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ORIGINAL ARTICLE

Analysis of amino acids by HPLC/electrospray negative ion tandem mass spectrometry using 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) derivatization

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Abstract A new method for the determination of amino acids is presented. It combines established methods for the derivatization of primary and secondary amino groups with 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) with the subsequent amino acid specific detection of the derivatives by LC–ESI–MS/MS using multiple reaction monitoring (MRM). The derivatization proceeds within 5 min, and the resulting amino acid derivatives can be rapidly purified from matrix by solid-phase extraction (SPE) on HR-X resin and separated by reversed-phase HPLC. The Fmoc derivatives yield several amino acid specific fragment ions which opened the possibility to select amino acid specific MRM transitions. The method was applied to all 20 proteinogenic amino acids, and the quantification was performed using L-norvaline as standard. A limit of detection as low as 1 fmol/µl with a linear range of up to 125 pmol/µl could be obtained. Intraday and interday precisions were lower than 10 % relative standard deviations for most of the amino acids. Quantification using L-norvaline as internal standard gave very similar results compared to the quantification using deuterated amino acid as internal standards. Using this protocol, it was possible to record the amino acid profiles of only a single root from *Arabidopsis thaliana* seedlings and to compare it with the amino acid profiles of 20 dissected root meristems (200 µm).

Keywords *Arabidopsis thaliana* · Amino acids · Mass spectrometry · Derivatization · Root meristems

Introduction

Amino acids serve as building blocks for proteins, are precursors for the biosynthesis of nucleic acids (Stryer 1988; Buchanan et al. 2000; Wu et al. 2013), for many pharmaceutically active or health promoting substances (Buchanan et al. 2000; Ziegler and Facchini 2008; Dewick 2009), and for the production of biofuel with higher energy content (Zhang et al. 2008). Furthermore, a set of essential amino acids (e.g., nine compounds for humans including histidine, methionine, and tryptophan) must be supplied to mammalian organisms by the diet in order to avoid malnutrition. Based on these and other numerous applications for amino acids, there is a high interest in the modification of amino acid contents in living organisms, especially in plants (Binder 2010). Amino acid biosynthetic pathways are interconnected and co-regulated, so that modifications in one specific amino acid also affect other amino acids. Therefore, methods able to record the entire set of proteinogenic amino acids are required. Additionally, the huge chemical diversity, especially in plants, demands highly selective methods, in order to discriminate amino acids from the large number of other compounds. Screening or breeding programs to obtain organisms with altered amino acid content and composition preferentially demand very small amounts of sample. Furthermore, in order to obtain a deeper understanding of regulatory processes in cells, methods to perform single cell type analysis are being developed (Wang and Bodovitz 2010). Both approaches require sensitive detection methods. A recent report analyzing metabolite profiles of specific cell types in *Arabidopsis*

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thaliana roots required several hundred thousand cells (isolated from 5,000 to 12,000 plants) for the detection of some amino acids (Moussaieff et al. 2013).

Several methods are commonly applied for amino acid analysis (Kaspar et al. 2009; Mandrioli et al. 2013). For measurements using gas chromatography coupled to mass spectrometry (GC–MS), amino acids are derivatized in order to reduce the polarities of functional groups (Kvitvang et al. 2011). However, for different analytes different derivatization procedures have to be employed to achieve best results. Depending on the number of functional groups, derivatization can lead to the formation of multiple products, thereby complicating quantification. Additionally, derivatization can lead to artifact formation and conversion of analytes, as discussed for the conversion of arginine to ornithine (Halket et al. 2005). High performance liquid chromatography (HPLC) coupled with optical detection such as UV (HPLC–UV) or fluorescence (HPLC-FLD) is often applied. For optical detection, the amino groups are derivatized either before or after separation with an UV absorbing or fluorescent reagent (Le Boucher et al. 1997; Toyo'oka 1999; Rigas 2013). In some cases, the dyes can be simultaneously used for fluorescence, UV, and electrochemical detection (Joseph and Davies 1983; Pappa-Louisi et al. 2007). Derivatization of amino groups with appropriate dyes is rapidly accomplished, and the development of dyes led to increasing sensitivity in the low nanomolar range (Kaspar et al. 2009; Rigas 2013). An advantage of those methods is the high reproducibility which enables high level of automatization (Le Boucher et al. 1997; Kaspar et al. 2009). However, UV or fluorescent signal do not allow discrimination between individual amino acids, so that all derivatized amino acids need to be chromatographically separated, which requires longer analysis times (Le Boucher et al. 1997). Additionally, discrimination between amino acids and non-specific fluorescent signals (e.g., other amines, reagent impurities) is not possible (Jambor and Molnár-Perl 2009; Kaspar et al. 2009; Rigas 2013). Additionally, the fluorescent property of tryptophan interferes with fluorescence from the fluorescent label leading to a poor detection of this amino acid.

Capillary electrophoresis (CE) has also been applied for amino acid analysis with similar sensitivity compared to liquid chromatography. However, if optical detection is performed, the constraints in terms of selectivity are the same as described for liquid chromatography (Kaspar et al. 2009; Poinsot et al. 2010).

Recently, LC–MS/MS-based methods using multiple reaction monitoring (MRM) are considered as a convenient alternative to enhance selectivity (Piraud et al. 2003; Nagy et al. 2003; Piraud et al. 2005; Gu et al. 2007; Thiele et al. 2008). However, the wide range of charges and polarities of underivatised amino acids imposes difficulties identifying the right separation system, which is also compatible with MS detection. This has been overcome by ion-pair chromatography on reverse phase columns using different eluent additives (Piraud et al. 2003, 2005; Armstrong et al. 2007; Gu et al. 2007; de Person et al. 2008), cation exchange chromatography (Thiele et al. 2008), or hydrophilic interaction chromatography (HILIC) (Langrock et al. 2006; Zhou et al. 2013). Jander et al. (2004) developed a high throughput HPLC–MS/MS assay with separation times of only 1.5 min on a Phenyl-Hexyl column. However, separation of isoleucine and leucine could not be achieved. All these methods exhibit high ion suppression, caused either by the presence of high concentrations of the ion-pair reagent, and/or interference with signals derived from the matrix (Kaspar et al. 2009; Pande et al. 2014), which leads to lower sensitivity. In order to increase signal to noise ratios for the analytes, it is beneficial to detect compounds exhibiting a high *m/z* ratio. However, the molecular masses of most amino acids are in the same range as unspecific signals derived from eluents or matrix. Furthermore, the small size of amino acids limits the choice of suitable and specific fragments for MRM. As such, most MRM methods rely on the detection of fragments derived after the loss of CO_2, ammonia, water, or formic acid (Jander et al. 2004; Gu et al. 2007; de Person et al. 2008; Zhou et al. 2013). Several derivatization techniques have been employed to facilitate the analysis of amines or amino acids by LC–MS/MS (Casetta et al. 2000; Leavens et al. 2002; Shimbo et al. 2009a, b; Inagaki et al. 2010). Reagents were chosen in order to improve LC-separations and/or ionization efficiencies, and very high sensitivity could be achieved. However, these methods are based on the detection of fragment ions derived from the reagent instead of the derivatized molecule, and structural information about the amino acid is not taken into account.

In addition to ions derived from the chromatographic system, ions derived from unrelated compounds in the sample are the major source of ion suppression leading to increased detection limits. Therefore, efficient sample cleanup is required to achieve maximum sensitivity. However, the wide range of charges and polarities of amino acids imposes difficulties to develop efficient and simple protocols for the quantitative recovery of all amino acids from matrix.

This study was performed to develop a method, which circumvents the limitations with respect to selectivity, sensitivity, and comprehensiveness compared to previous methods. We used a combination of derivatization and LC–MS/MS to selectively and sensitively detect and quantify amino acids from very low amounts of sample. Using an apolar and bulky derivatization reagent of high specificity for amino groups, we were able to enrich all amino acids using a single solid-phase extraction step, to separate the derivatives by reversed-phase HPLC without eluent additives, and to perform MRM with amino acid specific fragments.

Note: During preparation of this manuscript another study describing amino acid determination after Fmoc-Cl derivatization followed by LC–MS/MS detection appeared (Pande et al. 2014), however, without SPE purification of derivatized samples.

Experimental

Chemicals and materials

All L-amino acid standards, ammonium formiate, *n*-pentane (reagent grade), and 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) were commercial products from Sigma-Aldrich (St. Louis, MO, USA). Boric acid, formic acid, and methanol (Rotisolv HPLC Gradient Grade) were purchased from Roth (Karlsruhe, Germany). Acetonitrile (Ultra Gradient HPLC Grade) was acquired from Baker (Deventer, The Netherlands). Deuterium-labeled L-amino acid standards ((5,5,5-D_3)-*L*-Leucine, (Ring-D_5)-*L*-Phenylalanine, (2,4,4-D_3)-*L*-Glutamic acid, (Indole-D_5)-*L*-Tryptophan, (2,3,3,-D_3)-*L*-Serine)) were obtained from Cambridge Isotope Laboratories (Andovar, MA, USA). Ultrapure water (resistivity 18.2 MΩ) was supplied by a TKA X-CAD ultrapure water purification system (Thermo Fisher Scientific, Waltham, MA, USA). Chromabond® Multi 96 filter plates and Chromabond® Sorbent HR-X were from Macherey–Nagel (Düren, Germany).

Standard solutions and plant extracts

All standard solutions were prepared in ultrapure water. The standard stock solution contained 500 pmol/µl of each amino acid and was further diluted in order to record calibration curves.

Plant extracts for determination of reproducibility (intraday, interday), ion suppression, and stability of the amino acid derivatives were prepared from frozen *A. thaliana* seedlings grown on 0.8 % (w/v) agar plates (Murashige and Skoog Medium Caisson Laboratories, North Logan, UT, USA) for 5 days under long day conditions (16 h light, 8 h dark) at room temperature. Seedlings (about 5 mg) were homogenized with a mixer mill MM301 (Retsch, Haan, Germany) at a frequency of 25/s for 50 s with a single 5-mm diameter steel bead in a 2 ml Eppendorf cup. The resulting powder was extracted with 250 µl 20 % (v/v) methanol, 200 mM KCl, 5 mM β-mercaptoethanol, and 10 mM potassium phosphate buffer pH 6 for 20 min with vigorous shaking, and the slurry was centrifuged twice at 10,000g for 5 min. The final supernatant was used for derivatization. *A. thaliana* plants for the harvest of roots or root tips were grown as described above. One root or 20 root tips (about 200 µm in length) were cut under a microscope (Nikon SMZ1000) and frozen in liquid nitrogen. The extraction was performed as described above using 100 µl of extraction buffer. For quantification, 100 pmol to 1 nmol (depending on the amount of starting material) of L-norvaline was added as standard to the extraction buffer. To evaluate the accuracy of the L-norvaline-based quantification method, 100 pmol each of norvaline, and deuterated standards were added to the extraction buffer.

Sample derivatization and processing

Derivatization of the samples (25 µl) was performed by adding 50 µl of 0.5 M sodium borate buffer pH 7.9 and 100 µl of a 3 mM Fmoc-Cl solution in acetone. After about 5 min, the reactions were extracted three times with 500 µl *n*-pentane. After removal of the organic layer after the last extraction step, the remaining organic solvent was allowed to evaporate for 10 min. Before loading onto the SPE resin, 500 µl 5 % (v/v) of acetonitrile was added to the remaining aqueous phase. The SPE 96-well plate was prepared by distributing dry HR-X-resin into the wells of the 96-well filtration plate (50 mg/well). The resin was conditioned by 1 ml of methanol followed by 1 ml of water. In this and all subsequent steps, the liquid was passed through the resin by centrifugation at 250 g for 5 min using a JS5.3 swing-out rotor in an Avanti J-26XP centrifuge (Beckman Coulter, Fullerton, CA, USA). After loading the samples, the resin was washed with 1 ml of water, and eluted with 1 ml of methanol into a 96-deep well plate (Roth, Karlsruhe, Germany). The eluates were transferred to 2 ml Eppendorf vials, and the solvent was evaporated under vacuum in an Eppendorf Concentrator 5301 at 45 °C (Eppendorf, Hamburg, Germany). The dry residue was dissolved in 50 µl 30 % (v/v) acetonitrile, which was optimal for resuspension of the analytes. After dilution with 50 µl of water and centrifugation at 10,000g for 10 min, the samples were transferred to autosampler vials for LC–MS/MS analysis. The dilution with water was performed in order to obtain a percentage of organic solvent in the sample to a value, which is lower than the starting conditions of the chromatography. This improved the peak shape of early eluting compounds.

LC–ESI–MS/MS analysis

Separations were performed on a Nucleoshell RP18 column (50 × 3 mm, particle size 2.7 µm; Macherey–Nagel (Düren, Germany)) at 30 °C using an Agilent 1100 series HPLC system. Eluents A and B were 10 mM aqueous ammonium formate buffer pH 4.5 and acetonitrile, respectively. From the starting condition with 25 % B, the percentage of B was increased to 50 % over the first 6 min, further increased to 98 % in 0.5 min followed by an isocratic period of 0.5 min at 98 % B. The starting conditions were restored within the next 0.5 min, and the column was allowed to re-equilibrate for 3.5 min at 25 % B. The flow rate was set to 0.9 ml/min. The analytes were detected on-line by ESI–MS/MS using an API 3200 triple-quadrupole LC–MS/MS system equipped with an ESI Turbo Ion Spray interface, operated in the negative ion mode (AB Sciex, Darmstadt, Germany). The ion source parameters were set as follows: curtain gas: 30 psi, ion spray voltage: −4,500 V, ion source temperature: 350 °C, nebulizing, and drying gas: 50 psi. Triple-quadrupole scans were acquired in the MRM mode with Q1 and Q3 set at "unit" resolution. Scheduled MRM was performed with a window of 90 s and a target scan time of 0.5 s. MS parameters describing the MRMs for each amino acid are shown in Table S1.

Compound-specific parameters were optimized individually for each analyte in infusion experiments. For these experiments, derivatized standards (1 pmol/µl in 25 % (v/v) acetonitrile) were infused into the ion source via a syringe pump (Hamilton OEM Syringe Pump, 1 ml syringe, Hamilton Bonaduz AG, Bonaduz, Switzerland) operating at a flow rate of 10 µl/min. The ion source parameters were set to: curtain gas: 10 psi, ion spray voltage: −4,500 V, ion source temperature: room temperature, nebulizing gas 30 psi, and drying gas: 0 psi.

Peak areas were calculated automatically using the IntelliQuant algorithm of the Analyst 1.6 software (AB Sciex, Darmstadt, Germany) and manually adjusted if necessary. All subsequent calculations were performed with Excel (Microsoft Office Professional Plus 2010).

For each series of measurements calibration curves for each amino acid were recorded using at least six serial dilutions of the amino acid standard solution. The resulting response factor for each amino acid was used to correct for the differences in derivatization, sample cleanup, and detection efficiencies between different series of measurements. The corrected values were then used to calculate the amounts based on the area of the added standards (L-norvaline or deuterium-labeled standards, respectively). For histidine and tyrosine, the signals of the mono as well as the double-derivatized amino acid were calculated independently based on their response factors. Then, the values for Tyr-1 and Tyr-2 as well as His-1 and His-2 were summed, yielding the final value for histidine and tyrosine, respectively. Similarly, the signals for methionine and methionine-oxide were treated independently and combined to give the final value for methionine.

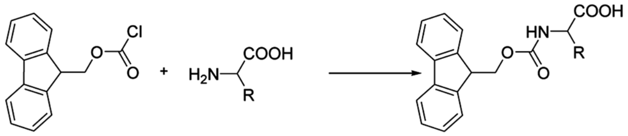

Fig. 1 Fmoc-derivatization reaction

Results and discussion

Fragmentation of Fmoc-derivatized amino acids under negative ion collision-induced dissociation (CID)

We used Fmoc-Cl as derivatization reagent for the amino acids (Fig. 1), because (i) it has been used successfully and routinely for amino acid analysis for HPLC-FLD detection providing many data regarding reaction conditions, (ii) it reacts readily with both primary and secondary amino groups, (iii) it has been shown to be sufficiently stable, and (iv) its hydrophobic core renders all amino acids hydrophobic for purification and separation on reverse phase resins.

In the negative ionization mode, derivatized amino acids showed an increased mass of 222 amu compared to the native amino acid, indicating a single derivatization. Lysine derivatization resulted in an $[M–H]^-$ ion at *m/z* 589, as expected for the labeling of the α and ε nitrogen (increase by 444 amu compared to the $[M–H]^-$ ion of lysine at *m/z* 145. Corroborating previous results using Fmoc-Cl as derivatization reagent, prolonged reaction times increased the proportion of doubly derivatized His ($[M–H]^-$ at *m/z* 598) and Tyr ($[M–H]^-$ at *m/z* 624) compared to the mono derivatized amino acids ($[M–H]^-$ at *m/z* 376 for His and *m/z* 402 for Tyr) (Jambor and Molnár-Perl 2009). Under positive ionization, sodium adduct ions ($[M + 23]^+$) were observed. However, in case of histidine and arginine, only the $[M + H]^+$ ions were detected, but sodium adducts could not be observed. Since most of the derivatized amino acid showed sodium adducts in the positive ionization mode displaying only a poor fragmentation, we decided to create an MRM method based on fragment ions formed under negative ion electrospray MS/MS. The mass spectral fragmentation of the Fmoc derivatives under negative ion CID conditions resulted in two prominent fragments **a** and **b** being typical for all amino acids (Figs. 2, 3, Table S2). While fragment **a** comprises the amino acid moiety and a CO unit derived from the Fmoc moiety ($[M–H–C_{14}H_{12}O]$, corresponding to $[M–H]_{\text{aminoacid}} + 26$ amu), the key ion **b** corresponds to the $[M–H]^-$ ion of the amino acid moiety ($[M–H–C_{15}H_{10}O_2]^-$. Only proline did not yield a fragment of type **a**, instead, a fragment at *m/z* 158 corresponding to $[M–H–C_{14}H_{10}]^-$ (formally proline + CO_2) appeared. Except for glycine, proline, and threonine fragment **e** corresponding to $[M–H–C_{15}H_{13}NO_2]^-$ (formally $[\text{amino acid-}NH_3]^-$)

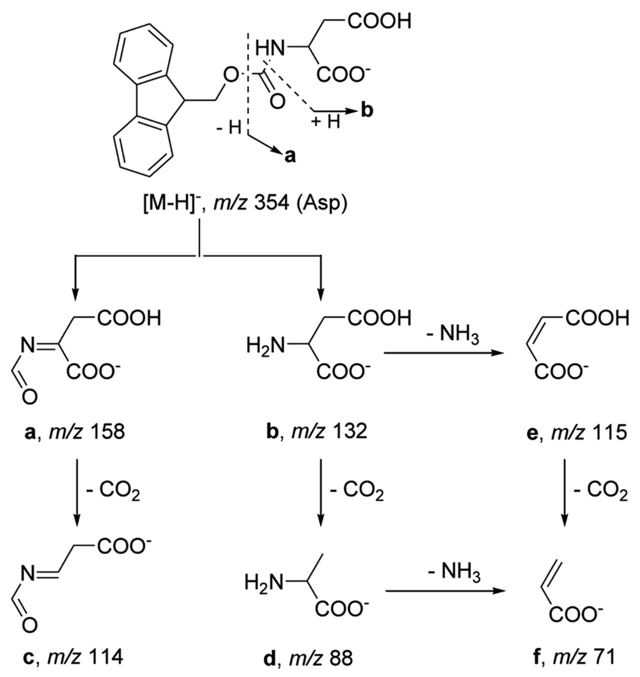

Fig. 2 Mass spectral fragmentation of the [M–H]$^-$ ion of aspartate (Asp) under ion CID conditions

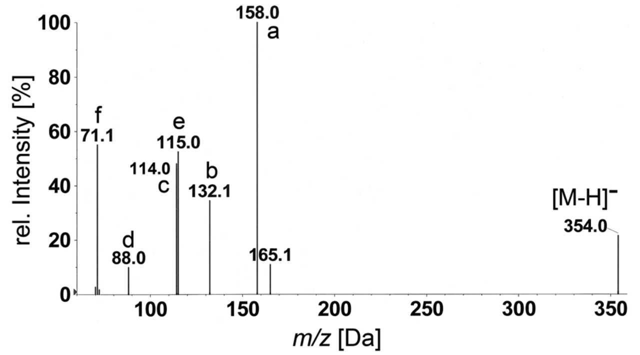

Fig. 3 CID mass spectrum (collision potential −50 V) of Fmoc-derivatized aspartate

was consistently observed. A loss of CO_2 from fragment **a** resulting in fragment **c** ([M–H–$C_{15}H_{12}O_3$]$^-$) was observed for aspartate, glutamate, asparagine, glutamine, serine, and valine, whereas a loss of CO_2 from fragment **b** appeared in case of aspartate and glutamate leading to ion **d** ([M–H–$C_{16}H_{10}O_4$]$^-$). The amino acids with a hydroxyl group (serine and threonine) indicated an ion at *m/z* 100 as base peak. This ion corresponds to [M–H–$C_{15}H_{14}O_2$]$^-$ (serine) and [M–H–$C_{15}H_{12}O_2$]$^-$ (threonine), respectively, and is formed from fragment **a** of serine by the loss of formaldehyde, or from fragment **b** of threonine by the loss of water. Specific fragment ions indicating the loss of cyanamide (*m/z* 131 and *m/z* 114) and methanethiol (*m/z* 126) were detected for arginine and methionine, respectively. Interestingly, the doubly derivatized amino acids histidine, lysine and tyrosine never yielded a fragment indicating the loss of only one Fmoc moiety, but rather fragments derived after the loss of both Fmoc moieties.

The fragment at *m/z* 165 was present at a low intensity in the CID spectra of all derivatized amino acids and probably comprises the fluorenyl anion [$C_{13}H_9$]$^-$ of the Fmoc moiety

Derivatization and solid-phase extraction (SPE)

Derivatization conditions were based on the experiments by Jambor and Monar-Perl (2009). They reported that higher Fmoc-Cl concentrations in acetone accelerates the reaction but led to higher noise in subsequent FLD runs because of higher abundance of impurities derived from the Fmoc-Cl solution and its degradation products. Since these peaks are not of concern in our MRM-based detection method, we chose a concentration of Fmoc-Cl of 3 mM in acetone, since the reaction time can be reduced.

In FLD-based methods, the presence of the remaining Fmoc reagent is of major concern for the subsequent detection and quantification. Although the remaining Fmoc would not interfere with analyte detection in MRM-based methods, we decided to remove it in order to increase the lifetime of the HPLC column. For FLD detection, the remaining reagent is often removed by extraction with organic solvents, although it has been reported to result in losses of the very apolar Fmoc derivatives such as Lys, His-2, and Tyr-2 (Jambor and Molnár-Perl 2009). We found 1.4 ± 0.38 (n = 4)-fold higher signal intensities after extraction with *n*-pentane compared to non-extracted samples. Possibly, the excess of Fmoc-Cl reagent in non-extracted samples competed with the recovery of derivatized amino acids during solid-phase extraction (see below). However, after normalization with the internal standard norvaline, 96 % of signal intensities (lowest value of 77 % for Tyr-2) were obtained after extraction with *n*-pentane compared to the values without *n*-pentane extraction.

After derivatization and extraction with *n*-pentane, the samples contain high concentration of sodium borate, which led to strong ion suppression during LC–MS. Therefore, sample cleanup by SPE was performed. As the Fmoc label renders all amino acids apolar, reverse phase SPE was applied. Apolar polymeric as well as C18-substituted silica phases were tested and both performed equally well. In order to increase sample throughput, the hydrophobic polysterol-divinylbenzol copolymer HR-X was chosen as it can be used in a centrifugal device in a 96-well format. Analyte recoveries could not be estimated since ion suppression was too strong in unprocessed samples.

Chromatography

Since all derivatized amino acids are detected according to their specific MRM transitions, it was only necessary to separate leucine and isoleucine, as well as valine and norvaline, as they showed identical MRM transitions. This separation was achieved using the gradient described in the Experimental section with a total run time of 11 min including re-equilibration of the column (Fig. 4; Table 1). The use of ammonium formate buffer as polar solvent was crucial to separate leucine/isoleucine and valine/norvaline. As expected, amino acids with two Fmoc groups (His-2, Lys, and Tyr-2) eluted at the end of the gradient. Interestingly, also cysteine eluted as if it was derivatized twice, although its mass ($[M–H]^-$ at *m/z* 342) indicated a single derivatization. This mass was not detected at earlier retention times. It is conceivable, that during sample processing either cysteine is oxidized to cystine before derivatization, or, after derivatization, cysteine-Fmoc is oxidized to cystine-Fmoc leading to the observed retention. However, we did not detect the corresponding $[M–H]^-$ ion (*m/z* 683) for cystine-Fmoc. We assume that cystine-Fmoc eluting late from the column might undergo in-source fragmentation to cysteine-Fmoc.

After evaporation of the SPE eluates, we consistently observed low recoveries of methionine-Fmoc ($[M–H]^-$ at *m/z* 370) which eluted in the center of the gradient. Instead, with decreasing intensities of the methionine-Fmoc signal, we observed increasing signal intensities for a signal at *m/z* 386 ($[M–H]^-$) which eluted early. The mass spectrum of this signal indicated an *S*-oxidation of methionine (Table S2). Obviously, methionine-Fmoc is oxidized to methionine-oxide-Fmoc during evaporation.

Limit of detection, limit of quantification, and linear range

The protocol was applied to serial dilutions of 21 amino acids ranging from 250 pM to 125 µM. Limit of detection (LOD) was determined as the lowest concentration which was higher than the controls without amino acids, whereas the limit of quantification (LOQ) was defined as the lowest concentration at which linearity started. For most amino acids, LOD and LOQ were 1 and 4 nM, respectively. For cysteine, glutamate, and glutamine, both values were 15 nM. To our knowledge, this sensitivity is higher than the sensitivities reported for FLD (Rigas 2013), GC–MS (Badaway et al. 2008; Kvitvang et al. 2011), and LC–MS/MS without derivatization detection methods (Nagy et al. 2003; Piraud et al. 2005; de Person et al. 2008; Thiele et al. 2008; Zhou et al. 2013; for comparison of different methods see Kaspar et al. 2009). Superior sensitivities have been reported for derivatizations using trimethylphosphonium or positively charged carbamate reagents, which strongly increased ionization efficiencies (Shimbo et al. 2009a, b; Inagaki et al. 2010). In both cases, single reaction monitoring detecting a characteristic, positively charged fragment from the reagent moiety was used instead of fragments indicative of the amino acid moiety. In a recent investigation, which was published during the preparation of this manuscript, Fmoc-Cl derivatization followed by LC–MS/MS detection was used for amino acid determination (Pande et al. 2014). However, after derivatization and extraction of residual Fmoc-Cl, the samples were directly applied to the LC–MS/MS system without SPE purification. If the SPE step was omitted, we obtained LODs between 200 and 500 nM. At concentrations of 500 nM and 2.5 µM, the average signals intensities for all amino acids decreased by 98.8 % (±1.8 %) and 94.7 % (±4.4 %), respectively, if the reactions were directly applied to the LC–MS/MS system without SPE purification. This result shows that the SPE step is crucial to minimize ion suppression, which is probably due to the high concentration of borate buffer in the reaction mix, and to achieve the observed sensitivities between 1 and 15 nM. However, it is worth mentioning that at concentrations of 200 µM and higher, the effect of the SPE was negligible.

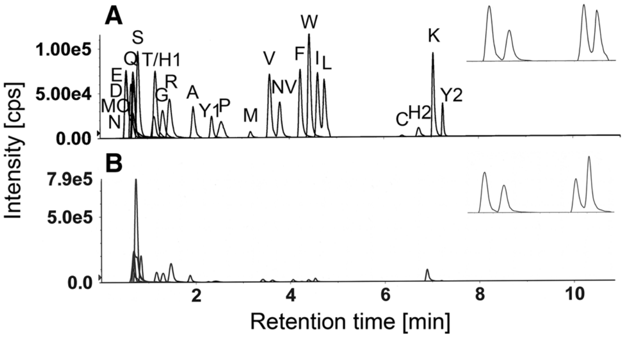

Fig. 4 Chromatograms of *A* an amino acid standard mixture, and *B* an *Arabidopsis thaliana* seedling extract. *Insets* Separation of valine/norvaline (*front*) and isoleucine/leucine)

Calibration curves were linear ($r^2 > 0.98$) up to the maximum concentration of 125 µM, with the exception of cysteine, aspartate, glutamate, lysine, asparagine, and glutamine, which were linear up to 65 µM. The slope of the calibration curves differed between amino acids. As expected under negative ionization conditions in the ion source, the slopes (response factors) of the negatively charged amino acids glutamate and aspartate were about three-fold higher compared to uncharged amino acids such norvaline (Table 1). Since the proportion of mono- and double-derivatized histidine and tyrosine varies depending on duration of derivatization, the response factors across different experiments showed large variations. Therefore, an average response factor for histidine and tyrosine is

Table 1 Retention times, quantifier MRM transitions, MS-responsiveness, intraday and interday precision, and ratio between quantitation methods for each amino acid

Amino acid	Retention time (min)	MRM transition^{A}	Slope vs norvaline^{B}	Intraday precision rel. SD (%)^{C}	Interday precision rel. SD (%)^{D}	Ratio between quantitation methods^{E}
Ala	1.71	$310 \rightarrow 88^{b}$	0.998 ± 0.065	4.9	6.8	
Cys	6.45	$342 \rightarrow 146^{a}$	0.322 ± 0.047	12.0	4.5	
Asp	0.64	$354 \rightarrow 158^{a}$	2.856 ± 0.193	5.5	7.4	
Glu	0.66	$368 \rightarrow 128^{c}$	2.777 ± 0.251	8.2	5.4	0.914 ± 0.127
Phe	3.87	$386 \rightarrow 164^{b}$	1.324 ± 0.122	7.6	2.8	0.989 ± 0.038
Gly	1.19	$296 \rightarrow 74^{b}$	0.964 ± 0.072	6.4	8.2	
His-1	1.12	$376 \rightarrow 154^{b}$	n.d.	15.5	16.5	
His-2	6.07	$598 \rightarrow 154^{b}$				
Ile	4.16	$352 \rightarrow 130^{b}$	1.608 ± 0.103	8.2	8.5	
Lys	6.77	$589 \rightarrow 145^{b}$	1.415 ± 0.171	8.0	8.8	
Leu	4.30	$352 \rightarrow 130^{b}$	1.309 ± 0.064	6.0	7.3	0.968 ± 0.063
Met	2.85	$370 \rightarrow 174^{b}$	n.d.	6.4	5.7	
Met-oxide	0.72	$386 \rightarrow 190^{b}$				
Asn	0.67	$353 \rightarrow 113^{c}$	1.158 ± 0.125	6.6	8.0	
Pro	2.20	$336 \rightarrow 114^{b}$	0.767 ± 0.063	5.1	5.3	
Gln	0.70	$367 \rightarrow 171^{a}$	1.528 ± 0.181	3.5	6.7	
Arg	1.44	$395 \rightarrow 173^{b}$	1.356 ± 0.081	5.1	5.9	
Ser	0.80	$326 \rightarrow 130^{a}$	1.942 ± 0.133	5.2	5.3	0.897 ± 0.026
Thr	1.03	$340 \rightarrow 100^{*}$	2.111 ± 0.134	6.2	6.6	
Val	3.19	$338 \rightarrow 116^{b}$	1.742 ± 0.099	6.3	6.7	
Trp	4.06	$425 \rightarrow 203^{b}$	2.287 ± 0.184	6.4	7.3	0.906 ± 0.063
Tyr-1	2.10	$402 \rightarrow 180^{b}$	n.d.	7.9	12.0	
Tyr-2	7.16	$624 \rightarrow 180^{b}$				
Norvaline	3.38	$338 \rightarrow 116^{b}$	N/A	N/A	N/A	

n.d. Not determined, since the proportions of both transitions varied between experiments

[A] MRM transitions used for quantification are indicated. For additional MRM transitions see Table S2; lowercase superscripts indicate the fragments according to Fig. 2 and Table S1, * = m/z 100 = $[C_4H_6NO_2]^-$ (formally Thr-H_2O)

[B] the slopes of the calibration curves for each amino acid divided by the slope of the calibration curve for norvaline, $n = 10$

[C] $n = 10$

[D] $n = 5$

[E] quantitation using deuterated standards divided by quantitation using norvaline; $n = 5$

not indicated in Table 1. As the proportion of methionine and methionine-oxide was variable depending on evaporation time, consequently leading to considerable variations in response factors for those compounds, they are also not included in the table.

Quantification, ion suppression, stability, and intraday and interday reproducibility

Methods based on mass spectrometry generally allow quantification using labeled internal standards. However, the investment to purchase all amino acids in their deuterated or ^{13}C labeled form would be considerable. Therefore, we decided to perform quantification by adding the non-natural amino acid norvaline as internal standard. As mentioned above, the amino acids exhibited differences in their response factors. In order to account for this, a correction factor was determined. For every series of experiments, standard curves for every amino acid were recorded. The correction factor was then calculated by dividing the slope of the calibration curve for each amino acid by the slope of the calibration curve for norvaline. Finally, the amino acids were quantified (i) by dividing the peak area of the analyte by the peak area of the internal standard, and (ii) by dividing the resulting value by the correction factor. For histidine, tyrosine, and methionine, both signals were calculated independently based on their individual response factors in each experiment. The final values for these three amino acids are the results of the sum of the respective two signals. We validated this approach by comparing the results for glutamate, phenylalanine, leucine, serine, and tryptophan obtained using norvaline with those obtained using the respective deuterated internal standards. As seen in Table 1, both quantification methods showed differences of about 10 %.

Pande et al. (2014) performed quantification by spiking each sample with a ^{13}C, ^{15}N-labeled amino acid mix from an algal amino acid. The omission of the SPE step probably led to strong differences in ion suppression between the analytes, which demands quantification of each amino acid by the respective labeled amino acid. In our method, ion suppression is equally low for all analytes (see next paragraph) and allows quantification of all amino acids using the unnatural amino acid norvaline. This enables a more convenient and less expensive possibility for amino acid quantification.

Sensitive detection of analytes is often compromised by ion suppression derived from the sample matrix. In order to estimate the ion suppression effects for the protocol, we compared the response factors for each amino acid in the presence and absence of *Arabidopsis* seedling extracts. The concentration of seedling extract was 5 mg of tissue/250 µl extraction buffer or a 1:5 dilution thereof. The concentration of the diluted sample is equivalent to the concentration of extracts which was used for measurements. In the concentrated seedling extract, ion suppression of less than 5 % was observed for most amino acids, only the signals for aspartate, glutamate, and asparagine were suppressed between 5 and 10 %. In the diluted extract, no ion suppression could be detected.

Stability of the derivatives was determined by storing an injection-ready (after SPE and evaporation) derivatized sample of *Arabidopsis* seedling extracts for up to 12 days at room temperature and at −20 °C. Most derivatives were stable up to 6 days at RT, and all were stable up to 12 days at −20 °C. Only cysteine, histidine and lysine exhibited decreases by 60 % in signal intensities after storage at room temperature for 2 days.

Intraday and interday reproducibility was estimated by applying the protocol for the same *Arabidopsis* seedlings extract 10 times at a single day or in triplicates on five different days, respectively. As shown in Table 1, relative standard deviations were below 10 % for most amino acids, which are comparable to other methods with similar sensitivity (Shimbo et al. 2009a, b; Inagaki et al. 2010). Cysteine, histidine, and tyrosine showed higher relative standard deviations around 15 %.

Amino acid profiling of minute amount of tissue with low

In order to demonstrate the applicability and sensitivity of the method, we compared the amino acids content and composition of excised root meristems with whole primary roots of *A. thaliana* seedlings. In many reports, amino acid profiling was performed with more than 50 mg of plant material (Jander et al. 2004; Thiele et al. 2008; Pant et al. 2014), which would be equivalent to about 100 whole roots of young *A. thaliana* seedlings. For this protocol, we extracted only one single root (15 mm in length) or 20 root meristems (0.2 mm, consisting of about 3,000 cells each (personal communication Dr. Jens Müller)). Since the very low amount of material does not allow accurate determination of sample weight, we expressed the amino acid content based on number of tissue. As seen in Fig. 5, glutamine was the most abundant amino acid with 1,500 pmol per primary root, followed by glutamate, serine, asparagine, alanine,

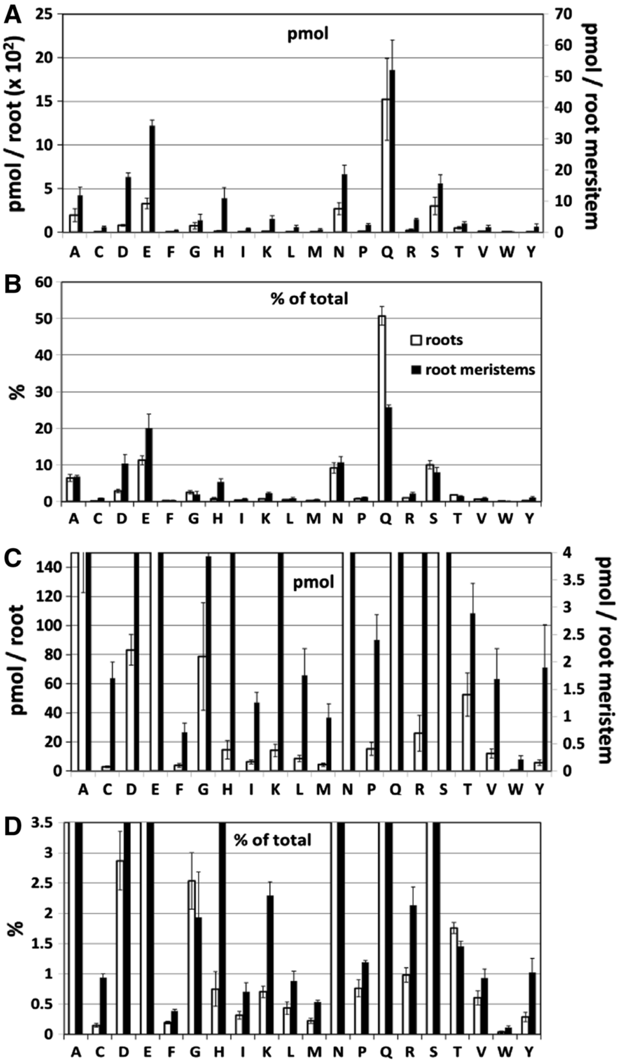

Fig. 5 Amino acid content in whole roots (*left y axis*) or root meristems (*right y axis*) of 5 days old *Arabidopsis thaliana* seedlings (**a**, **c**). Proportion of each amino acid in the total amino acid pool (**b**, **d**). *Panels c* and *d* represent an expanded view of *panels a* and *b*, respectively. *White bars* whole roots, *black bars* root meristems, and *Error bars* denote ± SD ($n = 4$)

aspartate, and glycine. Tryptophan was the least abundant amino acid with 0.7 pmol per root. In root tips, glutamine still constituted the most abundant amino acid with 50 pmol per root tip, but the next most abundant amino acids were glutamate, aspartate, asparagine, serine, alanine, and histidine. The difference in content of each amino acid between whole roots and root tips became more obvious when the contribution of each amino acid to the total amino acid pool was considered. Whereas there was a strong decrease in the proportion of glutamine (from 50 to 25 %) and modest changes for asparagine, alanine, threonine, serine, and glycine, there was an increase by up to three-fold for glutamate, tryptophan, methionine, isoleucine, arginine, leucine, phenylalanine, proline, and valine, and up to seven-fold for histidine, cysteine, aspartate, tyrosine, and lysine (Fig. 5).

Conclusion

The presented method offers several advantages compared to previous methods. Compared to optical detection methods it enables discrimination from unspecific signals. Furthermore, analysis time is shortened, since LC–MS/MS detection obviates the need for separation of every single amino acid. Compared to GC–MS methods, it is more comprehensive, since all proteinogenic amino acids can be detected. The efficient and facile removal of contaminants by a single SPE step as well as the shift to a higher mass range of the analytes minimizes interference of background signals derived from sample matrix and eluents. This increases the sensitivity compared to methods employing LC–MS/MS detection of underivatized amino acids. The low ion suppression facilitates quantification using only norvaline as internal standard instead of internal standards for every amino acid. Higher selectivity is obtained, since amino acid specific fragments rather than reagent specific signals are detected, as in other LC–MS/MS methods using derivatized amino acids (Shimbo et al. 2009a, b, 2010). Furthermore, derivatization with Fmoc-Cl is readily accomplished at room temperature without the need of special equipment, and the reagent is constantly commercially available and comparably inexpensive. It should be noted, however, that the low intensity of the fragment derived from the Fmoc moiety (*m/z* 165) would impede sensitive precursor ion scans for amines. The analysis of the amino acid profile of minute amounts of plant material, such as root meristems, exemplifies the high sensitivity and selectivity of the method. Generally, the discussed procedure can be applied to other compounds with primary and secondary amino groups with negatively chargeable functionalities since adduct formation in the positive ionization mode results in poor fragmentation. Although the protocol was optimized for measurement of plant samples, it should be applicable to other biological samples. This, however, needs to be tested, and might require modifications of the method.

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Conflict of interest The authors declare that they have no conflict of interest.

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