Determination of soil amino acids by high performance liquid chromatography-electro spray ionization-mass spectrometry derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate

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Abstract

Determination of amino acids by mass spectrometry (MS) is an important technique to investigate soil nitrogen transformation and cycling as amino acids being the major nitrogen-containing compounds in soil organic matter. However, researchers have long faced a critical problem in coupling an efficient separation technique to a sensitive MS detection system simultaneously. In this context, we established a new method of liquid chromatography coupled to mass spectrometry based on the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatization method for convenient and accurate quantification of amino acids in soil samples. Baseline separation of 17 amino acid AQC-derivatives was achieved on an XTerra\textsuperscript{®} MS C\textsubscript{18} column using ammonium formate as a mobile phase modifier. The concentration of ammonium formate and the pH of the mobile phase were optimized in order to obtain sensitive MS signals. The response curves were linear over the range of 50–800 \textmu mol L\textsuperscript{−1} amino acids. The detection limits were 0.20–0.60 pmol L\textsuperscript{−1} on column and 0.07–0.24 \textmu g g\textsuperscript{−1} soil under the optimized conditions. The method has been applied successfully for the first time to determine amino acids in 4 types of soil samples, in which 15 amino acids were quantified by MS detector but methionine and cystine were below the detection limits. Both the recovery and the precision were satisfactory. Hence, this proposed technique shows a potential for the identification of amino acids in soil as well as tracing the transformation of soil amino acids with isotope dilution technique in nitrogen cycling investigation.

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1. Introduction

Amino acids are the main component in soil organic matter and play important roles in soil nitrogen (N) biogeochemical cycling in terrestrial ecosystems [1]. Quantitatively speaking, around 20–50% of total N in soil organic matter was found in the form of amino acids, which are mainly present in peptide or protein and can be released by acid hydrolysis [2–5]. Hence, the determination of amino acids in soil and tracing the transformation process of amino acids became a powerful tool in characterizing N turnover in soil.

Although various methods have been applied to quantify amino acids in soil samples, mass spectrometry (MS) is still a promising technique especially for the selective measurement of the compounds at low concentrations in complex matrix and has a great potential for the application to the compound-specific isotope enrichment investigation [6,7]. Theoretically, it is unnecessary to separate the target compounds in MS detection; however, there are still some limitations mainly occurred in the following three cases: (1) the isomass compounds cannot be separated by mass; (2) the “chemical suppression” phenomenon in ion trap is brought when several molecules are co-eluted and ionized together; and (3) in a lesser extent, the presence of isotopes can interfere with other molecules, especially when the mass difference is 1 or 2 only [8]. Therefore, the good chromatography separation is still essential for sensitive MS analysis.

Gas chromatography–mass spectrometry (GC–MS) has been used to identify amino acids and trace isotope enrichment in target compounds [9–13]. However, a complex and laborious derivatization procedure has to be conducted before GC determination. For example, two steps of reactions at 110 and 150 °C are required to form pentfluoropropionyl isopropyl ester derivatives of amino acids. Even though, it is still difficult to ensure complete derivatization of arginine and histidine. As a result, the reproducibility of the...
derivatization is not always satisfied under such rigorous reaction conditions [13].

Compared with GC–MS, liquid chromatography–mass spectrometry (LC–MS) is more convenient for both amino acid separation and isotope enrichment measurement due to easier sample preparation. Traditionally, amino acid isolation can be achieved by ion-exchange liquid chromatography [14–16], but the high salt concentration in modifier is not suitable for MS detection. Hence, amino acid analyses without derivatization have been re-updated for MS detector [16] and the separation under this condition is usually achieved by ion-pair reversed-phase liquid chromatography [8,17–22]. Although the procedure without derivatization can reduce the errors introduced by side reactions and reagent interferences, there are still some notable disadvantages that cannot be overcome at present. Firstly, because the molecular weight of most amino acids is below 200, the interference from the mobile phase and sample matrix are obvious for the fragmentation acquisition of individual amino acids [8,17,19,23]. Hence the specific MS/MS detection has to be adopted for a satisfactory quantification of amino acids in the complex matrix, for instance soil samples [17,23]. Secondly, the detection limits of most amino acids are bigger than 1 pmol, much higher than that of UV or fluorescence detection [23]. Thirdly, some widely used ion-pair reagents, such as perfluoroheptanoic acid (PFHA) [19] can be accumulated in the column slowly. PFHA would change the surface of the stationary phase of the column, and furthermore, influence the reproducibility and separation significantly. As a result, the column has to be flushed frequently with pure organic solvent during analysis process, and this prolongs the analysis time and makes the process more complicated.

In this context, the pre-column derivatization method is more promising for amino acid analysis by liquid chromatography. The commonly used derivatization reagents include ortho-phthalaldehyde (OPA) [24,25], phenyl isothiocyanate (PITC) [26–29], 2-(9-fluorenyl)-ethyl chloroformate (FMOC-Cl) [26–29], 2-(9-carbazole)-ethyl chloroformate (CEOC) [34] and 6-aminocinolinyl-N-hydroxysuccinimidyl carbamate (AQC) [35]. The AQC derivatization technique for the analysis of amino acids was established by Cohen and Michard [35] and was commercialized by the Waters Co. Ltd. Notable advantages of this method included rapid, sensitive, quantitative and linear reactions [36]. In the past 15 years, AQC derivatization has been used in a wide variety of samples including amino acid nutrients in feed grain hydrolyzates [37,38], non-amino acid analytes such as polyamines [39] and incompletely hydrolyzed peptides [40,41] as well as nicotianamine and amino acids in some metal-hyperaccumulating plants [42]. Compared with other derivatization reagents, the compatibility of derivatization in the presence of salts makes the AQC reagent as the preferred choice for the amino acid analysis in complex samples. However, the identification and quantification of AQC–amino acid derivatives with mass spectrometer has not been reported until today, especially in soil samples. The reason, to our knowledge, is just due to the use of the non-volatile modifier in the mobile phase for obtaining good separation [35–37]. The non-volatile components will bring noisy background, and consequently, decrease the sensitivity of the MS system and affect the accuracy of quantification seriously [43]. In practice, the non-volatile modifiers in mobile phase are either removed by using on-line membrane suppressor or replaced by volatile modifier in order to be compatible with MS, but unfortunately, this attempt does not always give identical resolution for the analyzed compounds.

In a word, there is still a need to optimize the mobile phase for achieving both good resolution and high sensitivity of amino acid identification and quantification if AQC–derivatization technique is applied. Our objective is, therefore, to develop a new LC–MS method for the determination of amino acids in soil samples, providing a new choice for tracing the dynamics of amino acids in soil.

2. Experimental

2.1. Soils and reagents

2.1.1. Soils

Four types of surface soil samples (0–20 cm) were collected in 2006 across four provinces in China for amino acid determination. The physical and chemical characteristics of the tested soils are significantly different and main properties were listed in Table 1.

2.1.2. Reagents

AQC fluor reagent kit (consisting of AQC reagent, acetonitrile and 0.2 mmol L−1 sodium borate buffer, pH 8.8) as well as glass reaction tubes (6 x 50 mm) were provided by Waters Co. Ltd. (Milford, MA, USA). Amino acid standard solution was purchased from Pierce (Rockford, IL, USA) and it contains 17 amino acids including alanine (Ala), arginine (Arg), aspartic acid (Asp), cysteine (Cys–Cys), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr), and valine (Val). The concentration of each amino acid is 2.5 mmol L−1. Ultra-pure water was generated using a Milli-Q water purification system (Millipore, Milford, MA, USA). HPLC-grade acetonitrile (ACN) and formic acid (>96.0%) were purchased from DIKMA Tech. Inc. (Richmond, Hill, ON, USA). L-2-aminobutyric acid (ABA) (>99.0%) and Dowex 50WX8 cation exchange resin (100–200 mesh) was bought from Sigma–Aldrich Inc. (St. Louis, MO, USA). Parafilm® was the product of Alcan Packaging Co. Ltd. (Neenah, WI, Canada). Ammonium formate was purchased from Yili fine chemical Co. Ltd. (China). HCl, NH3, H2O and oxalic acid were also purchased in China and are all analytical-grade (Shenyang, LN, China).

2.2. Pre-column derivatization with AQC

A set of amino acid standard mixture solutions containing 200 μmol L−1 of internal standard was prepared at a concentration range of 50–800 μmol L−1. A portion of 10 μL of each standard mixture solution was pipetted into the bottom of a glass tube, and then 110 μL sodium borate buffer (0.2 mmol L−1 at pH 8.8) was added. After 15 μL of AQC (10 mmol L−1 in acetonitrile) was added, the glass tube was closed with Parafilm® and heated for 20 min at 55 °C to form AQC–amino acid. Thereafter, the solution was cooled down to room temperature and 10 μL portion of each solution was injected into the HPLC-ESI-MS system for amino acid analysis without further purification. It was reported that di-AQC substituted derivatives were dominantly formed for lysine and cystine, while the other tested amino acids reacted with AQC at 1:1 ratio [42].

2.3. Samples hydrolysis and purification

Soil amino acids in bound forms were liberated by acid hydrolysis and the hydrolyte was purified before derivatization to remove the interfering organic components and metal ions in soil matrix. The hydrolysis and purification process was conducted according to the method of Amelung and Zhang [13]. Briefly, 200–250 mg of the air-dried and sieved (< 0.25 mm) soil samples was weighted accurately, and then hydrolyzed with 20 mL of 6 mol L−1 HCl for 12 h (105 °C). After 80 μL of 2.5 mmol L−1 l-2-aminobutyric acid addition, the hydrolyte was filtered. The filtrate was dried using a rotary evaporator (<45 °C) and the residue was re-dissolved in 4 mL of 0.05 mol L−1 HCl. The solution was then transferred into a polypropylene column of 25 mL (BioRad Laboratories, Munich, Germany), which was filled with 3 g Dowex 50WX8 cation exchange
resin for sample purification. After being rinsed with 5 × 5 mL of 0.1 mol L\(^{-1}\) oxalic acid (pH 1.6–1.8, adjusted with NH\(_3\)·H\(_2\)O), 5 × 5 mL of 0.01 mol L\(^{-1}\) HCl and 5 mL of de-ionized water in turn, the amino acids were eluted with 5 × 5 mL of 2.5 mol L\(^{-1}\) NH\(_3\)·H\(_2\)O. The eluted solution was also dried by rotary evaporator (<45 °C), and re-dissolved in 4 mL 0.1 mol L\(^{-1}\) HCl. After being centrifuged, the supernatant was freeze-dried and then was dissolved with 700 μL of 0.02 mol L\(^{-1}\) HCl. Finally, a portion of 10 μL solution was used for derivatization as described in Section 2.2.

2.4. LC–MS system

The Waters 2695 LC–MS systems (USA) was used and it is equipped with four pumps, Waters 996 photodiode array detector for ultraviolet detection and ion trap mass spectrometer with electrospray ionization (ESI) source (Waters micromass ZQ4000, USA). Waters MassLynx 3.5 software was used for data analysis. The separation of amino acid derivatives was carried out on an Xterra® MS C\(_18\) column (3.5 μm, 150 mm × 2.1 mm L.D.) maintained at 35 °C. L-2-aminobutyric acid was used as internal standard and the injection volume of sample solution was 10 μL. The mobile phase consisted of three solvents: 1.5 mmol L\(^{-1}\) ammonium formate in water (solvent A, pH 3.0, adjusted with formic acid), acetonitrile (solvent B), and water (solvent C). The gradient profile was listed in Table 2. The wavelength \(\lambda = 248\) nm was used for ultraviolet detection and the analysis time was 50 min.

The operation conditions of MS detector were optimized using a 17 AQC–amino acid standard at 50 μmol L\(^{-1}\) and the derivatives were infused into the ESI-MS system with the flow rate of mobile phase at 0.2 mL min\(^{-1}\). The MS detection conditions were set as: capillary voltage, 3000 V; cone voltage, 25 V; extractor and RF lens voltage, 4 and 0.4 V, respectively; ion source temperature, 105 °C; desolvation temperature, 120 °C; desolvation gas flow (N\(_2\)), 2.5 L min\(^{-1}\); and cone gas flow (N\(_2\)), 1.7 L min\(^{-1}\). Under the full scan mode (the mass range was 220–900), the signal intensity of \([M + H]^+\) of the positive ion mode was maximal for most amino acids, but for lysine and cystine derivatives, \(m/z 244\) and 291 was the most abundant, respectively.

2.5. Quantification technique and method validation

The internal standard method was applied for amino acid quantification and it was done by calculating the ratio between the integration of the most abundance peak (i.e., \(m/z [M + H]^+\)) of the target amino acid and that of AAAB, but \(m/z 244\) for lysine and 291 for cystine were better than \(m/z [M + H]^+\). The same procedure was employed to plot calibration curves and six standard mixtures at concentrations of 50, 100, 200, 400, 600 and 800 μmol L\(^{-1}\) were prepared for the linearity calculation of the MS detector. The detection limits (LOD) were determined by the direct injection of the amino acids standard solution at 5 μmol L\(^{-1}\) and then calculated based on the signal to noise ratio of 3. Ten μL of 2.5 mmol L\(^{-1}\) amino acid standard mixture solution was spiked to a Mollisol-1 sample to evaluate the recovery of individual amino acids. The determination procedure of amino acids in the spiked soil was the same as described above.

In addition, the amino acids in soil samples was determined by a amino acid auto analyzer (Hitachi L-8800, Japan), an independent technique, to test the accuracy of the proposed MS method. The operation conditions of the instrument were set as follows: column type: 4.6 mm × 60 mm (#2622 resin); analysis column temperature: 50 °C; reaction column temperature: 135 °C; flow rate of the buffer: 0.4 mL min\(^{-1}\); flow rate of chromatogenic agent (Ninhydrin): 0.35 mL min\(^{-1}\); injection volume: 20 μL; detection wavelength: 570 nm (440 nm for proline).

3. Results and discussion

3.1. Preliminary modification of the AQC derivatization method

As developed by the Waters Co., Ltd., sodium acetate (CH\(_3\)COONa) used in the traditional AQC method [35] has been replaced by ammonium acetate (CH\(_3\)COONH\(_4\)) for coupling to MS detector and the mobile phase contained 140 mmol L\(^{-1}\) CH\(_3\)COONH\(_4\) (pH 4.95–5.05, titrated with acetic acid), acetonitrile and water, respectively. Unfortunately, under such conditions, no intensive MS response was acquired at the scan mode although the separation was excellent (Fig. 1). If CH\(_3\)COONH\(_4\) was no longer used, while other conditions were kept unchanged, the intensity of MS response was enhanced significantly. However, the separation of 17 AQC–amino acids became poor (see Fig. 2).

The findings indicated that 140 mmol L\(^{-1}\) CH\(_3\)COONH\(_4\) at pH 4.95–5.05 was essential for good resolution of AQC–amino acids, but the MS response of the derivatives was restrained significantly due to high concentration CH\(_3\)COONH\(_4\) addition. Therefore, more work should be conducted to optimize the pH and volatile modifier of the mobile phase in order to improve both the sensitivity of MS response and the resolution of AQC–amino acids.

3.2. Effect of the mobile phase pH on MS response and separation of AQC–amino acid derivatives

Mass spectrometry is a universal detection mode, and the ionization of a compound is the precondition for determination. Among different ionization modes, ESI is especially appropriate for the ionization of polar or ionic compounds such as amino acids [44]. Unlike most of other ionization processes occurring in the gas phase,
electrospray ionization is the transfer of the ions present in a liquid phase into their gas phase [44]. Therefore, a prerequisite for the ionization with ESI is that the analyte exist in ion form in the solution, i.e., the AQC–amino acids in the mobile phase should be ionized intensively in order to obtain the sensitive MS response. On the basis of the reaction principle, the AQC–amino acid derivatives contain both NH and COOH groups (Fig. 3) and the ionization of the compounds was highly depended on solution pH [42]. When the pH of mobile phase A (water in this case) was adjusted by formic acid or NH$_3$·H$_2$O and other conditions were kept the same as shown in Fig. 1, the ionization degree of AQC–amino acids was affected by the pH of the mobile phase A. As shown in Fig. 4, when the pH value was increased from 2.5 to 9.9, the intensity of MS signal changed greatly and the highest response was obtained between

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Fig. 1. LC-UV-MS analysis of AQC–amino acids (a) MS detection and (b) ultraviolet detection. Mobile phase: (A) 140 mmol L$^{-1}$ of ammonium acetate in water (pH 4.95–5.00, titrated with acetic acid); (B) acetonitrile; and (C) water. Gradient: Initial = 100% A, 0.5 min = 99% A and 1% B, 18 min = 95% A and 5% B, 19 min = 91% A and 9% B, 29.5 min = 83% A and 17% B, hold for 4.5 min, washed with 60% acetonitrile in water for 3 min, and equilibrate with 100% A for 9 min before subsequent injection (step gradient at 0.5 min, wash and equilibration steps, liner steps for all other elution).

Fig. 2. Primarily modified LC-UV-MS analysis of AQC–amino acids (a) MS detection and (b) ultraviolet detection. Mobile phase: A, water solution (pH 4.95–5.00, adjusted with acetic acid); B, acetonitrile; and C, water. Gradient: Initial = 100% A, 0.5 min = 99% A and 1% B, 18 min = 95% A and 5% B, 19 min = 91% A and 9% B, 29.5 min = 83% A and 17% B, hold for 4.5 min, washed with 60% acetonitrile in water for 3 min, and equilibrate with 100% A for 9 min before subsequent injection (step gradient at 0.5 min, wash and equilibration steps, liner steps for all other elution).

Fig. 3. The derivatization reaction model of AQC and amino acids.
pH 3 and 3.5, while the lowest was found at pH 5.9, suggesting that pH was a key factor to influence the sensitivity of MS response due to the occurrence of isoelectric point in amino acids. Hence lower pH of the mobile phase enhanced the ionization of the AQC–amino acids to produce positive ions in the solution [35,36]. According to the results in Fig. 4, the pH of the mobile phase was optimal between 3 and 3.5 for the MS detection of AQC–amino acid derivatives.

Not only ionization but also the retention behavior of AQC–amino acids on the column was highly related to the mobile phase pH. The separation of amino acids was improved significantly when the pH of the mobile phase was decreased from 5.0 to 3.5 even without any modifier addition. Ten of the 17 amino acids in the standard mixture were fully separated under the same gradient condition, while Arg, Ser, Ile, Leu, and Phe were partly separated and the other two pairs were co-eluted (Cys–Cys with Lys and Val with Met) (Fig. 5a). It is known that Leu and Ile should be separated absolutely for accurate quantification because they cannot be distinguished by their mass in MS detector. When the pH of the mobile phase was decreased to 3.0, only Met and Val were still co-eluted, while Leu, Ile and the other AQC–amino acids were all in the baseline separation (Fig. 5b). The results showed that pH 3.0 of mobile phase A is optimal, but the overall separation is still unacceptable, hence further optimization should be done.

3.3. Effect of ammonium formate in mobile phase on the resolution and MS response

In principle, it is unnecessary for the target compound to be totally separated from the co-eluted components when identified by MS due to different m/z acquirement, but the co-eluted ions may severely disturb the ionization process of the analyte to be measured due to so called chemical suppression effect, and consequently, it might influence the accuracy of quantification. Therefore, the baseline separation was preferred by adjusting the modifier in mobile phase. Considering excess inorganic ion can deteriorate the MS response severely even if it was volatile and beneficial to the resolution of amino acid derivatives, we attempted to add low concentration gradients of volatile modifier such as ammonium formate (the concentration is 1.0–2.5 mmol L\(^{-1}\)) to solvent A to improve the resolution of the AQC–amino acids. It was found that the separation of Cys–Cys-, Lys-, Tyr-, Met-, and Val-AQC derivatives was improved significantly with the modifier addition at 1.5–2.0 mmol L\(^{-1}\) and the sensitive MS response was also acquired at the same time (data not shown). Therefore, the concentration of 1.5 mmol L\(^{-1}\) of ammonium formate was preferred for the derivative resolution and MS detection. The determination of AQC–amino acids in both standard and soil hydrolysate under the optimized conditions was shown in Fig. 6. The separation of individual amino acids was improved due to the enhanced hydrophobic character of AQC–amino acids which results in increased retention on a C18 column [42]. Consequently, the interference of mobile phase on the ionization of AQC–amino acids was reduced because of the significant increase in the derivative mass (246–581) [8,17,19,23].

3.4. Validation and applicability of the method

The validation of our method was conducted at the optimized condition, i.e., the pH of mobile phase A was 3.0 by using 1.5 mmol L\(^{-1}\) ammonium formate as the modifier. In the concentration range of 50–800 \(\mu\)mol L\(^{-1}\), the MS response of the AQC-amino acids showed excellent linearity (\(r^2 = 0.988–0.999\), as listed in Table 3). The detection limits were determined by using a 5 \(\mu\)mol L\(^{-1}\) of a standard mixture of 17 AQC–amino acids and calculated based on the signal to noise ratio of 3. The detection limits for all amino acids were in the range of 0.20–0.60 pmol L\(^{-1}\) (Table 3).
Furthermore, we spiked a mixture of amino acid standard (10 μL of 2.5 mmol L⁻¹ amino acid) to Mollisol-1 sample and the mean recovery of all amino acids ranged from 82.8% to 111.0% (Table 4). On the basis of the LOD on column, recovery and aliquot of hydrolyte solution for derivatization, the calculated detection limits of amino acids in the soil samples were in the range of 0.07–0.24 μg g⁻¹ soil (Table 4), suggesting that the proposed MS method is sensitive and quantitative. The precision of the method was firstly tested using a mixed standard of 17 AQC–amino acids at the concentration of 100 μmol L⁻¹. The mixture was analyzed for six consecutive times in 1 day (intraday, n = 6) and 6 times in three different days (interday, n = 6). The relative standard deviation (RSD%) for the intraday and interday analyses ranged from 1.1% to 4.0% and from 2.3% to 8.7%, respectively (Table 3). The RSD of the LC–MS determination of the soil samples (n = 4) among different assays was below 10% (except tyrosine) for the very heterogeneous and complex matrix, indicating that this method was highly precise (Table 4).

### Table 3
Calibration linearity, precision and detection limits of the MS method for the determination of AQC–amino acids.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>[M + H]⁺ ion of AQC–amino acid derivative (m/z)</th>
<th>Calibration linearity (r²)</th>
<th>Detection limit (pmol μL⁻¹)</th>
<th>Precision (RSD%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intraday (n = 6)</td>
<td>Interday (n = 6 in 3 days)</td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>326</td>
<td>0.988</td>
<td>0.35</td>
<td>3.0</td>
</tr>
<tr>
<td>Ser</td>
<td>276</td>
<td>0.996</td>
<td>0.23</td>
<td>1.9</td>
</tr>
<tr>
<td>Arg</td>
<td>345</td>
<td>0.998</td>
<td>0.35</td>
<td>3.4</td>
</tr>
<tr>
<td>Gly</td>
<td>246</td>
<td>0.989</td>
<td>0.60</td>
<td>3.6</td>
</tr>
<tr>
<td>Asp</td>
<td>304</td>
<td>0.993</td>
<td>0.49</td>
<td>2.5</td>
</tr>
<tr>
<td>Glu</td>
<td>318</td>
<td>0.993</td>
<td>0.55</td>
<td>1.8</td>
</tr>
<tr>
<td>Thr</td>
<td>290</td>
<td>1.000</td>
<td>0.57</td>
<td>2.5</td>
</tr>
<tr>
<td>Ala</td>
<td>280</td>
<td>0.999</td>
<td>0.42</td>
<td>1.5</td>
</tr>
<tr>
<td>Pro</td>
<td>286</td>
<td>0.996</td>
<td>0.43</td>
<td>3.0</td>
</tr>
<tr>
<td>Cys–Cys</td>
<td>581</td>
<td>0.999</td>
<td>0.35</td>
<td>3.3</td>
</tr>
<tr>
<td>Lys</td>
<td>487</td>
<td>0.998</td>
<td>0.33</td>
<td>4.0</td>
</tr>
<tr>
<td>Tyr</td>
<td>352</td>
<td>0.991</td>
<td>0.24</td>
<td>1.9</td>
</tr>
<tr>
<td>Met</td>
<td>320</td>
<td>0.996</td>
<td>0.20</td>
<td>3.0</td>
</tr>
<tr>
<td>Val</td>
<td>288</td>
<td>0.994</td>
<td>0.41</td>
<td>2.1</td>
</tr>
<tr>
<td>Ile</td>
<td>302</td>
<td>0.997</td>
<td>0.42</td>
<td>1.8</td>
</tr>
<tr>
<td>Leu</td>
<td>302</td>
<td>0.997</td>
<td>0.30</td>
<td>1.1</td>
</tr>
<tr>
<td>Phe</td>
<td>336</td>
<td>0.999</td>
<td>0.27</td>
<td>1.2</td>
</tr>
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</table>

**Notes:**
- A The molecule weight of AQC combined with amino acid is 171.

### Table 4
Recovery, precision and detection limit of amino acids in soil samples and concentrations of amino acids detected by MS and UV in Mollisol-1.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Precision (RSD%)</th>
<th>Recovery (%)</th>
<th>Detection limit (μg g⁻¹ soil)</th>
<th>Concentrations of amino acids (μg g⁻¹ soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MS detection</td>
</tr>
<tr>
<td>His</td>
<td>10.0</td>
<td>82.8</td>
<td>0.15</td>
<td>40.7 ± 4.8</td>
</tr>
<tr>
<td>Ser</td>
<td>8.6</td>
<td>95.1</td>
<td>0.07</td>
<td>178.6 ± 17.3</td>
</tr>
<tr>
<td>Arg</td>
<td>9.8</td>
<td>87.7</td>
<td>0.17</td>
<td>70.2 ± 7.7</td>
</tr>
<tr>
<td>Gly</td>
<td>6.7</td>
<td>100.0</td>
<td>0.13</td>
<td>203.7 ± 7.3</td>
</tr>
<tr>
<td>Asp</td>
<td>5.9</td>
<td>111.0</td>
<td>0.18</td>
<td>294.6 ± 44.8</td>
</tr>
<tr>
<td>Glu</td>
<td>6.3</td>
<td>107.3</td>
<td>0.27</td>
<td>215.9 ± 18.1</td>
</tr>
<tr>
<td>Thr</td>
<td>8.7</td>
<td>101.0</td>
<td>0.19</td>
<td>132.5 ± 18.6</td>
</tr>
<tr>
<td>Ala</td>
<td>6.4</td>
<td>101.6</td>
<td>0.10</td>
<td>196.5 ± 21.6</td>
</tr>
<tr>
<td>Pro</td>
<td>8.1</td>
<td>97.4</td>
<td>0.14</td>
<td>95.3 ± 9.9</td>
</tr>
<tr>
<td>Cys–Cys</td>
<td>–</td>
<td>108.7</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>9.9</td>
<td>94.4</td>
<td>0.13</td>
<td>144.6 ± 14.2</td>
</tr>
<tr>
<td>Tyr</td>
<td>12.5</td>
<td>94.0</td>
<td>0.12</td>
<td>13.8 ± 1.8</td>
</tr>
<tr>
<td>Met</td>
<td>5.9</td>
<td>101.8</td>
<td>0.08</td>
<td>91.9 ± 12.1</td>
</tr>
<tr>
<td>Ile</td>
<td>5.7</td>
<td>103.1</td>
<td>0.15</td>
<td>43.6 ± 1.7</td>
</tr>
<tr>
<td>Leu</td>
<td>4.9</td>
<td>109.9</td>
<td>0.11</td>
<td>90.3 ± 9.0</td>
</tr>
<tr>
<td>Phe</td>
<td>6.3</td>
<td>104.1</td>
<td>0.12</td>
<td>49.6 ± 6.9</td>
</tr>
</tbody>
</table>

**Notes:**
- a The recovery evaluation was conducted by adding a amino acid standard with known concentration to the soil samples and then to determine total amount of the amino acids in the spiked samples and the same soil samples without spiking were also determined in the same manner. The recovery was calculated by the following formula: recovery% = the amount of AAs in the spiked soils/(the amount of AAs in the original soil plus that of added AA) × 100%.
- b The detection limits of amino acids in soil samples were calculated based on the detection limit on column and the recovery of amino acids in soil samples as well as the aliquot of hydrolyte solution for derivatization.
- c The soil samples were determined in four replicates (n = 4) and the confidence level was 95%.
- d Concentrations were below detection limits.

AABA was recommended for the quantification of amino acids by LC analysis, while the application in MS detection should be further validated due to the possible chemical suppression in complex samples which may affect the reliability of quantification. When AABA concentration was kept as 200 μmol L⁻¹ and amino acid standard in the range of 50–800 μmol L⁻¹, the intensity ratio between the selected ion of the target amino acid and AABA was strictly proportional to the corresponding concentration. And furthermore, the purification of soil amino acids was effective and the baseline separation was obtained in our method, which enables the quantitative determination of amino acids in soil samples. Thereby, we recommend using AABA as an internal standard without isotopic labeling for routine analysis. Nevertheless, the correction of chemical suppression by stable isotope labeled amino acids is still preferred; especially when the target compounds were not well separated from their complex matrix.
Thr 70.7 ± 7.5 73.6 ± 6.1 109.5 ± 12.9 119.6 ± 11.5 275.7 ± 17.6 271.4 ± 21.2 236.1 ± 15.4 202.9 ± 14.6

Arg 58.5 ± 4.8 51.4 ± 5.1 98.1 ± 7.5 111.6 ± 10.9 251.5 ± 16.6 283.7 ± 20.4 226.1 ± 15.4 202.9 ± 14.6

Gly 83.2 ± 8.5 89.1 ± 8.4 166.4 ± 17.0 178.4 ± 14.3 455.6 ± 28.2 421.1 ± 26.1 875.3 ± 36.8 937.8 ± 76.9

Asp 113.7 ± 11.1 109.5 ± 9.2 233.7 ± 18.7 244.7 ± 17.1 630.7 ± 44.1 548.2 ± 46.0 1140.7 ± 54.8 1098.0 ± 59.3

Glu 151.8 ± 17.3 137.9 ± 12.7 236.3 ± 19.4 234.4 ± 17.3 592.7 ± 48.6 548.2 ± 37.3 990.0 ± 39.4 949.1 ± 47.5

Thr 70.7 ± 8.5 69.1 ± 6.4 102.7 ± 7.2 115.1 ± 9.4 284.2 ± 21.0 307.4 ± 17.8 491.4 ± 31.4 480.6 ± 43.3

Ala 102.2 ± 10.6 102.4 ± 8.6 151.4 ± 15.4 164.6 ± 12.5 409.9 ± 29.5 389.2 ± 24.1 978.0 ± 66.5 947.5 ± 70.1

Pro 74.5 ± 8.2 74.8 ± 6.1 91.9 ± 8.6 100.4 ± 10.4 258.8 ± 23.8 315.0 ± 23.3 536.3 ± 45.0 538.5 ± 44.2

Fig. 6. The total ion current chromatogram of amino acids in standard (a) and soil samples (b) under the optimized mobile phase conditions. Mobile phase: (A) 1.5 mmol L⁻¹ of ammonium formate in water (pH 3.0, titrated with formic acid); (B) Acetonitrile; and (C) water. Gradient conditions: Initial = 100% A, 0.5 min = 99% A and 15% B, 21 min = 91% A and 9% B, 25 min = 83% A and 17% B, hold for 2 min, washed with 60% acetonitrile in water for 3 min, and equilibrate with 100% C for 10 min before subsequent injection (step gradient at 0.5 min, wash and equilibration steps, liner steps for all other elution). The flow rate of mobile phase was set at 0.2 ml min⁻¹ and the analysis time was 50 min.

Table 5

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration of amino acids (µg g⁻¹ soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ultrasol</td>
</tr>
<tr>
<td></td>
<td>Data 1</td>
</tr>
<tr>
<td>His</td>
<td>25.7 ± 2.8a</td>
</tr>
<tr>
<td>Ser</td>
<td>78.4 ± 7.5</td>
</tr>
<tr>
<td>Arg</td>
<td>58.5 ± 4.8</td>
</tr>
<tr>
<td>Gly</td>
<td>83.2 ± 8.5</td>
</tr>
<tr>
<td>Asp</td>
<td>113.7 ± 11.1</td>
</tr>
<tr>
<td>Glu</td>
<td>151.8 ± 17.3</td>
</tr>
<tr>
<td>Thr</td>
<td>70.7 ± 8.5</td>
</tr>
<tr>
<td>Ala</td>
<td>102.2 ± 10.6</td>
</tr>
<tr>
<td>Pro</td>
<td>74.5 ± 8.2</td>
</tr>
</tbody>
</table>

Table 5

Concentrations of amino acids in four different soils determined by the proposed method (data 1) and amino acid automatic analyzer (data 2).

To test the applicability of the method for a broad range of soils, we analyzed amino acids in five samples representing different types of soil with significantly different properties, especially soil organic matter (SOM) and total nitrogen (TN) contents. The soil samples are classified as Ultrasol, Alfisol, Inceptisol, and Mollisols (Mollisol-1 and 2), respectively. The amino acid contents in the soil samples were determined firstly by our MS method (Table 5) and it was found that the concentration of all amino acids was in different range, which is accordant with that from other studies [5]. In order to test the reliability of the method, the amino acids in Mollisol-1 sample was also analyzed by the UV detector online with the MS detector on the same condition (see Table 4). Both total and individual amino acid concentrations determined by the two detectors were very close, suggesting that the quantitative results by the MS detector are reliable. In addition, the contents of amino acids in soil samples were determined independently by using a widely accepted method, the amino acid automatic analyzer. We found that the concentrations of most amino acids are identical between the two independent methods, though significant difference was observed for lysine, isoleucine and tyrosine (Table 5). The reason, to our opinion, is mainly derived from the different principles for amino acid determination. Each amino acid was quantified by the selective ion ([M + H]⁺) or the fragment with the most abundance in our method, while it was evaluated by the total area of its chromatogram peak in the amino acid automatic analyzer technique. The separation of isoleucine was excellent in both standard and soil samples under the two detection techniques, but its concentrations in the Inceptisol was significantly higher detected by amino acid automatic analyzer. This might have been attributed to the interference from the co-elution of soil impurities because not all the impurities in soil hydrolysis can be removed during purification. If this happens, it is known for sure that the quantification from amino acid automatic analyzer detection should be influenced more significantly than those from our MS detection. In this context, the amino acid automatic analyzer method could result in overestimation of isoleucine. On the other hand, chemical suppression may influence the accuracy of MS quantification for the co-eluted amino acids (lysine and tyrosine in our case), especially for those with low concentrations, so the concentration of tyrosine might be erratically determined by our MS method. However, tyrosine is a minor amino in soils and its quantification result does not influence the interpretation of the whole picture of soil amino acids. The concentrations of cystine and methionine in the tested soils were below detection limits from both methods, but the recoveries and sensitivity of the two amino acids were quite high, inferring that it is still possible to quantify the two amino acids while increasing soil sample amounts.

4. Conclusion

A new method of liquid chromatography coupled to mass spectrometry was established for convenient and accurate quantification of amino acids in soil samples. The method is based on the use of pre-column AQC derivatization as well as the optimization of mobile phase conditions, especially of pH and ammonium formate concentration. In addition, the sensitivity, linearity and detection limits of amino acids by using MS detector were assessed strictly. Finally, the method is validated by the calculation of the recoveries of the amino acids in spiked soil and the comparison of the results quantified by the MS detector and UV detector as well as by the two independent methods. All findings indicate that the
The proposed MS method can be practically applied for the determination of amino acids in a wide range of soil samples. It is the first time that 15 amino acids in soil have been quantified by HPLC-MS and thus, the technique shows a potential for tracing the transformation of soil amino acids with isotope technique for N transformation and cycling investigation.

Acknowledgements

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References