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Antioxidant response of cucumber (*Cucumis sativus*) exposed to nano copper pesticide: Quantitative determination via LC-MS/MS

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ABSTRACT

Targeted metabolomics aims to provide a new approach to investigate metabolites and gather both qualitative and quantitative information. We describe a protocol for extraction and analysis of plant metabolites, specifically 13 secondary metabolites (antioxidants) using liquid chromatography coupled to triple quadrupole mass spectrometry (LC-MS/MS), with high linearity (R2 > 0.99) and reproducibility (0.23–6.23 R%) with low limits of detection (> 0.001 ng/mL) and quantification (> 0.2 ng/mL). The protocol was applied to study the antioxidant response of cucumber plants exposed to nanocopper pesticide. Dose-dependent changes in antioxidant concentrations were found, and 10 antioxidants were significantly consumed to scavenge reactive oxygen species, protecting plants from damage. Levels of three antioxidants were up-regulated, as a response to the depletion of the other antioxidants, signaling activation of the defense system. We demonstrated that the reported LC-MS/MS method provides a quantitative analysis of antioxidants in plant tissues, for example to investigate interactions between plants and nanomaterials.

1. Introduction

Antioxidants in plant cells and tissue play an important role in the response to environmental stressors that induce reactive oxygen species (ROS); these compounds are generally present in relatively low concentrations but contribute to inhibiting or delaying the oxidation of substrates (Matkowski, 2008). In plants, phenolic acids (e.g. flavonoids such as ferulic, caffeic, p-coumaric, and vanillic acids) and vitamins (e.g. α-tocopherol) are important low molecular weight antioxidants, which play an important role in the defense to hierarchical oxidative stress (Blokhina, Virolainen, & Fagerstedt, 2003). Some antioxidants (e.g. α-tocopherol) act as chain-breaking inhibitors of lipid peroxidation when ROS are generated *in vivo*, and interfere with free radical propagation cascades (Salah et al., 1995). Previous studies have semi-quantitatively demonstrated that ROS stress influences the levels of antioxidant in various plants (Soria, Montes, Bisson, Atilla-Gokcumen, & Aga, 2017; Zhao, Huang, Adeleye, & Keller, 2017; Zhao, Ortiz, et al., 2016).

The rapid development of nanotechnology in the past decade has resulted in increased consideration and use of nanoscale fertilizers and pesticides in agriculture (Khot, Sankaran, Maja, Ehsani, & Schuster, 2012). In particular copper-containing nanopesticides (Cu NPs) are being introduced to the market due to their excellent antimicrobial and antifungal properties (Keller et al., 2017). However, there is an increasing concern on the environmental fate, bioavailability and toxicity of engineered nanomaterials (ENMs) to terrestrial plants (Kah & Hofmann, 2014). Some metallic ENMs (e.g. Cu NPs) and/or released ions (e.g. Cu^{2+}) can induce the formation of ROS within plant cells, and their over-accumulation in plants can result in oxidative damage of membrane lipids, proteins, and nucleic acids (Gill & Tuteja, 2010). A number of studies have shown, via indirect methods such as antioxidant assays, that Cu NPs can induce oxidative stress in various crop species such as alfalfa (Hong et al., 2015), barley (Shaw et al., 2014), cilantro (Zuverza-Mena et al., 2015), chickpeas and soybeans (Adhikari, Kundu, Biswas, Tarafdar, & Rao, 2012), mung beans and wheat (W. M. Lee, An, Yoon, & Kweon, 2008), radish (Atha et al., 2012), carrot (Ebbs et al., 2016) and corn (Zhao, Hu, Huang, & Keller, 2017). As a result, the metabolic pathways may be up- or down-regulated due to exposure to these ENM stressors (Hasler-Sheetal, Castorani, Glud, Canfield, & Holmer, 2016). Low molecular weight metabolites are the end products

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of cellular regulatory processes, and their levels reflect the ultimate response of biological systems to environmental changes (Fiehn, 2002). Monitoring the changes in concentrations of low molecular mass metabolites can provide a more holistic view of plant response to these environmental stressors.

Untargeted metabolomics can be used to perform a rapid screening of the metabolites that are over- or under-expressed due to a change in conditions. It generates a large list of metabolites that are significantly affected by a stressor. For example, recently we applied untargeted gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) and ^{1}H nuclear magnetic resonance (NMR)-based metabolomics to detect and evaluate the responses induced by various Cu NPs (i.e. nano-Cu, nano-CuO and nano-$Cu(OH)_2$) on various crop plants (i.e. cucumber, lettuce, spinach and corn) (Zhao, Hu, Huang, Fulton, et al., 2017; Zhao, Huang, et al., 2017; Zhao, Huang, Hannah-Bick, Fulton, & Keller, 2016; Zhao, Ortiz, et al., 2016). In some instances, up to 357 unique metabolites were detected via GC-TOF-MS, and around 150 metabolites were identified on the basis of their mass spectral fingerprints and retention-index matches. Of these, levels of around 30 were significantly ($p < 0.05$) altered (Zhao, Huang, et al., 2017), including several anti-oxidants. Other non-targeted metabolomics studies also indicated that anti-oxidants are almost always affected by exposure to these ENMs (Zhao, Hu, Huang, Fulton, et al., 2017; Zhao, Huang, et al., 2017; Zhao, Huang, Hannah-Bick, et al., 2016; Zhao, Ortiz, et al., 2016). 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. Thus, there is a need for a quantitative determination of the changes using rigorous quantitation methods.

Based on the previous untargeted metabolomics studies, levels of several antioxidants (e.g. ferulic acid, α-tocopherol) showed significant changes in cucumber plants exposed to Cu NPs (Gill & Tuteja, 2010; Zhao, Huang, et al., 2017; Zhao, Huang, Hannah-Bick, et al., 2016; Zhao, Ortiz, et al., 2016). Thus, 13 compounds that are highly related to the anti-oxidative defense of these crop plants were chosen as analytes (Table S1) to be evaluated in this study. Since most of the analytes possess relatively high polarities, liquid chromatography was considered as a separation method. LC–MS/MS can provide high sensitivity to analyze many metabolites and generally requires less sample pretreatment (Junot, Fenaille, Colsch, & Becher, 2014). Thus, LC-MS/MS based mass spectrometric technique may be used as a quantitative environmental metabolomics platform.

2. Materials and methods

2.1. Chemicals and reagents

All analytical standards used during the study were at least > 96% purity and every effort was made to use standards of the highest purity commercially available. Most chemicals were purchased from Sigma-Aldrich (St. Louis, MO), including: p-coumaric acid (purity ≥ 98%), caffeic acid (≥98%), benzoic acid (99.5%), 2-hydroxycinnamic acid, predominantly trans (97%), l-glutathione reduced (≥98.0%); curcumin (≥98%), α-d-glucose 1-phosphate disodium salt hydrate (≥98%), and (±)-α-tocopherol (≥96%)). Vanillic acid (98%) was obtained from Alfa Aesar (Ward Hill, MA), gallic acid hydrate (> 98%) from TCI Chemicals (Japan), ferulic acid (99.6%) from MP Biomedicals (Solon, OH) and 4-(trifluoromethyl)cinnamic acid (98%) from Matrix scientific (Columbia, SC) (Table S1). Isotopically-labeled internal standards (benzoic acid-^{13}C, 99%) and ferulic acid-$^{13}C_3$, 99%) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Methanol, acetonitrile (ACN), isopropyl alcohol (IPA) and LC-MS grade water were purchased from Burdick and Jackson (Muskegon, MI), while formic acid (LC-MS grade), and ammonium acetate (LC-MS grade) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Characteristics of nCu

Uncoated nCu (U.S. Research Nanomaterials) was employed here; a detailed characterization was presented in previous studies (Adeleye, Conway, Perez, Rutten, & Keller, 2014). Briefly, the primary particle size is 40 nm and the hydrodynamic diameter (HDD) is 2590 ± 1138 nm in deionized (DI, Barnstead nanopure) water at pH 7 (0.5 mM phosphate buffer), at similar concentrations as in this study. 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 −29.4 ± 0.8 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 (Sedgwick soil). The final concentration of nCu in soil (mg/kg) was 0 (Control), 400 (low) and 800 (high). This total Cu concentration is within the range predicted for biosolids applied to soils or due to the application of copper-based nanopesticides. 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 for 60 days in the greenhouse at a controlled temperature of 25.5–30.0 °C during the day and 17.7–18.9 °C at night.

2.4. Extraction of antioxidants

At harvest, the fresh cucumber leaves were immediately placed in liquid nitrogen for rapid freezing. The frozen tissues were homogenized in liquid nitrogen into a fine powder using a pestle and mortar, and then stored at −85 °C in a freezer (VIP Series Ultra-Low Temperature Freezer, Sanyo Scientific, Bensenville, IL). For the extractions, 100 mg of frozen cucumber leaf powder was weighted into 2 mL Eppendorf microcentrifuge tubes, and then a 1 mL extraction solution (80:20 methanol and water with 2% formic acid) was added. The microcentrifuge tubes were vortexed at 3000 rpm for 30 min, and then sonicated in a 25 °C water bath for 30 min. A final centrifugation at 20,000*g* was done for 20 min. The supernatant (0.5 mL) was used for LC-MS/MS analysis.

To determine the recovery of antioxidants during the extraction, two levels of mixed antioxidants standards (50 and 100 ng/mL) were spiked into cucumber leaves tissues samples before and after the extraction process, to obtain pre- and post-extraction spike recovery, respectively. The recovery was calculated using:

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

where $C_{observed}$ is the concentration of pre- or post-extraction spiked sample with mix antioxidants standards; C_{neat} is the concentration of non-spiked (control) sample; $C_{expected}$ is the concentration that was spiked into samples.

2.5. Liquid chromatography

An Agilent 1260 UHPLC binary pump was used to perform liquid chromatography for all analyses. An Agilent ZORBAX StableBond 80 Å C18 (4.6 mm × 50 mm, 3.5 μm) column was used for chromatographic separation of all analytes. The column was maintained at 30 °C throughout the run. A dual eluent mobile phase comprised of water with 0.1% formic acid and 5 mM ammonia formate (A) and methanol (B) at 500 μL/min was used for separation. The mobile phase was held at 2% solvent B for 2 min. At 2 min, solvent B was linearly increased to 100% for 4 min. This gradient was held for 3 min, before returning to the initial condition after a total of 10 min. A 4.5 min post-run column

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

Compound Name	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator Voltage (V)	Retention Time (min)	ESI Polarity
2-hydroxycinnamic acid	163	117.1	81	28	4	7.14	Negative
4-(trifuoromethyl) cinnamic acid	215	171.1	87	12	4	8.08	Negative
	215	151.1	87	20	4	8.08	Negative
α-tocopherol	431.4	165.1	142	24	4	11.35	Positive
	431.4	69.1	142	40	4	11.35	Positive
	431.4	57.2	142	28	4	11.35	Positive
benzoic acid	121	77.1	77	12	4	7.21	Negative
caffeic acid	179	135.1	94	16	4	6.32	Negative
chlorogenic acid	353.1	191.1	102	16	4	6.18	Negative
curcumin	367.1	217.1	112	8	4	8.18	Negative
	367.1	149.1	112	16	4	8.18	Negative
	367.1	134	112	40	4	8.18	Negative
ferulic acid	193.1	134.1	87	16	4	6.8	Negative
	193.1	134.1	87	16	4	6.8	Negative
gallic acid hydrate	169	125.1	97	16	4	3.99	Negative
reduced glutathione	308.1	162	91	16	4	1.25	Positive
	308.1	162	91	16	4	1.25	Positive
	308.1	76.1	91	28	4	1.25	Positive
L-dehydroascorbic acid	173	158.1	174	12	4	8.17	Negative
p-coumaric acid	163	119.1	81	16	4	6.74	Negative
	163	93.1	81	40	4	6.74	Negative
vanillic acid	167	152.1	82	12	4	6.35	Negative
	167	108	82	20	4	6.35	Negative

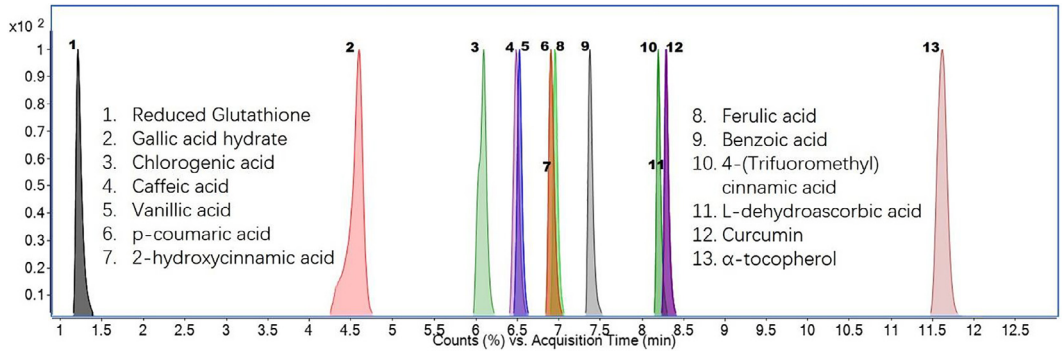

Fig. 1. Overlaid and normalized LC-MS/MS chromatograms of the investigated analytes. The peaks are the transitions of the following compounds: 1. reduced glutathione; 2. gallic acid; hydrate; 3. chlorogenic acid; 4. caffeic acid; 5. vanillic acid; 6. p-coumaric acid; 7. 2-hydroxycinnamic acid; 8. ferulic acid; 9. benzoic acid; 10. 4-(trifuoromethyl) cinnamic acid; 11. L-dehydroascorbic acid; 12. curcumin and 13. α-tocopherol. The peak heights are scaled to the highest in the chromatogram, and there was an overlap between Peak 6 and Peak 7 as well as Peak 10 and Peak 11.

re-equilibration at 2% solvent B was added before the next analysis. This resulted in a total cycle time of 14.5 min per sample.

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. The optimized compound parameters and retention times (RT) are shown in Table 1 while source-dependent parameters for both electrospray (ESI) positive and negative modes (run simultaneously) are shown in Table S2.

The mass spectrometer was run in dynamic multiple reaction monitoring (DMRM) mode with a delta RT of 0.8 min for each compound. Fast polarity switching allowed for simultaneous analysis in ESI positive and negative in the same run. Generally, two transitions were used for most of the compounds to increase specificity of the method: a quantifier (most-abundant product) and qualifier; however, for some compounds only one transition was identified (Table 1). Data acquisition and analysis was performed using the Agilent MassHunter software (version Rev B.06.00). RT locking and product ion ratio monitoring reduced the possibility of false positives in the method.

2.7. Determination of LOD, LOQ and MRL

Instrumental limit of detection (LOD) and quantification (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 0.001 to 1000 ng/mL was analyzed in order to determine LODs and LOQs. To determine the method reporting limits (MRLs), eight injections of cucumber leaf extraction samples spiked with target analytes standards at 2–3 times the estimated LOQs were analyzed. The standard deviation of the eight replicates was multiplied by the student's *t*-test value for $n-1$ degrees of freedom at 99% confidence levels

to determine the MRL of each target compound.

3. Results and discussion

3.1. Method development

Separation and identification of the antioxidants was effective (Table 1, Figs. 1 and S5), via differences in RT and product ion(s) ratio (s).

Optimization of the extraction of the antioxidants from leaf tissues required testing various solution matrixes. Different ratios of organic solvent and water (e.g. 100:0 methanol/water, 80:20 methanol/water, 50:50 methanol/water, 100:0 acetonitrile/water, 80:20 acetonitrile/water, 50:50 acetonitrile/water, and 80:20 acetone/water) were employed for the extraction matrix. In addition, different additives, such as formic acid (0.1%, 1% or 2%) and ammonium formate (5 mM) were added to the extraction matrixes to adjust and control pH to optimize the recovery. The recovery using different extraction matrixes are presented in the Supporting Information (Table S3). Comparing across 11 different extraction matrixes, 80:20 methanol and water with 2% formic acid was chosen as the best extraction solution since it extracted most of the analytes efficiently with high recovery. In this matrix, 12 of the 13 analytes exhibited recovery values within the range of 65–120% at the tested spiking level (discussed in more detail below). Reduced glutathione has the highest polarity, and showed low pre-extraction recovery, with a maximum of 17.9%. However, in the post-extraction spike study, a recovery of 87% was achieved for reduced glutathione, indicating that most of the losses occur during sample preparation, maybe due to the high polarity of reduced glutathione and its low solubility in organic solvent (e.g. methanol). The observed recovery result from a combination of losses during sample preparation (e.g. tissue extraction) and of matrix effects in the LC-MS/MS measurements. The levels for reduced glutathione in the real plant samples though were very high and allowed for easy quantification with the lower recovery too.

The only available isotopically labeled internal standards on the market are benzoic acid-^{13}C and ferulic acid-$^{13}C_3$. However, they are not “ideal” internal standards for the current method since they exhibit somewhat limited recovery (~40–50%) and may experience sample preparation losses and matrix effects. Instead, to correct the potential losses during the extraction and matrix effects, matrix-matched standards were prepared.

3.2. Determination of LOD, LOQ and MRL

Other than α-tocopherol, LOD ranged from 0.001 to 6 ng/mL, and LOQ ranged from 0.01 to 1.7 ng/mL (Table 2). Notably, α-tocopherol exhibited the highest LOD (500 ng/mL) and LOQ (1665 ng/mL), but since α-tocopherol usually exists in high abundance in plant tissues it was not a concern in these studies. Overall, the present method can be considered as highly sensitive, with detection and quantification of very low concentrations of antioxidants in plant tissues.

MRLs for most of the analytes ranged from 0.007 ng/mL to 15 ng/mL, with the exception of ferulic acid and α-tocopherol (Table 2). Given the high abundance of ferulic acid and α-tocopherol in cucumber leaves, their MRLs could not be determined accurately but we estimate based on abundance and signal to noise that they would be < 1 ng/mL.

3.3. Matrix spike and recoveries

Four replicates of blank cucumber leaf extraction samples were spiked with all target analytes at two levels (50 ng/mL and 100 ng/L). As shown in Table 3, the recovery were within the required range (60–120%) for all analytes with the exception of reduced glutathione (17.91% in the low spike of 50 ng/mL, and 20.86% in the high spike of 100 ng/mL), which was partially lost during sample preparation due to its high polarity. The R% for both spiking levels in cucumber leaf tissue extraction was < 10% for 12 out of 13 target analytes (including reduced gluthathione even with its low recovery) with most compounds having an R% < 5%, indicating very good reproducibility of the current extraction approach. α-tocopherol exhibited high R% in both spiking levels (~15%), due to the relatively high concentration of α-tocopherol present in cucumber leaves. 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 our previous studies (Zhao, Hu, Huang, Wang, et al., 2017; Zhao, Huang, Zhou, et al., 2016).

Table 2
LOD, LOQ and MRL for all analytes.

Compound Name	LOD (ng/mL)	LOQ (ng/mL)	MRL (ng/mL)
2-Hydroxycinnamic acid	0.005	0.02	15
4-(Trifluoromethyl) cinnamic acid	0.001	0.003	0.17
α-Tocopherol	500	1665	N/A[a]
Benzoic acid	0.01	0.03	2.5
Caffeic acid	0.5	1.7	0.93
Chlorogenic acid	0.005	0.02	0.010
Curcumin	0.004	0.01	0.007
Ferulic acid	0.1	0.3	N/A[a]
Gallic acid hydrate	0.05	0.2	0.031
Glutathione reduced	0.05	0.2	4.1
L-Dehydroascorbic acid	0.01	0.033	0.73
p-Coumaric acid	0.4	1.3	5.1
Vanillic acid	0.6	2	3.7

[a] Given the high abundance of ferulic acid and α-tocopherol in cucumber leaves, their MRLs could not be determined accurately, reported as N/A.

Table 3
Matrix recovery rates (n = 4 for each analyte).

Compound Name	50 ng/mL		100 ng/mL	
	Recovery %	RSD%	Recovery %	RSD%
2-hydroxycinnamic acid	92.0	5.6	84.6	6.0
4-(trifuoromethyl) cinnamic acid	76.8	6.1	75.0	2.0
α-tocopherol	62.1	14.7	65.5	15.4
benzoic acid	78.2	2.7	74.3	5.4
caffeic acid	64.7	7.1	68.5	7.7
chlorogenic acid	67.5	2.9	66.1	6.3
curcumin	81.3	1.9	70.8	4.7
ferulic acid	70.4	2.9	87.9	5.4
gallic acid hydrate	64.8	2.1	73.9	6.9
reduced glutathione	17.9	0.88	20.9	5.8
L-dehydroascorbic acid	72.1	1.8	69.8	5.1
p-coumaric acid	73.6	3.4	72.0	2.5
vanillic acid	79.5	3.4	77.9	3.1

3.4. Linearity and precision

Linearity for each calibration curve was evaluated via the correlation coefficient (R^2). The calibration curve started at (or just above) the LOQ of each analyte and ended at 2000 μg/L. All analytes had an $R^2 > 0.99$ (Table 4), with an 11 point calibration curve with linear fitting. The precision of the method was validated by inter-day and intra-day variation of a cucumber extraction sample spiked with 1 μg/mL analyte standards, expressed as relative standard deviation (R%). Intra-day variation was determined using a cucumber extraction sample spiked with 1 μg/mL analyte standards run four times on the same day within 3 h of each other. Inter-day variation was determined using a cucumber extraction sample spiked with 1 μg/mL analyte standards run four times on five different days. The results for the linearity and precision values are presented in Table 4. All 13 analytes have inter-day variability < 7% with 11 having R%s < 5%. α-tocopherol and reduced

Table 4
Linearity and precision values for target analytes.

Compound Name	Linearity	Inter-day variability (n = 4) R%	Intra-day variability (n = 5) R%
2-hydroxycinnamic acid	0.99	4.62	2.97
4-(trifuoromethyl)cinnamic acid	0.99	0.52	0.40
α-Tocopherol	0.99	6.23	16.38
benzoic acid	0.99	1.19	0.64
caffeic acid	0.99	1.30	4.12
chlorogenic acid	0.99	0.97	6.18
curcumin	0.99	0.99	4.26
ferulic acid	0.99	0.77	1.79
gallic acid hydrate	0.99	2.37	1.26
reduced glutathione	0.99	6.17	18.61
L-dehydroascorbic acid	0.99	0.80	4.04
p-coumaric acid	0.99	0.23	2.45
vanillic acid	0.99	0.38	0.41

glutathione had R%s of 6.23 and 6.17% respectively. For the intra-day variability, most of the analytes were under 5%, expect the α-tocopherol (16.38%), chlorogenic acid (6.18%) and glutathione reduced (18.61%), which could in part be due to more rapid oxidation of these antioxidants when exposed to air over time. Since all analytes had inter-day R%'s < 10%, the precision is acceptable.

3.5. Analysis of changes antioxidant levels in cucumber plants exposed to Cu NPs

The optimized method was used to determine the concentration of the 13 antioxidants in cucumber plant leaves exposed to nCu. All targeted antioxidants were detected in the unexposed control group, but the levels of p-coumaric acid in cucumber leaf tissues was lower than the LOD.

After exposure to different levels of nCu, it was observed that 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. S2). Cu concentrations in all cucumber tissues, including root, stem, leaf and fruit, were higher compared to control ($p < 0.05$) (Fig. S3), which indicates Cu is taken up by the roots and translocated even to the fruits (Zhao, Huang, Hannah-Bick, et al., 2016; Zhao, Huang, Zhou, et al., 2016). The biomass of cucumber root, stem, leaf and fruit also showed obvious decrease after exposure to nCu (Fig. S4).

The cucumber leaf tissue concentrations of 10 of the 13 targeted antioxidants decreased, while leaf tissue concentrations of benzoic acid, gallic acid hydrate and p-coumaric acid were up-regulated compared to the control, statistically (Table 5). In particular levels of p-coumaric acid and benzoic acid showed very significant increases, while α-tocopherol, reduced glutathione and vanillic acid decreased considerably. However, not all changes were dose-dependent, indicating that a maximum or minimum level may have already been reached even at the lower dose (400 mg/kg nCu).

Benzoic acid plays an important role in the antioxidative defense system of plants, as it contributes to scavenging H_2O_2 as well as neutralizing free radicals (S.-H. Lee et al., 2007); the up-regulated benzoic acid (~80% increment in concentration) in exposed cucumber leaf tissues indicates the activation of the defense system (Zhao, Huang, Hannah-Bick, et al., 2016; Zhao, Huang, Zhou, et al., 2016). The concentration of gallic acid, a derivative of benzoic acid, also increased up to 3-fold after exposure to nCu, and the increment was dose dependent. Surprisingly, even though the level of p-coumaric acid (4-hydroxycinnamic acid) was below LOD (0.4 ng/g fresh leaf) in the control, it increased to 680–900 ng/g, indicating that p-coumaric acid is a key part of the oxidative defense system of cucumber plants. These phenolic acids can inhibit the synthesis of or scavenge ROS in plant cells under stress to protect plants from damage (Dixon et al., 2002). p-coumaric acid is involved in the shikimate-phenylpropanoid metabolic pathway, suggesting this pathway was up-regulated, which is a sign of the activation of the defense system (Zhao, Hu, Huang, & Keller, et al., 2017). The plant may up-regulate these antioxidants (benzoic acid, gallic acid and p-coumaric acid) once the other antioxidants reach a minimum level, but more work is needed to confirm this hypothesis.

Five derivatives of cinnamic acid (2-hydroxycinnamic acid, 4-(trifuoromethyl)cinnamic acid, caffeic acid, ferulic acid and chlorogenic acid) significantly decreased in plants exposed to nCu. These polyphenols are beneficial to plants due to their radical scavenging properties (Thavasi, Leong, & Bettens, 2006). The hydroxyl groups on the polyphenols can donate hydrogen to react with reactive oxygen and reactive nitrogen species, which would terminate the radicals generation cycle. Thus, the decreased concentrations of cinnamic acid derivatives are the initial response of the plants to ROS induced by excess Cu content. Furthermore, these polyphenols, for example, ferulic acid, participate in the phenylpropanoid pathway biosynthesis (Hura et al., 2007). The decreased levels of the antioxidants in these tissues can lower their nutritional benefits (Boeing et al., 2012; Cai, Luo, Sun, & Corke, 2004; Chong, Macdonald, & Lovegrove, 2010).

4. Conclusions

The quantitative LC-MS/MS method developed here for the analysis of antioxidants 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 antioxidants, providing a robust quantitative analysis not available from untargeted metabolomics. The reported LC-MS/MS method exhibited high linearity

Table 5
Change in antioxidant levels in cucumber leaf tissues after exposure to nCu.

Compound Name	Control (ng/g fresh leaf)	Exposed to 400 mg/kg nCu (ng/g fresh leaf)	Exposed to 800 mg/kg nCu (ng/g fresh leaf)
2-hydroxycinnamic acid	5.1 ± 0.3	< 0.05	1.0 ± 0.3**
4-(trifuoromethyl)cinnamic acid	19.1 ± 4.4	5.6 ± 1.1*	5.3 ± 1.7*
α-tocopherol	122,500 ± 4384	86,810 ± 1,238**	102,800 ± 4638**
benzoic acid	211.7 ± 41.9	378.7 ± 25.1**	361.8 ± 25.8**
caffeic acid	48.6 ± 7.2	19.1 ± 1.3**	41.2 ± 8.2
chlorogenic acid	15.3 ± 1.8	4.1 ± 0.2**	4.1 ± 0.2**
curcumin	52.8 ± 5.2	8.4 ± 1.9**	5.2 ± 0.5**
ferulic acid	6,876 ± 129.6	4,018 ± 309.4**	5,966 ± 502.1
gallic acid hydrate	23.7 ± 2.5	31.0 ± 2.4**	63.1 ± 5.8**
reduced glutathione	4,511 ± 169.6	3,521 ± 56.0**	3,790 ± 86.7**
L-dehydroascorbic acid	36.0 ± 4.0	5.3 ± 0.7**	3.5 ± 0.6**
p-coumaric acid	< 4	900.0 + 91.9**	682.1 ± 77.6
vanillic acid	223.2 ± 8.3	149.0 ± 8.7**	125.3 ± 8.4**

All data are mean ± SD (n = 4). *P < 0.05, **P < 0.01, as compared to the control.

(R^2 > 0.99) and reproducibility (0.23–6.23 R%) with low LOD (≥0.001 ng/mL) and LOQ (≥0.2 ng/mL). As a case study, significant changes in the antioxidants were detected in cucumber leaf tissues after exposure to nCu. Due to the overproduction of ROS induced by excess Cu content in plant cells, a significant amount of 10 of the 13 antioxidants was consumed to scavenge or inhibit the synthesis of ROS to protect plants organism from damage. Levels of benzoic acid, gallic acid hydrate and p-coumaric acid were up-regulated, as a response to the depletion of the other antioxidants, signaling activation of the defense system. This quantitative LC-MS/MS method can be widely applied to study oxidative stress response, detoxification mechanisms and nutritional content of plants subjected to stressors such as ENMs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.07.069.

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