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Research paper

Quantitative analysis of changes in amino acids levels for cucumber (*Cucumis sativus*) exposed to nano copper

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ABSTRACT

The increasing usage of nanopesticides in agriculture poses a concern to plant crops due to unknown implications of engineered nanomaterials (ENMs). Targeted metabolomics can provide both qualitative and quantitative information at a molecular level to investigate the response of plants to emerging environmental stressors, such as nanopesticides. Here we describe a detailed protocol for the extraction and analysis of plant metabolites, specifically 23 amino acids in plants, using hydrophilic interaction liquid chromatography coupled to triple quadrupole mass spectrometry (HILIC-LC-MS/MS). Sufficient separation of 23 amino acids was achieved, without the need for derivatization, on an HILIC column with an MS/MS detector in a single run of 12 min with high sensitivity, selectivity and robustness and low LOD (0.005–15 ng/mL) and LOQ (0.02–50 ng/mL). A simple and efficient method to effectively extract amino acids from plant tissues was developed with a high recovery rate (80–120%). The protocol was then applied to determine the levels of amino acids in cucumber plants exposed to various environmentally-relevant levels of nano copper (nCu at 0, 200, 400, and 800 mg/kg soil; harvested in 60 days). Dose-dependent changes in amino acid levels were found; 13 amino acids were upregulated due to nCu stress, particularly, tyrosine increased 6.1, 8.2, and 11.0 fold after exposure to 200, 400 and 800 mg/kg nCu, respectively. The change in amino acid levels suggests an active defense response of the cucumber plant to nCu stress. We demonstrate that the HILIC-LC-MS/MS method is an effective and efficient technique to analyze underivatized amino acids in plant samples.

1. Introduction

Recently, there has been an increasing use of nanoscale fertilizers and pesticides in agriculture (Raliya et al., 2018; Dimkpa and Bindraban, 2018), and copper-containing nanopesticides (Cu NPs) are one of the most popular products on the market because of their excellent antimicrobial and antifungal properties (Bergeson, 2010; Kiaune and Singhasemanon, 2011; Keller et al., 2017). However, due to the unique physicochemical properties of engineered nanomaterials (ENMs), e.g., ultra-fine particle size, high reactivity and etc., studies indicated they can be considered as potential environmental stressors to terrestrial plants (Conway et al., 2015; Rizwan et al., 2017; Du et al., 2017). Some metallic ENMs (e.g. Cu NPs) and/or released ions (e.g. Cu^{2+}) can induce the stress to plant, e.g. the formation of reactive oxygen species (ROS) within plant cells to induce oxidative stress (Zhao et al., 2016a; Shaw et al., 2014). The levels of low molecular weight metabolites, including amino acids, represent the ultimate response of biological systems to environmental changes (Fiehn, 2002). Furthermore, by studying these metabolites, we can understand better the metabolic pathways and networks that are up- or down-regulated due to exposure to these ENM stressors (Hasler-Sheetal et al., 2016). Thus, the quantitative determination of amino acids is important in mapping the metabolomic profile and evaluating the pathway of key metabolites, as well as the nutritional supplies from plant tissues; metabolomics provides a more holistic view of plant response to these environmental stressors. In addition, since Cu^{2+} exhibits strong binding to amino acids forming complexes, increased levels of some amino acids may induce the transformation of Cu NPs within the plants and/or in soil (Huang et al., 2017). Monitoring the changes in amino acids can also serve to better understand the underlying mechanisms behind plant-ENMs interactions at a molecular level.

In our previous studies, untargeted gas chromatography-time of

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flight-mass spectrometry (GC-TOF-MS) and ^{1}H nuclear magnetic resonance (NMR)-based metabolomics were applied for a rapid screening of metabolite changes within crop plants as responses to the stress induced by Cu NPs (Zhao et al., 2016a, b, c, d, 2017a, b, c, d, e). Amino acid levels within plant tissues were significantly ($p < 0.05$) altered after exposure to Cu NPs (Zhao et al., 2016a, c, 2017a, c, d, e). For example, eleven amino acids (e.g., alanine, glycine, proline and etc.) were significantly up-regulated in the root exudate of cucumber, suggesting an active defense mechanism against Cu NPs stress (Zhao et al., 2016a). With untargeted analytical techniques, an overall metabolic profile can provide a molecular-scale perspective on the response of plants to stressors, such as nanopesticides. However, the untargeted metabolomics analysis provides semi-quantitative information on the changes in metabolite levels, since there is no rigorous assessment of the recovery of metabolites during extraction, or calibration of the GC-TOF-MS responses. Targeted metabolomics, for example liquid chromatography coupled to triple quadrupole mass spectrometry (LC-MS/MS), can quantitatively determine the changes in secondary metabolite concentrations in plants exposed to ENMs, which provides a more-sensitive and mechanistic understanding of the biological response to a stressor (Huang et al., 2019).

Hydrophilic interaction liquid chromatography (HILIC) can separate and help quantitatively analyze a wide range of polar compounds, including amino acids (Gao et al., 2016; Dell'mour et al., 2010), peptides (Le Maux et al., 2015), carbohydrates (Schulze et al., 2017), metabolites and other biologically important compounds (Mackay et al., 2015; Buszewski and Noga, 2012). Compared to other analytical techniques (e.g., reversed-phase high-performance liquid chromatography (RP-HPLC) and/or capillary electrophoresis (CE) coupled with optical or mass spectrometry (MS) detection), no pre-treatment (derivatization with strong chromophore groups, for example, ninhydrin (Bidlingmeyer et al., 1984), *o*-phthalaldehyde (Nimura and Kinoshita, 1986), etc.) is required to analyze amino acids on HILIC. This avoids time-consuming derivatization procedures, and can minimize issues such as derivative instability, insufficient reproducibility of derivative yield, and interferences caused by the reagent (Kaspar et al., 2009). Furthermore, coupled with tandem mass spectrometry (MS/MS), HILIC-MS/MS can offer gains in selectivity and sensitivity by reaction monitoring (MRM), while avoiding issues of instrument contamination and downtime that occur with the use of derivatizing agents.

Previous LC methods exhibited long retention time for underivatized amino acids analysis (Prinsen et al., 2016; Krumpochova et al., 2015). In this study, A newly released HILIC column with small particle size (2.7 μm) was used to develop a fast and sensitive HILIC-MS/MS method for direct quantitative analysis of underivatized amino acids. A single transition was used for quantification (Prinsen et al., 2016), and a second transition was employed as qualifier for confirmation of the identity of the targeted amino acids. The HILIC-MS/MS method presented here is capable of performing quantitation at trace levels (e.g., μg/L) of these amino acids in food, biological or environmental matrices. Using the quantitative secondary metabolites (e.g., amino acids) data, targeted metabolomics were conducted to investigate the response of plants (e.g., cucumber) exposed to ENMs (e.g., Cu NPs).

2. Materials and methods

2.1. Chemicals and reagents

All analytical standards used during the study had at least > 96% purity. All amino acid standards were purchased from Sigma-Aldrich (St. Louis, MO), including: L-tyrosine (≥99.0%), L-proline (≥99.0%), L-alanine (≥98.5%), L-valine (≥98.5%), L-phenylalanine (≥98.5%), L-lysine (≥98%), L-threonine (≥99.0%), glycine (≥99.0%), L-asparagine (≥98%), L-ornithine monohydrochloride (≥99.0%), L-arginine (≥98.5%), L-glutamic acid (≥98.5%), L-tryptophan (≥99.0%), L-isoleucine (≥98.5%), L-glutamine (≥99.0%), L-leucine (≥98.5%), L-methionine (≥99.0%), L-histidine (≥99.0%), L-aspartic acid (≥99.0%), L-cysteine (≥97%), L-citrulline (≥98%), L-serine (≥99.0%) and L-homoserine (≥98%) (Table S1). Isotopically labeled internal standards (ISTD), L-isoleucine-^{15}N (98%), L-methionine-2,3,3,4,4-d_5-methyl-d_3 (98%), L-glutamic acid-^{15}N (98%), Glycine-2,2-d_2 (98%), and L-alanine-3,3,3-d_3 (99%) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). DL-Lysine-3,3,4,4,5,5,6,6-d_8 dihydrochloride (99.6%) was purchased from CDN Isotopes (Pointe-Claire, Québec, Canada), used as isotopically labeled internal standard (ILIS). LC-MS grade acetonitrile (ACN) and water were purchased from Burdick and Jackson (Muskegon, MI), while LC-MS grade formic acid and ammonium formate were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Characteristics of nCu NPs

Uncoated nCu (U.S. Research Nanomaterials) was employed here; a detailed characterization was presented in a previous study (Adeleye et al., 2014). In this study, the primary particle size is 40 nm and the hydrodynamic diameter (HDD) was measured as 2432 ± 484 nm in deionized (DI, Barnstead nanopure) water at pH 7 (0.5 mM phosphate buffer). Scanning electron microscope (SEM) and transmission electron microscopy (TEM) images of nCu are presented in the Supporting Information (Fig. S1). The surface charge, expressed as zeta potential in 0.5 mM phosphate buffer solution, is −28.8 ± 0.6 mV at pH 7.

2.3. Plant exposure and growth conditions

Cucumber (*Cucumis sativus*) seeds were purchased from Seed Savers Exchange (Iowa, USA). nCu was suspended in DI water and sonicated for 30 min before being applied to the soil surface without mixing (top soil collected from Sedgwick Reserve, CA, USA; and the characteristics of the soil were provided in SI, as Table S3). The final concentration of nCu in soil (mg/kg) was 0 (Control), 200 (low), 400 (medium) and 800 (high). This total Cu concentration is within the range predicted for biosolids applied to soils (Lazareva and Keller, 2014) or due to the application of copper-based nanopesticides (Conway et al., 2015). Each treatment had four replicates. In each replicate, pairs of cucumber seedlings were grown in 3.0 L Poly-Tainer containers. The cucumber plants were grown 60 days in the greenhouse at a controlled temperature of 25.5 to 30.0 °C during the day and 17.7 to 18.9 °C at night.

2.4. Extraction of amino acids

Cucumber leaf tissue extracts were prepared from freshly harvested cucumber leaves, which were immediately placed in liquid nitrogen for rapid freezing. The frozen cucumber tissues were homogenized in liquid nitrogen into a fine powder using a mortar and pestle, then stored in a freezer at −85 °C. For extraction, 100 mg of frozen cucumber leaf powder was weighed into 2-mL Eppendorf microcentrifuge tubes, and 1 mL of 0.5 M aqueous HCl was added. The tubes were vortexed at 8000 rpm for 20 min, sonicated in a 25 °C water bath for 20 min, then centrifuged at 20,000 × *g* for 20 min. Finally, 250 μL of the extraction supernatant was transferred into LC vials with ISTD already added, and the mixture was diluted to 1 mL with 20% water in acetonitrile.

To determine the extraction recovery rates of amino acids, three levels of mixed amino acid standards were spiked into cucumber leaf tissue samples before and after the extraction process, to obtain pre- and post-extraction spike recovery rates, respectively. The spiked concentrations of amino acids were 20, 40 and 80 μg/g (cucumber leaf tissues).

The recovery rates were calculated using:

$$\text{Recovery rate } (\%) = \frac{C_{observed} - C_{neat}}{C_{expected}} \times 100$$

where, $C_{observed}$ is the concentration of pre- or post-extraction spiked

sample with a mixture of amino acid standards; C_{neat} the concentration of non-spiked (control) sample; $C_{expected}$ the concentration that was spiked into the samples.

2.5. Liquid chromatography

An Agilent 1260 UHPLC binary pump was used to perform liquid chromatography for all analyses. An Agilent InfinityLab Poroshell 120 HILIC-Z (2.1 × 100 mm, 2.7 μm) column was used for chromatographic separation of all analytes. The column was maintained at 25 °C throughout the run. A dual eluent mobile phase was run at 500 μL/min for separation. Stock aqueous solution was prepared in water with 200 mM ammonium formate and adjusted to pH 3 with formic acid. Mobile phase A (aqueous) was prepared by diluting the stock solution 9:1 in water, and mobile phase B (organic) was prepared by diluting the stock solution 9:1 in acetonitrile (final ionic strength of both mobile phases = 20 mM). The mobile phase B was linearly decreased from 100% to 70% for 11.5 min, and returned to the initial condition after a total of 12 min. A 3 min post-run column re-equilibration at 100% solvent B was added before the next analysis. This resulted in a total cycle time of 15 min per sample. The injection volume for each sample was 1 μL.

2.6. Mass spectrometry

Mass spectrometry was performed on an Agilent 6470 triple quadrupole mass spectrometer. The optimization of the mass spectrometer was divided into two: (i) compound-specific optimization and (ii) source-dependent optimization. Details of the optimization procedure have been published previously (Anumol et al., 2013). The optimized compound parameters and retention times (RT) are shown in Table 1 while source-dependent parameters for electrospray (ESI) positive mode are shown in Table S2.

While the RTs were very stable and did not vary much, for safety so as not to miss any peaks or co-eluents, the mass spectrometer was run in dynamic multiple reaction monitoring (DMRM) mode with a delta RT of 2 min for each compound. Two transitions: a quantifier (most-abundant product) and qualifier were used for most of the compounds to increase specificity of the method. Data acquisition and analysis was performed using the Agilent MassHunter software (version Rev. B.06.00). RT validation and product ion ratio monitoring reduced the possibility of false positives in the method.

The precision of the method was validated by retention time and analyte response variation of the calibration standards, as well as the inter-day and intra-day variation of a cucumber extraction sample, expressed as relative standard deviation (RSD). Retention time and analyte response variation was determined via 15 continuous injections of calibration standards. Intra-day and inter-day variation was determined using a cucumber extraction sample run three times on the same day within 6 h of each other and six times on six different days, respectively.

2.7. Method validation

The proposed LC-MS/MS method for quantitative analysis of underivatized amino acids was evaluated with regards to linearity, limit of detection (LOD) and quantification (LOQ), intraday and interday precisions, stability, accuracy, and matrix effects. Instrumental LOD, LOQ were determined to be the lowest concentration in which the signal to noise ratios (SNR) are 3:1 and 10:1, respectively. A set of standards ranging from blank to 100 ng/mL was analyzed in order to determine LODs and LOQs.

Table 1
Optimized compound-specific parameters and retention times for LC-MS/MS.

Compound	ISTD	Retention time (min)	Precursor ion (*m/z*)	Product ions				
				Quant ion (*m/z*)	Collision energy (V)	Qual ion (*m/z*)	Collision energy (V)	Fragmentor (V)
Amino acids								
Phenylalanine	Isoleucine-$^{15}N_1$	2.95	166.1	120.1	13	103	29	80
Leucine	Isoleucine-$^{15}N_1$	3.38	132.1	86.1	9	30.2	17	75
Tryptophan	Isoleucine-$^{15}N_1$	3.41	205.1	188.0	8	146	20	80
Isoleucine	Isoleucine-$^{15}N_1$	3.75	132.1	86.1	9	44.2	25	75
Methionine	Methionine-d_8	4.22	150.1	104.0	9	56.1	17	75
Valine	Alanine-d_3	4.95	118.1	72.1	9	55.1	25	70
Proline	Alanine-d_3	4.96	116.1	70.1	17	43.2	37	75
Tyrosine	Alanine-d_3	5.01	182.1	136.1	13	91.1	33	85
Cysteine	Alanine-d_3	5.63	122.0	59.1	29	76	13	65
Alanine	Alanine-d_3	6.61	90.1	44.2	9	45.3	40	40
Threonine	Alanine-d_3	6.72	120.1	74.1	9	56.1	17	75
Homoserine	Alanine-d_3	6.91	120.1	74.1	9	56.1	21	70
Glycine	Glycine-d_2	7.00	76.0	30.3	12	NA	NA	35
Glutamine	Glycine-d_2	7.23	147.1	84.1	17	130.1	9	80
Serine	Glycine-d_2	7.26	106.1	88.1	8	42.2	24	67
Asparagine	Glycine-d_2	7.31	133.1	87.1	5	74	17	75
Glutamic acid	Glutamic acid-$^{15}N_1$	7.68	148.1	84.1	17	130	5	75
Citrulline	Glutamic acid-$^{15}N_1$	7.89	176.1	159.1	9	70.1	25	80
Aspartic acid	Aspartic acid-d_3	8.38	134.0	88.1	9	74	13	70
Histidine	Aspartic acid-d_3	9.06	156.1	110.1	13	83.1	29	90
Arginine	Aspartic acid-d_3	9.54	175.1	70.1	24	60.1	12	100
Lysine	Lysine-d_8	10.16	147.1	84.1	17	130.1	9	75
Ornithine	Lysine-d_8	10.28	133.1	116	8	70	20	76
Internal standards								
Isoleucine-$^{15}N_1$		3.75	133.1	87.1	8	NA	NA	75
Methionine-d_8		4.26	158.1	112.1	8	NA	NA	75
Alanine-d_3		6.61	93.1	47.2	12	NA	NA	40
Glycine-d_2		7.00	78.1	32.2	12	NA	NA	40
Glutamic acid-$^{15}N_1$		7.68	149.1	85.1	16	NA	NA	75
Aspartic acid-d_3		8.37	137.1	75.0	16	NA	NA	60
Lysine-d_8		10.16	155.2	92.1	20	NA	NA	80

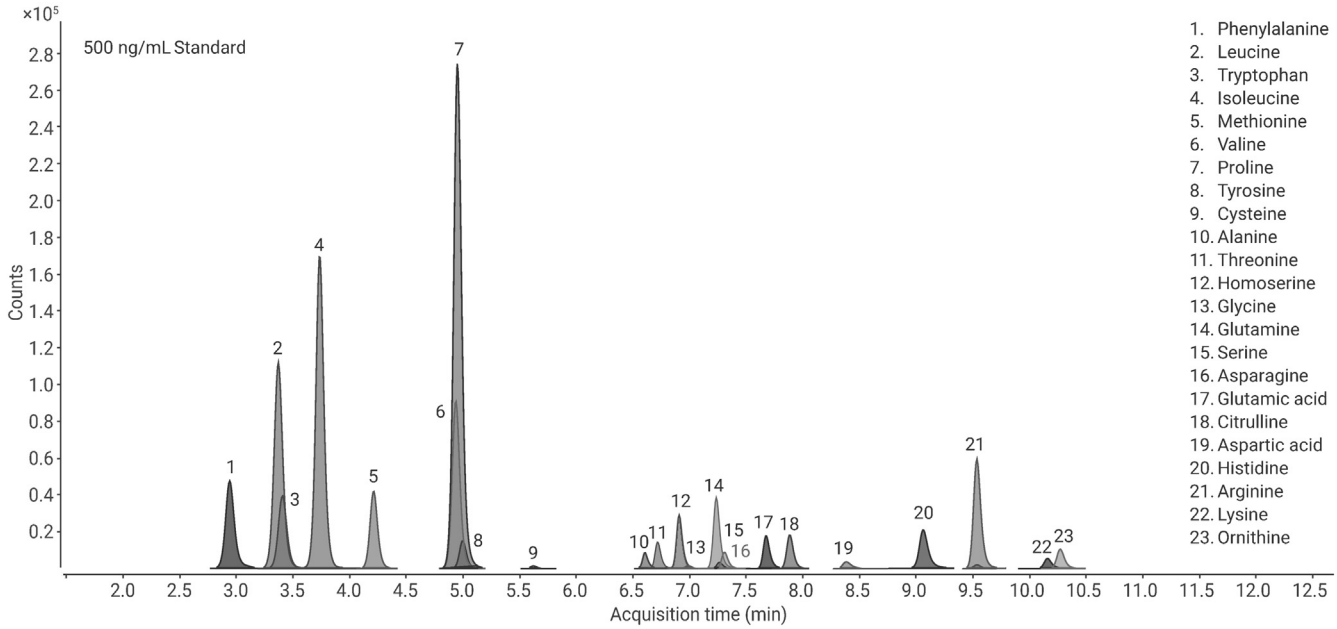

Fig. 1. LC-MS/MS chromatograms of the investigated analytes overlaid and (B) normalized (peak heights scaled to the highest peak in the chromatogram). The peaks are the transitions of the following compounds: 1. Phe; 2. Leu; 3. Trp; 4. Ile; 5. Met; 6. Val; 7. Pro; 8. Tyr; 9. Cys; 10. Ala; 11. Thr; 12. Hse; 13. Gly; 14. Gln; 15. Ser; 16. Asn; 17. Glu; 18. Cit; 19. Asp; 20. His; 21. Arg; 22. Lys; and 23. Orn.

2.8. Data processing and statistical analyses

Data were processed with Agilent MassHunter Workstation Software Quantitative Analysis (Version B.07.01/Build 7.1.524.0). Principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were conducted using MetaboAnalyst 4.0 (Xia and Wishart, 2016).

3. Results and discussion

3.1. LC-MS method development

Separation and identification of the AAs was effective under 11 min (Fig. 1 and Fig. S2), via differences in RT and product ion ratio(s) (Table 2).

The HILIC can retain and separate polar analytes due to the strong hydrophilic interaction between the polar compounds and the hydrophilic polar stationary phase (Jandera, 2011). In this reported method, the new Agilent InfinityLab Poroshell 120 HILIC-Z column with small particle size provides selectivity on the quantitative analysis of amino acids with sharp peaks and short retention time. The current method can separate and identify 23 amino acids within 12 min, providing faster quantitative analysis compared to previous studies (Prinsen et al., 2016; Krumpochova et al., 2015). Notably, baseline separation was also achieved for both the leucine/isoleucine isobars and threonine/homoserine isobars (Fig. S3), which overcome challenges in previous studies (Gokmen et al., 2012). The choice of ammonium formate (20 mM) as a salt additive into the mobile phase with lower pH also helped to further improve the peak shapes in the amino acids analysis (Guo et al., 2013).

3.2. Method validation

The proposed LC-MS/MS method for quantitative analysis of underivatized amino acids was evaluated with regards to linearity, LOD, LOQ, intraday and interday precisions, stability, accuracy, and matrix effects. The results are summarized in Table 2. Isotopically labeled ISTDs were chosen based on RT across the chromatography spectrum to correct the response of the analytes. Seven ISTDs exhibited good representation of different polarities for all 23 amino acids, resulting in good linearity in a wide range of concentrations (50 to 10,000 ng/mL). The coefficient values (R^2) of the calibration curves were higher than 0.9950 for all analytes. Other than serine, LOD ranged from 0.005 to 15 ng/mL, and LOQ ranged from 0.01 to 50 ng/mL (Table 2). Notably, serine exhibited the highest LOD (50 ng/mL) and LOQ (167 ng/mL) in our current method. Serine usually requires pre-column derivatization to improve the LOD and LOQ (Sakamoto et al., 2016; Xie et al., 2014). The current method, a direct quantitative analysis of underivatized serine, had a better LOD when compared to the values reported in previous studies (LOD = 262–436 ng/mL) (Vilches et al., 2017; Nemkov et al., 2015). Thus, the present method is highly sensitive, with detection and quantification of very low concentrations of amino acids.

The results for the repeatability and precision values are presented in Table 2. All 23 analytes in the calibration standards have retention time variability < 0.5%, indicating no peak shifting. The variability in the responses of the calibration standards were < 5% for all target amino acids except tyrosine was slightly higher (RSD = 8.77%). For cucumber leaf tissues extract intra-day variability, most of the analytes were under 5%, except cysteine (8.8) and glutamine (12.1). Similar results were obtained for cucumber leaf tissues extract inter-day response variability: only cysteine (10.8) and glutamine (29.9) showed an RSD higher than 5%, which could be partly due to the fast oxidation of cysteine to cystine and the instability of glutamine in various matrices (Grossie et al., 1993). All other analytes have inter-day RSDs < 5%, indicating good precision and stability.

3.3. Matrix spike and recoveries

Optimization of the extraction of the AAs from leaf tissues required testing various solution matrixes. Different ratios of organic solvent and water (e.g. 75:25 acetonitrile/water, 50:50 acetonitrile/water, 25:75 acetonitrile/water, and water) were employed for the extraction matrix. In addition, different additives, such as HCl (0.01 M, 0.1 M, 0.5 M, 0.7 M, 0.9 M and 1 M) and ammonium formate (20 mM) were added to the extraction matrixes to adjust and control pH and optimize the recovery rates. The recovery rates using different extraction matrixes are presented in the Supporting Information (Table S4). Comparing across

Table 2
Linearity, LOD and LOQ, repeatabilities, precisions and stabilities of target analytes.

Compound name	LOD (ng/mL)	LOQ (ng/mL)	R^2 for calibration curve (50–10,000 ng/mL)	Retention time variability RSD (n = 15) (%)	Calibration standard response variability (n = 15) RSD (%)	Intra-day response variability (n = 3) RSD (%)	Inter-day response variability (n = 6) RSD (%)
Phenylalanine	0.5	1.67	0.9999	0.35	0.99	0.9	4.9
Leucine	0.005	0.02	0.9979	0.43	2.97	0.8	4.1
Tryptophan	0.005	0.02	0.9999	0.24	0.65	1.6	2.6
Isoleucine	0.005	0.02	0.9978	0.37	2.08	0.2	0.4
Methionine	0.05	0.17	0.9996	0.35	0.96	0.4	2.2
Valine	0.4	1.33	0.9981	0.73	1.21	3.2	4.0
Proline	1	3.33	0.9983	0.58	1.04	1.5	3.3
Tyrosine	0.15	0.50	0.9987	0.69	8.77	3.1	4.0
Cysteine	5	16.67	0.9999	0.07	1.25	8.8	10.8
Alanine	0.005	0.02	0.9994	0.00	3.76	0.5	0.5
Threonine	0.4	1.33	0.9987	0.10	1.51	2.6	3.6
Homoserine	0.15	0.50	0.9969	0.00	1.20	3.6	4.3
Glycine	15	50.00	0.9999	0.06	1.82	1.5	3.9
Glutamine	0.5	1.67	0.9993	0.06	0.73	12.1	29.9
Serine	50	166.67	0.9957	0.08	1.08	4.5	4.5
Asparagine	1	3.33	0.9994	0.00	1.38	1.2	1.2
Glutamic acid	0.005	0.02	0.9978	0.00	1.19	0.3	0.6
Citrulline	0.15	0.50	0.9997	0.05	1.29	4.1	3.4
Aspartic acid	0.005	0.02	0.9999	0.13	2.77	0.4	1.3
Histidine	0.25	0.83	0.9961	0.06	0.70	3.6	3.7
Arginine	0.01	0.03	0.9972	0.09	0.68	2.9	3.5
Lysine	0.005	0.02	0.9992	0.06	1.02	1.1	1.9
Ornithine	0.01	0.03	0.9998	0.08	0.63	2.1	2.1

11 different extraction matrixes, water with 0.5 M HCl was chosen as the best extraction solution since it extracted most of the analytes efficiently with high recovery rates. In this matrix, all of the analytes exhibited recovery values within the range of 65–135% at the tested spiking level (Table S4); and notably, 22 of the 23 analytes achieved 80–120% recovery rates. High recovery rates in this extraction matrix (0.5 M aqueous HCl) resulted from higher solubilities of amino acids in acidic aqueous solution compared to organic solvents (e.g., methanol) (Fuchs et al., 2006).

To validate our extraction method, three replicates of frozen cucumber leaf powder (100 mg) were spiked with all target analytes at three levels (20, 40 and 80 μg/g) and then extracted using the optimized extraction method (pre-extraction-spike). As shown in Table 3, the recovery rates were within the required range (80–120%) for all analytes with the exception of cysteine (57–66%) and ornithine (134% in the medium spike of 40 μg/g, and 163% in the high spike of 80 μg/g). However, in the post-extraction-spike study (samples were spiked after extraction at same concentration as pre-extraction-spike, Table S5), a recovery rate of 94–105% was achieved for cysteine. It suggested that the current optimized extraction method was still not optimal for cysteine, although it was the best for cysteine among the 11 extraction methods; there was < 40% recovery of cysteine using the other 10 methods (Table S6). For ornithine, similar higher recovery rates (138% in the medium spike of 40 μg/g, and 160% in the high spike of 80 μg/g) were observed in the post-extraction-spike study (Table S5), while in the lower spike level (20 μg/g), the recovery rates in both pre- and post-extraction spike were < 120%. The high recovery in both pre- and post-spike study of ornithine indicates a matrix effect, and further clean-up after sample extraction may be needed. However, since we aimed to develop a rapid method that can test most amino acids directly after extraction, and the HILIC-Z column provides effective separation of analytes, no additional clean-up steps were developed in this study. Overall, this extraction method is a simple and effective approach for these secondary metabolites, and may be useful for many other metabolites previously semi-quantitatively determined via untargeted metabolomics in previous studies (Zhao et al., 2016c, 2017f; De Vos et al., 2007) (Table 3).

3.4. Response of cucumber plant leaves after exposure to Cu NPs

After exposure to different levels of nCu, we found that the photosynthetic rate and instantaneous water use efficiency of cucumber leaves decreased compared to the control, while the transpiration and stomatal conductance rates tended to increase (Fig. S4), although not all the changes were statistically significant at $p < 0.05$. Cu concentrations in all cucumber tissues, including roots, stems, leaves and fruits, were significantly higher compared to the control ($p < 0.05$) (Fig. S5), which indicates Cu was taken up by the roots and translocated to upper tissue (e.g., the fruits) (Zhao et al., 2016c). The translocation factor (Cu in shoots/Cu in roots) of Cu ions/nCu in the control (0 mg/kg nCu) group was 0.33, but it decreased to 0.21, 0.22 and 0.23 for plants exposed to 200, 400 and 800 mg/kg nCu, respectively. The reduced translocation rate suggests that cucumber plants grown in nCu treated soils may uptake both Cu ions and nCu (Zhao et al., 2016c). Although the biomass of cucumber roots, stem, leaves and fruits decreased after exposure to nCu, the difference was not statistically significantly (Fig. S6).

The optimized method was used to determine the concentration of the 23 amino acids in cucumber plant leaves exposed to nCu and unexposed control samples. All targeted amino acids were detected in the unexposed control group, but the levels of ornithine in cucumber leaf tissues were lower than the LOD (Table 4). In all three (low, medium and high) nCu exposure treatment groups, all the amino acids (except ornithine) exhibited significant change ($p < 0.01$) as compared to the unexposed control group. Some of the amino acids were clearly up-regulated (e.g. tyrosine, citrulline, glutamine) and others were down-regulated (e.g. threonine, methionine, serine) mostly in a dose dependent manner. Compared to the control, tyrosine increased 6.1, 8.2, and 11.0 fold, while serine decreased 10.8, 8.1 and 3.9 fold after exposure to 200, 400 and 800 mg/kg nCu, respectively. In other cases, although there is a significant change in levels, the response was not clearly dose-dependent. Thus, we performed an analysis of the changes in a more comprehensive manner as discussed below.

Unsupervised PCA was performed for the 23 amino acids in control and nCu treated cucumber leaf tissues (Fig. 2A). The first two principal

Table 3
Extraction method validation results (n = 4 for each analyte).

Compound name	Spike level					
	20 μg/g		40 μg/g		80 μg/g	
	% recovery	%RSD	% recovery	%RSD	% recovery	%RSD
Phenylalanine	107 ± 1	0.9	106 ± 2	1.9	102 ± 3	2.9
Leucine	126 ± 1	0.8	122 ± 2	1.6	112 ± 1	0.9
Tryptophan	132 ± 1	0.8	126 ± 3	2.4	116 ± 4	3.4
Isoleucine	102 ± 0	0.0	99 ± 3	3.0	96 ± 1	1.0
Methionine	80 ± 2	2.5	78 ± 3	3.8	78 ± 1	1.3
Valine	124 ± 1	0.8	117 ± 6	5.1	99 ± 7	7.1
Proline	104 ± 1	1.0	105 ± 5	4.8	102 ± 7	6.8
Tyrosine	106 ± 7	6.6	100 ± 5	5.0	91 ± 3	3.3
Cysteine	57 ± 8	14.1	66 ± 2	3.0	61 ± 3	4.9
Alanine	85 ± 4	4.7	89 ± 2	2.2	83 ± 3	3.6
Threonine	107 ± 4	3.7	105 ± 5	4.8	100 ± 6	6.0
Homoserine	102 ± 1	1.0	108 ± 6	5.5	105 ± 5	4.8
Glycine	96 ± 1	1.0	94 ± 2	2.1	89 ± 2	2.3
Glutamine	134 ± 1	0.7	130 ± 3	2.3	119 ± 3	2.5
Serine	107 ± 5	4.7	102 ± 2	2.0	94 ± 2	2.1
Asparagine	99 ± 2	2.0	97 ± 2	2.1	93 ± 3	3.2
Glutamic acid	80 ± 14	17.4	97 ± 1	1.0	88 ± 1	1.1
Citrulline	87 ± 1	1.2	85 ± 1	1.2	84 ± 2	2.4
Aspartic acid	82 ± 4	4.9	92 ± 1	1.1	93 ± 4	4.3
Histidine	119 ± 5	4.2	120 ± 5	4.2	119 ± 6	5.0
Arginine	98 ± 1	1.0	96 ± 2	2.1	91 ± 3	3.3
Lysine	92 ± 2	2.2	96 ± 3	3.1	92 ± 2	2.2
Ornithine	112 ± 2	1.8	134 ± 5	3.7	163 ± 6	3.7

components (PC 1 and PC 2) accounted for > 97% of the variance; PC 1 and PC 2 accounted for 90.6% and 7.2% of the total variance, respectively. The remaining principal components, which had a minor effect on the model, were discarded. PCA analysis revealed a clear separation in amino acid profiles collected from control and the three different levels of nCu treatment.

PLS-DA, a supervised multivariate analytical method, was also applied to determine the separation between groups. From a score plot of PLS-DA (Fig. 2B), the control samples were clearly separated from different levels of nCu treated sample (particularly the group treated with 800 mg/kg nCu) along PC1, which explained 86.3% of the total variability. This indicates that exposure to nCu significantly changed the amino acid profiles in cucumber plants. Using variable importance in projection (VIP) scores, 4 amino acids (lysine, glutamine, alanine and glutamic acid) with VIP > 1were found to be responsible for this separation (data not shown).

Overall, 13 amino acids, including phenylalanine, leucine, tryptophan, isoleucine, valine, tyrosine, cysteine, glycine, glutamine,

Table 4
Change in amino acid levels in cucumber leaf tissues after exposure to nCu.

Compound name	Control		Low		Medium		High	
	Conc. (ng/g leave)	%RSD	Conc. (ng/g leave)	%RSD	Conc. (ng/g leave)	%RSD	Conc. (ng/g leave)	%RSD
Phenylalanine	4776.8 ± 125.1	2.6	6973.1 ± 380.8	5.5	7093.0 ± 495.5	7.0	9737.7 ± 370.6	3.8
Leucine	20,045.3 ± 531.7	2.7	22,940.5 ± 986.1	4.3	22,156.0 ± 1062.6	4.8	31,834.2 ± 863.8	2.7
Tryptophan	7918.7 ± 357.7	4.5	11,345.1 ± 677.3	6.0	8972.4 ± 468.5	5.2	12,211.0 ± 535.0	4.4
Isoleucine	3615.8 ± 125.2	3.5	7965.9 ± 356.7	4.5	6327.1 ± 284.1	4.5	10,080.8 ± 209.3	2.1
Methionine	1378.5 ± 50.1	3.6	561.3 ± 18.6	3.3	613.8 ± 14.2	2.3	753.6 ± 9.4	1.2
Valine	6790.1 ± 331.0	4.9	12,793.9 ± 916.6	7.2	10,135.7 ± 825.4	8.1	15,780.6 ± 815.6	5.2
Proline	3236.3 ± 171.6	5.3	2130.4 ± 159.9	7.5	2790.8 ± 221.8	7.9	4909.2 ± 259.6	5.3
Tyrosine	850.9 ± 21.0	2.5	4380.2 ± 267.4	6.1	5858.9 ± 449.0	7.7	7943.9 ± 350.2	4.4
Cysteine	200.1 ± 16.4	8.2	416.8 ± 32.2	7.7	239.9 ± 23.3	9.7	564.5 ± 45.3	8.0
Alanine	21,486.0 ± 660.3	3.1	20,437.4 ± 948.4	4.6	13,312.8 ± 549.4	4.1	19,349.7 ± 342.2	1.8
Threonine	809.9 ± 56.7	7.0	135.1 ± 7.4	5.4	120.3 ± 11.2	9.3	139.1 ± 6.9	5.0
Homoserine	132.0 ± 16.6	12.6	142.6 ± 11.0	7.7	94.2 ± 6.3	6.7	102.8 ± 6.3	6.1
Glycine	2965.9 ± 87.1	2.9	4593.3 ± 257.5	5.6	3506.8 ± 197.1	5.6	4542.6 ± 147.0	3.2
Glutamine	7860.3 ± 229.6	2.9	15,200.6 ± 686.6	4.5	19,163.9 ± 829.6	4.3	23,116.1 ± 731.4	3.2
Serine	2654.4 ± 138.6	5.2	205.5 ± 36.5	17.8	319.2 ± 26.4	8.3	677.2 ± 51.4	7.6
Asparagine	4340.3 ± 125.5	2.9	2730.7 ± 141.1	5.2	2417.3 ± 104.5	4.3	4215.2 ± 88.5	2.1
Glutamic acid	11,732.7 ± 384.5	3.3	33,985.8 ± 1596.0	4.7	19,140.9 ± 1000.2	5.2	37,048.5 ± 442.6	1.2
Citrulline	505.2 ± 31.8	6.3	2705.9 ± 154.0	5.7	1904.6 ± 114.6	6.0	2998.0 ± 87.7	2.9
Aspartic acid	7073.6 ± 299.9	4.2	14,416.1 ± 623.3	4.3	8128.6 ± 471.6	5.8	20,373.5 ± 826.5	4.1
Histidine	6562.1 ± 127.6	1.9	5526.7 ± 397.5	7.2	4110.9 ± 271.6	6.6	6926.4 ± 270.6	3.9
Arginine	6288.0 ± 119.1	1.9	4551.9 ± 144.9	3.2	4657.1 ± 129.3	2.8	4992.1 ± 56.7	1.1
Lysine	11,414.9 ± 517.1	4.5	8977.7 ± 389.5	4.3	9580.8 ± 545.4	5.7	81,781.6 ± 6179.3	7.6
Ornithine	< 0. 04	N/A[a]	< 0.04	N/A[a]	< 0.04	N/A[a]	< 0.04	N/A[a]

All data are mean ± SD (n = 4). All the data in the low, medium and high exposure group exhibited a $p < 0.01$, as compared to the control.
[a] The concentration of ornithine in cucumber leaf is lower than LOD, and %RSD was reported as N/A.

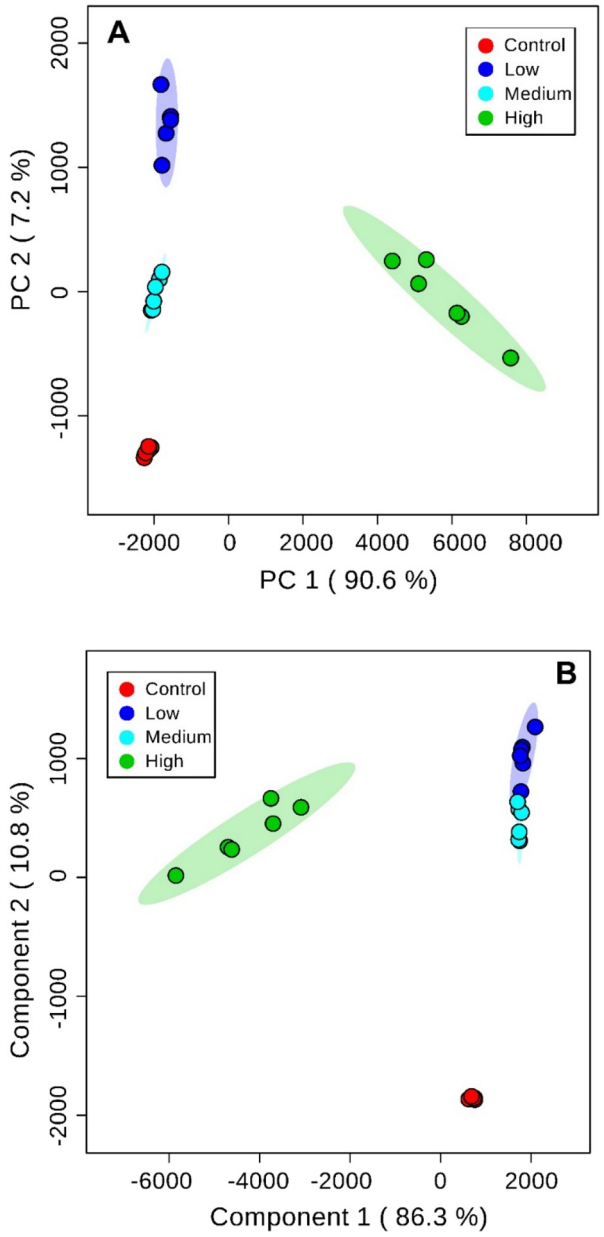

Fig. 2. (A) PCA and (B) PLS-DA score plots of amino acids level in cucumber leaf tissues after exposure to nCu different doses. The shaded area around each data point represents the 95% confidence region.

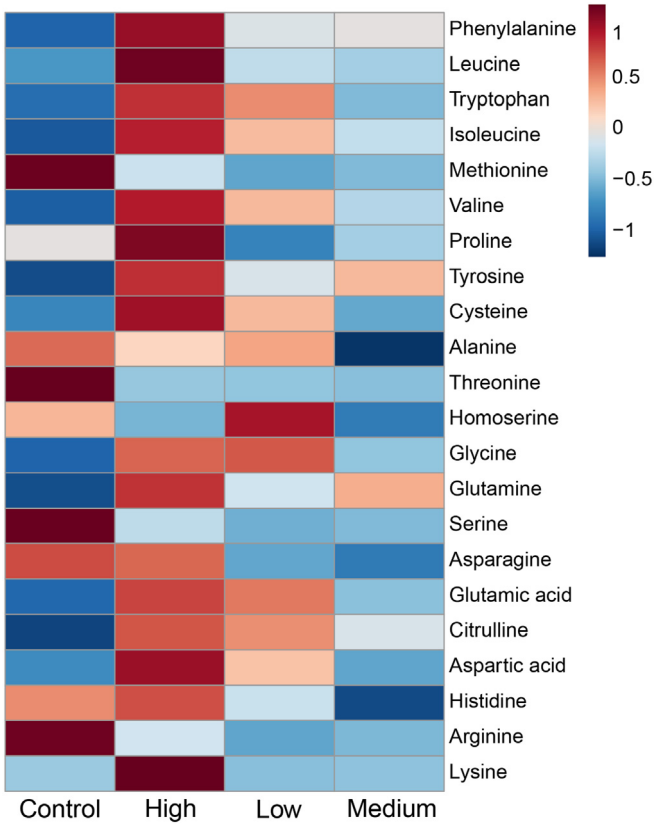

Fig. 3. Heatmap of the changes in amino acid levels (in rows) in cucumber leaf tissues after exposure to different dosage of nCu (in columns). Red colors indicate higher concentrations of amino acids, while blue colors indicate lower concentrations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

glutamic acid, citrulline, aspartic acid and lysine, were generally up-regulated in response to nCu in a dose-dependent way (Fig. 3). The increased levels of amino acids is likely part of the active defense response of the cucumber plant to the stress induced by nCu (Zhao et al., 2016a). For example, the upregulated phenylalanine and tyrosine, precursors of the shikimate-phenylpropanoid biosynthesis pathway, are an indication of the activation of the plant defense and detoxification systems (Rai, 2002). As shown in a previous study (Huang et al., 2017), many amino acids strongly bind with copper ions, which could hinder the translocation of Cu^{2+} inside the plant. The upregulated amino acids can also contribute to chelating Cu^{2+} as well as other metals (e.g. Ni) (Liao et al., 2000; Sharma and Dietz, 2006; Homer et al., 1997; Zhao et al., 2018).

Furthermore, some amino acids (e.g. glutamate) can serve as signaling molecules and have an antioxidant defense function (Sharma and Dietz, 2006). The increased levels of valine and leucine (branched-chain amino acids (BCAA)), may serve as an oxidative phosphorylation energy source during plant stress (Taylor et al., 2004), indicating an adaptation process of cucumber plants to stress induced by nCu.

Change in amino acid levels can also indicate impacts to metabolic pathways. Glutamine is involved in nitrogen metabolism that regulates ammonium assimilation in plants (Forde and Lea, 2007). The increase in glutamine might imply a higher capacity of nitrogen assimilation through the glutamine synthetase pathway (Forde and Lea, 2007). The up-regulation of glutamic acid might be a mechanism to improve the chlorophyll content. The increase in amino acids may also contribute to increased protein synthesis (Less and Galili, 2008). The increased levels in glycine and serine, two essential amino acids formed during photo-respiration (Novitskaya et al., 2002), may reflect an upregulation of this process.

4. Conclusions

The quantitative LC-MS/MS method developed here for the analysis of amino acids in plant tissues is a powerful tool for studying changes induced by exposure to stressors, such as ENMs. The method provides rapid screening and low-level (ng/L) quantification of amino acids, providing a robust quantitative analysis not available from untargeted metabolomics. The reported LC-MS/MS method exhibited high linearity ($R^2 > 0.99$) and reproducibility (0.63–8.77% RSD) with low LOD (0.005–15 ng/mL) and LOQ (0.02–50 ng/mL) for all analytes except serine (LOD = 50 ng/mL and LOQ = 167 ng/mL). We also developed a simple and efficient method to effectively extract most of these 23 amino acids from plant tissues with high recovery rate (80–120%). As a case study, significant changes in the amino acids were detected in cucumber leaf tissues after exposure to nCu, in a dose-dependent way. As part of an active defense to the response of the cucumber plant to the

stress of nCu, a significant amount (13 out 23 amino acids) were up-regulated. The change in amino acid levels can serve to indicate responses in signaling molecules and antioxidant defense metabolites, as well as impacts to metabolic pathways. The quantitative LC-MS/MS method developed here can be widely applied to study oxidative stress response, detoxification mechanisms and nutritional content of plants.

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Appendix A. Supplementary data

Table S1 presents the chemical structures of the amino acids. Table S2 provides the optimized source parameters. Table S3 contains the characteristics of soil. Fig. S1 shows the SEM and TEM image of nCu. Fig. S2 displays the LC-MS/MS chromatograms of the investigated analytes normalized. Fig. S3 presents the spectrum separation of isobars. Table S4 shows the extraction recovery rates. Figs. S4–S6 present the photosynthesis activity, biomass accumulation and Cu accumulation in cucumber plant tissues after exposure to nCu. Supplementary data to this article can be found online at https://doi.org/10.1016/j.impact.2018.08.008.

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