex AAC). The second one contained only three  
339 “branched-chain amino acids” (BCAA, containing L-Valine, L-Leucine and L-Isoleucine), as  
340 these are usually known by sportsmen to boost muscle growth and enhance exercise  
341 performance. The third one contained two non-proteinogenic amino acids, Taurine and L-  
342 Theanine (Taurine & Theanine Complex, TTC). Taurine is thought to have anti-oxidant  
343 properties and is often found in dietary supplements like energy drinks, while L-Theanine is  
344 believed to affect mood and stress levels favorably.

345

#### 346 3.2.1 External calibration with internal standard

347

348 First, a simple external calibration was attempted, using L-Tryptophan as an internal  
349 standard as it was not present in any of the three samples. Limits of detection and  
350 quantification (LOD and LOQ) were determined from the signal-to-noise ratio through  
351 successive dilutions of standard solutions. The results are presented in Table 3. LOD values  
352 ranged from 1.0 to 33.4 ng (per injected solution), with the lowest LOD being obtained for  
353 Isoleucine and Proline, and the highest LOD for Glycine. The average LOD value was about  
354 5.8 ng. LOQ values ranged from 3.3 to 111.3 ng, with an average LOQ value of 19.2 ng. In  
355 comparison with validated methods in the literature, these values are in the same range. For  
356 example, Sanchez-Hernandez *et al.* found that for the 3 native amino acids comprising an  
357 aromatic ring (Tryptophan, Phenylalanine and Tyrosine) LOD values ranged from 10 to 40 ng  
358 [39]. However, these results are worst compared to those found in LC-MS / MS [40], which is  
359 natural as MS/MS methods should be more sensitive than a single-quadrupole MS detection.

360

361 Excellent calibration curves were obtained as appears in Table 3, with  $r^2$  values mostly above  
362 0.99, with a single exception of 0.9857 for Aspartic acid. However, the calculated  
363 concentrations in the samples were significantly different from the ones expected, based on  
364 the information provided by the manufacturers. Very strong positive and negative matrix

365 effects were responsible for the deviations observed. A second method was then explored,  
366 with standard additions and the same internal standard, to eliminate matrix effects.

367

### 368 *3.2.2 Standard addition with internal standard*

369

370 The standard addition method was carried out and validated on the BCAA sample and on  
371 Taurine in the TTC sample, to demonstrate the feasibility of the method in different samples.  
372 The results are presented in Table 4.

373

374 Repeatability of the method (expressed in %RSD) was determined with three replicate  
375 injections of each calibration solution and measured for retention time and peak areas.  
376 Repeatability of retention times was excellent, with %RSD being inferior to 0.1%. This is an  
377 interesting observation as the Acquity UPC<sup>2</sup> instrument was not necessarily designed to  
378 operate under such wide elution gradients. Repeatability of relative peak areas was inferior  
379 to 2% in all cases.

380

381 Precision of the method was demonstrated for four concentration levels (no addition and  
382 three levels of standard addition). Intra-day precision was assessed using six separately  
383 prepared sample solutions at each concentration level and each of them was analyzed three  
384 times. Inter-day precision was evaluated over four days using the same protocol. In the worst  
385 cases, intra-day and inter-day precision were as high as 13 and 15% respectively, but were  
386 mostly below 6 and 12% respectively. In any case, this was still acceptable in this context.  
387 For example, intra-day and inter-day precision on the sample solutions with one standard  
388 addition is shown in Table 4. Compared to the literature, these values are equivalent. For  
389 instance, Liyanaarachchi *et al.* found that the intra-day and inter-day precision were below  
390 10% with LC-MS analysis of rice samples [40].

391

392 While linear calibration curves had been obtained with the external calibration method (Table  
393 3), the standard addition method yielded curves that were better fitted with polynomial  
394 regression (Table 4). This is not uncommon for mass spectrometric detection [41]. The  
395 determination coefficient for polynomial regression was higher than 0.992 for all studied  
396 compounds.

397

398 Finally, accuracy was largely improved with the standard addition method over the external  
399 calibration method as appears in Table 4. Deviations from the theoretical concentrations (as  
400 indicated on the sample packagings) ranged from 8 to 22%.

401

402       **4. Conclusions**

403  
404       The use of an uncommon mobile phase additive (methanesulfonic acid) and a broad elution  
405       gradient ranging from supercritical fluid to liquid mobile phase allowed the elution of 20 free  
406       amino acids with satisfying peak shapes from the zwitterionic column. Furthermore, the  
407       application of a reversed pressure gradient and a reversed flow rate gradient permitted a fast  
408       method with total cycle time of 10 minutes. Application to dietary supplements proved to be  
409       difficult, due to strong matrix effects that were circumvented by a standard addition method.  
410       The proposed method permits the separation of isobaric compounds (Leucine and  
411       Isoleucine) and offers simple sample preparation (no derivatization is required) followed by a  
412       fast analysis, which should be advantageous when large quantities of samples must be  
413       analyzed. Finally, it is worth mentioning that, although no thorough study of column stability  
414       was performed, no impact of the acidic analyte on stationary phase was observed in the  
415       course of this project. It is possible that the degradation may be lessened by the very low  
416       concentration of water (2% in the co-solvent).

417  
418

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425

426       **Compliance with ethical standards**

427       The authors declare they have no conflict of interest.

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581 **Figure captions**

582

583 **Figure 1:** Gradient profile in the optimized method showing the variation of co-solvent (blue  
584 lines), back-pressure (orange lines) and flow rate (green lines).

585

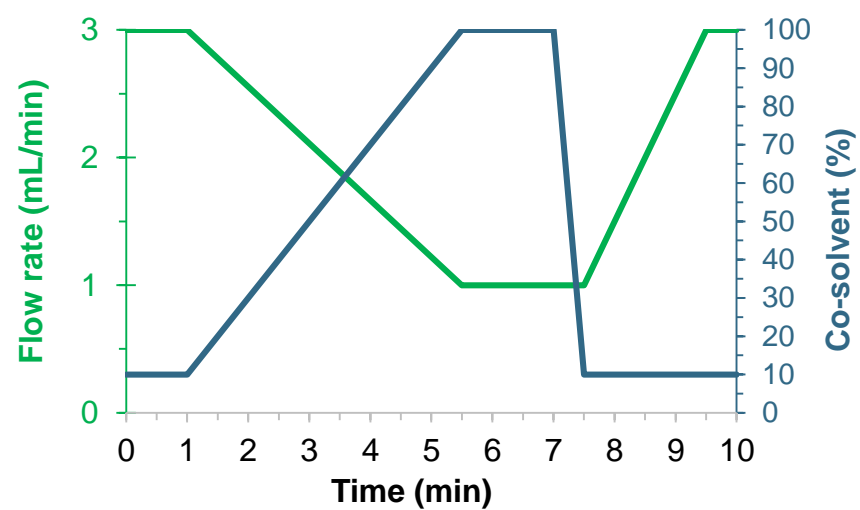
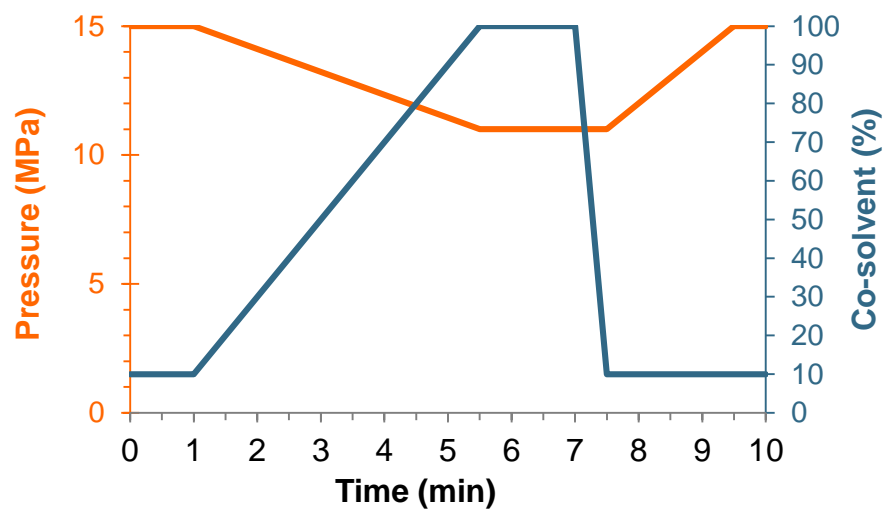
586 **Figure 2:** Chromatograms of all amino acid standards with the optimal gradient program  
587 (defined in figure 1). Other conditions: Chiralpak ZWIX(+) (150 x 3.0, 3  $\mu$ m), 25°C. ESI-MS  
588 detection with single-ion recording (see table 1). (a) Hydrophobic side chains (b) Polar side  
589 chains and special cases (c) Acidic and basic side chains.

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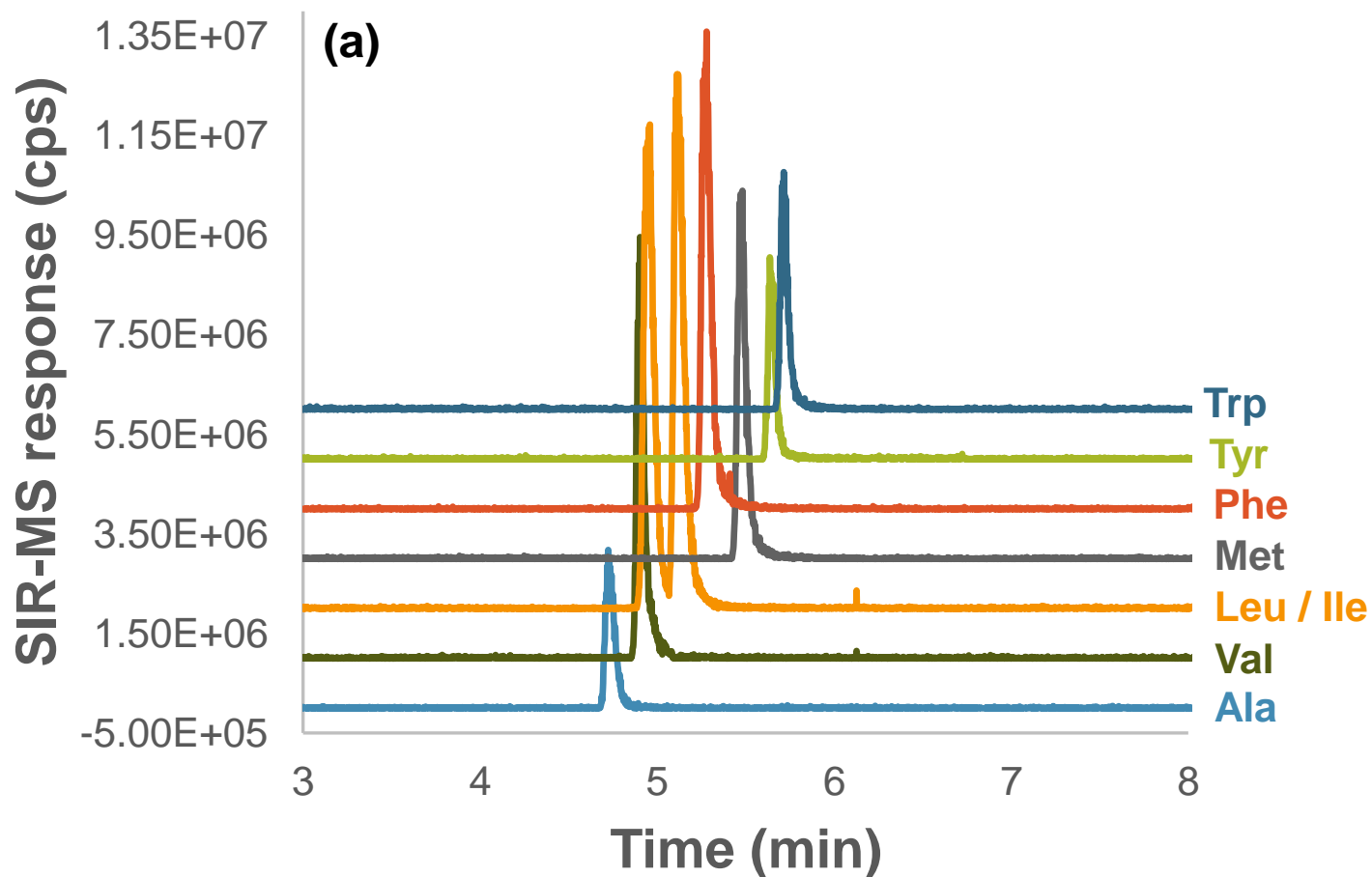
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592 isoleucine) with the (a) initial and (b) optimal gradient program (see text for details). Other  
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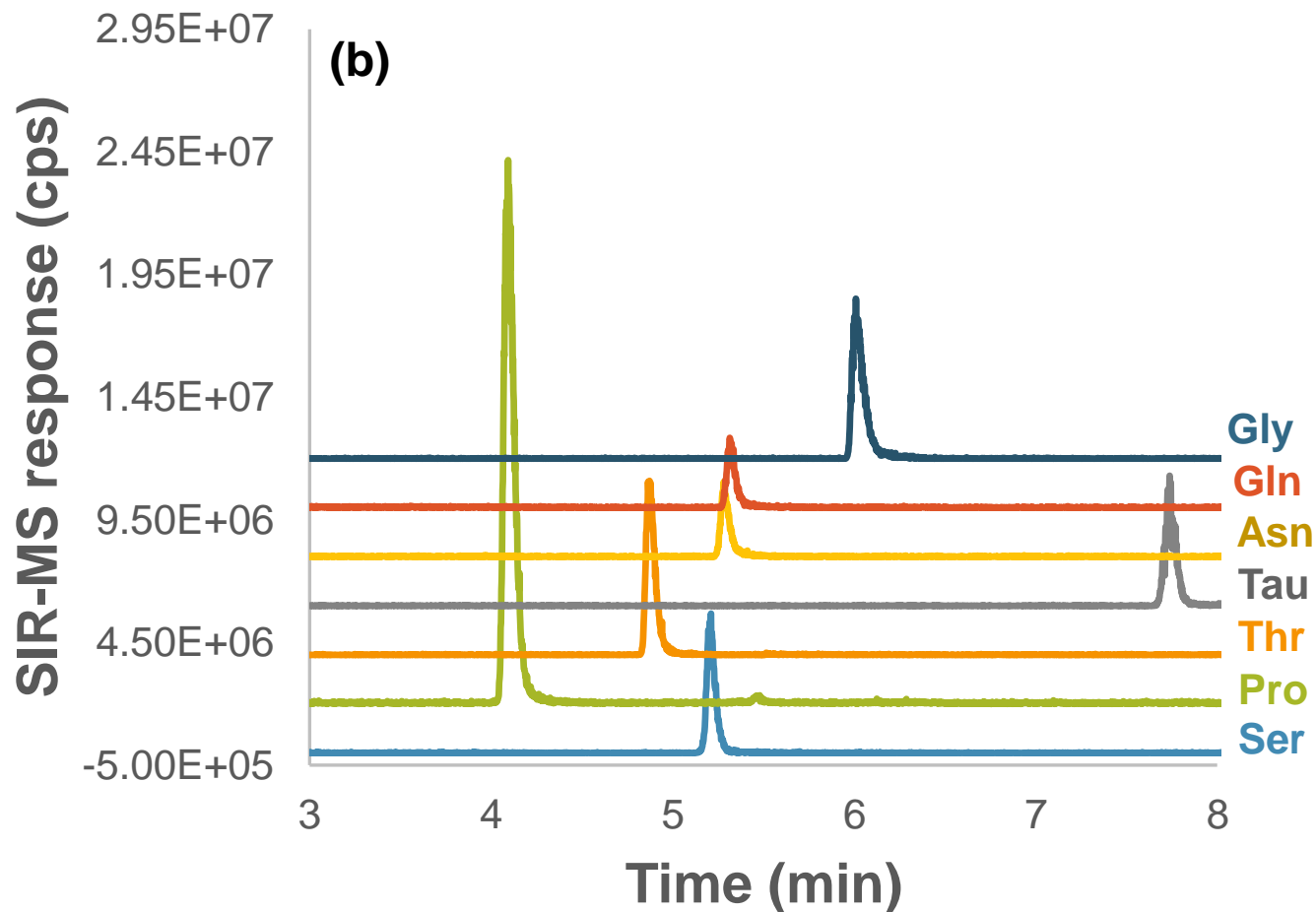
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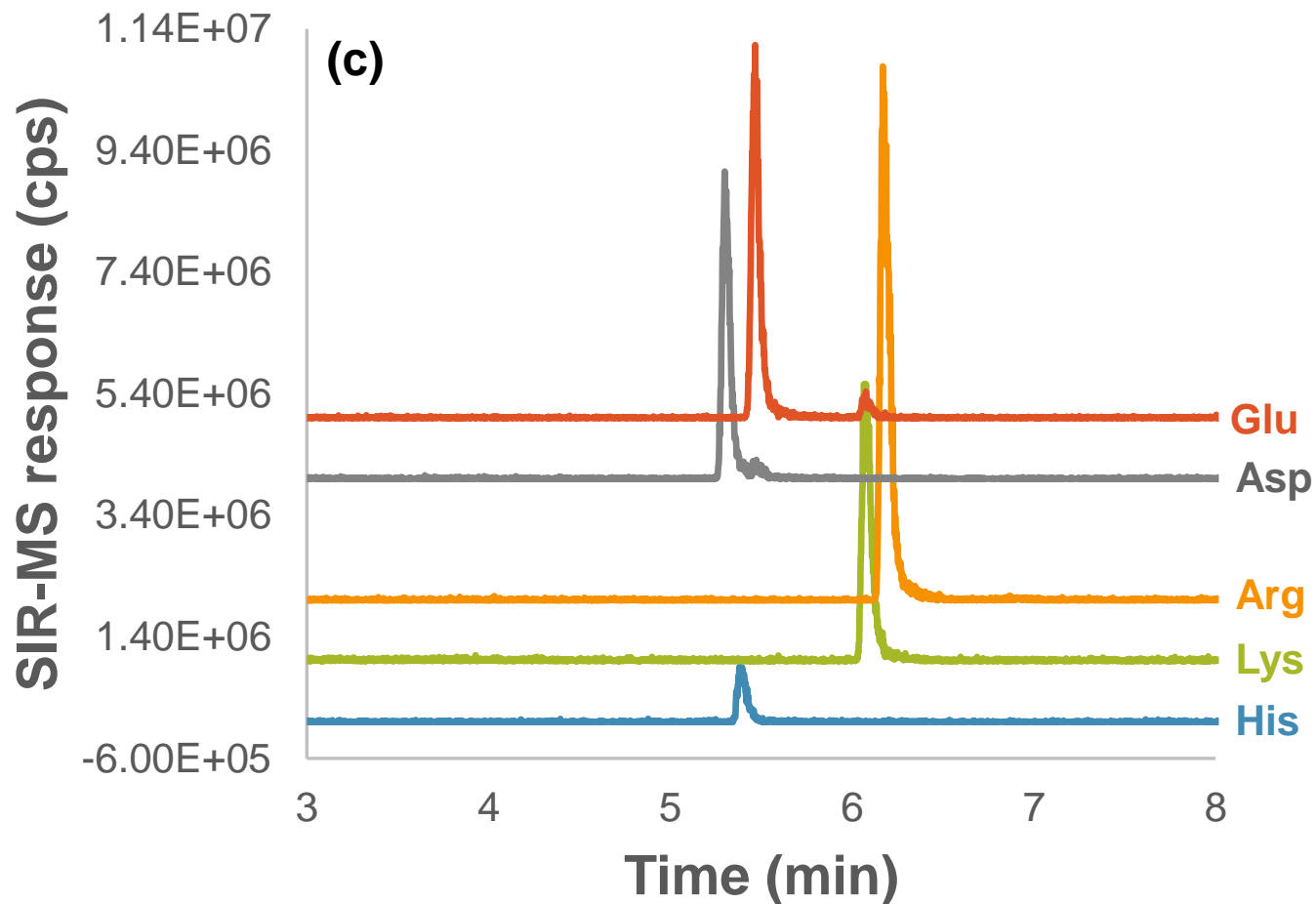
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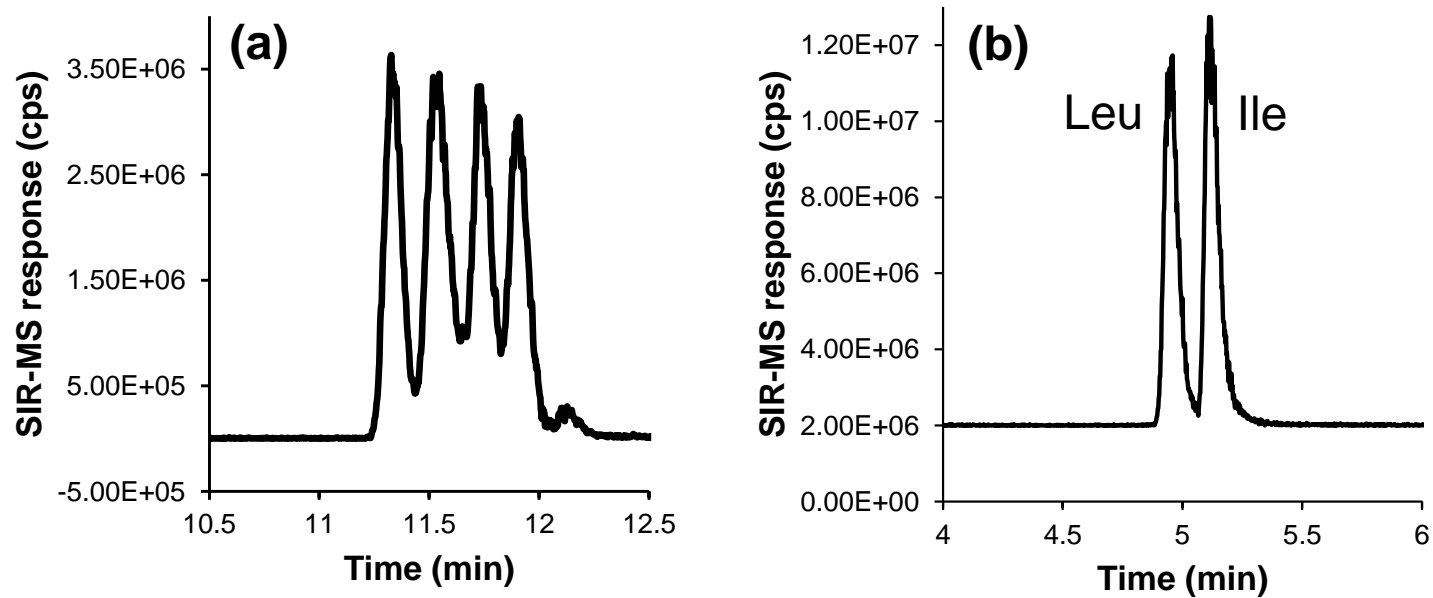
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**Figure 2 (continued).** Chromatograms of all amino acid standards with the optimal gradient program (defined in figure 1). Other conditions: Chiralpak ZWIX(+) (150 x 3.0, 3  $\mu$ m), 25°C. ESI-MS detection (see table 1). (a) Hydrophobic side chains (b) Polar side chains and special cases (c) Acidic and basic side chains.

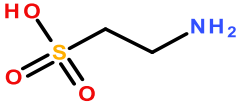
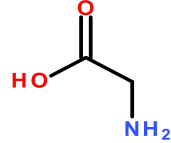
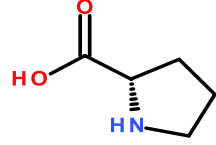
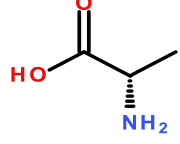
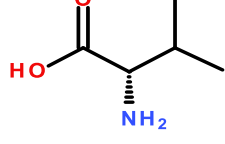
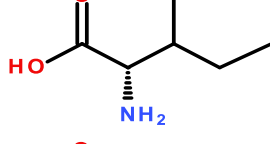
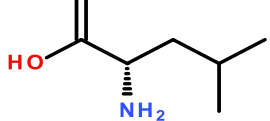


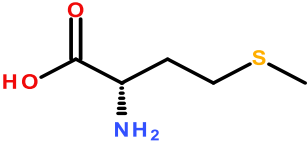
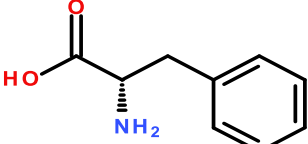
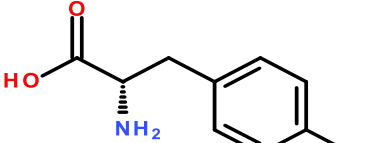
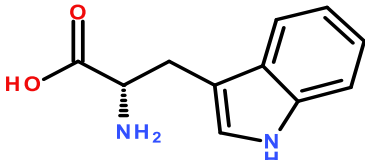
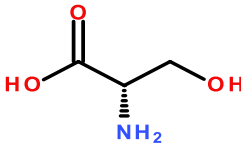
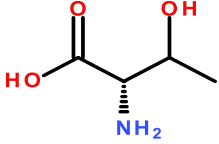
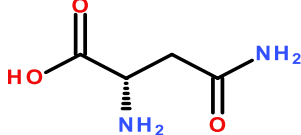
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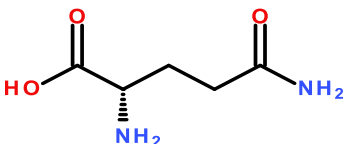
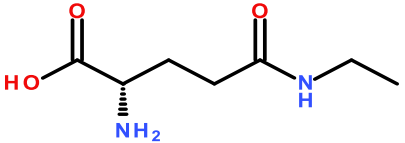
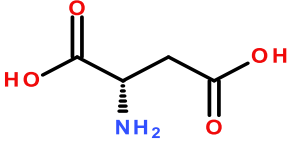
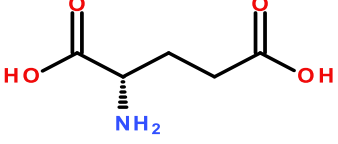
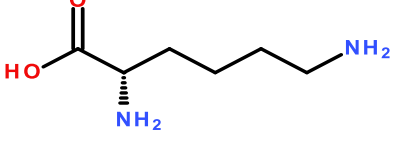
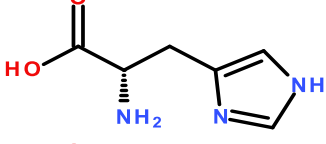
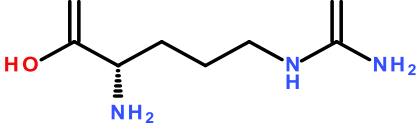
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**Table 1.** Amino acids examined in this work

Sample	Name	Abbreviation	Structure	Amino acids class	tR (min)	MW (g/mol)	ESI(+) m/z
TTC	Taurine	Tau		Special cases	7.69	125.15	126
AAC	Glycine	Gly		Special cases	5.41	75.07	76
AAC	L-Proline	Pro		Special cases	4.05	115.13	116
AAC	L-Alanine	Ala		Hydrophobic side chain	4.73	89.09	90
AAC / BCAA	L-Valine	Val		Hydrophobic side chain	4.92	117.15	118
AAC / BCAA	L-Isoleucine	Ile		Hydrophobic side chain	4.96	131.17	132
AAC / BCAA	L-Leucine	Leu		Hydrophobic side chain	5.14	131.17	132

AAC	L-Methionine	Met		Hydrophobic side chain	5.50	149.21	150
AAC	L-Phenylalanine	Phe		Hydrophobic side chain	5.30	165.19	166
AAC	L-Tyrosine	Tyr		Hydrophobic side chain	5.67	181.19	182
Internal standard	L-Tryptophan	Trp		Hydrophobic side chain	5.74	204.23	205
AAC	L-Serine	Ser		Polar uncharged side chain	5.23	105.09	106
AAC	L-Threonine	Thr		Polar uncharged side chain	4.89	119.12	120
X	L-Asparagine	Asn		Polar uncharged side chain	5.28	132.12	133



AAC	L-Glutamine	Gln		Polar uncharged side chain	5.32	146.14	147
TTC	L-Theanine	The		Polar uncharged side chain	4.86	174.2	175
AAC	L-Aspartic Acid	Asp		Acidic side chain	5.33	133.11	134
X	L-Glutamic Acid	Glu		Acidic side chain	5.50	147.13	148
AAC	L-Lysine	Lys		Basic side chain	6.11	146.19	147
AAC	L-Histidine	His		Basic side chain	6.05	155.16	156
AAC	L-Arginine	Arg		Basic side chain	6.21	174.2	175

**Table 2.** Preparation of standard solutions for quantitation of amino acids.

Group	Amino acid	Concentration (ppm)	MW (g/mol)	C1 (ppm)	C2 (ppm)	C3 (ppm)	C4 (ppm)	C5 (ppm)	C6 (ppm)
1	Glycine	150	75.07	10	50	100	200	300	500
	L-Arginine	200	174.2	10	50	100	200	300	500
	L-Tyrosine	250	181.19	10	50	100	200	300	500
	L-Asparagine	/	132.12	10	50	100	200	300	500
2	L-Serine	400	105.09	100	300	400	500	800	1000
	L-Proline	400	115.13	100	300	400	500	800	1000
	L-Valine	400	117.15	100	300	400	500	800	1000
	L-Alanine	420	89.09	100	300	400	500	800	1000
	L-Threonine	500	119.12	100	300	400	500	800	1000
3	L-Isoleucine	400	131.17	100	300	500	700	800	1500
4	L-Leucine	1600	131.17	200	700	800	1500	1700	2000
5	L-Lysine	750	146.19	200	500	700	800	1000	1500
	L-Methionine	750	149.21	200	500	700	800	1000	1500
	L-Histidine	750	155.16	200	500	700	800	1000	1500
	L-Phenylalanine	750	165.19	200	500	700	800	1000	1500
	Acid L-Aspartic	800	133.11	200	500	700	800	1000	1500
6	Taurine	1000	125.15	500	750	1000	1250	1500	2000
7	L-Glutamine	1400	146.14	750	1000	1500	1750	2000	2500

**Table 3.** Linearity, limits of detection and quantification with external calibration method  
 LOD and LOQ are expressed in terms of amount detected/quantified per analysis

Amino acid	LOD (ng)	LOQ (ng)	Linearity	
			Regression equation	r <sup>2</sup>
Val	12.5	41.7	$y = 0.00049x + 0.03419$	0.9997
Ile	1.0	3.3	$y = 0.00080x + 0.08880$	0.9990
Leu	1.3	4.2	$y = 0.00096x + 0.17094$	0.9999
Ala	4.0	13.3	$y = 0.00015x + 0.01043$	0.9992
Ser	2.5	8.3	$y = 0.00035x + 0.00775$	0.9997
Gln	7.0	23.3	$y = 0.00042x + 0.06272$	0.9992
Thr	2.0	6.7	$y = 0.00042x + 0.03137$	0.9979
Asn	4.5	15.0	$y = 0.00024x + 0.00032$	0.9998
Phe	2.0	6.7	$y = 0.00065x + 0.15310$	0.9939
Asp	6.3	20.8	$y = 0.00021x + 0.06617$	0.9857
Met	2.5	8.3	$y = 0.00067x + 0.03820$	0.9976
Arg	6.3	20.8	$y = 0.00079x - 0.00985$	0.9976
Gly	33.4	111.3	$y = 0.00004x + 0.00065$	0.9987
His	6.3	20.8	$y = 0.00078x + 0.04876$	0.9966
Tyr	5.0	16.7	$y = 0.00039x - 0.00240$	0.9993
Pro	1.0	3.3	$y = 0.00106x + 0.11285$	0.9986
Lys	10.0	33.3	$y = 0.00077x - 0.02023$	0.9973
Tau	5.0	16.7	$y = 0.00071x + 0.05083$	0.9992
Glu	1.5	5.0	Not present in samples	
Trp	1.5	5.0	Internal standard	

**Table 4.** Features of the standard addition method

Amino acid	Polynomial regression		Repeatability (RSD, %, n=3)		Intermediate precision (RSD, %)		Weight in one capsule (mg)		Recovery (%)
	Regression equation	r <sup>2</sup>	Retention time	Peak area	Intra-day (n=6)	Inter-day (n=4)	Theoretical	Determined	
Val	$y = -2E-05x^2 + 0.0194x + 0.9374$	0.9927	0.05	0.9	2.3	14.6	160.0	185.7	116.1
Leu	$y = -2E-05x^2 + 0.0312x + 1.1716$	0.9974	0.03	1.0	2.5	12.3	160.0	146.4	91.5
Ile	$y = -2E-05x^2 + 0.0304x + 0.7506$	0.9970	0.06	1.5	2.4	13.8	90.0	97.2	108.0
Tau	$y = -2E-06x^2 + 0.0054x + 0.1661$	0.9998	0.02	1.1	3.6	11.0	100.0	121.7	121.7