



A non-derivatized method for simultaneous quantitation of proteinogenic, urea-cycle, and acetylated amino acids by liquid chromatography–high-resolution mass spectrometry

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Abstract

The quantification of amino acids as freely dissolved compounds or from hydrolyzed peptides and proteins is a common endeavor in biomedical and environmental sciences. In order to avoid the drawbacks of derivatization and application challenges of tandem mass spectrometry, we present here a robust 13-min liquid chromatography coupled with a full-scan mass spectrometry method to achieve rapid detection and quantification of 30 amino acids without derivatization. We combined hydrophilic interaction liquid chromatography with heated electrospray ionization and high-resolution mass spectrometry operated with polarity switching to analyze the 20 proteinogenic amino acids, ornithine, citrulline, homoserine, cystine, and six acetylated amino acids. We obtained high mass accuracy and good precision of the targeted amino acids. Limits of detection ranged from 0.017 to 1.3 μM . Noteworthy for environmental samples, we found comparable ionization efficiency and quantitative detection for the majority of the analytes prepared with pure water versus a high-salt solution. We applied the method to profile carbon source-dependent secretions of amino acids by *Pseudomonas protegens* Pf-5, a well-known plant-beneficial bacterium.

Keywords Polarity switching · Non-targeted analysis · Acetylated amino acids · Urea-cycle amino acids · High-resolution mass spectrometry

Introduction

Analysis of amino acids as free compounds or as constituents of proteins and small peptides in biological and environmental samples is important for gaining insights into nitrogen cycling, dissolved organic nitrogen (Berto et al. 2018; Tappin et al. 2007; Trubetskaya et al. 2016), transformation products in treatment systems (Sharma and Sohn

2012), physiological responses to toxicity (Dubey et al. 2018), and biological aerosol particle sources (Abe et al. 2016). To achieve amino acid analysis via optical spectroscopy, a chemical derivatization step is required (Kaspar et al. 2009), which is time-consuming and costly. Furthermore, derivatization can transform or decompose amino acids, decreasing the number of quantifiable analytes (Abe et al. 2016). Mass spectrometry, which can be employed without derivatization, has become the preferred detection method for the analysis of free amino acids (Kaspar et al. 2009). The common tandem mass spectrometry approach requires laborious prediction of parent–daughter transitions, and chromatogram quality decreases with increasing transitions. Here, we present a non-derivatized hydrophilic interaction liquid chromatography method coupled with a full-scan mass spectrometry approach capable of monitoring simultaneously a total of 30 amino acids.

Due to its suitability to separate polar analytes (Buszewski and Noga 2012), non-derivatized liquid chromatography–mass spectrometry methods for amino acid analysis have been employed primarily hydrophilic interaction

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liquid chromatography separation (Aviram et al. 2016; Chen et al. 2017; Gao et al. 2016; Gökmen et al. 2012; Guo et al. 2013; Huang et al. 2018; Prinsen et al. 2016; Qi et al. 2015; Vilches et al. 2017; Wang et al. 2013; Yao et al. 2013). These methods focused on analyzing the proteinogenic amino acids and seldom included citrulline and ornithine (Guo et al. 2013; Huang et al. 2018; Prinsen et al. 2016; Qi et al. 2015), which are two non-proteinogenic amino acids associated with urea-cycle metabolism and nitric oxide synthesis (Joshi

et al. 2019). Furthermore, despite N-terminal acetylation of 80–90% of soluble proteins (Lindner et al. 2008), previous methods developed for the proteinogenic amino acids do not typically include acetylated amino acids.

We developed our method to target, in addition to the 20 common proteinogenic amino acids, homoserine (an isomer of threonine), cystine (a cysteine dimer), citrulline and ornithine from the urea cycle, and six acetylated amino acids (Table 1). We performed validation of the method by

Table 1 Liquid chromatography–mass spectrometry parameters and limit of detection values

Compound	Retention time (mins)	Neutral stoichiometric composition	m/z ($\pm H$)	ppm error ^a	Limit of detection (μM) ^b
<i>Nonpolar amino acids</i>					
L-alanine	7.65	C ₃ H ₇ NO ₂	90.05496 (+)	4.9	1.2
Glycine	7.94	C ₂ H ₅ NO ₂	76.03930 (+)	7.5	1.3
L-isoleucine	4.44	C ₆ H ₁₃ NO ₂	132.10191 (+)	0.73	0.063
L-leucine	4.05	C ₆ H ₁₃ NO ₂	132.10191 (+)	0.76	0.086
L-methionine	4.81	C ₅ H ₁₁ NO ₂ S	150.05833 (+)	1.1	0.073
L-proline	5.68 & 6.38	C ₅ H ₉ NO ₂	116.07061 (+)	1.2	0.017
L-valine	4.97	C ₅ H ₁₁ NO ₂	118.08626 (+)	0.80	1.2
<i>Aromatic amino acids</i>					
L-phenylalanine	3.86	C ₉ H ₁₁ NO ₂	166.08626 (+)	0.88	0.078
L-tryptophan	3.83	C ₁₁ H ₁₂ N ₂ O ₂	205.09715 (+)	0.73	0.40
L-tyrosine	5.25	C ₉ H ₁₁ NO ₃	182.08117 (+)	0.48	0.061
<i>Polar amino acids</i>					
L-arginine	8.95	C ₆ H ₁₄ N ₄ O ₂	175.11895 (+)	1.1	0.19
L-asparagine	8.25	C ₄ H ₈ N ₂ O ₃	131.04622 (–)	1.0	0.13
L-aspartic acid	8.68	C ₄ H ₇ NO ₄	132.03023 (–)	1.1	0.16
L-citrulline	8.34	C ₆ H ₁₃ N ₃ O ₃	176.10297 (+)	0.95	0.070
L-cysteine	7.20	C ₃ H ₇ NO ₂ S	120.01247 (–)	1.1	1.3
L-cystine	9.70	C ₆ H ₁₂ N ₂ O ₄ S ₂	239.01657 (–)	1.4	0.096
L-glutamine	8.12	C ₅ H ₁₀ N ₂ O ₃	145.06187 (–)	0.98	0.051
L-glutamic acid	8.32	C ₅ H ₉ NO ₄	146.04588 (–)	0.96	0.099
L-histidine	8.93	C ₆ H ₉ N ₃ O ₂	156.07675 (+)	0.95	0.11
L-homoserine ^c	7.91	C ₄ H ₉ NO ₃	120.06552 (+)	0.53	0.071
L-lysine	9.09	C ₆ H ₁₄ N ₂ O ₂	147.11280 (+)	1.4	0.27
L-ornithine	9.14	C ₅ H ₁₂ N ₂ O ₂	133.09715 (+)	0.98	0.31
L-serine	8.18	C ₃ H ₇ NO ₃	104.03532 (–)	1.3	0.22
L-threonine	7.83	C ₄ H ₉ NO ₃	118.05097 (–)	1.2	0.20
<i>Acetylated amino acids</i>					
N-acetyl-L-alanine	2.35	C ₅ H ₉ NO ₃	130.05097 (–)	2.9	0.038
N-acetyl-L-glutamine	4.88	C ₇ H ₁₂ N ₂ O ₄	187.07243 (–)	2.8	0.14
N-acetyl-L-glutamic acid	4.31	C ₇ H ₁₁ NO ₅	188.05645 (–)	2.7	0.15
N ^α -acetyl-L-lysine	7.83	C ₈ H ₁₆ N ₂ O ₃	189.12337 (+)	2.8	0.14
N-acetyl-L-ornithine	8.01	C ₇ H ₁₄ N ₂ O ₃	175.10772 (+)	2.6	0.16
O-acetyl-L-serine	5.74 & 6.35	C ₅ H ₉ NO ₄	148.06043 (+)	2.5	0.73

^aThe m/z error (in ppm) was calculated as: $(\text{theoretical value} - \text{experimental value})/\text{theoretical value} \times 10^6$

^bAmino acids: $n = 62$; acetylated amino acids: $n = 92$

^cAnalyzed separately due to overlap with threonine

determining limit of detection (LOD) and limit of quantitation (LOQ), precision and mass errors in standards prepared in pure water, followed by a comparison of ionization efficiency of amino acid standards prepared with a high-salt solution representative of biological or environmental samples. Finally, the method was applied to determine the extracellular composition of amino acids secreted by a soil bacterium, *Pseudomonas protegens* Pf-5, isolated from the rhizosphere.

Experimental

Materials

Details on the materials are provided in Appendix A (Supplementary Information).

Liquid chromatography–mass spectrometry and method application

Analysis was performed on a Dionex Ultimate 3000 liquid chromatography system coupled to a Q-Exactive mass spectrometer controlled with XCalibur software (Thermo Scientific; Waltham, Massachusetts). The amide column was a $4.6 \times 100 \text{ mm} \times 3.5 \mu\text{m}$ XBridge™ (Waters; Milford, Massachusetts) maintained at 35 °C. The injection volume was 50 μL with the sample matrix consisting of 50% v/v acetonitrile. The chromatography, at a constant flow rate of 1 mL min^{-1} , was a 13-min binary gradient of eluent A (90:10 v/v acetonitrile/water, 0.15% v/v formic acid, 10 mM ammonium formate) and eluent B (0.15% v/v formic acid in water and 10 mM ammonium formate); chromatography grade water was used to prepare the eluents. The gradient for eluent A was: 0 min, 93%; 4.6 min, 93%; 5.5 min, 90%; 6 min, 75%; 9.25 min, 60%; 10.25 min, 60%; 10.35 min, 93%; 13 min, 93%. The heated electrospray ionization parameters were: sheath, auxiliary, and sweep gas flow rates at, respectively, 60, 15, and 0 arbitrary units; a spray voltage of 3 kV; capillary temperature of 380 °C; and auxiliary gas heater temperature of 400 °C. Full mass spectrometry spectra, with the scan range of 60–300 mass-over-charge (m/z) and resolution of 70,000, were acquired in both positive and negative ionization modes using polarity switching. Details on LOD and LOQ determinations are in Appendix B, Supplementary Information.

To evaluate the effect of solution matrix, standard solutions were prepared with purified water or a high-salt bacterial growth medium (Appendix C, Supplementary Information). For method application, *P. protegens* Pf-5 cells (ATCC® BAA-477™) were grown with either 3 g L^{-1} (16.6 mM) glucose or 3.2 g L^{-1} (16.6 mM) citrate in the growth medium (Wilkes et al. 2019). Culture aliquots taken

during exponential and stationary growth phases were pelleted by centrifugation. The supernatants were stored at $-20 \text{ }^{\circ}\text{C}$ until analysis. In between runs, we also applied our in-house metabolomics liquid chromatography–mass spectrometry method (Aristilde et al. 2017), which uses tributylamine (m/z $[\text{M} + \text{H}]^+$ 186.22163) as an ion-pairing agent. Due to detection interference in positive mode by the tributylamine residue, the positive ionization mode was divided into the following three m/z ranges to exclude the two most abundant contaminant ions (m/z 130.1591 and 186.2216): (1) m/z 100–250, negative ionization; (2) m/z 70–125, positive ionization; (3) m/z 131.5–185, positive ionization; and (4) m/z 188.5–250, positive ionization.

Results and discussion

Chromatographic separation

The 13-min duration of the chromatographic separation is comparable to previously published methods (Aviram et al. 2016; Chen et al. 2017; Gao et al. 2016; Guo et al. 2013; Huang et al. 2018; Konn et al. 2015; Prinsen et al. 2016; Qi et al. 2015; Van Helmond et al. 2017; Wang et al. 2013; Yao et al. 2013; Yin et al. 2017) but identified a greater diversity of different types of amino acids (Fig. 1a). In accordance with the hydrophilic interaction liquid chromatography conditions, the nonpolar and aromatic amino acids eluted first from the column (3.8–8 min), followed by the uncharged polar amino acids (asparagine, citrulline, cysteine, glutamine, homoserine, serine and threonine; 7–8.25 min), and the charged polar amino acids (arginine, aspartate, glutamate, histidine, lysine and ornithine) at relatively longer retention times (8.3–9.2 min); cystine had the longest retention time of 9.7 min (Table 1; Fig. 1b). Acetylated amino acids eluted between 2 and 8 min, depending on their polarity. Chromatographic separation of homoserine and threonine was not achieved, but we obtained partial separation of leucine and isoleucine, which can be quantifiable using their top peak areas.

Limits of detection and quantitation, mass accuracy, and precision

Of the 30 amino acids targeted by our method using the acetonitrile/water matrix, 25 were detectable with limits of detections (LODs) at or below $0.4 \mu\text{M}$ (Table 1) and 24 were quantifiable at less than $1 \mu\text{M}$ (Appendix B, Supplementary Information). The majority of our LOD values were comparable to previous studies using non-derivatized liquid chromatography–mass spectrometry methods and full mass spectrometry acquisition mode (Appendix B, Supplementary Information), except for alanine,

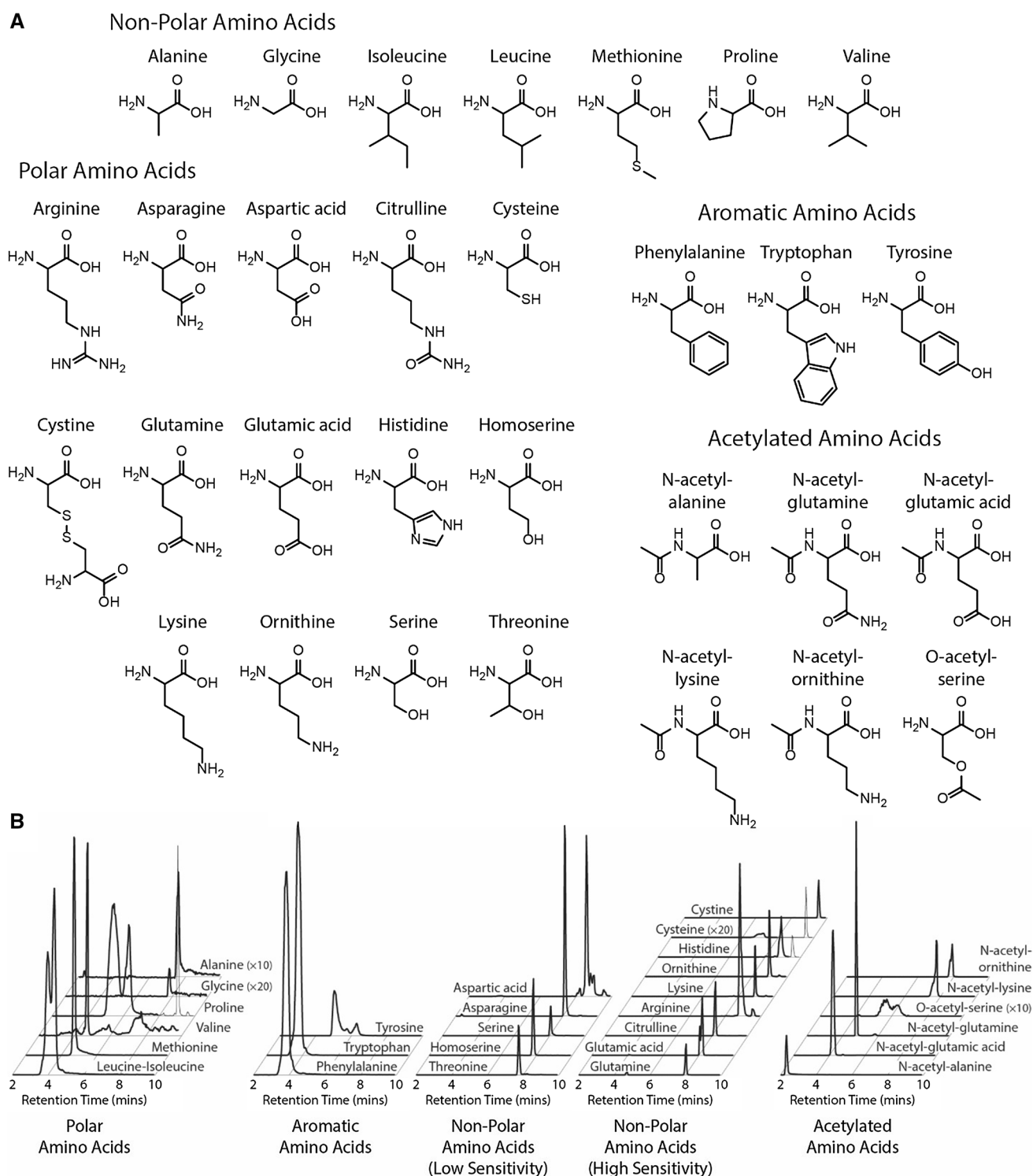


Fig. 1 **a** Chemical structures of the 30 amino acids categorized by the type of R groups and **b** representative extracted ion chromatograms from a 10 μ M standard mixture (acetonitrile/water matrix). Selected amino acids were magnified (by 10 $\times$ or 20 $\times$) to improve graphi-

cal presentation. The hydrophilic interaction liquid chromatography approach separates the different amino acid structure in accordance with their different polarity. The nonpolar and aromatic amino acids eluted first, and the charged polar amino acids eluted last

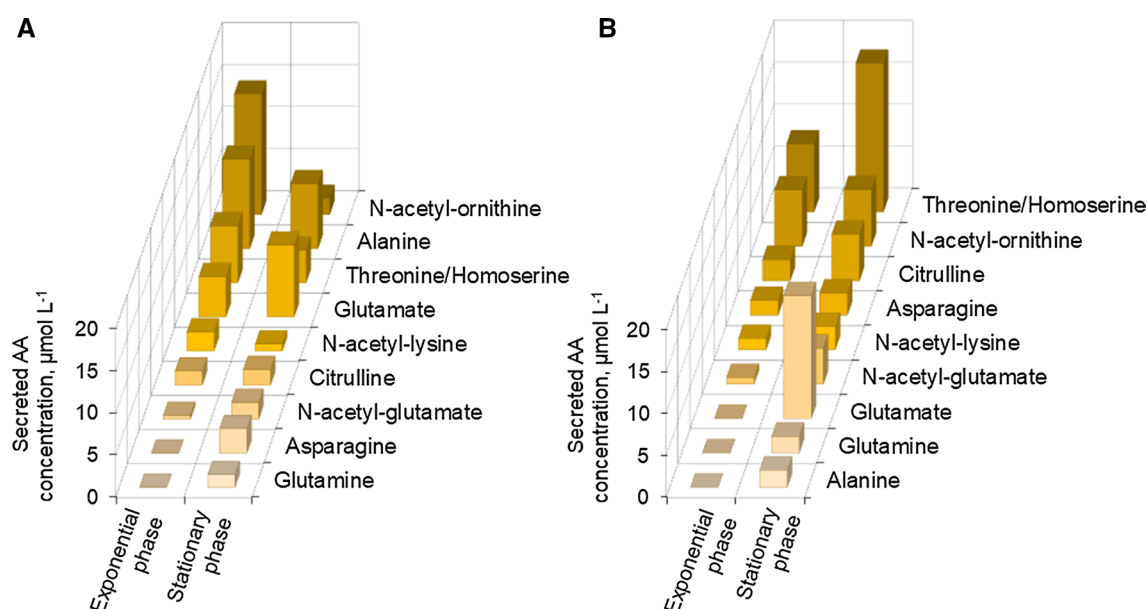


Fig. 2 Profiled amino acids in the extracellular matrix of *P. protegens* Pf-5 cells grown on **a** citrate or **b** glucose. Samples were taken during exponential or stationary growth phase. Full data (average $\pm$ standard deviation) are provided in Appendix D (Supplementary Information). The secreted amino acids were dependent on both the carbon source and bacterial growth stage. For citrate-grown cells, the highest amino

acid concentration was for N-acetyl-ornithine at exponential growth, but glutamate has the highest concentration at the stationary phase. For glucose-grown cells, the isomers threonine/homoserine had the most elevated concentrations; glutamate was also a major fraction at the stationary phase

valine, tryptophan, cysteine, and lysine (Aviram et al. 2016; Gökmen et al. 2012; Konn et al. 2015). Also, our LOD values were in the ranges of previous reports using tandem mass spectrometry, with the exception of alanine, valine, phenylalanine, tryptophan, cysteine, homoserine, and ornithine (Chen et al. 2017; Gao et al. 2016; Guo et al. 2013; Huang et al. 2018; Prinsen et al. 2016; Qi et al. 2015; Vilches et al. 2017; Wang et al. 2013; Yao et al. 2013). No previous reports of LOD values for acetylated amino acids using non-derivatized methods were available.

Our full mass spectrometry acquisition mode identified the compounds based on high-accuracy mass detection with a mass error of ~ 2.7 ppm for the acetylated amino acids, and ~ 1 ppm for the other amino acids, except for alanine (4.9 ppm) and glycine (7.5 ppm). The precision of the method was determined using percent relative standard deviation (%RSD). Except for cysteine (29% RSD), all amino acids had a RSD less than 15%, with most less than 8% (Appendix B, Supplementary Information). Previously reported %RSD values with non-derivatized liquid chromatography–mass spectrometry methods were typically less than 10 (Chen et al. 2017; Gökmen et al. 2012; Guo et al. 2013; Huang et al. 2018; Wang et al. 2013; Yao et al. 2013).

Amino acid profiling in high-salt solutions

The influence of sample matrix on the ionization efficiency for each amino acid is presented in Appendix C (Supplementary Information). We applied our method by quantifying the extracellular amino acids in high-salt secretions from *P. protegens* Pf-5 grown on either glucose or citrate as the carbon source (Fig. 2); the growth medium contained no amino acids in the absence of the bacterial cells. In response to metabolism of the different carbon substrates, *P. protegens* Pf-5 was shown to secrete different types of short-chain carboxylic acids (Wilkes et al. 2019). Here, our analysis highlighted that the extracellular amino acid profile by *P. protegens* Pf-5 was dependent on both the carbon source and the cell growth stage, reflecting the dynamic nature of coupled carbon and nitrogen metabolisms. Notably, there was a twofold higher amino acid concentration in the media of exponentially growing cells on citrate compared to exponentially growing cells on glucose. However, at stationary phase, the amino acid concentration of glucose-grown cells was increased by threefold from the level obtained at exponential growth. In glucose-grown cells, the amino acid composition contained primarily N-acetyl-ornithine, citrulline, and threonine/homoserine during both exponential and stationary phases; glutamate and N-acetyl-glutamate also

became major fractions at stationary phase. In citrate-grown cells, alanine, glutamate, and threonine/homoserine were consistently abundant during both stationary and exponential phases; N-acetyl-ornithine was also a dominant fraction during the exponential stage. At stationary growth phase for both carbon conditions, glutamate became a major fraction of the extracellular amino acid composition.

Conclusion

Amino acids found as free compounds or within proteins and small peptides represent an important component of dissolved organic nitrogen. For instance, approximately half of the protein-like fluorescence in dissolved organic matter has been attributed to the aromatic amino acids tryptophan and tyrosine (Trubetskaya et al. 2016). Until recently, method development to measure free amino acids mostly required a chemical derivatization step or employed tandem mass spectrometry technology. To circumvent the challenges of implementing tandem mass spectrometry acquisition, the method presented here makes use of a full mass spectrometry detection mode. Moreover, our method saves time and experimental costs by eliminating the derivatization step and expands the diversity of amino acid compounds analyzed in a single method by including detection of urea-cycle and acetylated amino acids, in addition to the proteinogenic amino acids. Given the successful application of our method with a high-salt matrix, this method is suitable to conduct amino acid profiling in environmental samples such as soil and water extracts.

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Compliance with ethical standards

Conflicts of Interest The authors declare no conflicts of interest.

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