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探討大鼠呼吸暴露奈米氧化鋅微粒的系統性反應

Lipidomic approach to investigate systemic effects of zinc oxide nanoparticles in serum of rats via inhalation

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摘要

美國科學技術委員會（United States National Research Council）指出，奈米技術將成為下一次工業革命的核心，且先前研究指出，奈米顆粒會引發發炎反應，並與心血管等系統性疾病有關。奈米氧化鋅顆粒是台灣工業界最常使用的材料之一經研究發現會引發肺部發炎反應，健康大鼠在暴露低於目前氧化鋅燻煙之容許濃度標準(PEL, 5 mg/m3)之奈米氧化鋅微粒會誘使健康大鼠的急性肺部發炎與傷害以及系統性發炎反應。因此，探討氧化鋅顆粒與系統性生物反應的關係具有研究價值，而生物性系統反應與代謝物在生物中的調節與變化有關，特別是脂質在生物系統中扮演重要的角色，然而脂質體學研究領域中關於氧化鋅顆粒的研究還十分有限。

本研究的目標是，以脂質體學的研究方法探討氧化鋅顆粒的系統生物反應。本研究所分析的樣本是Spraque-Dawley雄性大鼠透過呼吸暴露高、中、低三種濃度與粒徑分別為250 nm、35 nm之氧化鋅顆粒(6hrs/day)後犧牲取得之大鼠血清。經Folch's萃取得到脂類代謝物後以超效能液相層析串聯飛行時間質譜儀進行脂質定性分析，經正交偏最小方差判別分析(Orthoganal partial least square discriminant analysis, OPLS-DA) 結果顯示磷脂醯膽鹼類是貢獻暴露組與控制組間差異的主要脂質；使用超效能液相層串聯質譜儀進行半定量與結構鑑定以探討磷脂醯膽鹼類含量在氧化鋅顆粒暴露與控制組差異及可能的氧化鋅顆粒暴露生物指標。

35 nm氧化鋅顆粒高、中、低暴露組和各劑量控制組的OPLS-DA結果顯示，雙碳鍊磷脂醯膽鹼類 PC(P-20:5/18:0) (*m/z* 792.88)在各劑量組間存在正相關劑量效

應；250 nm米顆粒高、中、低暴露組和各劑量控制組的OPLS-DA結果顯示雙碳鍊磷脂醯膽鹼類 PC(37:1)有可能作為氧化鋅顆粒暴露的系統性反應生物指標。

關鍵字:氧化鋅顆粒、氧化鋅、質譜儀、脂質體學、磷脂醯膽鹼類

ENGLISH ABSTRACT

Nano-sized zinc oxide (ZnO) was widely applied in industrial, and ZnO material is one of the most important nanotechnology products in Taiwan owing to its wide application. However, there are literatures indicating that nano-size particles may induce inflammation, thrombosis and cardiovascular diseases, or even penetrate into systemic circulation; research has found ZnO nano-particles could induce lung inflammation and systemic inflammation responses in animals. Human studies also demonstrated that inhalation of ZnO particles can cause metal fume fever. So far, there is little research using lipidomic approach to investigate the systemic effects of ZnO particles.

To conduct ZnO induced systemic effect, male Spraque-Dawley rats were exposed to high, moderate, or low concentration of either 35 nm or 250 nm ZnO particles via inhalation for 6 hours, and serum samples were collected. After extracted by Folch's method, serum samples were analyzed by ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-Qtof/MS) for lipids profiling; following ultra- performance liquid chromatography triple quadrupole mass spectrometry (UPLC-TQMS) for glycerophosphocholine (PC) profiling, structural identification and semi-quantification. According to OPLS-DA from UPLC-Qtof/MS lipid profiling results, we found PCs are major lipid species that can illustrate the effect of ZnO particle exposure. Combining PC profiling, semi-quantification and structural analysis from UPLC-TQMS, PC(P-20:5/18:0) has positive regulation after 35 nm ZnO particle treatment; PC(37:1) was discovered a negative regulation from OPLS-DA of 250 nm ZnO particle treatment. Other PCs modulations between various doses were also observed in either 35 nm or 250 nm groups.

In conclusion, this study takes a leading role in conducting ZnO particle exposure using lipidomics research approach that has proved PCs demonstrate dose-response potential biomarkers.

Key words: Zinc Oxide particle, Zinc Oxide, Mass Spectrometry, Lipidomics, glycerophosphocholines

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CHAPTER 1

INTRODUCTION

1.1 Zinc-oxide nanoparticles

Nanoparticle Zinc Oxide (ZnO) has been applied in a various of industrial applications, environmental science and medical sciences (Seaton et al., 2005). Same as other oxidized metal such as silver, titanium, carbon and silica; ZnO is one of the commonly used nano-materials based on the statistical survey conducted by consumer product inventory constructed for Project on Emerging Nanotechnologies by the Woodrow Wilson International Center for Scholars (http://www.nanotechproject.org/inventories/consumer/).

Occupational exposure studies pointed out the zinc smelting industrial exposure may induce flu like symptoms, namely metal fume fever (Fine et al., 2000). Researches demonstrated ZnO particle exposure could induce lung inflammation in exposed animals (Kuschner et al., 1997). Due to the characteristics of nano-particles, including high rate of pulmonary particle deposition (Cioffi et al., 2005); traveling from the lung to systemic sites (Chen et al., 2006); dermal barriers penetrations (Lei et al., 2008), entering the brain through olfactory nerves resulting in extensive cellular damages, and a high systemic inflammatory (Seaton et al., 2005).

Ho et al. have used a rat inhalation model to conduct the impact of ZnO particle exposure. For lung injury markers, Ho et al. found significantly higher total protein and lactate dehydrogenase (LDH) in bronchoalveolar lavage fluid *(*BAL) fluid were higher in higher dose ZnO particle exposure groups (either in 35 nm or 250 nm ZnO particle). In systemic effect related marker, they found the number of white blood cells were significantly higher in treatment groups than in control groups. However, there was no difference in other blood (Ho et al., 2011).

Since ZnO is a common metal element on earth's crust, essential mineral in human nutrition and its appearance in ambient air particles from made ZnO environmental exposure an issue to date (Kodavanti et al., 2000; Pagan, Costa, McGee, Richards, & Dye, 2003). As a result, systemic effects due to ZnO particle exposure in organism post a high value for investigation no matter either industrial or environmental exposure perspectives.

1.2 Metabolomics

1.2.1 Metabolomics Introduction

Metabolome consists of a complex set of metabolites, non-genetically encoded substrates, intermediates, and products of metabolic pathways within an organism or biological system (Nielsen et al., 1993). By representing integrative information across multiple functional levels and by linking DNA encoded processes, metabolome offers a window to map core attributes responsible for different phenotypes. Given increasing demand to quantitatively identification of the metabolome and understanding the trafficking of these metabolites throughout the metabolic network impact cellular behavior, metabolomics emerged as an important complementary technique to the cell-wide measurements of mRNA, proteins, and their interactions (e.g. protein-DNA). Metabolomics is known to be a powerful tool in drug discovery and development in metabolic engineering. While maintaining these strengths, the field promises to play a heightened role in systems biology research, which is transforming the practice of medicine and our ability to engineer living organisms.

Coming decades, robust and powerful analytical instrumentations have spurred widespread applications in metabolomic researches (Vinayavekhin et al., 2010). With

innovative methodological, statistical, computational applications, characterizing the plethora of low molecular weight endogenous metabolites within a biological sample enabled researchers to discover related biomarkers; study the underlying physiological or pathological mechanistic (Vinayavekhin et al., 2010).

1.2.2 Analytical Methods

The metabolic fingerprints of bio-fluid obtained by mass spectrometry- (MS) or nuclear magnetic resonance (NMR)- based profiling contain hundreds to thousands of metabolic signals contributed both by genetic and environmental (Roux et al, 2011). However, accurate identification and sufficient separation are required for metabolomics analysis owing to the complexity and diversity of metabolites, such as (Li et al, 2011). Liquid chromatography (LC) coupled with MS technology has a lot of advantage in biological field over NMR due to great sensitivity in MS and less amounts of sample required (Fiehn, 2002; Roux et al., 2011). Electrospray techniques combined with LC technology applied, which gave good sensitivity, high dynamic range and versatility to provide access to large range of biological molecules (Roux et al., 2011). All the advanced technology mentioned above, bringing us one of most the widely used analytical methods in metabolomics to date— ultra performance liquid chromatography (UPLC) coupled with MS system, which have improved chromatographic resolution, peak capacity and sensitivity. UPLC coupled with high-resolution mass spectrometers such as time-of-flight (tof)/MS tended to enhance the peaks' resolutions (Wilson et al., 2005), became a powerful tool in systems biology, functional genomics, and biomarker discovery studies among others (Bino et al., 1988; Katajamaa et al., 2006).

1.2.3 Data analysis

Highly complex data needs proper processing to help us to understand how the endogenous molecules and metabolites may affect external perturbation. In metabolomics research (Goodacre et al., 2004), unsupervised methods such as principal components analysis (PCA), supervised analysis like partial least-square-discriminate analysis (PLS-DA) and orthogonal PLS-DA (OPLS-DA) are major concepts of data analyzing to have data summarized and visualized (Wiklund et al., 2008).

PCA reduces the complex metabolomics data dimension while retain as much variation as possible (Barker et al., 2003). Variables that illustrating PCA

patterns are integrated into two or more principal components in the scores plots. Variables that have the strongest power to explain the variation between treatment groups or control groups are defined as principal component one. In other words, variables in principle component one have stronger abilities to explain the pattern on PCA scores plots than principle component two's (Scholz et al., 2005).

Both the PLS-DA and OPLS-DA models provide powerful tools to successfully fit and yield good diagnostics in (Sadeghi et al., 2011). PLS-DA allows collinear investigation in X-variables while classification in Y-variables (dependent variables) of complex data. PLS-DA is used for finding latent variable with a maximum covariance in predictor variables (i.e., X, - variables) and response variables (i.e., Y, -classes) where these latent variables are linear combination of original X-variables. PLS-DA is used for detecting hidden variables which contribute to class separation (Eriksson et al., 2001).

While, the OPLS-DA proved to be preferable model where it has better interpretability compared to PLS-DA. Since OPLS-DA solved the problem in PLS models in interpretation, which is due to increases in proportion to the amount of Y-orthononal variation present in X, systematic variation from the matrix X that is unrelated (orthogonal) to the response matrix Y (Sadeghi et al., 2011). As a result, OPLS-DA may perform more ideal visualized patterns in data analysis.

1.3 Lipidomics

1.3.1 Lipidomics Introduction

A comprehensive analysis of lipid molecules is now called lipidomics (Fahy et al., 2005). Lipidomics, part of metabolomics, is defined the characteristics of different lipid species and their physiological functions in cellular systems (Spencer et al., 2003).

In a functional cellular system, thousands of lipids pose important and distinguishing roles. Specific properties of cellular membranes are determined by a certain lipid molecular composition to maintain physiological (Bielawski et al., 2010). In presence, specific lipids in the cell may pose an impact on life processes, where the condition, state, and dysfunction of a cell can be detected via lipid composition changes. (Bielawski et al., 2010; Halket et al., 2005; Sullards, Wang, Peng, & Merrill, 2003).As a result, analyzing the composition of lipid species has become a trendy methods in biochemistry, and lipidomics has become a major research target of postgenomics revolution and system biology (Fahy et al., 2005).

Lipidomics research includes the identification and quantification of numerous cellular lipid molecular *species* and their interactions with other species in cellular

systems. To understand the relationships between lipid molecular species, comprehensive understanding of lipids classification is required. The most commonly used classification of lipids proposed by Fahy et al. , divides lipid into eight categories with fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR), saccharolipids (SL), and polyketides (PK). And each category consists of diverse classes and subclasses (Fahy et al., 2005) (Fig. 1).

Analytical strategy of lipid molecules to do lipidomics research can be divided into untargeted lipidomics, focused lipidomics, and the target lipidomics. In this study, UPLC-Qtof/MS was applied to untargeted lipidomics approach, and triple quadrupole (TQ) MS coupled with UPLC was used for focused lipidomics that suggested from UPLC-Qtof/MS (Nalatie et al., 2009).

1.4 Proposed Studies

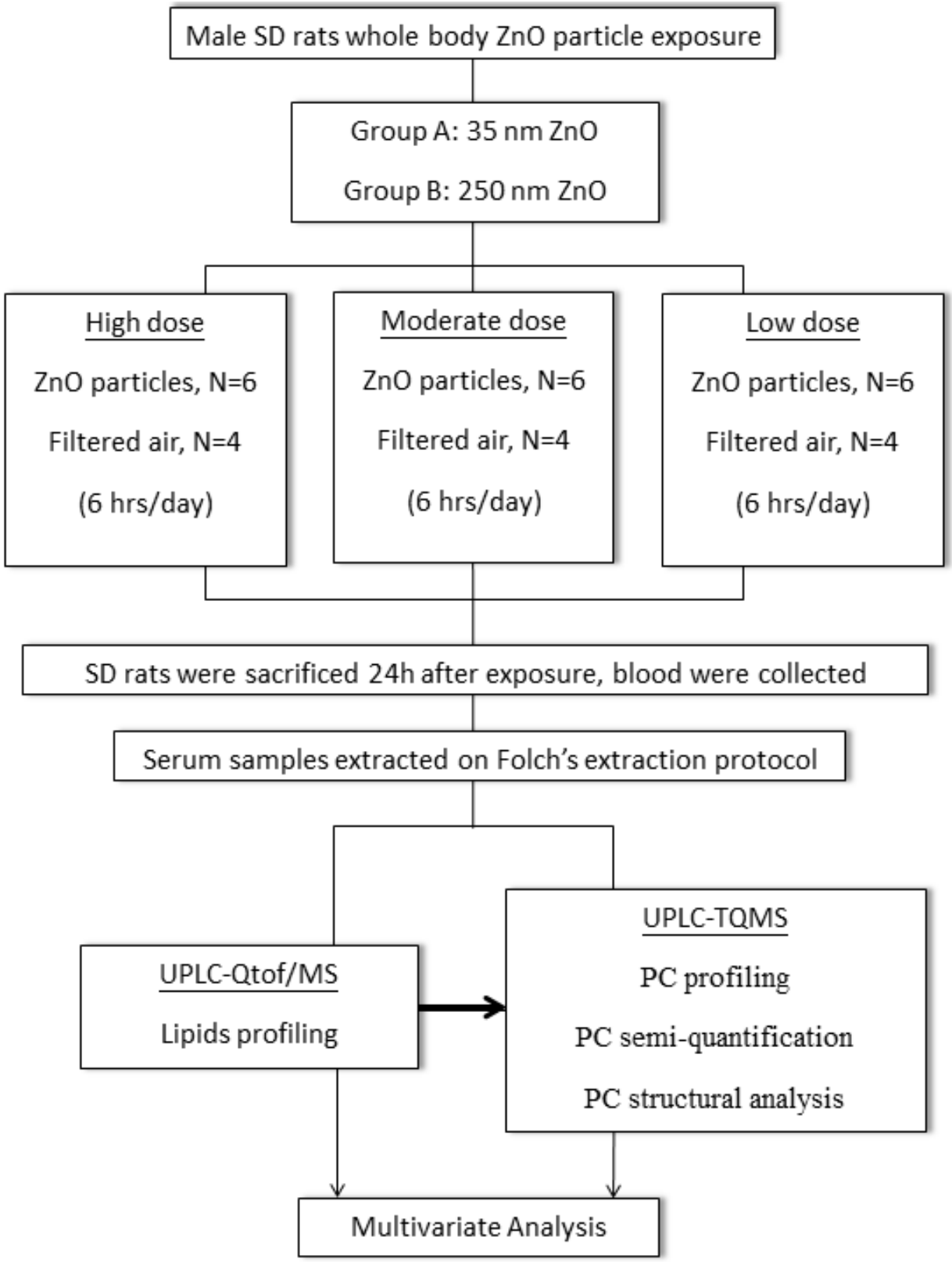
Since there were literatures suggesting the adverse health effects of ZnO particles, I intend to examine the systemic lipid perturbation caused by ZnO particles using a rat inhalation model. Throughout this research, potential ZnO systemic markers which are able to illustrate the overall lipid responses post ZnO exposure could be identified. Furthermore, the detected lipids will undergo a series of structural analysis to confirm the lipids structure.

To conduct ZnO induced systemic effect, rats were exposed to either 35 nm or 250 nm ZnO and serum samples were collected for lipid analysis. UPLC-Qtof/MS was applied for metabolites profiling, suggesting important lipid category to UPLC-TQMS for following up distinct lipid category semi-quantification and structural analysis. To achieve the goal to determine nano- (35 nm) and fine- (250 nm) particles of ZnO induced lipid turbulence biomarkers.

CHAPTER 2

MATERIALS AND METHODS

2.1 Experiment Design



2.2 SD rats ZnO particle exposure experiment

2.2.1 Particle Generation

The particle generator was originally designed by Energy & Environment Research Laboratories, Industrial Technology Research Institute of Taiwan, R.O.C. (http://www.itri.org.tw/eng/). The system generates particles under the concept of "furnace flow reactor" and evaporation-coagulation mechanism, which is similar with the concept described by Fine et al (Fine et al., 1997). Figure 2 shows the schematic representation of the nanoparticle generation and exposure system, the zinc powder was heated in furnace to turn into zinc vapor and carried by nitrogen gas to react with oxygen yield to generate ideal size ZnO nano-particles (35 nm) and ZnO fine particles (250 nm) and diluted with filtered air before entering the whole body exposure chamber (Maciejczyk et al., 2005). Operation parameters were shown in Table 1. Average ZnO particle exposure condition were shown as Table 2 (Ho et al., 2011). Transmission electron microscope (TEM, JEOL JSM-1200EX II) was used to observe particle morphology in animal exposure experiments. Particles synthesized in the low-, moderate- and high-groups were collected and pooled to determine the material's purity by X-ray diffraction (XRD, RICAKU, D/MAX-2200) (Ho et al., 2011).

2.2.2 Animals handling

All the animal experiments were approved and conducted in accordance with the National Taiwan University's Institutional Animal Care and Use Committee guidelines on animal care. The research was conducted in conformity with the Public Health

Service Policy on Humane Care and Use of Laboratory Animals. Seven-week old Spraque-Dawley (SD) rats were acclimated for one week before exposed to ZnO. They were housed in plastic cages (three rats per cage) on chip bedding, provided with Lab Diet 5001 and water in a room with controlled temperature (23 ± 1℃) and humidity (55 ± 10%) with a 12-h light/dark cycle. During twenty-four hours post whole-body inhalation exposure, rats were anesthetized using pentobarbital (50mg/kg). Whole blood was recovered from the aorta. Following centrifuged at 2500 g at 4℃ for 10 min. The entire upper transparent layer (serum) was collected for further analysis (Ho et al., 2011).

2.3 Sample Preparation

Serum samples were followed by lipid extraction based on Folch's protocol (Folch et al., 1957) with appropriate modifications. Fifty μL of rats' serum sample was transferred to a glass tube and 6 mL extraction solvent (Chloroform: Methanol: H_2O=8:4:3). The mixture was vortexed for 30 seconds and went through a 30 minutes centrifugation which is set to 2500 rpm under 4℃. The entire lower layer was transferred and vaporized in room temperature. These lipid extracts were kept frozen at -80℃ until further analysis.

Summary of samples is as following. Table 3 summarizes the experiment dates, numbers of SD rats used and final analyzed sample numbers of each treatment and control groups. Total of 58 samples were detected based on quadruplicate on each sample in UPLC-Qtof/MS analysis; triplicate on each sample in UPLC-TQMS analysis.

2.4 UPLC-Qtof/MS

2.4.1 Chromatography

Reversed-phase liquid chromatography was performed on Waters ACQUITY UPLC Ultra Performance LC system. Reverse-phase column, BEH C_8 column 2.1 x 100 mm, 1.7 μm was applied for the analysis. During the entire analysis, the column temperature was set at 45°C. A solvent gradient system was applied in the analysis where the solvent A consists of water with 2 mM ammonium added and solvent B, acetonitrile with the flow rate 0.5 mL/min. The gradient elution, solvent B was programmed to increase from 40 % to 80 % in 2 min, then to 99 % in 6 min, and hold at 100 % for 8.4 min, decrease from 99 % to 40 %and hold at 40 % for 1.5 minutes.

2.4.2 Qtof/MS

The UPLC system was coupled to Waters SYNAPT high definition mass spectrometry (HDMS) Qtof/MS, equipped with an electrospray ionization (ESI) source. The ESI source was tuned to acquire maximum precursor ions, and positive ion mode was adopted in this study. The capillary and cone voltage were set at 3.0 kV and 35 V respectively. The desolvation gas temperature and flow rate was set at 300 °C and 0.7 mL/min respectively. The source temperature was conducted at 80 °C. The Qtof/MS was operated over a mass range of *m/z* 20 to *m/z* 900 was carried out. Spectrum data were collected using Markerlynx V 4.1 software.

2.5 UPLC-TQMS

2.5.1 Chromatography

Reversed-phase liquid chromatography was performed on Waters ACQUITY UPLC system. Reverse-phase column, BEH C_{18} column 2.1 × 100 mm, 1.7 μm was applied for the analysis. During the entire analysis, the column temperature was set at 70°C. A solvent gradient system was applied in the analysis where the solvent A consists of water with 10 mM ammonium acetate and solvent B, acetonitrile/methanlol (65/35 v/v) with 1% 1.0M ammonium acetate added. The flow rate 700 L/hr was applied during the entire sample analysis. The gradient elution, solvent B was programmed to increase from 35 % to 70 % in 0.1 min, then to 100 % in 1.4 min, and hold at 100 % for 4.5 min, decrease from 100 % to 35 %and hold at 35 % for 4 minutes.

2.5.2 TQMS

2.5.2.1 PC profiling

Mass Spectrometry was performed on a Waters Quattro Premier XE TQMS System (Waters, Milford, MA, USA) operated in positive ion mode. The capillary and cone voltage were set at 2500 V and 35 V respectively with collision energy of 30eV. The desolvation temperature was set to 450 °C and ion source temperature to 120 °C; the desolvation gas was set to a flow rate of 0.7 mL/min and cone gas flow was set to 50L/h; acquisition range was set from *m/z* 200 to *m/z* 900 (Tang et al., 2011).

2.5.2.2 PC structural identification

According to the nature of the phosphocholines head group, which was the formation of *m/z* 184 , preliminary PC profiling was underwent under precursor ion scan of *m/z* 184 under $[M+H]^+$ mode (Cheng et al., 2011). Based on the preliminary phosphocholines profiling, constructed by the pair of m/z values and retention times, the product ion spectra was acquired for the description for the molecular structure determination. LysoPCs are usually eluted before two minutes in the UPLC system in our study and PCs are usually late than 2 minutes (Tang et al., 2011) so that lysoPC can be distinguished from PC based on the retention time.

For further PC structure illustration, the product ion spectra of $[M+Na]^+$ was employed to determine the PC subclass (Tang et al., 2011). Each PC subclass had common product ions *m/z* 86 (vinyltrimethylamine) and *m/z* 147 (sodiated five-member cyclophosphane) in the spectrum acquired form $[M+Na]^+$ fragmentation. However, The relative intensity of $[M+Na-183]^+$ ($[M+Na-HPO_4(CH_2)_2N(CH_3)_3]^+$)and$[M+Na-205]^+$ ($[M+Na-NaPO_4(CH_2)_2N(CH_3)_3]^+$) ions were observed (Tang et al., 2011) for PCs subclasses divisions. In diacyl-PC product ion spectra, dominant of [M+Na-183]+ with minor $[M+Na-205]^+$ were observed; alkylacyl-PC and alk-1-enylacyl-PC sodium adducts pose relative minor intensity of $[M+Na-183]^+$ and $[M+Na-205]^+$ ions compared with diacyl-PC. But $[M+Na-183]^+$ and $[M+Na-205]^+$ ions relative intensities are still different. For alk-1-enylacyl-PC sodium adducts intensities, $[M+Na-183]^+$ are often higher than $[M+Na-205]^+$ ions; alkylacyl-PC in contrast (Tang et al., 2011).

The fatty acid substituent on sn-1 or sn-2was decided via product ion spectrum of $[M-Me]^-$. In addition, the carbon number and the degree of unsaturation could be illustrated in $[M-Me]^-$ product ion scan .(Tang et al., 2011)

2.6 Spectral processing

2.6.1 UPLC-Qtof/MS spectral processing

The parameters were set in Markerlynx V 4.1 (Waters, CA, USA) pertain to the peak detection algorithm, alignment of detected. Parameters are related to peak properties. In this study, peak width was at 5 % height is 12, MS detection range was *m/z* 20 to *m/z* 999 with mas window 0.05 Dalton and mass tolerance 0.01. Peak to peak base-line baseline was set to 200, intensity threshold is 10 counts. Retention time window was 0.2 second, acquired by the liquid. Noise elimination level is at 6. Smoothing and data deisotope were applied in UPLC-Qtof/MS spectral processing.

2.6.2 UPLC-TQMS spectral processing

Acquired raw data must be transferred into NetCDF in Masslynx V4.1 (Waters, CA, USA). Converted file will then re-import to non-commercialized software, MZmine 2.9.1for peak detection, peak list alignment and normalization.

The UPLC-Qtof/MS data preprocessing tool, MZmine main modules are raw data processing, peak detection, peak list alignment and normalization (Briones et al., 2010). Raw data processing includes smoothing and filtering methods; peak detection includes chromatography construction, peak deconvolution, peak remodeling; peak list alignment includes join aligner using retention time and *m/z*; using linear normalization in normalization procedure in my study (Pluskal et al., 2010).

2.7 Data Analysis

Multivariate analysis of UPLC-Qtof/MS data were processed with Markerlynx V 4.1 (Waters, CA, USA) to obtain unsupervised PCA and supervised OPLS-DA results; UPLC-TQMS data were processed with Simca-P^+13.0 (Umetrics, Inc., Umeåa, Sweden. In PCA and OPLS-DA modeling; $R^2X[1]$ and $R^2X[2]$ are assessed the goodness of fit; Q^2 is assessed the predictive power of the model, Q^2 measures over 0.5 represent much more reliable in analysis models (Muñoz et al.1997). Variable importance in the projection can be assessed by (*variable i*mportance for projection) VIP measures to validate the influence of variables (markers that are interested in studies). Lipids with top 50 highest VIP measures were collected in each group to analyze the potential dose-response biomarkers and to process semi-quantification. The semi-quantification values are all given as mean ± SD (standard deviation). And the difference of between semi-quantification values of treatment and control were examined with t-test using SPSS Statistics 17.0 software. To obtain further understanding of the interactions between metabolites and ZnO various exposure treatments, Venn diagram is used for obtain intersection set which contain metabolites that are able to describe the fold-change (metabolites average concentration of treatment / control) variation in 3 doses treatment ZnO exposure groups (Muller et al., 2005).

CHAPTER 3

RESULTS

3.1 UPLC-Qtof/MS analysis on systemic effects of ZnO on rat

Serum samples of rat exposed to ZnO particles were analyzed using UPLC-Qtof/MS analysis method which is described in chapter 2. And the total ion current chromatography is shown as Figure 26.

3.1.1 Effect of 35 nm ZnO exposure on lipid profiling of rat serum

PCA scores plot illustrated various metabolome among treatments and controls SD rats' serum sample lipid profiling analysis results of high, moderate and low 35 nm ZnO particle dosage groups. We have discovered a trend of separation, t[1] (first components) scores of the treatment are tend to be larger than their control (Fig. 3) and 223 analytes were isolated from UPLC-Qtof/MS spectra of PCA score plot (Table 4).

However, the pattern controls of 3 dosage is separated so that I used OPLS-DA to conduct lipids profiling differences between each 35 nm ZnO treatment group (Fig. 4~Fig. 6). Lipids with top 50 VIP values were selected and to generate intersection set using Venn diagram, where the common metabolites from different analysis could be identified (Fig. 7).

Lipid selected based on VIP value were further analyzed by t-test to examine the average concentration differences between treatments and controls. By combining the results of VIP values and t-test, important lipids that illustrate significant differences of lipids profiling in each dose treatment were generated (Table. 5~7).

According to OPLS-DA between the control and high dosage of ZnO particle exposure (Fig. 4), PCs were selected accounted for the majority in the selected metabolites based on VIP, presenting 32 PC and lysoPC in a total 48 kinds of

metabolites suggested based on VIP. Among 48 lipids, significant (p< 0.05) up regulations were observed in PC(34:1), PC(16:0/16:0), PC(37:4), lysoPC(18:0), lysoPC(20:1), tetracosanoic acid, PE(42:4), PC(37:4), and huperzine B. Contrary, significant (p<0.05) down regulations were observed in PC(38:3), lysoPC(18:2), lysoPC(18:1), lysoPC(22:6), lysoPC(16:1), lysoPC(22:5), and N-Lignoceroylsphingosine (Table 5).

Based on OPLS-DA of moderate dose ZnO exposure group (Fig. 5), 29 PCs and lysoPCs in a total 47 kinds of metabolites were suggested based on VIP in 35 nm ZnO moderate dosage group. Among 47 lipids, significant (p<0.05) up regulations were observed in PC (42:9), 1alpha,25-dihydroxy-2alpha-(3-hydroxypropoxy)-19-norvitamin D3 32,35-anhydrobacteriohopaneterol, goyaglycoside, GPSer(28:0), hericene A, and sabadelin. Contrary, significant (p<0.05) down regulations were observed in PC(18:3), PC(34:1), PC(36:3), PC(36:1), PC(32:1), PC(36:5), PC(38:2), PC(33:1) and PC(40:7) (Table 6).

The OPLS-DA in low dose ZnO exposure group (Fig. 6), PC metabolites was the majority of selected metabolites with the top 50 VIP values. There are 17 PC and lysoPC molecules in a total 47 kinds of metabolites suggested based on VIP. Among 47 lipids, significant (p<0.05) up regulations were observed in PC(38:4), PC(36:2), PC(37:4), PC(40:7), PC(36:4), PC(38:3), PC(36:0), TG(48:3), and SM(40:1) . Contrary, significant (p<0.05) down regulations were observed in PC(34:2), PC(34:1), PC(36:1), PC(33:2), lysoPC(18:1), 1-tetradecanyl-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine (Table 7).

3.1.2 Effects of 250 nm ZnO exposure on lipid profiling of rat serum

PCA scores plot illustrates various metabolome among controls of 3 doses of 250 nm ZnO. Figure 8 shows the pattern of lipids on PCA scores plot. We have discovered a that moderate groups are separated from high and low dosage groups on t [1] (first components) (Fig. 8), and controls of 3 dosages groups are also divided. By comparing the treatment to its own control, 270 markers were selected from the results of PCA from the analysis of UPLC-Qtof/MS (Table 9). To obtain more details on variables that each treatment group (high, moderate or low doses), OPLS-DA were used to extract important lipids that differentiate treatment and control groups (Fig. 9~Fig. 11). Lipids with top 50 VIP values were selected from each treatment (Table 10~12) to generate intersection set using Venn diagram. Lipids in intersection set were evaluated the average concentration fold-changes.

17 PCs included lysoPC from a total 50 kinds of metabolites suggested based on VIP values generated from OPLS-DA in high dose treatment group (Fig. 9). Among 50 lipids, significant (p<0.05) up regulations were observed in PC(36:2), PC(40:6), PC(36:1), DG(40:8), DG(44:1), PE(28:0), DG(36:4), 1-tetradecanyl-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine, arachidonic acid, ajmaline, DG(36:2), DG(38:7), retinoic acid; significant (p<0.05) down regulations were observed in PC(34:3), PC(33:0), PC(32:0), PC(38:3), anhydroeschscholtzxanthin, 1alpha,3alpha-Dihydroxy-5beta-cholan-24-oic Acid, 17a-Ethynylestradiol, PC(19:0)(Table. 10).

According to OPLS-DA in moderate dose ZnO exposure group (Fig. 10), PCs metabolites were major in selected lipids based on VIP values. 22 PC included lysoPC from a total 50 kinds of metabolites suggested based on VIP. Among 50 lipids, significant (p<0.05) up regulations were observed in PC(16:0/16:0), PE(38:3),

citramalic acid; significant (p<0.05) down regulations were observed in PC(38:3), PC(34:3), lysoPC(22:6), 1alpha,3alpha-Dihydroxy-5beta-cholan-24-oic Acid, arachidonic acid, PE(28:0), tetracosanoic acid, 3-Hydroxy-linoleyl carnitine, PC(42:1), 3-Carboxy-4-methyl-5-pentyl-2-furanpropionic acid (Table 11).

Table 12 shows 21 metabolites with top 50 values from OPLS-DA of low dosage group of 35 nm ZnO (Fig. 11). 21 PC and lysoPC in a total 47 kinds of metabolites suggested based on VIP. Among them, significant (p<0.05) up regulations were observed in PC(36:4), PC(40:8), PC(38:3), Lactosylceramide (30:1), PE(42:4), tetracosanoic acid were listed in table 12; T-test was used to test the significance of the average concentration differences between treatments and controls from each dose. Significant (p<0.05) down regulations were observed in PC(36:2), PC(38:3), PC(36:3), lysoPC(18:2), lysoPC(18:1), lysoPC(20:2), lysoPC(20:3), 1alpha,3alpha-Dihydroxy-5beta-cholan-24-oic Acid, PE(38:5), dihydrothymine, tetradecanedioic acid,tetradecenoyl carnitine,fexofenadine.

3.1.3 Markers with dose-response identified from UPLC-Qtof/MS

A. Markers with 35 nm ZnO dose-response

There are 16 metabolites collected in Venn diagram intersection set generated from lipids suggested from OPLS-DA VIP values of high, moderate and low concentration 35 nm ZnO particle exposure groups , which contain metabolites that are able to describe fold-changes (treatment/ control) variation between high, moderate and low dosage treatment groups (Fig. 7). Table 8 shows the 16 potential 35 nm ZnO particle exposure bio-markers in SD rat serum. Except lactosylceremide (30:1), TG (48:3), PC and lyso PC are the most.

B. Markers with 250 nm ZnO dose-response

To further explore the PC effects of ZnO particles, OPLS-DA results were collected to conduct common lipids for a further illustration of dose-response relationships between each dose treatment and lipids using Venn diagrams (Kestler et al., 2008). Intersection set in Venn diagram resents 14 lipids that commonly existed in high dose, moderate dose and low dose were able to describe dose response between the 3 various doses treatment groups (Fig. 12). Table 13 shows the dose response relationship of 14 lipids. Except lactosylceremide (30:1), TG (48:3), anhydroeschscholzxanthin, 1alpha,3alpha-Dihydroxy-5beta-cholan-24-oic Acid, tetracosanoic acid , PE(38:5), PC and lyso PC are the majority in intersection set of 3 doses treatment groups.

3.2 UPLC-TQMS analysis on systemic effects of ZnO on rat

Serum samples of rat exposed to ZnO particles were analyzed using UPLC-TQMS analysis method which is described in chapter 2. And the total ion current chromatography is shown as Figure 28.

3.2.1 Effect of 35 nm ZnO exposure on PC profiling of rat serum

PCA scores plot from analysis of UPLC-TQMS spectra demonstrates large sample variation in the high-dose group. (Fig.13). To reduce the variation from each animal experiment, OPLS-DA were used to extract important lipids that differentiate each treatment (high, moderate or low doses) and control group (Fig. 14~Fig. 16). VIPs of each 3 various doses OPLS-DA results were collected to conduct common lipids for a further illustration of dose-response relationships between each dose treatment and lipids (Fig. 17).

Lipids selected sased on VIP values from OPLS-DA results, t-test were employed on each selected VIPs to examine semi-quantification differences of lipids with top 50 VIP values from OPLS-DA results (Table. 14~16).

According to OPLS-DA in high dose ZnO exposure group (Fig. 14), PCs metabolites were major in the most selected metabolites suggested based on VIP. There are 18 identified PC and lysoPC metabolites (Tang et al., 2011). Significant (p<0.05) up regulations were observed in alk-1-enylacyl-PC, PC(16:0/22:1), PC(P-38:3), PC(18:0/18:0) and an unknown PC m/z 818.61; significant (p<0.05) down regulations were observed in PC(17:0/18:0), PC(18:1/22:6), PC(18:0/22:6) and unknown PCs of *m/z* 835.04, *m/z* 546.44, *m/z* 522.63, *m/z* 867.08 and *m/z* 746.98 (Table 14).

Selected metabolites based on VIP values from OPLS-DA in moderate dose ZnO exposure group (Fig. 15), PCs metabolites were major in the most selected. There are 22 identified PC (PCs) and lysoPC metabolites (Tang et al., 2011). Significant (p<0.05) down regulations were observed in PC(16:0/0:0),PC(16:0/16:0), PC(16:0/18:2), PC(16:0/20:4), PC(20:3/0:0), PC(22:0/18:1), PC(a21:2/0:0), unknown PC of *m/z* 806.92, *m/z* 839.15 and m/z 856.85 (Table 15).

PCs metabolites were also major in the most selected metabolites suggested based on VIP from OPLS-DA in low dose ZnO exposure group (Fig. 16). There are 24 identified PC and lyso PC metabolites (Tang et al., 2011). Significant (p<0.05) up regulations were observed in PC(16:0/20:3), PC(P-38:3), PC(18:0/20:3), PC(18:1/22:6), PC *m/z* 770.992, known PC of *m/z* 818.21 and m/z 843.9; significant (p<0.05) down regulations were observed in PC(18:0/18:1), PC(P-20:5/18:0) and unknown PC m/z 819.01 (Table 16).

3.2.2 Effects of 250 nm ZnO exposure on PC profiling of rat serum

PCA scores plot generated from 250 nm ZnO particle exposure groups did not show significant separation (Fig. 20). To obtain a more detailed factors that differentiate 3 various doses treatment groups (high, moderate or low doses), OPLS-DA were used to extract important lipids that differentiate treatment and control groups (Fig. 21~Fig. 23), and lipids selected based on VIP values of each 3 various doses OPLS-DA results were collected to conduct common lipids from Venn diagram (Fig. 24).

Lipids selected based on VIP values were further analyzed by combining the results of VIP values and using t-test to exam significant differences of average concentration in important lipids in each doses treatment and control groups (Table 18~20).

There are 16 identified PC and lysoPC (Tang et al., 2011) in OPLS-DA result of high dose ZnO exposure group. Significant (p<0.05) up regulations were observed in unknown PC *m/z* 822.86; significant (p<0.05) down regulations were observed in PC(18:0/18:0), PC(a16:0/16:0), PC(18:0/18:1), unknown PC of *m/z* 815.11, *m/z* 845.02, *m/z* 818.11, m/z, *m/z* 789.1, *m/z* 844.97 and *m/z* 793 (Table 18).

According to OPLS-DA in moderate dose nZnO exposure group, PCs metabolites were major in the most selected metabolites. There are 23 identified PC and lysoPC metabolites (Tang et al., 2011). Significant (p<0.05) up regulations were observed in PC(16:1/22:6), PC(18:0/18:0), PC(18:0/22:4), PC(18:1/22:6), PC(22:5/18:0), PC(a18:0/16:0), PC(a25:5/15:1) and PC m/z 799.407 and unknown PC of m/z 722.9, m/z 818.11, m/z 835.04, m/z 776.99, m/z 764.96; significant (p<0.05) down regulations were observed in PC(16:1/22:6) (Table 19).

PCs (PCs) metabolites were also major in the most selected metabolites based on VIP values form OPLS-DA in low dose ZnO exposure group,. There are 19 identified PC and lysoPC metabolites (Tang et al., 2011). Significant (p<0.05) up regulations were observed in PC(16:0/22:1), PC(16:1/22:6), PC(17:0/18:0), PC(18:0/18:0), PC(20:4/20:4), PC(a25:5/15:1), PC(P-20:0/16:0), PC(P-38:5), PC of m/z 990.92 and m/z of 848.81; significant (p<0.05) down regulations were observed in PC(22:0/18:1) and unknown PC of m/z 843.02 (Table 20).

3.2.3 Markers with dose-response identified from UPLC-TQMS

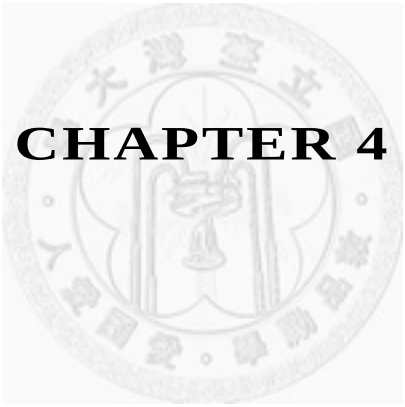
A. Markers with 35 nm ZnO dose-response

To obtain PC the interaction with various dosage of 35 nm ZnO exposure, OPLS-DA results were collected for common lipid in the intersection set on Venn diagrams (Kestler et al., 2008). 4 lipids that commonly existed in high dose, moderate dose and low dose were able to describe dose response between the 3 various doses treatment groups (Fig. 17). Table 17 shows the potential dose response biomarkers of 35 nm ZnO particle exposure. Among 4 dose response biomarkers, PC (P-20:5/18:0) shows a clear positive trend dose response which the concentration increases with increasing doses.

B. Markers with 250 nm ZnO dose-response

To further explore the PC effects of ZnO particles, OPLS-DA results were collected to conduct common lipids for a further illustration of dose-response relationships between each dose treatment and lipids using Venn diagrams (Kestler et al., 2008). 3 lipids that commonly existed in high dose, moderate dose and low dose were able to describe dose response between the 3 various doses treatment groups (Fig. 24).

Table 21 shows the dose response relationship that each lipid metabolite presented. Among 3 dose response biomarkers, PC(37:1) shows a clear negative trend dose response which the concentration increases with decreasing doses.



CHAPTER 4

DISCUSSION

4.1 UPLC-Qtof/MS analysis on systemic effect of ZnO on rat lipid profiling

Tumor necrosis factor-alpha, as a multifunction cytokine has been demonstrate to regulate energy metabolism and lipid homeostasis (Chen et al., 2009). Studies have evaluated the toxicity of ZnO nano-particles on normal primary human cells and their potential immune-modulatory effects (Hanley et al., 2009). ZnO nanoparticles trigger and induce the production of the pro-inflammatory cytokines, interferon (IFN)- c , TNF-alpha, and interleukin-12, at concentrations below causing cell death. Lipids are small molecules which own hydrophobic or amphipathic property that may originate entirely or in part by carbonion-based condensations of thioesters (fatty acids, poly-ketides, etc.) and/or by carbocation-based condensations of isoprene units (prenols, sterols, etc.). Lipid signaling and inflammation are convoluted modulators of homeostasis and immunity. Both positive and negative regulations in inflammatory responses have been reported by Glass CK et al (Glass et al., 2012). The classification scheme presented in the statement which has published by Fahy et al. organizes lipids into well-defined and presented chemically based the distinct hydrophobic and hydrophilic elements that compose the lipid. Owing to historical and bioinformatics advantages as previous review, scientists separated fatty acyls from other polyketides, the glycerophospholipids from the other glycerolipids, and sterol lipids from other prenols, resulting in a total of eight primary categories, shown as Figure 1.

PC and lyso PC metabolites are identified as dominant markers in serum of rats. 35 nm and 250 nm ZnO particle exposure experiments which were underwent in two periods, resulting in the separation of two size ZnO exposure pattern on PCA score plot.

The clustering based on particle size was observed in the control groups. This may be caused various physiological conditions between two groups of rats (Table 3).

Owing to limited exposure chambers, each dose exposure (high, moderate, and low) experiment was conducted separately, resulting in the control from various doses groups are appeared to be different based on PCA. As a result, the effects of exposure cannot be easily observed. Therefore, OPLS -DA were applied to find important lipids that differentiate treatments (high, moderate, and low) and control groups of two ZnO particles sizes (35 nm and 250 nm).

As a result, to look into the important markers that really differentiate treatments and controls, OPLS-DA provide good method to obtain lipids selected based on VIP values that illustrate lipids concentration variations between treatments and controls groups. All above are inferring that PC are very important molecular to interpret the exposure of ZnO particles exposure.

4.2 UPLC-TQMS analysis on systemic effect of ZnO on rat PC profiling

Further UPLC-TQMS analysis focusing on glycerophosphocholine structure and semi-quantifications can strength our findings from UPLC- TQMS results. Metabolites selected from UPLC-Qtof/MS need to be confirmed their structure either by comparing the LC-MS results of standards or by using Qtof/MS.

According to UPLC- TQMS lipids screening results, glycrophosphocholine take the most proportion than other lipids species, furthermore, PC take even more proportion than lyso PC. And the potential ZnO particle exposure biomarkers of 35 nm

and 250 nm analysis results from UPLC- TQMS all are PCs. Moreover, TQMS provides more ideal quantification ability compared with Qtof/MS.

Systemic effect triggered by ZnO exposure has been studied in previous studies. Gordon T. et al have reported that ZnO NPs could induce lung inflammation in animals. Human studies also found that inhalation of ZnO particles can cause metal fume fever, a flu-like symptom such as myalgias, cough, fatigue, and induce lung and systemic inflammation responses. But there are few studies that demonstrate interaction between lipids and ZnO particle exposure.

In this study,PC (31:0), PC (18:0/ 18:0) and PC(P-20:5/18:0) are commonly suggested metabolites from PC profiling OPLS-DA results of 35 nm treatments; and fold-changes with regulation was observed in PC(P-20:5/18:0). PC (42:6), PC(37:1) and PC(18:0/18:0) (*m/z* 790.78) are lipids suggested based on OPLS-DA results of 3 various dosed of 250 nm ZnO exposure treatments. PCs and other glycerophospholipids are the major structural lipids in eukaryotic membranes. PC has been pointed out own over 50% of the phospholipids in most eukaryotic membranes (Van et al., 2008). In addition to serve as a primary component of cellular membranes and binding sites for intracellular and intercellular proteins, some glycerophospholipids in eukaryotic cells are either precursors of, or are themselves, membrane-derived second messengers. PC is storage form for choline within cytosol. Choline ubiquitously distributes in all cells, mostly in the form of the, lysophosphatidylcholine, choline plasmalogens, and sphingomyeline essential components of all membranes (Zeisel et al., 1990). Rohlfs et al. have discovered the interaction with enzymes (phospholipases A_1, phospholipases A_2, PC desterase were discovered to play a crucial role in cellular systems (Zeisel et al., 1996; Kanfer et al., 1988). Gland, such as mammary gland has the ability to synthesis

and secrete PC (Rohlfs et al., 1993). Systemic effects such as chronic inflammation, atherosclerosis, aging and cancer are related to hypochlorous acid-mediation generated of PC from fatty acyl residues of phospholipids (Lessig et al., 2007). PC up-regulation was reported to induces membrane rupture, nuclear expansion, cell lysis, and enhance intracellular Ca^{+} level, furthermore, the production of ROS results in cytotoxic injuries and necrosis (Zhou et al., 2006). All the advances prove that the perturbations of PC are important when conduct systemic effects. Platelet-activating factor (PAF), lipid mediator has been implicated in cutaneous inflammation. Findings in literature have proved PC species can activate PAF agonistic activity (Travers et al., 1998). PC species is the product of phospholipase-B mediated de-acylation of phosphatidylcholine and phosphatidylinositol. And studies have evaluated the toxicity of ZnO nano-particles on normal primary human cells and their potential immune-modulatory effects. ZnO nanoparticles trigger and induce the production of the pro-inflammatory cytokines, interferon (IFN)- c , TNF-alpha, and interleukin-12, at concentrations below causing cell death. Consequently, concentration variation of PCs represents systemic effects turbulence occurs in organism (Almaguer et al., 2006) and have potential relationship with ZnO nano-particle exposure (Hanley et al., 2009). However, Oberdorster G. et al. summarized the effects on the body of inhalation nano-scaled ultrafine particle, which that translocate particles then can induce various damages in different parts of the body.

This study takes a leading role in conducting ZnO particle exposure using lipidomics research approach that has proved PCs demonstrate dose-response potential biomarkers.

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Figures

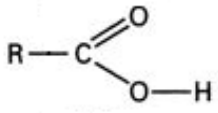
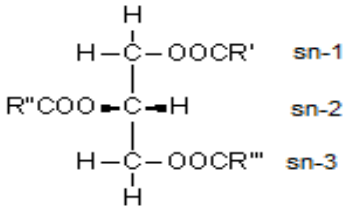
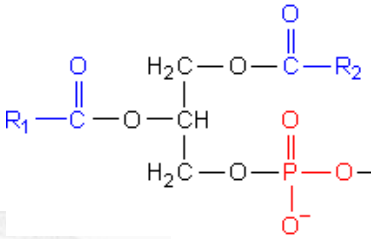
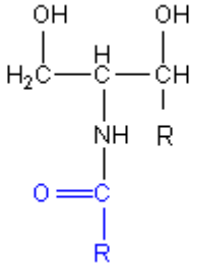
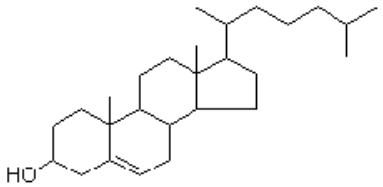
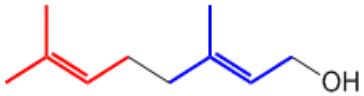
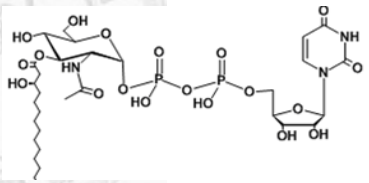
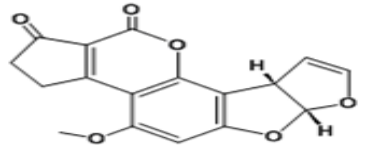
1 Fatty Acyls	2 Glycerolipids	3 Glycerophospholipids	4 Sphingolipids
			
5 Sterol Lipids	6 Prenol Lipids	7 Saccharolipids	8 Polyketides
			

Figure 1. Primary categories of lipid.

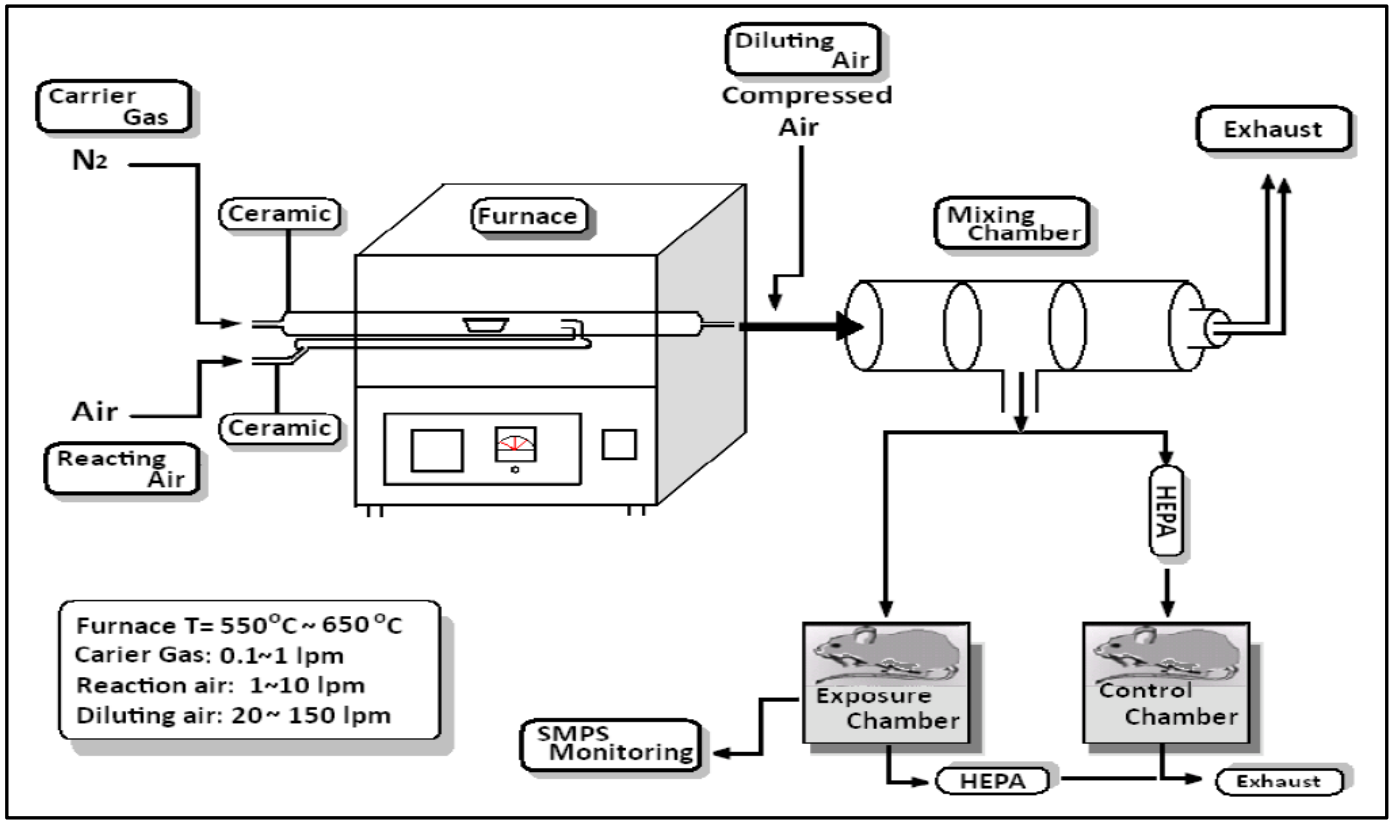

Carrier Gas
N2
Ceramic
Furnace
Diluting Air
Compressed Air
Exhaust
Mixing Chamber
Air
Ceramic
Reacting Air
HEPA
Furnace T= 550°C ~ 650°C
Carier Gas: 0.1~1 lpm
Reaction air: 1~10 lpm
Diluting air: 20~ 150 lpm
SMPS Monitoring
Exposure Chamber
Control Chamber
HEPA
Exhaust

Figure 2 Schematic representation of the nanoparticle generation and exposure system

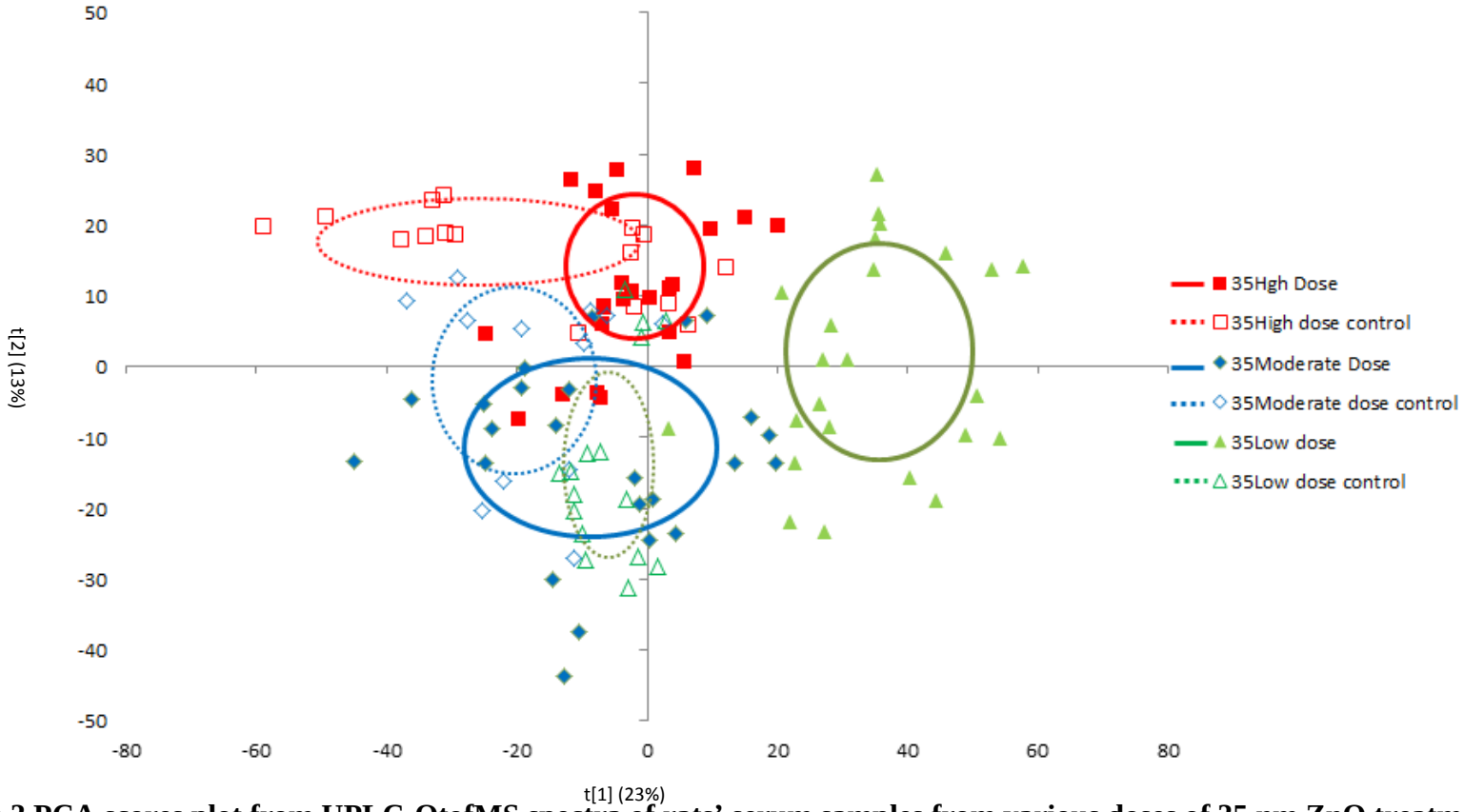


Figure 3 PCA scores plot from UPLC-QtofMS spectra of rats' serum samples from various doses of 35 nm ZnO treatment. Each point on PCA scores plot represents one sample run.

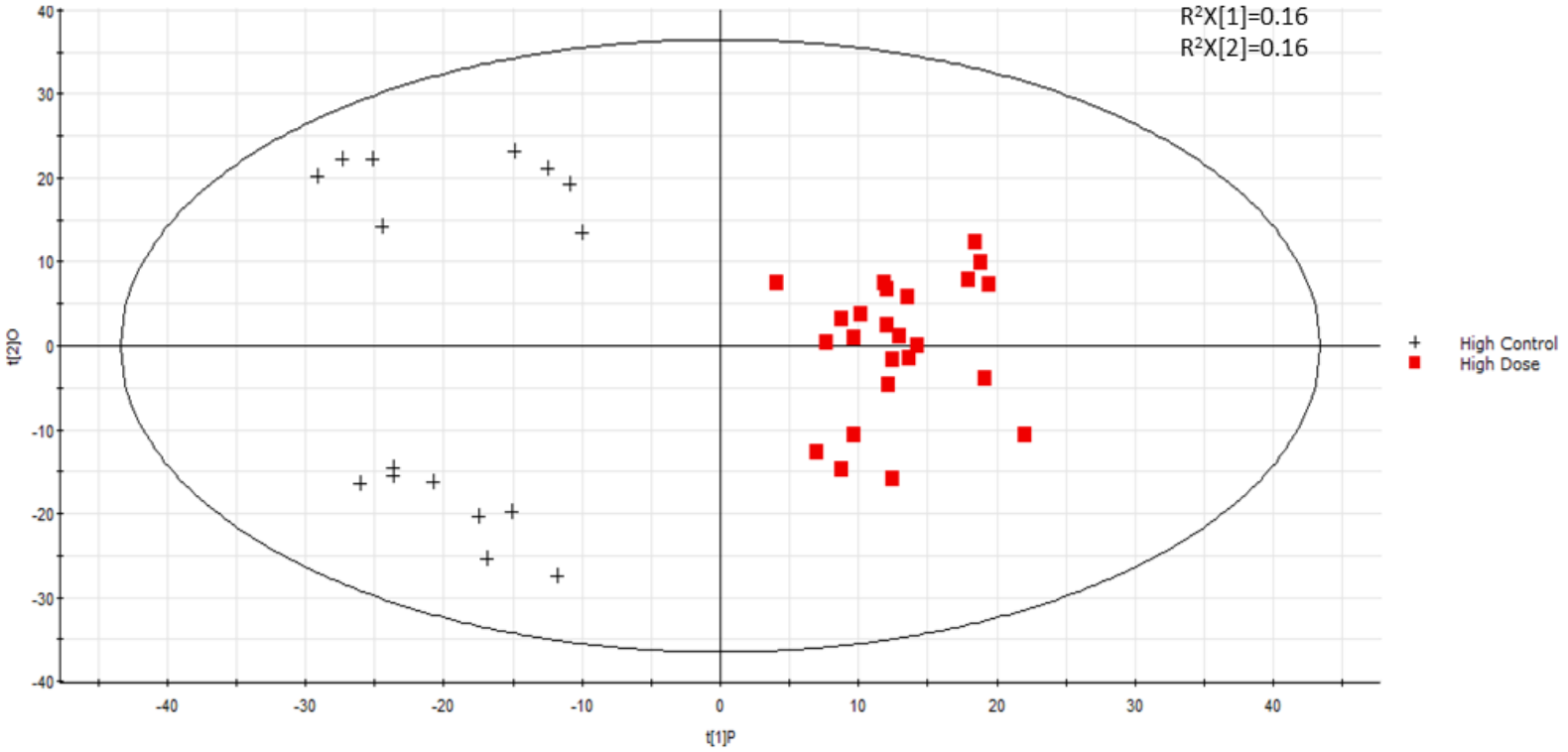


Figure 4 OPLS-DA score plot (high dose/ high dose control) generated from UPLC-Qtof/MS spectra of high dose 35 nm ZnO exposure group. (Q^2=0.68)

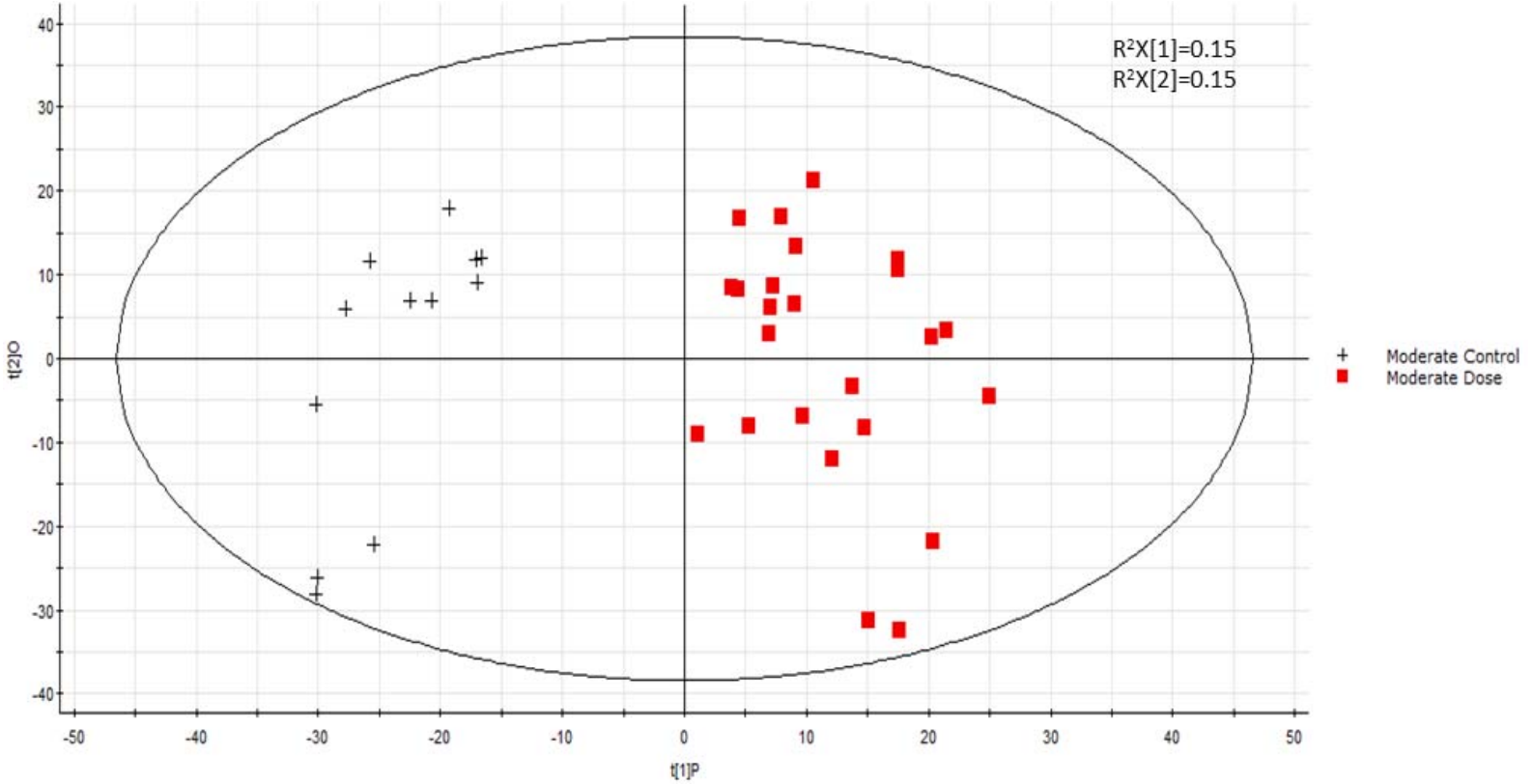


Figure 5 OPLS-DA score plot (moderate dose/ moderate dose control) generated from UPLC-Qtof/MS spectra of moderate dose 35 nm ZnO exposure group. (Q^2=0.73)

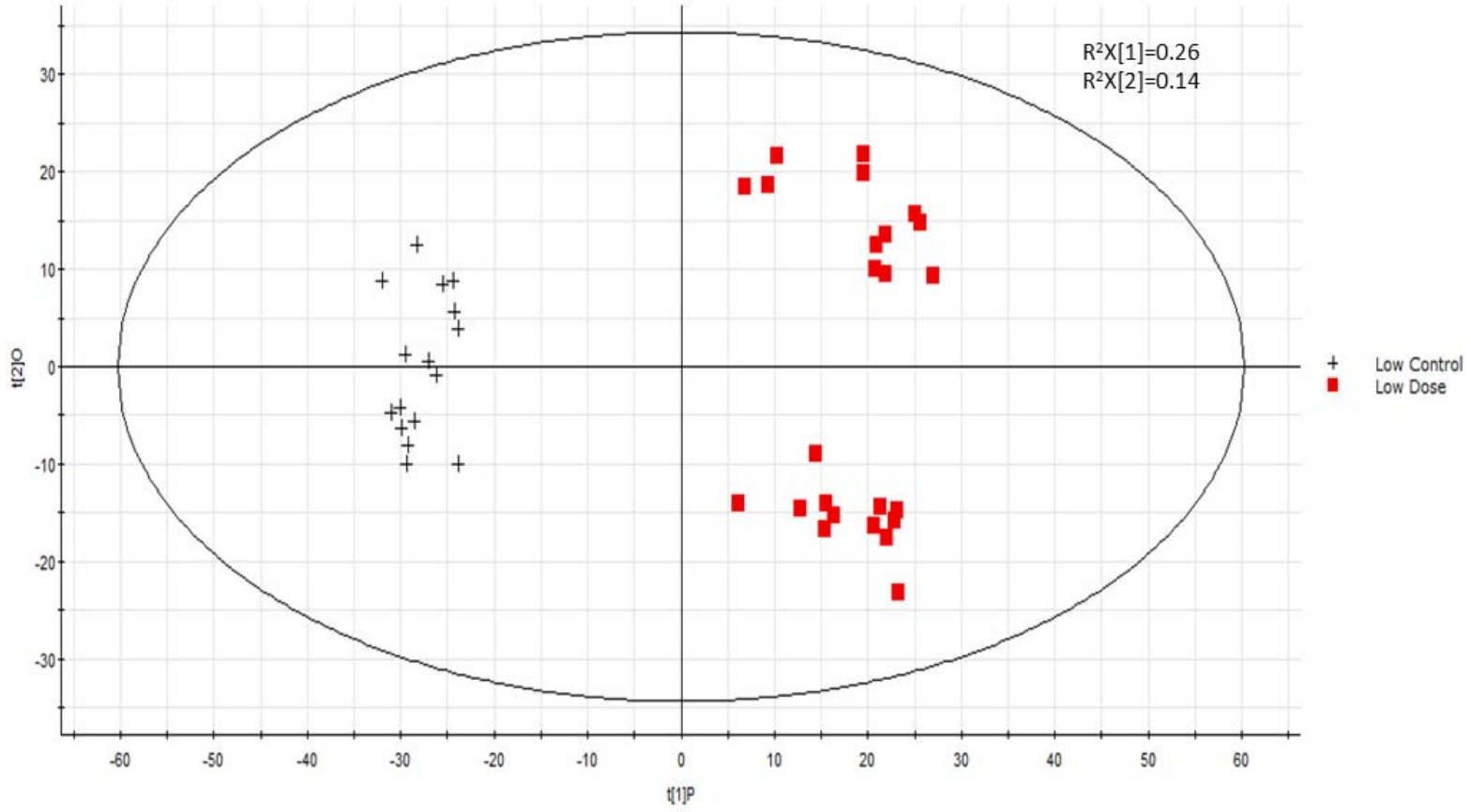


Figure 6 OPLS-DA score plot (low dose/ low dose control) generated from UPLC-Qtof/MS spectra of low dose 35 nm ZnO exposure group. (Q^2=0.80)

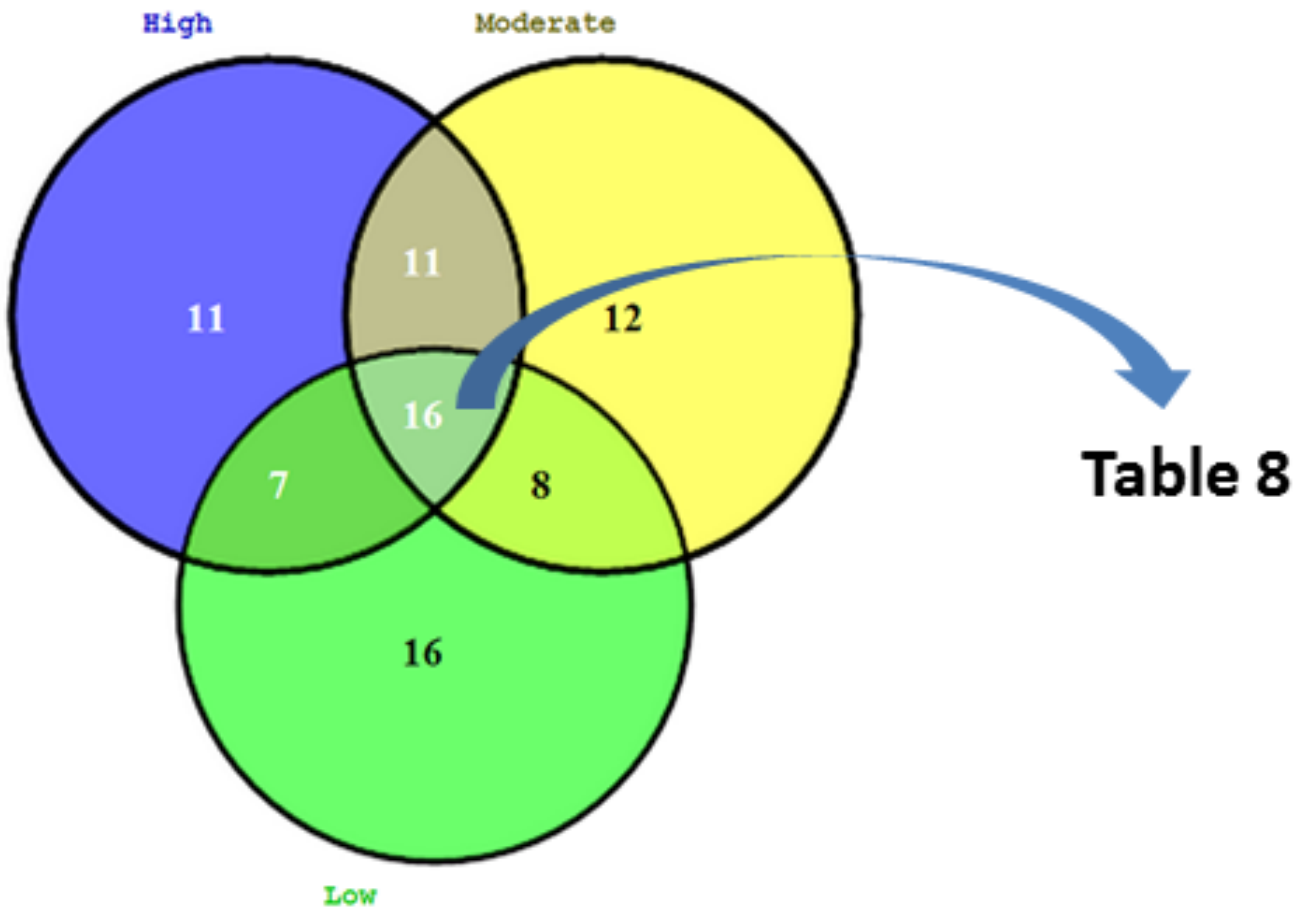


Figure 7 UPLC-QtofMS analysis result. 16 common lipids selected from (high-, moderate- and low-dose/ high-, moderate- and low-dose control) OPLS-DA top 50 VIP in 35 nm ZnO exposure groups.

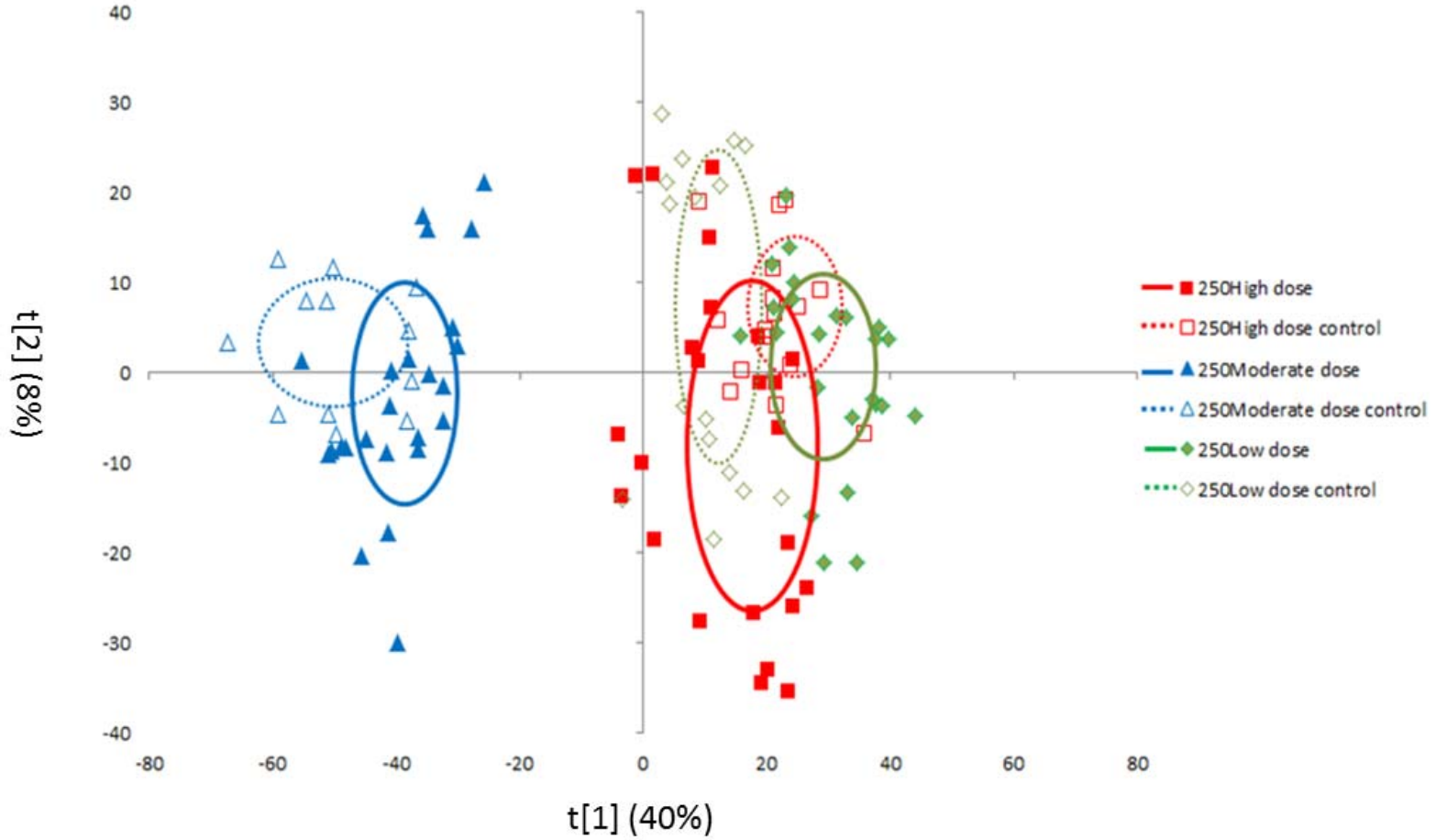


Figure 8 PCA scores plot from UPLC-Qtof/MS spectra of rats' serum samples from various doses of 250 nm ZnO treatment. Each point on PCA scores plot represents one sample run.

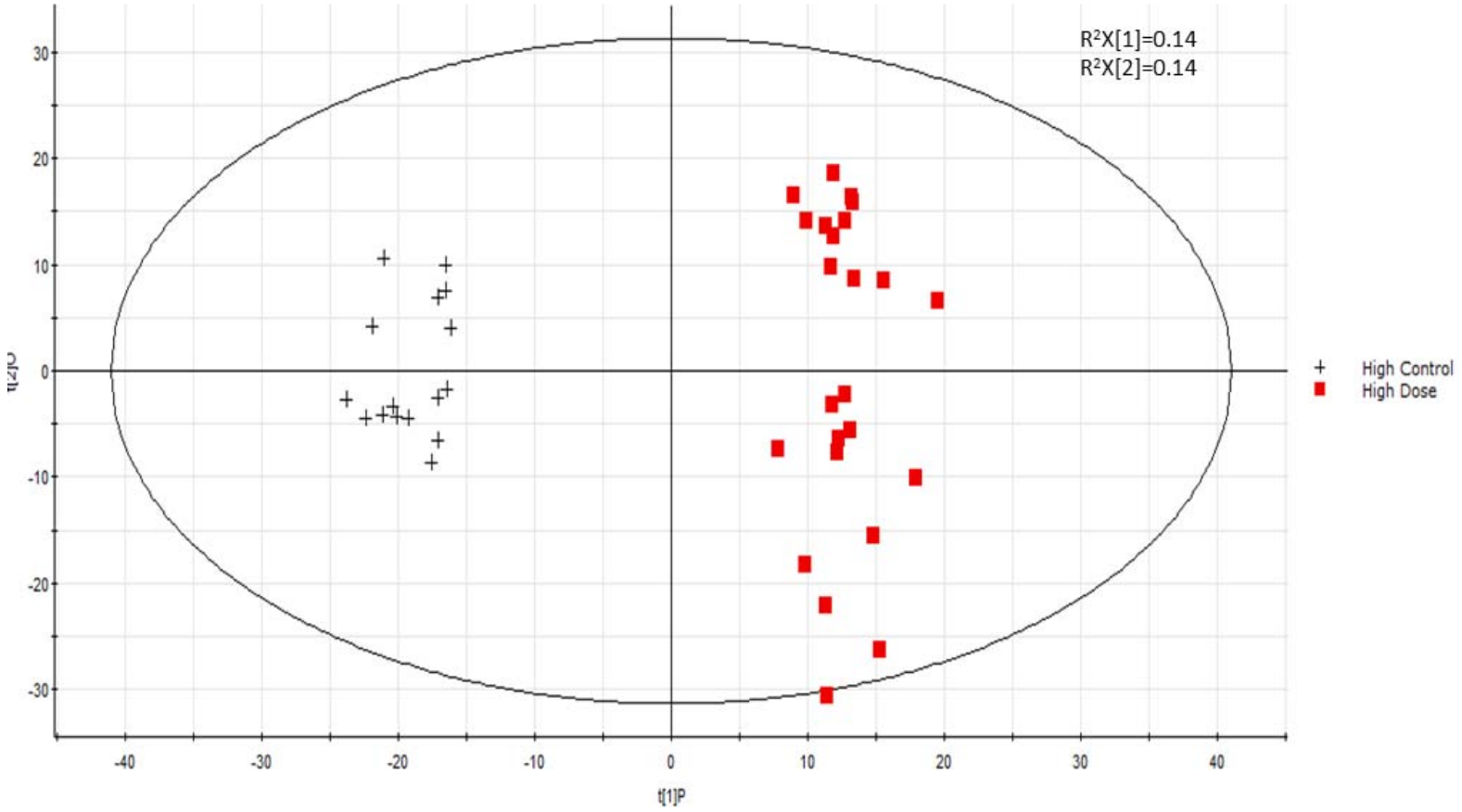


Figure 9 OPLS-DA score plot (high dose/ high dose control) generated from UPLC-Qtof/MS spectra of high dose 250 nm ZnO exposure group (Q^2=0.69).

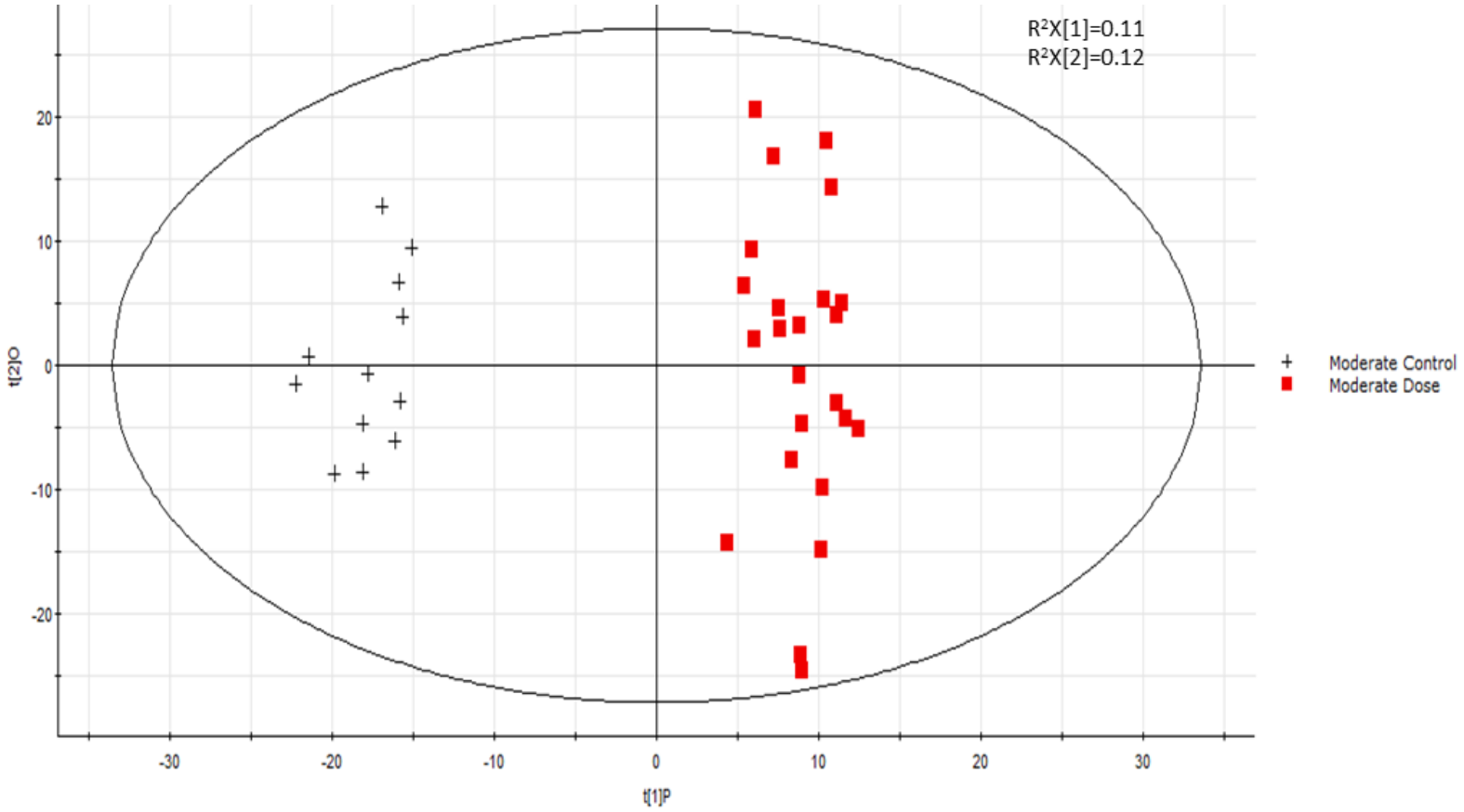


Figure 10 OPLS-DA score plot (moderate dose/control) generated from UPLC-Qtof/MS spectra of moderate dose 250 nm ZnO exposure group. (Q^2=0.62)

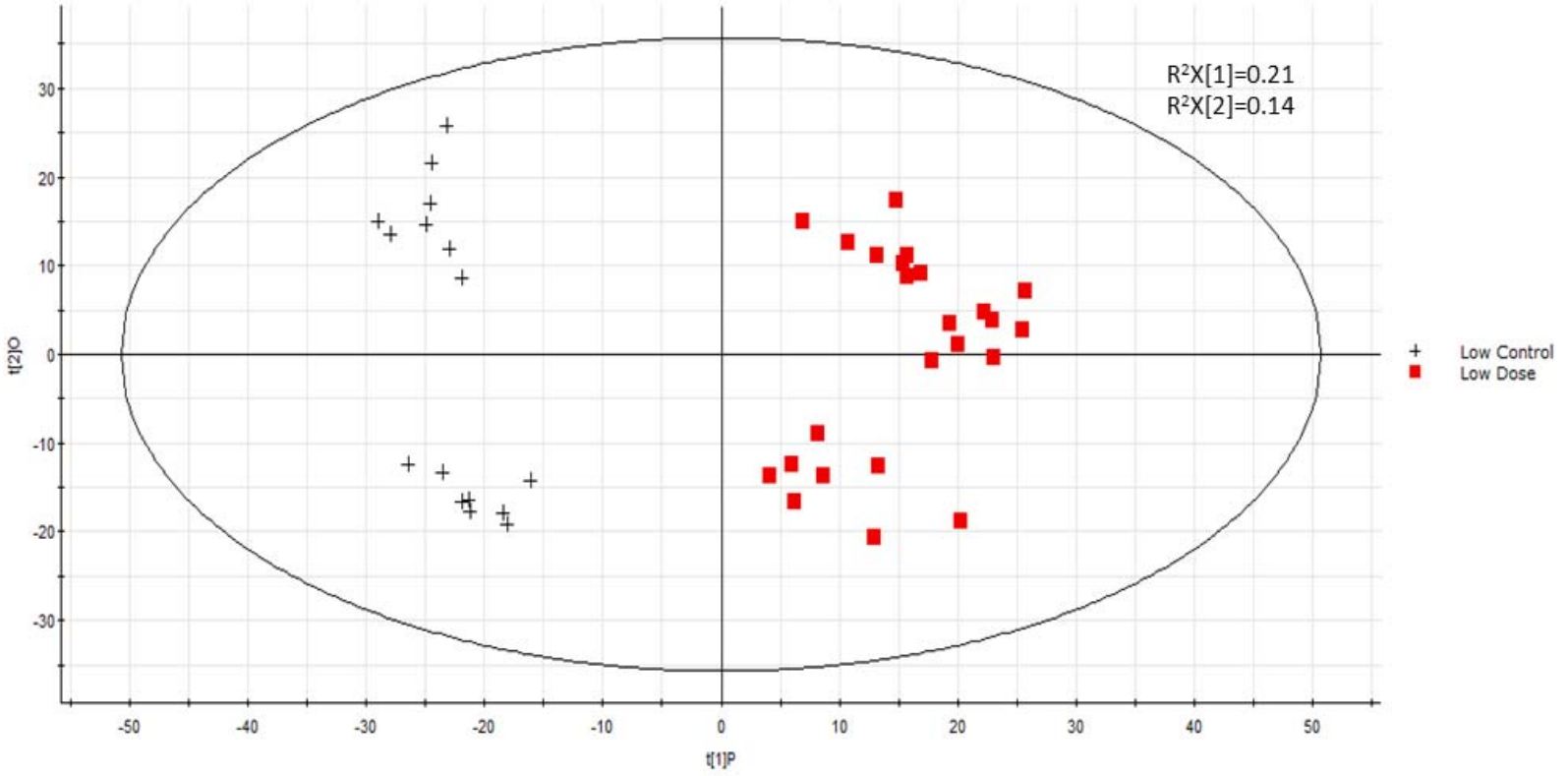


Figure 11 OPLS-DA score plot (low dose/ low dose control) generated from UPLC-Qtof/MS spectra of low dose 250 nm ZnO exposure group. (Q^2=0.83)

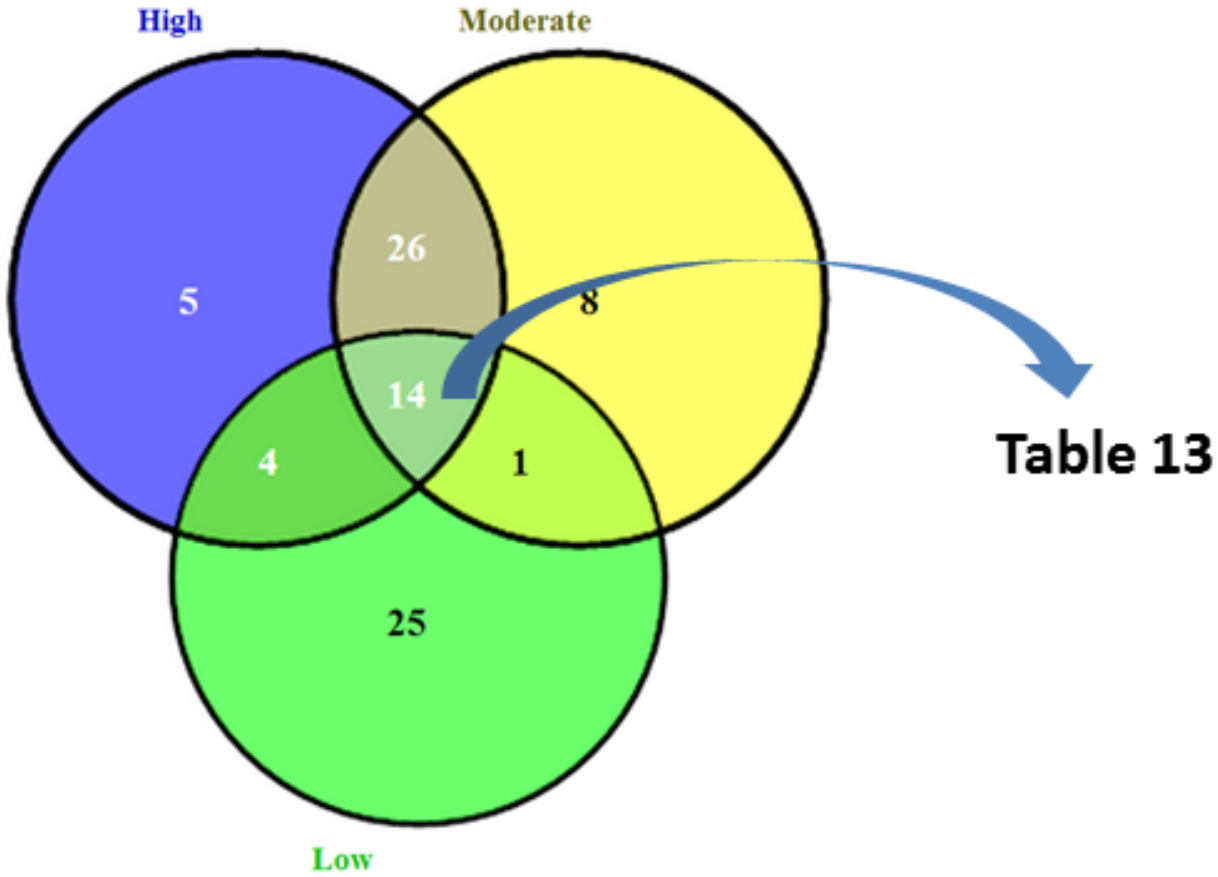


Figure 12 UPLC-Qtof/MS analysis result. 14 common lipids selected from (high-, moderate- and low-dose/ high-, moderate- and low-dose control) OPLS-DA top 50 VIP in 250 nm ZnO exposure groups.

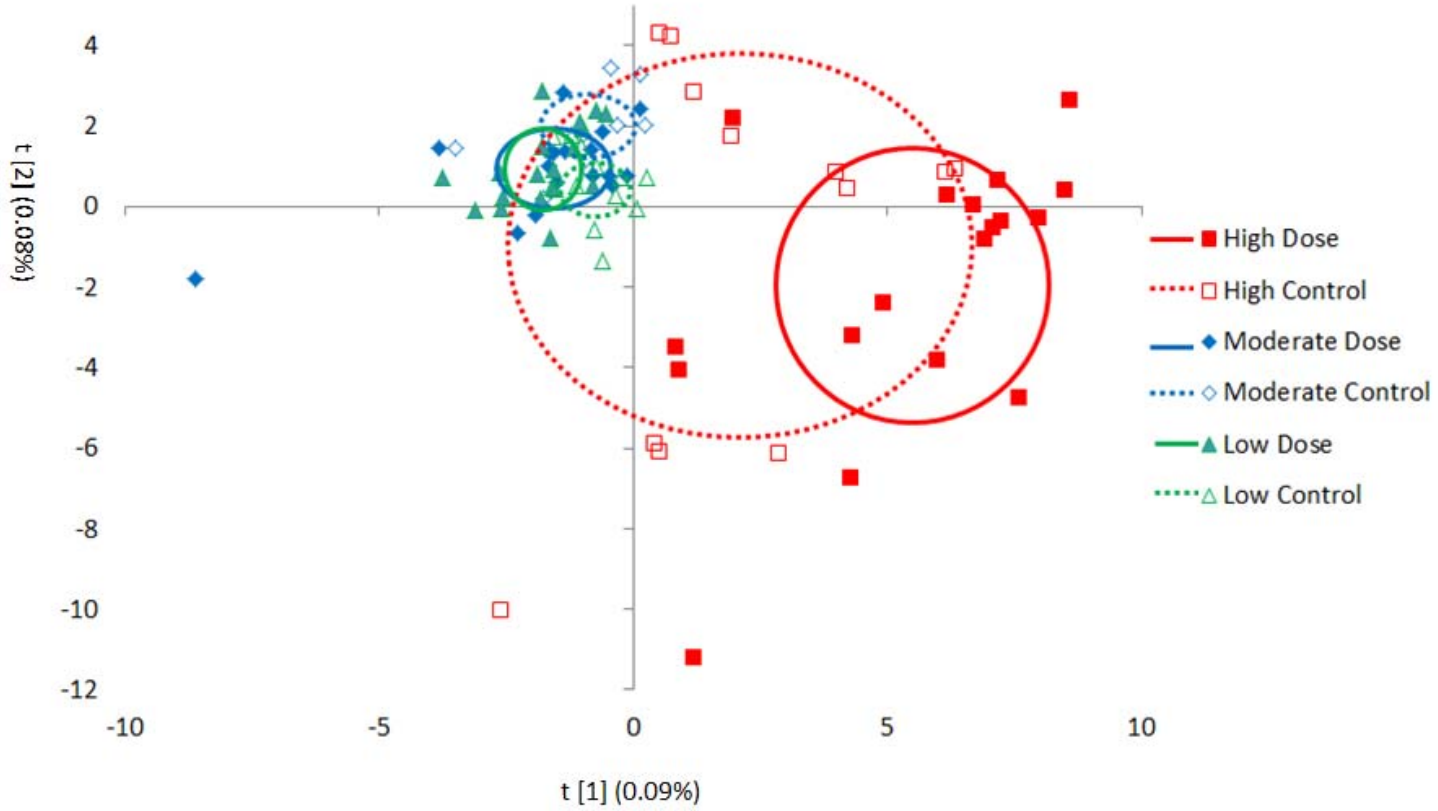


Figure 13 PCA scores plot from UPLC-TQMS spectra of rats' serum samples from various doses of 35 nm ZnO treatment. Each point on PCA scores plot represents one sample run. (Q^2=0.09)

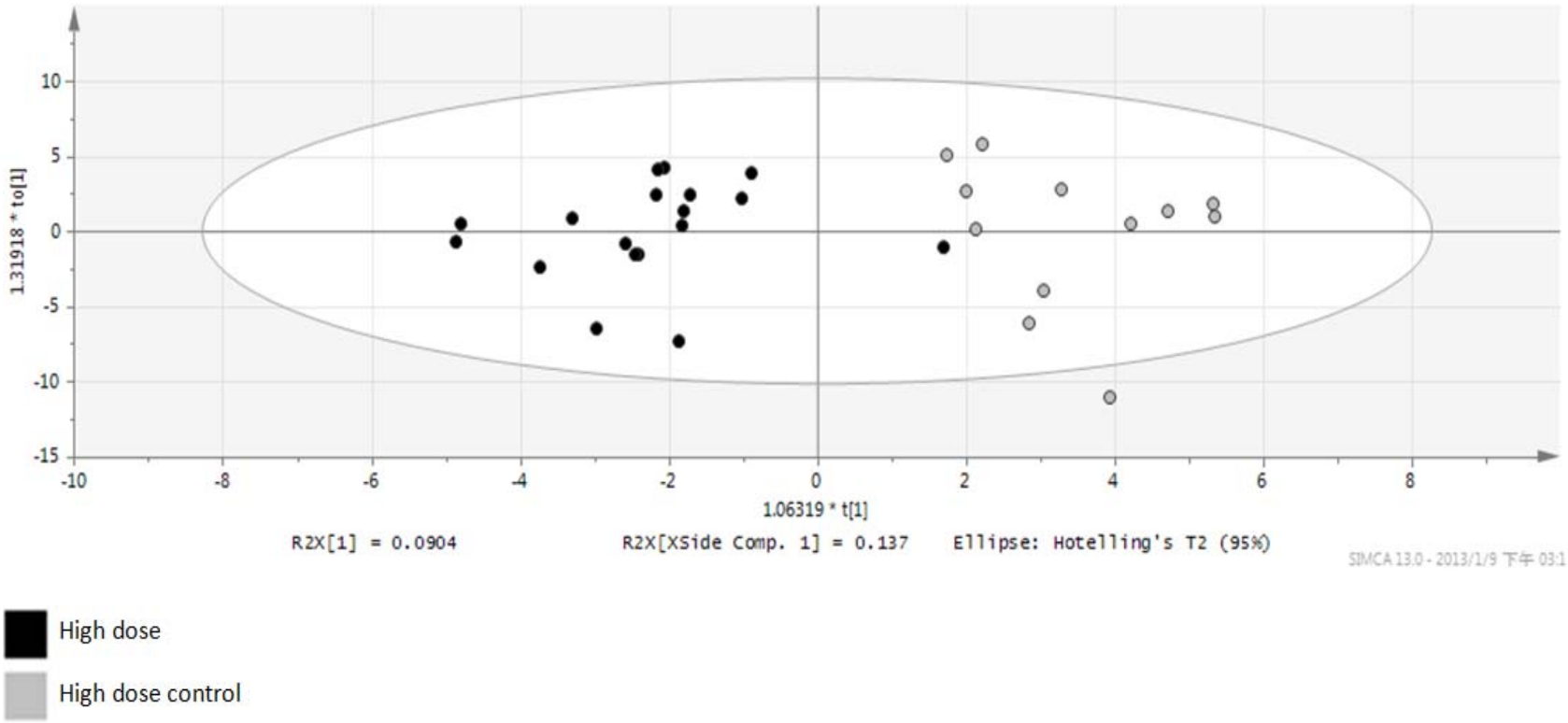


Figure 14 OPLS-DA score plot (high dose/ high dose control) generated from UPLC-TQMS spectra of high dose 35 nm ZnO exposure group. (Q^2=0.32)

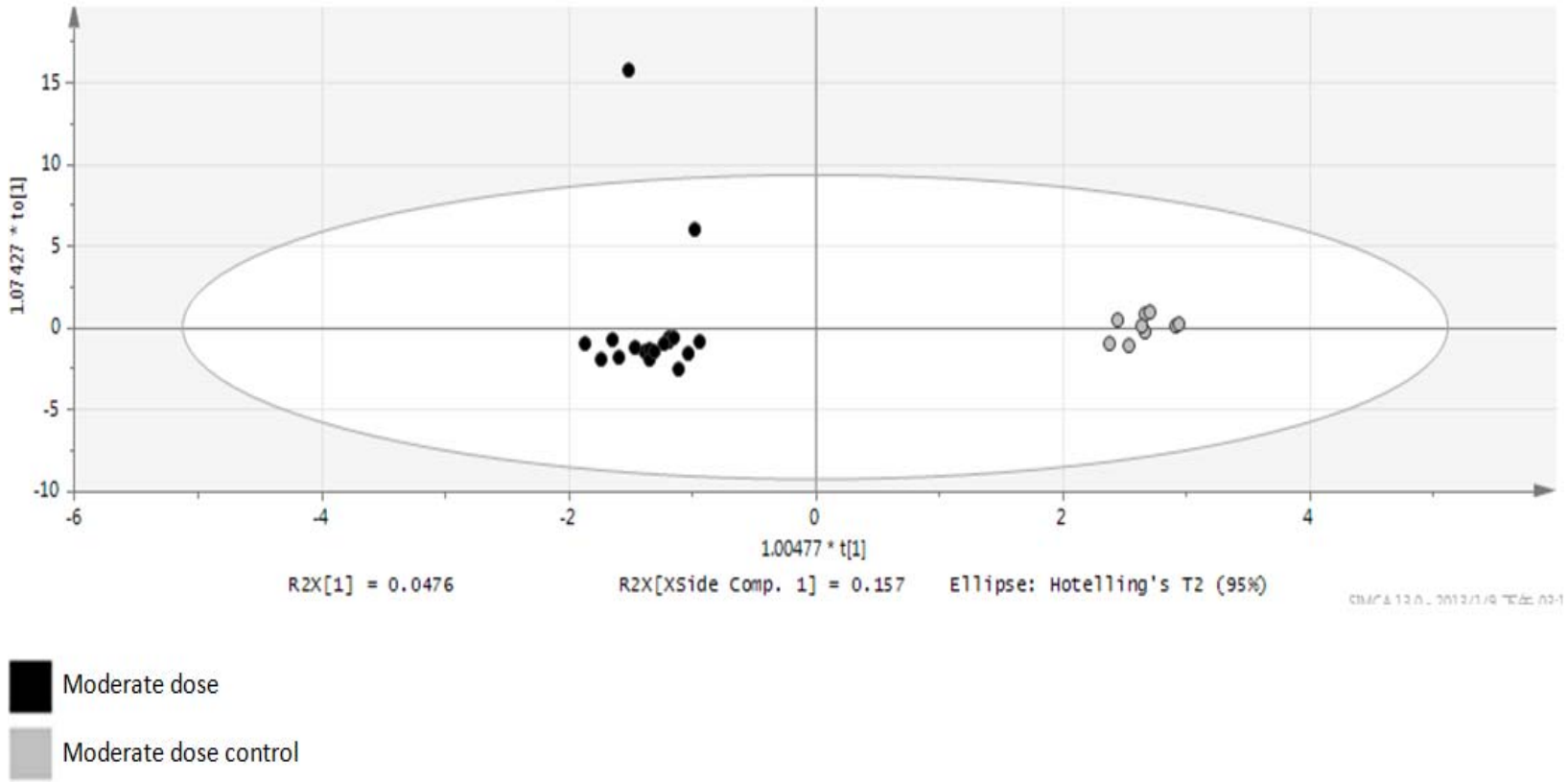


Figure 15 OPLS-DA score plot (moderate dose/ moderate dose control) generated from UPLC-TQMS spectra of moderate dose 35 nm ZnO exposure group. (Q^2=0.32)

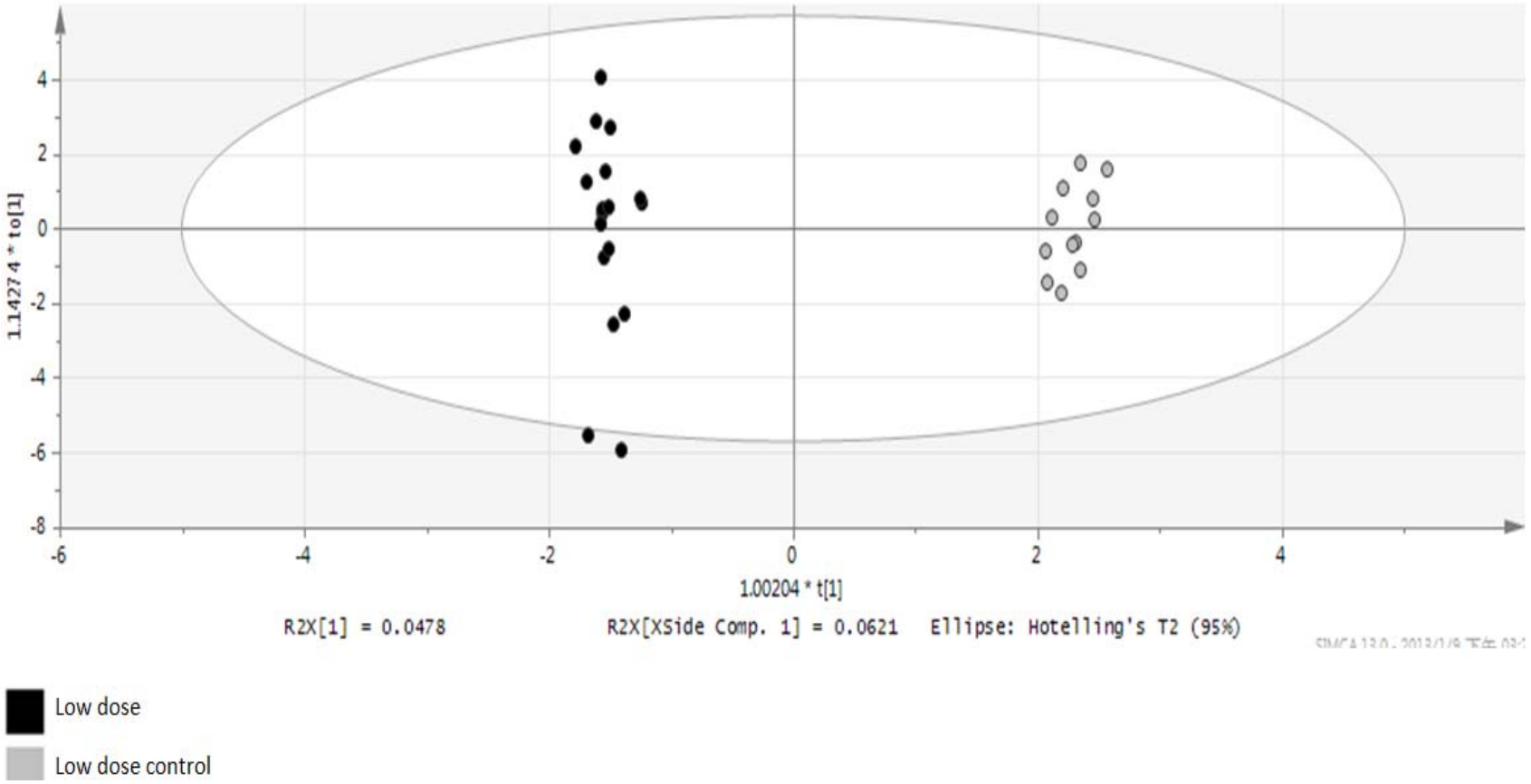


Figure 16 OPLS-DA score plot (low dose/ low dose control) generated from UPLC-TQMS spectra of low dose 35 nm ZnO exposure group. (Q^2=0.31)

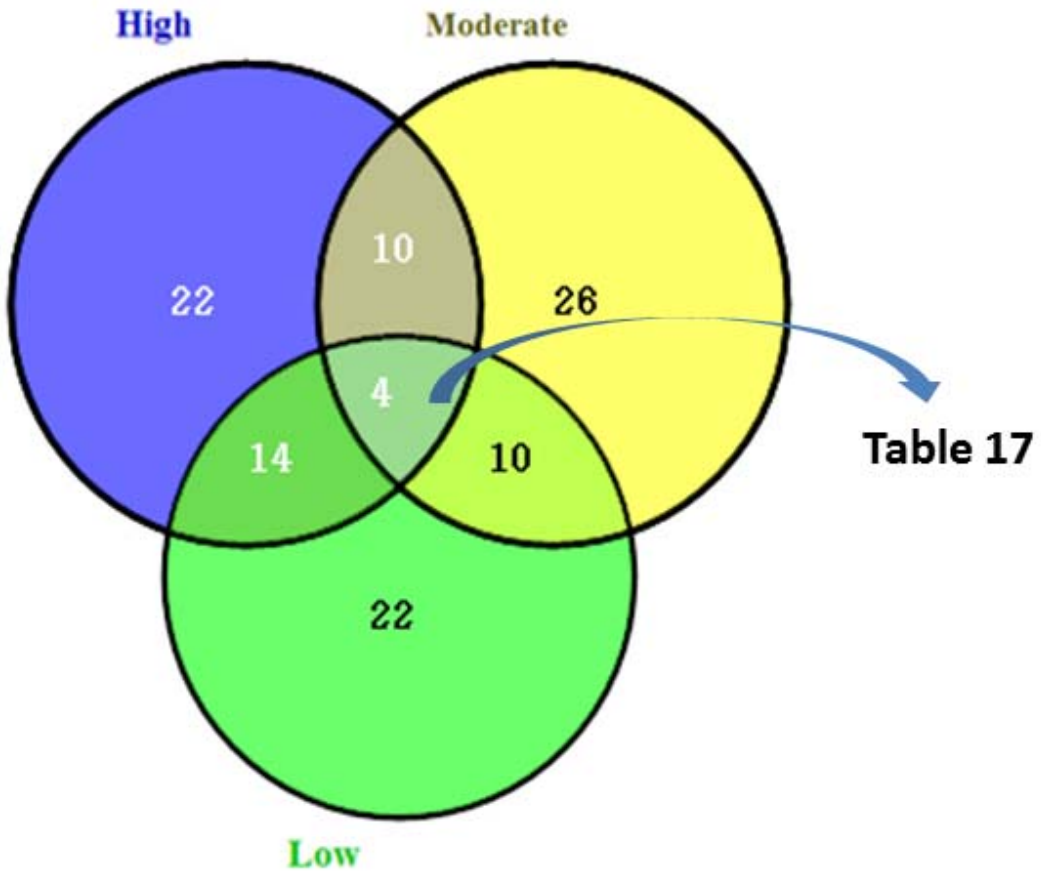


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Figure 17 UPLC-TQMS analysis result. 4 common lipids selected from (high-, moderate- and low-dose/ high-, moderate- and low-dose control) OPLS-DA top 50 VIP in 35 nm ZnO exposure groups.

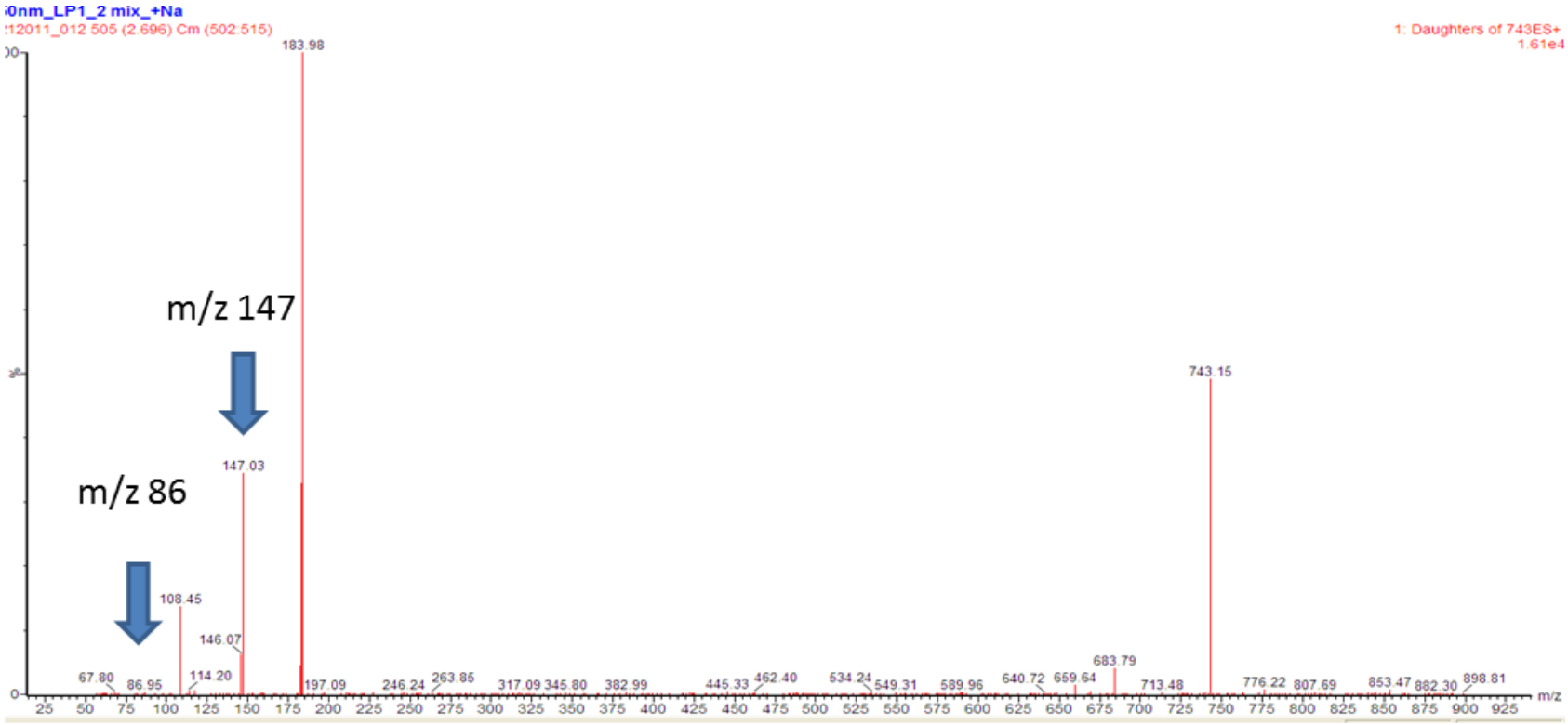

Figure 18 Collision-induced fragmentation spectra of the $[M+Na]^+$ PC (31:0) molecular ion was detected by UPLC-TQMS.

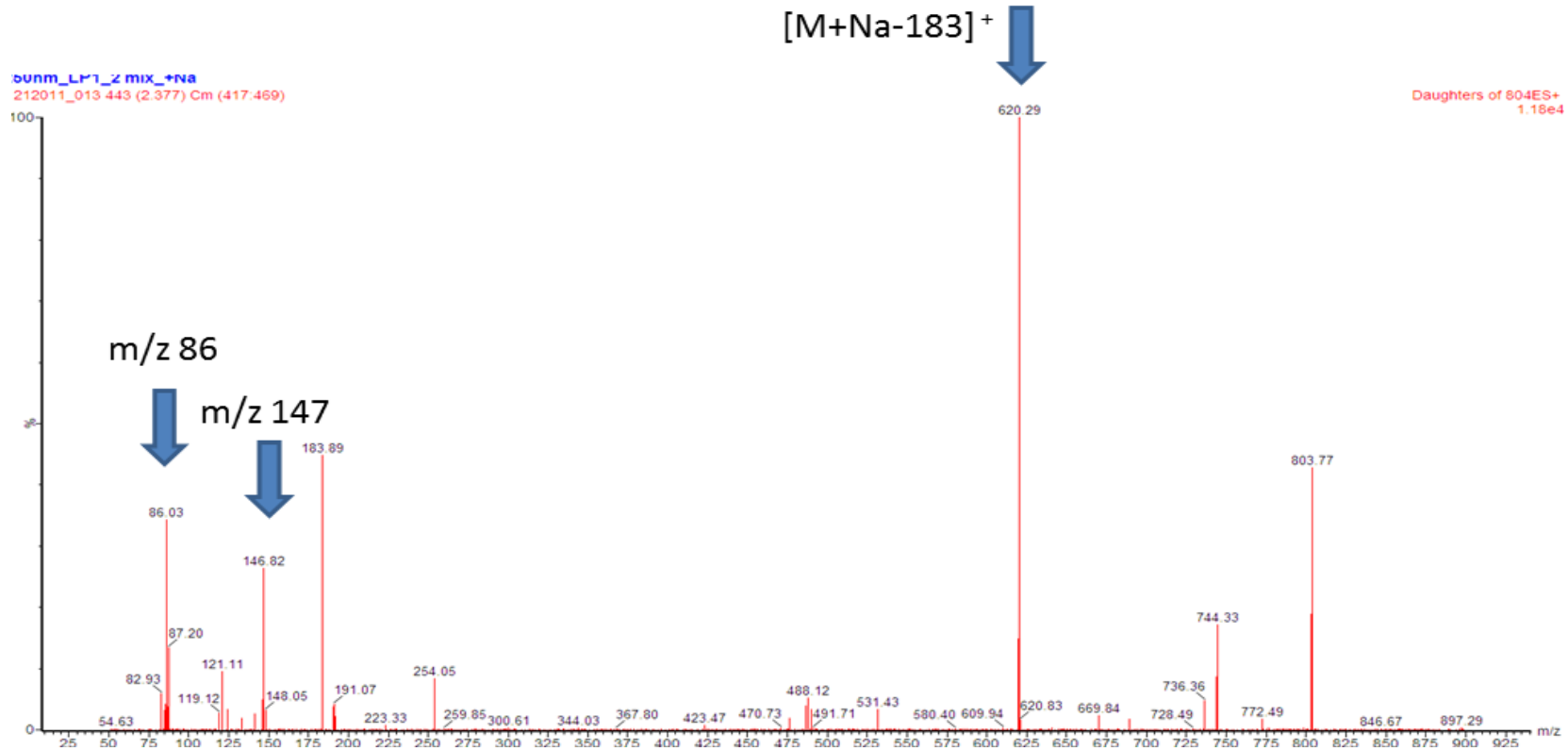

Figure 19 Collision-induced fragmentation spectra of the $[M+Na]^+$ m/z PC(36:4) molecular ion was detected by UPLC-TQMS.

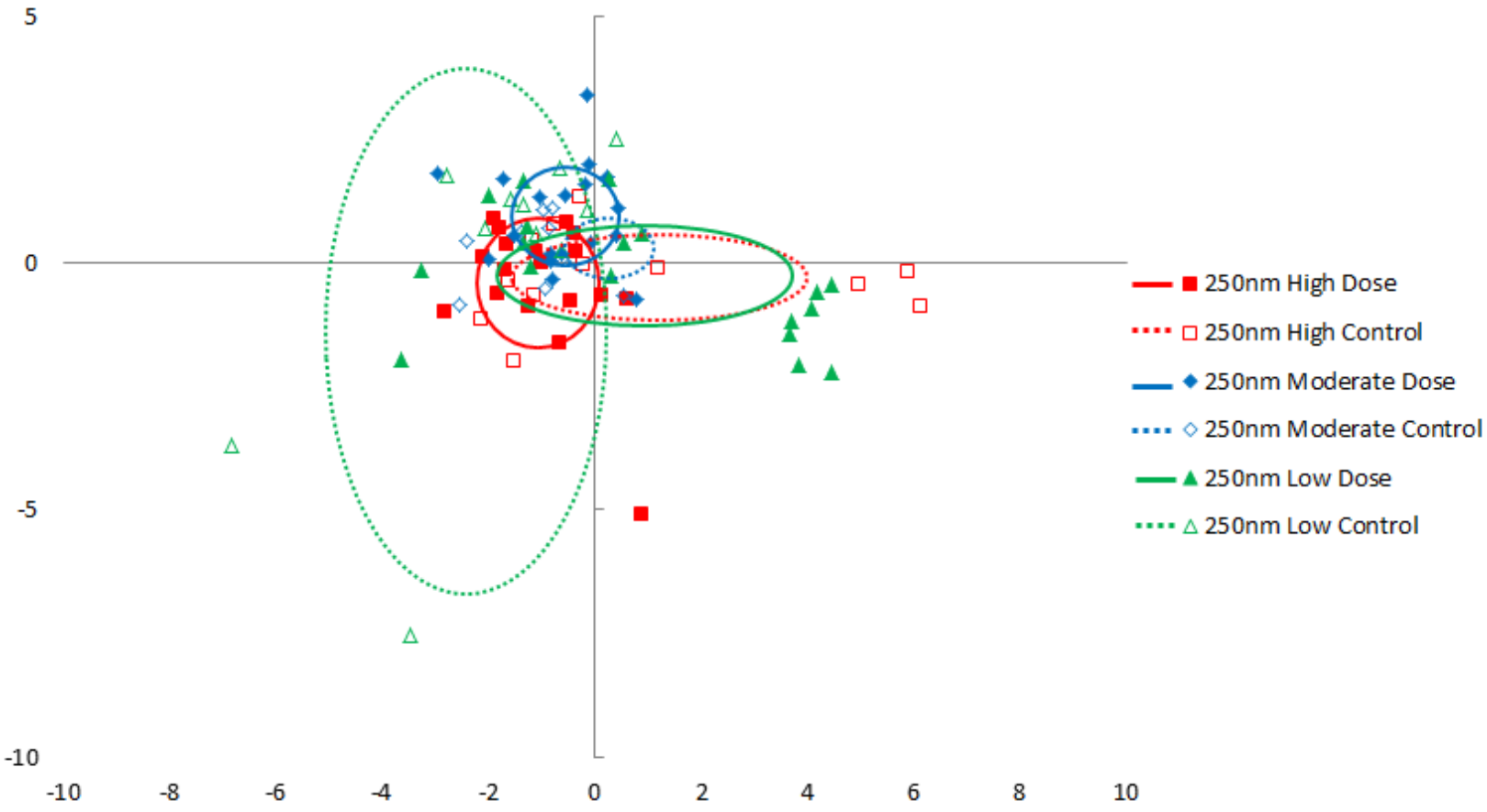

Figure 20 PCA scores plot from UPLC-TQMS spectra of rats' serum samples from various doses of 250 nm ZnO treatment. Each point on PCA scores plot represents one sample run.

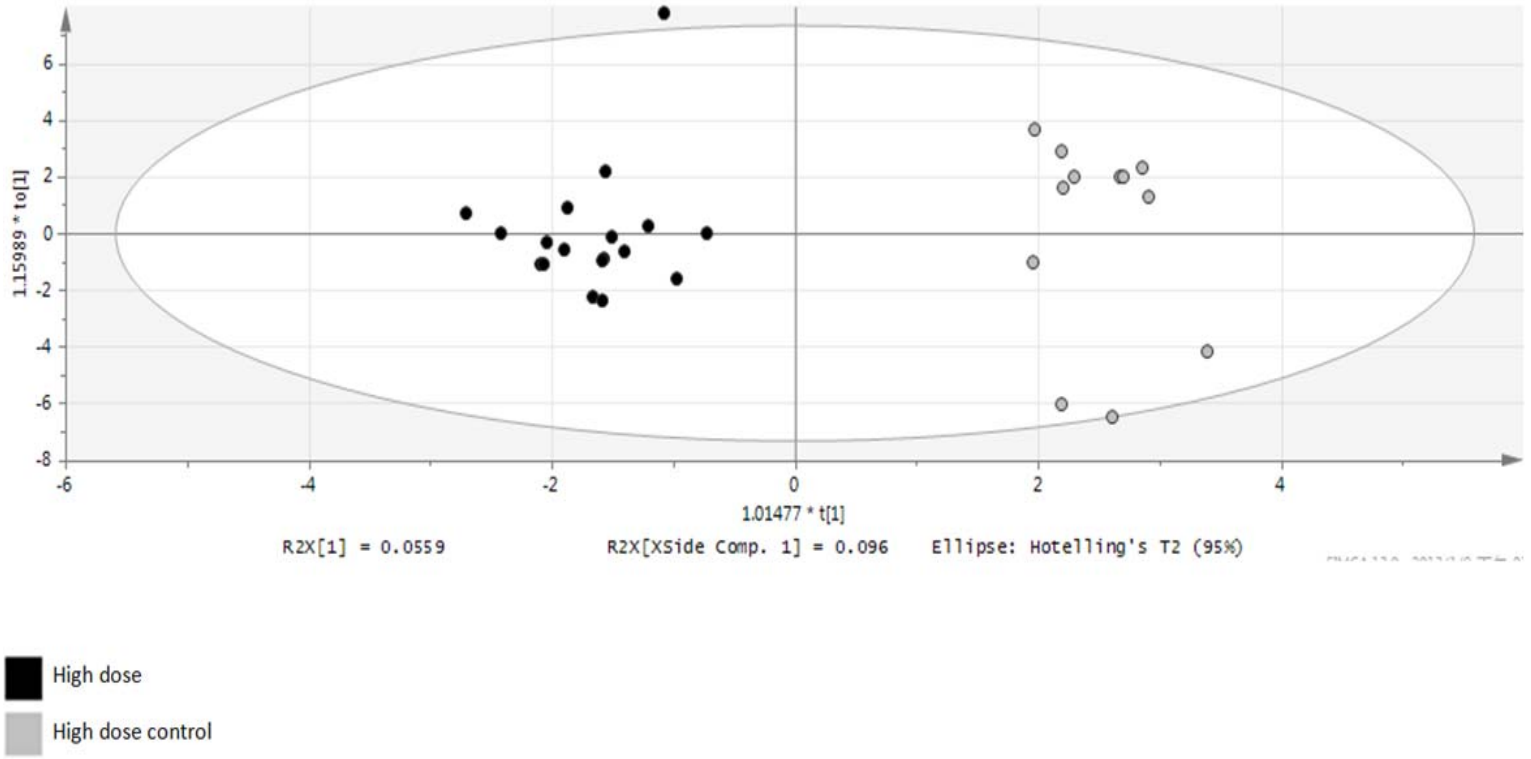

Figure 21 OPLS-DA score plot (high dose/ high dose control) generated from UPLC-TQMS spectra of low dose 250 nm ZnO exposure group.

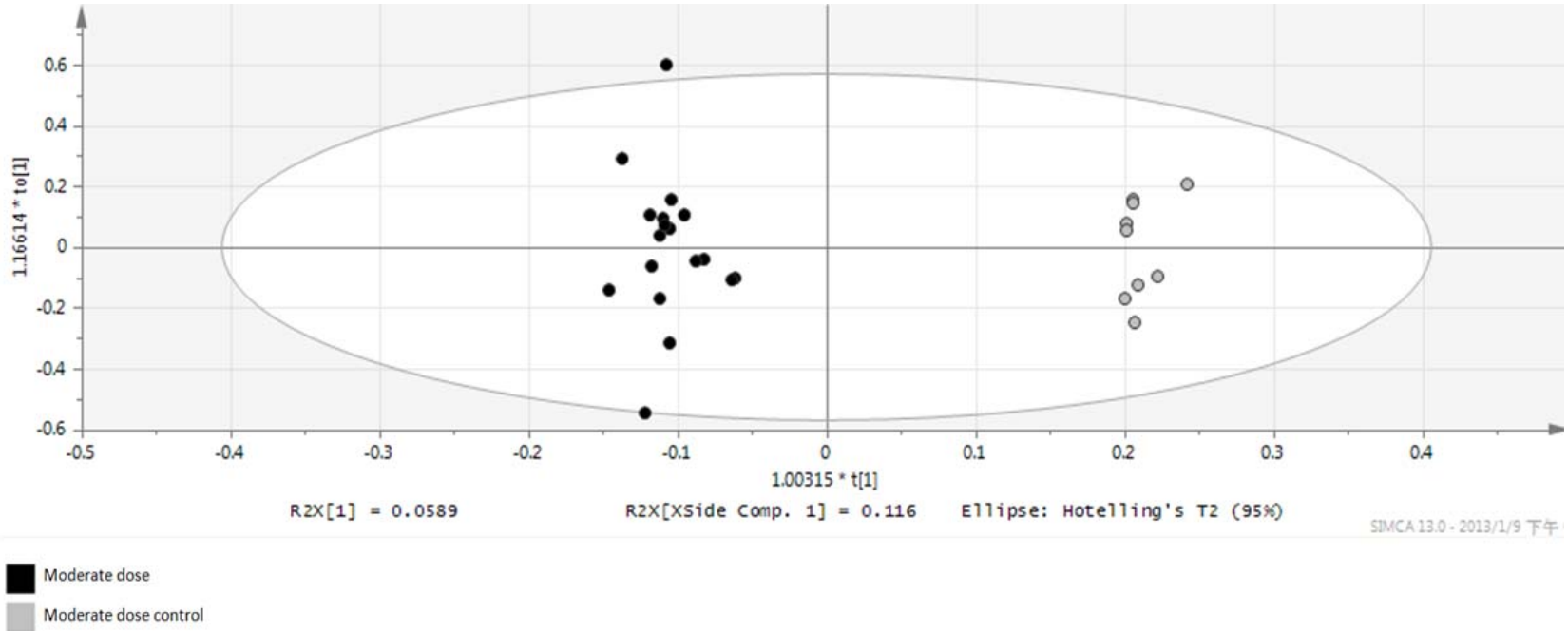

Figure 22 OPLS-DA score plot (moderate dose/control) generated from UPLC-TQMS spectra of high dose 250 nm ZnO exposure group.

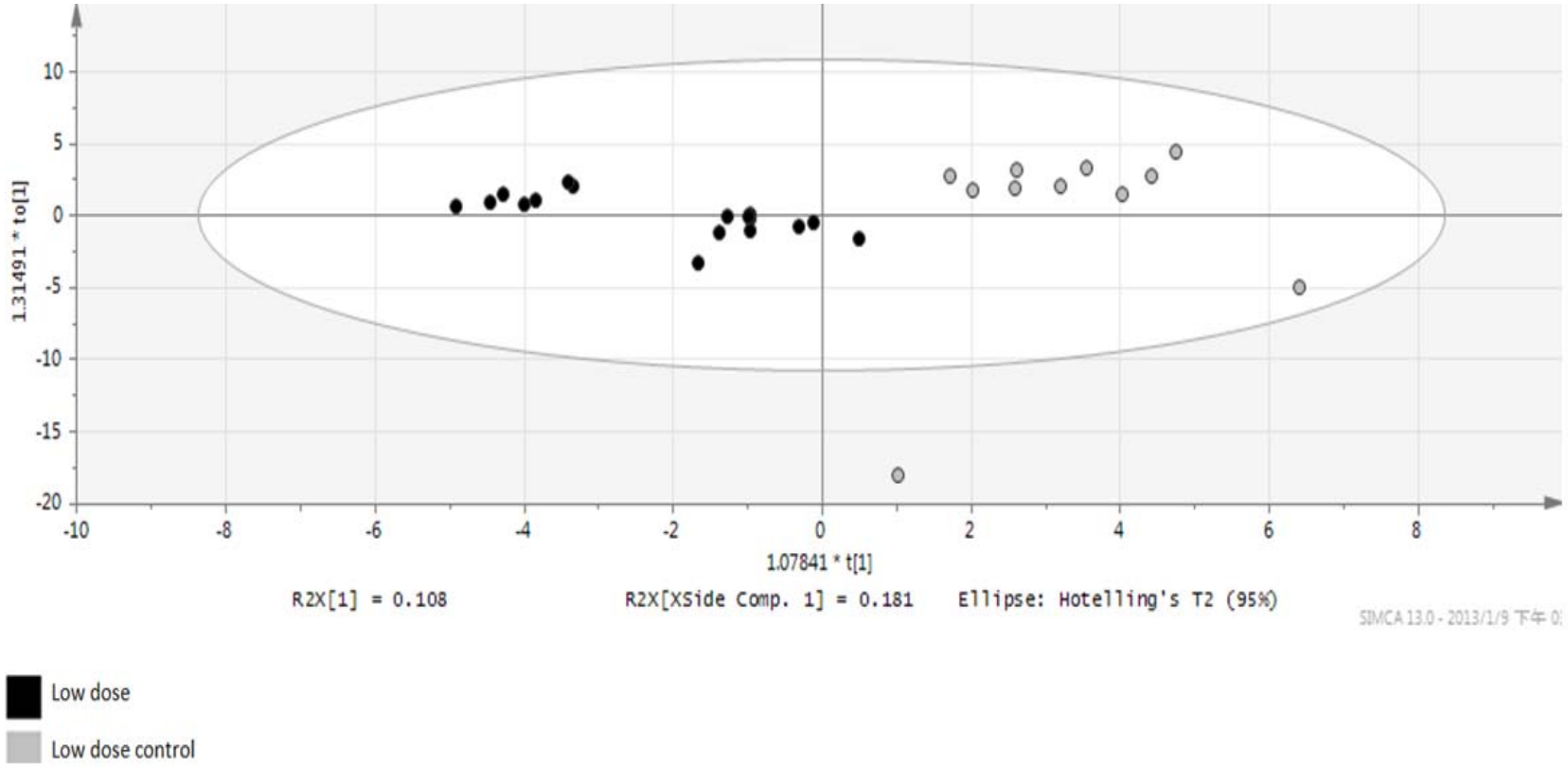

Figure 23 OPLS-DA score plot (low dose/ low dose control) generated from UPLC-TQMS spectra of high dose 250 nm ZnO exposure group.

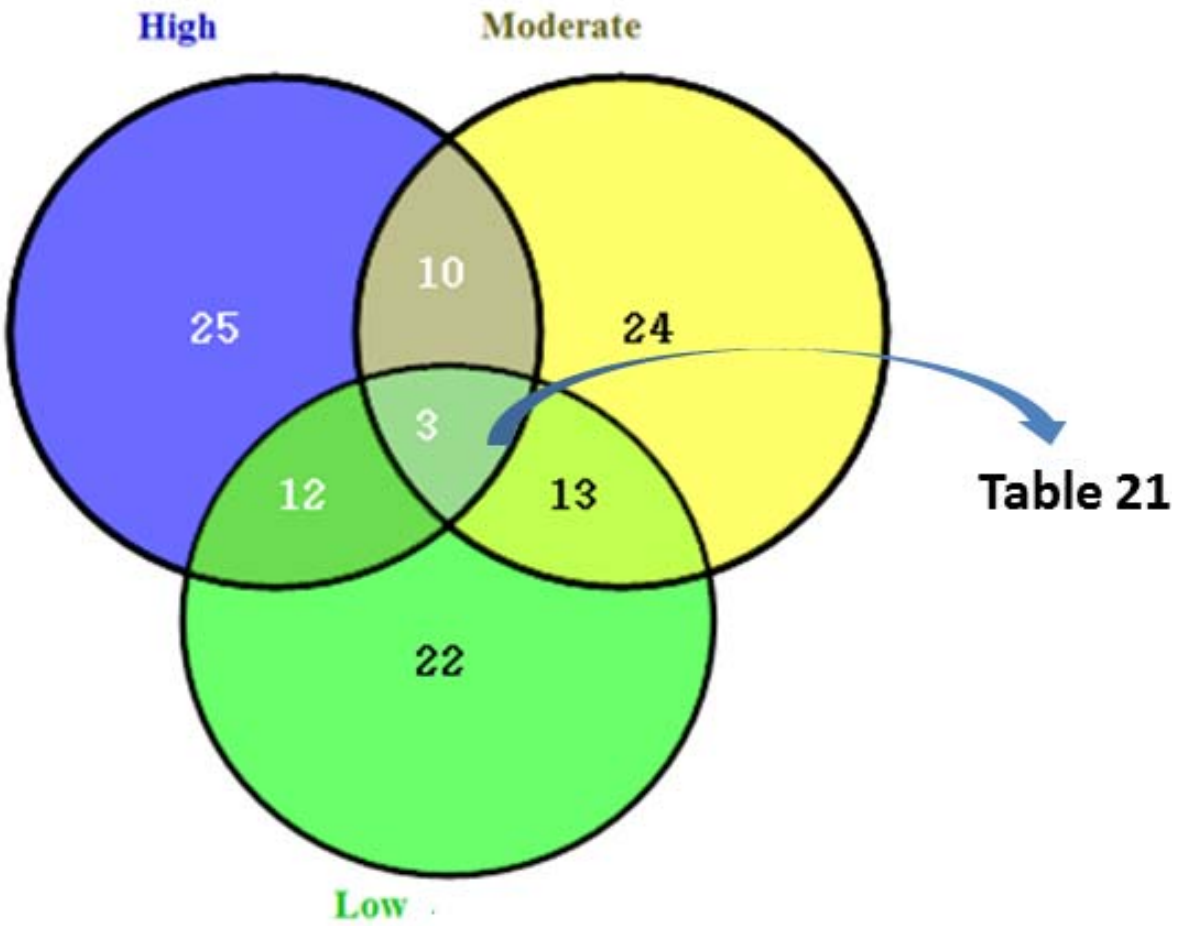

Figure 24 UPLC-TQMS analysis result. 3 common lipids selected from (high-, moderate- and low-dose/ high-, moderate- and low-dose control) OPLS-DA top 50 VIP in 250 nm ZnO exposure groups.

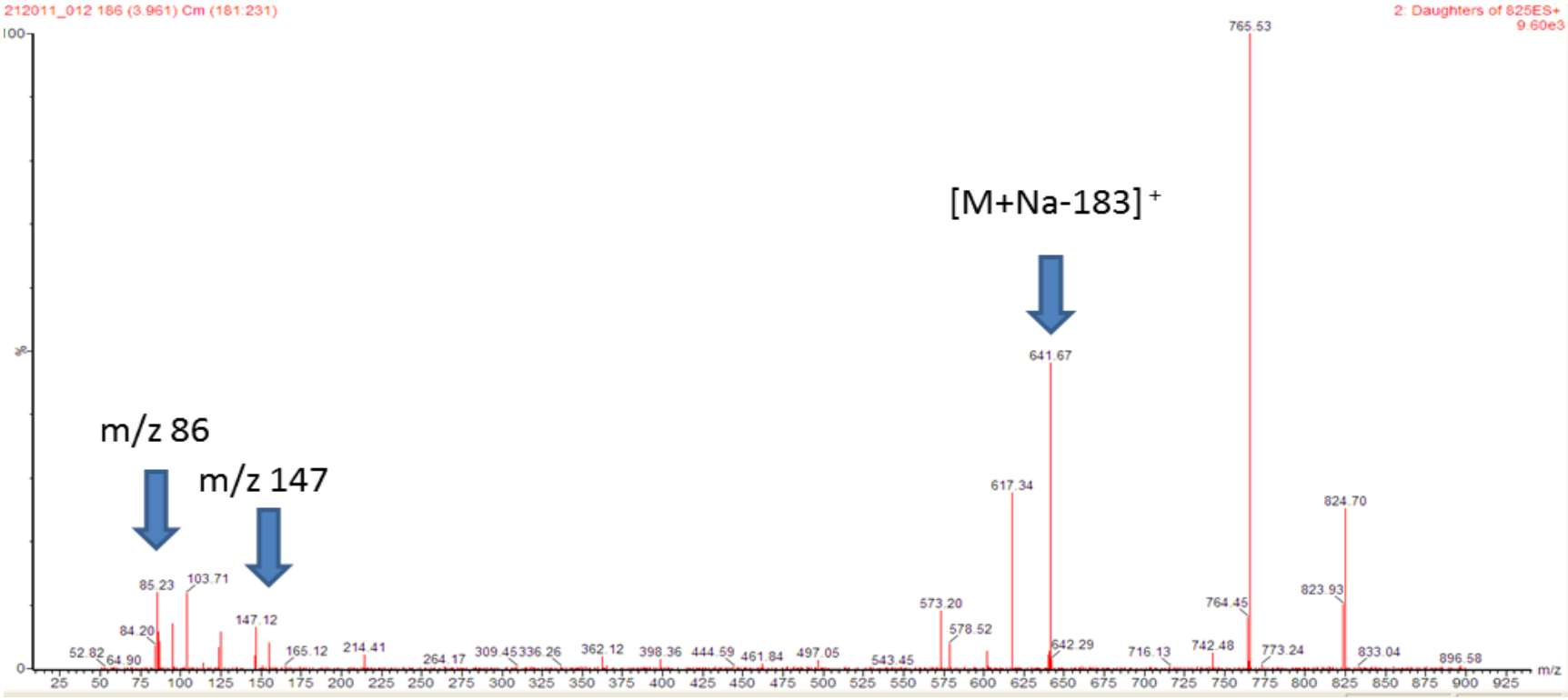

Figure 25 Collision-induced fragmentation spectra of the $[M+Na]^+$ PC(37:1) molecular ion was detected by UPLC-TQMS.

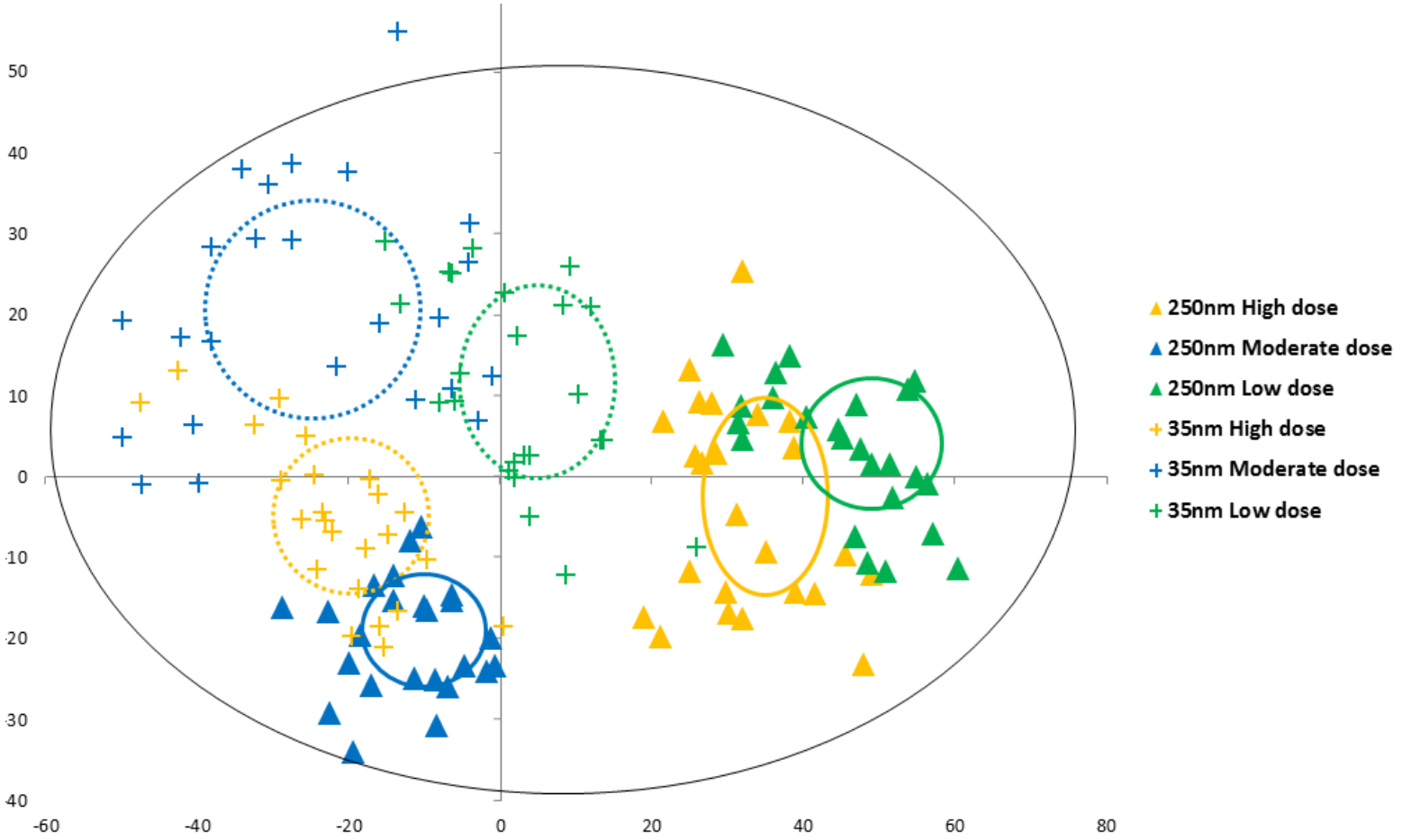

Figure 26 PCA scores plot from UPLC-Qtof/MS spectra of rats' serum samples from various doses of 250 nm ZnO and 35 nm treatment. Each point on PCA scores plot represents one sample run.

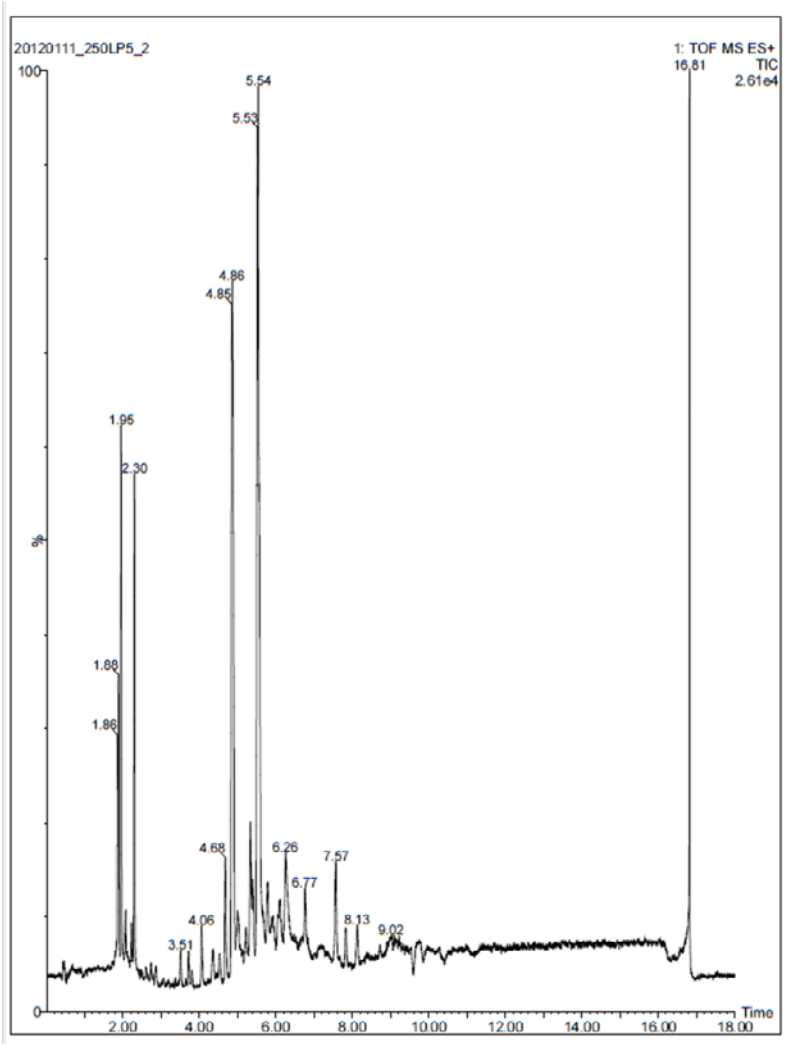

Figure 27 Total ion current (TIC) chromatogram of UPLC-Qtof/MS separation of rat's serum sample after extraction.

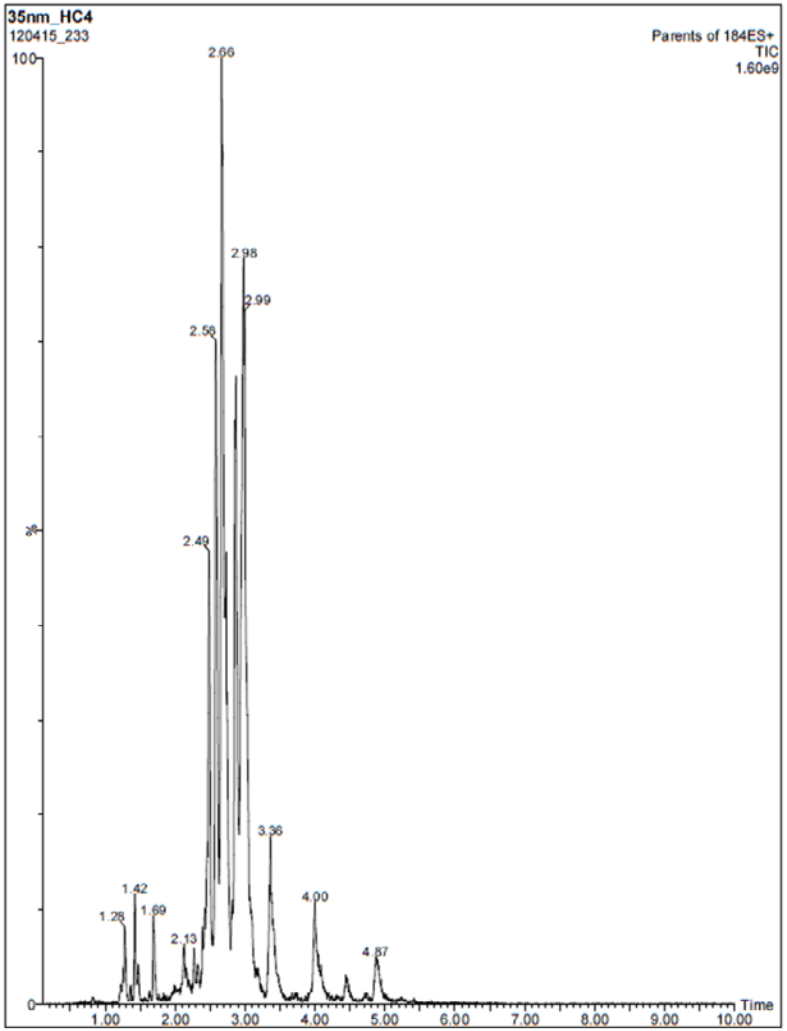

Figure 28 Total ion current (TIC) chromatogram of UPLC-TQMS separation of rat's serum sample after extraction.

TABLES

Table 1 Operation parameters in ZnO exposure experiment.

	Low dose	Moderate dose	High dose
35 nm			
Furnace temp. (°C)	5775	5775	5775
N^2 (L/min)	0.75	1	1
Reacting air (L/min)	7.5	10	10
Diluting air (L/min)	120	150	50
250 nm			
Furnace temp. (°C)	650	650	650
N^2 (L/min)	0.2	0.3	0.7
Reacting air (L/min)	2	3	7
Diluting air (L/min)	120	100	50

Table 2 Average exposure condition in each experiment.

	Low dose	Moderate dose	High dose
35 nm			
Diameter (nm) (Count Geo. Mean)	37.5	37.9	35.6
Geo. Std. dev.	2	1.9	2
Estimated surface area conc. (mm2/m3)	1.70E+04	2.50E+04	1.00E+04
Number conc. (#/cm3)	1.50E+04	2.10E+04	7.90E+04
Mass conc. (mg/m3)	2.4	3.7	12.1
250 nm			
Diameter (nm) (Count Geo. Mean)	262	242.4	250
Geo. Std. dev.	1.7	1.6	1.6
Estimated surface area conc. (mm2/m3)	2.00E+04	4.20E+04	1.20E+04
Number conc. (#/cm3)	6.20E+04	1.50E+04	4.00E+04
Mass conc. (mg/m3)	7.2	11.5	45.2

Table 3 ZnO particle exposure date, number of SD rat used and sample number for MS analysis of each group.

ZnO particle exposure experiment	Exposure date	# of SD rat	# of sample taken for MS analysis
35 nm high dose	November 27, 2008	6	6
high dose control		4	4
35 nm moderate dose	December 4, 2008	6	6
moderate dose control		4	3
35 nm low dose	December 11, 2008	6	6
low dose control		4	4
250 nm high dose	February 19, 2009	6	6
high dose control		4	4
250 nm moderate dose	February 27, 2009	6	6
moderate dose control		4	3
250 nm low dose	March 6, 2009	6	6
low dose control		4	4

Table 4 223 markers selected from the results of PCA from the analysis of UPLC-Qtof/MS spectra of serum of rats exposed to 35 nm ZnO.

ID	Retention time	m/z
Oxalicunknownacid	0.48	90.98
Senecioicunknownacid	3.80	101.06
L-Valinol	1.95	104.11
Pyrocatechol	3.80	111.04
Niacinamide	0.43	123.08
4-Methylcatechol	0.54	125.06
unknown	0.47	128.96
Dihydrothymine	3.80	129.06
2-Methylglutaricunknownacid	3.80	147.07
Citramalicunknownacid	2.22	149.02
unknown	0.48	158.96
trans-4-Decenoicunknownacid	2.89	171.14
Cysteinylglycine	1.09	179.06
4-Pyridoxicunknownacid	1.88	184.07
L-Kynurenine	5.79	209.08

Prilocaine	1.87	221.15
unknown	0.48	226.95
unknown	1.20	240.23
Pirbuterol	3.80	241.18
HuperzineunknownB	0.68	257.18
Tetradecanedioicunknownacid	3.80	259.19
Acetyl-N-formyl-5-methoxykynurenamine	1.84	265.14
Selenocystathionine	1.34	271.00
Sulfametopyrazine	6.76	281.05
octadecanamide	3.03	284.29
17a-Ethynylestradiol	0.67	297.17
PC(O-2:0/O-1:0)	2.21	301.14
Retinoicunknownacid	2.61	303.23
Arachidonicunknownacid	2.87	305.24
unknown	3.54	312.32
ArachidonicunknownAcidunknown	2.68	313.27
Diampromide	2.61	325.21
Ajmaline	2.59	327.23

Ajmaline	2.87	327.23
Docosapentaenoicunknownacid	2.68	331.28
unknown	3.51	338.34
2-oxo-heneicosanoicunknownacid	3.08	341.30
MG(18:3)	2.68	353.27
5beta-Chol-9-en-24-oicunknownAcid	3.07	359.31
N-butylunknownarachidonoylunknownamine	3.51	360.32
unknown	0.48	362.93
trans-3-Hydroxycotinineunknownglucuronide	2.37	369.12
Tetracosanoicunknownacid	2.50	369.35
Tetradecenoylunknowncarnitine	3.80	371.31
24-Nor-5beta-cholane-3alpha,7alpha,12alpha,23-tetrol	3.09	381.30
1alpha,3alpha-Dihydroxy-5beta-cholan-24-oicunknownAcid	3.80	393.30
Etonitazene	3.50	397.24
2-(2-{2-[2-(2-{2-[2-(2-Ethoxy-Ethoxy)-Ethoxy]-Ethoxy}-Ethoxy)-Ethoxy]-Ethoxy}-Ethoxy)-Ethanol,unknownPolyethyleneglycolunknownPeg400	3.18	399.25
L-Palmitoylcarnitine	1.97	400.34
7b-Hydroxy-3-oxo-5b-cholanoicunknownacid	2.89	403.31
unknown	4.35	404.39

N1-(1-Dimethylcarbamoyl-2-Phenyl-Ethyl)-2-Oxo-N4-(2-Pyridin-2-Yl-Ethyl)-Succinamide	2.79	411.17
LysoPC(10:0)	3.82	413.27
Eplerenone	1.59	415.21
Palmitoylunknownglucuronide	4.31	419.32
5beta-Cholest-25-ene-3alpha,7alpha,12alpha-triol	5.70	419.35
Stearidonylunknowncarnitine	2.89	420.33
1beta,3alpha,7alpha,12alpha-Tetrahydroxy-5beta-cholan-24-oicunknownAcid	2.89	425.29
1beta,3alpha,7alpha,12alpha-Tetrahydroxy-5beta-cholan-24-oicunknownAcid	3.37	425.30
Elaidicunknowncarnitine	2.10	426.35
Cholesterylunknownacetate	6.09	429.37
Cholesterylunknownacetate	6.27	429.37
phenyl{bis[(trifluoroacetyl)oxy]}-lambda~3~-iodane	0.48	430.91
27-Nor-5b-cholestane-3a,7a,12a,24,25-pentol	4.95	439.32
L-Alfa-Lysophosphatidylcholine,unknownLauroyl	4.30	441.30
3-Hydroxy-linoleylunknowncarnitine	4.37	441.33
LysoPE(0:0/16:0)	1.93	454.29
Sulfolithocholicunknownacid	2.09	457.28
LysoPC(14:0)	1.60	468.31

unknown	2.09	474.31
unknown	5.69	475.41
unknown	7.02	475.42
Gentamicin	1.95	478.33
Iloprost	2.09	479.26
1alpha,25-dihydroxy-2alpha-(3-hydroxypropoxy)-19-norvitaminunknownD3unknown/unknown1alpha,25-dihydroxy-2alpha-(3-hydroxypropoxy)-19-norcholecalciferol	5.80	479.37
LysoPC(15:0)	2.28	482.32
LysoPC(15:0)	1.78	482.33
PC(O-16:0/0:0)	2.03	482.36
unknown	5.69	492.44
LysoPC(16:1)	1.73	494.32
LysoPC(16:0)	1.95	496.34
Fexofenadine	1.87	502.30
unknown	1.86	502.33
CP-526423	6.76	503.11
1alpha-hydroxy-2beta-(5-hydroxypentoxy)vitaminunknownD3unknown/unknown1alpha-hydroxy-2beta-(5-hydroxypentoxy)cholecalciferol	5.80	503.41

LysoPC(18:1)	2.30	506.36
PC(O-18:1/0:0)	2.15	508.38
LysoPE(0:0/20:0)	2.12	510.36
LysoPC(18:3)	1.95	518.32
Genisteinunknown7-O-glucoside-6"-malonate	6.77	519.14
LysoPC(18:2)	1.85	520.34
unknown	5.48	520.51
unknown	5.24	521.42
LysoPC(18:1)	2.07	522.35
LysoPC(18:0)	2.30	524.37
32,35-anhydrobacteriohopaneterol	6.84	529.46
Anhydroeschscholtzxanthin	4.65	531.41
Oleoylunknownestrone	5.80	535.44
Malvidinunknown3-(6"-acetylglucoside)	6.77	536.16
LysoPE(20:0)	2.47	538.39
LysoPC(20:5)	1.83	542.32
LysoPC(20:4)	2.07	544.34
LysoPC(20:3)	2.30	546.35

LysoPC(20:2)	2.16	548.37
unknown	7.02	548.50
2-hexaprenyl-6-methoxy-1,4-benzoquinol	5.86	549.42
LysoPC(20:1)	2.38	550.39
LysoPC(20:0)	2.63	552.40
PC(10:0/9:0)	4.65	553.39
unknown	5.80	557.42
DG(32:3)	6.30	563.47
octatriacontanoicunknownacid	6.34	565.57
unknown	1.89	566.32
unknown	0.48	566.89
LysoPC(22:6)	1.87	568.34
LysoPC(22:5)	1.96	570.36
unknown	7.60	579.54
unknown	6.10	580.49
unknown	5.35	584.45
3,4-Dihydrospirilloxanthin	3.63	599.50
unknown	7.53	604.60

Peonidinunknown3-rutinoside	7.83	610.18
unknown	6.78	610.54
unknown	7.84	618.62
Ceramideunknown(18:1/22:0)	7.53	622.61
unknown	4.21	627.53
unknown	3.86	627.53
unknown	4.08	627.53
DG(18:0/0:0/18:0)	7.58	630.62
unknown	8.14	632.63
unknown	6.40	634.54
PE(28:0)	6.90	636.55
Cer(18:1/23:0)	7.84	636.63
unknown	7.38	638.57
unknown	8.38	646.65
Ceramideunknown(d18:1/24:1(15Z))	7.59	648.63
N-Lignoceroylsphingosine	8.14	650.64
Cer(d18:0/24:0)	8.26	652.67
unknown	6.47	654.51

unknown	7.57	663.45
Ceramideunknown(d18:1/25:0)	8.38	664.66
N-(24-hydroxytetracosanyl)sphinganine	7.66	668.65
unknown	3.51	675.68
GPSer(14:0/14:0)[U]	7.57	680.48
unknown	6.06	682.54
DG(19:0/22:6/0:0)	2.68	683.54
Petunidinunknown3-glucoside-5-(6"-acetylglucoside)	8.73	684.20
1-tetradecanyl-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine	6.55	684.55
unknown	8.30	696.59
unknown	3.65	701.56
unknown	2.62	702.44
unknown	0.48	702.86
2-demethylmenaquinone-8	4.06	703.57
unknown	2.63	707.40
PC(32:0)	5.53	720.59
unknown	4.06	725.55
PC(14:0/18:2)	4.25	730.54

Ubiquinol	4.71	731.60
PC(14:0/18:1)	4.73	732.56
PC(16:0/16:0)	5.22	734.57
unknown	3.09	739.60
PC(33:2)	4.56	744.55
PC(33:1)	5.06	746.57
PC(33:0)	5.67	746.60
PE(38:5)	5.84	752.56
PE(38:3)	5.88	754.58
PC(34:3)	5.23	756.55
PC(34:3)	4.40	756.55
PC(34:2)	4.88	758.57
PC(O-18:1/O-18:1)	5.45	759.64
PC(34:1)	5.40	760.58
PC(14:0/20:0)	5.95	762.60
PC(10:0/24:0)	3.80	763.61
PC(36:4)	5.12	766.58
PC(15:0/20:4)	5.54	768.55

PC(18:2/18:1)	5.14	768.59
PC(15:0/20:2)	5.21	772.59
PC(15:0/20:1)	5.76	774.60
PE(22:5/18:1)	5.63	776.57
PC(14:0/22:5)	4.92	780.55
PC(36:4)	4.53	782.57
PC(36:4)	4.85	782.57
unknown	5.65	785.66
PC(36:2)	5.57	786.60
SM(18:1/22:0)	6.23	787.67
PC(36:1)	6.11	788.61
PC(36:0)	6.66	790.63
PC(15:0/22:6)	4.37	792.55
PC(20:4/18:1)	4.95	792.59
TG(38:4)	5.28	794.61
PC(37:4)	5.19	796.59
PC(38:3)	5.61	796.62
unknown	6.06	799.67

PC(15:0/22:2)	5.92	800.62
TG(48:3)	6.73	801.69
Lactosylceramideunknown(18:1/12:0)	4.67	806.57
PC(16:0/22:5)	5.59	808.59
PC(38:4)	5.53	810.60
unknown	5.70	811.66
PC(38:3)	5.82	812.62
Glucosylceramide	7.24	812.70
TG(49:3)	6.27	813.68
PC(14:1/24:1)	6.19	814.64
TG(49:3)	7.19	815.70
PE(20:0/22:6)	5.00	820.59
PE(42:4)	5.87	824.62
PC(18:3/22:6)	4.68	828.55
PC(40:8)	4.43	830.57
PC(40:7)	5.51	832.59
PC(40:6)	5.77	834.60
3-decaprenyl-4,5-dihydroxybenzoicunknownacid	6.27	835.67

PC(18:0/22:5)	5.81	836.62
PC(18:0/22:4)	6.00	838.63
PC(18:0/22:4)	6.23	838.64
PC(20:4/22:6)	4.31	854.57
PC(20:3/22:6)	5.32	856.59
unknown	3.48	876.56
unknown	3.63	876.57
PI(16:0/22:4)	4.21	887.57
unknown	4.10	904.60
unknown	3.86	904.60
unknown	8.88	940.75
unknown	8.97	966.76

Table 5 The metabolites with top 50 VIPs values selected from the results of OPLS-DA (high dose vs. high dose control) of UPLC-Qtof/MS spectra from serum of rats exposed to high dose of 35 nm ZnO.

VIP score	Lipid	m/z	Mean ± SD (Control)	Mean ± SD (Treatment)	t-test
6.01	PC(34:1)	760.59	447.28±66.43	348.2461±35.06	0.014 [a]
5.19	LysoPC(18:0)	524.37	550.23±53.12	635.14±53.93	0.05 [a]
4.98	LysoPC(18:2)	520.34	491.93±42.57	421.15±29.42	0.01 [b]
3.52	LysoPC(18:1)	522.35	194.5391±14.58714	159.0461±20.77115	0.02 [b]
3.49	LysoPC(16:0)	496.34	722.06±14.32	776.61±67.84	0.16
2.77	Anhydroeschscholtzxanthin	531.41	35.47±43.48	5.35±9.84	0.131
2.47	PC(36:4)	782.57	469.61±55.51	507.17±46.42	0.32
2.46	Lactosylceramide (30:1)	806.57	217.23±19.25	19.25±18.82	0.07
2.44	PC(36:1)	788.61	273.31±41.82	245.80±20.65	0.2
2.22	PC(38:3)	812.62	159.27± 17.85	128.04±20.33	0.038 [b]
2.2	PC(36:2)	786.60	1097.6±71.17	1066.12±24.51	0.336
2.18	LysoPC(22:6)	568.34	44.68±4.04	33.54±2.65	0.005 [b]
2.11	PC(32:0)	734.57	54.6146±4.42769	67.8793±9.04513	0.03 [a]
2.1	CerP(44:1)	799.67	11.81±9.16	27.88±6.63	0.012 [a]
2.02	PC(34:0)	762.60	69.71±7.41	82.2±7.80	0.39

2	PC(34:2)	758.57	1086.48±67.58	1117.68±44.89	0.4
1.91	PC(36:4)	782.57	73.14±9.74	89.62±12.14	0.054
1.68	PC(36:3)	784.59	27.24±11.07	15.32±5.56	0.05
1.56	TG(49:3)	815.70	105.58±27.09	123.93±15.91	0.21
1.55	PC(32:1)	732.56	24.4922±9.25880	15.4260±5.37342	0.08
1.52	PC(38:3)	812.62	10.6799±6.00374	2.7716±1.89446	0.02 [b]
1.48	LysoPC(16:1)	494.32	19.31±5.09	13.041.72	0.02 [b]
1.36	PC(19:0)	553.39	8.62±8.34	1.78±3.51	0.106
1.32	Tetracosanoic acid	369.35	6.94±3.23	14.42±4.69	0.025 [a]
1.31	PC(38:2)	814.64	44.22±4.98	37.98±4.32	0.07
1.3	PC(40:6)	834.60	214.66±31.61	200.92±9.49	0.34
1.3	N-Lignoceroylsphingosine	650.64	42.69±5.42	36.28±3.21	0.045 [b]
1.23	PC(14:0/22:5)	780.55	33.2550±5.95650	26.9832±6.21458	0.15
1.21	PE(42:4)	824.62	16.57±2.54	20.94±2.23	0.02 [a]
1.15	MG(24:0/0:0/0:0)	521.42	2.55±1.71	7.46±4.89	0.094
1.14	PC(37:4)	796.59	20.04±1.95	23.7±1.30	0.007 [a]
1.13	TG(48:3)	801.69	80.61±14.75	89.45±8.47	0.26
1.1	LysoPC(22:5)	570.36	6.76±0.46	3.86±1.04	0.001 [b]

1.05	PC(33:1)	746.57	9.19±1.71	5.54±2.10	0.02 [b]
1	PC(35:1)	774.60	14.95±7.45	10.78±2.74	0.07
0.98	LysoPC(20:3)	546.36	11.43±9.07	4.83±3.25	0.13
0.91	SM(40:2)	785.66	0±0	4.69±4.34	0.067
0.9	PE(38:5)	752.56	8.59±1.64	11.58±2.80	0.093
0.88	PC(40:5)	836.62	14.6±7.45	7.96±8.80	0.25
0.86	Huperzine B	257.18	9.42±1.40	12.11±1.26	0.013 [a]
0.85	PC(33:0)	746.61	17.15±3.00	20.41±3.71	0.182
0.84	Traumatic acid	474.31	1.14±2.10	5.41±8.23	0.347
0.81	LysoPE(20:0)	510.36	15.43±1.05	18.2±3.29	0.147
0.81	Cholesteryl acetate	429.37	1.17±1.68	5.65±9.22	0.37
0.76	LysoPC(20:1)	550.39	5.49±0.46	7.22±1.10	0.018 [a]
0.76	Iloprost	479.26	0.97±1.81	4.34±4.34	0.332

[a] increased average concentration compared to control with significant *p* value with t-test.

[b] decreased average concentration compared to control with significant *p* value with t-test.

Table 6 The metabolites with top 50 VIP values selected from the results of OPLS-DA (moderate dose vs. moderate dose control) of UPLC-Qtof/MS spectra from serum of rats exposed to high dose of 35 nm ZnO.

VIP score	Lipid	m/z	Mean ± SD (Control)	Mean ± SD (Treatment)	t-test
5.42	PC(38:3)	812.62	177.6±26.52	74±22.37	<0.0001 [b]
5.27	PC(38:4)	810.60	584.25±105.59	712.14±110.07	0.14
4.79	PC(34:1)	760.58	485.2±53.69	390±38.89	0.018 [b]
3.97	PC(34:2)	758.57	1065.33±68.95	1156.92±116.18	0.257
3.77	LysoPC(18:0)	524.37	639.17±54.92	718.47±100.76	0.253
3.20	PC(36:3)	784.59	31.84±7.26	5.1±3.21	<0.0001 [b]
3.13	PC(36:1)	788.61	274.29±29.18	240.36±11.63	0.035 [b]
3.06	Goyaglycoside	663.45	152.42±13.83	183.19±14.19	0.033 [a]
2.53	PC(40:6)	834.60	223.8±25.04	182.63±65.57	0.341
2.28	LysoPC(18:1)	522.35	199.02±5.24	176.02±21.60	0.122
2.26	PC(32:1)	732.56	25.95±7.52	10.8±5.34	0.009 [b]
2.14	PC(36:5)	780.55	31.62±5.74	18.28±5.31	0.03 [b]
1.94	trans-4-Decenoic acid	171.14	0±0	21.9±31.84	0.288
1.93	PC(32:0)	734.57	70.54±4.10	58.64±6.24	0.22
1.83	Lactosylceramide (30:1)	806.57	226.87±28.04	201.03±46.92	0.417

1.79	PC(38:2)	814.64	40.01±2.29	30.83.63	0.006 [b]
1.70	LysoPC(16:1)	494.32	19.86±2.87	11.86±2.45	0.019 [b]
1.61	LysoPC(16:0)	496.34	841.27±71.65	871.7±79.85	0.596
1.53	32,35-anhydrobacteriohopaneterol	529.46	29.11±2.56	36.12±2.78	0.008 [a]
1.38	PC(36:4)	782.57	430.93±57.07	455.83±74.53	0.63
1.37	LysoPC(18:2)	520.34	437.85±38.15	418.76±42.33	0.533
1.37	1alpha,25-dihydroxy-2alpha-(3-hydroxypropoxy)-19-norvitamin D3	479.37	27.23±2.42	33.07±2.75	0.025 [a]
1.33	TG(49:3)	815.70	144±27.51	123.80±25.89	0.35
1.32	GPSer(28:0)	680.48	28.53±3.04	34.49±2.78	0.04 [a]
1.32	PC(33:1)	746.57	9.65±0.83	4.47±1.97	0.004 [b]
1.30	Malvidin 3-(6"-acetylglucoside)	536.16	62.21±41.16	83.03±32.57	0.431
1.25	LysoPC(20:4)	544.34	207.59±33.94	220.52±14.05	0.427
1.22	SM(40:1)	787.67	99.16±10.17	88.6±9.84	0.177
1.21	LysoPC(22:6)	568.34	44.12±9.76	36.5±7.29	0.224
1.21	TG(48:3)	801.69	93.31±8.78	104.334±8.07	0.146
1.15	LysoPC(20:3)	546.35	12.48±8.01	19.2±2.40	0.085
1.13	Palmitoyl glucuronide	419.32	7.55±9.03	1.66±2.14	0.15

1.11	Cholesteryl acetate	429.37	1±0.87	8.03±8.41	0.206
1.09	PC(16:0/22:5)	808.59	100.4±25.83	86.39±21.07	0.408
1.04	Hericene A	557.42	5.46±1.80	8.804±21.08	0.009 [a]
1.00	PE(42:4)	824.62	10.00±2.95	15.55±5.94	0.18
0.99	LysoPC(20:5)	542.32	7.32±7.74	11.75±1.41	0.06
0.97	PC(18:0/22:5)	836.62	8.41±2.35	11.34±1.41	0.055
0.95	PC(40:7)	832.59	15.83±1.06	11.93±3.37	0.015 [b]
0.94	PC(35:1)	774.60	0.12±0.10	6.4±11.53	0.099
0.94	1beta,3alpha,7alpha,12alpha-Tetrahydroxy-5beta-cholan-24-oic Acid	425.29	1.81±0.75	6.84±7.30	0.392
0.92	MG(18:3)	353.27	1.23±0.31	4.52±2.73	0.287
0.91	DG(36:4)	634.54	19.08±2.21	23.38±2.95	0.085
0.89	PC(20:3/22:6)	856.59	10.22±1.02	12.99±1.42	0.009 [a]
0.87	Sabadelin	548.50	10.23±1.02	13±1.42	0.021 [a]
0.85	PC(34:3)	756.55	19.14±2.32	15.34±3.62	0.148
0.85	PC(34:0)	762.60	70.21±5.14	73.48±2.67	0.166

[a] increased average concentration compared to control with significant p value ($p<0.05$).

[b] decreased average concentration compared to control with significant p value ($p<0.05$).

Table 7 The metabolites with top 50 VIP values selected from the results of OPLS- DA (low dose vs. low dose control) of UPLC-Qtof/MS spectra from serum of rats exposed to high dose of 35 nm ZnO.

VIP score	Lipid	m/z	Mean+±SD (Control)	Mean ± SD (Treatment)	t-test
7.63	PC(38:4)	810.60	733.34±25.79	998.36±54.37	<0.0001 [a]
5.65	PC(34:2)	758.57	1127.15±74.76	950.26±78.82	0.009 [b]
5.23	PC(36:2)	786.60	1101.8±42.58	943.71±93.90	0.014 [a]
4.27	LysoPC(18:2)	520.34	475.52±290.	377.3±45.78	0.006 [b]
3.20	PC(34:1)	760.58	326.96±10.40	276.45±18.44	0.001 [b]
2.54	LysoPC(16:0)	496.34	836.8±84.75	768.98±85.58	0.259
2.53	PC(40:6)	834.60	190.62±18.30	234.6±35.97	0.056
2.53	LysoPC(18:1)	522.35	170.24±17.38	136.03±11.84	0.006 [b]
1.98	PC(36:3)	784.58	182.58±24.40	130.88±42.00	0.059
1.97	PC(16:0/22:5)	808.59	70.08±27.80	110.59±51.14	0.19
1.91	PC(36:1)	788.61	231.93±6.52	210.93±11.83	0.013 [b]
1.82	Lactosylceramide (30:1)	806.57	180.6±23.94	212.9±41.83	0.204
1.71	LysoPC(18:0)	524.37	717.39±60.29	760.38±85.79	0.414
1.63	TG(48:3)	801.69	76.15±11.46	97.4±9.76	0.014 [b]
1.45	LysoPC(20:4)	544.34	253.87±32.00	277.87±32.29	0.287

1.32	SM(40:1)	787.67	89.57±4.12	101.51±8.05	0.027 [a]
1.30	PC(37:4)	796.59	17.14±4.05	27.51±6.84	0.027 [a]
1.25	PC(38:2)	814.64	38.29±5.21	28.47±6.12	0.031 [b]
1.16	Anhydroeschscholtzxanthin	531.41	26.49±7.92	14.75±13.91	0.169
1.08	2-demethylmenaquinone-8	703.57	99.2±9.51	113.94±25.22	0.304
1.05	SM(40:0)	811.66	78.13±9.00	88.87±13.29	0.199
1.05	PE(42:4)	824.62	13.63±5.57	21.97±6.20	0.062
0.99	Goyaglycoside	663.45	191.88±25.78	174.51±44.15	0.502
0.95	PC(40:7)	832.59	6.73±3.16	12.88±2.27	0.007 [a]
0.95	1-tetradecanyl-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine	684.55	6.96±3.33	1.75±1.64	0.01 b
0.95	PC(36:3)	768.59	18.29±2.50	24.96±6.61	0.094
0.92	TG(49:3)	813.68	247.1±19.57	266.4±58.38	0.548
0.92	LysoPC(22:6)	568.34	43.38±4.56	37.16±4.76	0.074
0.89	TG(38:4)	794.61	13.54±2.71	19.47±5.36	0.078
0.87	Tetracosanoic acid	369.35	3.27±1.76	10.33±7.44	0.105
0.85	PE(28:0)	636.55	10.92±4.68	5.22±5.04	0.11
0.84	PC(32:0)	734.57	54.02±8.37	62.71±15.41	0.338
0.80	PC(36:4)	766.58	3.75±2.73	8.11±2.94	0.048 [a]

0.76	32,35-anhydrobacteriohopaneterol	529.46	40±5.95	34.3±6.59	0.199
0.76	Petunidin 3-glucoside-5-(6"-acetylglucoside)	684.20	43.8±5.84	36.53±11.59	0.229
0.75	PC(33:0)	746.60	16±2.19	20.81±5.29	0.128
0.75	PC(19:0)	553.39	9.63±2.09	5.13±4.84	0.122
0.73	Ubiquinol	731.60	5.46±1.82	9.62±3.53	0.064
0.72	PC(38:3)	796.62	11.21±0.68	14.47±2.41	0.04 [a]
0.70	PC(33:2)	744.55	12.74±2.74	9.33±1.61	0.032 [b]
0.67	PC(36:0)	790.63	20.45±3.14	24.85±4.93	0.036 [a]
0.65	PC(35:2)	772.59	35.01±3.32	30.23±7.78	0.287
0.64	DG(36:2)	610.54	4.51±3.41	1.39±12.30	0.071
0.64	Ceramide (42:2)	648.63	5.64±1.01	9.5±3.56	0.072
0.63	PC(40:8)	830.57	3.16±2.60	7.5±5.98	0.214
0.61	MG(18:3)	353.27	8.86±11.68	16.3±22.67	0.567

[a] increased average concentration compared to control with significant p value ($p<0.05$).

[b] decreased average concentration compared to control with significant p value ($p<0.05$).

Table 8 Fold-changes (high-, moderate- and low-dose/ high-, moderate- and low-dose control) of 17 common lipids selected from high-, moderate- and low-dose of 35 nm ZnO treatment from UPLC-Qtof/MS results.

Lipid	High dose / High dose control	Moderate dose/ Moderate dose control	Low Dose/ Low dose control
PC(38:5)	0.61	0.86	1.58
PC(34:1)	0.78	0.80	0.85
PC(34:2)	1.03	1.09	0.84
LysoPC(18:0) [a]	1.15 *	1.12	1.06
PC(36:3)	0.56	0.16	0.72
PC(36:1)	0.90	0.88	0.91
PC(40:6)	0.94	0.82	1.23
Lactosylceramide (30:1) [b]	0.09	0.89	1.18
PC(38:2) [a]	0.86	0.77 *	0.74
LysoPC(16:0) [a]	1.08	1.04	0.92
PC(36:4)	1.08	1.06	1.28
LysoPC(18:2)	0.86	0.96	0.79
LysoPC(22:6) [b]	0.75 *	0.83	0.86
TG(48:3) [b]	1.11	1.12	1.28 *
PE(42:4) [b]	1.26 *	1.56	1.61

PC(34:1) [b]	0.78 *	0.80 *	0.85 *
PC(34:2)	1.03	1.09	0.84
PC(36:3)	0.56	0.16	0.72
PC(36:1)	0.90	0.88	0.91
PC(40:6)	0.94	0.82	1.23
LysoPC(18:1)	0.82	0.88	0.80
PC(32:0)	1.24	0.83	1.16
Lactosylceramide (30:1)	0.09	0.89	1.18
PC(36:4)	1.08	1.06	1.28
LysoPC(18:2)	0.86	0.96	0.79

[*] significant p value with t-test ($p<0.05$).

[a] increased average concentration compared to control with significant p value with t-test($p<0.05$).

[b] decreased average concentration compared to control with significant p value with t-test ($p<0.05$).

Table 9 270 markers selected from the results of PCA from the analysis of UPLC-Qtof/MS spectra of serum of rats exposed to 250 nm ZnO.

ID	Retention Time	m/z
Unkonwn	5.33	55.44
Unkonwn	5.33	55.63
Oxalic acid	0.49	90.98
L-Valinol	1.95	104.11
L-Valinol	2.29	104.11
Betaine	0.46	118.09
Niacinamide	0.43	123.08
2,5-Dimethylpyrimidin-4-Amine	8.93	124.09
Unkonwn	0.49	128.95
Dihydrothymine	3.80	129.06
Unkonwn	5.52	134.87
L-Malic acid	5.52	135.01
2-Methylglutaric acid	3.80	147.06
Citramalic acid	4.36	149.02
Unkonwn	0.49	158.97

3-(3-Hydroxyphenyl)propanoic acid	5.79	167.07
Pyridoxamine	0.43	169.09
4-Pyridoxic acid	1.88	184.07
cis-stilbene oxide	1.85	197.12
L-Kynurenine	5.79	209.08
Prilocaine	1.87	221.15
Unkonwn	0.49	226.95
Traumatic acid	0.56	229.14
Talbutal	2.19	253.18
Huper ine B	0.69	257.18
Tetradecanedioic acid	3.80	259.19
Acetyl-N-formyl-5-methoxykynurenamine	1.84	265.14
Atenolol	2.28	267.16
Diethylstilbestrol	0.56	269.14
3-Hydroxytetradecanedioic acid	4.31	275.16
Alpha-Linolenic acid	2.74	279.23
Sulfametopyra ine	6.76	281.05
octadecanamide	3.03	284.29

Unkonwn	2.73	284.33
17a-Ethynylestradiol	0.69	297.17
Unkonwn	2.84	298.35
PC(O-2:0/O-1:0)	2.21	301.14
9-cis-Retinoic acid	2.74	301.21
Retinoic acid	2.60	303.23
Arachidonic acid	2.86	305.25
Unkonwn	3.54	312.32
Escitalopram	1.89	325.20
Diampromide	2.60	325.21
Ajmaline	2.86	327.23
Docosahexaenoic acid	3.20	329.24
Docosahexaenoic acid	2.74	329.25
Unkonwn	2.45	336.33
N-Dodecyl-N,N-Dimethyl-3-Ammonio-1-Propanesulfonate	6.05	337.27
Unkonwn	3.51	338.34
3b,15b,17a-Trihydroxy-pregnenone	2.58	349.21
Tetrahydrocorticosterone	2.51	351.23

MG(18:3)	2.64	353.27
5beta-Chola-3,8(14),11-trien-24-oic Acid	2.88	355.26
N-butyl arachidonoyl amine	3.51	360.32
Unkonwn	0.49	362.93
3-Oxochola-1,4,6-trien-24-oic Acid	2.80	369.24
Tetracosanoic acid	4.35	369.35
Tetradecenoyl carnitine	3.80	371.32
Biocytin	2.51	373.21
MG(0:0/20:5/0:0)	2.51	377.27
24-Nor-5beta-cholane-3alpha,7alpha,12alpha,23-tetrol	3.07	381.30
Clonita ene	1.34	387.18
1alpha,3alpha-Dihydroxy-5beta-cholan-24-oic Acid	3.80	393.30
Etonita ene	3.49	397.24
2-(2-{2-[2-(2-{2-[2-(2-Ethoxy-Ethoxy)-Ethoxy]-Ethoxy}-Ethoxy)-Ethoxy]-Ethoxy}-Ethoxy)-Ethanol, Polyethyleneglycol Peg400	2.20	399.25
L-Palmitoylcarnitine	1.97	400.34
Unkonwn	4.34	404.39
LysoPC(10:0)	3.81	413.27
Palmitoyl glucuronide	4.31	419.32

5beta-Cholest-25-ene-3alpha,7alpha,12alpha-triol	5.69	419.35
Linoelaidyl carnitine	1.88	424.34
1beta,3alpha,7alpha,12alpha-Tetrahydroxy-5beta-cholan-24-oic Acid	3.37	425.30
Elaidic carnitine	2.10	426.36
Stearoylcarnitine	2.33	428.37
Cholesteryl acetate	6.08	429.37
Unkonwn	5.47	430.38
phenyl{bis[(trifluoroacetyl)oxy]}-lambda~3~-iodane	0.49	430.91
27-Nor-5b-cholestane-3a,7a,12a,24,25-pentol	4.95	439.32
L-Alfa-Lysophosphatidylcholine, Lauroyl	4.30	441.30
3-Hydroxy-linoleyl carnitine	4.37	441.33
LysoPE(0:0/16:0)	1.93	454.29
Sulfolithocholic acid	2.09	457.28
LysoPC(14:0)	1.60	468.31
Unkonwn	2.09	474.31
Unkonwn	5.68	475.41
Unkonwn	7.01	475.41
LysoPE(18:2)	1.84	478.29

Gentamicin	1.95	478.33
Iloprost	2.09	479.26
1alpha,25-dihydroxy-2alpha-(3-hydroxypropoxy)-19-norvitamin D3 / 1alpha,25-dihydroxy-2alpha-(3-hydroxypropoxy)-19-norcholecalciferol	5.79	479.37
LysoPC(dm16:0)	2.06	480.34
LysoPC(15:0)	2.28	482.32
PC(16:0)	2.03	482.36
1alpha-hydroxy-2beta-(4-hydroxybutoxy)vitamin D3 / 1alpha-hydroxy-2beta-(4-hydroxybutoxy)cholecalciferol	5.30	489.40
Unkonwn	5.69	492.44
LysoPC(16:1)	1.73	494.32
LysoPC(16:0)	1.95	496.34
Fexofenadine	1.86	502.32
CP-526423	6.76	503.11
1alpha-hydroxy-2beta-(5-hydroxypentoxy)vitamin D3 / 1alpha-hydroxy-2beta-(5-hydroxypentoxy)cholecalciferol	5.79	503.41
LysoPC(18:1)	2.29	506.36
LysoPE(20:1)	1.89	508.34
PC(18:1)	2.15	508.38

LysoPE(0:0/20:0)	2.12	510.36
LysoPC(18:3)	1.71	518.32
Genistein 7-O-glucoside-6"-malonate	6.76	519.14
LysoPC(18:2)	1.85	520.34
Unkonwn	5.47	520.51
Unkonwn	5.27	521.42
LysoPC(18:1)	2.07	522.35
LysoPC(18:0)	2.30	524.37
LysoPE(22:6)	1.86	526.29
GPSer(18:0/0:0)	1.88	526.33
32,35-anhydrobacteriohopaneterol	6.83	529.46
Anhydroeschscholt xanthin	4.65	531.41
4-Amino-N-{4-[2-(2,6-Dimethyl-Phenoxy)-Acetylamino]-3-Hydroxy-1-Isobutyl-5-Phenyl-Pentyl}-Ben amide	2.12	532.34
SP2456	2.87	533.19
2-Hexaprenyl-6-methoxyphenol	7.36	533.46
Oleoyl estrone	5.79	535.44
Malvidin 3-(6"-acetylglucoside)	6.76	536.16
LysoPE(20:0)	2.47	538.39

LysoPC(20:5)	1.84	542.32
LysoPC(20:4)	1.88	544.34
31-hydroxy-32,35-anhydrobacteriohopanetetrol	6.45	545.46
LysoPC(20:3)	2.30	546.35
LysoPC(20:2)	2.16	548.37
Unkonwn	7.01	548.50
2-hexaprenyl-6-methoxy-1,4-ben oquinol	5.85	549.42
LysoPC(20:1)	2.38	550.39
LysoPC(20:0)	2.63	552.40
Unkonwn	5.79	557.42
DG(32:3)	6.29	563.47
octatriacontanoic acid	6.33	565.57
Unkonwn	1.89	566.32
LysoPC(22:6)	1.87	568.34
Unkonwn	6.10	580.50
Unkonwn	5.34	584.45
3,4-Dihydrospirilloxanthin	3.32	599.50
Unkonwn	7.52	604.60

LysoPC(24:0)	3.48	608.47
Peonidin 3-rutinoside	7.83	610.18
Unkonwn	6.15	610.50
Unkonwn	6.77	610.54
Unkonwn	7.26	612.56
Unkonwn	7.84	618.62
Ceramide (d18:1/22:0)	7.52	622.61
Cer(d18:0/22:0)	7.66	624.63
Unkonwn	3.71	627.53
DG(18:0/0:0/18:0)	7.59	630.62
Unkonwn	8.13	632.63
Unkonwn	6.39	634.54
PE(28:0)	6.89	636.56
Cer(d18:1/23:0)	7.83	636.63
Unkonwn	7.37	638.57
Unkonwn	8.38	646.65
Ceramide (d18:1/24:1)	7.59	648.63
N-Lignoceroylsphingosine	8.13	650.64

N-Lignoceroylsphingosine	7.71	650.65
Cer(d18:0/24:0)	8.26	652.66
Unkonwn	6.47	654.51
Unkonwn	5.81	656.52
Unkonwn	6.26	658.54
PE(dm16:0/dm16:0)	6.75	660.55
Unkonwn	7.57	663.45
Ceramide (d18:1/25:0)	8.38	664.66
N-(24-hydroxytetracosanyl)sphinganine	7.66	668.65
Unkonwn	3.51	675.68
Ceramide (d18:1/26:0)	8.69	678.68
GPSer(14:0/14:0)	7.57	680.48
Unkonwn	6.05	682.54
Petunidin 3-glucoside-5-(6"-acetylglucoside)	8.72	684.20
1-tetradecanyl-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine	6.54	684.55
PE(dm16:0/dm18:1)	6.78	686.57
Etn-1-P-Cer(d14:1/18:0)	3.78	689.56
Unkonwn	8.29	696.60

Unkonwn	3.64	701.56
Unkonwn	2.62	702.44
2-demethylmenaquinone-8	4.06	703.57
PC(14:0/16:0)	4.56	706.54
Unkonwn	2.63	707.40
PC(15:0/16:0)	4.89	720.55
PC(32:0)	5.53	720.59
PE(18:3/dm18:1)	5.15	724.53
Unkonwn	4.06	725.56
PC(14:0/18:2)	4.25	730.54
Ubiquinol	4.71	731.60
PC(14:0/18:1)	4.73	732.57
PC(16:0/16:0)	5.22	734.57
PC(33:2)	5.59	744.55
PC(33:2)	4.56	744.55
PC(16:0/18:1)	5.17	744.60
PC(33:1)	5.06	746.57
PC(33:0)	5.67	746.61

PE(O-16:1/22:6)	4.95	748.53
PC(O-16:0/18:0)	6.27	748.63
PE(20:4/18:1)	5.29	750.55
PE(38:5)	5.84	752.56
PE(38:3)	5.88	754.58
PC(34:3)	4.39	756.55
PC(34:2)	4.88	758.57
PC(O-18:1(9E)/O-18:1)	5.44	759.64
PC(34:1)	5.40	760.58
PC(14:0/20:0)	5.95	762.60
PC(10:0/24:0)	3.80	763.61
PC(36:4)	5.12	766.58
PC(15:0/20:4)	4.53	768.56
PC(18:2/18:1)	5.13	768.59
PC(15:0/20:2)	5.21	772.59
Unkonwn	5.87	773.65
PC(15:0/20:1)	5.75	774.60
PE(22:5/18:1)	5.63	776.57

PC(14:0/22:5)	4.40	780.56
PE(22:4/18:0)	6.32	780.59
PC(36:4)	4.53	782.57
PC(36:4)	5.40	782.57
PE(22:2/18:1)	6.34	782.61
PC(36:3)	5.02	784.59
Glucosylceramide (d18:1/22:0)	6.57	784.67
Unkonwn	5.63	785.66
PC(36:2)	5.57	786.60
SM(40:1)	6.41	787.67
SM(18:1/22:0)	6.23	787.67
PC(36:1)	6.00	788.61
PC(36:0)	6.66	790.63
PC(15:0/22:6)	4.37	792.56
PC(20:4/18:1)	4.94	792.59
PC(20:3/18:1)	5.81	794.61
PC(37:4)	5.19	796.59
PC(38:3)	5.84	796.62

Unkonwn	6.91	798.68
Unkonwn	6.04	799.67
PC(15:0/22:2)	5.92	800.63
TG(48:3)	6.69	801.69
PC(16:1/22:6)	4.82	804.55
Lactosylceramide (30:1)	4.67	806.57
PC(16:0/22:5)	5.59	808.59
PC(16:0/22:5)	5.11	808.60
Unkonwn	5.13	809.62
PC(38:4)	5.13	810.61
GlucosylCeramide (42:2)	6.62	810.68
Unkonwn	5.69	811.66
Unkonwn	5.81	811.67
PC(38:3)	6.03	812.62
Glucosylceramide	7.24	812.70
TG(49:3)	6.68	813.68
TG(49:3)	6.45	813.69
PC(14:1/24:1)	6.19	814.64

TG(49:3)	7.18	815.70
TG(49:3)	7.62	815.71
PC(22:5/18:1)	5.11	818.59
PE(20:0/22:6)	5.00	820.59
PE(42:4)	5.85	824.62
PC(18:3/22:6)	4.67	828.55
PC(40:8)	4.98	830.57
PC(18:1 /22:6)	5.50	832.59
PC(40:7)	4.81	832.59
PC(40:6)	5.10	834.60
3-decaprenyl-4,5-dihydroxyben oic acid	6.26	835.67
PC(18:0/22:5)	5.56	836.62
PC(18:0/22:4)	6.00	838.64
PC(18:0/22:4)	6.22	838.64
PC(20:4/22:6)	4.31	854.57
PC(20:3/22:6)	5.32	856.59
Unkonwn	4.30	859.61
Unkonwn	3.32	876.57

PI(16:0/22:4)	3.71	887.57
Unkonwn	3.72	904.60
Unkonwn	8.87	940.75
Unkonwn	8.97	966.76

[a] increased average concentration compared to control with significant p value with t-test ($p<0.05$).

[b] decreased average concentration compared to control with significant p value with t-test($p<0.05$).

Table 10 The metabolites with top 50 VIP values selected from the results of OPLS-DA (high dose vs. high dose control) of UPLC-Qtof/MS spectra from serum of rats exposed to high dose of 250 nm ZnO.

VIPS score	Lipid	m/z	Mean ± SD (Control)	Mean ± SD (Treatment)	t-test
6.93	PC(36:2)	786.60	767.85±33.31	876.67±62.47	0.013 [a]
6.03	PC(40:6)	834.60	238.84±24.20	328.73±69.09	0.039 [a]
5.01	PC(16:0/16:0)	734.57	123.82±61.63	62.55±9.99	0.039
4.11	PC(36:4)	782.57	707.36±57.30	646.81±32.28	0.063
3.57	PC(36:1)	788.61	195.68±10.83	225.05±18.20	0.021 [a]
3.17	LysoPC(16:0)	496.34	719.1±70.25	666.37±96.03	0.377
2.57	PC(38:4)	810.60	1109.67±27.30	1071.98±66.14	0.319
2.56	Anhydroeschscholtzxanthin	531.41	16.6±7.39	3.61±2.98	0.004 [b]
2.38	PC(14:0/20:0)	762.60	78.53±15.74	63.8±3.51	0.053
2.34	Lactosylceramide (30:1)	806.57	244.14±37.12	272.87±50.53	0.333
2.23	PC(34:2)	758.57	870.97±1.93	901.38±62.05	0.365
2.15	PC(38:3)	812.62	87.1±4.11	112.01±26.03	0.1
2.11	Huperzine B	257.18	21.07±13.47	9.23±2.78	0.063
2.05	1alpha,3alpha-Dihydroxy-5beta-cholan-24-oic Acid	393.30	11.38±5.66	2.69±2.12	0.008 [b]

1.96	DG(40:8)	682.54	7.22±1.46	15.78±5.55	0.018 [a]
1.85	17a-Ethynylestradiol	297.17	9.51±7.61	1.38±0.46	0.027 [b]
1.74	PE(O-18:1/20:4(5Z)	752.56	22.87±18.94	12.03±2.51	0.191
1.61	Goyaglycoside	663.45	186.16±15.15	173.41±20.80	0.326
1.59	PC(10:0/9:0)	553.39	5.99±2.45	1.15±0.93	0.002 [b]
1.57	DG(44:1)	940.75	7.8±1.77	13.1±2.66	0.008 [a]
1.54	PE(28:0)	636.56	1.74±0.42	6.86±2.89	0.009 [a]
1.53	DG(36:4)	634.54	1.86±0.10	6.43±2.07	0.003 [a]
1.48	L-Alfa-Lysophosphatidylcholine, Lauroyl	441.30	12.34±9.18	4.83±5.64	0.144
1.47	1-tetradecanyl-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine	684.55	1.92±0.73	6.57±2.53	0.008 [a]
1.42	PC(34:3)	756.55	11.16±3.28	6.69±1.36	0.016 [b]
1.41	Thromboxane	338.34	8.55±2.27	16.35±11.90	0.24
1.41	Tetracosanoic acid	369.35	24.49±9.23	17.04±3.77	0.109
1.34	PC(33:0)	746.61	27.92±4.46	23.36±1.48	0.045 [b]
1.32	PC(32:0)	720.59	10.98±3.45	6.98±0.80	0.023 [b]
1.29	TG(49:3)	815.70	131.09±16.16	116.25±23.47	0.306
1.28	LysoPC(18:2)	520.34	235.12±24.14	249.01±45.10	0.592
1.25	Palmitoyl glucuronide	419.32	8.11±12.23	1.7±3.46	0.248

1.24	PE(38:3)	754.58	7.18±9.05	1.58±1.15	0.161
1.23	LysoPC(22:6)	568.34	25.11±2.30	29.7±4.42	0.096
1.19	3-Hydroxy-linoleyl carnitine	441.33	12.7±12.23	6.65±3.31	0.271
1.17	PC(15:0/22:2)	800.63	30.86±1.50	35.76±6.11	0.161
1.16	Arachidonic acid	305.25	1.6±0.63	4.43±1.26	0.003 [b]
1.15	PC(38:3)	812.62	2.81±3.52	8.05±4.45	0.074
1.12	LysoPC(18:0)	524.37	612.61±31.44	626.22±52.46	0.657
1.11	Ajmaline	327.23	2.09±0.19	4.8±0.84	<0.0001 [a]
1.11	Ceramide (42:2)	648.63	17.66±2.32	13.64±3.63	0.088
1.09	DG(36:2)	610.54	0.61±0.20	3.16±1.20	0.003 [a]
1.06	PC(18:2/18:1)	768.59	23.97±3.69	20.73±1.47	0.084
1.05	Citramalic acid	149.02	3.7±5.83	0	0.147
1.05	PC(24:1/P-16:0)	904.60	20.31±5.95	24.19±1.91	0.167
1.05	DG(38:7)	656.52	1.72±0.50	4.03±0.72	0.001 [a]
1.04	PC(38:3)	796.62	14.32±1.32	11.87±0.96	0.009 [b]
1.03	Retinoic acid	303.23	2.55±0.44	4.93±1.33	0.009 [a]
1.03	DG(32:3)	563.47	12.04±3.63	8.82±2.15	0.113
1.02	TG(48:3)	801.69	106.19±9.20	115.12±12.98	0.271

[a] increased average concentration compared to control with significant p value with t-test ($p<0.05$).

[b] decreased average concentration compared to control with significant p value with t-test(p<0.05).

Table 11The metabolites with top 50 VIP values selected from the results of OPLS-DA (moderate dose vs. high dose control) of UPLC-Qtof/MS spectra from serum of rats exposed to high dose of 250 nm ZnO.

VIP score	Lipid	m/z	Mean ± SD (Control)	Mean ± SD (Treatment)	t-test
7.18	1alpha,3alpha-Dihydroxy-5beta-cholan-24-oic Acid	758.57	1055.1±32.45	965.07±60.67	0.049 [b]
5.26	PC(38:4)	810.60	688.88±36.72	758.15±79.78	0.206
5.20	PC(40:6)	834.60	205.34±27.53	257.16±42.61	0.102
4.20	LysoPC(16:0)	496.34	793.09±26.00	752.98±33.39	0.114
3.66	LysoPC(18:2)	520.34	515.81±52.53	480.82±30.08	0.231
3.40	PC(32:0)	787.67	68.61±13.82	86.44±5.62	0.024 [a]
3.19	PC(38:3)	734.57	77.67±14.80	61.7±4.01	0.034 [b]
3.00	PC(36:4)	782.57	443.2±27.30	472.87±43.76	0.326
2.67	PC(36:3)	788.61	218.44±2.70	235.68±20.61	0.205
2.62	LysoPC(22:6)	257.18	15.1±7.57	5.3±2.01	0.016 [b]
2.55	PC(34:2)	524.37	630.33±50.51	660.69±70.61	0.533
2.55	TG(49:3)	524.37	630.33±50.51	660.69±70.61	0.533
2.49	PC(18:4/18:0)	782.57	84.97±9.42	67.39±9.68	0.036 [b]
2.27	PC(14:0/20:0)	531.41	14.63±25.33	1.9±2.93	0.232

2.12	Lactosylceramide (30:1)	812.62	95.33±12.06	117.61±27.35	0.231
2.07	PC(38:4)	808.59	99.92±20.87	117.7±12.22	0.142
2.01	17a-Ethynylestradiol	762.60	84.67±1.21	76.65±7.24	0.108
1.95	Arachidonic acid	752.56	17.6±1.23	12.4±2.28	0.009 [b]
1.91	PE(28:0)	744.55	8.05±2.02	3.12±1.34	0.003 [b]
1.85	Anhydroeschscholtzxanthin	806.57	239.16±14.88	256.09±42.61	0.537
1.66	PC(36:2)	786.60	1042.7±4.55	1024.79±52.46	0.586
1.60	PC(38:3)	801.69	68.13±10.25	76.02±4.93	0.149
1.60	PC(34:3)	429.37	6.08±4.40	1.07±1.30	0.029 [b]
1.58	Tetracosanoic acid	806.57	17.83±2.34	11.67±3.18	0.022 [b]
1.36	LysoPC(18:0)	522.35	187.82±19.40	179.39±15.35	0.497
1.30	PC(33:0)	904.60	18.22±2.62	21.13±1.23	0.051
1.30	PC(32:0)	768.55	6.61±1.31	3.35±2.69	0.093
1.24	4alpha-formyl-4beta-methyl-5alpha-cholesta-8-en-3beta-ol	429.37	3.96±6.86	0±0	0.17
1.24	DG(36:2)	610.54	7.56±0.99	4.93±1.80	0.055
1.23	Palmitoyl glucuronide	703.57	93.24±13.27	87.2610.02	0.469
1.22	3-Hydroxy-linoleyl carnitine	441.30	4.38±2.16	1.5±0.62	0.015 [b]
1.19	PC(19:0)	553.39	4.22±7.28	0.64±0.97	0.245

1.18	PE(38:3)	627.53	15.53±2.10	17.84±0.75	0.039 [a]
1.14	L-Alfa-Lysophosphatidylcholine, Lauroyl	563.47	7.1±0.52	10.18±3.75	0.213
1.09	PE(38:5)	338.34	6.92±1.63	11.49±9.64	0.456
1.08	TG(48:3)	634.54	8.78±1.14	11.49±9.64	0.192
1.07	Ajmaline	545.46	0.037±0.63	2.29±2.08	0.114
1.04	Ceramide (42:2)	664.66	11.54±3.45	9.18±1.29	0.163
1.03	Huperzine B	784.59	233.9±21.12	225.15±35.55	0.711
1.02	PC(42:1)	812.70	8.13±0.70	6.16±0.75	0.007 [b]
1.02	DG(32:3)	830.57	3.04±2.65	7.56±6.63	0.305
1.01	PE- NMe(36:2)	758.57	0±0	2.96±2.70	0.109
1.00	PC(38:3)	646.65	10.51±3.42	8.2±1.45	0.182
0.99	Citramalic acid	887.57	9.58±0.78	11.12±0.53	0.009 [a]
0.98	1-tetradecanyl-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine	799.67	16.24±7.80	20.71±5.05	0.324
0.98	PC(36:1)	772.59	39.81±6.65	36.33±5.35	0.482
0.98	SM(42:1)	815.70	0.11±0.13	4.76±7.73	0.348
0.96	PC(37:2)	832.59	14.29±0.62	16.67±2.57	0.17
0.94	Retinoic acid	668.65	9.29±2.03	7.3±1.60	0.149
0.93	3-Carboxy-4-methyl-5-pentyl-2-furanpropionic	269.14	1.54±1.30	0.18±0.21	0.031 [b]

acid

[a] increased average concentration compared to control with significant *p* value ($p<0.05$).

[b] decreased average concentration compared to control with significant *p* value($p<0.05$).

Table 12 The metabolites with top 50 VIP values selected from the results of OPLS-DA (low dose vs. low dose control) of UPLC-Qtof/MS spectra from serum of rats exposed to high dose of 250 nm ZnO.

VIPS score	Lipid	m/z	Mean ± SD (Control)	Mean ± SD (Treatment)	t-test
7.48	PC(36:4)	782.57	6363.81±46.38	796.31±63.90	0.003 [a]
7.42	PC(38:4)	810.60	1070±69.23	1247.73±94.42	0.009 [a]
5.33	PC(36:2)	786.60	846.05±66.77	747.2547.02	0.024 [b]
5.11	LysoPC(18:2)	520.34	306.71±35.07	231.42±23.84	0.004 [b]
3.38	TG(49:3)	813.68	325.65±45.12	266.54±42.01	0.067
3.08	PC(38:3)	812.62	130.89±9.78	83.82±26.46	0.01 [b]
3.03	PC(36:3)	784.59	120.54±30.29	72.07±31.03	0.045 [b]
3.00	LysoPC(18:1)	522.35	115.46±2.45	91.67±8.32	0.001 [b]
2.65	Lactosylceramide (30:1)	806.57	250.48±42.70	296.29±60.68	0.308 [a]
2.44	PC(34:1)	760.58	258.08±18.04	285.03±26.77	0.118 [a]
2.22	PC(38:2)	814.64	33.98±2.77	21.02±3.80	<0.0001 [b]
2.13	Anhydroeschscholtzxanthin	531.41	30.05±53.81	3.66±4.53	0.252
2.13	LysoPC(22:6)	568.34	51.7±29.38	31.22±6.86	0.13
2.03	PC(34:2)	758.57	801.12±68.20	834.97±47.91	0.38
1.76	1alpha,3alpha-Dihydroxy-5beta-cholan-24-	393.30	14.29±3.44	5.92±2.00	0.001 [b]

	oic Acid				
1.55	PC(36:3)	784.59	13.96±6.13	3.91±3.11	0.008 [b]
1.51	DG(38:6)	658.54	3.5±3.19	12.19±7.44	0.062
1.46	TG(49:3)	815.70	102.36±9.14	122.16±20.87	0.116
1.32	LysoPC(20:4)	544.34	278.48±59.67	261.23±14.01	0.504
1.30	TG(49:3)	813.69	16.32±3.24	29.4±16.68	0.167
1.28	Goyaglycoside	663.45	182.15±19.77	169.71±19.85	0.365
1.23	PE(42:4)	824.62	24.87±4.01	31.81±5.09	0.004 [a]
1.19	PC(16:0/22:5)	808.59	134.69±15.62	123.97±10.65	0.229
1.16	L-Alfa-Lysophosphatidylcholine	441.30	7.16±5.22	1.93±1.13	0.138
1.15	PC(40:7)	832.59	17.93±7.06	12.21±2.05	0.091
1.14	PE(38:5)	752.56	14.3±1.13	10.31±2.02	0.007 [b]
1.14	TG(48:3)	801.69	87±11.55	99.14±15.46	0.22
1.08	PC(40:8)	830.57	3.37±3.99	8.83±2.19	0.022 [a]
1.05	PC(38:4)	810.60	10.09±3.95	4.18±2.74	0.023 [b]
1.01	PC(36:1)	788.61	197.38±12.87	189.85±10.81	0.344
1.00	DG(44:1)	940.75	12.64±3.50	8.6±2.34	0.058
0.98	Tetracosanoic acid	369.35	24.15±0.80	27.55±2.41	0.028 [a]

0.97	DG(40:8)	682.54	10.13±10.27	15.81±4.81	0.265
0.97	LysoPC(20:2)	548.37	5.02±0.25	2.63±0.63	<0.0001 [b]
0.93	PC(15:0/20:2)	772.59	23.56±4.18	19.8±2.04	0.091
0.93	PC(36:4)	782.57	24.83±6.02	19.53±5.04	0.168
0.93	SM(40:0)	811.66	96.39±14.71	104.96±10.35	0.307
0.89	Dihydrothymine	129.06	3.53±1.44	1.15±0.51	0.005 [b]
0.88	PC(10:0/9:0)	553.39	5.78±9.02	1.3±1.52	0.255
0.86	Tetradecanedioic acid	259.19	2.77±0.91	0.74±0.28	0.001 [b]
0.86	32,35-anhydrobacteriohopaneterol	529.46	34.12±4.01	30.4±3.53	0.182
0.85	PC(38:3)	796.62	13±1.87	15.63±1.27	0.027 [a]
0.83	Tetradecenoyl carnitine	371.32	3.34±1.59	1±0.61	0.01 [b]
0.82	TG(48:3)	801.69	21.35±11.60	14.22±13.68	0.404
0.79	PC(40:6)	834.60	292.2±75.48	306.07±67.97	0.769
0.79	PC(36:4)	766.58	6.63±.84	8.91±1.65	0.035 [a]
0.79	Fexofenadine	502.32	4.44±2.02	2.16±0.76	0.033 [b]
0.79	LysoPC(20:3)	546.36	3.95±3.06	0.24±0.59	0.018 [b]

[a] increased average concentration compared to control with significant p value ($p<0.05$).

[b] decreased average concentration compared to control with significant p value ($p<0.05$).

Table 13 Fold-change (high-, moderate- and low-dose/ high-, moderate- and low-dose control) of 19 common lipids selected from high-, moderate- and low-dose of 250 nm ZnO treatment.

Lipid	Low dose/ Low dose control	Moderate dose/ Moderate dose control	High dose/ High dose control
PC(36:4) [b]	1.25	1.07	0.91
PC(38:4) [b]	1.17	1.10	0.97
PC(36:2)[a]	0.88	0.98	1.14
PC(36:4) [a]	0.75	0.93	1.06
PC(38:3) [a]	0.64	0.79	1.29
Lactosylceramide (30:1)	1.18	1.23	1.12
Anhydroeschscholtzxanthin	0.12	1.07	0.22
LysoPC(22:6)	0.60	0.35	1.18
PC(34:2)	1.04	1.05	1.03
1alpha,3alpha-Dihydroxy-5beta-cholan-24-oic Acid	0.41	0.91	0.24
TG(49:3) [b]	1.19	1.05	0.89
Tetracosanoic acid	1.14	0.65	0.70
LysoPC	0.27	1.43	0.39
PE(38:5)	0.72	1.66	0.53

TG(48:3)	1.14	1.31	1.08
PC(36:1)	0.96	0.91	1.15
PC (10:0/9:0)	0.22	0.15	0.19
PC(38:3)	1.20	0.78	0.83
PC(40:6) [a]	1.05	1.25	1.38

[*] significant p value with t-test.

[a] increased average concentration compared to control with significant p value with t-test.

[b] decreased average concentration compared to control with significant p value with t-test.

Table 14 The metabolites with top 50 VIP values selected from the results of OPLS-DA (high dose vs. high dose control) of UPLC-TQMS spectra from serum of rats exposed to high dose of 35 nm ZnO.

VIP score	Lipid	m/z	Retention time	Mean ± SD (Control)	Mean ± SD (Treatment)	t-test
3.21	Unknown	819.22	4.91	287.19±339.40	894.25±1288.92	0.124
2.97	Unknown	876.90	4.95	459.37±782.61	1419.97±1524.94	0.032
2.79	PC(17:0/18:1) [a]	774.82	2.83	305.37±517.74	158.47±131.62	0.355
2.50	Unknown	570.61	1.24	1172.24±1646.43	178.88±265.61	0.062
2.48	Unknown	818.61	4..92	1035.48±2215.49	4475.55±5159.44	0.019↑
2.46	PC(P-20:5/18:0) [a]	792.88	2.42	2388.86±3093.94	800.57±1025.81	0.11
2.35	Alk-1-enylacyl-PC[a]	848.81	2.88	170.83±202.96	816.77±996.57	0.015↑
2.26	PC [a]	800.91	2.82	46125.82±62578.57	65920.92±50717.31	0.348
1.96	PC(36:4) [b]	781.66	2.52	162.59±188.78	46.00±58.94	0.06
1.90	PC(17:0/18:0) [a]	776.94	3.04	3597.71±3410.36	1213.18±1162.36	0.037 ↓
1.89	PC(18:0/20:3) [a]	812.73	2.73	175.11±85.8	167.93±150.99	0.883
1.82	Unknown	835.04	2.49	4617.65±4180.85	1804.01±2826.96	0.036 ↓
1.78	PC(31:0) [c]	720.95	2.89	1013.01±2045.28	3790.77±4020.02	0.019↑
1.77	Unknown	546.60	1.25	7535.53±6788.19	2658.97±3024.35	0.35

1.77	Unknown	546.44	1.26	3348.12±2959.37	1123.83±870.54	0.026↓
1.76	Unknown	522.63	1.29	8836.82±6668.01	3208.62±2647.84	0.015↓
1.75	PC(17:0/18:0) [a]	776.39	3.04	177.88±213.94	348.81±701.21	0.422
1.74	Unknown	819.04	4.92	309.39±348.30	806.49±1492.02	0.269
1.71	PC(a21:2/0:0) [a]	548.63	1.42	366.82±800.7	267.59±235.01	0.622
1.70	PC(18:1/22:6) [a]	832.91	2.32	3993.6±4070.93	924.02±1668.18	0.027↓
1.70	Unknown	736.79	2.98	5322.08±6979.90	7740.75±6226.65	0.329
1.62	PC(16:0/20:4) [a]	782.90	2.42	2936.98±4675.88	3473.61±5328.05	0.779
1.60	Unknown	841.03	3.32	2539.17±2564.03	863.66±1195.05	0.052
1.59	Unknown	802.88	3.2	1228.02±1826.94	2109.31±2282.30	0.273
1.54	Unknown	709.09	2.64	362.78±329.07	499.15±727.85	0.549
1.53	Unknown	867.08	2.5	3853.26±3038.56	1873.55±2258.04	0.5↓
1.48	Unknown	799.04	2.73	2805.58±2716.19	1112.82±1560.68	0.068
1.45	PC(22:0/18:1) [a]	844.97	3.85	1902.25±2887	3700.96±4210.34	0.208
1.43	PC(16:0/22:1) [a]	816.39	3.25	1830.85±2518.49	4397.74±4260.32	0.048↑
1.42	Unknown	814.05	2.89	13794.93±12751.16	5011.53±7445.06	0.047↑
1.42	PC(39:6) [a]	820.85	2.4	1400.33±1724.8	1955.06±2185.60	0.467

1.39	Unknown	818.88	4.93	954.86±1233.29	770.40±925.11	0.643
1.38	Unknown	746.98	2.56	1181.93±1438.96	225.81±185.59	0.042↓
1.38	Unknown	795.01	2.64	905.50±1484.72	864.68±902.85	0.926
1.36	PC(37:1)	803.07	3.19	1434.79±2258.62	1511.43±1652.34	0.915
1.33	PC(18:0/22:6) [a]	834.96	2.54	1257.04±880.92	599.65±692.40	0.03↓
1.32	PC(0:0/18:1) [a]	522.59	1.29	1083.65±1386.54	456.50±1040.71	0.168
1.31	PC(P-38:3) [a]	796.65	2.71	825.90±1330.87	2323.00±2502.95	0.043↑
1.30	Unknown	832.22	2.34	1799.11±3048.16	1336.65±1998.02	0.619
1.28	Unknown	708.30	2.83	452.78±797.45	495.11±763.46	0.885
1.24	Unknown	815.11	3.08	3220.42±5932.24	2167.20±4208.84	0.573
1.19	Unknown	845.08	2.06	398.93±248.96	725.85±744.64	0.155
1.18	Unknown	707.81	1.27	1008.91±1202.06	1063.67±1145.29	0.901
1.17	Unknown	736.72	2.97	5267.47±4215.13	6098.81±4852.31	0.632
1.12	Unknown	826.90	3.08	1400.33±1724.80	1955.06±2185.60	0.467
1.12	Unknown	843.02	2.6	7381.40±5848.23	4541.01±4482.46	0.143
1.10	PC(42:6)	862.80	3.36	149.17±115.35	431.22±713.07	0.117
1.09	PC(22:5/20:3) [a]	858.85	2.33	670.76±947.42	1228.41±1672.45	0.255

1.07	PC(18:0/18:0) [a]	790.02	3.22	1450.15±2570.30	4192.85±4013.31	0.03↑
1.07	Unknown	822.77	2.62	936.61±1202.83	833.35±1911.75	0.869
1.05	Unknown	818.11	4.92	12242.98±26632.52	9369.46±13287.72	0.698
1.03	Unknown	840.91	3.2	1491.79±1970.72	727.34±873.59	0.157

[a] PC identified based on Tang et al. 2011.

[b]35 nm ZnO exposure dose response biomarker. Structural analysis result shown as Figure 20.

c 35 nm ZnO exposure dose response biomarker. Structural analysis result shown as Figure 21.

↑increased average concentration compared to control with significant p value with t-test.

↓decreased average concentration compared to control with significant p value with t-test.

Table 15 The metabolites with top 50 VIP values selected from the results of OPLS-DA (moderate dose vs. moderate dose control) of UPLC-TQMS spectra from serum of rats exposed to high dose of 35 nm ZnO.

VIP score	Lipid	m/z	Retention time	Mean ± SD (control)	Mean ± SD (treatment)	P-value
3.01	Unknown	895.09	2.76	3910.1993±976.33	1467.2944±1548.40	<0.0001 ↓
2.76	PC(36:4) [c]	781.66	2.32	301.9±536.73	60.86±60.16	0.066
2.57	Unknown	809.25	2.48	25900.4442±9749.83	19027.7456±15256.39	0.232
2.36	Unknown	804.08	4.41	7102.0795±5842.39	2861.9972±3539.53	0.071
2.21	Unknown	806.92	2.8	2233.0157±1822.78	605.0449±696.09	0.029↓
2.17	Unknown	762.09	2.66	2534.4749±928.19	1078.9817±965.03	0.001↓
2.13	PC(16:0/18:2) [a]	758.85	2.45	2676.5788±1229.56	1162.9816±863.17	0.001↓
2.10	Unknown	722.80	2.76	1551.1005±1392.39	506.8862±609.50	0.058
2.09	Unknown	802.88	3.08	105.1205±60.61	83.048±89.70	0.513
2.03	PC(22:0/18:1) [a]	845.02	3.85	2470.8554±1581.50	1098.3989±1373.49	0.028↓
2.02	PC(16:0/0:0) [a]	496.64	1.29	539.2581±425.00	226.4894±237.65	0.021↓
2.01	Unknown	842.90	2.58	80.0267±73.90	44.0657±58.41	0.179
1.85	PC(22:0/18:1) [a]	844.37	3.65	206.0113±159.01	117.8416±94.91	0.082
1.84	PC(P-38:5) [a]	792.88	2.44	160.4122±135.26	116.6483±96.38	0.341
1.83	PC(16:1/22:6) [a]	804.21	2.17	365.5313±306.54	745.8879±597.79	0.087
1.74	Unknown	804.08	4.41	8650.0387±5763.04	11190.0068±4059.49	0.195
1.70	Unknown	818.07	3.25	14254.3692±12059.14	20262.0626±12422.58	0.243
1.69	PC(16:0/16:0) [a]	734.88	2.7	7685.8744±2332.37	3937.8186±1540.15	0.001↓
1.66	PC(16:0/18:1) [a]	760.98	2.68	42753.297±59166.69	47269.187±40400.87	0.84

1.66	Unknown	677.95	2.41	161.1484±87.53	237.146±139.14	0.149
1.59	Unknown	803.02	3.72	2691.1682±2804.45	3798.3143±2919.74	0.356
1.55	Unknown	736.72	2.93	4742.8682±2634.00	6331.714±2534.44	0.142
1.54	PC(37:1)	803.07	3.72	3300.9916±2798.73	1472.109±2320.12	0.083
1.50	PC(16:1/18:2) [a]	756.71	2.29	486.8626±380.36	204.5587±93.80	0.058
1.49	Unknown	869.11	2.58	1166.2231±963.41	508.8829±518.13	0.084
1.49	PC(18:1/22:6) [a]	832.22	2.32	2128.47±1300.67	2758.7997±1069.15	0.191
1.46	Unknown	849.08	2.97	695.8759±860.86	248.4129±189.67	0.16
1.45	PC(16:0/20:4) [a]	782.90	2.38	4722.1046±2442.15	2399.1361±1333.97	0.004↓
1.41	PC(P-16:1/20:3) [a]	776.39	2.46	361.531±307.73	209.4909±170.63	0.109
1.40	Unknown	749.74	2.77	900.292±406.48	550.7655±444.61	0.059
1.37	Unknown	845.08	2.65	271.1023±82.46	401.0116±250.00	0.058
1.36	Unknown	722.90	3.18	2498.9984±1733.45	1339.526±1413.04	0.074
1.33	Unknown	839.15	2.72	3192.689±1524.29	1662.0363±872.53	0.018↓
1.31	PC(18:0/18:0) [a]	790.02	2.9	4957.8864±4562.73	2185.9002±1789.78	0.112
1.29	Unknown	848.81	2.94	52.4293±94.69	46.0403±69.74	0.844
1.27	PC(18:0/18:0) [a]	790.66	3.26	116.4164±123.27	65.4096±57.59	0.151
1.27	PC(20:3/0:0) [a]	546.44	1.25	5608.4805±1332.04	3459.4167±1629.66	0.002↓
1.26	Unknown	812.72	2.85	94902.1285±40895.61	64129.278±48861.19	0.117
1.24	Unknown	856.85	2.27	933.3624±926.52	413.5133±267.35	0.034↓

1.24	PC(16:0/22:1) [a]	816.20	3.25	151.8927±112.04	98.6342±111.85	0.255
1.23	Unknown	512.57	1.56	446.7727±379.74	577.6194±521.55	0.511
1.21	Unknown	860.89	2.51	178.7642±59.20	229.181±123.65	0.261
1.20	Unknown	843.90	2.57	200.6671±151.03	250.3172±164.60	0.455
1.19	PC(31:0) [b]	720.95	2.89	266.6061±203.55	180.9334±125.82	0.188
1.19	PC(799) [a]	800.91	2.82	722.6052±1127.04	197.5341±373.25	0.207
1.17	Unknown	811.23	2.57	6568.068±4421.41	8791.9108±6398.79	0.36
1.16	PC(P-16:1/20:3) [a]	766.25	2.46	939.0928±622.42	644.6791±537.65	0.214
1.16	Unknown	790.02	3.22	8591.5693±5258.79	8964.2181±3286.26	0.822
1.15	PC(a21:2/0:0) [a]	548.63	1.42	994.2119±677.22	560.2233±404.09	0.047↓
1.15	PC(20:0/0:0) [a]	552.60	1.81	214.2241±146.42	276.4147±209.82	0.435

[a] PC identified based on Tang et al. 2011.

[b]35 nm ZnO exposure dose response biomarker. Structural analysis result shown as Figure 20.

c 35 nm ZnO exposure dose response biomarker. Structural analysis result shown as Figure 21.

↑increased average concentration compared to control with significant p value with t-test.

↓decreased average concentration compared to control with significant p value with t-test.

Table 16 The metabolites with top 50 VIP values selected from the results of OPLS-DA (low dose vs. low dose control) of UPLC-TQMS spectra from serum of rats exposed to high dose of 35 nm ZnO.

VIP score	Lipid	m/z	Retention time	Mean ± SD (control)	Mean ± SD (treatment)	t-test
1.24	PC(a21:2/0:0) [a]	548.633	1.42	695.73±574.72	582.14±805.14	0.677
1.17	Unknown	705.83	2.7	6258.36±2984.17	9128.32±4505.62	0.063
1.14	Unknown	708.30	2.69	391.07±239.15	1250.11±2835.00	0.307
1.58	PC(31:0)[c]	720.95	2.82	211.00±112.53	147.91±112.76	0.144
1.58	Unknown	722.80	3.18	283.00±144.40	792.78±955.05	0.039
1.15	Unknown	722.90	3.18	1049.31±1006.17	1793.50±1332.97	0.111
1.37	PC(16:0/16:0) [a]	733.987	2.63	2776.21±1976.95	4140.91±2094.02	0.085
1.65	Unknown	736.79	2.92	11368.99±2542.83	8079.19±4075.40	0.019
2.64	Unknown	736.80	2.92	5185.47±2756.03	11640.13±3918.16	0*↑
1.49	Unknown	748.80	2.82	2048.29±1659.62	1318.73±1652.06	0.247
1.35	PC(16:0/16:0) [a]	756.707	2.7	267.81±242.6	489.52±476.09	0.149
1.12	PC(16:0/18:1) [a]	760.975	2.68	55635.28±42073.33	43867.7±41767.68	0.457
1.26	PC [a]	770.922	2.58	5721.24±2140.16	7458.25±1588.99	0.016*↑
1.82	PC(P-18:0/18:1) [a]	772.859	3.02	1360.7±1216.98	730.25±862.42	0.107
1.54	PC(17:0/18:1) [a]	774.818	2.76	113.25±106.47	62.09±57.7	0.148
1.72	PC(36:4) [b]	781.66	3.16	75.40±61.20	112.96±91.34	0.223
1.42	PC(16:0/20:3) [a]	784.873	2.48	50741.51±23511.64	65602.74±12403.24	0.032*↑

1.22	Unknown	787.50	2.69	13179.31±9818.93	7757.59±3329.68	0.089
1.55	PC(18:0/18:1) [a]	788.849	2.9	115677.92±23730.8	84530.5±38991.54	0.02*↓
1.58	PC(18:0/18:0) [a]	790.018	3.22	3280.12±3452.8	2274.66±3596.78	0.452
1.59	PC(P-20:5/18:0) [a]	792.879	2.44	189.35±81.87	119.81±58.48	0.011*↓
1.29	PC(37:5) [a]	794.932	2.51	1285.35±1039.66	1603.38±1143.83	0.446
2.03	PC(P-38:3) [a]	796.648	2.71	1580.42±1086.96	2703.31±1128.22	0.011*↑
1.19	PC(P-20:0/18:2) [a]	798.834	2.56	2283.94±2995.08	2857.02±1703.74	0.509
1.95	Unknown	799.04	2.69	1919.48±1725.31	2880.69±1566.07	0.125
1.19	Unknown	799.41	2.69	2268.27±3097.73	3029.12±2983.26	0.506
1.14	PC(16:1/22:6) [a]	804.084	2.16	7656.83±6087.77	9813.69±4263.49	0.262
1.09	Unknown	805.14	4.35	184.56±111.10	140.52±91.68	0.246
1.46	Unknown	811.23	2.57	9630.98±6137.22	5951.77±4387.69	0.065
2.07	Unknown	812.72	2.85	77440.89±32000.17	57843.82±52075.24	0.213
1.46	PC(18:0/20:3) [a]	812.784	2.77	112690.86±25561.01	146410.96±22857.83	0.001*↑
1.15	PC(16:0/22:1) [a]	816.203	3.21	67.96±66.11	111.16±147.48	0.286
1.45	PC(16:0/22:1) [a]	816.852	3.21	192.92±207.67	131.28±102.54	0.288
2.74	Unknown	818.21	4.8	1161.02±1204.31	3128.43±2394.08	0.014*↑
1.17	Unknown	818.61	4.8	562.87±844.84	197.24±130.49	0.164
1.22	Unknown	819.01	4.81	8828.41±2717.99	6664.35±2556.68	0.035*↓
1.95	Unknown	819.04	4.88	452.92±1011.64	123.79±98.27	0.285
1.52	Unknown	819.22	4.86	72.35±46.84	80.50±96.95	0.789
1.66	PC(a25:5/15:1) [a]	820.854	2.62	182.34±116.85	158.7±184.22	0.697
1.38	Unknown	821.02	2.92	3375.09±1201.82	2655.35±1863.48	0.248
1.84	PC	826.908	2.83	1781.83±1346.95	1523.61±1139.99	0.576
1.62	PC(18:1/22:6) [a]	832.905	2.32	4328.74±2120.84	6101.39±1458.36	0.011*↑
1.51	Unknown	843.90	2.57	167.74±58.85	295.41±160.76	0.005*↑

1.98	PC(22:0/18:1) [a]	844.828	3.85	331.36±176.45	507.68±292.06	0.072
1.12	Unknown	849.08	2.99	194.65±76.59	612.28±944.05	0.079

[a] PC identified based on Tang et al. 2011.

[b]35 nm ZnO exposure dose response biomarker. Structural analysis result shown as Figure 20.

c 35 nm ZnO exposure dose response biomarker. Structural analysis result shown as Figure 21.

↑increased average concentration compared to control with significant p value with t-test.

↓decreased average concentration compared to control with significant p value with t-test.

Table 17 Fold-changes (high-, moderate- and low-dose/ high-, moderate- and low-dose control) of 4 common lipids selected from high-, moderate- and low-dose of 35 nm ZnO treatment.

Lipid	m/z	High dose /High dose control	Moderate dose/ Moderate dose control	Low dose /Low dose control
PC(31:0)	720.95	1.92	0.68	0.70
PC(36:4)	781.66	0.28	0.20	1.50
PC(18:0/18:0)	790.02	1.28	0.44	0.69
PC(P-20:5/18:0) [a]	792.88	2.66	0.73	0.63

[a] increased average concentration compared to control with significant p value with t-test.

[b] decreased average concentration compared to control with significant p value with t-test.

Table 18 The metabolites with top 50 VIP values selected from the results of OPLS-DA (high dose vs. high dose control) of UPLC-TQMS spectra from serum of rats exposed to high dose of 250 nm ZnO.

VIP score	Lipid	m/z	Retention time	Mean ± SD (control)	Mean ± SD (treatment)	P-value
2.85	Unknown	816.39	4.07	2412.09±5266.98	244.78±388.97	0.182
2.71	Unknown	815.11	2.88	515.8±808.59	2612.59±2142.83	0.001*↓
2.70	Unknown	845.02	3.89	1473.8±1214.53	401.63±548.14	0.012*↓
2.59	PC(37:1) [b]	803.07	3.24	1431.03±1480.18	428.31±1058.24	0.057
2.49	Unknown	848.81	3.45	470.31±870.00	56.48±84.41	0.128
2.43	Unknown	800.91	2.74	263.56±198.35	127.28±166.49	0.051
2.35	Unknown	818.11	5.02	18710.45±9693.33	8327.67±8501.39	0.004*↓
2.33	Unknown	876.90	3.98	833.72±1510.89	22.76±39.18	0.09
2.33	Unknown	839.01	2.86	3644.1±2913.34	7602.86±4096.09	0.007*↑
2.23	Unknown	876.43	2.12	58.92±65.76	101.2±72.51	0.116
2.14	Unknown	789.10	3.02	31633.79±16949.23	16665.38±20115.28	0.043*↓
2.13	Unknown	844.97	3.89	1007.37±1089.32	295.12±217.24	0.046*↓
2.06	Unknown	793.00	2.74	2140.37±1301.20	955.48±1145.17	0.014*↓
2.04	Unknown	822.86	2.62	1042.65±1091.92	1897.74±1089.26	0.044*↑

1.94	Unknown	790.77	3.00	73.39±78.57	121.97±105.20	0.184
1.91	PC(P-38:5) [a]	792.945	2.44	1245.57±1960.91	167.02±134.51	0.083
1.74	PC(18:0/18:0) [a]	790.018	3.2	2111.35±1157.93	1139.34±643.85	0.018*↓
1.70	PC(22:5/20:3) [a]	858.709	2.33	676.06±1069.91	168.2±68.75	0.129
1.69	PC(a18:0/16:0) [a]	748.967	3.24	2448.02±2011.16	3820.775±1913.38	0.07
1.61	Unknown	841.26	3.17	173.8±127.49	118.23±156.25	0.315
1.60	PC(a16:0/16:0) [a]	720.952	2.89	295.29±200.71	141.40±97.92	0.027*↓
1.59	Unknown	821.02	4.92	2976.13±1621.53	1766.75±1314.70	0.033*↓
1.52	PC(18:1/22:6) [a]	832.905	2.3	3341.56±2259.41	4419.71±1327.96	0.155
1.51	PC(a25:5/15:1) [a]	820.935	2.62	1549.58±1936.29	480.3±267.66	0.083
1.50	PC(18:0/18:1) [a]	788.09	2.9	5617.25±2213.14	3616.47±1984.87	0.015*↓
1.48	Unknown	839.06	2.88	6811.29±3217.69	3936.1±3309.33	0.026
1.46	PC(42:6)	862.80	2.85	277.89±139.65	166.96±86.31	0.012*↓
1.43	PC(22:0/18:1) [a]	844.828	3.85	528.46±333.68	342.59±213.89	0.073
1.37	Unknown	722.80	3.26	798.69±843.72	293.84±199.07	0.065
1.37	PC(22:5/18:0) [a]	836.818	2.5	33725.27±11788.01	40831.01±8016.67	0.059
1.29	Unknown	809.25	2.50	9684.18±8800.65	13703.61±8758.24	0.229

1.25	Unknown	874.02	4.08	212.73±115.85	159.15±151.30	0.308
1.24	PC(20:4/20:4) [a]	830.262	2.19	401.68±546.14	163.51±91.75	0.162
1.23	Unknown	736.79	2.99	6234.28±2380.48	4306.39±2363.05	0.038*↓
1.22	PC(16:0/18:1) [a]	760.975	2.68	44403.96±30533.29	32689.82±32061.55	0.326
1.20	PC(16:0/16:0) [a]	734.876	2.7	3452.90±991.52	2655.54±1818.27	0.133
1.19	Unknown	525.83	1.70	311.9±210.49	477.07±317.88	0.126
1.17	PC(22:5/20:3) [a]	858.846	2.33	424.97±659.56	166.91±84.03	0.204
1.17	Unknown	512.57	1.58	482.64±448.67	294.72±245.09	0.149
1.13	Unknown	819.22	4.92	107.04±96.26	117.78±150.42	0.829
1.13	Unknown	748.80	3.12	354.42±407.80	610.21±624.75	0.222
1.11	Unknown	788.78	3.00	94762.4±20715.72	105899.04±17504.93	0.124
1.11	PC(16:0/22:1) [a]	816.091	3.24	7580.41±11573.97	6493.02±6635.97	0.746
1.11	Unknown	801.03	2.37	322.19±148.75	229.88±169.12	0.136
1.09	Unknown	833.73	2.38	1646.7±1941.27	704.51±650.09	0.129
1.06	Unknown	818.08	4.95	9673.2±9676.58	14596.5±11497.45	0.232
1.04	Unknown	818.21	5.01	1684.87±1291.78	1150.21±1064.03	0.226
1.00	Unknown	844.83	2.60	528.46±333.68	342.59±213.89	0.073

0.99	Unknown	548.63	1.38	255.66±235.68	298.57±193.17	0.589

[a] PC identified based on Tang et al. 2011.

[b] 250 nm ZnO exposure dose response biomarker. Structural analysis result shown as Figure 27.

↑increased average concentration compared to control with significant p value with t-test.

↑decreased average concentration compared to control with significant p value with t-test.

Table 19 The metabolites with top 50 VIP values selected from the results of OPLS-DA (moderate dose vs. moderate dose control) of UPLC-TQMS spectra from serum of rats exposed to high dose of 250 nm ZnO.

VIP score	Lipid	m/z	Retention time	Mean ± SD (control)	Mean ± SD (treatment)	P-value
4.41	Unknown	876.43	5.03	28.73±85.52	89.03±110.05	0.163
4.05	Unknown	790.78	3.4	51.67±75.71	181.17±207.83	0.085
2.70	Unknown	733.99	3.11	1954.97±1965.88	4084.65±1406.75	0.013*↑
2.42	Unknown	815.11	2.9	268.1±223.40	2169.46±2349.72	0.003
2.23	PC(a18:0/16:0) [a]	748.797	3.24	880.87±650.28	2516.9±1122.32	0*↑
2.16	PC(16:1/22:6) [a]	804.078	2.16	4002.67±3646.73	8253.84±2724.43	0.002*↑
2.12	PC(18:0/18:0) [a]	790.66	3.37	70.5±69.12	124.03±115.95	0.198
2.09	Unknown	722.90	2.86	465.64±466.52	1423.2±1220.86	0.007*↑
1.99	Unknown	496.64	1.23	167.69±159.26	436.68±381.99	0.055
1.95	PC(16:0/18:1) [a]	760.975	2.68	41481.8±39136.78	59486.67±31002.71	0.198
1.94	Unknown	806.92	2.28	637.59±992.69	1396.36±1040.84	0.082
1.81	Unknown	818.11	5.09	5066.89±4451.24	12509±9336.10	0.01*↑
1.69	Unknown	835.04	2.49	4067.53±2389.89	6815.62±1902.06	0.003*↑
1.68	PC(16:0/18:1) [a]	834.955	2.68	1361.01±1187.68	2137.25±1113.13	0.1

1.67	PC(18:1/22:6) [a]	832.905	2.323	4291.08±1976.00	6560.36±1615.66	0.003*↑
1.65	PC(37:1) [b]	803.07	3.23	252.17±529.73	96.63±68.75	0.406
1.64	PC(16:0/17:1) [a]	746.977	2.52	663.48±662.59	356.66±253.01	0.189
1.61	Unknown	776.99	2.87	789.42±809.92	2105.29±1665.32	0.011*↑
1.58	Unknown	787.21	2.69	7276.95±5011.25	14104±10647.54	0.082
1.55	PC(17:0/18:0) [a]	776.944	3.03	138.93±116.11	126.82±118.89	0.799
1.54	Unknown	837.01	2.77	11087.32±6513.20	8314.55±9255.95	0.43
1.52	PC(42:6)	862.80	2.65	201.28±138.48	338.51±180.75	0.057
1.51	PC(17:0/18:0) [a]	776.387	3.03	96.63±81.79	170.43±134.06	0.129
1.50	Unknown	843.90	2.6	214.29±118.05	138.46±41.59	0.094
1.49	PC(18:1/22:6) [a]	832.224	2.32	1047.75±1254.10	401.58±228.76	0.14
1.45	Unknown	570.61	1.22	1986.88±1323.07	1301.64±999.81	0.144
1.43	PC(a18:0/16:0) [a]	748.973	3.24	2213.47±1646.85	3348.12±1121.63	0.074
1.39	Unknown	802.88	3.25	106.3±125.28	125.16±80.76	0.639
1.37	PC(22:5/18:0) [a]	836.818	2.5	29204.44±11498.64	40840.98±8512.75	0.006*↑
1.34	PC(16:1/22:6) [a]	804.084	2.16	4511.75±3457.24	1983.53±1033.83	0.048*↓
1.33	Unknown	522.63	1.28	7562.53±1695.85	6105.97±2241.70	0.099

1.33	Unknown	841.03	3.18	1612.69±1300.85	1260.55±1364.99	0.527
1.31	PC(17:0/18:2) [a]	772.859	2.6	433.66±452.33	861.36±874.38	0.107
1.30	Unknown	841.26	3.2	92.16±60.96	170.31±135.15	0.049
1.26	PC(18:0/22:4) [a]	839.057	2.82	2096.47±990.83	3383.31±1805.29	0.025*↑
1.24	Unknown	813.04	2.93	21744.24±19828.83	16531.18±22159.51	0.557
1.24	PC [a]	799.407	2.82	1501.02±1226.72	3542.34±2596.11	0.011*↑
1.24	PC(16:0/22:1) [a]	816.203	3.25	152.01±174.67	67.92±79.82	0.178
1.23	Unknown	814.05	2.92	1622.93±1039.98	2719.4±1716.86	0.092
1.23	Unknown	832.22	2.36	1106.28±1316.57	408.21±223.71	0.152
1.23	PC(p20:0/18:2) [a]	797.687	2.56	5313.28±1295.92	4399.39±2444.25	0.217
1.19	PC(a25:5/15:1) [a]	820.935	2.62	356.95±125.83	662.78±383.44	0.006*↑
1.16	Unknown	822.77	2.65	222.26±158.70	323.31±138.50	0.101
1.15	PC(16:0/22:1) [a]	816.059	3.25	19773.03±8049.02	15011.64±11544.37	0.22
1.13	Unknown	818.61	5.16	123.46±60.82	294.42±387.74	0.205
1.13	Unknown	860.89	2.48	365.06±234.73	285.02±231.62	0.407
1.13	PC(19:0/20:4) [a]	824.487	2.7	98.8±129.97	96.74±58.16	0.963
1.09	Unknown	764.96	3.01	2611.85±1345.20	4924.12±3433.09	0.02*↑

1.07	PC(18:0/18:0) [a]	790.023	3.26	5652.29±2586.67	8246.8±3274.85	0.042*↑
1.05	Unknown	842.90	2.63	77.2±93.87	145.16±140.63	0.204
1.03	Unknown	849.08	3.43	249.99±147.50	503.53±506.30	0.157

[a] PC identified based on Tang et al. 2011.

[b] 250 nm ZnO exposure dose response biomarker. Structural analysis result shown as Figure 27.

↑increased average concentration compared to control with significant p value with t-test.

↓decreased average concentration compared to control with significant p value with t-test.

Table 20 The metabolites with top 50 VIP values selected from the results of OPLS-DA (low dose vs. low dose control) of UPLC-TQMS spectra from serum of rats exposed to high dose of 250 nm ZnO.

VIP score	Lipid	m/z	Retention time	Mean±SD (control)	Mean±SD (treatment)	P-value
1.17	Unknown	498.59	1.41	14072.57±6606.29	18078.59±2921.51	0.081
1.24	Unknown	782.79	2.42	3505.39±4122.94	2184.35±2761.92	0.318
1.62	Unknown	784.87	2.55	59783.19±43126.21	105773.76±22118.63	0.006*↑
1.48	Unknown	787.21	2.64	9505.32±12477.02	4670.89±3050.96	0.135
1.40	Unknown	799.04	2.69	1610.69±1330.15	2841.12±1137.73	0.015
3.08	Unknown	802.88	3.13	105.48±164.14	422.55±483.81	0.021*↑
1.40	PC(37:1) [b]	803.07	3.16	1428.17±1962.63	1282.04±1462.17	0.823
1.15	Unknown	804.08	4.38	6991.49±6400.45	4797.31±3646.04	0.257
1.41	Unknown	804.21	4.37	1590.56±2118.11	1092.82±1843.22	0.516
1.66	Unknown	806.92	2.25	1083.85±1048.98	614.76±847.84	0.204
1.14	Unknown	809.25	2.49	23749.99±13478.71	22951.63±22124.27	0.916
1.26	Unknown	811.23	2.55	10590.45±67.65	13399.86±30650.54	0.768
1.74	Unknown	815.00	3.06	24579.72±14152.70	14830.3±7770.76	0.055
1.29	Unknown	818.66	2.66	340.43±180.05	259.69±107.90	0.148

1.87	Unknown	819.22	2.64	70.52±64.27	213.98±183.14	0.007*↑
2.13	Unknown	821.02	2.93	2423.06±2133.73	4118.55±1280.57	0.032
1.36	Unknown	822.77	2.59	1344.88±2328.22	574.71±711.32	0.309
1.23	Unknown	826.90	2.95	1102.45±895.93	2451.61±1759.62	0.013*↑
1.14	Unknown	826.91	2.94	3029.94±1949.31	5210.22±2489.63	0.021*↑
1.36	Unknown	833.73	2.33	3524.88±3247.81	2250.21±2578.65	0.259
1.70	Unknown	835.04	2.46	6268.35±3813.20	4007.1±2738.13	0.079
2.32	Unknown	842.90	2.59	100.22±102.57	946.11±1331.58	0.019*↑
1.16	Unknown	843.02	2.57	6122.27±3139.39	8552.2±2353.87	0.027*↓
1.22	Unknown	843.90	2.58	282.19±211.59	352.7±212.64	0.398
1.38	Unknown	856.85	2.25	503.88±315.44	391.84±474.52	0.497
1.15	Unknown	858.85	2.25	194.07±152.96	494.79±716.47	0.184
1.16	PC(42:6)	862.80	2.72	896.11±1050.81	318.61±328.71	0.104
1.68	Unknown	867.08	2.48	2551.35	4590.67±1565.37	0.008*↑
1.17	Unknown	872.45	2.86	1488.85±2438.42	726.03±497.90	0.329
3.24	Unknown	876.90	4.83	28.02±43.28	285.4±560.55	0.143
1.59	Unknown	895.09	2.74	2360.07±2121.67	3697.34±1502.58	0.088

1.17	PC(20:0/0:0) [a]	552.60	1.81	187.32±130.21	257±143.2	0.18
1.33	PC [a]	770.92	2.58	5356.38±3792.33	8835.07±3030.29	0.009*↑
2.64	PC(P-20:0/16:0) [a]	774.82	3.23	37.52±42.39	535.45±861.79	0.025*↑
1.63	PC(17:0/18:0) [a]	776.94	3.04	132.67±97.69	230.13±191.54	0.078*↑
1.17	PC(18:0/18:0) [a]	790.77	3.37	53.01±61.98	138.08±146.97	0.039*↑
2.83	PC(P-38:5) [a]	792.88	2.44	90.79±110.9	922.41±1364.49	0.02*↑
1.19	PC(P-20:0/18:2) [a]	798.83	2.56	2640.2±2092.8	5028.94±3964.51	0.066
1.57	PC [a]	800.91	2.82	120.19±106.55	409.13±655.18	0.143
1.16	PC(16:1/22:6) [a]	804.08	2.16	7524.19±6678.73	13888.77±7789.02	0.028*↑
1.61	PC(16:0/22:1) [a]	816.39	3.21	354.96±937.13	801.61±1334.5	0.324
1.46	PC(16:0/22:1) [a]	816.85	3.21	193.85±162.6	1835.09±3017.3	0.034*↑
2.26	PC(a25:5/15:1) [a]	820.85	2.62	146.15±187.01	867.54±1024.52	0.009*↑
2.56	PC(19:0/20:4) [a]	824.49	2.7	119.37±136.96	241.71±214.53	0.092
1.97	PC(20:4/20:4) [a]	830.26	2.19	250.43±254.2	704.48±708.73	0.021*↑
1.36	PC(18:1/22:6) [a]	832.22	2.32	1417.51±1593.16	608.65±870.88	0.129
1.29	PC(18:0/22:6) [a]	834.96	2.54	1464.67±1289.09	970.06±776.4	0.199
2.04	PC(22:0/18:1) [a]	844.97	3.85	362.81±262.38	3052.52±3652.42	0.006*↓

2.14	PC[a]	848.81	2.88	37.16±67.77	166.84±193.61	0.016*↑

[a] PC identified based on Tang et al. 2011.

[b] 250 nm ZnO exposure dose response biomarker. Structural analysis result shown as Figure 27.

↑increased average concentration compared to control with significant p value with t-test.

↓decreased average concentration compared to control with significant p value with t-test.

Table 21 Fold-change (high-, moderate- and low-dose/ high-, moderate- and low-dose control) of 3 common lipids selected from high-, moderate- and low- of 250 nm ZnO treatment.

Lipid	m/z of lipids	High dose	Moderate dose	Low dose
PC(18:0/18:0)	790.78	1.66	3.51	2.60
PC(37:1) [b]	803.07	0.30	0.38	0.92
PC (42:6)	862.80	0.60	1.68	0.38

[b] decreased average concentration compared to control with significant p value with t-test.