Interactive adsorption of phenolic acids and amino acids on soil minerals

Implications for the formation and properties of soil mineral–organic associations

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Chapter 3

Robust analysis of underivatized free amino acids in soil by hydrophilic interaction liquid chromatography coupled with electrospray tandem mass spectrometry

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Abstract

Amino acids are an important and highly dynamic fraction of organic N in soils and their determination in soil without derivatization is challenging due to the difficulties in separation and detection of trace amounts of these polar analytes. In the present work, we developed an analytical method to quantify 20 free amino acids in aqueous soil extracts without derivatization. The method employed hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC–MS/MS) technique combined with a cation exchange solid phase extraction (SPE). Four stable isotope labelled amino acids were used as internal standards to improve the method performance. Good separation of 20 underivatized amino acids was achieved within 12 min. The limit of detection (LODs) and limit of quantification (LOQs) were in the range of 13–384 ng g^{-1} and 43–1267 ng g^{-1} (dry soil basis), respectively. The results showed that overall recoveries with high precision were obtained for the extracted free amino acids from ten different soils. The overall recoveries of 18 amino acids were similar for the ten soils used, which differed substantially in organic C content and in other properties as soil texture and pH. For most of the amino acids, the average recoveries from soil extracts were between 74% and 117%, with the exception of Met (31%), Pro (52%) and Arg (68%). Variability was within acceptable limits (relative standard deviations were between 4% and 13%), with the exception of Met (relative standard deviation = 90%) and Arg (relative standard deviation = 53%). Thus the proposed method with high throughout and high analyte specificity shows great promise for consistent analysis of free amino acids extracted from soils and offers new horizons for the analysis of amino acids in terrestrial and aquatic ecosystem.

3.1 Introduction

Amino compounds (amino acids and amino sugars) play an important role in soil N cycling. These compounds account for 30–80% of total soil N and constitute the majority of soil organic N (Martens *et al*., 2006; Olk, 2008; Olk *et al*., 2008; Creamer *et al*., 2012; Creamer *et al*., 2013). Amino acids are the key component of soil dissolved organic nitrogen (DON) and they are closely related with the soil organic carbon (SOC) pool in terms of the dynamics and stabilization of dissolved organic carbon (Perakis & Hedin, 2002; Knicker, 2011). Amino acids not only represent a crucial N source for microorganism but also can be directly utilized by some plants (Näsholm *et al*., 2000; Jones *et al*., 2004). Additionally, these compounds can serve as biomarker to indicate their sources (e.g. microbial vs. plant residues) (Creamer *et al*., 2013).

Amino acids are zwitterionic compounds, i.e. they can exist as an overall neutral form, a weak acid or a weak base. Due to the lack of specific chromophores for ultraviolet (UV) or fluorescence detection for most of amino acids (Kaspar *et al*., 2009), derivatization is widely used to improve the separation and the sensitivity of detection for analysing amino acids in biological and environmental samples (Kielland, 1995; Callejón *et al*., 2010; Inagaki *et al*., 2010; Kvitvang *et al*., 2011; Sarazin *et al*., 2011; Sánchez-Hernández *et al*., 2011). Overall these techniques can guarantee good separation, sensitive detection and high reproducibility but they have some drawbacks, such as being time-consuming and displaying instable derivatives, insufficient reproducibility of derivative yield, lack of analyte specificity and side effects of reagents (Langrock *et al*., 2006; Kaspar *et al*., 2009). Therefore, recently, the techniques for direct analysis of underivatized amino acids have gained more attention and several methodologies have been developed. Casella and Contursi (2003) measured 12 amino acids in milk using ion chromatography (IC) coupled to amperometric detection. High performance anion exchange chromatography combined with pulsed amperometric detection (HPAEC-PAD) was successfully applied to analyse soil amino acids (Martens & Loeffelmann, 2003; Olk *et al*., 2008; Creamer *et al*., 2012). Desiderio *et al*. (2010) demonstrated that capillary electrophoresis (CE) linked to mass spectrometry (MS) detection enabled analysis of amino acids without previous derivatization. Ion-pairing liquid chromatography (LC) coupled to MS detection is another alternative approach to analyse underivatized amino acids (Piraud *et al*., 2005; Armstrong *et al*., 2007; Liu *et al*., 2008; Le *et al*., 2014). However, there are still serious drawbacks connected to all described techniques, including low throughput, reagents-induced retention time shift and contamination of analytical system (Liu *et al*., 2008; Kaspar *et al*., 2009).

Most recently, hydrophilic interaction liquid chromatography (HILIC) has attracted considerable attention because of the many advantages over conventional normal phase LC and reverse phase LC (Buszewski & Noga, 2012). HILIC is capable of separating a broad range of polar compounds, including amino acids, peptides, carbohydrates, polar drugs, metabolites and biologically important compounds in proteomics, glycomics and clinical analysis (Dell'mour *et al*., 2010; Jandera, 2011; Buszewski & Noga, 2012; Guo *et al*., 2013). Guo *et al*. (2013) have demonstrated that HILIC is a reliable technique to analyse amino acids in food. The successful application of HILIC in detecting medicine and cellular metabolites was displayed as well (Preinerstorfer *et al*., 2010; Zhou *et al*., 2013). As part of these efforts, various HILIC columns have been tested for separation of amino acids, such as silica, amide, bridged ethyl hybrid (BEH) amide and zwitterion (Dell'mour *et al*., 2010; Guo *et al*., 2013; Gökmen *et al*., 2012). While successfully applied in the before-mentioned matrices, the applicability of HILIC has yet to be tested for the analysis of amino acids from soils, which form a complex and difficult matrix because of the plethora of binding sites for various compounds including amino acids. In the mineral soil compartment, many interfering compounds can be co-extracted from soils together with the targeted amino acids (McDowell & Likens, 1988; Guggenberger & Zech, 1994; Kaiser *et al*., 2001), and the free amino acid concentrations in soil solutions are generally low (Jones *et al*., 2002). Therefore, in contrast to the application in matrices such as food, purification and concentration of soil extracts are most likely necessary prior to the analysis of soil amino acids. Solid phase extraction (SPE) has become a widely-used technique to clean up and concentrate extracted amino acids in complex samples through isolating analytes of interest from a wide variety of matrices, such as biological matrices (Armstrong *et al*., 2007; Calderón-Santiago *et al*., 2012; Tang *et al*., 2014) and sediment (Yang *et al*., 2011). However, only a few studies have reported the performance of the SPE technique in purifying and concentrating soil extracts prior to the measurement of free amino acids. Dell'mour *et al*. (2010) reported that the overall recoveries of 12 free amino acids in soil extracts through SPE procedure ranged between 13% and 70%.

Therefore, the aim of the present study was to develop and test a novel, robust and fast procedure to extract and analyse amino acids from soils through combining HILIC-HPLC–MS/MS and SPE technique. For this purpose, ten soils representing a broad range of compositions (pH, texture class, organic C content, etc.) were augmented with 20 amino acids that are common in soils. These were subsequently used to develop and test the new procedure.

3.2 Material and methods

3.2.1 Chemicals

Acetonitrile (ACN, LC/MS grade), methanol (MeOH, ULC/MS grade) and formic acid (FA, ULC/MS grade) were purchased from Biosolve B.V. (Valkenswaard, The Netherlands). Analytical grade ammonium formate (NH_4HCO_2) and ammonium acetate (NH_4OAc) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Analytical grade hydrochloric acid (37%), ammonium hydroxide (25 wt%) and acetic acid (AcOH) were purchased from Merck KGaA (Darmstadt, Germany). Analytical grade sodium azide (NaN_3) was purchased from Janssen Chimica (Beerse, Belgium). All mobile phases and solutions were prepared using subboiled water (sub-boiling distilled water). Ultrapure deionized water produced by a Purelab Ultra system (MK2-Analytic, ELGA, High Wycombe, United Kingdom) was used to extract soil free amino acids.

Targeted amino acids and internal standards (IS) are listed in Table 3.1. Those amino acids were divided into three groups: acidic, basic and neutral amino acids. The purity of each compound was higher than 98%.

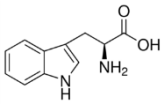
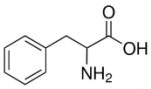
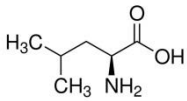
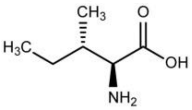
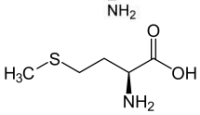
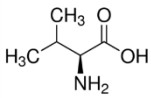
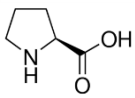
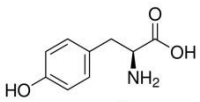
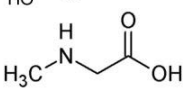
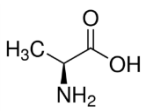
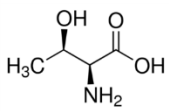
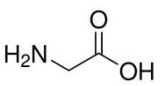
3.2.2 Instrumentation

The separation system employed a Prominence fast LC (Shimadzu, Kyoto, Japan), consisting of two LC-20AD XR pumps, a SIL-20AC XR auto-sampler and a CTO-20AC column oven. HILIC separation was performed on an ACQUITY UPLC BEH Amide separation column (2.1 mm × 100 mm, 1.7 µm, Waters), together with an ACQUITY UPLC BEH Amide 1.7 µm VanGuard guard column. BEH amide column was selected because it can provide great retention and selectivity (Guo *et al*., 2013; Kumar *et al*., 2013).The injection volume was 20 µl. Sample was eluted at a flow rate of 0.4 ml min^{-1}. The column temperature was set at 35 °C.

Mass spectrometry detection was carried out on a 4000 QTRAP LC–MS/MS System (AB SCIEX, MA, USA), coupled with an ESI interface. Multiple reaction monitoring (MRM) method was used to determine all the analytes. Data acquisition and analysis were performed with AB SCIEX Analyst software (ver. 1.5.1).

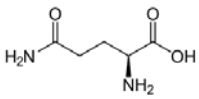
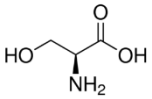
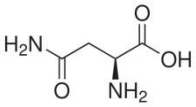
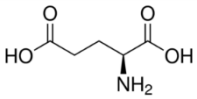
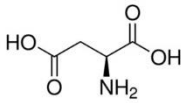
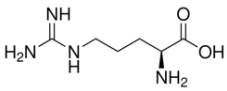
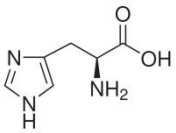
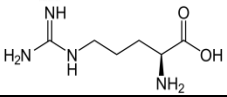
A bench-top freeze dryer (Scala Scientific, The Netherlands) was used to freeze-dry soil extracts.

Table 3.1 Properties and supplier of the targeted amino acids and internal standards.

Amino acids		Abb.[a]	Structure	MW	pI[b]	Supplier
Neutral						
1	L-tryptophan	Trp		204.23	5.89	SA[c]
2	DL-phenylalanine	Phe		165.19	5.48	SA
3	L-leucine	Leu		131.18	5.98	SA
4	L-isoleucine	Ile		131.18	6.02	SA
5	L-methionine	Met		149.21	5.74	SA
6	L-valine	Val		117.15	5.96	SA
7	L-proline	Pro		115.13	6.30	SA
8	L-tyrosine	Tyr		181.19	5.66	SA
9	Sarcosine	Sar		89.09	6.20	SA
10	L-alanine	Ala		89.09	6.01	SA
11	L-threonine	Thr		119.12	6.16	SA
12	Glycine	Gly		75.07	5.97	SA

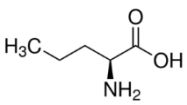
(Continued)

Table 3.1 Properties and supplier of the targeted amino acids and internal standards (continued).

Amino acids		Abb.[a]	Structure	MW	pI[b]	Supplier
Neutral						
13	L-glutamine	Gln		146.15	5.65	SA[c]
14	L-serine	Ser		105.09	5.68	SA
15	L-asparagine	Asn		132.12	5.41	SA
Acid						
16	L-glutamic acid	Glu		147.13	3.24	SA
17	L-aspartic acid	Asp		133.1	2.77	SA
Basic						
18	L-arginine	Arg		174.2	10.76	SA
19	L-histidine	His		155.16	7.59	SA
20	L-lysine	Lys		146.19	9.82	SA

(Continued)

Table 3.1 Properties and supplier of the targeted amino acids and internal standards (continued).

Amino acids		Abb.[a]	Structure	MW	pI[b]	Supplier
Internal standards (IS)						
21	DL-norvaline	Nva		117.15		SA[c]
22	L-lysine HCl (4,4,5,5-D4)	Lys-D4		150.21		SA
23	L-alanine (2,3,3,3-D4)	Ala-D4		93.12		CIL[d]
24	Glycine (D5)	Gly-D5		80.1		CIL
25	L-glutamic acid (2,3,3,4,4-D5)	Glu-D5		152.16		CIL

[a] Abbreviation; [b] Isoelectric point, from Barrett (1985) and Cernei *et al*. (2012); [c] Sigma-Aldrich (Zwijndrecht, the Netherlands); [d] Cambridge Isotope Laboratories (Andover, MA, USA).

3.2.3 Preparation of test solutions

Separate stock standard solutions of targeted amino acids (2000 µg ml^{-1}) were prepared by dissolving each compound in 0.1 M HCl. The concentration of Tyr was set at 500 µg ml^{-1} because of its low solubility in water. Separate IS stock solutions (500 µg ml^{-1}) were also prepared in 0.1 M HCl. All the stock solutions were stored at −20 °C before use. Working solutions were prepared by mixing and diluting the stock standard solutions. According to our results, the stock standard solutions were stable for at least one month at −20 °C (data not shown).

3.2.4 Extraction of soil free amino acids

Table 3.2 shows the tested soil samples in this study. Ten different soils from around the world ranging from Podzols to Luvisols were selected to represent the broad range of complex matrices that could be encountered when extracting amino acids from soils. The effects of different pre-treatment of soil samples

were also considered in this study to cover the methods used by people in quantifying soil amino acids. Prior to the extraction some soils (1–6) were dried and ground, some soils (9 and 10) were only dried and sieved (< 2 mm), and soil 7 and 8 with field moisture were stored at 4 °C and directly extracted. Based on soil organic carbon (SOC) contents, different ratios of soil to water were used in extracting soil amino acids. A suspension of soil/ultrapure H_2O (1:20, w/v, for soil 1; 1:10, w/v, for soil 2–10) was horizontally shaken for 1 h (120 rpm) at ambient temperature, centrifuged (4000 *g*, 20 min) and filtrated through 0.2 µm mixed cellulose ester membrane filter (ME24, GE Healthcare Life Sciences, Whatman). The loss of the 20 AAs during the filtration was negligible (the filtration recovery for each of them was between 94% and 102%). To inhibit microbial activity NaN_3 (final concentration 1 mM) was added before shaking. The soil extracts were stored at 4 °C before use.

Another set of extracts was prepared using the same way as before but without addition of NaN_3 for the measurement of total organic carbon (TOC), total nitrogen (TN), dissolved organic nitrogen (DON) and solution pH. TOC and TN were determined by a TOC analyser (TOC-V_{CPH}, Shimadzu, Kyoto, Japan). Solution pH was measured with a multi-parameter analyser (CON-SORT C832, Abcoude, The Netherlands). The inorganic N in the extracts was analysed with a SAN^{++} auto-analyser fitted with a 1074 auto-sampler (Skalar, Breda, The Netherlands). The DON was calculated as the difference between the TN and inorganic N contents. The C and N contents of bulk soils were determined using a C and N analyser (Elementar VarioEL, Hanau, Germany).

3.2.5 Sample concentration and clean up

Freeze-drying and an SPE technique were tested in this study to concentrate and clean up soil extracts. The three tested SPE cartridges (Waters Milford, MA, USA) and the corresponding extraction procedure are presented in Table 3.3. The initial SPE procedure was based on the suggestions of the supplier and reported methods (Yang *et al*., 2011). All the eluted solutions were evaporated under N_2 gas flow on a water bath (50 °C) and reconstituted in ACN/H_2O (85:15, v/v).

After selecting the proper SPE sorbent, several wash steps, various eluent pH (1.25%, 3.75%, or 7.5% NH_4OH in methanol, v/v) and different final volumes (0.2 and 0.5 ml) were tested. Samples were filtered through a 0.2 µm

Table 3.2 Sources, classification, and properties of soil samples.

Code	Soil	Specification	Site	TC /%	TN /%	Texture (sand-silt-clay) /%	Extraction ratio (W/V)	pH	DOC /mg C l^{-1}	DON /mg N l^{-1}
1	Fimic Anthrosol[a]	black humus layer (H)	Buunderkamp (The Netherlands)	12.65	0.62	NI	1:20	4.27	95.8	4.27
2	Fimic Anthrosol[a]	illuvial (Bs) horizon	Buunderkamp (The Netherlands)	0.65	0.03	NI	1:10	4.84	24.7	0.91
3	Haplic Luvisol[b]	Ap horizon (0–10 cm)	Southern Limburg (The Netherlands)	0.92	0.11	8.6-82.2-9.2	1:10	6.24	17.0	1.13
4	Endogleyic Stagnosol[c]	1000 years old Paddy soil, Alp (0–10 cm)	Cixi (China)	1.49	0.16	silt loam	1:10	6.25	35.7	2.32
5	Petric Calcisol[d]	eroded sediment	Alqueria (Spain)	1.05	0.07	43.2-47.3-9.5	1:10	8.34	33.7	2.60
6	Leptic dystric regosol	A horizon (0–10/12 cm)	Courmayeur - Aosta (Italy)	1.52	0.12	68.9-27.0-4.2	1:10	7.57	46.2	3.45
7	Dystric regosol[e]	Ap1 horizon (0–15 cm)	Parco del Ticino, Lombardia (Italy)	1.14	0.09	52-43-5	1:10	5.6	2.3	ND
8	Brunic dystric regosol[e]	A2 horizon (6–13 cm)	Bosco Siro Negri, Lombardia (Italy)	1.59	0.14	74-24-2	1:10	4.66	4.6	0.23
9	Haplic Luvisol[f]	A_p/A_h horizon (10–25 cm)	Speuld (The Netherlands)	1.04	0.03	NI	1:10	4.84	16.9	0.69
10	Haplic podzol[g]	E1 horizon (10–25 cm)	Klosterhede (Denmark)	2.72	0.10	NI	1:10	4.67	13.2	0.47

[a] Jansen *et al*. (2005). [b] Wang *et al*. (2014). [c] Hanke *et al*. (2013). [d] Cammeraat (2004). [e] Cerli *et al*. (2009). [f] Koopmans *et al*. (1997). [g] Ingerslev (1997). ND: not detectable. NI: not included in the literature.

Table 3.3 Three different solid phase extraction (SPE) cartridges and the corresponding extraction procedure.

Cartridge	Size and properties	Extraction procedure			
		Equilibration	Loading	Washing	Elution
		Initial			
Oasis MCX	150 mg, 60 µm, strong cation exchange sorbent	a. 3 ml of MeOH b. 3 ml of acidified water (2% FA)	10 ml of acidified standard mixture (2% FA)	3 ml of MeOH	3 ml of basified MeOH (1.25% NH_4OH)
		Optimized			
		a. 3 ml of MeOH b. 3 ml of acidified water (2% FA)	10 ml of acidified standard mixture (2% FA)	a. 3 ml of MeOH b. 3 ml of acidified H_2O/MeOH (1:1, v/v, 0.1% FA)	3 ml of basified MeOH (7.5% NH_4OH)
Oasis MAX	60 mg, 30 µm, strong anion exchange sorbent	a. 1 ml of MeOH b. 1 ml of basified water (0.25% NH_4OH)	10 ml of basified standard mixture (1.25% NH_4OH)	1 ml of MeOH	1 ml of acidified MeOH (5% FA)
Oasis WAX	60 mg, 30 µm, weak anion exchange sorbent	a. 1 ml of MeOH b. 1 ml of acidified water (2% FA)	10 ml of standard mixture	1 ml of MeOH	1 ml of basified MeOH (1.25% NH_4OH)

hydrophilic polypropylene (GHP) syringe filter (Acrodisc, Pall Life Science, East Hills, NY) prior to injection.

3.2.6 Analytical method optimization

Both positive and negative ionization mode were tested for MS/MS detection. The optimal MS conditions for each analyte and IS were obtained by infusing the analytes according to the following steps: (1) isolation and selection of a precursor ion for each analyte and IS, (2) optimization of the declustering potential, (3) optimization of the collision energies and collision cell exit potential for the most abundant product ions. Afterwards, the source/gas parameters were optimized through flow injection analysis, including curtain gas (10 l h^{-1}), collision gas (6 l h^{-1}), ion-spray voltage (5500 V), temperature (450 °C), ion source gas 1 (40 l h^{-1}) and ion source gas 2 (60 l h^{-1}).

The HPLC gradient was based on the methodology reported by Guo *et al*. (2013). To improve separation, peak shape and intensity, the influences of buffer concentration (2–10 mM NH_4HCO_2 in both solvent A and B, see the composition of the mobile phase in Table A1 of appendix), mobile phase pH (3, 5, 7 and 9) and organic modifier (MeOH) were investigated under the optimal MS/MS condition. Modification of the HPLC gradient method was tested as well.

3.2.7 Method validation for amino acids in soil

The standard addition method was adopted to test the overall recovery of the SPE procedure, including extraction efficiency and matrix effects, with the 10 soil extracts and ultrapure H_2O. For this, 10 ml of the soil extracts or ultrapure H_2O were spiked with only IS or both IS and the amino acids standard mixture (the final concentration in the spiked extracts were 0.05 µg ml^{-1} Ala, Asp, Gln, Glu, Gly, Ile, Leu, Phe, Pro, Ser, Thr, Tyr, Val and 0.01 µg ml^{-1} Asn, His, Lys, Met, Sar, Trp). The entire samples were homogenised and purified using the optimized SPE technique described in the results section. All the experiments were performed in triplicate. Only the reconstituted sample of soil 6 was diluted 6 times in ACN/H_2O (85:15, v/v) before measurement because the concentrations of some amino acids were outside the range of the calibration line. Both non-labelled (Nva) and stable isotope labelled IS (Ala-D4, Glu-D5, Lys-D4 and Gly-D5) were tested in this study to quantify free amino acids in soil

Table 3.4 Precursor/product ion pairs and optimal parameters for multiple reaction monitoring (MRM) of analytes and internal standards.

Code	Amino acids	Retention time /min	MRM transition (precursor/product)	DP[d] / V	CE[e] / V	CCEP[f] / V
1	Trp	2.5	205/188[a]	49	15	11
			205/146[c]	49	24	13
2	Phe	2.5	166/120[a]	45	22	9
			166/120[b]	45	35	9
			166/103[c]	45	38	8
3	Leu	2.6	132/86[a]	51	16	5
			132/86[b]	51	25	5
4	Ile	2.8	132/86[a]	54	14	3
			132/86[b]	54	25	3
			132/69[c]	54	26	5
5	Met	3.2	150/133[a]	44	15	6
			150/61[c]	44	33	1
6	Val	3.6	118/72[a]	50	17	2
			118/72[b]	50	27	2
			118/55[c]	50	28	3
7	Pro	3.7	116/70[a]	51	21	4
			116/70[b]	51	47	4
			116/43[c]	51	42	5
8	Tyr	3.7	182/165[a]	44	13	8
			182/136[c]	44	19	7
9	Sar	4.6	90/44[a]	44	18	6
10	Ala	5.2	90/44[a]	39	21	2
11	Thr	5.8	120/74[a]	51	16	2
			120/102[c]	51	13	4
12	Gly	6.1	76/48[a]	44	10	8
			76/30[c]	44	19	3
13	Gln	7	147/84[a]	30	23	3
			147/130[c]	30	11	4
14	Ser	7.1	106/60[a]	20	14	2
			106/70[c]	20	20	2

(Continued)

Table 3.4 Precursor/product ion pairs and optimal parameters for multiple reaction monitoring (MRM) of analytes and internal standards (continued).

Code	Amino acids	Retention time /min	MRM transition (precursor/product)	DP[d] / V	CE[e] / V	CCEP[f] / V
15	Asn	7.5	133/74[a]	47	21	5
			133/87[c]	47	14	6
16	Glu	7.8	148/84[a]	54	23	6
			148/130[c]	54	14	5
17	Asp	9.1	134/74[a]	67	23	5
			134/88[c]	67	11	4
18	Arg	9.3	175/70[a]	54	33	5
			175/116[c]	54	21	5
19	His	9.5	156/110[a]	53	21	6
			156/83[c]	53	35	6
20	Lys	9.5	147/84[a]	30	23	3
			147/130[c]	30	14	5
21	Nva	3.3	118/72[a]	41	14	4
			118/55[c]	41	32	8
22	Lys-D4	9.5	151/88[a]	51	23	3
			151/134[c]	51	14	6
23	Ala-D4	5.2	94/48[a]	42	17	7
24	Gly-D5	6.1	78/32[a]	44	19	3
			78/50[c]	44	10	7
25	Glu-D5	7.8	153/88[a]	44	25	3
			153/135[c]	44	14	6

[a] quantifier with optimal parameters. [b] quantifier (with less optimal parameters) used in quantifying soil amino acids. [c] quanlifier. [d] declustering potential. [e] Collision energy. [f] Collision cell exit potential.

extracts. The concentration of Nva in the spiked soil extracts or ultrapure H_2O before loading was 0.025 μg ml^{-1} and the concentrations of stable isotope labelled IS were 0.025 μg ml^{-1} (Ala-D4, Glu-D5 and Lys-D4) or 0.125 μg ml^{-1} (Gly-D5).

Intra-day precision of the method was examined by repeatedly measuring the SPE extraction samples of soil 3 for 5 times within a day and inter-day precision was examined by measure the same samples for three consecutive days.

3.2.8 Quality control

The standard mixture solution of 20 amino acids and IS for calibration were prepared in solvent B of the mobile phase. Calibration lines were made by plotting the ratio of analyte to IS concentration versus analyte to IS peak area. Quantification of all measurements was performed with a ten point calibration line. For each different level of analyte concentration, the IS concentration was kept the same. The concentration of Nva in the standard mixture solution for calibration was 0.2 μg ml^{-1} and the concentrations of the stable isotope labelled IS were 0.2 μg ml^{-1} (Ala-D4, Glu-D5 and Lys-D4) or 1 μg ml^{-1} (Gly-D5). Calibration lines only included the levels with accuracies lying between 70% and 130%. Meanwhile analogous transition ratios of quantifier to qualifier were guaranteed for different concentrations of each analyte except for Leu, Ala and Sar, which only had one sensitive transition.

The limit of quantification (LOQ) for each analyte equalled the lowest concentration of the calibration line and the limit of detection (LOD) was calculated by dividing the LOQ by 3.3. LOQs and LODs for free amino acids in a given soil were calculated assuming the extraction ratio of soil to water was 1:10 (v/v) and took into account the recoveries obtained in this study.

All tests were performed in triplicate.

3.3 Results and discussion

3.3.1 Optimization of MS/MS condition

The retention time, transitions and optimal MS conditions for all the targeted amino acids and IS are listed in Table 3.4. Positive ionization mode was selected in MRM detection in line with the study of Guo *et al*. (2013). For all amino acids except Gly-D5, the protonated molecular ion $[M+H]^+$ was selected as the precursor ion (Table 3.4). The precursor mass of Gly-D5 was based on the $[M\text{-}D_3+H_3+H^+]^+$ ion because its acidic deuterium atoms are exchanged with H upon aqueous dissolution (Štefanić *et al*., 2009). The qualifier and quantifier ions of each analyte and IS were selected by direct infusion mode. The transition with higher intensity was selected as quantifier ion. Only one sensitive transition could be obtained for Leu, Ala, Sar and Ala-D4.

Almost all amino acids tested can generate several dominant product ions in MRM detection, especially those with relative high molecular weight (e.g. Trp,

Tyr and Arg). Among the dominant product ions, a neutral loss of the ion represented by m/z 46 was observed for the majority of the amino acids and attributed to the loss of formic acid (FA) (Langrock *et al*., 2006). However, the product ion $[M+H-46]^+$ was not necessarily the most dominant for all these amino acids. Fragment ions $[M+H-17]^+$ and $[M+H-46-17]^+$ were the most intensive product ions for several amino acids, such as Trp, Met, Gln and Lys. Fragment ion $[M+H-17]^+$ was attributed to neutral loss of NH_3 (Liu *et al*., 2008; Guo *et al*., 2013). Because of the difficulty in detecting the above mentioned three fragment ions, the product ions of m/z 70 and 84 were selected to quantify Arg and Glu, respectively. Since the maximum MS detection limit was reached for Phe, Leu, Ile, Val and Pro in soils, no parameter optimization was necessary to quantify these amino acids.

3.3.2 Optimization of HPLC condition

The results revealed that raising the buffer concentration resulted in narrower and therefore higher peaks for Asn, Asp, Glu, Lys, Met and His, while it caused a loss in intensity (peak height) for other amino acids (Figure A1, Appendix). However, all amino acids showed slightly decreasing peak areas with increasing buffer concentration (Figure A2, Appendix), probably due to the ion suppression effect of the buffer (Annesley, 2003). In addition, increasing the buffer concentration from 2 to 10 mM resulted in relatively shorter retention time (shift 0.50–1.33 min) for a few late eluting basic or acidic amino acids (Arg, Asp, His and Lys), while small shifts (0.03–0.19 min) were observed for all other amino acids. Given the overall positive effects of higher buffer concentration, especially on Asp and His (Figure A3, Appendix), a concentration of 8 mM NH_4HCO_2 (0.12% FA) was selected as the optimum.

NH_4OAc, AcOH and NH_4OH were used to prepare a mobile phase with different pH values for testing the influence of mobile phase pH (Table A2, Appendix). The best peak shapes for all the amino acids were observed at pH 3 as compared with pH 5, 7 and 9 (data not shown). Moreover, the retention time of all the tested amino acids increased when raising the pH from 3 to 5 and few differences were observed between pH 5 and 9 (data not shown). Specifically, basic (Arg, His and Lys), acidic (Asp and Glu) and some neutral amino acids (Ser, Thr, Asn and Gln) exhibited relatively large retention time shifts (0.91–2.53 min) over the pH range of 3–9, while other neutral amino acids had small retention time shifts (0.28–0.80 min). Because all the above mentioned amino acids have charged or polar uncharged side chains, stronger hydrophilic

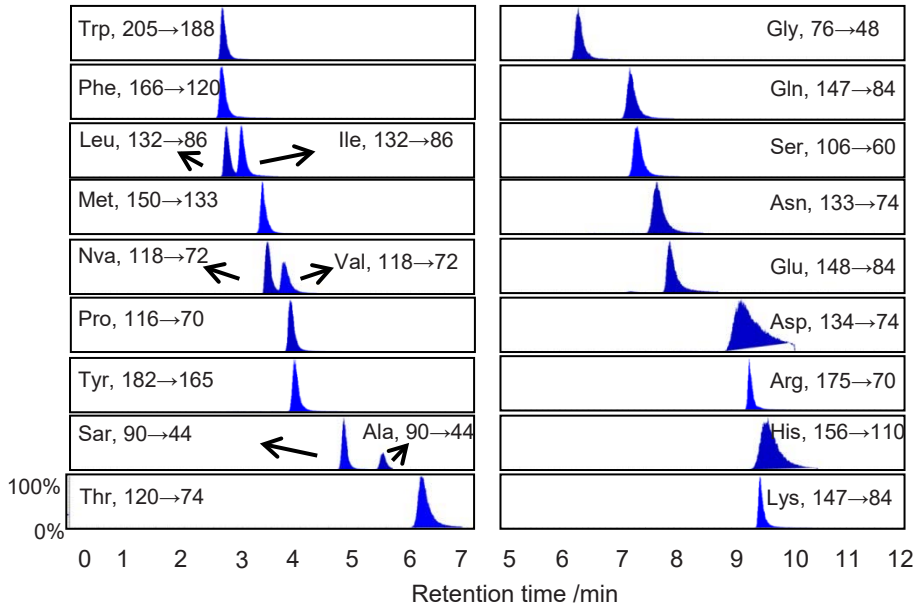


Figure 3.1 Typical chromatograms of the targeted amino acids (transitions of quantifier) obtained from standard mixture (2.5 µg ml^{-1} for Asp and Gly, 0.5 µg ml^{-1} for the rest). The involved mobile phase included solvent A (90% H_2O, 10% ACN, 8 mM NH_4HCO_2, 0.12% FA) and solvent B (90% ACN, 10% H_2O, 8 mM NH_4HCO_2, 0.12% FA). The gradient method was 94–88% B from 0 to 6 min, 88–50% B from 6 to 11 min, constant 50% B from 11 to 12 min.

interactions could occur between these compounds and the column sorbent under neutral and basic condition. The remaining amino acids (Gly, Pro, Ala, Leu, Ile, Met, Phe, Sar, Trp, Tyr and Val) have no or only hydrophobic side chains, hence, little change of retention time occurred for them.

Overall, the mobile phase with pH 3 produced the best peak shape with shortest retention time (respecting dead volume) for all tested amino acids. In addition, at pH 3 clear separation of the isomer pairs, Leu/Ile and Val/Nva was obtained. Therefore, pH 3 was used as optimum pH in our study, also in line with previous results of Guo *et al*. (2013).

Compared with other amino acids, Asp and His still had relatively wide peaks under optimum buffer concentration and mobile phase pH. Thus, to improve their peak shape, modifications of solvent A or B with different concentration of MeOH (2.5–10%) were tested. The results illustrated that adding MeOH in

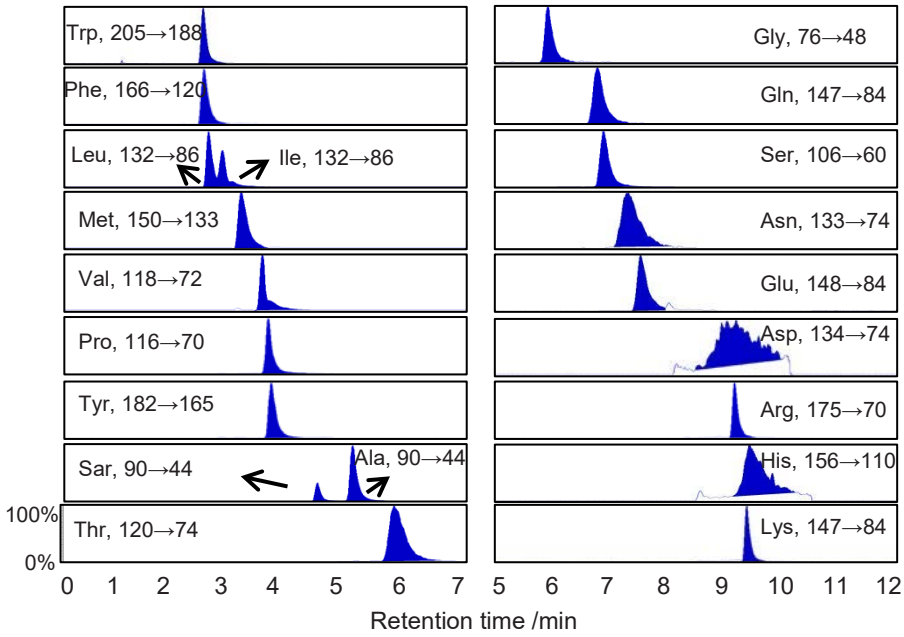


Figure 3.2 Typical chromatograms of the targeted amino acids (transitions of quantifier) obtained from purified soil extract. The involved mobile phase included solvent A (90% H_2O, 10% ACN, 8 mM NH_4HCO_2, 0.12% FA) and solvent B (90% ACN, 10% H_2O, 8 mM NH_4HCO_2, 0.12% FA). The gradient method was 94–88% B from 0 to 6 min, 88–50% B from 6 to 11 min, constant 50% B from 11 to 12 min.

solvent A or B not only impaired the separation of Leu/Ile and Val/Nva but also worsened the peak shape of several amino acids, i.e. Asp, Lys, His and Arg.

Further modification of the elution program showed that increasing the H_2O proportion from 40% to 50% over the period of 12–13 min produced narrower peaks for some amino acids (Asp, Glu, Lys, Gln, Arg and Ser) with reduced retention time (data not shown). Finally solvent A of mobile phase (100% H_2O, 8 mM NH_4HCO_2, 0.12% FA, v/v) was adjusted to contain 10% ACN to prevent growth of microorganism. Solvent B remained the same (90% ACN, 10% H_2O, 8 mM NH_4HCO_2, 0.12% FA, v/v). Following these adjustments, the finalized gradient method was 94–88% B from 0 to 6 min, 88–50% B from 6 to 11 min, constant 50% B from 11 to 12 min. The separation system was reconstituted to the start condition within 1 min and equilibrated for 10 min between injections. The typical chromatograms of targeted amino acids obtained from standard mixture are shown in Figure 3.1.

Table 3.5 Regression linear range, coefficients of determination (r^2), transition peak area ratios, limits of detection (LODs) and limits of quantification (LOQs) of the 20 targeted amino acids.

Code	Analyte	IS	Linear range /μg ml^{-1}	r^2	Transition ratio (quantifier[a]/quanlifier)		LOD /ng ml^{-1}	LOD_{soil} /ng g^{-1}	LOQ /ng ml^{-1}	LOQ_{soil} /ng g^{-1}
					Average	RSD /%				
1	Trp	Ala-D4	0.005–0.1	0.9986	2.7	4.0	1.5	17	5	56
			0.05–2	0.9984						
2	Phe	Ala-D4	0.005–2	0.9986	1.1	2.5	1.5	17	5	57
3	Leu	Ala-D4	0.005–1.0	0.9980	NA		1.5	16	5	53
			0.1–2	0.9930						
4	Ile	Ala-D4	0.005–0.1	0.9984	2.9	5.2	1.5	17	5	56
			0.02–2	0.9980						
5	Met	Ala-D4	0.005–0.1	0.9992	1.8	4.0	1.5	49	5	163
			0.2–2	0.9978						
6	Val	Ala-D4	0.005–0.1	0.9982	1.9	4.8	1.5	20	5	67
			0.02–2	0.9980						
7	Pro	Ala-D4	0.02–0.1	0.9956	17.1	7.9	6.1	116	20	381
			0.1–2	0.9922						
8	Tyr	Ala-D4	0.01–0.2	0.9996	1.1	7.4	3.0	31	10	104
			0.05–2	0.9968						

(Continued)

Table 3.5 Regression linear range, coefficients of determination (r^2), transition peak area ratios, limits of detection (LODs) and limits of quantification (LOQs) of the 20 targeted amino acids (continued).

Code	Analyte	IS	Linear range /μg ml^{-1}	r^2	Transition ratio (quantifier[a]/quanlifier)		LOD /ng ml^{-1}	LOD$_{soil}$ /ng g^{-1}	LOQ /ng ml^{-1}	LOQ$_{soil}$ /ng g^{-1}
					Average	RSD /%				
9	Sar	Ala-D4	0.005–0.5	0.9990	NA		1.5	20	5	67
			0.02–2	0.9984						
10	Ala	Ala-D4	0.005–0.5	0.9998	NA		1.5	16	5	53
			0.1–2	0.9990						
11	Thr	Gly-D5	0.005–0.1	0.9984	2.2	5.7	1.5	13	5	43
			0.05–2	0.9988						
12	Gly	Gly-D5	0.025–1	0.9926	0.9	3.7	7.6	78	25	257
			0.5–10	0.9982						
13	Gln	Ala-D4	0.005–0.1	0.9980	1.3	7.0	1.5	19	5	64
			0.05–2	0.9972						
14	Ser	Gly-D5	0.02–0.2	0.9932	12.9	9.3	6.1	75	20	248
			0.05–2	0.9948						
15	Asn	Gly-D5	0.01–0.1	0.9976	1.1	5.3	3.0	35	10	114
			0.02–2	0.9958						
16	Glu	Glu-D5	0.005–0.1	0.9992	1.8	9.5	1.5	14	5	46
			0.05–2	0.9984						

(Continued)

Table 3.5 Regression linear range, coefficients of determination (r^2), transition peak area ratios, limits of detection (LODs) and limits of quantification (LOQs) of the 20 targeted amino acids (continued).

Code	Analyte	IS	Linear range /μg ml^{-1}	r^2	Transition ratio (quantifier[a]/quanlifier)		LOD /ng ml^{-1}	LOD$_{soil}$ /ng g^{-1}	LOQ /ng ml^{-1}	LOQ$_{soil}$ /ng g^{-1}
					Average	RSD /%				
17	Asp	Glu-D5	0.1–2.5	0.9986	1.6	7.5	30.3	384	100	1267
			1–10	0.9954						
18	Arg	Lys-D4	0.005–0.2	0.9992	2.4	6.3	1.5	22	5	73
			0.02–2	0.9952						
19	His	Lys-D4	0.02–0.5	0.9992	4.9	6.4	6.1	68	20	223
			0.1–2	0.9956						
20	Lys	Lys-D4	0.005–2	0.9992	1.4	7.0	1.5	14	5	48

[a] The quantifier of Phe, Leu, Ile, Val and Pro were transitions with less optimal parameters (Table 3.4). NA: not applicable.

3.3.3 Optimization of SPE procedure

Most free amino acids in soil extracts were too low in concentration to be measured directly and pre-concentration was needed to enable their analysis. Therefore, an SPE technique was adopted to purify and concentrate free amino acids in the soil extracts. Comparison of various SPE cartridges indicated that the best recoveries were obtained on MCX cartridges for most of the amino acids (data not shown), therefore this sorbent was selected for further evaluation of the clean-up procedure.

Washing loaded cartridges once with neutral MeOH was found insufficient to purify soil extracts. The eluted solvent could have a slightly yellowish colour, probably derived from soil organic matter. Therefore, acidified MeOH/H_2O (1:1, 0.1% FA, v/v) was used to wash the cartridge, following neutral MeOH to wash away more polar compounds. Multiple elution experiments with varied eluent pH (1.25%, 3.75%, and 7.5% NH_4OH in MeOH for the first, second and third elution step, respectively) showed that for most of the amino acids, 1.25% NH_4OH was enough to yield nearly complete elution. However, increasing eluent pH with 7.5% NH_4OH would increase elution of His, Arg and Lys (Table A3, Appendix). Accordingly, the optimum was 7.5% NH_4OH in MeOH. Yang *et al*. (2011) also found poor recovery of Lys and Arg under 3% NH_4OH in MeOH and our results indicated increasing the eluent pH to 7.5% NH_4OH in MeOH would improve the recovery of Lys and Arg.

The absolute recovery of Nva was quite different from the majority of the targeted amino acids (data not shown) and thus deemed unsuitable for quantifying the targeted amino acids. Based on similarity of the absolute recoveries from the preliminary test (data not shown), the 20 amino acids could be divided into three groups, i.e. basic, acidic and neutral. Consequently, four stable isotope labelled amino acids, including one basic (Lys-D4), one acidic (Glu-D5) and two neutral amino acids (Ala-D4 and Gly-D5), were used as the IS to evaluate the SPE method.

When dried samples were reconstituted in a final volume of 0.2 ml, an aqueous layer and an organic layer were visually separated because of formation of an aqueous phase with a high concentration of buffer salts (i.e. NH_4HCO_2) produced during the SPE procedure. Increasing the final volume to 0.5 ml prevented this problem, and reduced other matrix effects. Hence, a volume of 0.5 ml was used to validate the SPE method.

3.3.4 Method performance

A separation of the linear range into two parts was necessary to build a reliable calibration line with a wide concentration range for most of the amino acids. The results are presented in Table 3.5. The coefficients of determination (r^2) were higher than 0.9922 for all the calibration lines (Table 3.5). The relative standard deviation (RSD) of the transition ratios were below 10% for all the amino acids with two sensitive transitions. The LODs ranged from 1.5 to 30.3 ng ml^{-1} and the LOQs ranged from 5 to 100 ng ml^{-1}. These results indicated that the detection limits of the less sensitive amino acids, such as Thr, Glu and Lys, were clearly improved in comparison with the results of Guo *et al*. (2013). The corresponding LODs and LOQs for free amino acids in soil ranged from 13 to 384 ng g^{-1} and from 43 to 1267 ng g^{-1} (dry basis), respectively. In addition, for all the 10 different soils, the transition ratios of all the amino acids were similar to the standard results in pure H_2O (the differences were lower than 30%). The intra-day precision (RSD) was between 1% and 5%, and the inter-day precision (RSD) was between 1% and 14% (Table A4, Appendix).

3.3.5 Application to measurement of soil free amino acids

Table 3.6 shows the overall recoveries of spiked amino acids in ultrapure H_2O and soil extracts. Except for Val, Pro, Met and Arg the overall recoveries of the amino acids were similar for both the pure H_2O and soil extracts and ranged between 74% and 117% (Table 3.6). For Val and Pro, the overall recoveries were relatively consistent for soil extracts (74% for Val and 52% for Pro), and lower compared to pure H_2O (97% for Val and 94% for Pro). The overall recoveries of Met and Arg had large variation among pure H_2O and different soil extracts (Table 3.6). The overall recoveries of Val and Pro were normalized to the same IS of Ala-D4. The differences of the overall recoveries of Val or Pro between pure H_2O and soil extracts could be attributed to the weaker bonding of Val or Pro with the existence of other compounds extracted from soils. Conversely, some extracted compounds from soil probably enhanced the bonding of Met. Thus, the overall recoveries of Met for some soils were much higher than that for pure H_2O. Other soils did not contain the same compounds, therefore the overall recoveries of Met for these soils showed little change compared to pure H_2O. The large variation of the overall recoveries of Arg could be explained by the varied interaction between positively charged Arg and negatively charged organic compounds extracted from soil. These results indicated that the recovery process of some amino acids (Val, Pro, Met and Arg)

Table 3.6 Overall percent recoveries of spiked amino acids in ultrapure H_2O and soil extracts.

Analyte	H_2O	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6	Soil 7	Soil 8	Soil 9	Soil 10	Mean
Trp	90±1	77±2	83±4	82±2	86±4	89±0	96±7	85±5	92±6	103±7	94±7	89±8
Phe	90±1	83±2	86±5	83±3	81±2	80±3	109±14	92±2	96±5	90±5	85±6	88±9
Leu	94±3	88±1	94±5	80±4	84±5	86±3	125±24	95±1	99±7	92±8	92±9	94±12
Ile	96±5	86±5	87±2	76±3	81±3	77±4	114±11	89±1	96±5	92±7	93±8	89±11
Met	4±4	71±4	66±2	5±0	26±4	4±1	30±21	0	5±2	63±5	36±6	31±28
Val	97±4	80±2	75±0	69±3	70±2	72±4	120±19*	71±2	75±3	79±8	79±8	74±4
Pro	94±5	60±1	54±1	57±1	47±3	51±2	49±4	50±1	51±3	53±4	51±6	52±4
Tyr	128±3	112±3	93±2	113±2	93±6	93±1	98±11	103±1	104±4	79±3	77±3	97±12
Sar	80±0	74±4	79±4	79±1	72±2	75±1	65±2	79±1	81±1	69±1	77±2	75±5
Ala	98±1	92±3	92±3	98±3	91±2	88±1	95±6	101±1	101±1	95±1	95±1	95±4

(Continued)

Table 3.6 Overall percent recoveries of spiked amino acids in ultrapure H_2O and soil extracts (continued).

Analyte	H_2O	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6	Soil 7	Soil 8	Soil 9	Soil 10	Mean
Thr	120±2	119±6	117±3	127±2	108±5	111±4	108±6	121±2	123±2	113±4	121±1	117±7
Gly	98±2	99±3	97±6	100±4	94±6	93±3	91±2	98±4	97±1	100±6	103±12	97±4
Gln	83±1	77±2	81±5	85±1	77±5	83±1	70±3	77±2	82±2	72±10	78±2	78±5
Ser	80±0	79±2	84±4	83±2	79±2	79±5	85±8	82±1	85±3	73±4	79±0	81±4
Asn	83±2	93±7	94±10	77±4	87±12	77±9	113±3	85±2	89±3	79±14	85±5	88±11
Glu	117±4	105±1	107±1	108±5	124±17	103±6	99±68	114±2	115±4	109±1	108±1	109±7
Asp	80±7	88±11	79±5	77±4	76±20	83±8	111±12*	81±6	77±7	71±6	79±4	79±5
Arg	88±2	90±5	92±6	29±1	57±11	26±2	16±20	57±1	94±6	113±22	108±20	68±36
His	85±8	72±2	92±7	93±1	97±3	99±4	210±49*	87±10	98±3	89±12	81±4	90±9
Lys	100±2	102±3	104±4	108±1	107±4	107±13	115±13	99±4	107±2	104±4	96±4	105±5

Note: the results are presented as means with standard deviation (n=3). The values with an asterisk are taken as outliers. The data in the mean column are the averaged results of soil sample.

was highly dependent on the composition of soil extracts. More stable and accurate results could be obtained if stable isotope labelled analogues were used to quantify those amino acids. Typical chromatograms of the targeted amino acids obtained from purified soil extract are shown in Figure 3.2. The contents of soil free amino acids are presented in Table A5 (Appendix).

HPAEC-PAD has been widely used to analyse amino acids in soil digestions without derivatization and purification (Martens and Loeffelmann, 2003; Olk, 2008; Creamer *et al*., 2012), but this method suffered from long runtime (35 min) and lack of analyte specificity. The HILIC method coupled with an SPE technique was first applied in rhizosphere samples by Dell'mour *et al*. (2010). However, in addition to a different matrix, Dell'mour *et al*. (2010) only tested 11 amino acids and their internal standard (^{13}C-tyrosine) was not suitable to quantify all the tested amino acids. In this study, good separation of 20 underivatized amino acids was achieved on a HILIC column within 12 min upon prior SPE extraction incorporating improvements in the technique as reported by Yang *et al*. (2011) but using more cost-efficient H-labelled instead of C/N labelled IS.

3.4 Conclusion

For the first time a reliable method for quantifying 20 common amino acids in 10 different soils spanning a wide range of matrix composition (texture, pH, organic C content etc.) was developed and validated. The method that consists of a combination of extraction and pre-concentration with SPE and subsequent analysis with HILIC-HPLC–MS/MS yielded good separation and accurate determination of the 20 amino acids without derivatization for all soils and all amino acids, with the exception of Arg and Met. With respect to extraction from the complex soil matrices, specifically, the cation exchange SPE technique was able to purify and concentrate amino acids in soil extracts, which are generally lower than the detection limit or not abundant enough for accurate analysis. For Arg and Met in spite of SPE extraction, significant differences in the recoveries between some of the soils were found that warrant further investigation. For all other amino acids, the newly developed method paves the way for broad routine application to analyse their presence and abundance in soils of varying composition. Added advantage is that the new methodology is cost-effective as only four deuterated internal standards were used for quantification of the targeted amino acids.