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ORIGINAL ARTICLE

Rapid and sensitive method for determining free amino acids in plant tissue by high-performance liquid chromatography with fluorescence detection

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Abstract This paper describes a reliable and rapid method for the complete separation and quantitation of twenty-five amino acids typically found in plants, based on reversed phase high-performance liquid chromatography–linked fluorescence detector using a 150 × 4.6 mm Zorbax Eclipse AAA column. Plant tissue free amino acids (FAA) were extracted by ultrasonication with 5% (v/v) aqueous trifluoroacetic acid followed by ultrafiltration of extracts. The following analysis of amino acids was performed through programmed precolumn derivatization with ortho-phthalaldehyde and 9-fluorenylmethyl chloroformate reagents and efficient elution of derivatives within 26 min using binary gradient scheme. The method was validated over a concentration range of 4.5–450 μmol L^{-1} (μM). Separation analysis showed good selectivity (resolution > 1.5) for most amino acids. The average repeatability (RSD%, relative standard deviation) of the analysis at seven calibration concentrations was below 4% and ranged from 1.13% to 12.04%. The intra-day mean coefficient of variation at two concentrations (22.5 and 90 μM) was within 2%, and the intermediate precision was less than 4%. The limits of detection were between 0.012 and 6.68 μM. The coefficients of determination (R^2) of the linear calibration curves were from 0.9989 to 0.9999. When the method was applied to plant samples, the FAA recoveries at two spiked levels (25 and 100 μM) ranged from 67.0% to 108.9% with an average of 94.4%, and the precision was 0.26%–12.31% RSD. A specific application combining this method with optimized extraction and interference removal procedures was successfully used to determine the FAA pools in different plant tissues. Finally, a PLS-DA multivariate statistics model was validated for the classification of three plant species according to their FAA profiles.

Keywords HPLC · OPA · FMOC · Free amino acids · Plant · PLS-DA

1 Introduction

Amino acid (AA) analysis has become commonplace and been frequently used in biomedical analysis, ecology, metabolomics, food science and environmental science (Divino Filho et al. 1997; Fritz et al. 2006; Hermosín et al. 2003; Jones et al. 2008; Mandrioli et al. 2013; Obata and Fernie 2012). Free amino acids (FAAs), an important class of small polar nitrogen metabolites of plants, provide indicative information about biological responses to environmental or physiological metabolism changes triggered by fluctuation in the N status, water shortage or other stress (Bouché and Fromm 2004; Calanni et al. 1999; Fritz et al. 2006; Obata and Fernie 2012; Szabados and Savoure 2010). However, the diverse physicochemical properties of AAs as well as their wide concentration range pose significant challenges in the simultaneous sensitive detection

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and precise and accurate quantitation of multiple FAAs in various biological samples. Gas chromatography (GC) and liquid chromatography (LC) are the two most commonly used separation techniques and are typically coupled to flame ionization (FID), photodiode array (DAD), fluorescence (FLD) or mass spectrometric (MS) detectors for quantitative analysis (Cooper et al. 2001; Noctor et al. 2007; Silva et al. 2004; Sobolevsky et al. 2003).

The GC based methods have well-known limitations and drawbacks, such as lengthy sample preparation steps (matrix clean-up) and issues related to analyte size and volatility (Fábián et al. 1991; Labadarios et al. 1984). GC requires indispensable derivatization of the functional groups in AAs to form suitable volatile derivatives under strictly controlled conditions, e.g., anhydrous and heated (Orata 2012; Woo and Lee 1995). Several methods have been developed for AA analysis using LC systems, including chromatographic ion-pair separation (Chaimbault et al. 1999) and ion-exchange chromatography (IEC) with spectrophotometric detection of post-column ninhydrin derivatives (Cooper et al. 2001; Dupont et al. 1989; Meyer 1957). The disadvantages of the previous liquid separation techniques include high background signals or ion suppression effects, high limit of detections, and large sample amounts required for analysis. Low sample throughput and long analysis times (up to 60 min) complicate the analysis of large sample batches in limited amounts of time. Coelution of the target analytes with one another or with matrix compounds is another typical problem affecting the LC separation efficiency. These coelutions problem can be tackled using different strategies. MS allows for mass spectral deconvolution to generate a "pure" mass spectrum for each overlapping peak in metabolomics profiling (Dettmer et al. 2007). However, for MS systems, the analysis of analytes in complex matrices can be handicapped by salts ion suppression or signal suppression (Choi et al. 2001). Alternative strategies include selective sample preparation for targeted metabolites, improvement of chromatographic resolution (stationary phase) or column efficiency (longer column). Fortunately, reversed-phase (RP) columns with smaller internal diameters and particle sizes achieve such improvements in analytical separation performance that co-elution is typically not a serious problem with LC-FLD (Jones and Gilligan 1983). Reversed phase (RP) high-performance liquid chromatography (HPLC) is the chromatographic technique of choice for most modern biochemists studying limited sets of target metabolites. Automatic precolumn derivatization and HPLC tandem DAD or FLD methods have the advantages of reduced analysis times, enhanced sensitivity and selectivity, greater flexibility, and less instrumentation upkeep than dedicated amino acid analyzers or MS platforms.

A typical HPLC method uses precolumn modification of AAs to form the corresponding derivatives. A variety of techniques have been tested to convert AAs into analyzable UV-absorbing and fluorescence-emitting derivatives for sensitive analysis. In addition to the colorimetric ninhydrin method (Cooper et al. 2001;), popular reagents employed for the derivatization include 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride, DANS-Cl) (De Jong et al. 1982; Tapuhi et al. 1981), 4-fluoro-7-nitorobenzo-2-oxa-1,3-diazole (NBD-F) (Watanabe and Imai 1983), phenyl isothiocyanate (PITC) (Bidlingmeyer et al. 1984), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Cohen and Michaud 1993), ortho-phthalaldehyde (OPA) (Hill et al. 1979; Roth 1971) and 9-fluorenylmethyl chloroformate (FMOC-Cl) (Einarsson et al. 1983). DANS-Cl reacts with both primary and secondary amines but requires high temperatures and long reaction times. In addition to poor sensitivity, successive drying steps are required with PITC, which makes this derivatization method quite laborious (Fürst et al. 1990). The OPA and FMOC reagents enable fast precolumn derivatization of AAs to yield highly fluorescent derivatives without any interfering artifacts; thus, high-sensitivity analyses can be performed accurately at the lower picomolar level by means of fluorescence detection (Schuster 1988; Schwarz et al. 2005; Woodward et al. 2007).

The quantitative extraction and purification of plant FAAs was studied based on published methods in order to obtain the highest recoveries and cleaner chromatograms (Álvarez-Sánchez et al. 2010; Hyötyläinen 2009; Kim and Verpoorte 2010; Molero et al. 2014; Mushtaq et al. 2014; Pérez-Palacios et al. 2014). There is no universal sample extraction technique for all types of samples and analytes. Removal of interfering compounds, such as proteins, lipids, sugars, colored matter and salts, is an effective strategy often employed to improve metabolite coverage and reproducibility. Various media including organic solvents (acetone, acetonitrile, chloroform or alcohols) and acidic water (5-sulphosalicylic acid, perchloric acid, trichloroacetic acid or 0.01 M HCl at pH 2.2), which have somewhat diverse polarities, have been used for proteinic enzyme inactivation, deproteinization, delipidation and extraction of nonprotein nitrogen from biological materials (Bhatty and Finlayson 1973; Noctor et al. 2007; Persson and Näsholm 2001; Weckwerth and Kahl 2013). In addition, liquid–liquid extraction (LLE), solid-phase extraction (SPE) and cation-exchange resin chromatography are effective pre-treatment methods for sample clean-up purposes and for analyte enrichment (Amelung and Zhang 2001; Takano et al. 2010; Wang et al. 2010). Cold ethanol (EtOH)-water mixtures or aqueous solution of trichloroacetic acid (TCA) or trifluoroacetic acid (TFA) have great abilities in denaturating proteins and eliminating high

molecular mass compounds (Blanchard 1981; Rajalingam et al. 2009; Sivaraman et al. 1997). The combination of these extraction solvents with centrifugation and membrane-based techniques (such as ultrafiltration) for the removal of interfering substances are quick and very simple methods that present good reproducibility and recovery of polar and semi-polar metabolites (Bhatty 1972; Molero et al. 2011; Weckwerth and Kahl 2013).

The main purposes of this work were to establish a convenient, rapid and precisely quantitative analysis method for AAs and to optimize a FAA extraction procedure for various plant tissues that yields stable extracts containing as many AAs as possible, which can be readily analyzed by HPLC-FLD. The method was evaluated in terms of the precision; linearity of the calibration; sensitivity, as detection and quantification limits; and accuracy, as recovery percentages of standards spiked into plant matrices (Prichard and Barwick 2007). The method was then applied to investigate the FAA content profiles in three plant species and to discriminate FAA metabolism patterns using multivariate statistics.

2 Materials and methods

2.1 Chemicals

Twenty-two individual high-purity L-amino acid standards were obtained from Sigma-Aldrich (St Louis, MO, USA): aspartic acid (Asp), glutamic acid (Glu), serine (Ser), histidine (His), glycine (Gly), threonine (Thr), citrulline (Cit), arginine (Arg), alanine (Ala), γ-aminobutyric acid (Gaba), theanine (Thea), tyrosine (Tyr), α-aminobutyric acid (Aaba), cystine (Cy2), valine (Val), methionine (Met), phenylalanine (Phe), isoleucine (Ile), ornithine (Orn), leucine (Leu), lysine (Lys) and proline (Pro). Another six AAs, asparagine (Asn), glutamine (Gln), norvaline (Nva), tryptophan (Trp), hydroxyproline (Hyp) and sarcosine (Sar), were provided in an auxiliary amino acid kit (PN 5062-2478) purchased from Agilent (Agilent Technologies, Palo Alto, CA). The derivatization reagents, 9-fluorenylmethyl chloroformate (FMOC) and o-phthalaldehyde 3-mercaptopropionic acid (OPA-3MPA), were also from Agilent Technologies. The Acetonitrile (ACN 99.9%) and methanol (MeOH, 99.9%) used in the mobile phases were HPLC-grade reagents supplied by J&K Technology (Shanghai, China). Borate buffer, hydrogen phosphate (NaH_2PO_4) and all other chemicals used were analytical reagent grade and purchased from Aladdin (Shanghai, China). Deionized water produced using a Milli-Q water system (Millipore, Billerica, MA, USA) was degassed under vacuum and filtered through a 0.22-micron nylon membrane before use in HPLC analysis. Fresh aliquots of the derivatization reagents were used for each set of samples every day.

2.2 Analyte solutions

Stock standard solutions of the analytes were prepared by dissolving appropriate amounts of crystalline AAs in 0.1 M hydrochloric acid (solution sonicated until dissolved), in which the final concentration of each AA was 1 mM, except for Asn, Gln, Trp and Hyp, which were prepared in deionized water at concentrations of 9 mM as extended amino acid (EAA) stock solutions. Internal standard (IS, nonprotein AAs) stock solutions contained Aaba and Sar at 1 mM and Nva at 2 mM in deionized water. These stock solutions were stored at − 20 °C and were stable for approximately 3 weeks of continual use before Met and Gln converted to methionine sulphoxide and pyroglutamic acid, respectively (Jones and Gilligan 1983; Jones et al. 1981; Woo and Lee 1995).

Calibration standards containing the 28 analytes were prepared by mixing and diluting stock AA standard solutions with freshly deionized water on a weekly basis. Finally, solutions containing seven different concentrations, 4.5, 9.0, 22.5, 45.0, 90.0, 225 and 450 μM, of the AA mixture and fixed amounts of Aaba (25 μM), Nva (50 μM) and Sar (25 μM) as ISs, were prepared to construct the calibration curves. Solutions at two concentrations (22.5 and 90 μM) were also used as quality control standard solutions (QC). All AA mixtures were divided into small aliquots, stored in 2 mL capped autosampler vials, and kept in darkness and frozen at − 20 °C until further use.

2.3 Apparatus and operating conditions

The analyses were performed on an Agilent 1260 HPLC system (Agilent Technologies, Palo Alto, CA) consisting of G1311B quaternary pump with an in-line 4-channel vacuum degasser, G1329B autosampler, G1316A thermostatic column oven, G1315C photodiode array detector set at a wavelength of 338 nm, G1321B FLD set at excitation and emission wavelengths of 340 and 450 nm, respectively, and changed to 266 and 305 nm at 14.5 min. Agilent ChemStation software was used for instrumental control and data acquisition. The automatic precolumn in-loop derivatization procedure by OPA-3MPA and FMOC at ambient temperature was adjusted according to the injector programs recommended by Agilent (Henderson et al. 2000).

The derivatives were separated on a Zorbax Eclipse AAA column (150 × 4.6 mm i.d., 5 micron particle size, Agilent), protected by a C_{18} guard column cartridge (12.5 × 4.6 mm, 5 micron, Agilent, PN 820950-931). Column ageing, possibly as a result of silica dissolution at

the top of the column under high pH conditions, is less evident for C_{18} than C_8 columns. The column temperature was maintained at 40 °C. The chromatographic separation was obtained using binary mobile-phase gradient elution at a flow rate of 2.0 mL/min (Table 1), and the total necessary time was 26 min, which included the column regeneration and returning to initial conditions. Mobile phase A and B were degassed in an ultrasonic bath and vacuum-filtered through a 0.22-micron membrane filter before use. The pH of eluent A was particularly critical for the steadiness of the elution of the compounds throughout the analyses. To maintain column integrity, a mixture of 60% MeOH and 40% water was run for 60 min to clean the column at the end of each batch of samples or every 25–30 injections.

2.4 Analytical method validation

For the selectivity criterion, analyte peaks had to have a chromatographic baseline with acceptable resolution ($Rs \geq 1.5$). The limits of detection (LOD) and quantification (LOQ) were calculated at signal-to-noise ratios (S/N) of 3 and 10, respectively (Shimbo et al. 2009; Zhao et al. 2013). Calibration curves were constructed from triplicate HPLC analyses of the AA mixtures at seven concentrations (4.5, 9.0, 22.5, 45.0, 90.0, 225, 450 μM). The slopes and intercepts of the calibration equations were determined through least-squares linear regression of AA response peak areas and concentrations. The quality of fit was determined using the found (back-calculated) concentration-to-nominal concentration ratios. The linearity of the calibration curves was verified using a 'lack-of-fit' test and residual plots (Analytical Methods Committee 1994; Cuadros Rodríguez et al. 1996).

The repeatability was determined from triplicate injections of solutions at seven concentrations covering the analyte calibration range, for a total of 21 continuous analyses. The orders of these injections were randomly arranged to minimize interferences between the analyses (Prichard and Barwick 2007). The intermediate precision of the method for all AAs was determined by injecting two QC standards at regular intervals on four consecutive days. The QC standards were also employed to detect any trends within the HPLC analysis batches, e.g., fluctuation in signal intensity, changes in peak shapes, inertness of the analytical column, and retention time (RT) drift. The precision of the sample analysis was determined from the extraction and analysis of plant materials in triplicate.

Likewise, to measure the 'trueness' of the method, analyte recovery experiments were performed by adding the AA standard mixtures (25 and 100 μM) to original plant materials that were analyzed alongside the same samples unspiked. Three replicates were analyzed for each spiked level. The average extraction recoveries, used to determine the method accuracy (trueness) and to assess the matrix effect in the plant extracts, were calculated using the formula, recovery (%) = [(amount found − original amount)/amount spiked] × 100% (Magnusson 2014).

Table 1 Mobile phases and gradient elution conditions

Time (min)	Mobile phase A (%) 40 mM Na_2HPO_4 pH = 7.8	Mobile phase B (%) 45% methanol 45% acetonitrile 10% water
0.0	100	0
1.9	100	0
18.1	43	57
18.6	0	100
24.3	0	100
25.2	100	0
26.0	100	0

2.5 Plant material and extraction

Three plant species materials were sampled in the growing season (July, 2015): a moss, a coniferous gymnosperm and an evergreen broad-leaved angiosperm. Twigs of *Pinus massoniana Lamb* and *Cinnamomum camphora* were cut off from the middle crown region using a tree pruner. Equivalent volumes of mature leaves were stripped from several first- and second-order branches (Huhn and Schulz 1996). An epilithic moss (*Haplocladium microphyllum*) was collected at the same site; sampling and preparation were conducted according to the guidelines described by Liu (Liu et al. 2008, 2012). Mixed foliage specimens (from each of the 3 trees) and moss tissue were immediately frozen and transported on ice to avoid senescence-induced metabolic changes (Gidman et al. 2005). Fresh and frozen plant tissues were stored at − 80 °C or freeze-dried prior to homogenization and extraction.

All clean plant tissues were lyophilized (low-temperature vacuum cryodesiccation) to complete dryness, milled, and homogenized into a very fine power using a grinder (or a mortar and pestle) and 100 mesh ring sieve. The freeze-dried plant powder samples were stored in evacuated desiccators filled with silica gel beads at low temperatures to avoid water absorption. A dark, cool and dry environment ensured that enzymes and transporters were inactive in the complete absence of water, which avoided degradation and modification of metabolites in the sample (Villas-Boas et al. 2007).

A 100 mg (dry weight) portion of the lyophilized powder was transferred into a 5 mL microcentrifuge tube, followed by 100 µL of the IS stock solution, prior to extraction. The IS was added to the samples as early as possible to mirror and compensate for AA losses throughout the process of sample preparation and analysis (e.g., due to protein binding). Thereafter, FAAs were extracted using 2*2 mL precooled 80% (v/v) aqueous EtOH, 5% (w/v) aqueous TCA or 5% (v/v) aqueous TFA, separately. These three different extraction mediums were studied to compare the extraction efficiency, repeatability, analyte yield and analyte recovery. After 10 min of ultrasonic extraction at low temperature, the slurry was centrifuged at 15,000*g*/min for 20 min (at 4 °C). Ultrasonication facilitated metabolite release from the sample, resulting in increased extraction efficiency (Huie 2002; Kim and Verpoorte 2010). The gathered and pooled supernatant containing the target metabolites was filtered through a 0.22-micron PTFE syringe filter, followed by purification of the filtrate with a membrane filter-Amicon® Ultra-15 10 K NMWL centrifugal filter device (Millipore, Bedford, MA, USA). The fresh extract was centrifuged and filtered into a 2-mL autosampler vial before an aliquot of the extract was directly introduced into the LC system to avoid clogging the HPLC system or reducing the lifetime of the LC guard and separation columns.

2.6 Statistics

All statistical tests and visualizations in this work were conducted in Origin (version 8.1, OriginLab Corporation, Northampton, MA) or the R software environment (version 3.1.2) (R Core Team 2016) for univariate and multivariate data analysis. Tukey's honestly significant difference (Tukey's HSD) multiple comparisons test was used for post hoc testing when the null hypothesis was rejected by one-factor analysis of variance (ANOVA). Differences were considered to be significant when $P < 0.05$. Partial least squares discriminant analysis (PLS-DA), a supervised machine learning method of multivariate statistics analysis, was used to discriminate among the three plant species on the basis of their AA profiles (Barker and Rayens 2003; Brereton and Lloyd 2014). The data matrix consisted of the plant samples in rows and the corresponding FAA contents in columns. Proper data preprocessing prior to the multivariate modeling was necessary (Craig et al. 2006; Gromski et al. 2015). Each variable (column) was log-transformed to obtain a more symmetric distribution and minimize possible effects of outliers. Most multivariate approaches worked better when distributions were normal rather than skewed. Column scaling such as standardization (centering and unit variance scaling) ensured that the variation in each variable had a comparable scale and an approximate equal importance in pattern recognition (Berg et al. 2006). Row scaling converted each AA to a relative level (percent %) by dividing by the total FAA pool, which minimized the inter-sample unnecessary variability caused by the distinguishing tissue structures of different plant species (Brereton 2009).

3 Results and discussion

3.1 Separation and identification

Typical HPLC chromatograms of a mixed standard solution and plant extracts of the AAs are represented in Fig. 1. The method demonstrated good chromatographic separation of the 28 AAs (Fig. 1, 90 µM), including the ISs, within the 19 min. All AAs apart from Phe and Ile ($Rs = 1.31$) were baseline resolved with resolutions ≥ 1.5 (Fig. 1, Table 2). Notably, our method can separate more AAs with better resolution and less time than Wang et al. (2010) which only analyzed 17 AAs using Zorbax Eclipse XDB-C_{18} column in 35 min, and was comparable with Heems et al. (1998) of separating 27 AAs using Hypersil BDS C_{18} column in 24 min. The peaks corresponded to Aaba never overlapped with the inference peak while those at Sar slightly overlapped; however, ammonia ions could interfere with the Nva peak. Furthermore, Aaba and Sar were almost non-existent in the plant samples, so only Aaba and Sar were selected as ISs for calibration (Noctor et al. 2007; Tada et al. 1998). Highly consistent RTs (mean RSD $\leq 1\%$) for all the analyzed AAs, including the ISs, are shown in Tables 2 and 4. The repeatability of the RTs analyzed at seven concentrations ranged from 0.15% (Arg) to 1.19% (Asp) (IS $< 0.4\%$) comparable with that of Heems et al. (1998) (0.1%–0.3%). As observed in Fig. 1, blank response levels were minimized through the careful handling of samples and with reagent preparation techniques. The representative chromatograms obtained for the three plant species (moss and trees) show that the analytical peaks for the individual FAAs can be easily distinguished from superfluous or interferential peaks, and the identifications were confirmed visually or by matching the FAA RTs with those of authentic standards injected within the sample batches and comparing the relative RTs to the IS RTs (Aaba, Nva and Sar).

Because the absolute peak areas fluctuated greatly, the average coefficient of variation (CV) of the seven analyzed concentrations was above 6% (Table 2) higher than that of Heems et al. (1998) at AA concentration of 10 mg/L (1.2%–4.7%) and Noctor et al. (2007) (1.4%–4.2%). The relative area ratios ($R_{AA/IS}$) obtained by dividing the peak area of the AA by that of an IS were used to correct analytical losses and to internally standardize the volume

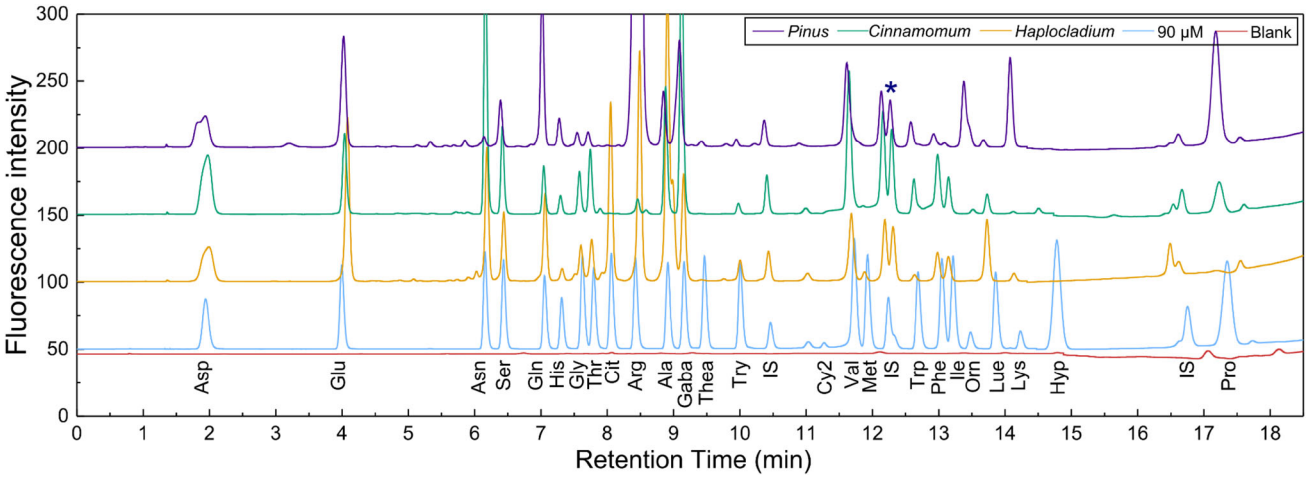

Fig. 1 Typical chromatographic profiles of the 28 standard AAs in a 90 μM solution and the plant extracts of three species, *Haplocladium*, *Cinnamomum* and *Pinus*. Order of elution: glutamic acid (Glu), aspartic acid (Asp), asparagine (Asn), serine (Ser), glutamine (Gln), histidine (His), glycine (Gly), threonine (Thr), citrulline (Cit), arginine (Arg), alanine (Ala), γ-aminobutyric acid (Gaba), theanine (Thea), tyrosine (Tyr), IS-α-aminobutyric acid (Aaba), cystine (Cy2), valine (Val), methionine (Met), IS-norvaline (Nva), tryptophan (Trp), phenylalanine (Phe), isoleucine (Ile), ornithine (Orn), leucine (Leu), lysine (Lys), hydroxyproline (Hyp), IS-sarcosine (Sar) and proline (Pro); asterisk denotes ammonium ion (NH_4^+)

variations during extraction and analysis. Aaba and Sar were used for identifying and correcting primary and secondary AAs (Hyp and Pro), respectively. Table 2 shows the superior consistency in the $R_{AA/IS}$ compared to the absolute peak area for all AAs. The average RSD was below 3%, and only three (Cy2, Orn and Hyp) were above 4%. The intensity ratio of two ISs (Sar/Aaba) was stable (2.04–2.66), with CV % = 7.14% (n = 21).

3.2 Repeatability and intermediate precision

The analytical repeatability was dependent on the AA and sample type. As shown in Table 3, the repeatability of the analysis of each calibration standard within 1 day resulted in an average RSD % of the mean found concentrations (mean FCs, back-calculated concentrations from the calibration curves) below 4% for all AAs and concentration levels, apart from Lys (5.33%), Cy2 (11.0%) and Orn (12.4%), which were above 5%. Most of the RSD % values were less than 2% at 450 μM and expanded to 6%–10% at the lowest measured concentration of 4.5 μM, which indicates good repeatability of the method. AAs with lower responses (e.g., Gln, His, Cy2, Orn and Lys—Fig. 1) tended to have higher % CVs at all concentrations.

The intra and inter-day variations in the RT, relative peak area ($R_{AA/IS}$) and mean FCs for the two QC standard solutions (22.5 and 90 μM) are summarized in Table 4. The precision tests demonstrated good intermediate precision, with average within-day RSDs of < 0.3% and < 2.0% and between-day mean RSDs of < 1.0% and < 5.0% for the RTs and relative area ratios, respectively, which were very close to Zhao et al. (2013) with corresponding precision of 0.1% and < 4.0% for RTs and < 3.0% and < 6.0% for peak areas. The mean FCs of all AAs had average CVs within 2% for intra-day measurements and frequently no more than 3.5% for inter-day measurements, with the highest imprecision (excluding Cy2 and Orn) corresponding to Lys (inter-day 7.69%) at the 22.5 μM level. Cy2, Orn and Lys were not reproducible because their OPA derivatives have considerably shorter lifetimes and lower fluorescent response intensities (not UV response); as a result, their chromatographic peak heights were much lower than those of the other AAs (Fig. 1). Another major factor influencing the intermediate precision or inter-batch precision of the method was the indispensable column washes, performed every 25–30 injections with a mixture of MeOH/water (60/40) to thoroughly regenerate the column. Additionally, the mean FCs at each concentration level were very close to their nominal amounts for almost all AAs.

3.3 Linear calibration curves, LODs and LOQs

The relative correction factor (RCF) was calculated for each AA with reference to the IS, obtained as $(A_{IS} \times C_{AA})/(A_{AA} \times C_{IS})$, where A = peak area, C = concentration, AA = amino acid, IS = internal standard. The CVs for the RCF of the individual AAs obtained from the injection of external amino acid standards at different concentrations were < 13% (n = 21, mean = 8.97%), comparable to previous findings (Amelung and Zhang 2001; Mustafa et al. 2007), and ranged from 3.01% (Met) to 12.58% (Gln), except for Cy2, Orn and Lys, which had higher CVs. Nonetheless, most of the AAs had inconsistent RCFs

Table 2 Precision of the retention times (RTs), absolute peak areas and relative area ratios ($R_{AA/IS}$)

Amino acid	Retention time (min)	Resolution	FLD detector RSD% (n = 21)		
			RT	Area	$R_{AA/IS}$
Aspartic acid	2.06		1.00	7.25	3.51
Glutamic acid	4.14	13.97	0.34	6.74	3.11
Asparagine	6.26	19.91	0.22	5.57	2.21
Serine	6.53	2.64	0.27	5.93	1.98
Glutamine	7.15	5.77	0.25	6.61	2.98
Histidine	7.41	2.44	0.25	8.26	3.72
Glycine	7.71	2.86	0.32	4.92	0.92
Threonine	7.86	1.51	0.32	6.73	2.94
Citrulline	8.15	2.40	0.28	5.57	1.98
Arginine	8.56	3.32	0.15	5.85	2.11
Alanine	9.02	4.83	0.34	5.32	1.27
Gaba	9.27	2.11	0.33	5.15	1.34
Theanine	9.58	2.65	0.30	5.24	1.55
Tyrosine	10.13	4.69	0.29	5.95	1.91
Aaba[a]	10.56	3.88	0.31	4.94	1
Cystine[b]	11.39	6.79	0.25	5.49	8.06
Valine	11.83	3.64	0.29	5.49	2.33
Methionine	12.03	1.56	0.29	5.15	1.51
Norvaline[a]	12.34	2.45	0.29	4.85	1.83
Tryptophan	12.80	3.51	0.28	6.01	2.50
Phenylalanine	13.14	2.84	0.29	6.60	3.14
Isoleucine	13.30	1.31	0.29	5.50	1.69
Ornithine[b]	13.59	2.07	0.30	8.94	4.72
Leucine	13.89	2.49	0.30	5.52	1.48
Lysine[b]	14.29	2.87	0.27	9.36	3.36
Hydroxyproline	14.85	3.14	0.21	8.12	4.93
Sarcosine[a]	16.82	10.22	0.25	6.36	1
Proline	17.40	2.76	0.30	8.26	3.89
Average RSD%			0.31	6.35	2.53

[a]Internal standard (IS)

[b]AA has a much lower detector response factor

$R_{AA/IS}$: relative area ratio = target compound area/IS area, using Aaba for primary AAs and Sar for imino acids (Hyp and Pro)

between high and low concentration levels ($P < 0.05$), while the RCFs of some AAs, such as Gaba, Thea, Met, Trp and Ile, showed no significant differences among each concentration level, with RSD% values below 4%. In such a situation, internal standard calibration curves were constructed through linear regression of the peak area ratio (y-axis) versus the concentration ratio (x-axis) to an IS. The calibration curves can often be expected to be linear over a wide concentration range, even if the detector sensitivity may change with the analyte concentration.

The parameters of the linear calibration curves are shown in Table 5. All compounds showed excellent linearity with determination coefficient (R^2) close to unity, ranging from 0.9989 to 0.9999 over the calibration concentration range from 4.5 to 450 μM for most AAs. These results could be largely explained by the use of superior ISs to control for the variations due to the low sample injection volumes. The intercepts in all calibration curves were nearly 0, indicating low noise-signal of the AA chromatographic peaks. The slopes (regression coefficients) were similar and approached one for all AAs except His, Cy2, Orn and Lys, which had lower slopes, indicating more insensitive FLD responses than Aaba and the other analyzed AAs. The "lack-of-fit" test showed no significant deviation from linearity. The residuals were randomly distributed around zero in the residual-concentration plots (Hibbert and Gooding 2005). Linear regression between the back-calculated and nominal concentrations provided

Table 3 Repeatability (RSD, in %) of the mean found concentrations (mean FCs) over 3 runs within 1 day for each calibration standard mixture

Amino acid	4.5 μM		9.0 μM		22.5 μM		45.0 μM		90.0 μM		225.0 μM		450.0 μM		Mean RSD%
	Mean FC	RSD%	Mean FC	RSD%	Mean FC	RSD%	Mean FC	RSD%	Mean FC	RSD%	Mean FC	RSD%	Mean FC	RSD%	
Aspartic acid	4.64	9.49	9.57	11.66	22.15	2.77	44.26	1.43	90.38	0.96	221.09	0.28	451.10	0.26	3.84
Glutamic acid	5.13	7.34	9.56	8.62	21.87	2.94	42.88	1.61	90.99	1.55	219.21	0.19	451.49	0.51	3.25
Asparagine	3.92	4.58	8.96	3.80	22.37	1.87	45.63	0.47	89.70	1.80	230.15	0.17	447.44	0.47	1.88
Serine	4.24	6.19	9.86	4.78	21.72	2.25	45.15	2.34	90.03	1.27	219.69	0.03	452.22	0.12	2.43
Glutamine	2.74	6.89	8.54	3.16	23.48	1.83	47.39	0.47	88.53	0.32	227.87	0.05	449.68	1.57	2.04
Histidine	5.44	3.94	8.69	1.66	22.63	4.94	43.34	2.75	90.72	2.59	227.11	1.69	433.22	4.71	3.18
Glycine	4.26	2.44	9.64	0.60	21.58	1.20	44.76	2.51	90.22	0.67	219.19	0.28	452.82	0.19	1.13
Threonine	4.21	2.76	9.35	4.35	22.35	2.17	44.48	1.65	90.23	1.91	236.65	1.24	442.59	1.57	2.24
Citrulline	4.41	3.96	9.05	4.91	22.53	2.21	44.46	0.76	90.22	2.38	220.43	0.23	451.72	0.21	2.09
Arginine	4.68	6.30	8.47	1.23	22.99	2.54	44.21	0.59	90.27	2.83	219.81	0.25	451.72	0.84	2.08
Alanine	4.36	3.37	9.50	2.46	21.91	1.63	44.64	2.03	90.23	0.26	220.50	0.18	451.97	0.14	1.44
Gaba	4.90	2.84	8.74	0.40	22.54	0.98	44.65	2.56	90.16	0.69	219.13	0.38	452.82	0.20	1.15
Theanine	4.25	2.35	8.97	3.30	22.65	1.80	44.83	0.47	90.04	2.59	220.95	0.65	451.58	0.45	1.66
Tyrosine	4.84	2.17	9.40	3.95	22.35	2.06	43.90	0.38	90.51	1.45	222.13	0.80	450.20	1.33	1.74
Cystine	6.62	38.89	9.73	7.28	19.70	5.19	43.94	13.07	91.99	6.09	224.81	2.75	447.84	3.74	11.00
Valine	4.83	2.53	9.29	3.67	22.06	0.42	44.06	3.84	90.49	1.95	214.86	0.42	454.46	2.20	2.15
Methionine	4.53	1.31	9.14	3.00	22.36	1.52	44.46	1.21	90.26	0.82	220.05	0.21	451.92	0.41	1.21
Tryptophan	4.40	0.10	8.96	2.56	22.14	2.97	44.93	1.32	90.08	3.38	227.84	0.36	448.49	0.07	1.54
Phenylalanine	4.61	9.80	9.26	8.18	22.22	2.36	43.92	1.32	90.51	1.89	221.29	0.17	450.99	0.34	3.44
Isoleucine	4.58	0.54	9.33	3.62	22.17	2.54	44.41	1.47	90.31	1.93	221.98	0.48	450.78	0.24	1.54
Ornithine	6.16	39.80	9.90	11.74	21.25	15.47	40.64	5.82	91.85	5.45	226.98	3.81	447.95	2.20	12.04
Leucine	4.74	1.62	9.35	3.82	22.15	0.56	44.02	0.85	90.49	2.19	220.13	0.43	451.45	0.48	1.42
Lysine	6.49	16.70	8.43	10.27	20.82	2.19	45.26	3.62	90.18	0.25	229.17	3.34	446.96	0.94	5.33
Hydroxyproline	4.73	12.96	9.22	6.21	22.94	5.86	43.25	15.37	90.67	4.93	223.34	1.43	451.59	3.48	7.18
Proline	4.51	9.16	9.35	1.42	22.50	1.79	43.77	8.98	90.52	6.51	217.30	1.45	453.57	2.36	4.53

Table 4 Intermediate precision (RSD, in %) of retention times (RT), relative area ratios ($R_{AA/IS}$) and mean found concentrations (mean FCs) over 6 runs (intra-day) and over 24 runs over 4 days (inter-day) for two quality control (QC) standard solutions

Amino acid	RT (min)	Mean FC		Intra-day RSD % (n = 6)						Inter-day RSD% (n = 24)					
				22.5 μM			90 μM			22.5 μM			90 μM		
		22.5 μM	90 μM	RT	$R_{AA/IS}$	Mean FC	RT	$R_{AA/IS}$	Mean FC	RT	$R_{AA/IS}$	Mean FC	RT	$R_{AA/IS}$	Mean FC
Aspartic acid	2.06	22.55	90.47	0.61	0.95	0.98	1.34	0.72	1.32	3.37	3.26	3.38	2.69	1.60	1.54
Glutamic acid	4.14	22.08	88.74	0.33	1.03	1.03	0.52	0.36	1.34	2.10	1.85	1.85	1.64	1.90	2.36
Asparagine	6.26	22.65	91.51	0.10	0.70	0.74	0.27	0.78	1.53	1.12	2.01	2.11	0.97	1.64	2.01
Serine	6.53	22.47	90.88	0.12	0.60	0.65	0.31	0.48	1.45	1.16	5.25	5.72	1.03	1.91	1.98
Glutamine	7.15	22.57	86.90	0.12	0.65	0.74	0.28	2.05	2.25	1.04	5.72	2.67	0.94	5.95	4.19
Histidine	7.41	24.86	95.92	0.10	3.13	3.05	0.28	2.51	3.92	1.04	6.64	4.83	0.96	5.23	5.11
Glycine	7.71	21.28	88.87	0.13	0.57	0.66	0.32	0.33	0.86	1.10	4.03	1.66	1.03	1.28	1.34
Threonine	7.86	23.17	93.00	0.13	1.08	1.11	0.30	1.24	2.08	0.97	3.49	3.60	0.94	2.74	3.09
Citrulline	8.15	22.45	90.56	0.12	0.99	1.01	0.29	0.88	0.98	0.94	2.05	2.09	0.90	1.46	1.63
Arginine	8.56	22.75	89.38	0.10	0.86	0.86	0.23	1.28	1.60	0.84	2.24	2.23	0.77	2.32	2.26
Alanine	9.02	22.37	90.61	0.14	0.68	0.72	0.33	0.36	0.90	0.96	2.87	3.05	0.97	0.92	0.90
Gaba	9.27	22.29	88.98	0.13	0.72	0.72	0.32	1.02	0.78	0.98	1.35	1.35	0.99	1.27	1.37
Theanine	9.58	22.58	90.23	0.11	0.78	0.80	0.28	1.20	1.13	0.80	1.78	1.83	0.84	1.52	1.71
Tyrosine	10.13	23.47	93.56	0.10	0.94	0.93	0.28	0.64	1.75	0.90	3.87	3.85	0.90	2.67	2.89
Cystine	11.39	19.28	78.59	0.09	13.88	8.42	0.19	4.40	15.29	0.88	18.06	9.90	0.79	28.94	28.40
Valine	11.83	22.51	90.33	0.09	0.65	2.27	0.22	1.88	1.13	0.80	6.11	4.73	0.82	2.95	2.88
Methionine	12.03	22.48	89.95	0.09	0.64	0.64	0.24	0.41	0.82	0.81	1.60	1.60	0.84	1.38	1.32
Tryptophan	12.80	22.36	91.20	0.09	0.90	0.91	0.23	1.12	1.28	0.79	2.01	2.03	0.82	1.89	2.22
Phenylalanine	13.14	22.40	90.38	0.09	0.98	1.01	0.23	1.31	1.15	0.76	1.80	1.86	0.80	1.60	1.58
Isoleucine	13.30	22.60	90.39	0.09	0.76	0.76	0.23	0.99	1.57	0.75	1.95	1.96	0.80	2.01	1.98
Ornithine	13.59	22.02	93.68	0.10	4.99	9.20	0.21	3.95	9.92	0.76	8.98	9.04	0.77	12.90	12.84
Leucine	13.89	22.28	89.61	0.10	0.29	0.29	0.23	1.33	1.33	0.81	1.59	1.59	0.79	1.58	1.96
Lysine	14.29	21.84	86.55	0.06	3.69	3.51	0.22	1.76	4.22	0.75	16.10	7.69	0.75	15.20	4.29
Hydroxyproline	14.85	21.44	85.13	0.07	4.65	4.79	0.18	2.56	5.43	0.65	10.08	7.67	0.67	11.55	5.67
Proline	17.40	22.30	87.84	0.09	3.88	3.32	0.23	3.32	4.67	0.66	10.08	6.66	0.68	9.00	4.97
Average		22.36	89.73	0.13	1.77	1.37	0.30	1.35	1.89	1.00	4.56	3.31	0.94	4.74	2.58

slopes equal to 1.00 ($P = 0.2186$, t test) and intercepts close to 0 ($P = 0.6176$). The accuracy (%) of a calibration curve was evaluated as a mean of the ratio of measured concentration to nominal concentration, which for each calibration point was approximately 100%. Only Cy2 (102.2%) and Lys (103.8%) were slight overestimated.

The LODs and LOQs of the analytical procedure ranged from 0.12 μM (Glu) to 6.75 μM (Cy2) and from 0.39 to 22.28 μM, respectively (Table 5). Cy2 and Orn had relatively higher LODs due to their low fluorescence responses, and all the other AAs had LODs below 2 μM, except the LOD of Lys was 3.44 μM. Likewise, Zhao et al. (2013) analyzed 21 FAA in tea using HPLC-FLD, and LODs of method ranged from 0.2 μM (Gaba) to 19.2 μM (Arg) and LOQs from 0.7 μM to 63.9 μM. Typically, FLD has higher sensitivity than DAD and FID, but less in comparison with MS detector (Mohabbat and Drew 2008; Shimbo et al. 2009; Wang et al. 2010). Although the highest LODs of approximately 7 μM were obtained for Cy2 and Orn, this concentration only corresponded to an absolute injection amount of 2 pmol. These results demonstrate that the method presented satisfactory analytical sensitivity for quantifying FAAs in plant tissues.

3.4 Extraction method and recovery

Figure 2 shows the FAA extraction efficiency obtained using three solvents from the foliage of two higher plants, *Pinus* (a) and *Cinnamomum* (b), which contained more complex components than moss tissue. TCA and TFA extracted almost equivalent levels of each FAA and remarkably more total FAAs (TAA) than EtOH.

Table 5 Parameters of the linear calibration curves, the LODs and LOQs of 25 AAs

Amino acid	Linearity range μM	$A_{AA}/A_{IS} = C_{AA}/C_{IS} \times a+b$[a]		Determination coefficient R^2	Accuracy[b]		LOD μM	LOQ μM
		Slope a	Intercept b		(%)	RSD %		
Aspartic acid	4.5–450	0.761	0.065	0.9998	101.4	4.50	0.19	0.64
Glutamic acid	4.5–450	0.802	0.066	0.9995	100.4	4.31	0.12	0.39
Asparagine	4.5–450	0.885	0.073	0.9998	100.0	3.14	0.49	1.64
Serine	4.5–450	0.861	0.063	0.9998	100.0	5.03	0.42	1.40
Glutamine	4.5–450	0.855	0.005	0.9998	100.0	4.15	0.50	1.67
Histidine	4.5–450	0.453	0.053	0.9996	100.0	2.95	1.00	3.33
Glycine	4.5–450	0.926	0.073	0.9997	100.0	3.27	0.35	1.17
Threonine	4.5–450	0.739	0.179	0.9983	100.0	2.90	0.60	1.99
Citrulline	4.5–450	0.927	0.030	0.9998	100.0	2.78	0.51	1.70
Arginine	4.5–450	0.901	0.031	0.9997	100.0	3.38	0.58	1.93
Alanine	4.5–450	0.873	0.034	0.9998	100.0	2.43	0.50	1.67
Gaba	4.5–450	0.926	− 0.046	0.9998	100.0	1.38	0.56	1.87
Theanine	4.5–450	0.950	0.028	0.9999	100.0	2.23	0.53	1.77
Tyrosine	4.5–450	0.828	0.078	0.9997	100.0	2.47	0.59	1.96
Cystine	22.5–450	0.061	0.007	0.9989	102.2	8.63	6.75	22.28
Valine	4.5–450	1.417	− 0.047	0.9992	100.0	2.46	0.92	3.06
Methionine	4.5–450	1.043	0.014	0.9998	100.0	1.83	1.22	4.05
Tryptophan	4.5–450	0.883	0.038	0.9999	100.0	3.40	1.41	4.69
Phenylalanine	4.5–450	0.979	0.086	0.9998	100.0	4.64	1.21	4.02
Isoleucine	4.5–450	1.050	0.055	0.9999	100.0	1.86	1.19	3.98
Ornithine	22.5–450	0.165	0.051	0.9994	101.3	9.16	6.68	21.40
Leucine	4.5–450	0.8810	0.046	0.9997	100.0	1.98	1.41	4.69
Lysine	9.0–450	0.189	0.028	0.9996	103.8	7.01	3.44	11.48
Hydroxyproline	4.5–450	0.966	− 0.083	0.9998	100.0	7.14	1.05	3.52
Proline	4.5–450	0.835	− 0.020	0.9995	100.0	5.99	1.42	4.74

[a]*A* peak area, *C* concentration, *AA* amino acid, *IS* internal standard

[b]Accuracy: (mean of measured concentration/nominal concentration) × 100 (%)

Nevertheless, TCA ineluctably led to a split of the Glu peak into two peaks in chromatogram (not shown here). The extraction of AAs with R groups with a range of polarities was significantly affected by the solvent polarity (Lin et al. 2007; Mushtaq et al. 2014; Yang and Smetena 1993). The aqueous solutions of TCA and TFA containing a relatively high ratio of a highly polar solvent (water) preferentially extracted more hydrophilic AAs, especially acidic Asp and Glu (R group: CH_2–COOH). The 80% aqueous EtOH solution was less polar, resulting in a higher extraction efficiency for more hydrophobic AAs, such as nonpolar Phe and Pro. However, for most extracted FAAs, the differences in the peak intensity were less noticeable. Furthermore, organic solvents (EtOH and acetone) always extracted abundant plant pigments, such as chlorophyll, flavones, and polyphenolic compounds, which tended to irreversibly adsorb on the silica surface of the analytical columns, negatively impacting subsequent purification and analysis steps of extraction The extraction precision of TFA was superior to that of TCA and EtOH, especially for low-concentration AAs, such as Trp, Ile, Leu and Pro. The consistency of the FAA extraction by TFA was represented by a mean RSD of 3.34% and 1.30% for *Pinus* and *Cinnamomum*, respectively, and ranges of 0.19%–9.98% and 0.02%–4.74%. The between-extract variability of our method was slightly lower than Noctor et al. (2007) of 4.6%–17% who extracted leaf FAA with 2 mL 80% methanol.

Figure 3 shows the FAA recoveries obtained using 5% (v/v) TFA from *Pinus* (a) and *Cinnamomum* (b) foliage at two spiked levels (25 and 100 μM). The recovery quotients of the individual AAs from both plant species were similar, with most AAs above 90% and none exceeding 110%, showing that the extraction procedure itself did not cause appreciable loss or transformation of amino acids. Only three to five of the 25 analytical AAs had recoveries below

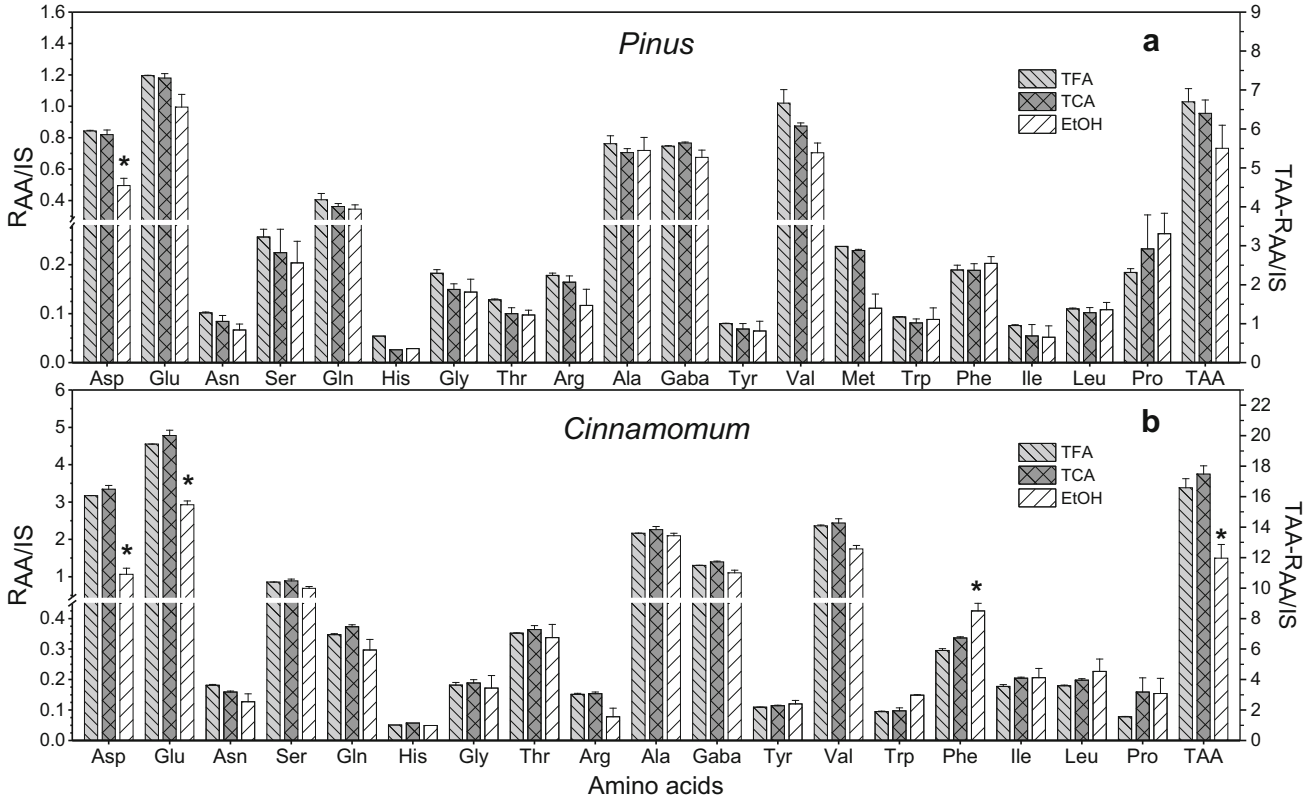

Fig. 2 FAA extraction of **a** *Pinus* and **b** *Cinnamomum* samples using 5% (v/v) TFA, 5% (w/v) TCA or 80% (v/v) EtOH. Bar heights indicate the mean of all three biological replicates, and error bars show the standard deviations. Asterisk denotes the mean extraction efficiency of EtOH was significantly different ($P < 0.05$) from those of the two other solvents. Abbreviations as in Fig. 1

the 80% criterion; Trp (71%) and Hyp (67%) were notably underestimated when spiked at 25 μM. The recoveries when spiking at 100 μM were slightly higher than those at 25 μM for both plant species, but the difference was less noticeable. Similarly, several analytes with lower recoveries were those AAs that were less abundant in the sample or were presumably relatively instable in the extraction process, e.g., Trp is destroyed during acid hydrolysis (Cooper et al. 2001). The recovery of all AAs calculated over all spiked levels and for all plant replicates averaged 94.4% and ranged from 67.0% (Hyp) to 108.9% (Tyr). The result is comparable with previous research for FAA extraction of plant foliage. For instance, the recovery values 80%–120% were obtained by 80% methanol extraction and 89.8%–107.4% by distilled water at 90 °C followed SPE (Noctor et al. 2007; Wang et al. 2010). The method also had satisfactory recovery repeatability, with an average RSD of ca. 3%, ranging from 0.20% for Gln at 100 μM to 9.34% for Orn at 25 μM spiked level in *Cinnamomum* extract.

3.5 Application

The plant FAA peak areas were normalized by correcting for the IS response. The FAA contents (μmol g^{-1} DW) in the plant samples were calculated using the corresponding calibration equations and were normalized to the sample dry weight. The FAA contents obtained by applying these procedures to the plant specimens are summarized in Table 6. Nineteen common FAAs were found in all 136 samples of the three species, *Haplocladium*, *Cinnamomum* and *Pinus*. The amplitudes (max/min) of fluctuation of the content for individual FAA in each plant species varied from few to hundreds, and exhibited large amplitudes for most of the AAs, especially Asn, Arg, Gly, His and Trp. The average percent contents (%) of Arg, Asn, Glu and Gln, the most abundant FAAs, surpassed 10% in *Haplocladium. Cinnamomum* was characterized by higher absolute contents of Asp, Glu, Gaba and Val. Generally, the four most abundant FAAs in *Pinus* were Arg, Aps, Glu, and Gaba. FAAs such as Cit, Met, Cy2 and Orn were infrequently present in very low amounts (< 1%). The average total FAA content in *Haplocladium* (67.39 μmol g^{-1} DW) was approximately six times that in *Cinnamomum* (9.66) and *Pinus* (10.79). *Haplocladium*

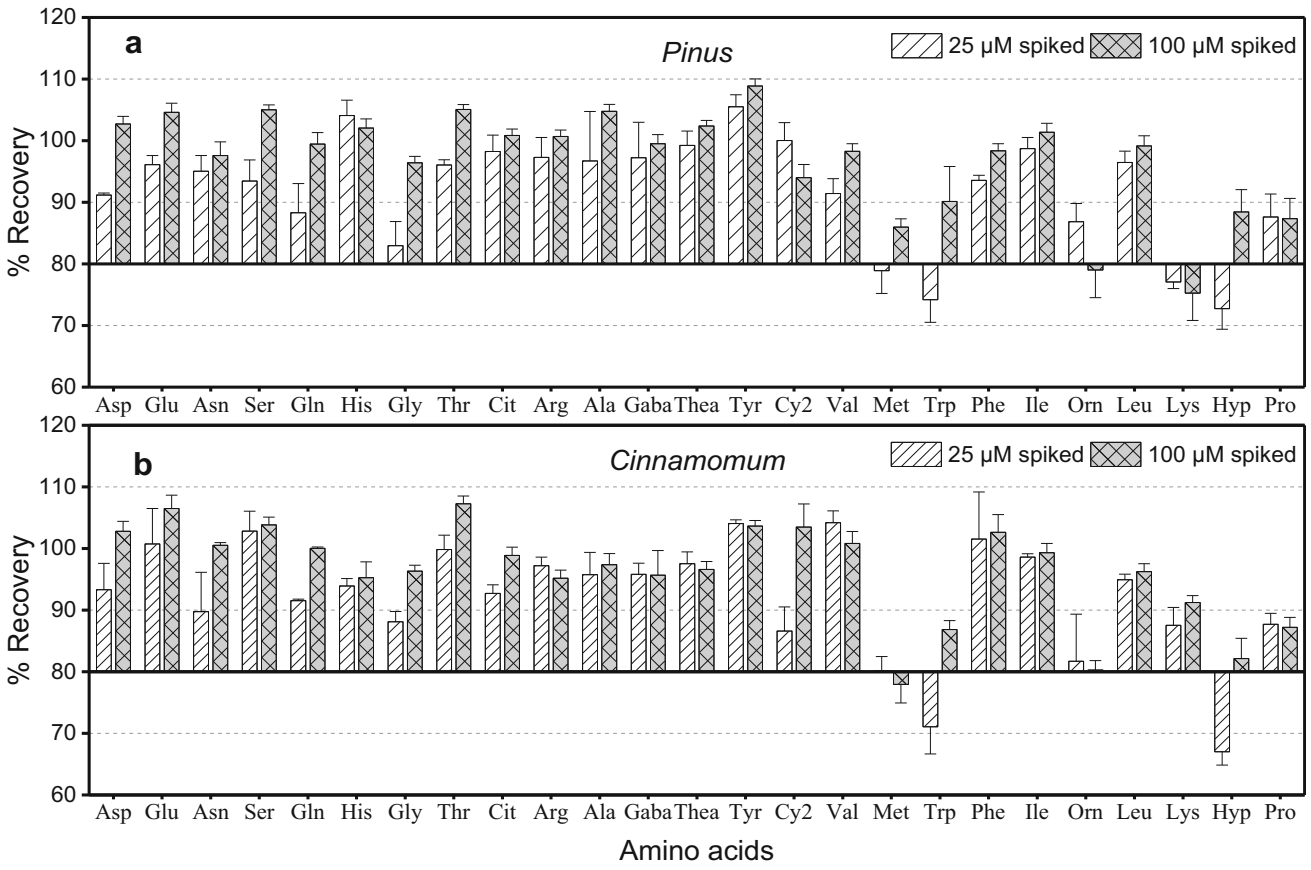

Fig. 3 FAA recoveries (%) at two spiked concentrations from **a** *Pinus* and **b** *Cinnamomum* extracted using 5% (v/v) TFA. Abbreviations as in Fig. 1

contained highest abundances of almost all AAs based on dry weight compared to the other plants, which may have been caused by the different proportions of mesophyll cells and wood fiber in the plant tissues of different species.

A PLS-DA classification model was applied to reduce the dimensionality of the data and to facilitate interpretation of the FAA patterns. The resampling approach randomly split the samples into a training set (104, approximately three-quarters of the data) for building a model and a test set (32) for externally validating the predictive capability. Ten times repeated 7-cross valuation (rCV) was used to optimize the model complexity (model selection) in terms of the number of PLS components by maximizing the criteria of "Accuracy" (agreement between the observed and predicted classes) and "Kappa" (Cohen's Kappa) (Ferri et al. 2009; Filzmoser et al. 2009; Kuhn and Johnson 2013; Pérez-Castaño et al. 2015). Once the model parameters had been tuned, the complete training data were used for model construction and used to predict the data in the test set. We performed 100 repetitions of data split so that the power of the classifiers could be assessed using the mean classification accurate rate % (100 splits) of the test set to avoid the pitfalls of over-fitting (Brereton 2009).

The statistical significance of the final PLS-DA models was evaluated through a Monte Carlo permutation test (Hesterberg et al. 2005; Pesarin and Salmaso 2010; Westerhuis et al. 2008). The null hypothesis H_0 of the permutation tests assumed that there was no difference among groups. By randomly reordering (shuffling) the labels of the samples enough times, we obtained the null distribution of H_0 for the diagnostic parameters of new models with the permuted data sets. As demonstrated in Fig. 4, a clear distinction between the statistical values was obtained from the permutation distribution and the original data. Of the 2000 permutations, none model had a value above the original values of 0.96 (Accuracy) or 0.95 (Kappa), leading to $P < 0.0005$ and providing strong evidence that the null hypothesis H_0 could be rejected. Therefore, the observed differences between groups were considered significant in the statistical sense.

Figure 5 depicts the discriminant biplot of the final PLS-DA classifiers. The total variance explained by the first three components was 60% (pls1: 32%, pls2: 13%, and pls3: 15%). The PLS scores of the species samples

Table 6 FAA contents (μmol•g^{-1} DW) in the plant specimens: minimum–maximum (mean)

Amino acids	*Haplocladium (22)*	*Pinus (47)*	*Cinnamomum (67)*
Aspartic acid	1.12–13.06 (6.50)	0.56–3.80 (1.66)	0.38–4.91 (1.68)
Glutamic acid	3.11–17.97 (8.76)	0.49–2.61 (1.52)	0.85–4.18 (1.83)
Asparagine	1.28–33.25 (11.81)	0.01–1.33 (0.20)	0.02–0.35 (0.09)
Serine	1.24–6.69 (3.38)	0.13–1.18 (0.35)	0.17–1.10 (0.45)
Glutamine	2.49–15.39 (7.04)	0.13–2.08 (0.65)	0.08–0.94 (0.27)
Histidine	0.37–2.62 (1.18)	0.06–0.90 (0.18)	0.04–0.15 (0.08)
Glycine	0.18–1.61 (0.63)	0.02–0.20 (0.06)	0.01–0.21 (0.08)
Threonine	0.69–2.33 (1.28)	0.06–0.31 (0.13)	0.07–0.51 (0.20)
Arginine	2.48–31.45 (11.99)	0.06–19.67 (2.12)	0.04–0.27 (0.10)
Alanine	2.96–12.35 (6.21)	0.38–3.02 (0.97)	0.33–2.47 (1.06)
Gaba	0.92–9.15 (4.05)	0.34–3.75 (1.24)	0.26–4.36 (1.57)
Tyrosine	0.16–0.78 (0.39)	0.03–0.19 (0.06)	0.03–0.16 (0.06)
Valine	0.76–2.12 (1.38)	0.38–1.31 (0.69)	0.76–1.64 (1.14)
Tryptophan	0.06–0.43 (0.2)	0.02–0.43 (0.11)	0.03–0.26 (0.10)
Phenylalanine	0.14–1.27 (0.47)	0.05–0.33 (0.13)	0.05–1.31 (0.46)
Isoleucine	0.10–0.88 (0.31)	0.02–0.14 (0.04)	0.03–0.21 (0.09)
Leucine	0.21–2.42 (0.88)	0.04–0.32 (0.1)	0.03–0.21 (0.09)
Lysine	0.25–1.44 (0.62)	0.07–0.54 (0.16)	0.08–0.28 (0.14)
Proline	0.33–1.65 (0.70)	0.20–0.95 (0.41)	0.05–0.51 (0.17)
$\sum$	67.79	10.79	9.66

$\sum$: sum of the mean FAAs determined in each species

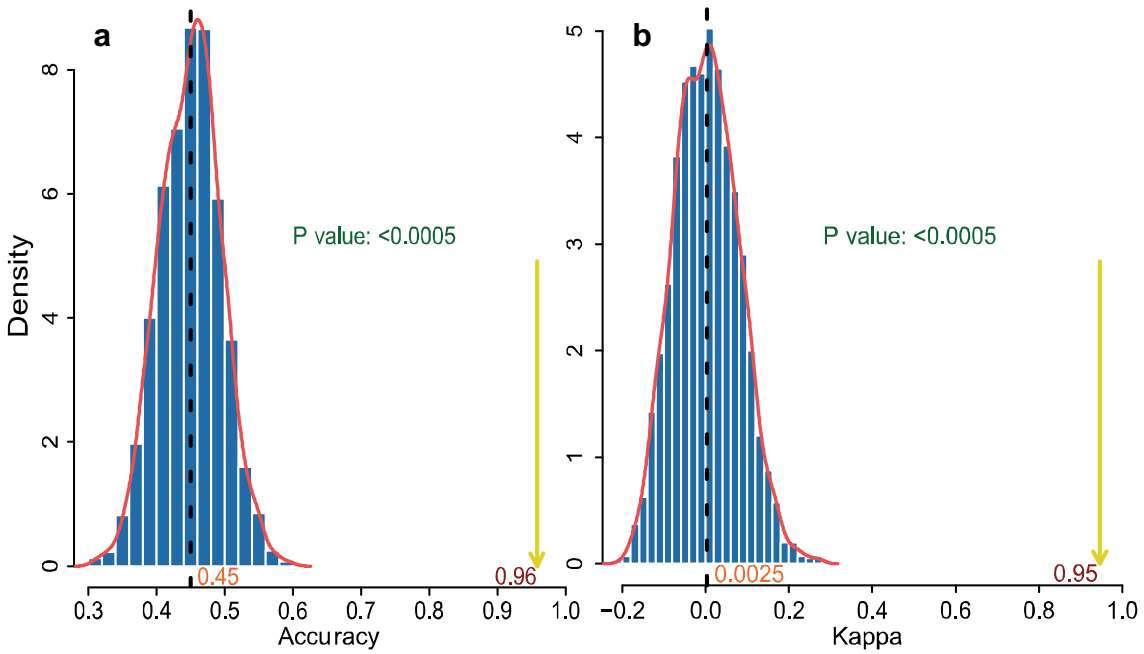

Fig. 4 Statistical significance of the PLS-DA model. The permutation distribution of the diagnostic parameters for **a** accuracy and **b** kappa. The dashed line (black) marks the mean of the permutation distribution. The solid vertical line (yellow) marks the original value; its location outside the right tail indicates that this large value is unlikely to occur when the null hypothesis H_0 is true

unambiguously formed three strong clusters along the axis of pls1 and pls2. *Haplocladium* and *Cinnamomum* were well separated across the pls1 component and grouped in the opposite direction. Pls2 improved the separation of *Pinus* from the other samples. The farther that a loading vector is from the origin, the greater the influence a given

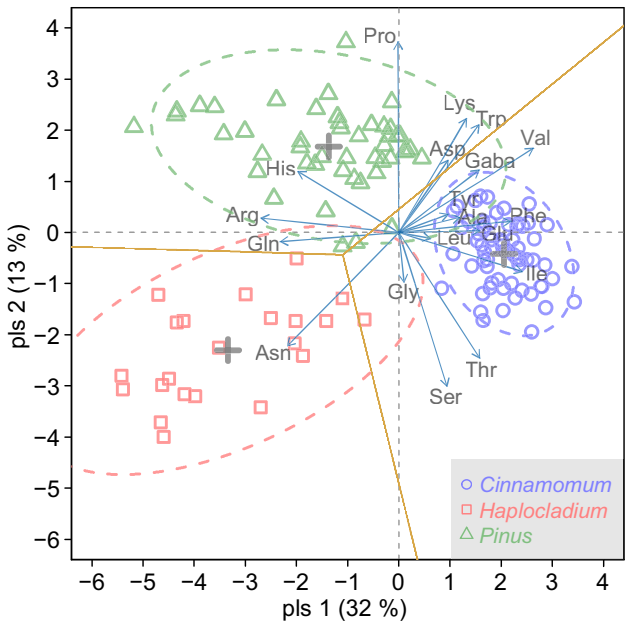

Fig. 5 Biplot of the PLS-DA classification model for the three plant species. The samples of *Cinnamomum* (empty circle), *Haplocladium* (empty square) and *Pinus* (empty triangle) in the two dimensional score-space indicate three strong groups. Loading vectors (left arrow) represent the contribution of individual AAs to the clustering. Ellipses (dashed line) and crosses (plus symbol) correspond to the 95% confidence intervals and space centers (centroids) of each cluster in the discriminatory space, respectively. The discriminant decision boundaries are indicated using solid lines. Abbreviations as in Fig. 1

AA has on the linear combination in structuring the discriminant axes. The loadings demonstrate that the samples were grouped in pls1 + (positive axis) mainly according to their relatively higher proportion (%) of Phe, Val, Ile and Gaba in *Cinnamomum*. On the other hand, the negative axis of pls1 was influenced by the relative contents of Arg, Gln, Asn and His, which had higher proportions in *Haplocladium* and *Pinus*. The samples that were mostly contained in quadrant of pls1- and pls2 + were explained by the percentage of Pro, Lys and His, which were mainly responsible for separating *Pinus* samples from *Cinnamomum* samples. The negative axis of pls2 was explained by the remaining variables studied, Ser, Thr and Gly. Confidence ellipses (confidence level set at 0.95) for each class were plotted, and their slight overlap highlights the strength of the discrimination. Only a few samples were slightly outside their own 95% confidence ellipse and were regarded as moderate outliers. The linear discriminant decision boundaries show that nearly all samples were correctly assigned to each plant species (average accuracy rate of the test sets was 99.12%), except for several *Haplocladium* and *Pinus* samples that might have been misclassified as *Cinnamomum*. The validated PLS-DA classifier and discriminant boundaries can be used as decision criteria for the classification of new samples and for finding potential discriminatory marker variables (Beckonert et al. 2007; Lloyd et al. 2009).

4 Conclusion

A proposed HPLC-FLD method was developed for the rapid, simultaneous and quantitative analysis of 25 AAs as their OPA and FMOC derivatives within 26 min run time. All evaluated validation indices showed that the HPLC method was sensitive, reproducible, accurate and robust for the trace determination of FAAs in plant tissues. In addition, the extraction solvent and extraction techniques played decisive roles in the accurate quantitation of FAAs. A comparison of three solvents (TFA, TCA and EtOH) for the extraction of FAAs from plant tissues revealed that a mixture of 5% (v/v) TFA/water was preferred over 5% (w/v) TCA and 80% (v/v) aqueous EtOH in terms of the extraction efficiency and cleanliness of the crude extracts. This extraction method makes a trade-off between the simplicity and ruggedness of the sample preparation technique. The main advantage of this method is that it requires comparatively little maintenance and is relatively easy, especially in terms of the fully automated online precolumn derivatization process, which makes it suitable for the routine AA analysis of various plant extracts.

Finally, three plant species were investigated, and inherently different FAA profiles were identified in these species through PLS-DA of 136 plant samples. This study serves as a basis for further biochemical characterization of the FAA metabolic patterns in plant tissue sampled from various environments and geographical origins.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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