

# Analytical Methods

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ARTICLE TYPE

# Fatty acid profile reveals toxic response in adipose tissue of C57BL/6J mice exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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Dioxin exposure tends to accumulate in adipose tissue and alters metabolism in mammals. In this study, gas chromatography coupled with mass spectrometry (GC-MS) in conjunction with multivariate statistical analysis was applied to profile small molecular metabolites in adipose tissue of aryl hydrocarbon receptor (AhR)-high affinity wild-type C57BL/6J mice exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Partial least squares discriminant analysis model was also constructed to map the discrimination between TCDD dosages and control group. As a result, a total of 16 differential metabolites were identified in high-dose TCDD group compared to control group, and 12 free fatty acids (FFAs) were highlighted among them. Both saturated and unsaturated FFA levels were significantly elevated in adipose tissues of TCDD-exposed mice. This promising tool for global characterization highlights FFAs which could be served as indicators for understanding the toxic responses to TCDD exposure in a dose-dependent manner. The data indicated that the use of GC-MS coupled with multivariate statistical analysis could provide new insight for fatty acid biosynthesis on AhR activation with TCDD exposure in wild-type mice.

## 1 Introduction

Metabolomics is an emerging technique of studying, profiling, and fingerprinting metabolites in various physiologic states. [1] Based on multivariate analysis of complex matrix profiles, metabolomics has been successfully applied in many fields including disease diagnosis, biomarker screening and nutrition research. [2] Mass spectrometry (MS) and nuclear magnetic resonance (NMR) are the most frequently employed methods in metabolome analysis. The important advantages of MS include its high sensitivity and high-throughput with the possibility to detect and confirm the identity of the components present in complex biological samples. Furthermore, the combination of separation techniques (e.g., chromatography) with MS expands the capability of the chemical analysis of highly complex samples. GC-MS has been applied to study metabolites including plasma fatty acids. [3]

Aryl hydrocarbon receptor (AhR) was believed to mediate the toxic effects of 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) in both animals and human. AhR activation might antagonize peroxisome proliferators-activated receptor gamma (PPAR $\gamma$ ) functions, suggesting that dioxin exposure might be a risk factor for diabetes through the antagonism of PPAR $\gamma$  functions, which might lead to insulin resistance. [4, 5] Another study suggested AhR had an inhibitory effect on PPAR $\gamma$  functions with a new pathway by which AhR ligands could disturb lipid metabolism in Wistar rats.

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The risk of developing diabetes might also be elicited in the presence of TCDD by continuous insulin release followed by beta cell exhaustion. To figure out the possible link between TCDD and type 2 diabetes, metabolomics has been applied to delineate the metabolic changes in blood, liver and skeletal muscle tissues in mice, revealing lipid accumulation, fatty acid beta-oxidation which also shed light on the pathogenesis associated with insulin resistance. [6-9] Human beings have been exposed to TCDD mainly via the food intake. [10] TCDD has a high solubility in lipid with a half-life estimated to be between 7 and 9 years in human body. [11] Association between TCDD exposure and various components of impaired carbohydrate metabolism has also been reported. [12]

Free fatty acids (FFAs) constitute an important energy source in most body tissues, representing the primary oxidative fuel for liver, resting skeletal muscle, renal cortex and myocardium. [13] When the demand for fuel rises, adipose tissue lipolysis is stimulated, thus increasing systemic FFA availability and preserving glucose for cerebral requirements. [14] In addition, FFAs have other important physiological functions. For instance, FFAs enhance both basal and glucose-stimulated insulin secretion and are essential for stimulus-secretion coupling in the pancreatic  $\beta$ -cell. [15, 16] Type 2 diabetes has been related to the exposure of TCDD. [17] TCDD exposure has been reported to increase risk of diabetes, modified glucose metabolism, or insulin resistance. [18-22] However, what role FFAs play in the link between TCDD exposure and the risk of developing diabetes is unclear. The application of GC-MS based metabolomics for investigation of FFA levels in adipose tissue of C57BL/6J mice exposed to TCDD has not been reported, to the best of our knowledge. In the present study, GC-MS coupled with multivariate statistical analysis was used to study profiles of FFAs in adipose tissue. Identification of the FFA allowed the separation between TCDD-

exposed mice and control mice group.

## 2 Experimental

### 2.1 Reagents and chemicals

Authentic standards of L-serine, decanoic acid, lauric acid, myristic acid, citric acid, palmitic acid, oleic acid, stearic acid, linoleic acid, 16-hydroxyhexadecanoic acid, eicosanoic acid, docosanoic acid, docosanedioic acid and 4-chloro-DL-phenylalanine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and chloroform were analytical grade from Lab-scan (Labscan Asia Co., Ltd., Bangkok, Thailand). Ultrapure water was prepared from a Milli-Q system (Millipore, USA). TCDD (purity >99%) was obtained from AccuStandard, Inc. (New Haven, CT, USA). Other reagents were of analytical grade.

### 2.2 Animal experiment

Eight week old male C57BL/6J (C6) mice were purchased from Chinese University of Hong Kong, Hong Kong SAR, China. The animal experiments were conducted according to the Government guidelines for the care and use of laboratory animals. Mice were treated with TCDD dissolved in corn oil ( $0.2 \mu\text{g mL}^{-1}$ ,  $2.0 \mu\text{g mL}^{-1}$ ) for three consecutive days by oral administration of  $2.0 \mu\text{g kg}^{-1}$  and  $20 \mu\text{g kg}^{-1}$  or corn oil  $10 \text{ mL kg}^{-1}$  (control group) body weight each day. The mice were divided into control group ( $n=7$ ), low dosage group ( $n=7$ ) and high dosage group ( $n=7$ ). On the seventh day after the first dosage, the mice were decapitated. The adipose tissue was collected and stored at  $-80 \text{ }^\circ\text{C}$ .

### 2.3 Sample preparation

Thirty mg of each sample of adipose tissue was weighed. The sample was homogenized after adding 1.2 mL chloroform/methanol (2:1, v/v) followed by centrifugation ( $8000 \text{ rpm} \times 5 \text{ min}$ ). The supernatant was collected and the extraction for the residue was repeated using the same method. The total supernatant was clarified for 5 min at  $13000 \text{ rpm}$  at  $4 \text{ }^\circ\text{C}$  and was dried with gentle nitrogen stream. Ten  $\mu\text{L}$  4-chloro-DL-phenylalanine ( $1.04 \text{ mg mL}^{-1}$ ) was added into each sample. Then, all samples were lyophilized, added with  $50 \mu\text{L}$  MSTFA for derivatization at  $60 \text{ }^\circ\text{C}$  for 2h. The sample extraction was conducted with  $100 \mu\text{L}$  chloroform. All samples were centrifuged for 5 min at  $8000 \text{ rpm}$  at  $4 \text{ }^\circ\text{C}$ . The supernatant was stored at  $-20 \text{ }^\circ\text{C}$  prior to GC-MS analysis.

### 2.4 GC-MS analysis

GC-MS analysis was performed on Agilent 6890N gas chromatography coupled with 5975B mass spectrometric detector. A  $1 \mu\text{L}$  aliquot was injected into HP-5 MS capillary column coated with 3% phenyl methyl siloxane ( $30\text{m} \times 250 \mu\text{m}$  i.d.,  $0.25 \mu\text{m}$ ) in splitless mode. Helium was used as the carrier gas with a flow rate of  $1.0 \text{ mL min}^{-1}$ . The temperature of the injector was  $280 \text{ }^\circ\text{C}$ . The derivatives of fatty acids were separated at constant flow with the following temperature program: held  $80 \text{ }^\circ\text{C}$  for 2 minutes; increased to  $200 \text{ }^\circ\text{C}$  at  $15 \text{ }^\circ\text{C min}^{-1}$ ; held  $200 \text{ }^\circ\text{C}$  for 10 min; increased to  $280 \text{ }^\circ\text{C}$  at  $10 \text{ }^\circ\text{C min}^{-1}$ . Solvent delay was set for 5 min. The measurements were made with electron impact (EI) ionization ( $70 \text{ eV}$ ) in the full scan mode ( $m/z$  50-500). Identification of the compounds was made by authentic standards and NIST library.

### 2.5 Data analysis

The GC-MS data in instrument-specific format were converted to CDF format files. The program mzmine2 was implemented for peak detection, peak recognition, and peak alignment. PLS-DA and score line plots were performed in SIMCA-P software version 12.0.1 (Umetrics AB, Umea, Sweden). The P-values were obtained by Originpro software version 8.0 (OriginLab, Co., MA).

Features selected based on the P-value and fold-change ( $P < 0.05$  with fold-change  $\geq 1.5$ ) of the resulting tab delimited table generated from MZmine 2 (<http://mzmine.sourceforge.net/>), then manual integrations of the peak areas were normalized by the peak area of the internal standard. The raw data was also performed in SIMCA-P after normalization to get score line plots.

## 3 Results and Discussion

### 3.1 Metabolic profiling analysis of free fatty acids

GC-MS analysis of metabolites in adipose tissue was completed with MSTFA derivatization. The typical total ion chromatograms of samples of three dosages group are shown in Fig. 1. After the GC-MS data were acquired, the program mzmine2 was implemented for the differentiation of ion peaks. The metabolome data sets of the adipose tissue of mice were globally characterized. A total of 765 features were observed and integrated in the analysis of C6 adipose tissue.

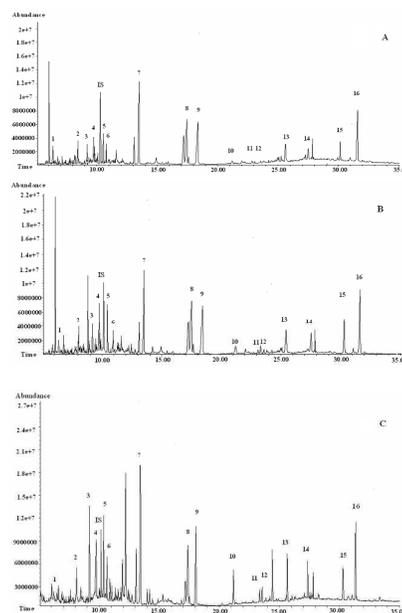


Figure 1.

**Fig.1. Typical GC-MS chromatograms from the FFA analysis of mice adipose tissues from (A) control group, (B) low dosage group and (C) high dosage group. The peaks assignment are: (1) L-serine; (2) Capric acid; (3) Lauric acid; (4) Palmitic acid; (5) Myristic acid; (6) Citric acid; (7) Palmitoleic acid; (8) Oleic acid; (9) Stearic acid; (10) Linoleic acid; (11) 16-Hydroxypalmitic acid; (12) Arachidic acid; (13) 3-Iodo-cholestane; (14) Behenic acid; (15) Cholest-4-ene-3, 6-dione; (16) Docosanedioic acid.**

The identification of the metabolites was carried out by comparing the analytical results of the detected FFAs with those from the analysis of authentic standards and by searching against NIST databases. It was possible to identify most compounds with

authentic standards from the comparisons of retention time and mass spectrum. When the standards were unavailable, the metabolite identification was conducted by searching against NIST library. The statistical data of significant compounds as the differentiating metabolites are summarized in Table 1. The obtained data demonstrated that most similarity index in NIST library were more than 93%.

Different responses to TCDD toxicity in mice with various dosages were investigated. Concentrations of FFA were found to be elevated in the TCDD-exposed mice. Notably, the FFA levels in the mice of high dosages group increased markedly compared to the control group, while the changes of FFAs between low dosage group and the control group were not significant. Levels of individual FFA were quantified by normalization to the internal standard and by reference to calibration curves for each individual component.

Table 1. Differential metabolites detected in adipose tissues of high dose TCDD-exposed mice by metabolomic analysis based on GC-MS.

No	Metabolites	RT (min)	Fold-Change <sup>a</sup>	P-value	Similarity index (%) <sup>b</sup>
1	L-serine	6.38	-1.41	1.07E-02	100
2	Capric acid	8.31	3.35	3.93E-04	100
3	Lauric acid	9.07	3.74	3.70E-03	100
4	Palmitic acid	9.76	7.52	1.03E-03	98
5	Myristic acid	10.43	3.34	9.23E-03	100
6	Citric acid	10.84	1.49	4.11E-02	100
7	Palmitoleic acid	13.53	3.50	1.19E-04	100
8	Oleic acid	17.46	2.94	1.37E-03	99
9	Stearic acid	18.37	2.91	1.33E-03	100
10	Linoleic acid	21.25	4.59	4.47E-04	100
11	16-Hydroxy palmitic acid	23.10	6.92	1.84E-04	100
12	Arachidic acid	23.62	3.47	5.78E-03	100
13	Cholestane, 3-iodo-	25.78	2.65	1.53E-02	95
14	Behenic acid	27.48	2.45	1.95E-03	93
15	Cholest-4-ene-3,6-dione	30.25	3.80	1.41E-02	96
16	Docosanedioic acid	31.43	8.29	2.34E-04	100

<sup>a</sup> Fold change (FC) with a positive value means that the concentration of a metabolite is relatively higher in dose group, while a negative value suggests a relatively lower concentration as compared to control group.

<sup>b</sup> Similarity index denotes the percentage of metabolite identification by searching against NIST library

### 3.2 Multivariate analysis

The data of three groups were imported into SIMCA 12.0.1 for data visualization. Profiles were implemented by PLS-DA and the score plots generated represent the distribution of all samples (Fig. 2). The separation of three groups was demonstrated. All subjects fell inside the 95% confidence interval (CI). The score t1 (first component) explains the largest variation of the X space, followed by t2 etc. Hence the scatter plot of t1 vs t2 is a window in the X space, displaying how the X observations are situated with respect to each other. The score plot is a map of the observations, showing the possible presence of outliers, groups,

similarities and other patterns in the data. Three groups appeared possible to be unambiguously discriminated. Based on the PLS-DA analysis the set of FFA for potential biomarkers were screened with capability of discrimination between control group and TCDD-exposed group.

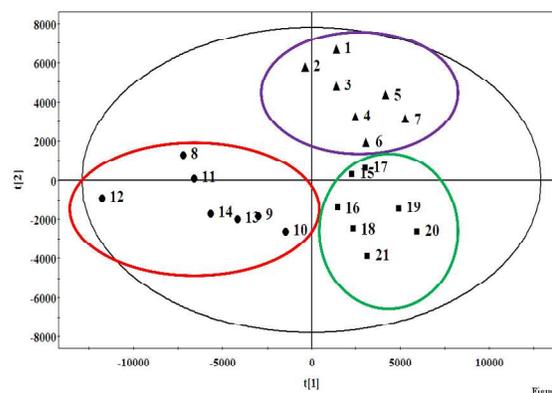


Fig.2. The score plot from partial least-squares-discriminant analysis classifying high dosage group (8-14), low dosage group (15-21) and control group (1-7).

Boxplot was used for comparing FFA changes among control, high and low dosage groups. The results shown in Fig. 3 indicated again that FFA levels of high dosage group increased markedly compared to the control group. By contrast, the FFA changes between the low dosage group and the control group were not obvious, which was consistent with the results shown in Fig. 1. For myristic acid and stearic acid, for instance, the relative intensity from the determination of their corresponding ions at  $m/z$  228.37 and  $m/z$  284.4772 increased in TCDD-treated group compared to the control group. Thus, the global characterization results of FFAs might be recognized to be a parameter for understanding the different responses to various dosages of TCDD exposure in mice.

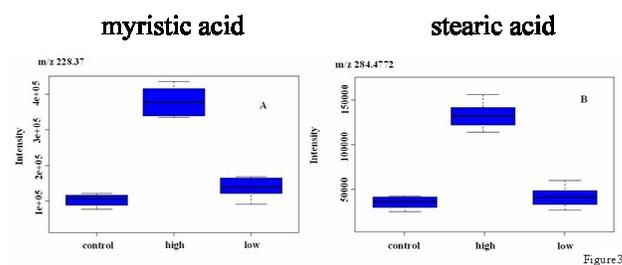


Fig.3. Boxplot of metabolites of (A) myristic acid and (B) stearic acid in control, low dosage versus high dosage groups.

To further compare data of one animal group with another, method with contribution of SIMCA was used. Fig. 4A shows low TCDD dosage group compared to the control group with the increased FFA levels in the low dosage group. Also, the results shown in Fig. 4B indicated that contents of FFA in high TCDD dosage group were higher than those in control group.

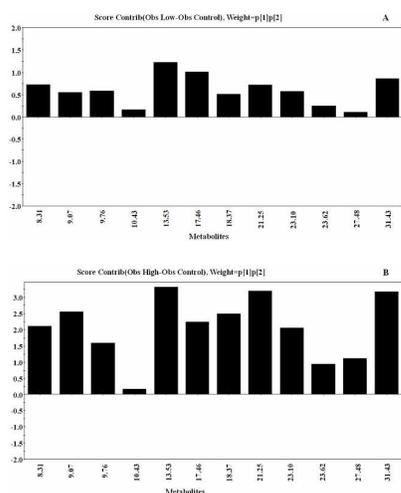


Figure 4.

Fig. 4. Contribution for one group to another group of (A) low-control and (B) high-control.

### 3.3 Analysis of metabolic function in C57BL/6J mice exposed to TCDD and control mice group

The difference of the FFA in adipose tissue between C57BL/6J mice exposed to TCDD and the control mice group were visualized by using PLS-DA. The metabolomics results revealed that TCDD exposure markedly induced an increase of FFAs in C57BL/6J mice. For example, capric acid, lauric acid, myristic acid, palmitic acid, oleic acid, stearic acid, linoleic acid, 16-hydroxypalmitic acid, arachidic acid, behenic acid and docosanedioic acid were elevated from 2.45-fold to 8.29-fold in the high dose TCDD-exposed mice compared to the control group.

It is known that the requisites for energy expenditure are covered mainly by two major substrates, namely glucose and FFA. High FFA levels decreased the ability of the liver to store sugars in blood and away from muscles that use FFA for energy. The increased use of FFA as a substrate for oxidation in muscle decreases glucose oxidation. Both pathways of glucose and fatty acid oxidation end up in the citric acid cycle. [23] The detected results on fatty acid levels might be related to the capacity of the TCA in cellular mitochondria. Thus, TCDD toxicity was the potential factor that caused the accumulation of fatty acids and the increased rate of fatty acid  $\beta$ -oxidation with mitochondrial TCA dysfunction. The increase rate of fatty acid  $\beta$ -oxidation, combined with the irregular lipid and fatty acid uptake, was the hallmark toxicant-specific perturbation in the C6 skeletal muscle exposed to TCDD, which manifested the diabetes-like metabolic phenotypes of insulin resistant onset. [6]

Adipose tissue has been proposed to be a site of insulin resistance. [24] Adipose tissue modulates metabolism by releasing non-esterified fatty acids and glycerol, hormones including leptin and adiponectin and proinflammatory cytokines. [25] A strong correlation is observed between increased plasma FFA, intramyocellular lipid accumulation and insulin resistance. [26-29] The higher levels of FFA and increased intracellular lipid inhibit insulin signaling in muscle. It is possible that elevations in FFAs may also contribute to  $\beta$ -cell insulin resistance. Abnormalities in  $\beta$ -cell function are critical in defining the risk and development of

type 2 diabetes. When the  $\beta$ -cell is healthy, the adaptive response to insulin resistance involves changes in both function and mass, and is so efficient that normal glucose tolerance is maintained. But when  $\beta$ -cell dysfunction is present, impaired glucose tolerance, impaired fasting glucose and, at the extreme, type 2 diabetes results. [24] The significant increase of FFA in C57BL/6J mice exposed to TCDD could possibly indicate that diabetes is accompanied by an underlying disorder of FFA metabolism. Further work will be necessary to study the relevance of increased levels of FFA in the etiology of diabetes. This work has revealed that the measurement of FFA in adipose tissue can be helpful for the primary diagnosis of diabetes.

### 4. Conclusions

This study reports 12 FFAs that differ significantly in levels between C57BL/6J mice exposed to TCDD and the control group of mice. Metabolic profiling revealed that the levels of FFA in the mice group with high dosage of TCDD were higher than those in control group. The global characterization results of FFAs could be regarded as a parameter for understanding the different responses to different dosages of TCDD exposure in mice. PLS-DA model was applied to refine differences between TCDD exposure and the control group. It was indicated that the use of GC-MS coupled with multivariate statistical analysis has the potential of metabolomic profiling of adipose tissue and therefore could provide holistic information on change of metabolic profile related to TCDD exposure in mice.

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