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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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Complete List of Authors:	Dziągwa, Magdalena; Institute of Soil Science and Plant Cultivation, Department of Weed Science and Tillage Systems Marin Ramos, Jose; Research Institute of Pesticides and Water, University Jaume I, Topolski, Jakub; Institute of Soil Science and Plant Cultivation, Department of Weed Science and Tillage Systems Oleszek, Wiesław; Institute of Soil Science and Plant Cultivation,, Department of Biochemistry and Crop Quality

STATEMENT OF SOCIETAL IMPACT

The manuscript " Determination of free amino acids in plants by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)" describes the analysis of 19 proteinogenic amino acids by UHPLC-MS/MS using the EZ:faast™ LC-MS Free Amino Acid kit commercially available from Phenomenex. Optimization of the MS as well as MS/MS parameters for confirmation and quantification transitions of each amino acid is presented. Validation of the method including the extraction step was performed with standard solutions and spiked weed plant samples with regard to the linear range, linearity, LODs, LOQs and intra/inter-day precision. Recoveries had been determined by weed samples spiked with amino acid internal standards from the EZ:faast™ LC-MS Free Amino Acid kit. The validated method has been applied to the analysis of *Stellaria media* weed samples.

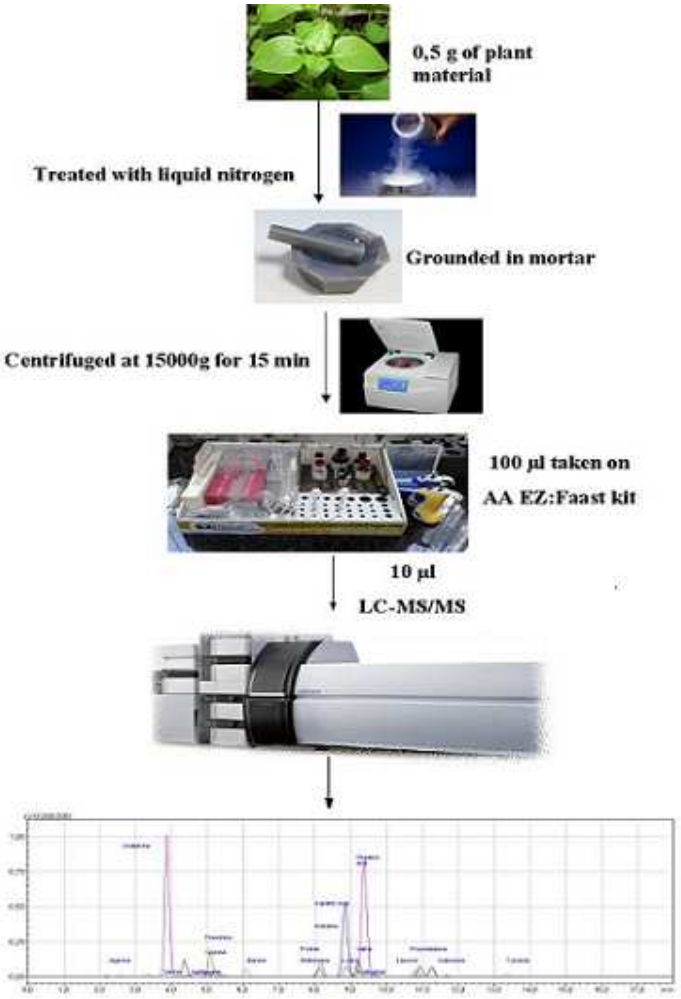
- ✓ Application of the EZ:faast™ LC-MS Free Amino Acid kit has been published by several other authors, however the novelty of this paper is its first application to plant samples after the comprehensive validation of the method in order to achieve accurate analysis of free amino acids.
- ✓ Our aim was to validate the method according to strict guidelines - ICH Q2 (R1) and also European SANCO, due to specific criteria concerning LC-MS/MS technique parameters in order to demonstrate that this analytical procedure is suitable for its intended purpose in plant matrices.
- ✓ As the EZ:faast™ kit is known and used widely among scientists, we think that complete validation for all proteinogenic amino acids in plants would be desirable, as we were not able not find any manuscript dealing with these issues, therefore societal impact is justified.
- ✓ Furthermore, many papers were published utilizing this kit, but most of them analyse only few amino acids. They neither present the MS/MS optimization parameters for all amino acids nor validation procedure, so the reliability of those methods is doubtful.
- ✓ Extraction using water when plants amino acids remain in native state is a merit for many scientists dealing with plant stress, when they are interested in non-altered plant composition in order to check how plants react after physiological stress. Moreover, not all amino acids are soluble in organic solvents or their solubility is hindered. As far as we are concerned there has not been any article incorporating water and the EZ:faast™ kit to study free amino acids so we feel it is worth and significant to fill this niche.

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

Magdalena M. Dziągwa[a]*, Jose M. Marin Ramos[b], Jakub K. Topolski[a], Wiesław A. Oleszek[c]

[a] Institute of Soil Science and Plant Cultivation, State Research Institute, Department of Weed Science and Tillage Systems, Wrocław, Poland
[b] Research Institute of Pesticides and Water, University Jaume I, Castellon, Spain
[c] Institute of Soil Science and Plant Cultivation, State Research Institute, Department of Biochemistry and Crop Quality, Puławy, Poland

***Corresponding author**: Tel.: +48 71 336 26 58; fax: +48 71 363 86 91; Wrocław ul. Orzechowa 61, 50-540, Poland, email: m.dziagwa@iung.wroclaw.pl



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, except for asparagine amounting 3000 pmol/mL. 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].

Twenty three 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[4]. 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 this is why their analysis in plant material creates some analytical problems. Irrespective of choice for the final determination instrument as LC or GC coupled to MS detectors, the sample preparation for AA is very laborious due to protein precipitation, extraction and very often derivatisation step[5–8] because AA are polar compounds and reverse phase separation is hindered [9–11].

There are a lot of 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 [10].

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. [12] to quantify free AA and dipeptides in human samples with their final determination 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 [9,13–15]. 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[16] report 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, the authors took MRM settings included in the EZ:faast protocol for granted, which are known to vary depending on the instrument, mobile phase and MS conditions and therefore should be verified before analysis, 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[17]. Nimbalkar et al. [18] present method for FAA profile in grain Amaranth, although not showing the whole validation study. Kıvrak et al.[19] state that their method, validated for giant puffball mushroom (*Calvatia gigantea*), could be applied to other food for studyig FAA content, although no example is provided.

This work presents the development, validation and application of a new method based on liquid chromatography coupled to tandem mass spectrometry LC-MS/MS for the identification, quantification and confirmation of 19 proteinogenic amino acids in weed matrice, with a total run time of 18 minutes.

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 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.

A mixed stock solution of AA at the concentration of 1 nmol/mL 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.

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 UHPLC system, equipped with a binary solvent manager, a degasser, column oven and an autosampler, 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 was used. 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, obtained from pressurized air in a N_2 LC-MS pump, working at a flow rate 15 L/min and 3 L/min, 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.

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 11000rpm 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 with some modifications [15,20]. Internal standards (Reagent 1) included in the EZ:faast$^{(TM)}$ kit: homoarginine (HARG), methionine-d3 (Met-d3) and homophenylalanine (HPHE) were diluted 100-fold obtaining the final concentration of 2 nmol/mL. Initial 200 nmol/mL concentration even after final aliquot dilution was causing detector saturation and unreliable results.

In brief, the analysis consists of solid phase extraction, derivatisation and finally liquid-liquid extraction step. 100 µL of weed extract and 100 µL of I.S. diluted 100-fold were passed slowly through the sorbent tip, which was attached to a 1.5 mL syringe. AA were bound on the sorbent, while interfering compounds flew through. No additional precipitation step was needed. Next, the sorbent with attached AA was ejected to a vial, where the derivatisation process took place using propyl chloroformate. The reaction derivatises both the amine and carboxyl groups of the amino acids forming a highly stable derivative. Derivatised amino acids simultaneously migrate to the organic layer for additional separation. 70 µL of the organic layer was transferred and 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.

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. 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 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) 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 in growth chamber were constant and equal in every experiment. Weeds meant for cognitive analysis were collected from field locations in the Lower Silesia region of Poland. 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 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 eliminate any carryover and 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, 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 and used for FIA.

All amino acids of a concentration of 200 nmol/mL water: methanol (1:2) with 10 mM ammonium formate were subjected to FIA at a flow rate of 0.4 mL/min, 30% water and 70% methanol and injection volume of 1 µL. Optimization was achieved by utilizing the 15000 u/sec 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). For 15 out of 22 compounds three transitions were acquired, whereas for 3 compounds two. In order to obtain specific transitions for ALA, ASP, LEU and ILE (see abbreviations in Table 1), only quantification transition was chosen. We paid special attention to non-specific transitions and tried to avoid them, not to report false positives. 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)[21] guidelines and European Union SANCO/12571/2013 guidelines[22]. 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 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). The square correlation coefficient (r^2) ≥ 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 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) 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, 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, 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[22].

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. 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, 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, 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 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, 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 for the 21 amino acids, except for asparagine amounting 3000 pmol/mL, 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, except for asparagine amounting 9132.0 pmol/mL. 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. [23] who quantified only 3 FAA in *S. media*. Moreover, they are not on a par with the one obtained by Shan et al.[24] who found only 16 free AA in *S. media*. GLN and ASP are present in high amounts, exceeding 580 mg/kg . However, the AA which is the most abundant in *S. media* is GLU, amounting 941 mg/kg. Similar results were presented by Arnáiz et al. [25] 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 [4]. In relation to the total content of AA in *S. media* it exceeded 3000 mg/kg. 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>156.05 303.20>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>115.10 243.20>211.30	0.8
Glycine	GLY	4.9	204.20>76.00	-16	204.20>102.00 204.20>144.10	1.0
Threonine	THR	5.1	248.20>74.05	-22	248.20>160.00 248.20>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>142.00 278.20>218.00	0.3
Proline	PRO	8.2	244.20>156.05	-14	244.20>70.20 244.20>113.95	0.4
Lysine	LYS	8.9	361.30>170.10	-22	361.30>301.05 361.30>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>196.15 369.90>284.20	0.7
Valine	VAL	9.2	246.20>158.15	-14	246.20>116.05 246.20>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>159.20 333.20>230.00	0.4
Leucine	LEU	10.9	260.20>172.15	-13	-	-
Phenylalanine	PHE	10.9	294.20>206.20	-14	294.20>120.05 294.20>163.95	0.8
Isoleucine	ILE	11.3	260.20>130.10	-20	-	-
Tyrosine	TYR	13.3	396.20>136.05	-33	396.20>222.00 396.20>308.10	0.9
Homoarginine[b]	HARG	3.6	317.3>84.15	-40	317.3>128.10 317.3>126.00	0.4
Methionine-d3[b]	Met-d3	8.1	281.20>193.00	-12	281.20>142.20 281.20>221.00	0.3
Homophenylalanine[b]	HPHE	12.2	308.20>117.15	-22	308.20>219.95 308.20>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]		RSD% t_R interday [nmol/mL]		RSD% C[a] intraday [nmol/mL]		RSD% C interday [nmol/mL]		LOD [pmol/mL]	LOQ [pmol/mL]
		0.2	2.0	0.2	2.0	0.2	2.0	0.2	2.0		
0.9897	GLY	1.1	0.1	1.1	0.1	5.1	0.1	5.1	5.1	1.6	5.7
0.9972	ALA	1.3	≈ 0	1.5	0.1	18.6	0.1	18.8	7.0	2.4	7.4
0.9907	SER	0.9	0.1	0.8	0.1	4.2	0.1	5.8	6.6	0.7	2.4
0.9902	ASN	1.0	0.8	0.9	1.1	13.8	1.1	12.9	3.2	3014.0	9132.0
0.9934	PRO	1.5	0.1	1.9	0.1	12.3	0.1	13.6	4.6	0.8	2.4
0.9883	VAL	1.6	0.1	2.1	0.1	15.9	0.1	15.3	7.8	2.4	7.3
0.9988	THR	1.1	0.1	1.1	0.1	5.1	0.1	6.8	4.4	1.7	5.3
0.9865	LEU	1.6	≈ 0	2.2	0.1	6.0	0.1	8.3	7.5	1.3	19.2
0.9962	ILE	1.6	0.1	2.1	0.1	11.5	0.1	10.7	5.1	1.0	16.3
0.9904	GLN	0.9	0.1	0.8	0.1	9.3	0.1	9.1	13.0	3.4	10.2
0.9802	MET	1.6	0.1	2.1	0.1	6.9	0.1	7.0	2.5	0.5	1.5
0.9977	PHE	1.6	≈ 0	2.2	0.1	6.6	0.1	6.3	3.5	0.6	1.8
0.9983	ARG	0.7	0.2	0.6	0.2	3.6	0.2	5.7	6.9	7.0	21.1
0.9758	ASP	1.7	0.1	2.2	0.1	1.8	0.1	7.4	6.0	0.7	2.0
0.9925	GLU	1.7	≈ 0	2.3	0.1	9.3	0.1	10.3	10.2	0.8	2.5
0.9829	TRP	1.7	0.1	2.4	0.1	4.1	0.1	4.8	3.8	1.6	4.8
0.9847	LYS	1.9	0.1	2.5	0.1	7.8	0.1	7.2	6.0	3.4	10.3
0.9970	HIS	1.8	0.1	2.3	0.1	6.6	0.1	5.0	5.2	0.9	13.8
0.9942	TYR	1.6	0.1	2.1	0.1	5.7	0.1	5.6	6.9	9.1	27.7
0.9989	HARG[b]	0.9	≈ 0	1.2	≈ 0	5.9	3.9	4.9	9.6	3.5	9.6
0.9953	Met-d3[b]	1.9	≈ 0	2.6	0.1	7.1	0.1	12.9	0.2	1.0	4.0
0.9997	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]
[b] internal standards

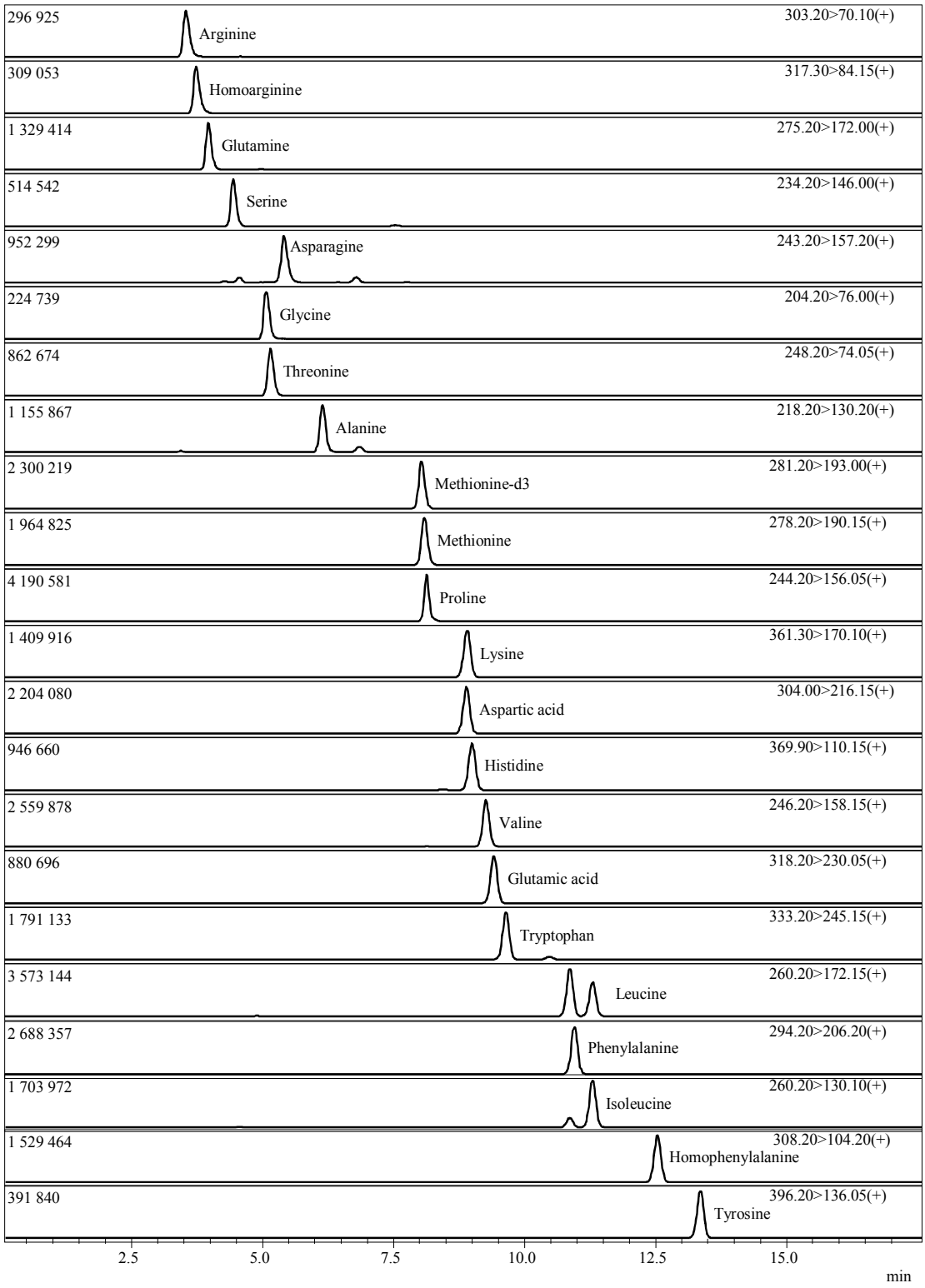


Fig.1 Selected reaction monitoring chromatograms of all 22 AA standards of the concentration 2 nmol/mL, based on quantification transition.

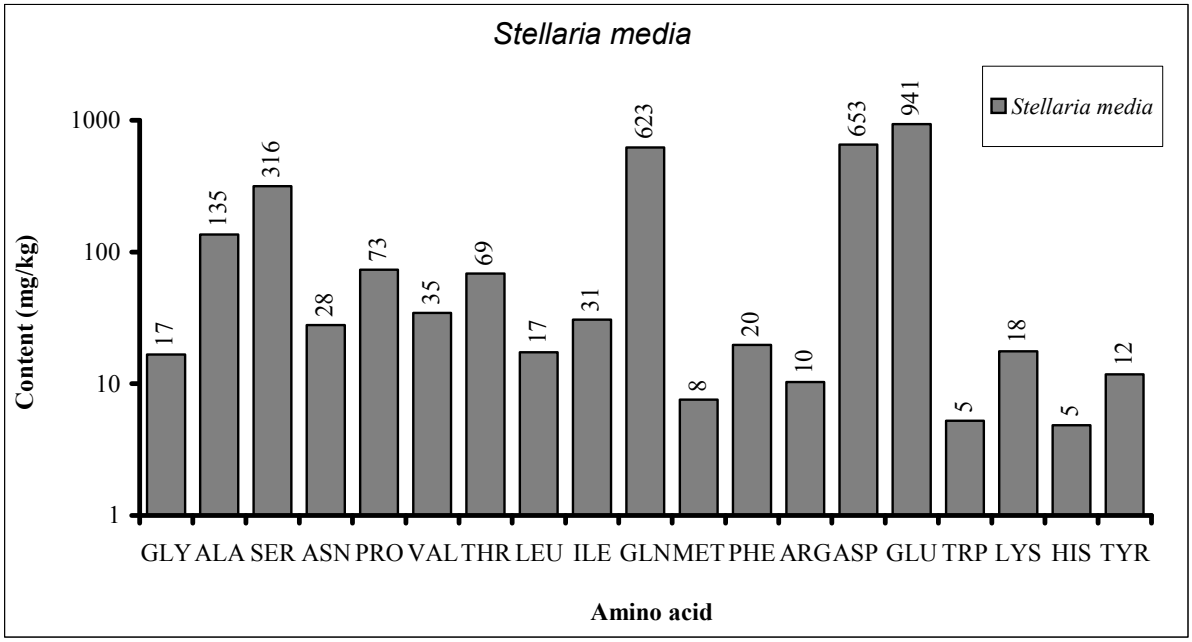
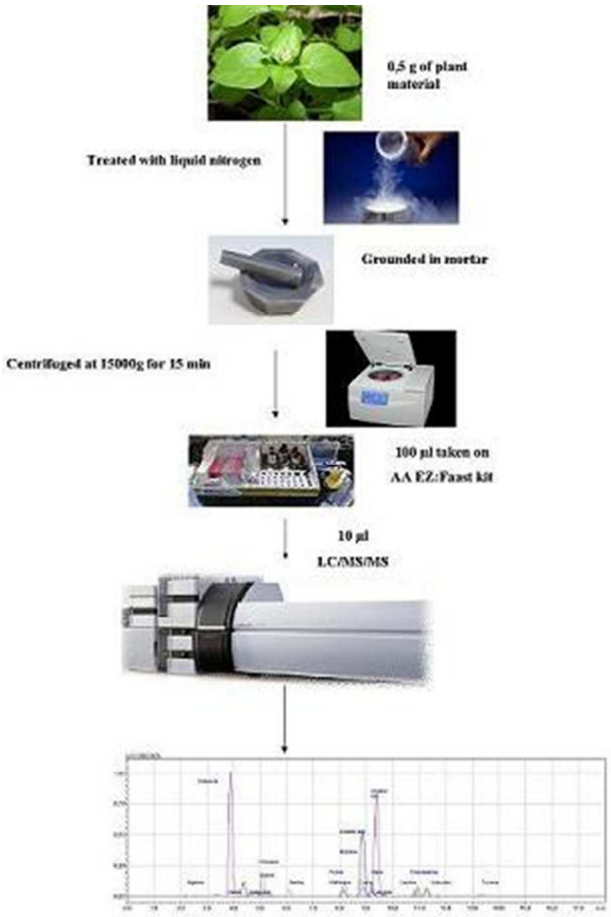


Fig.2 *Stellaria media* (n = 5) free amino acids content obtained from field locations.



A robust and sensitive method for identification (quantification and confirmation) of 19 free amino acids in plant matrice, based on liquid chromatography-electrospray ionization-triple quadrupole analyser (LC-ESI-MS/MS), is presented.
85x121mm (96 x 96 DPI)