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Metabolomic analysis of honey bee, *Apis mellifera* L. response to  
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## Abstract

The cyano-substituted neonicotinoid insecticide, thiacloprid, is nowadays widely used in agriculture for controlling insect pests. However, it also simultaneously has adverse effects on the health of important pollinators, such as honey bees. Previous studies have reported that sublethal doses of neonicotinoids impaired immunocompetence, learning and memory performance, and homing behaviour in honey bees. In the present study, using LC-MS-based combined with GC-MS-based metabolomic approaches, we profiled the metabolic changes that occur in the head of honey bee after subchronic exposure to 2 mg/L thiacloprid over 3 days. The estimated total dose of thiacloprid fed to each bee was 0.12 µg. The results showed that there were 115 metabolites significantly affected in thiacloprid-treated bees compared to control. The metabolites with high level of abundance enriched to wide range pathways associated with oxidative stress and detoxification suggest that the honey bees have activated their detoxification system to resistant toxicity of thiacloprid. While, the reduction of serotonin suggest thiacloprid may hinder the brain activity implicated in learning and behaviour development. Our study expand the understanding of the molecular basis of the complex interactions between neonicotinoids and honey bees.

Key words: *Apis mellifera*    thiacloprid    LC-MS    GC-MS    differential metabolites

## 1. Introduction

Honey bees are ideal pollinators and play an important role in pollination services of crops and wild plants. In recent years, the constant decline of bee populations has attracted the attention of the world. Despite the specific reasons for the current decline in bee populations still being unclear, many studies have shown that the heavy use of pesticides in agriculture may be one of the most significant contributing factors causing this phenomenon [1-4].

Neonicotinoid insecticides are systemic insecticides that bind to insects nicotinic acetylcholine receptors, causing abnormal excitation and leading to the death of insects through convulsive paralysis [5]. These insecticides are widely used in agricultural crops to control many types of pests [6]. Honey bees may also come into contact with these insecticides through the collection and feeding of contaminated nectar, pollen, and water. Many studies have found residues of neonicotinoid insecticides such as imidacloprid, thiamethoxam, and thiacloprid in pollen, honey, and beeswax [7-9]. Although most neonicotinoid pesticides are highly toxic or even hypertoxic to bees, it is generally believed that field doses do not lead to acute death [10]. However, sublethal doses of neonicotinoid insecticides can affect bee brain and midgut development [11,12], impair learning and memory abilities [13-15], and destroy their immune systems [16]. Many field trials have shown that neonicotinoid insecticides also reduce bee foraging and homing abilities [17-19].

Thiacloprid is a cyano-substituted neonicotinoid insecticide. In comparison with other nitro-substituted neonicotinoids, such as imidacloprid (3.7 to < 104 ng bee<sup>-1</sup>;

LD<sub>50</sub> 48h after oral exposure) and thiamethoxam (4-5 ng bee<sup>-1</sup>; LD<sub>50</sub> 48h after oral exposure), the inherent toxicity of thiacloprid to honey bees is substantially lower (17.3 µg bee<sup>-1</sup>; LD<sub>50</sub> 48h after oral exposure) [10]. To some extent, thiacloprid can be expeditiously detoxified by honey bees, and thus is classified safe for bees [20]. On the contrary, several studies have investigated the effects of sublethal concentrations of thiacloprid on honey bees, demonstrating negative impacts: on immunocompetence [16], learning behaviour [21], homing performance [19], and survival under pathological stress [22,23]. Whereas the latest research from Siede et al (2017) declare that the performance of honey bee colonies was not adversely affected by long-lasting exposure to sublethal doses of thiacloprid [24], thus still more studies are needed to evaluate the total risk of thiacloprid on honey bees.

During the past few years, genome-wide transcriptome analysis are carried out to investigate the molecular effects of neonicotinoid insecticides on honey bees [25-28]. In previous studies, we examined the influence of the neonicotinoid insecticide, thiamethoxam, on the transcriptome and miRNA expression in the honey bees [29,30]. Nevertheless, mRNA expression profiles do not always correlate well with protein concentrations and metabolic state. To further understand the molecular mechanisms of the complex interactions between neonicotinoids and honey bees, the metabolic profile differences between thiacloprid-treated bees and control bees were investigated using LC-MS- and GC-MS-based metabolomic approaches in the present study.

## 2. Materials and methods

## 2.1 Honey bee colonies

The honey bee (*Apis mellifera ligustica*) colonies were reared at the Institute of Apicultural Research, Anhui Agricultural University, Hefei, China from June 2017 to August 2017. Four colonies with 3-month-old sister queens and similar colony strength were selected as the experimental colonies.

## 2.2 Honey bees rearing

Frames with capped worker broods near adult emergence were taken from the above four colonies. The frames were held in an incubator under the following conditions:  $35 \pm 1^\circ\text{C}$ , a relative humidity (RH) of  $60 \pm 10\%$  and in darkness. We obtained the newly emerged honey bees and put them into wooden cages ( $11 \times 10 \times 8$  cm). They were fed with bee bread collected from the same apiary, 50% (w/v) sucrose–water solution, and maintained for 1 d at  $28 \pm 1^\circ\text{C}$ , an RH of  $60 \pm 10\%$ , and in darkness to suit the rearing conditions [29]. The dead bees in each cage were removed daily.

## 2.3 Thiacloprid preparation and exposure

Thiacloprid (> 99% purity) was purchased from aladdin (Shanghai, China). A stock solution of 1000 mg/L of thiacloprid was prepared in acetone. The 2 mg/L thiacloprid was selected as the treated concentration in this experiment. This dose of thiacloprid was confirmed as the highest concentration of thiacloprid that had no significant influence on survival of caged bees over a 10-d-oral exposure (Fig. S1, Supporting Information). A 2 mg/L of thiacloprid was prepared in a 50% sucrose–water solution with a 0.03% final concentration of acetone. A 50%

sucrose–water solution with the same concentration of acetone was also prepared as a control. Eight hundreds of 2-day-old bees were divided into two groups: thiacloprid-treated and control groups, 8 replicates in each group. Each replicate having 50 bees. These bees were equally selected from the four colonies. Bees were fed the control and experimental diets for 3 days, then immediately killed by liquid nitrogen and stored at -80°C until further analysis. Beacause generally each bee consume 20 µL of 50% sucrose–water solution daily, the estimated total dose of thiacloprid fed to the bees in the experiment group was 0.12 µg /bee [31].

#### 2.4 Metabolite extraction

Forty bee heads from each sample were ground into powder in liquid nitrogen with a pestle and mortar. For the liquid chromatography (LC) sample, 60 mg of powder was homogenized in 600 µL of a precooled methanol-water (v/v 4:1) solvent mixture with 20 µL of 0.3 mg/mL L-2-chloro-phenylalanine. After extracting for 10 min in an ice water bath and standing for 30 min at -20°C, the mixture was centrifuged at 13,000 rpm for 15 min at 4°C. 200 µL of supernatant was transferred to a new 1.5 mL polypropylene tube and processed through vacuum freeze drying before LC analysis. For the gas chromatography (GC) sample, 100 mg of powder was homogenized in 600 µL of a precooled methanol-water (v/v 4:1) solvent mixture with 20 µL of 0.3 mg/mL L-2-chloro-phenylalanine. After extracting for 10 min in an ice water bath and standing for 30 min at -20°C, the mixture was centrifuged at 13,000 rpm for 15 min at 4°C. 400 µL of supernatant was transferred to a new 1.5 mL polypropylene tube and processed through vacuum freeze drying before GC analysis.

## 2.5 UPLC-MS/MS analysis

Liquid chromatography was performed on a Waters UPLC I-class system equipped with a binary solvent delivery manager and a sample manager, coupled with a Waters VION IMS Q-TOF Mass Spectrometer equipped with an electrospray interface (Waters Corporation, Milford, USA). The separation of all samples was performed on an Acquity BEH C18 column (100 mm × 2.1 mm i.d., 1.7 µm) (Waters). The 3 µL of column was maintained at 45°C and separation was achieved using the following gradient: 5-20% acetonitrile (0.1% (v/v) formic acid) over 0-2 min, 20-60% acetonitrile (0.1% (v/v) formic acid) over 2-8 min, 60-100% acetonitrile (0.1% (v/v) formic acid) over 8-12 min, the composition was held at 100% acetonitrile (0.1% (v/v) formic acid) for 2 min, then 14-14.5 min, 100% to 5% acetonitrile (0.1% (v/v) formic acid), and 14.5-15.5 min holding at 5% acetonitrile (0.1% (v/v) formic acid) at a flow rate of 0.40 mL/min. The mass spectrometric data was collected using a Waters VION IMS Q-TOF Mass Spectrometer equipped with an electrospray ionization (ESI) source operating in either positive or negative ion mode. The source temperature and desolvation temperature was set at 120°C and 500°C, respectively, with a desolvation gas flow of 900 L/h. Centroid data was collected from 50 to 1,000 m/z with a scan time of 0.1 s and interscan delay of 0.02 s over a 13 min analysis time.

## 2.6 UPGC-MS/MS analysis

The derivatized samples were analyzed on an Agilent 7890A gas chromatography system coupled to an Agilent 5975C MSD system (Agilent, CA, USA). A HP-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 µm) (Agilent)

was utilized to separate the derivatives. Helium ( $> 99.999\%$ ) was used as the carrier gas at a constant flow rate of 6.0 mL/min through the column. The injector temperature was maintained at 280°C. Injection volume was 1  $\mu$ L by splitless mode. The initial oven temperature was 60°C, ramped to 125°C at a rate of 8°C/min, to 190°C at a rate of 10°C/min, to 210°C at a rate of 4°C/min, to 310°C at a rate of 20°C/min, and finally held at 310°C for 8.5 min. The temperature of MS quadrupole, and ion source (electron impact) was set to 150 and 230°C, respectively. The collision energy was 70 eV. Mass data was acquired in a full-scan mode (m/z 50-600), and the solvent delay time was set to 5 min.

## 2.7 Data analysis

Firstly, the LC-MS raw data and GC-MS raw data were preprocessed by Progenesis QI software (Waters) and ChromaTOF software (v 4.34, LECO, St Joseph, MI), respectively, within which data alignment, normalization, and peak picking were performed. Then, the data matrix was normalized to the total peak area of each sample in Excel 2007 (Microsoft, USA) and imported into a SIMCA (version 14.0, Umetrics, Umeå, Sweden), where principal component analysis (PCA), and partial least-squares discriminant analysis (PLS-DA), and orthogonal partial least-squares discriminant analysis (OPLS-DA) were performed. The quality of the models was described by the  $R^2X$  or  $R^2Y$  and  $Q^2$  values. The OPLS-DA model was also validated by a permutation analysis (200 times). The differential metabolites were selected on the basis of the combination of a statistically significant threshold of variable influence on projection (VIP) values obtained from the OPLS-DA model and a *p*-values from a two-tailed

Student's t-test on the normalized peak areas, where metabolites with VIP-values >1.0 and *p*-values < 0.05 were deemed statistically significant. Using the HMDB databases and METLIN databases, the putative differential metabolites of LC-MS were checked and confirmed. While, the NIST and Fiehn databases were used for the qualitative discrimination of the putative differential metabolites of GC-MS. A heat map was generated using the MetaboAnalyst 4.0 software based on the abundance of differential metabolite data. In the KEGG database, we used MetaboAnalyst 4.0 software to test the statistical enrichment of differential metabolite in KEGG pathways. KEGG pathways with *p*-values < 0.05 were considered significantly enriched by differential metabolites.

### 3. Results

#### 3.1 Data modeling

Using both LC-MS and GC-MS approaches, we examined the metabolomic profiles in the heads of honey bees after subchronic exposure to 2 mg/L thiacloprid over 3 d. Using LC-MS, a total of 18,360 ions (combined positive and negative ions) were identified in each sample profile. After removing low-quality ions (relative standard deviation (RSD) >30%), we obtained 13,832 ions. These ions were then further processed by principle component analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA). As shown in Fig. 1A, B, the first 2 principal components exhibited 39.4% of the total variance by PCA, and the supervised OPLS-DA models that emerged from the unsupervised PCA models showed good separation between the thiacloprid-treated bees and the control bees.

Using GC-MS, only 286 ions were detected and carried out for PCA and OPLS-DA. PCA models showed 32.4% of the total variance. OPLS-DA models exhibited good separation among these two groups (Fig. 2A, B).

### 3.2 Differential metabolites

These differential metabolites were chosen according to the combination of a statistically significant threshold of variable influence on projection (VIP) values obtained from the OPLS-DA model and a *p*-values from a two-tailed Student's t-test on the normalized peak areas, where metabolites with VIP-values >1.0 and *p*-values < 0.05 were deemed statistically significant. Using these significantly differential metabolites we constructed heat maps. Using LC-MS, 80 differential metabolites were identified in thiacloprid-treated bees compared with control bees (Fig. 3), while 35 differential metabolites were detected by GC-MS (Fig. 4). Interestingly, LC-MS and GC-MS were not detect the same differential metabolites. These differential metabolites detailed information is shown in Table S1 (Supporting Information). Among these 115 differential metabolites, 83 were up-regulated and 32 were down-regulated. A list of the 20 metabolites with the most significant differential expression are shown in Table 1; of which 19 were up-regulated and one was down-regulated. N-epsilon-Acetyl-L-lysine, D-erythro-sphingosine, and 1-Methyladenosine were the top three most significant differential metabolites with more than a 10-fold change, and all were up-regulated in thiacloprid-treated bees (Table 1).

### 3.3 Metabolic pathway analysis

A total of 48 metabolites were confirmed and mapped to 19 KEGG metabolic pathways, of which 39 metabolites were up-regulated in thiacloprid-treated bees, and 9 were down-regulated. To estimate the importance of the mapped KEGG metabolic pathways, the impact value and log (*p*-value) with metabolic pathway analysis was performed using MetaboAnalyst 4.0. Regarding these altered pathways, five metabolic pathways including glutathione metabolism, glycerophospholipid metabolism, alanine, aspartate and glutamate metabolism, arginine and proline metabolism, and cysteine and methionine metabolism all exhibited lower *p*-values and greater pathway impact (Fig 4), and glutathione metabolism and glycerophospholipid metabolism was significantly enriched with the *p*-values < 0.05 (Fig. 5).

#### 4. Discussion and conclusion

As an emerging and promising omics approach, metabolomics is used to understand the global metabolic information of biological samples [32]. Nowadays, it has been widely used in the study of diverse areas, including diseases, plants, microbiology, nutrition, toxicology and insects [33]. Recently, several studies have also used this approach to explore the interactions between honey bees and xenobiotics or parasites [31,34,35].

In the present study, using both LC-MS and GC-MS, we investigated the metabolome profile of honey bee heads after subchronic exposure to 2 mg/L thiacloprid over 3 d. A total of 115 differential metabolites, including diverse amino acids, fatty acids, phospholipids, and other compounds were confirmed, and more than 70% (83) of the differential metabolites were up-regulated. Previous studies have

indicated that the detoxification of nicotine, an alkaloid have similar modes of action with neonicotinoids, in honey bees was associated with increased energetic investment and also antioxidant and heat shock responses [31]. In our previous studies, we also found that the differentially-expressed genes induced by another neonicotinoid thiamethoxam in honey bees were mainly enriched in pathways linked to biosynthesis and metabolism, such as ribosomes, tyrosine metabolism, and drug metabolism pathway [29]. Our results here suggest that the honey bees might protect against thiacloprid stress by speeding up their metabolism. Surprisingly, among these differential metabolites, the insecticide thiacloprid or its metabolites were not detected. It might be because thiacloprid can be relatively quickly detoxified in the midgut after being absorbed by honey bees [20,36], thereby only allowing a minimum amount of thiacloprid to reach the honey bee heads.

N-epsilon-Acetyl-L-lysine and D-erythro-sphingosine were the two most up-regulated metabolites with 319.74- and 61.55-fold change in thiacloprid-treated bees compared with control, respectively. N-epsilon-Acetyl-L-lysine occurs frequently as a product of the post-translational modification of the N-epsilon-lysine acetylation [37-39]. The N-epsilon-lysine acetylation in eukaryotes has a major impact on the structure, function, location, and stability of thousands of proteins involved in diverse cellular processes [40]. Sphingosine is an 18-carbon amino alcohol with a long unsaturated hydrocarbon chain. Sphinganine, the derivative of sphingosine, is the basic structural core sphingolipid. Sphingolipids are complex and pervasive membrane lipids [41], which are responsible for the regulation of cellular metabolism, cell growth and cell

differentiation [42]. The alteration of D-erythro-sphingosine level may be involved in the destruction of sphingolipid metabolism [43]. In addition to the most changed metabolites, several other differential metabolites also deserved attention, such as 5-Methyltetrahydrofolic acid (5-M-THF), which is involved in one carbon pool by folate metabolism and methane metabolism. 5-M-THF is the predominant active form of folates in human blood serum [44], previous studies showed that folate deficiency is relevant to increased risk of neural tube defects [45]. The alteration of 5-M-THF here might advise the effects on nervous system in honey bees.

Several special differential metabolites interest us, such as 10-hydroxy-2E-decenoic acid (10-HDA), 9-oxo-2E-decenoic acid (9-ODA), and serotonin. 9-ODA and 10-HAD are two important components of royal jelly [46,47]. 10-HAD is considered to be important in bactericidal, fungicidal, and antitumor properties [46]. In addition, these two biologically active compounds, 9-ODA and 10-HAD, are also isolated from the queen substance, which are produced by the queen and play important roles in regulating ovarian development of worker bees [48-50]. Interestingly, 9-ODA and 10-HAD were up-regulated by thiacloprid in our study. Serotonin is an important biogenic amine in honey bees. In the past decades, diverse biological functions of serotonin have been found, including regulation of division of labour [51], aggressive behaviour [52,53], and appetitive learning [54,55]. The thiacloprid-induced reduction of serotonin might cause certain behaviour dysregulation in honey bees.

In order to further study the metabolic pathways influenced in honey bees after

thiacloprid exposure, 19 detailed related pathways of the differential metabolites were constructed using KEGG pathway analysis. Among these, two metabolic pathways, glutathione metabolism and glycerophospholipid metabolism were significantly affected ( $p$ -value < 0.05). Glutathione is the most abundant, low-molecular-weight thiol (0.5-10 mM) in plant and animal cells. Previous reviews have showed that glutathione plays an important role in diseases, antioxidant defense, xenobiotics detoxification, and regulation of cellular events [56-59]. In this study, four differential metabolites, including L-cysteine, L-glutamic acid, pyroglutamic acid, and ornithine were found to be involved in glutathione metabolism. L-cysteine is a non-essential amino acid which is synthesized from methionine [60]. In human beings, it plays important roles in detoxification, biologic defenses, signal transduction and diseases [61]. Previous reviews have indicated that pesticide-induced oxidative stress is a possible mechanism of toxicity in animals and insects [62,63]. The up-regulation of the above four differential metabolites, especially L-cysteine, might protect against oxidative stress induced by thiacloprid in honey bees. Glycerophospholipid is the structural components of cell membranes. Four differential metabolites here, including lysophosphatidylcholine (18:1(9Z)) (LysoPC(18:1(9Z))), ethanolamine, phosphatidylethanolamine, and glycerol 3-phosphate, participated in glycerophospholipid metabolism pathway. Previous studies showed that LysoPCs can release a pro-inflammatory fatty acid, arachidonic acid [64], and they were confirmed as etiological factors in certain chronic inflammatory diseases [65]. The thiacloprid-induced up-regulation of a LysoPC, LysoPC (18:1(9Z)), might cause an

inflammatory reaction in the honey bees [66].

In conclusion, a total of 115 differential metabolites, including fatty acids, amino acids, phospholipids, bioamines and other compounds, were identified in honey bee heads after subchronic exposure to 2 mg/L thiacloprid over 3 d, and most of the differential metabolites (83, 72.17%) were up-regulated. KEGG pathway analysis showed that the differential metabolites were mainly enriched in glutathione metabolism, glycerophospholipid metabolism, alanine, aspartate and glutamate metabolism, arginine and proline metabolism, cysteine and methionine metabolism, and D-Glutamine and D-glutamate metabolism, especially glutathione metabolism, glycerophospholipid metabolism pathway were significantly affected. Our results expand the understanding of the molecular basis of the complex interactions between neonicotinoids and honey bees.

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Table 1. The top 20 most significant differential metabolites between thiacloprid-treated bees and control bees.

Metabolites	Fold-change (Thiacloprid/Control)	VIP-value	p-value	up/down
N-epsilon-Acetyl-L-lysine	319.74	1.44355	0.0309	up
D-erythro-sphingosine	61.55	1.49919	0.0276	up
1-Methyladenosine	11.28	2.23262	0.000133	up
L-cysteine	5.79	1.70389	0.0098	up
2-ketobutyric acid	5.6	1.62932	0.0129	up
D-Fructose 1,6-bisphosphate	5.46	1.41985	0.0426	up
N-Ethylglycine	4.90	2.18515	0.000275	up
4-aminobutyric acid	3.75	1.62775	0.0139	up
Leucrose	3.33	1.68801	0.012	up
Guanidinosuccinic acid	3.04	1.74809	0.00827	up
Nicotinoylglycine	2.82	2.17584	0.000288	up
3b,17b-Dihydroxyethiocholane	2.57	1.00091	0.00522	up
hydroxylamine	2.47	1.77626	0.0061	up
PC(12:0/0:0)	2.20	1.12119	0.0263	up
2-hydroxypyridine	2.17	1.99054	0.00137	up
Lactobionic Acid	2.16	1.47804	0.0242	up
PA(O-16:0/20:1(11Z))	2.13	1.4628	0.0125	up
Threitol	2.02	1.6052	0.0169	up
Oleic acid	1.95	2.47232	0.0324	up
Maltotriose	0.18	1.42427	0.0344	down

Fig 1. PCA (A) and PLS-DA (B) score plot of thiacloprid-treated bees and control bees using LC-MS analysis. In the score plot, each data point represents one bee sample, and the distance between points indicates the similarity between samples.

Fig 2. PCA (A) and PLS-DA (B) score plot of thiacloprid-treated bees and control bees using GC-MS analysis. In the score plot, each data point represents one bee sample, and the distance between points indicates the similarity between samples.

Fig 3. Comparison of the metabolomes between thiacloprid-treated bees and control bees using LC-MS analysis. T: thiacloprid treated bees; C: Control bees.

Fig 4. Comparison of the metabolomes between thiacloprid-treated bees and control bees using GC-MS analysis. T: thiacloprid treated bees; C: Control bees.

Fig 5. Pathway analysis of the differential metabolites.

## Highlights

- There are 115 metabolites changed in honey bees after exposure to thiacloprid.
- Thiacloprid significantly affects glutathione metabolism and glycerophospholipid metabolism pathway.
- Thiacloprid might cause behaviour dysregulation in honey bees.
- Honey bees might protect against thiacloprid stress by speeding up metabolism.

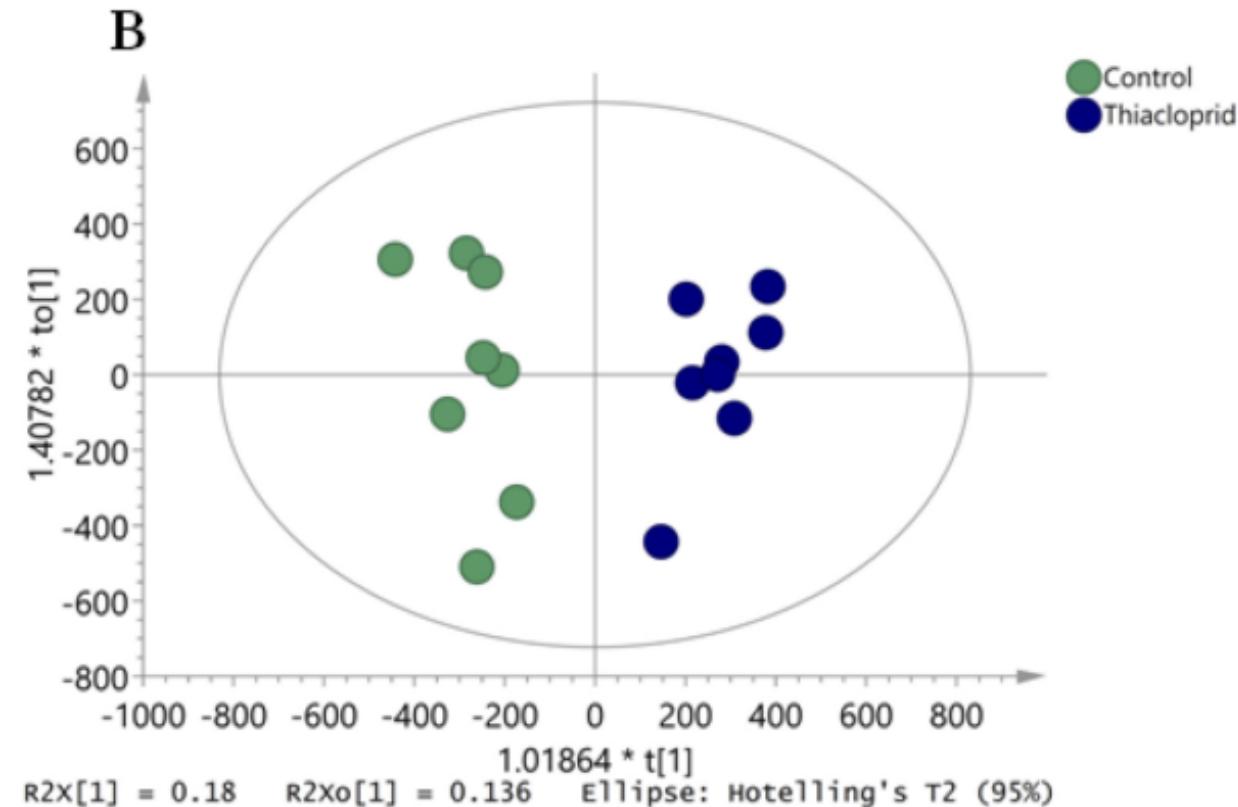
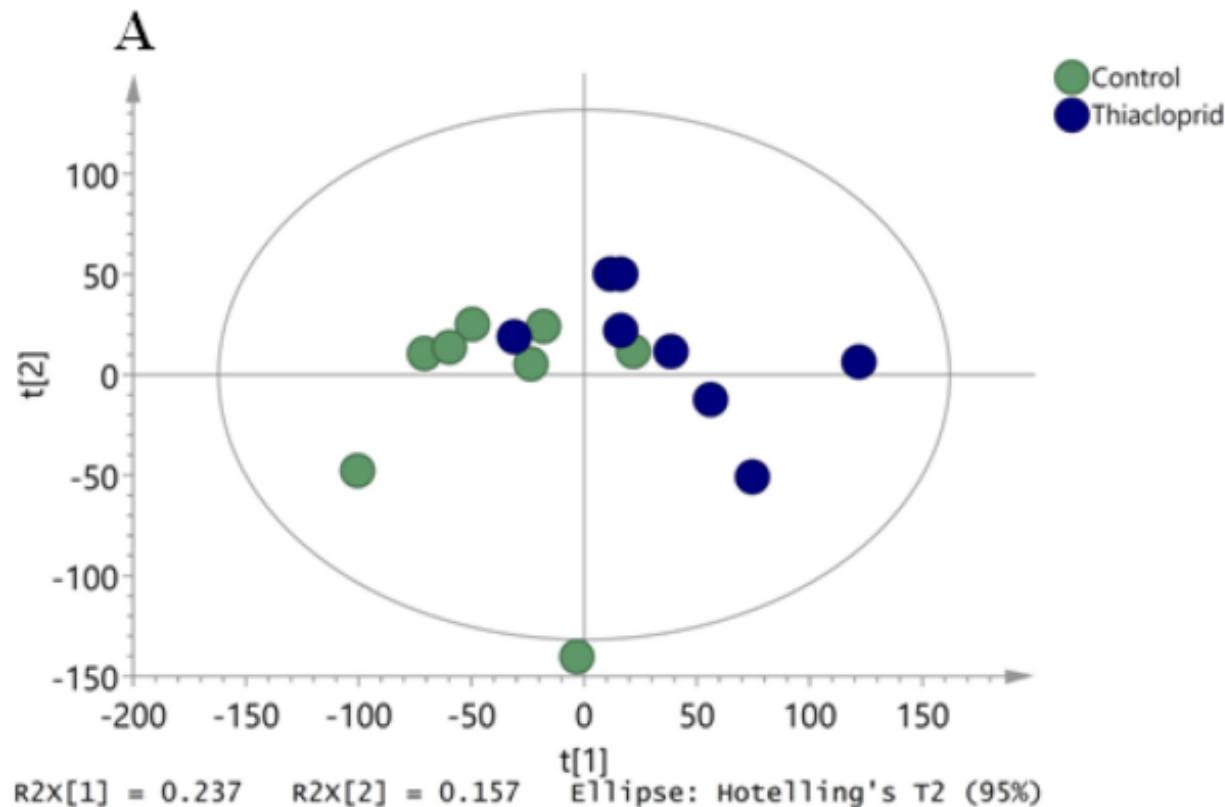


Figure 1

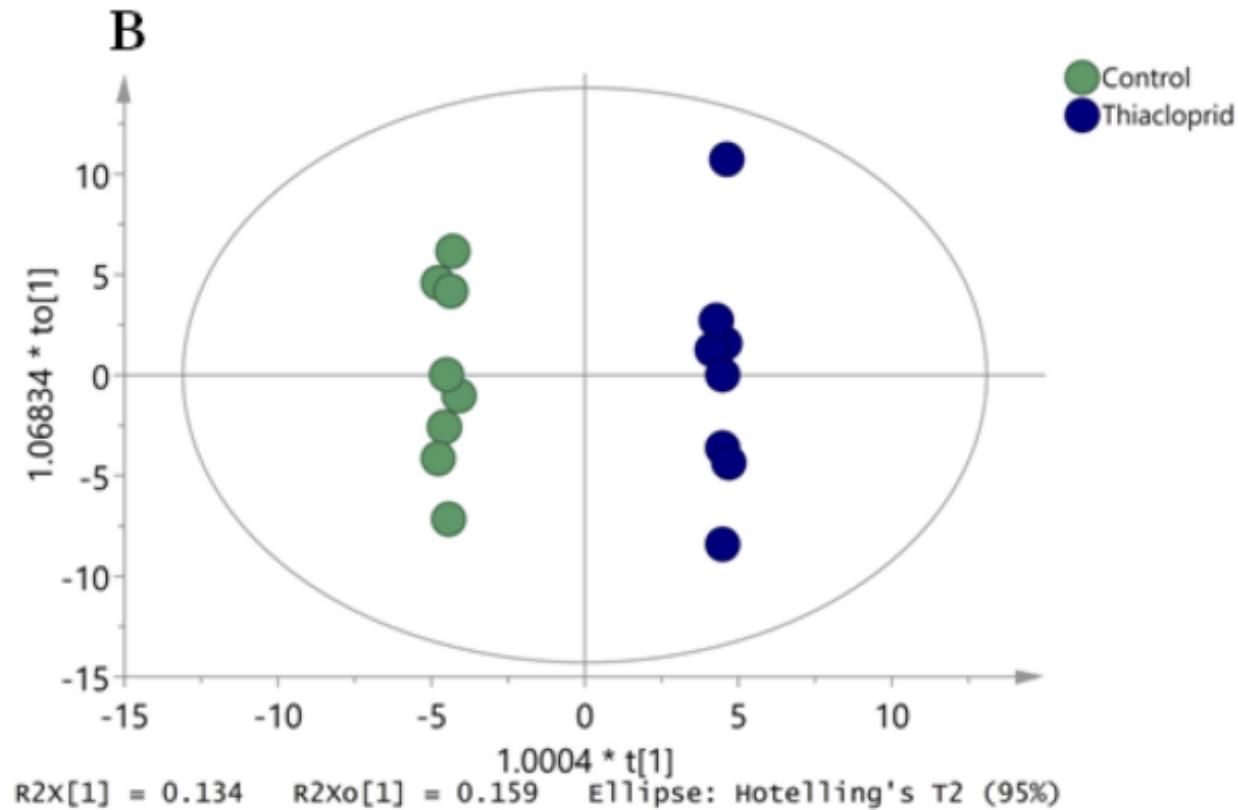
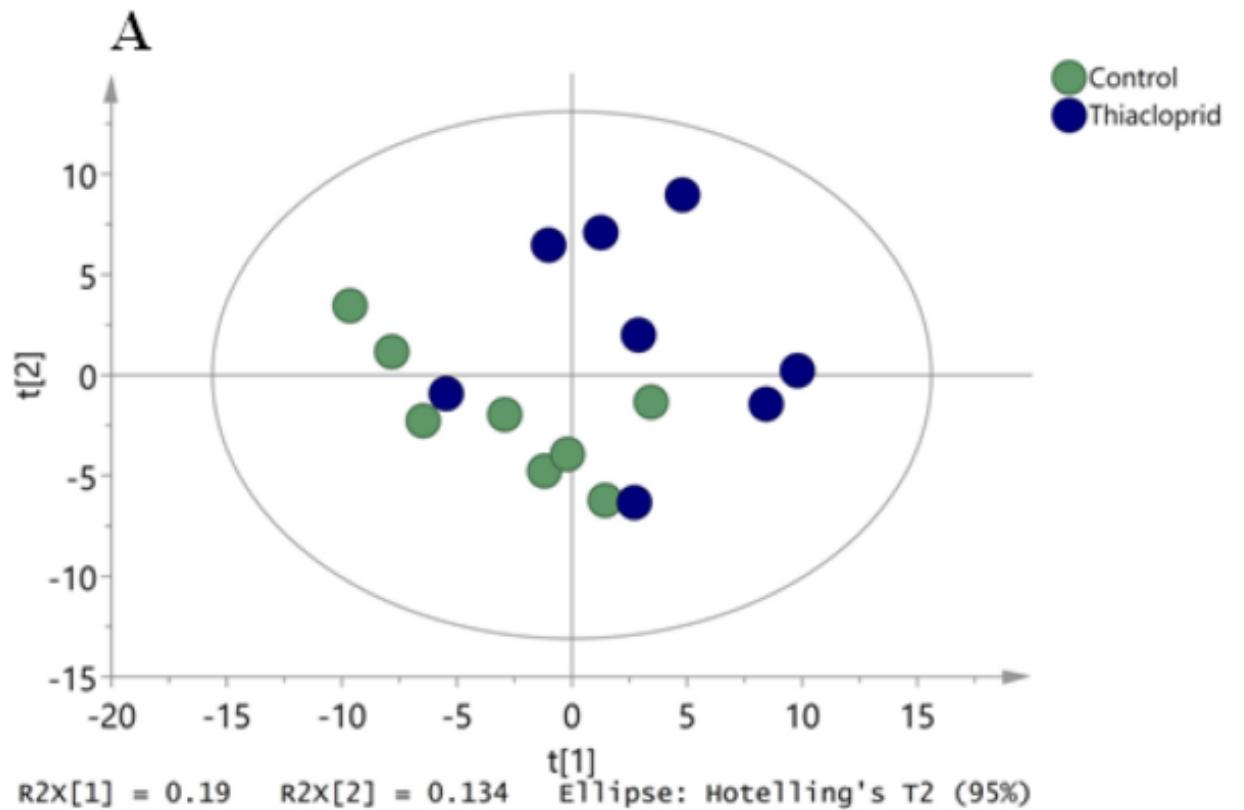


Figure 2

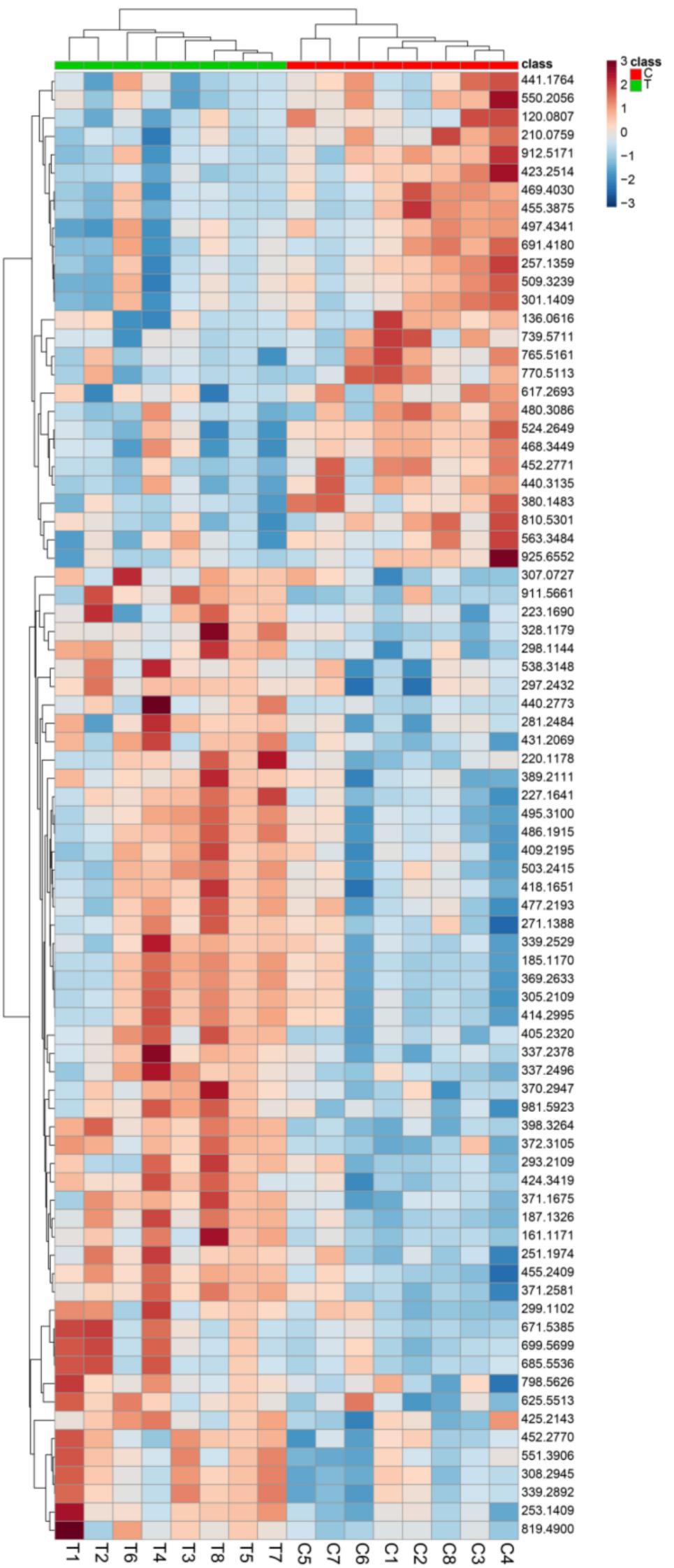


Figure 3

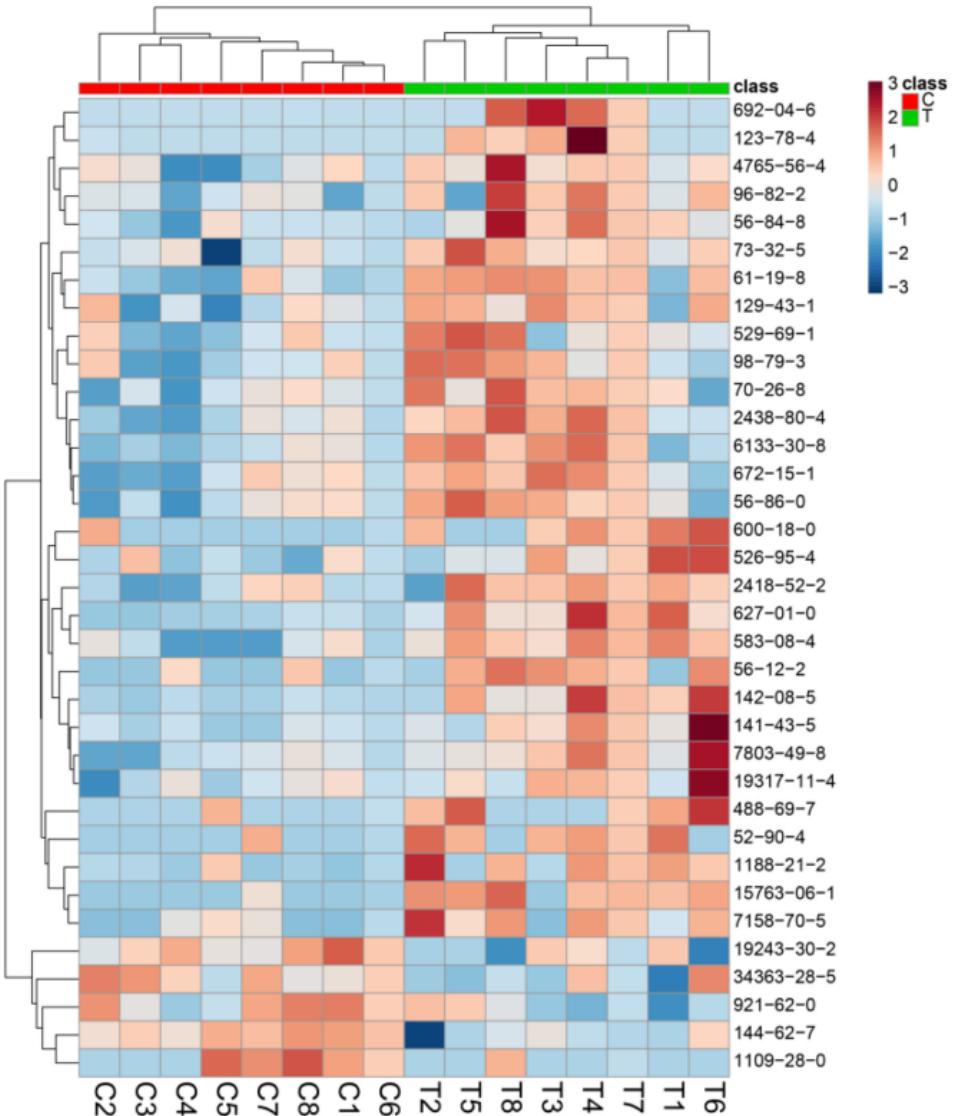


Figure 4

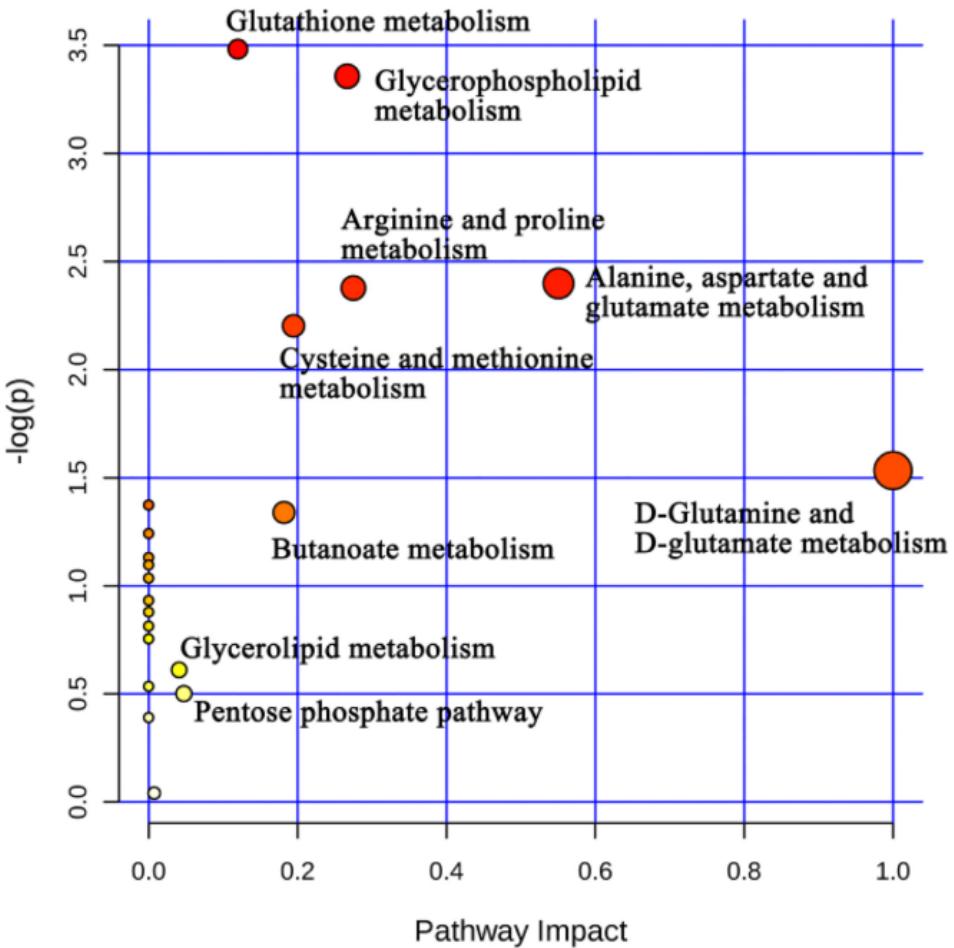


Figure 5