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H-1 NMR and GC-MS based metabolomics reveal nano-Cu altered cucumber (*Cucumis sativus*) fruit nutritional supply

### Permalink

<https://escholarship.org/uc/item/5c74m8qf>

### Journal

PLANT PHYSIOLOGY AND BIOCHEMISTRY, 110

### ISSN

0981-9428

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### Publication Date

2017

### DOI

10.1016/j.plaphy.2016.02.010

Peer reviewed



Contents lists available at ScienceDirect

## Plant Physiology and Biochemistry

journal homepage: [www.elsevier.com/locate/plaphy](http://www.elsevier.com/locate/plaphy)

## Research article

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## ARTICLE INFO

## Article history:

Received 16 December 2015

Received in revised form

6 February 2016

Accepted 7 February 2016

Available online xxx

## Keywords:

Nano-Cu

Cucumber

Metabolomics

NMR

GC–MS

## ABSTRACT

It is imperative to study the interaction of nanoparticles residuals with crop plants in agricultural soils, due to the increased application of nanotechnology in agriculture. So far, a few studies have focused on the impact of nanoparticles on fruit quality and nutritional supply. In this work, a thorough and comprehensive analysis of metabolite changes of cucumber fruits from plants under nano-Cu stress was possible through the use of both <sup>1</sup>H NMR and GC–MS. The results of supervised partial least-squares discriminant analysis from both platforms showed that cucumber fruit extracts samples were clearly grouped based on the nano-Cu level in soil. This indicates that the fruit metabolite profile was influenced by exposure to nano-Cu. GC–MS data showed concentrations of some sugars, organic acids, amino acids, and fatty acids were increased or decreased by nano-Cu. Several metabolites, such as methyl-nicotinamide (MNA), trigonelline, imidazole, quinoline were only detected and quantified by <sup>1</sup>H NMR. Our results showed that combining the two platforms provided a comprehensive understanding about the metabolites (nutrient supply) changes in cucumber fruits impacted by exposure to nano-Cu.

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## 1. Introduction

The implications of nanoparticles (NPs) on agriculture are receiving more and more attention, particularly with the increasing production and use of nanoparticle-based pesticides and fertilizers. Although a number of publications have begun to explore the interaction between NPs and plants (Rico et al., 2011; Deng et al., 2014; Ma et al., 2010; Priester et al., 2012; Majumdar et al., 2015; Musante and White, 2012), the knowledge of the implications of NPs on the nutritional value of crop fruit is still limited. Rico et al. showed that nCeO<sub>2</sub> decreased some of the nutrients in rice grains, including Fe, S, prolamin, glutelin, lauric and valeric acids, and starch. Moreover, nCeO<sub>2</sub> decreased the antioxidant properties of the rice grains (Rico et al., 2013). Our previous study (Zhao et al., 2014) also showed that ZnO NPs and CeO<sub>2</sub> NPs at certain doses induced sugar, starch, and protein content alterations in cucumber

fruits. Majumdar et al. (2015) reported that phaseolin and lectins in bean (*Phaseolus vulgaris*) seed, which are associated with nutrient storage and carbohydrate metabolism, were down-regulated in a dose dependent way due to exposure of the plants to nCeO<sub>2</sub>. However, those studies analyzed a limited number of target metabolites. A non-targeted metabolites profiling analysis would be a more powerful approach to characterize the changes in metabolites, and provide a deep insight into the response of complex biological systems to stress (Fiehn et al., 2000).

So far, the most commonly used analytical techniques for metabolomics studies are nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC–MS), and liquid chromatography-mass spectrometry (LC–MS). The NMR technique is a popular analytical platform for metabolite profiling due to simple sample preparation, which minimizes the change in the chemical composition and the loss of minor components during sample preparation (Koda et al., 2012). In addition, various metabolites can be detected simultaneously by NMR. However, NMR is generally considered to be less sensitive than GC–MS, and difficult to assign the output signal to specific compounds due to resonance

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overlap (Barding et al., 2013). The GC–MS analytical platform is much more sensitive, and particularly effective in the analysis of primary metabolites, which participate in central carbon metabolism (Barding et al., 2013).

In addition, most metabolomics studies are conducted using a single analytical platform. However, as mentioned before, each platform has inherent advantages and disadvantages that can influence the analytical coverage of the metabolome (Barding et al., 2013). But so far very few studies combined those two techniques to detect the metabolite profiling of crop fruit (Krishnan et al., 2005). Barding et al. compared two platforms in detecting the

response of rice to submergence stress (Barding et al., 2013). They found the sensitivity of GC–MS facilitated the quantitation of primary metabolites, such as sugars, organic acids, and amino acids, some of which were not detected by NMR, while several metabolites, such as S-methyl methionine and the dipeptide alanylglycine, were only detected and quantified by  $^1\text{H}$  NMR. Teo et al. (2013), also reported that  $^1\text{H}$  NMR can detect less thermal stable metabolites to provide additional information on the metabolic changes. In this study, both GC–MS and  $^1\text{H}$  NMR techniques were employed to provide a comprehensive insight into the changes in metabolites in cucumber fruits induced by nano-Cu.

**Table 1**

Primary metabolites detected in cucumber fruit exposed to different concentrations of nano-Cu.

Metabolites	Control	200 mg/kg nano-Cu	400 mg/kg nano-Cu	800 mg/kg nano-Cu
<b>Sugar</b>				
Glucose	1730412 ± 703980	2640435 ± 713824	1579289 ± 353966	2E+06 ± 388798
Fructose	1128642 ± 467504	1553420 ± 402029	1335231 ± 247984	1E+06 ± 259163
Sucrose	52193 ± 14570	47872 ± 31858	37929 ± 3022	35916 ± 24822
Xylose	5477 ± 2624	8316 ± 1284	6750 ± 328	6213 ± 1711
<b>Carbohydrate acid</b>				
Malic acid	161913 ± 71267	215443 ± 63268	122257 ± 50585	137639 ± 26052
Glyceric acid	17248 ± 4113	20748 ± 4072	16208 ± 2806	19154 ± 4682
Maleic acid	14961 ± 7947	16873 ± 9810	25633 ± 23040	40521 ± 27632
Citric acid	11668 ± 1701	9655 ± 2374	7297 ± 1075	9575 ± 5537
Pipecolic acid	9988 ± 5782	9776 ± 2370	8096 ± 1319	11255 ± 3771
Fumaric acid	2544 ± 1172	3155 ± 1106	4432 ± 3783	5762 ± 3509
Pelargonic acid	1392 ± 610	2077 ± 564	1691 ± 171	1641 ± 301
Xylonic acid	727 ± 224	820 ± 95	639 ± 119	805 ± 268
Benzoic acid	593 ± 264	867 ± 271	740 ± 164	715 ± 165
Lactic acid	443 ± 244	625 ± 134	519 ± 66	387 ± 81
Succinic acid	412 ± 84	545 ± 81	544 ± 120	474 ± 133
Citramalic acid	314 ± 182	383 ± 60	269 ± 19	304 ± 95
Pyruvic acid	294 ± 210	367 ± 216	427 ± 27	334 ± 238
Glycolic acid	274 ± 210	390 ± 190	328 ± 62	342 ± 88
Adipic acid	172 ± 77	195 ± 29	168 ± 62	192 ± 51
Glutaric acid	50 ± 28	79 ± 27	70 ± 17	58 ± 17
<b>Amino Acid</b>				
Glutamine	89085 ± 23592	101060 ± 12094	100556 ± 60848	92230 ± 30510
Oxoproline	66168 ± 11329	64219 ± 8539	57989 ± 25787	67504 ± 23725
Alanine	47320 ± 7913	51570 ± 25671	57118 ± 12413	46167 ± 11713
Glycine	17358 ± 7950	26110 ± 4651	29646 ± 10917	27688 ± 6723
Citrulline	16638 ± 25715	1354 ± 475	956 ± 287	9078 ± 18627
Leucine	16086 ± 3423	18255 ± 4203	22719 ± 7882	18206 ± 4277
Isoleucine	14684 ± 2249	16588 ± 3694	18816 ± 5880	16411 ± 3328
Valine	13794 ± 3067	16006 ± 4157	18275 ± 3458	16944 ± 2802
Tyrosine	12562 ± 3043	13272 ± 5960	16588 ± 6267	13319 ± 3057
Serine	11898 ± 2519	15214 ± 3163	12479 ± 3861	11542 ± 1959
Glutamic acid	11889 ± 2696	13553 ± 5673	13040 ± 3351	10756 ± 4341
GABA	10668 ± 6917	14113 ± 7833	13477 ± 1119	14118 ± 6481
Aspartic acid	8625 ± 4941	11217 ± 5744	3867 ± 254	7543 ± 5357
Proline	8262 ± 2199	11927 ± 4737	13276 ± 2173	11424 ± 3200
Phenylalanine	7063 ± 2399	6720 ± 1554	7365 ± 2568	6558 ± 1889
Threonine	3912 ± 1130	4207 ± 1576	4599 ± 1523	4189 ± 1107
Histidine	3473 ± 1749	3557 ± 2201	4175 ± 2763	3006 ± 737
Ornithine	3090 ± 625	2298 ± 748	1832 ± 412	2415 ± 613
Lysine	2142 ± 3283	843 ± 448	973 ± 345	760 ± 257
Methionine	2104 ± 635	1722 ± 552	1584 ± 1156	1839 ± 638
4-aminobutyric acid	505 ± 934	160 ± 134	103 ± 32	154 ± 72
Beta-alanine	235 ± 118	237 ± 71	213 ± 38	179 ± 52
Homoserine	109 ± 49	110 ± 26	116 ± 14	127 ± 39
<b>Fatty Acid</b>				
Stearic acid	6534 ± 2144	10186 ± 4258	7001 ± 167	7050 ± 815
Palmitic acid	2309 ± 911	2920 ± 725	2468 ± 398	2332 ± 480
Lauroic acid	748 ± 477	841 ± 161	707 ± 98	887 ± 576
Linolenic acid	745 ± 294	1062 ± 296	1008 ± 91	674 ± 247
Capric acid	125 ± 67	119 ± 49	100 ± 24	110 ± 29
Linoleic acid	214 ± 74	290 ± 74	273 ± 30	265 ± 118
Caprylic acid	392 ± 123	619 ± 145	500 ± 47	478 ± 178
<b>Others</b>				
Lignoceric acid	124 ± 72	173 ± 83	114 ± 40	154 ± 59
Myo-inositol	62903 ± 57351	32160 ± 19400	21766 ± 4824	38254 ± 15120
Nicotinic acid	190 ± 72	226 ± 62	165 ± 24	176 ± 56

## 2. Materials and methods

### 2.1. Nanoparticle and plant exposure

A detailed characterization of the nano-Cu (U.S. Research Nanomaterials) employed here was presented in a previous study (Adeleye et al., 2014). Briefly, the primary particle size is 40 nm and the hydrodynamic diameter in deionized water is  $2590 \pm 1138$  nm. The zeta potential is  $+10.9 \pm 4.0$ ,  $-29.4 \pm 0.8$  and  $-40.8 \pm 1.7$ , at pH 4, 7 and 11 respectively.

Cucumber (*Cucumis sativus*) seeds were planted in pots containing organic matter enriched soil. The soil was pre-treated with a suspension of nano-Cu to obtain the following final nano-Cu concentrations in soils: 0 (Control), 200 (Low), 400 (Medium), and 800 mg/kg (High). Each treatment had four replicates and each replicate contained two seedlings. The cucumber plants were grown in a greenhouse for 68 days until the cucumber fruits reached full maturity. The temperatures in the greenhouse were controlled at  $25.5\text{--}30^\circ\text{C}$  during the day and  $17.7\text{--}18.9^\circ\text{C}$  at night. At harvest, the weight and number of fruit from each treatment were recorded.

### 2.2. NMR sample preparation

Fresh harvested cucumber fruits were washed thoroughly using nano pure water. Then a 5-cm thick slice of cucumber was cut and

immediately frozen and ground in liquid  $\text{N}_2$ . The ground tissues were freeze-dried for 1 week using a lyophilizer (Labconco, MO, USA). The extraction method by Kim et al. (2010) was followed. Specifically, 50 mg of the freeze dried powdered fruit tissues were mixed with 0.75 ml of  $\text{CD}_3\text{OD}$  (deuterated methanol) and 0.75 ml of  $\text{KH}_2\text{PO}_4$  buffer in  $\text{D}_2\text{O}$  (pH 6.0) containing 0.1% TSP (3-(trimethylsilyl)propionic-2,2,3,3-d<sub>4</sub>, Aldrich) (wt/wt). The mixture was vortexed for 1 min, and ultrasonicated for 20 min at room temperature. After sonication, the mixture was centrifuged for 15 min. The entire 0.8 ml of supernatant was transferred to a 5 mm NMR tube.

### 2.3. NMR analysis and multivariate data analysis

One-dimensional (1D)  $^1\text{H}$  NMR spectra of fruit extracts were measured using a Bruker AVANCE III 800 MHz SB NMR Spectrometer, obtained using a  $14.5\text{ }\mu\text{s}$   $90^\circ$  pulse, 3 s relaxation delay, 20 ppm spectral width, and 2 s acquisition time, with 200 transients of 64k data points collected over a 16 min data accumulation time. The residual water signal was suppressed with pre-saturation during the relaxation delay. The resulting spectra were phase and baseline corrected and then the chemical shift was calibrated using the Bruker Topspin 3.1 software.

For the NMR data, the chemical shift region of significance between 0.5 ppm and 10.0 ppm was divided into small bins of equal width (0.04 ppm bin size). Finally about 240 buckets were

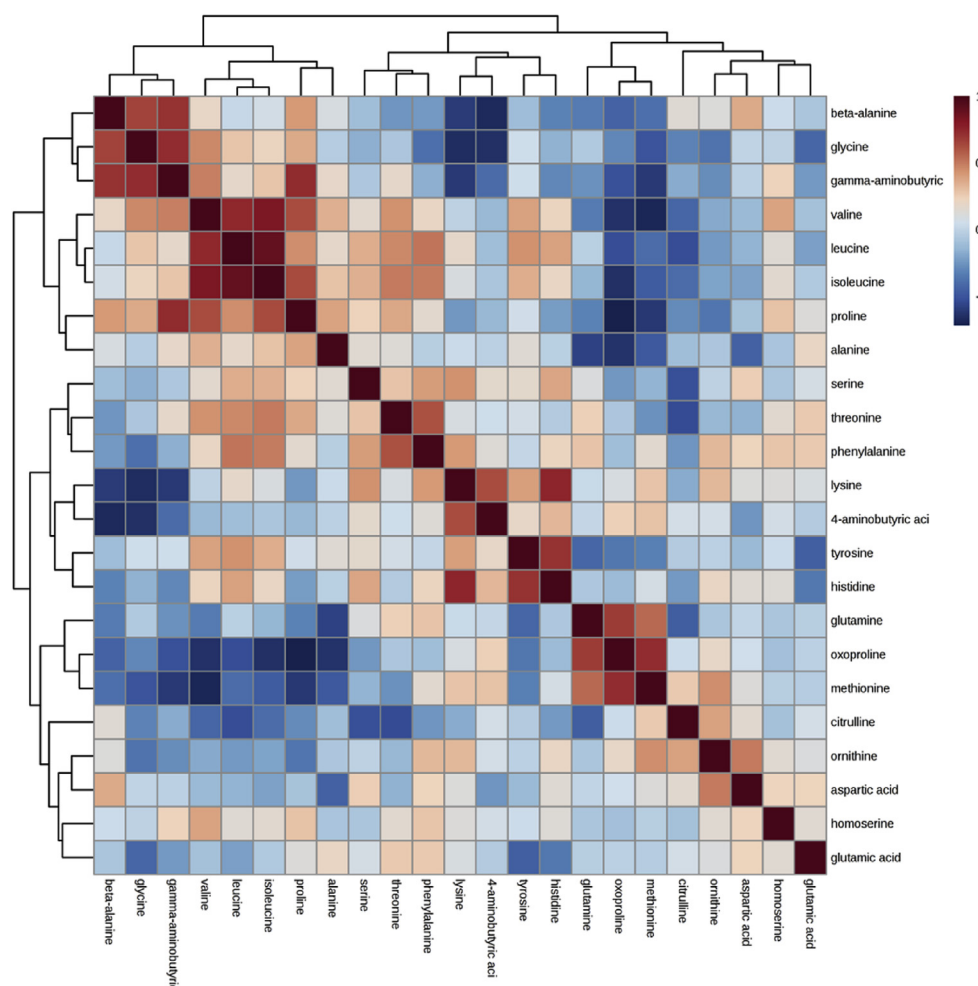


Fig. 1. Correlation map of amino acids in cucumber fruit extract.

generated for each sample. Partial least-squares discriminant analysis (PLS-DA), a supervised multivariate analysis of the binned NMR data was conducted using online resources (<http://www.metaboanalyst.ca>). For the bins with significant changes across a given set of samples, the responsible metabolites were identified with the Chenomx NMR Suite 7.0 (Chenomx Inc.).

#### 2.4. Gas chromatography-mass spectrometry (GC–MS) analysis of cucumber fruit

The freeze dried cucumber fruit samples were subjected to GC–MS analysis at the Genome Center Core Services, University of California Davis to identify the metabolites. Description of the instrument has been reported previously (Badri and Vivanco, 2009). All GC–MS data were further normalized before multivariate analysis.

### 3. Results and discussion

In this study, we characterized many primary metabolites using the GC–MS data, which can provide a fundamental view of the changes in nutritional supply due to plant exposure to nano-Cu.  $^1\text{H}$  NMR, as a powerful complementary technique for identifying and quantifying fruit metabolites, was also employed for additional insights.

#### 3.1. Metabolites in cucumber fruits determined by GC–MS

GC–MS detected and identified 107 metabolites in the cucumber fruit extract. Here, we emphasize the discussion on 53 metabolites related to nutritional supply, including sugars, amino acids, organic acids, and fatty acids. As shown in Table 1, the major sugars are glucose and fructose, followed by sucrose and xylose. The most abundant organic acids are malic, glyceric, maleic, citric, pipercolinic, fumaric, and pelargonic acids. The main fatty acids are stearic and palmitic acids. Cucumber fruit extract contained 23 amino acids, among them, histidine, isoleucine, leucine, lysine,

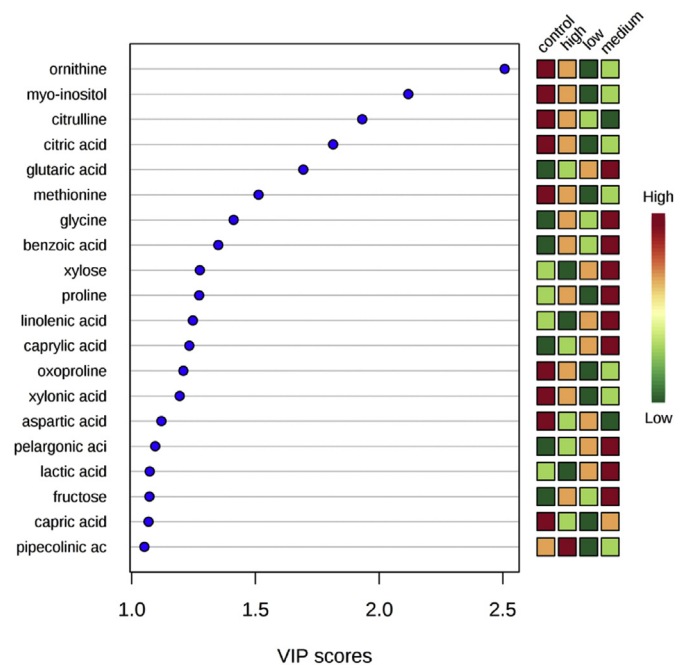


Fig. 3. VIP scores from PLS-DA analysis showing the discriminating metabolites induced the group separation. Control, low, medium and high in the figure means nano-Cu concentration as 200, 400, and 800 mg/kg.

methionine, phenylalanine, threonine, tryptophan, tryptophan, and valine, which are reported essential human amino acids. Humans cannot synthesize these amino acids, and thus they must be supplied via the diet (Shimomura et al., 2006). Among these essential amino acids, five amino acids (valine, leucine, isoleucine, threonine and tyrosine) were up-regulated for all nano-Cu treatments. Compared to the control, valine, leucine, isoleucine, threonine and tyrosine in fruits exposed to different concentrations of nano-Cu increased 16–32%, 13–41%, 12–28%, 0.1–18%, 0.1–32%,

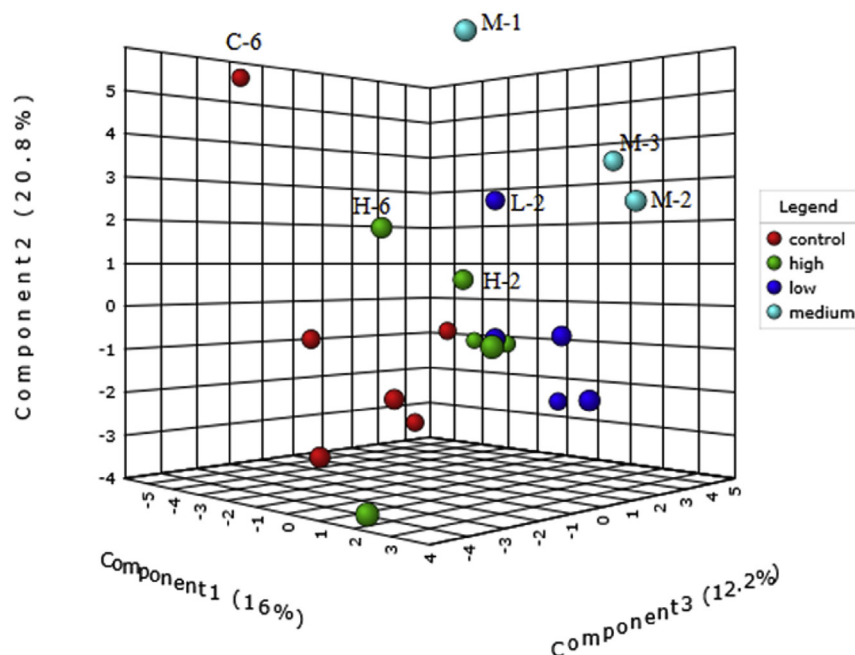


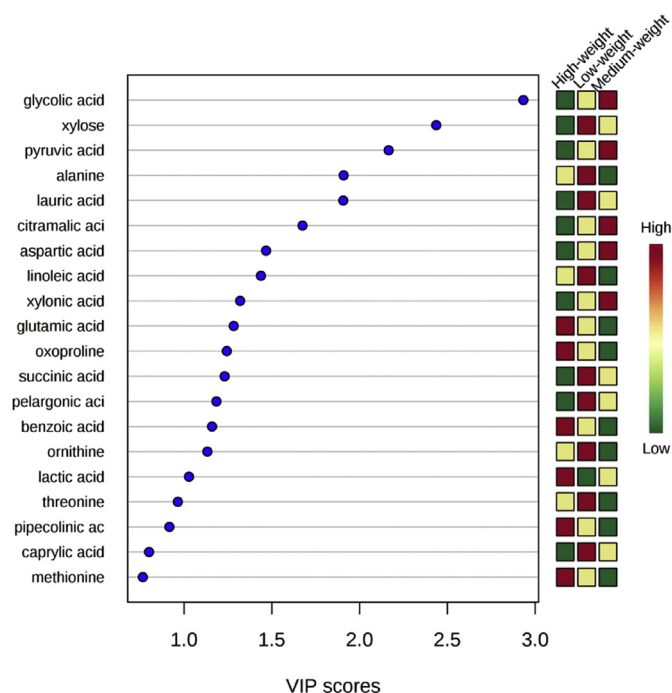
Fig. 2. PLS-DA score plots of 53 metabolites in cucumber fruit extract, data are from GC–MS. Cucumber fruits comes from cucumber plants grown in soil contains different concentrations of nano-Cu for two months. Control, low, medium and high in the figure means nano-Cu concentration = 0, 200, 400, and 800 mg/kg.

**Table 2**

Fresh weight of cucumber fruit at harvest. The number of matured cucumber from each replicate are 6, 5, 4, and 6 for control, Low (200 mg/kg), Medium (400 mg/kg), and High (800 mg/kg). The weight here means individual fruit. Bold mean the comparatively lower weight (<150 g).

Treatment	Abbr	Weight (g)
Control-1	C-1	310.63
Control-2	C-2	184.87
Control-3	C-3	167
Control-4	C-4	284
Control-5	C-5	203
<b>Control-6</b>	<b>C-6</b>	<b>110.66</b>
<b>Low-1</b>	<b>L-1</b>	<b>134.87</b>
<b>Low-2</b>	<b>L-2</b>	<b>128.54</b>
Low-3	L-3	194.82
Low-4	L-4	224.41
Low-5	L-5	173.68
Medium-1	M-1	170.4
<b>Medium-2</b>	<b>M-2</b>	<b>141.08</b>
Medium-3	M-3	173.03
High-1	H-1	172.13
<b>High-2</b>	<b>H-2</b>	<b>101.24</b>
High-3	H-3	207.9
High-4	H-4	179.3
High-5	H-5	154
<b>High-6</b>	<b>H-6</b>	<b>150</b>

respectively. It is noteworthy that for plants exposed to the medium concentration of nano-Cu, the accumulation of these amino acids in fruits were highest compared to the control and other treatments. Among the essential amino acids, the content of lysine and methionine in fruits, for plants treated with different concentrations of nano-Cu, decreased 55–61% and 13–25% respectively. However, none of changes in amino acids were statistically significant due to the large variation with groups. The correlation analysis (Fig. 1) showed valine, leucine, and isoleucine were clustered, indicating strong correlation. Those three amino acids are commonly referred to as branched-chain amino acids (BCAAs) due to their branched carbon skeletons and it has been reported that these amino acids play important roles in growth and development (Kochevenko et al., 2012). Yoshiharu et al. reported that supplements of branch chained amino acids may reduce exercise-induced

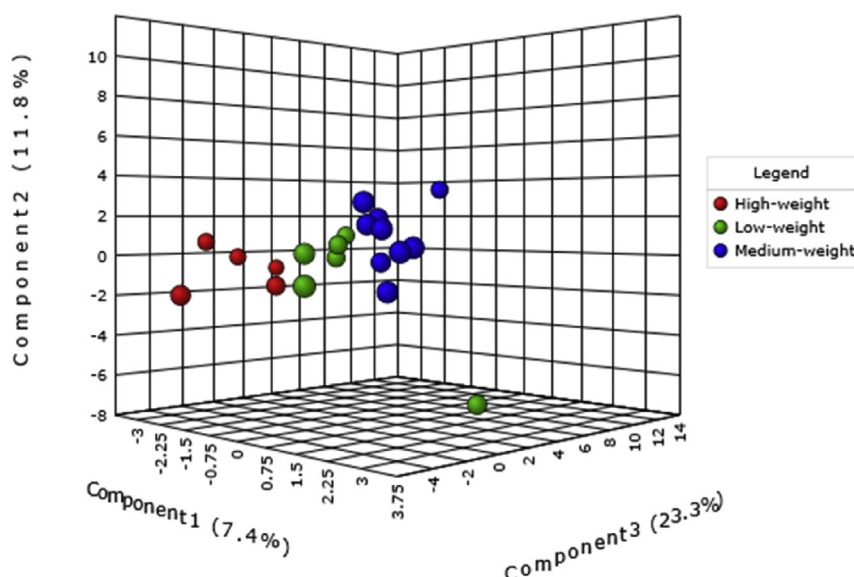


**Fig. 5.** VIP scores from PLS-DA analysis (grouped by weight) showing the discriminating metabolites induced the group separation.

muscle damage and promote the muscle recovery (Shimomura et al., 2006).

### 3.2. Metabolites profiles analysis

PLS-DA, a supervised multivariate analytical method, was applied to the GC–MS data of 53 metabolites to help identify general metabolite profile differences between the control and nano-Cu treated groups. From a score plot of PLS-DA (Fig. 2), the control samples were grouped with nano-Cu treated samples along PC1 dimension (PC1 explained 16% of the total variance). This



**Fig. 4.** PLS-DA score plots of 53 metabolites in cucumber fruit extract, data are from GC–MS. Cucumber fruits comes from cucumber plants grown in soil contains different concentrations of nano-Cu for two months. The grouping is based on fruit weight.



indicates that nano-Cu changed metabolite profiles and therefore altered nutrition supply of cucumber fruit. In order to further identify the metabolites responsible for this difference between the control and nano-Cu treated samples, the parameters of variable importance in projection (VIP) were determined. VIP is the weighted sum of the squares of the PLS-DA analysis, which indicates the importance of a variable to the entire model. A variable with a VIP above 1 is regarded as important. Twenty metabolites were identified as discriminating metabolites (VIP > 1) between treatments of different concentrations of nano-Cu (Fig. 3). Among them, several metabolites were down-regulated by nano-Cu at all concentrations, including myo-inositol, methionine, citric acid, ornithine and citrulline. Some sugars (xylose, fructose), amino acids (glycine, proline), organic acids (benzoic acid, glutaric acid), and fatty acids (caprylic acid, linolenic acid) were up-regulated by nano-Cu at all concentrations. Most of the down-regulated metabolites are not essential nutrients for humans, but they have been

added to “nutritional” foods or health care products. For instance, myo-inositol is a B complex vitamin. It has been shown that women who receive myo-inositol show a great improvement of the function and quality of ovulatory (Ciotta et al., 2011). Methionine, an  $\alpha$ -amino acid, has been shown to be associated with decreased risk of proximal colon cancer among men and rectal cancer among women (de Vogel et al., 2008). Ornithine and citrulline are non-essential amino acids, which participate in central reactions in the urea cycle (Suredda and Pons, 2012). Dietary interest for ornithine and citrulline has considerably increased during the last decade (Romero et al., 2006; Rouge et al., 2007). The up-regulation of a portion of metabolites will benefit the cucumber fruit, because most of them are carbohydrates (xylose, fructose) and necessary amino acids (glycine and proline). In addition, benzoic acid has been found to possess antioxidant and free radical scavenging activity (Velioglu et al., 1998). Oral supplementation of L-citrulline improved function in men with mild erectile dysfunction (Cormio

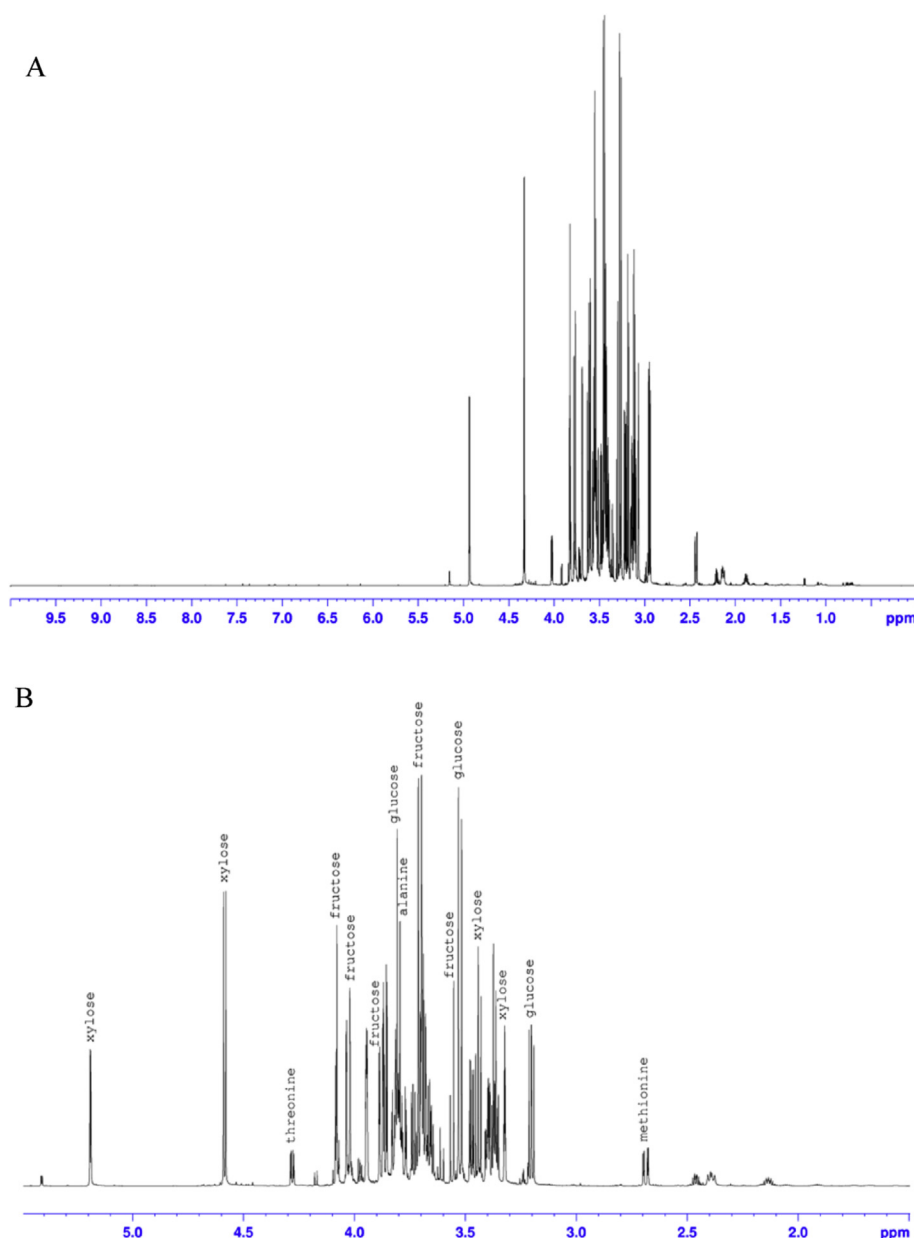


Fig. 6. Representative one-dimensional <sup>1</sup>H NMR spectra of cucumber fruit extracts (A) and spectra with main peaks labeled (B).

et al., 2011).

Two beneficial fatty acids in cucumber fruit were up-regulated by nano-Cu. Linolenic acid is the most common omega-3 fatty acid, which has a beneficial effect on liver fat content and other cardiovascular risk factors in women with polycystic ovary syndrome (PCOS) (Cussons et al., 2009). Hu et al. demonstrated that a higher intake of  $\alpha$ -linolenic acid is protective against fatal ischemic heart disease (Hu et al., 1999). Caprylic acid is an eight-carbon saturated fatty acid, and previous studies showed that caprylic acid can help to burn excess calories and therefore lose weight (Takeuchi et al., 2008).

It has been reported that some amino acids, e.g. arginine and proline increase gradually with maturity and ripening (Lamikanra and Kassa, 1999). Taking the weight into consideration (Table 2), we found that in Fig. 2, samples with comparatively lower weight (M-1, C-6, M-3, L-2, M-2, H-6, H-2) were distributed in the positive score along PC2 axis, and samples with higher weight had a negative score in the PC2 axis. We further classified all the samples according to weight: low-weight group (<150 g), medium-weight group (150–200 g), and high-weight group (above 200 g). PLS-DA was performed again based on fruit weight instead of nano-Cu concentration applied. Score plot shows samples from low, medium, and high weight groups were clearly grouped along PC1 (Fig. 4). This indicates that a portion of the metabolites were strongly impacted by maturity. The VIP scores presented in Fig. 5 were used to identify the metabolites strongly dependent on maturity. Comparing Figs. 3 and 5, the only overlap was xylose. These results indicate that the alteration of metabolites was mainly induced by plant exposure to nano-Cu. Also, the changes were independent of maturity.

### 3.3. $^1\text{H}$ NMR analysis

Representative  $^1\text{H}$  NMR spectra of cucumber fruit extract are presented in Fig. 6. Using Chenomx the main peaks were identified as fructose, glucose, xylose, methionine and alanine, which is generally consistent with the results of GC–MS analysis. In order to compare the overall metabolic profiling between samples, multivariate analysis (PLS-DA) of unassigned  $^1\text{H}$  NMR spectra was performed. The results showed that the control and exposed samples were clearly identified in separate groups along the PC1, which contributed 14.9% of the variability (Fig. 7). The grouping pattern is

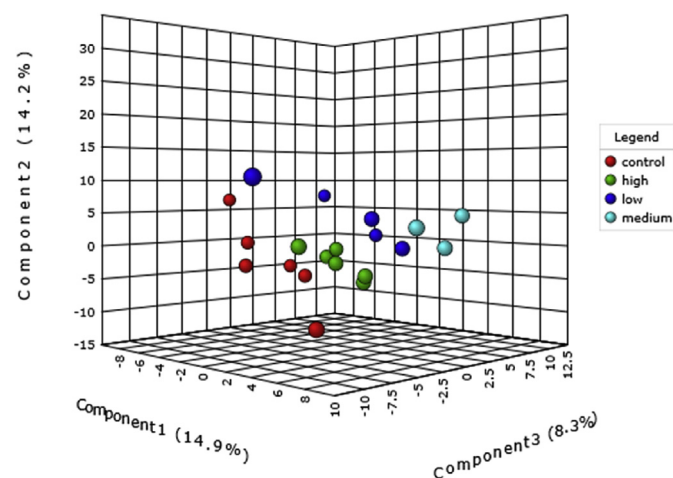


Fig. 7. PLS-DA score plots of 400 bin area from  $^1\text{H}$  NMR, showing clear separation of control, Low, Medium, and High. Control, low, medium and high in the figure means nano-Cu concentration = 0, 200, 400, and 800 mg/kg.

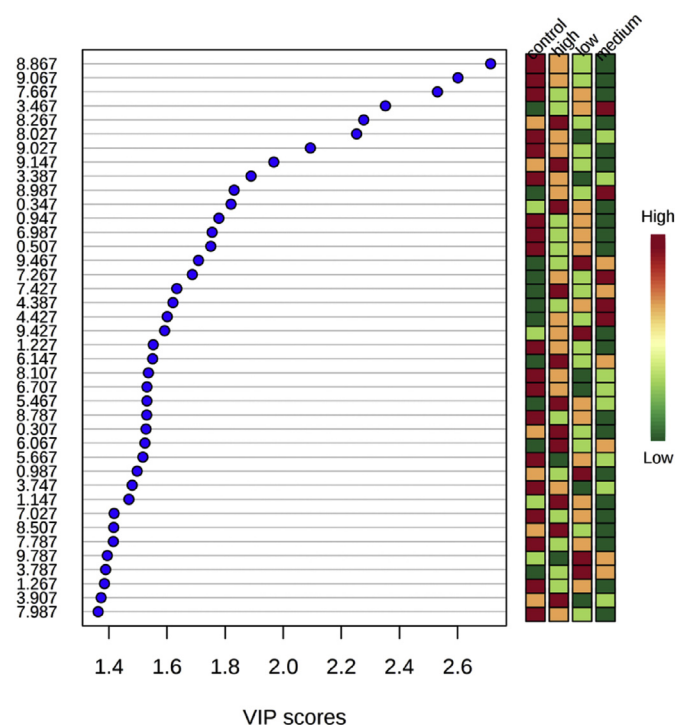


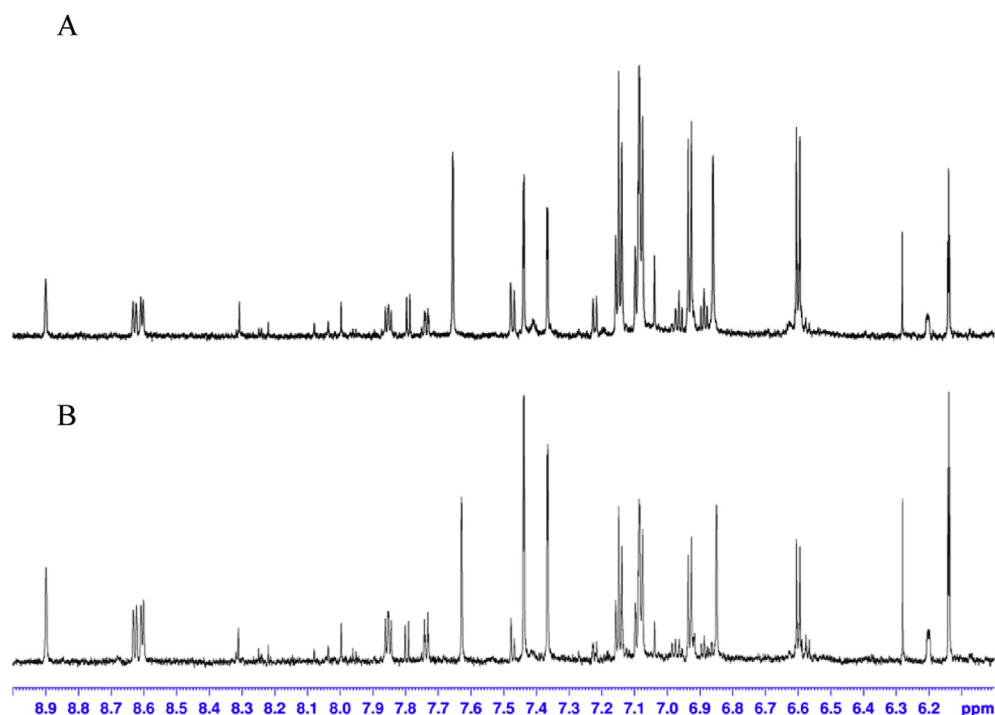
Fig. 8. VIP scores from PLS-DA analysis showing the discriminating  $^1\text{H}$  NMR bin area induced the group separation. Control, low, medium and high in the figure means nano-Cu concentration as 200, 400, and 800 mg/kg.

very similar with the GC–MS results reported before. The loading plot revealed there are 40 bins responsible for group separation (Fig. 8). For those bins, we identified metabolite peaks using Chenomx NMR suit 7.0 (Chenomx Inc.). Finally 10 metabolites were identified, including xylose, fructose, 2-aminobutyrate, and benzoate, which were detected by GC–MS. Furthermore, there were four significantly changed metabolites which were not detected by GC–MS (methylnicotinamide (MNA), trigonelline, imidazole, quinolinate). In the aromatic region ( $\delta$  9.50–6.00) (Fig. 9), the peak intensity is very weak, but there were many changes in this region. MNA (down-regulated) has been considered to be an inactive metabolite of nicotinamide. MNA displayed a profile of anti-thrombotic activity in vivo (Chlopicki et al., 2007). Trigonelline (down-regulated) is a plant hormone (Evans and Tramontano, 1981) which has been reported as having therapeutic potential in the prevention and treatment of diabetes and central nervous system disease (Zhou et al., 2012). Quinolinate (down-regulated) studies (Guillemin, 2012) showed that quinolinic acid has toxic effects, e.g., inducing mood depression. Imidazole was observed up-regulated. It is reported that the substituted imidazole derivatives are valuable in the treatment of many systemic fungal infections (Leon et al., 2004).

### 4. Conclusion

This study applied two platforms ( $^1\text{H}$  NMR and GC–MS) to detect metabolite changes in cucumber fruit for plants exposed to different concentrations of nano-Cu. GC–MS is a powerful tool to identify and quantify the primary metabolites. NMR supplied complementary information on the changes of these metabolites. Application of two analytical platforms to do non-target metabolomics provides a comprehensive insight into the metabolite changes impacted by nano-Cu. Results showed nano-Cu significantly altered some patterns in the metabolites, indicating that the





**Fig. 9.**  $^1\text{H}$  NMR spectra of aromatic region ( $\delta$  9.50–6.00), a representative sample spectra from the group of Medium (400 mg/kg nano-Cu) (A) and control (B). The detected metabolites responsible for group between control and nano-Cu treated samples are methylnicotinamide (MNA) ( $\delta$  9.3, 9.0, 8.9, 8.2), trigonelline ( $\delta$  9.1, 8.8, 8.1), imidazole ( $\delta$  8.3, 7.3), quinoline ( $\delta$  8.5, 8.0, 7.5).

nutrients supplied by the fruit will be changed. In several cases, the effect was to up-regulate metabolites that may be beneficial to human health, even though the intent of the application of nano-Cu was as a pesticide. Thus, the effects of using nanotechnology in agriculture need to be better understood.

## Acknowledgments

This work was supported by the National Science Foundation (NSF) and the U.S. Environmental Protection Agency (EPA) under NSF-EF0830117. Any opinions, findings, conclusions or recommendations expressed in this material are those of the authors do not necessarily reflect the views of the funding agencies. The MRL shared Experimental Facilities are supported by the MRSEC Program of the NSF under Award No. DMR 1121053; a member of the NSF-funded Materials Research Facilities Network.

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