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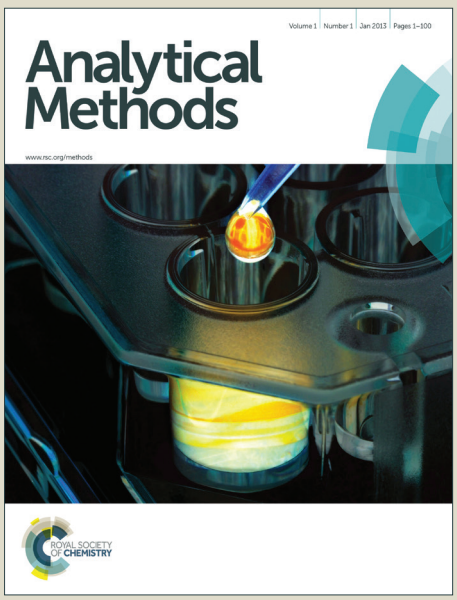





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# Determination of free amino acids in plants by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

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## ABSTRACT

**A robust and sensitive method for identification (quantification and confirmation) of 19 free amino acids in plant matrice - *Stellaria media*, based on liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), with a triple quadrupole analyser, has been developed.**
**Regarding MS optimization, the flow injection analysis (FIA) was used in Scan and selected reaction monitoring (SRM) mode. The collision energies optimized varied from -12 to -39 eV. The acquisition of three MS/MS transitions for most of compounds allowed the accurate confirmation of these analytes, which was supported by the accomplishment of ion intensity ratios and retention time as compared with the corresponding standards. The use of Phenomenex EZ:faast $^{(TM)}$Free (Physiological) Amino Acid kit speeds up the sample preparation immeasurably. Nineteen amino acids were separated within 18 minutes on reverse-phase column under a gradient stepwise programme using 10 mM ammonium formate both in water and methanol. The detection limit (LOD) of free amino acids varied from 0.4 to 9.1 pmol mL$^{-1}$, except for asparagine amounting 3000 pmol mL$^{-1}$. The quantification precision (RSD) of free amino acids for intra- and interday assays was 0.05 to 19% and 0.2 to 19% respectively, but for most of compounds did not exceed 5%. The optimized and validated method was subsequently utilized for free amino acids identification in weed collected from field location in Poland.**


**Keywords:** free amino acids, *Stellaria media*, LC-MS/MS, SRM, triple quadrupole

## INTRODUCTION

Weeds are considered as undesirable plants in crops. Some weeds are recognised as a direct threat to agricultural production and biodiversity all over the world. Because in most situations they are unwanted plants, they could be either chemically treated with the aid of herbicides or removed mechanically leading to a problem with their recycling [1]. In the latter situation, the collected biomass is usually discarded or sometimes utilized to produce feed. Another possible way of making use of weeds may be acquisition of bio-active metabolites [2]. *Stellaria media* (common chickweed) is considered as weed in many countries but it also possesses some distinctive features that are less known. It is medicinal plant, rich in valuable substances able to cure various diseases, it is substrate for cosmetics and finally it is edible, both for animals and for humans [3–5].

Twenty three amino acids (AA) are regarded as proteinogenic, meaning they are precursors to proteins. Human being and other animals can synthesise eleven of them, which means we have to obtain the rest from the food. Apart from AA building the protein chain, there is always a pool of free amino acids (FAA), synthesised from simple common intermediates, like pyruvate, ketoglutarate, 3-phosphoglycerate [6]. FAA are found in living cells, in amounts which vary according to the tissue and to the AA. Generally they occur in only trace amounts and plant matrices are very complex, so their analysis in plant material creates some analytical problems. Regardless of separation technique as liquid chromatography or gas chromatography coupled to different mass analysers, the sample preparation for AA determination is very laborious due to protein precipitation, extraction and very often derivatisation step [7–10] which is needed because AA are polar compounds and reverse phase separation is hindered [11–13].

There are many commercially available amino acids kits, generally aiming to speed up and facilitate sample preparation procedure i.e. Phenomenex EZ:faast$^{(TM)}$ , Waters Masstrak, AB Sciex aTRAQ, Perkin Elmer neogram AAAC kit, which are preferred by analitical chemists to traditional time-consuming methods [12].

One of them is EZ:faast$^{(TM)}$ LC-MS or GC-MS Free AA kit which allows rapid purification and derivatisation of the free AA in less than 20 minutes. It was successfully used by Fonteh et al. [14] to quantify free AA and dipeptides in human samples with their instrumental analysis on LC-MS/MS with the total run time of 35.5 min. and also by many other scientists to determine AA in biological fluids [11,15–17]. However, there are no reports to date on the application of EZ:faast$^{(TM)}$ and LC–MS/MS for the analysis of AA in plants. Only Mncwangi and Viljoen [18] reported a method for AA quantitation in *S.frutescens* (Cancer bush) utilising Phenomenex EZ:faast$^{(TM)}$kit and LC-MS/MS, however plant sample was treated with 50% acetonitrile and 0,1% formic acid solvent mixture, which altered the native state of AA. Furthermore, some MS conditions and Multiple Reaction Monitoring (MRM) settings included in the EZ:faast protocol can vary depending on the instrument, therefore should be checked in order to obtain the most reliable and optimum results. Unfortunately, the authors did not present the validation study, thus comparison between the methods is not possible.

Not rarely, authors alter the amino acid composition by using organic solvent for extraction [19]. Strong reagents either cause solubility differences or amino acids modifications. Our group was looking for a method that will show the native AA composition, which in further plants analysis like stress evaluation, will serve the best.

Nimbalkar et al. [20] present method for FAA profile in grain Amaranth, although not showing the whole validation study. Kıvrak et al. [21] state that their method, validated for giant puffball mushroom (*Calvatia gigantea*), could be applied to other food for studying FAA content, although no example is provided.

This work presents the first incorporation of EZ:faast$^{(TM)}$ kit and liquid chromatography coupled to tandem mass spectrometry (LC-ESI-MS/MS) to study FAA composition in plants in native state. The identification, quantification and confirmation of 19 proteinogenic amino acids in weed matrice, with a total run time of 18 minutes is presented. Due to important biological functions of amino acids, their accurate analysis is crucial to the life science and food industry.

## EXPERIMENTAL

### Chemicals and reagents

LC-MS grade methanol and water as well as eluent-additive LC-MS ultra ammonium formate ($NH_4HCO_2$) were purchased from Fluka Analytical (St. Louis, MO, USA). Amino acids standards at a concentration of 200 nmol mL$^{-1}$ and the derivatisation reagents were included in the EZ:faast$^{(TM)}$ LC-MS Free Amino Acid kit (Phenomenex, Torrance, CA, USA). The standard mixtures were stored in a freezer as some amino acids are not stable in solution[22].

A mixed stock solution of AA at the concentration of 1 nmol mL$^{-1}$ was prepared using LC-MS grade water and stored for a maximum time frame of three months at -20°C. For quantitation purposes, stock solution was diluted in water to prepare a working range of solutions for calibration from 0.01 to 40 nmol mL$^{-1}$.

Common chickweed (*S. media*) seeds were obtained from Department of Weed Science and Tillage Systems, Institute of Soil Science and Plant Cultivation (Wrocław, Poland), cultivated in greenhouse and harvested at flowering.

## Liquid chromatography

LC analysis was carried out using Shimadzu Prominence UFLC$_{XR}$ system, equipped with a LC-30ADXR binary solvent manager, a DGU-20A3 degasser, a CTO-10ASVP column oven, a SIL-20AXR autosampler and a CBM-20A system controller, and interfaced to a triple quadrupole analyser. For the chromatographic separation, an EZ:faast$^{(TM)}$4u AAA-MS column, 3µm, 250 x 2.0 mm (Phenomenex, Torrance, CA, USA) at a flow rate of 0.25 mL min$^{-1}$ was used. The separation was performed under reversed phase conditions. The column was kept at 35°C. Mobile phase consisted of water/methanol (A/B) gradient both 10 mM ammonium formate where the methanol percentage was changed linearly as follows: 0 min, 68%; 13 min, 83%; 13.01 min, 68%; 18 min, 68%. All samples were analysed under the above mentioned chromatographic conditions and the sample volume injected in the UHPLC system was 10 µL.

## Mass spectrometry

The tandem mass spectrometer LCMS-8030 (Shimadzu, Kyoto, Japan) with ultra fast polarity switching and ultra fast MRM transitions was used for analysis. Drying gas as well as nebulising gas was nitrogen, generated from pressurized air in a $N_2$ LC-MS pump, working at flows 15 L min$^{-1}$ and 3 L min$^{-1}$, respectively. Desolvation line temperature was maintained at 250°C and heat block temperature was 400°C. Collision induced dissociation gas (CID) was argon 99.999% (Linde, Wrocław, Poland) at a pressure of 230 kPa. Dwell time of 10 ms was selected. For UHPLC analysis, LabSolution Ver. 5.6 (Shimadzu, Kyoto, Japan) software was used to process quantitative data obtained from calibration standards and from weed samples.

## Additional equipment

An ultrasonic water bath was obtained from Polsonic (Poland), a vortex-mixer from VWR (Gdańsk, Poland) and centrifuge from Hettich (Kirchlengern, Germany). Ovation micropipettes were obtained from VistaLab Technologies (Mt Kisco, NY, USA). Additional consumables like syringes (0.6 and 1.5 mL), sample preparation vials, microdispenser, autosamples vials with inserts and sorbent tips were included in the EZ:faast$^{(TM)}$ kit (Phenomenex).

## Sample preparation

In order to determine AA content in plant samples (leaves and stalks), collected from pot experiments, they were placed in a mortar and ground with a pestle using liquid nitrogen. The samples (0.5 mg) were hand shaken with 10 mL LC-MS grade water for 2 min, followed by 15 min sonification in ultrasonic bath. Homogenates were centrifuged at 11000g for 15 min at 4ºC to obtain supernatants, ready to be analysed on the EZ:faast$^{(TM)}$ Free Amino Acid kit. Five replicates of each extract were done. The procedure of using EZ:faast$^{(TM)}$ kit is transparent and straightforward, however it was conducted according to the manufacturer's protocol [22] with some modifications [17]. Internal standards homoarginine (HARG), methionine-d3 (Met-d3) and homophenylalanine (HPHE) stock solution were diluted 100-fold obtaining the final concentration of 2 nmol mL$^{-1}$ in order to avoid detector saturation and unreliable results.

In brief, the procedure consists of solid phase extraction, derivatisation and finally liquid-liquid extraction step. 100 µL of aqueous plant extract and 100 µL of I.S. diluted 100-fold were passed slowly through the proprietary sorbent tip, which was attached to a 1.5 mL syringe. AA were bound on the sorbent medium, while interfering compounds were not retained. No additional precipitation step was needed, because they were excluded from the sample as it passeed through the sorbent tip. Next, the sorbent with attached AA was ejected to a vial, where the derivatisation process took place adding 50 µL of a solution of propyl chloroformate. The reaction derivatises both the amine and carboxyl groups of the amino acids forming a stable derivative. Derivatised amino acids simultaneously migrate to the organic layer for additional clean-up. 70 µL aliquot of the organic layer was evaporated under vacuum to dryness. Finally, amino acids were re-dissolved in 100 µL of a mixture of mobile phase A:B 1:2 (v/v) and analysed on the LC-MS/MS instrument. The whole procedure takes around 45 minutes [22].

## Validation study

The linearity of the method was studied by analyzing standard solutions in triplicate at 7 concentrations ranging from 0.01 to 40 nmol $mL^{-1}$. Adequate linearity, using weighted (1/X) least square regression was satisfactory, when square correlation coefficient ($R^2$) was higher than 0.99, based on peak area. Accuracy (estimated by means of recovery experiment) was evaluated by analysing five independent *S. media* samples spiked at two concentration levels each 0.2 and 2.0 nmol $mL^{-1}$ with amino acids internal standards included in the EZ:faast$^{(TM)}$ Free Amino Acid kit: homoarginine (HARG), methionine-d3 (Met-d3) and homophenylalanine (HPHE) at the beginning of the experiment. Precision (expressed as repeatability in terms of relative standard deviation) was evaluated by analysing five independent *S. media* samples spiked at two concentration levels each (0.2 and 2.0 nmol $mL^{-1}$) with all 22 amino acids. The instrumental limit of detection was estimated for a signal-to-noise ratio of three from chromatograms of standards. The LOQ was the lowest concentration for which the quantification transitions had a signal-to-noise ratio ≥ 10.

## Application to real samples

Common chickweed (*Stellaria media*) samples were cultivated in greenhouse. They were sown continuously every two weeks to obtain fresh plant material. The conditions were constant and equal in every experiment and kept to 21°C, pH=6.6, 60% water holding capacity and 14h of light exposure. Weeds meant for cognitive analysis were collected from field locations in the Lower Silesia region of Poland in September 2014. Samples were analysed immediately after collection.

In every sequence of analysis on LC-MS/MS, derivatised amino acids were injected by triplicate, preceded by calibration curve. Internal quality control i.e. *Stellaria media* sample fortified with all 3 I.S. at the concentration of 2 nmol $mL^{-1}$ was run every ten injections to allow system reproducibility monitoring and was considered satisfactory if the recoveries were in range 70-120%. Also pure methanol was injected every ten samples to check for carryover or contaminants during the analysis.

Confirmation of positive findings was carried out by calculating the peak area ratio between the confirmation (q) and quantification (Q) transition and comparing them with the corresponding reference standard. AA concentrations of *Stellaria media* were calculated from the calibration curves of each AA.

## RESULTS AND DISCUSSION

### MS and MS/MS optimization

Full scan and MS/MS spectra were obtained during flow injection analysis (FIA) of each derivatised amino acids. To obtain derivatised amino acids suitable for optimization, the following procedure was applied: The aliquots (200 µL) of Standard 1 (ALA, ARG, ASP, GLU, GLY, HIS, ILE, LEU, LYS, MET, PHE, PRO, SER, THR, TYR, VAL Standard Solution), Standard 2 (ASN, GLN, TRP Standard Solution) and Reagent 1 (I.S.) all of concentration 200 nmol $mL^{-1}$, were dispensed in two individual sample vials and the standard procedure described was applied. However, during the last step, organic layers from two sample vials were transferred to one vial and evaporated to dryness with a nitrogen stream. It was then reconstituted in 200 µL of mixture of mobile phase, obtaining derivatised AA concentration of 200 nmol $mL^{-1}$ and used for FIA.

All amino acids of a concentration of 200 nmol $mL^{-1}$ water: methanol (1:2) with 10 mM ammonium formate were subjected to FIA at a flow rate of 0.4 mL $min^{-1}$, 30% water and 70% methanol and injection volume of 1 µL. Optimization was achieved by utilizing the 15000 u $sec^{-1}$ high-speed performance in conjunction with the autosampler's FIA technique. Compared to the conventional infusion method, instrument contamination was decreased through reductions in sample concentration and injection volume. All AA were analysed under positive ionisation mode, showing an abundant $[M+H]^+$ ion, for each derivatised amino acid.

While working with triple quadrupole, it is well known that at least two specific transitions should be acquired for each compound. The first one used for quantification (Q) purposes and the second one as confirmation (q) [23]. For 15 out of 22 compounds even three transitions were acquired, whereas for 5 compounds two. For ALA and ASP (see abbreviations in Table 1), only one transition could be chosen. We paid special attention to non-specific transitions and tried to avoid them, not to report false positives. However, in the case of LEU and ILE, the isomeric AA, both shared the same transitions but with different ion-ratios. Thus, the quantification and confirmation transitions selected were interchanged. Nevertheless, the chromatographic conditions selected were able to fully resolved them. Furthermore, at least 10 points per peak are needed to provide a good peak shape with acceptable reproducibility. Under this work, a total of 55 Selected Reaction Monitoring (SRM) transitions were acquired during 18 min chromatographic run with low dwell times (10ms) without sensitivity losses. The use of a fast acquisition triple quadrupole mass analyser allows acquiring three simultaneous transitions per compound without sensitivity losses and cross-talk leading to reliable identification. Selected mass spectrometry parameters, like transitions, collision energy, $t_R$ and ion intensity ratios of selected transitions are summarized in Table 1.

### Method validation

In this work, validation of the method was based on the ICH Q2(R1) [24] guidelines and European Union SANCO/12571/2013 guidelines [25]. The latter were incorporated due to specific criteria concerning LC-MS/MS technique parameters, to demonstrate that this analytical procedure is suitable for its intended purpose. *S. media* was chosen for validation purposes.

## Calibration curve

Linearity of the method was studied in the range 0.01 to 40 nmol $mL^{-1}$ for all standards of selected AA, obtaining satisfactory results for most of compounds. Seven concentration points were assayed in triplicate (0.01; 0.1; 1.0; 2.0; 10.0; 20.0; 40.0 nmol $mL^{-1}$). The square correlation coefficient ($r^2$) $\geq$ 0.99 was achieved for 15 amino acids while for other 7 AA was very close ($r^2 \geq 0.97$) with residuals always lower than 20% (Table 2). It is worth mentioning that quantitation is based on external standardization. Twenty two SRM chromatograms of standards at the concentration of 2 nmol $mL^{-1}$ can be seen in Figure 1.

## Precision

Precision (expressed as repeatability in terms of relative standard deviation) was evaluated by analysing five independent *S. media* samples spiked at two concentration levels (0.2 and 2.0 nmol $mL^{-1}$) with all 22 AA. All experiments were performed in triplicate over three following days. The intraday quantification relative standard deviation was in the range from 1.8 to 18.6% and from 0.06 to 3.9% for 0.2 and 2.0 nmol $mL^{-1}$, respectively. The interday quantification relative standard deviation was in the range from 4.4 to 18.8% and from 0.2 to 13.0% for 0.2 and 2.0 nmol $mL^{-1}$, respectively. For most of compounds RSD did not exceed 5%. All obtained intra- and interday results are acceptable and show satisfactory precision needed for plant study. No significant changes in retention time expressed as relative standard deviation were observed (min. RSD% = 0.004% and max. RSD% = 2.5%), except for Met-d3 and HPHE amounting 2.6% (Table 2 ), which is in accordance with the 2.5% limit for LC analysis provided by European Union SANCO/12571/2013 guidelines [25].

## Recovery

In order to evaluate the robustness and accuracy of the developed method, recovery experiments were performed. Five *S. media* samples were spiked with amino acids internal standards included in the EZ:faast$^{(TM)}$ Free Amino Acid kit: homoarginine (HARG), methionine-d3 (Met-d3) and homophenylalanine (HPHE) at two different concentrations of 0.2 and 2.0 nmol $mL^{-1}$. Blank samples with no addition of internal standards were also prepared to subtract the levels of possible target compounds. Samples were measured in triplicate. No significant amounts of I.S. were present in blank matrices, i.e. HARG was not detected, Met-d3 and HPHE were present in higher amounts than LOD amounting 1.0 and 2.1 pmol $mL^{-1}$, respectively, but in lower amounts than LOQ amounting 4.0 and 6.0 pmol/mL, respectively.

Accuracy was regarded satisfactory if the recovery test for spiked sample was between 70 and 120%. The best results were obtained for HPHE with average recoveries amounting 108 and 101% for 0.2 and 2.0 nmol $mL^{-1}$, respectively, with the RSD for retention time not extending 1.8%. Also very good results were obtained for Met-d3, with average recoveries amounting 115 and 106% for 0.2 and 2.0 nmol $mL^{-1}$, respectively, with the RSD for retention time not extending 1.9%. HARG showed the lowest recoveries in all tests, although kept in acceptable range: for 0.2 and 2.0 nmol $mL^{-1}$, average recoveries amounted 70 and 77%, with the RSD for retention time of 0.9%. These results were supported by the accomplishment of ion intensity ratios and did not exceed ±10% which is in accordance with the maximum permitted tolerances for relative ion intensities using MS techniques (±30%) provided by European Union SANCO/12571/2013 guidelines. All of the recovery results were in range of 70 - 115%, meaning the accuracy of our method is suitable.

## Limit of detection

The instrumental limit of detection range was 0.4 – 9.1 pmol $mL^{-1}$ for the 21 amino acids, except for asparagine amounting 3000 pmol $mL^{-1}$, calculated according to a signal-to-noise ratio (S/N) of 3. The limit of quantitation (LOQ), defined as the concentration resulting in S/N ratio ≥ 10 was in range 1.5 to 27.7 pmol $mL^{-1}$, except for asparagine amounting 9132.0 pmol $mL^{-1}$. Furthermore, the method was found very sensitive and highly specific, as no relevant signals were observed to coelute with the individual amino acids standards, except for asparagine (Table 2).

## *Stellaria media* analysis

After method validation, *S. media* samples obtained from field locations in the Lower Silesia region of Poland were analysed (n=5) for the 19 free amino acids composition and quantification according to the presented procedure. Samples were injected by triplicate, preceded by calibration curve. Internal quality control and pure methanol were run every ten samples.

It can be observed that all of 19 analysed free AA is present in this plant. These results are more comprehensive than the one presented by Kieloch et al. [26] who quantified only 3 FAA in *S. media*. Moreover, they are not on a par with the one obtained by Shan et al. [27] who found only 16 free AA in *S. media*. GLN and ASP are present in high amounts, exceeding 580 mg $kg^{-1}$ . However, the AA which is the most abundant in *S. media* is GLU, amounting 941 mg $kg^{-1}$. Similar results were presented by Arnáiz et al. [28] who detected the highest amounts of GLN and PRO in broccoli leaves using supercritical fluid extraction. High amounts of GLN could be due to glutamate synthase cycle, where GLN is formed from simple compounds, like ammonia and only afterwards other AA are formed, like ARG, TRP, HIS or ASN. Three aromatic AA - PHE, TYR and TRP are synthesised through the shikimate pathway which is only found in microorganisms and plants and vary from 11 to 46 mg $kg^{-1}$ [6]. In relation to the total content of AA in *S. media* it exceeded 3000 mg $kg^{-1}$. and show that this weed could be a great and underestimated source of proteinogenic AA, especially GLU, GLN and ASP.

## CONCLUSIONS

To sum up, a robust and sensitive method using LC-ESI-MS/MS for the target measurement of free AA in weed matrix was optimized and further applied in plant samples. Satisfactory results have been obtained, both regarding quantitative and confirmative issues within the same chromatographic run. The method presented shows great repeatability, accuracy and sensitivity afforded by mass spectrometry at the same time reducing the sample preparation time to minimum, due to the use of EZ:faast$^{(TM)}$ Free Amino Acids kit. A big advantage of this kit is elimination of laborious precipitation procedure, because all interfering compounds are either retained on the sorbent tip or stay in water phase, meaning decreased time and the cost of the analysis. Once derivatised, AA are stable for several hours at room temperature and for 4 days if refrigerated, preventing sample loss by degradation.

Derivatisation step, although not indispensable in LC-MS/MS analysis, is a good choice, when working with complex plant matrix. It improves peak efficiency, stabilize the

amino acid concentration and lower the detection limit. Thus, together with short time needed for sample preparation utilizing liquid nitrogen followed by EZ:faast$^{(TM)}$ kit, this method is fully justified. Tandem mass spectrometry is the method of choice for metabolite profiling in complex natural extracts. To increase the confirmatory capability of this method, SRM mode was used for all AA and three respective transitions were monitored for most of compounds together with the ion intensity ratio (q/Q) which did not exceed 10%.

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**Table 1**. Amino acids, AA abbreviated name, retention time (min.), transitions chosen for each compound, collision energy used in LC-ESI-MS/MS to obtain quantification transition and ion ratio for confirmation and quantification transition (q/Q).

| Compound name | Abbreviated name | $t_R$ (min) | Quantification transition | Collision energy (eV) | Confirmation transition | Ion ratio 1 (q/Q) |
|---|---|---|---|---|---|---|
| Arginine | ARG | 3.3 | 303.20>70.10 | -39 | 303.20>156.05<br>303.20>114.05 | 0.5 |
| Glutamine | GLN | 3.8 | 275.20>172.00 | -31 | 275.20>84.00 | 0.8 |
| Serine | SER | 4.4 | 234.20>146.00 | -12 | 234.20>104.00 | 0.8 |
| Asparagine | ASN | 4.4 | 243.20>157.20 | -15 | 243.20>115.10<br>243.20>211.30 | 0.8 |
| Glycine | GLY | 4.9 | 204.20>76.00 | -16 | 204.20>102.00<br>204.20>144.10 | 1.0 |
| Threonine | THR | 5.1 | 248.20>74.05 | -22 | 248.20>160.00<br>248.20>188.10 | 0.7 |
| Alanine | ALA | 6.1 | 218.20>130.20 | -13 | -[a] | - |
| Methionine | MET | 8.1 | 278.20>190.15 | -12 | 278.20>142.00<br>278.20>218.00 | 0.3 |
| Proline | PRO | 8.2 | 244.20>156.05 | -14 | 244.20>70.20<br>244.20>113.95 | 0.4 |
| Lysine | LYS | 8.9 | 361.30>170.10 | -22 | 361.30>301.05<br>361.30>128.10 | 1.0 |
| Aspartic acid | ASP | 8.8 | 304.00>216.15 | -14 | - | - |
| Histidine | HIS | 8.9 | 369.90>110.15 | -36 | 369.90>196.15<br>369.90>284.20 | 0.7 |
| Valine | VAL | 9.2 | 246.20>158.15 | -14 | 246.20>116.05<br>246.20>186.05 | 0.8 |
| Glutamic acid | GLU | 9.4 | 318.20>230.05 | -14 | 318.20>258.10 | 0.8 |
| Tryptophan | TRP | 9.7 | 333.20>245.15 | -17 | 333.20>159.20<br>333.20>230.00 | 0.4 |
| Leucine | LEU | 10.9 | 260.20>172.15 | -13 | - | - |
| Phenylalanine | PHE | 10.9 | 294.20>206.20 | -14 | 294.20>120.05<br>294.20>163.95 | 0.8 |
| Isoleucine | ILE | 11.3 | 260.20>130.10 | -20 | - | - |
| Tyrosine | TYR | 13.3 | 396.20>136.05 | -33 | 396.20>222.00<br>396.20>308.10 | 0.9 |
| Homoarginine[b] | HARG | 3.6 | 317.3>84.15 | -40 | 317.3>128.10<br>317.3>126.00 | 0.4 |
| Methionine-d3[b] | Met-d3 | 8.1 | 281.20>193.00 | -12 | 281.20>142.20<br>281.20>221.00 | 0.3 |
| Homophenylalanine[b] | HPHE | 12.2 | 308.20>117.15 | -22 | 308.20>219.95<br>308.20>104.20 | 1.1 |

[a] The second and third transition were not monitored for this compound
[b] Internal standards

**Table 2.** Repeatability of LC-MS/MS analysis of amino acids in *Stellaria media* matrice using aliquots of 10 uL of sample (n=5), square correlation coefficient ($R^2$), limits of detection and quantification.

| $R^2$ | AA abbreviated name | RSD% $t_R$ intraday [nmol $mL^{-1}$] | | RSD% $t_R$ interday [nmol $mL^{-1}$] | | RSD% C[a] intraday [nmol $mL^{-1}$] | | RSD% C interday [nmol $mL^{-1}$] | | LOD [pmol $mL^{-1}$] | LOQ [pmol $mL^{-1}$] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.2 | 2.0 | 0.2 | 2.0 | 0.2 | 2.0 | 0.2 | 2.0 | | |
| 0.99 | GLY | 1.1 | 0.1 | 1.1 | 0.1 | 5.1 | 0.1 | 5.1 | 5.1 | 1.6 | 5.7 |
| 0.99 | ALA | 1.3 | ≈ 0 | 1.5 | 0.1 | 18.6 | 0.1 | 18.8 | 7.0 | 2.4 | 7.4 |
| 0.99 | SER | 0.9 | 0.1 | 0.8 | 0.1 | 4.2 | 0.1 | 5.8 | 6.6 | 0.7 | 2.4 |
| 0.99 | ASN | 1.0 | 0.8 | 0.9 | 1.1 | 13.8 | 1.1 | 12.9 | 3.2 | 3014.0 | 9132.0 |
| 0.99 | PRO | 1.5 | 0.1 | 1.9 | 0.1 | 12.3 | 0.1 | 13.6 | 4.6 | 0.8 | 2.4 |
| 0.99 | VAL | 1.6 | 0.1 | 2.1 | 0.1 | 15.9 | 0.1 | 15.3 | 7.8 | 2.4 | 7.3 |
| 0.99 | THR | 1.1 | 0.1 | 1.1 | 0.1 | 5.1 | 0.1 | 6.8 | 4.4 | 1.7 | 5.3 |
| 0.99 | LEU | 1.6 | ≈ 0 | 2.2 | 0.1 | 6.0 | 0.1 | 8.3 | 7.5 | 1.3 | 19.2 |
| 0.99 | ILE | 1.6 | 0.1 | 2.1 | 0.1 | 11.5 | 0.1 | 10.7 | 5.1 | 1.0 | 16.3 |
| 0.99 | GLN | 0.9 | 0.1 | 0.8 | 0.1 | 9.3 | 0.1 | 9.1 | 13.0 | 3.4 | 10.2 |
| 0.98 | MET | 1.6 | 0.1 | 2.1 | 0.1 | 6.9 | 0.1 | 7.0 | 2.5 | 0.5 | 1.5 |
| 0.99 | PHE | 1.6 | ≈ 0 | 2.2 | 0.1 | 6.6 | 0.1 | 6.3 | 3.5 | 0.6 | 1.8 |
| 0.99 | ARG | 0.7 | 0.2 | 0.6 | 0.2 | 3.6 | 0.2 | 5.7 | 6.9 | 7.0 | 21.1 |
| 0.98 | ASP | 1.7 | 0.1 | 2.2 | 0.1 | 1.8 | 0.1 | 7.4 | 6.0 | 0.7 | 2.0 |
| 0.99 | GLU | 1.7 | ≈ 0 | 2.3 | 0.1 | 9.3 | 0.1 | 10.3 | 10.2 | 0.8 | 2.5 |
| 0.98 | TRP | 1.7 | 0.1 | 2.4 | 0.1 | 4.1 | 0.1 | 4.8 | 3.8 | 1.6 | 4.8 |
| 0.98 | LYS | 1.9 | 0.1 | 2.5 | 0.1 | 7.8 | 0.1 | 7.2 | 6.0 | 3.4 | 10.3 |
| 0.99 | HIS | 1.8 | 0.1 | 2.3 | 0.1 | 6.6 | 0.1 | 5.0 | 5.2 | 0.9 | 13.8 |
| 0.99 | TYR | 1.6 | 0.1 | 2.1 | 0.1 | 5.7 | 0.1 | 5.6 | 6.9 | 9.1 | 27.7 |
| 0.99 | HARG[b] | 0.9 | ≈ 0 | 1.2 | ≈ 0 | 5.9 | 3.9 | 4.9 | 9.6 | 3.5 | 9.6 |
| 0.99 | Met-d3[b] | 1.9 | ≈ 0 | 2.6 | 0.1 | 7.1 | 0.1 | 12.9 | 0.2 | 1.0 | 4.0 |
| 0.99 | HPHE[b] | 1.8 | 0.1 | 2.6 | 0.1 | 5.2 | 2.6 | 4.4 | 1.9 | 2.1 | 6.0 |

[a] concentration of each AA [nmol $mL^{-1}$]
[b] internal standards

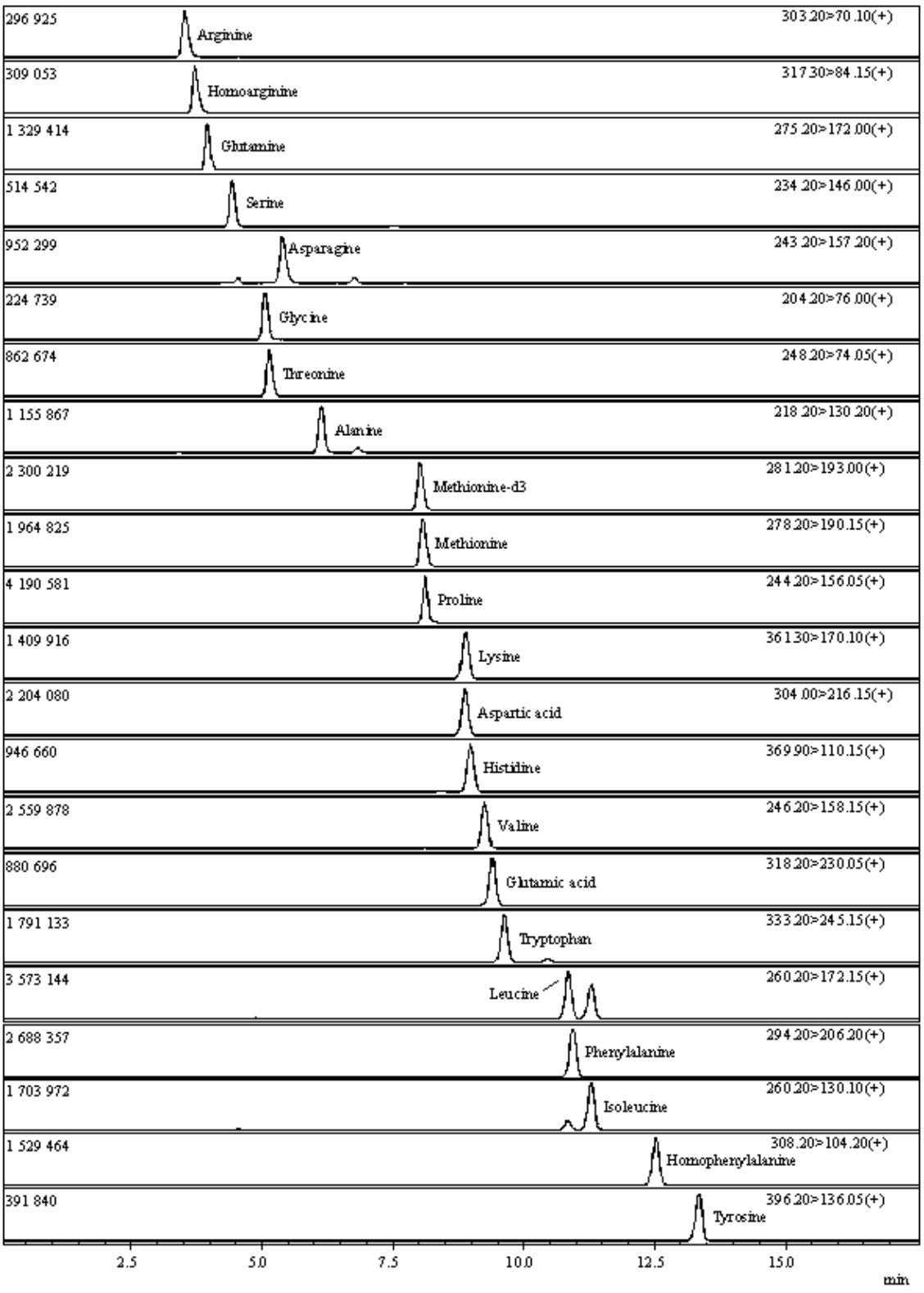


**Fig.1** Selected reaction monitoring chromatograms of all 22 AA standards of the concentration 2 nmol $mL^{-1}$, based on quantification transition.

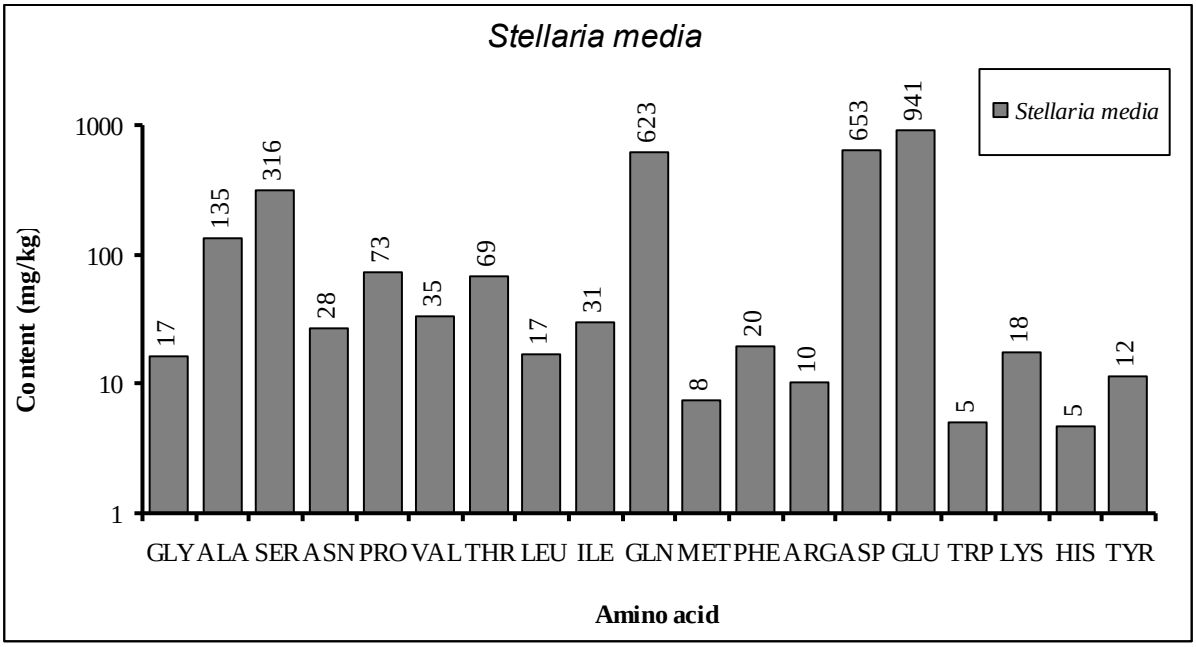


**Fig.2** *Stellaria media* (n = 5) free amino acids content obtained from field location.

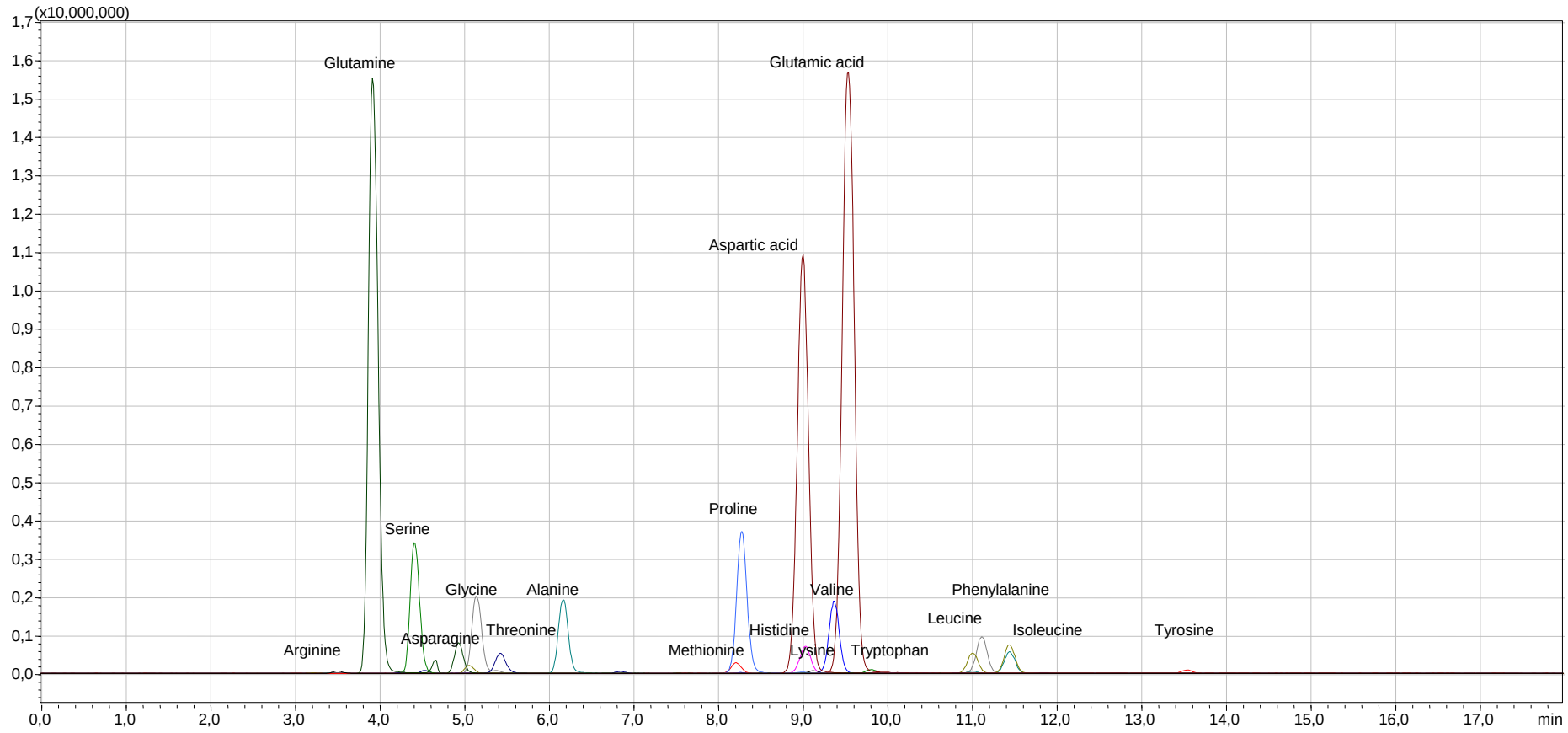


**Fig.3** Chromatogram illustrating *Stellaria media* AA content, obtained from field location.