ABSTRACT

Title of Document: ABSORPTION AND METABOLISM OF 3-MCPD 1-MONOPALMITATE IN RATS

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Fatty acid esters of 3-monochloropropane 1,2-diol (3-MCPD esters) are a group of potential chemical toxicants. Their toxic effects primarily include nephrotoxicity and hepatotoxicity. To understand the toxic mechanisms of 3-MCPD esters, one of the key points is to advance the understanding of their metabolic mechanisms in vivo. This dissertation investigated 1) the absorption and kinetics of 3-MCPD 1-monopalmitate in rats, 2) the possible metabolites of 3-MCPD 1-monopalmitate after oral administration to rats, and 3) the possible metabolic pathways of 3-MCPD 1-monopalmitate in vivo. The greatest concentration of 3-MCPD 1-monopalmitate in the plasma was 873.72 ng/mL (C_{max}) at about 1.67 hours (T_{max}) after oral administration. The concentration of 3-MCPD 1-monopalmitate reduced to half after 3.42 hours ($t_{1/2}$). No 3-MCPD 1-monopalmitate could be detected after 4 hours, which was its mean resident time (MRT). The

area under curve (AUC) for 3-MCPD 1-monopalmitate in rat plasma was 1676.15 h.ng/mL, which represented the maximum amount of 3-MCPD 1-monopalmitate absorbed into plasma under the testing conditions. Beside, 39 possible metabolites were tentatively identified in the liver, kidney, testis, brain, plasma and urine samples at 6, 12, 24 and 48 hours after oral administration of 3-MCPD 1-monopalmitate to rats. In addition, five major metabolic pathways of 3-MCPD esters were derivate to evaluate their metabolic conditions in vivo. These results can greatly enhance the understanding about the absorption, distribution and metabolism conditions of 3-MCPD esters in vivo, and promote further research about the biological actions of 3-MCPD esters.

ABSORPTION AND METABOLISM OF 3-MCPD 1-MONOPALMITATE IN RATS

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

In love and thanks to my parents, Jinjun Gao and Zhaohui Bi. Also to Fang and Edward of being my constant sources of support and encouragement in these years. I am truly thankful for having you in my life.

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Lastly, I thank the entire University of Maryland community for fostering a supportive, academic community.

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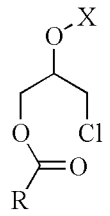
List of Abbreviation

ACN	Acetonitrile
AUC	Area under the curve
DAD	Diode array detector
DMPO	5,5-dimethyl-1-pyrroline
ESI	Electron spray ionization
ESR	Electron spin resonance
EtOH	Ethanol
FAME	Fatty acid methyl ester
FID	Flame ionization detector
GC	Gas chromatography
GSH	Glutathione
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IPA	Isopropyl alcohol
LC	Liquid chromatography
3-MCPD	3-monochloropropane 1,2-diol
MeOH	Methanol
MS	Mass spectrometry
PDA	Photodiode Array (detector)
RT	Retention time
RSD	Relative standard deviation

UPLC	Ultra performance liquid chromatography
UV/Vis	Ultraviolet / Visible light

Introduction



3-MCPD ester X = H, OR

Fatty acid esters of 3-monochloropropane 1,2-diol (3-MCPD esters), a group of chemical toxicants for kidney and testis, could be formed during the oil refining process and have been detected in many food categories, including infant and baby foods. In 2004, 3-MCPD esters were first reported together with free 3- MCPD in processed foods. With the ester form in a much higher concentration, especially in oils, fats, and fried foods. In 2013, the European Food Safety Authority estimated a tolerable daily value (TDI) of 2 μg/kg body weight for the total amount of free 3-MCPD.

Recent research has investigated the detection methods, possible formation mechanisms, and the toxicology of 3-MCPD esters in vivo and in vitro. However, there is little information about the absorption, distribution, metabolism, and excretion (ADME) of 3-MCPD esters, although these data are important to assess the risk of 3- MCPD ester intake. However, no systematic research about the ADME situation of 3-MCPD esters in vivo was reported.

ADME may alter the pharmacokinetics of food components and consequently influence their beneficial or toxicological activities at a selected organ or tissue. Only a few studies report the metabolism and metabonomics of 3-MCPD and its fatty acid

esters in vivo and their metabonomic results in rat urine and the cultured cells. Until now, no systematic research about the ADME situation of 3-MCPD esters in vivo was reported. Due to this fact, a systematic study on the absorption, distribution, metabolism, and excretion of 3-MCPD esters in vivo was performed.

The overarching goal of this study is to promote the understanding about the absorptive and metabolic conditions of 3-MPCD 1-monopalmitate in rats. The specific objectives by which this study approaches that goal are:

- To investigate the kinetics of 3-MPCD 1-monopalmitate in rats' plasma
- To identify the possible metabolites in different organs/tissues, plasma and urine after oral administration 3-MCPD 1-monopalmitate in rats
- To derivate the possible metabolic pathways of 3-MCPD 1-monopalmitate in vivo

This study represents the absorption, distribution, metabolism and excretion of 3-MCPD 1-monopalmitate in rats. The greatest concentration of 3-MCPD 1-monopalmitate in the plasma was 873.72 ng/mL (C_{max}) at about 1.67 hours (T_{max}) after oral administration. The concentration of 3-MCPD 1-monopalmitate reduced to half after 3.42 hours ($t_{1/2}$). No 3-MCPD 1-monopalmitate could be detected after 4 hours, which was its mean resident time (MRT). The area under curve (AUC) for 3-MCPD 1-monopalmitate in rat plasma was 1676.15 h.ng/mL, which represented the maximum amount of 3-MCPD

1-monopalmitate absorbed into plasma under the testing conditions. Beside, 39 possible metabolites were tentatively identified in the liver, kidney, testis, brain, plasma and urine samples at 6, 12, 24 and 48 hours after oral administration of 3-MCPD 1-monopalmitate to rats. In addition, five major metabolic pathways of 3-MCPD esters were derivate to evaluate their metabolic conditions in vivo. Besides, there are still large amount of metabolites remain in tissues 48 hours after oral administration of 3-MCPD 1-monopalmitate to rats, such as the acetylated 3-MCPD in liver and kidney, and the cysteine conjugated 3-MCPD in testis. Consider that people consume foods contained 3-MCPD esters everyday, these metabolites might be accumulated in the organs/tissues. Therefore, the toxicity effects of these metabolites should also be investigated. The present study might advance our understanding of the metabolism process of 3-MCPD esters and provide a base for further studies to have a better understanding about the toxicity effects and mechanisms of 3-MCPD esters in vivo.

Chapter 1: Literature Review

1.1. Overview

Fatty acid esters of 3-monochloropropane 1,2-diol (3-MCPD esters) are a new group of chemical compounds formed during oil refining process. These chemical compounds have nephrotoxicity, hepatotoxicity and testicular toxicity, and have been detected in many food categories including infant and baby foods (Hamlet et al., 2004; Liu et al., 2012; Zelinkova et al., 2006). In 2004, 3-MCPD ester was first reported together with free 3-MCPD in the processed foods, with the esters form in a much higher concentration, especially in oils and fats as well as in fried foods (Hamlet et al., 2004; Svejkovska et al., 2004). Later, 3-MCPD esters were detected in human breast milk, indicating their possible absorption and distribution in human organs and tissues (Zelinkova et al., 2008).

In addition, the amounts of 3-MCPD esters haven been quantified in some foodstuffs. In 1984, the analytical method by using thin layer chromatography (TLC) coupled with mass spectrum to identify the 3-MCPD diesters in goat's milk was established (Cerbulis et al., 1984). In 2005, the concentrations of 3-MCPD esters were determined between 145 and 1184 μg/kg in coffee surrogates products, whereas their amounts in malts were between 4 to 650 μg/kg in malts products, especially greater in roasted malts (463 to 650 μg/kg) (Dolezal et al., 2005). Vegetable oils are a group of food products reported has high level of 3-MCPD esters, especially in some oil products after high temperature process. In 2006, the amounts of 3-MCPD esters in oils were determined between < 100 to 2462 μg/kg, and the concentrations of 3-

MCPD esters were closed related with the oil refining temperature (Zelinkova et al., 2006). In 2012, the contaminations of 3-MCPD esters range from 0.3 to 8.8 μg/g in palm oils (Dobuis et al., 2012). In 2016, researchers reported that sunflower and olive oils contained the ester form of 3-MCPD in the range of 0.26 to 0.30 mg/kg, close to that reported earlier (Jedrkiewicz et al., 2016). Besides, 3-MCPD esters were detected and qualified in many other different types of foodstuffs, including biscuits (< 0.01 to 0.134 mg/kg), French fries (36.77 mg/kg fat), salty crackers (12.52 mg/kg fat) (Hamlet et al., 2002). More importantly, 3-MCPD esters have been detected in infant formula or infant food samples, with the concentrations of 62 to 588 μg/kg (Zelinkova, 2009) or around 0.4 mg/kg in 3-MCPD equivalent (Wohrlin et al., 2015). In 2008, Zelinkova and colleagues reported their study about the existence of 3-MCPD esters in human breast milk (from less than 300 to 2195 μg/kg fat, with a mean level of 1014 μg/kg milk fat) (Zelinkova et al., 2008). All these studies indicated a fact that 3-MCPD esters might be widely existed in many types of foodstuffs, and they might be absorbed into the human body and involved in the circulation system.

Recently, 3-MCPD esters became a food safety issue both in food research and food industry. In March 2011, *Europe Journal of Lipid Science and Technology* published a special issue about 3-MCPD esters and glycidol entitled “fatty acid esters of chloropropanols and glycidol”. In this special issue, a total of 17 reviews and research articles were published, including the chemical formation mechanism, analytical method development and toxic effects of 3-MCPD esters and glycidol. In 2013, the European Food Safety Authority estimated a tolerable daily value (TDI) of

2 μg/kg body weight for the total amount of free and fatty acid esters of 3-MCPD (EFSA, 2013). Together, these previous researches suggested that 3-MCPD esters are an important food safety concern and warrants additional research of their detection, toxicity and the biochemical mechanisms behind, their formation under different food formulation, processing and storage conditions, the chemical mechanism(s) involved in their formation, and the approaches for mitigation.

1.2. Analytical methods of 3-MCPD esters

Since 3-MCPD esters were reported and considered as potential food-source toxins in 2006 (Zelinkova et al., 2006), it is important to develop accurate, fast and high-sensitivity analytical methods to detect this group of chemical components in different types of food samples. All of the reported analytical methods can be separated into two major types. The first type method is to hydrolyze all the fatty acid esters of 3-MCPD to free 3-MCPD, quantify the amount of free 3-MCPD, and use the concentration of free 3-MCPD to represent the total amount of all the 3-MCPD esters indirectly, which is a convenient method. Another possible method is to characterize and quantify every 3-MCPD esters in food samples directly, which is straightforward and easy to understand, but is difficult in sample purification and method development. In the following paragraph, systematic literature reviews about these methods are summarized.

1.2.1. Indirect approaches in detecting 3-MCPD esters

The basic working mechanism of indirect analytical method is based on hydrolysis of all of the 3-MCPD esters into free 3-MCPD under acidic or alkaline

conditions, then convert free 3-MCPD into stable volatile derivatives and finally detected the amount of 3-MCPD derivatives using GC or GC-MS (Baer et al., 2010). The major differences between these methods are the transesterification conditions and derivatization agent different methods might choose. In 2006, Zelinková and colleagues reported their study results about the concentration of 3-MCPD esters and free 3-MCPD in 25 vegetable oil samples using the indirect analytical method (Zelinkova et al., 2006). In this study, 3-MCPD esters were extracted and hydrolyzed using sulphuric acid, neutralized with saturated $NaHCO_3$ solution, derivatized with phenylboronic acid and then injected into GC-MS for analysis. The results indicated that most of 3-MCPD that existed in oil samples are bound 3-MCPD (3-MCPD esters) rather than free 3-MCPD, and 3-MCPD di-esters are the major form of 3-MCPD esters. The results in this study also suggested the importance of researching ester form of 3-MCPD, since they are the major existence form of 3-MCPD in almost all types of foods. In 2008, Zelinková reported the occurrence of 3-MCPD esters in human breast milk using the similar analytical method, which confirmed the stability of this method (Zelinkova et al., 2008). Also in 2008, Seefelder designed and processed a novel method to hydrolyze the 3-MCPD esters to free 3-MCPD using the intestinal lipase in vitro, followed by the GC-MS analysis (Seefelder et al., 2008). The results represented the fact that only a small part (maximum 15%) of 3-MCPD bound in esters is mono-esters, and the rest part is di-esters. Still in 2008, Weißhaar reported a method to determine the total amount of 3-MCPD esters in edible fats and oil samples by transesterifing 3-MCPD esters with $NaOCH_3$/methanol, derivatizing with phenylboronic acid, and finally determined by GC-MS (Rüdiger, 2008). This method

was used to analyzing the ester form of 3-MCPD in different types of food products. In 2010, Baer and colleagues published a review article about 3-MCPD analyses for food samples (Baer et al., 2010). In this review, different derivatives including heptafluorobutyrylimidazole (HFBI) (Liu et al., 2013), phenylboronic acids (PBA) (Ermacora & Hrncirik, 2013; Küsters et al., 2010; Küsters et al., 2011), dioxolane were utilized to increase the volatility of free 3-MCPD. In 2016, Samaras and colleagues reported an indirect analytical method for the simultaneous quantification of 3-MCPD esters, 2-MCPD esters and glycidol esters in different types of food samples by purifing with pressurized liquid extraction (PLE) and determined using GC-MS. In order to differentiate glycidol esters from MCPD esters, all the glycidol esters were converted to monobromopropanediol esters (MBPD esters) in acid solution first. Then MCPD esters and MBPD esters were hydrolyzed to release their free forms in ethyl acetate with phenyl boronic acid. And the concentrations were finally quantified using isotopic labeled MCPD esters and glycidol esters analyzed in GC-MS (Samaras et al., 2016). Also in 2016, an indirect enzymatic method for the analysis of 3-MCPD esters, 2-MCPD esters and glycidol esters in edible oils and fats was reported. This enzymatic method utilized *Candida rugosa* lipase to hydrolyze the ester form of MCPDs and glycidols to their free form at ambient temperature in 30 min. Then the free form of MCPDs and glycidols were analyzed using GC-MS (Koyama et al., 2016). All these results indicated that the indirect approaches required less analytical standards, more applicable to all type of different oils and fats and other food products, and hence recommended to describe the total amount of 3-MCPD related components in food matrix. On the other hand, the indirect methods

need more sample preparation efforts with possible toxic chemical reagents. Furthermore, the indirect method can only represent the total amount of 3-MCPD esters, but not every specific 3-MCPD esters. These defects made the indirect analytical methods more and more uncommonly utilized after 2010, and more efforts were made to develop direct analytical methods to determine the concentrations of each 3-MCPD ester compounds individually.

1.2.2. Direct approaches in detecting 3-MCPD esters

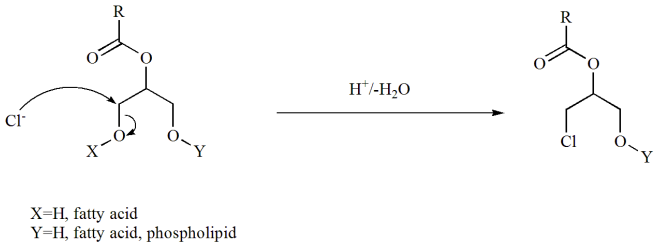
Many efforts have been made to develop chemical methods to analyze 3-MCPD esters directly without hydrolyze them to free 3-MCPD. This method was not widely applied before 2010. This might be mainly due to the non-polar nature and the relatively low volatility of 3-MCPD esters. Non-polar and low volatility compounds are generally not suitable for either LC or GC analysis. However, with the development of analytical technology and new types of LC columns, there are increasing research results about analyzing 3-MCPD esters directly in different food matrixes. In 2012, Dubois and colleagues made comparison of previous indirect methods with their newly developed direct method to analyze 3-MCPD esters in vegetable oils (Dubois et al., 2012). In this study, double solid phase extraction (SPE) and silicon gel column were utilized to separate and concentrate 3-MCPD esters, a HSS T3 normal phase LC column was utilized combined with ESI-time of flight (TOF) mass spectrometry. The comparison between indirect and direct methods showed that these two types of analytical methods had no difference in analyzing 29 oil samples. The indirect approach needs less chemical standards and relatively easier sample preparation, and is applicable to all type of commodities compared to the

direct approach to determine the total 3-MCPD esters content. On the other hand, direct method can provide detail information about the identification and concentration of each 3-MCPD ester, but with a longer sample pretreatment time. After 2012, increasing research articles reported modified direct method in analyzing the compositions and concentrations of 3-MCPD esters in food samples or organ/tissue samples for in vivo studies (Andreoli et al., 2015; Li et al., 2015; MacMahon et al., 2013a, 2013b; MacMahon et al., 2014). All these studies selected high performance liquid chromatography (HPLC) or ultra performance liquid chromatography (UPLC) combined with high-resolution mass spectrometry to detect 3-MCPD esters directly; these methods represented the development of 3-MCPD esters analysis research and played important role as references in our studies about the metabolism of 3-MCPD esters in vivo.

1.3. Formation mechanism of 3-MCPD esters

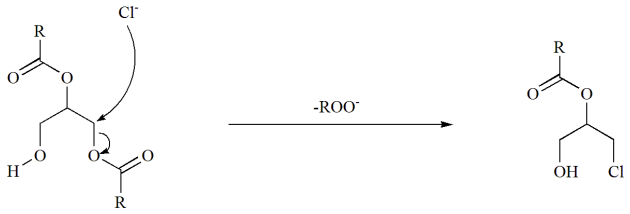
One of the important objectives about the 3-MCPD esters studies is to investigate the chemical and biochemical formation mechanisms during food processing, since it is the fundamental to reduce their level in foods. Several previous researches investigating the possible formation mechanism for 3-MCPD mono- and di-fatty acid esters were reviewed and summarized in four major possible mechanisms (Rahn & Yaylayan, 2011b), including the direct nucleophilic substitution of chlorine anion of a hydroxyl (pathway 1) or a fatty acid ester group at sn-3 carbon atom in the glycerol (pathway 2), as well as through the formation of an acyloxonium cation (pathway 3) or through an epoxide cation intermediate (pathway 4) and

followed by a nucleophilic attack by chlorine anion of the cation to open the 3 and 5 member rings.

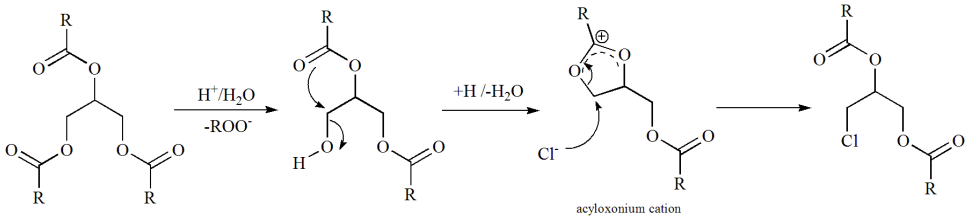


X=H, fatty acid
Y=H, fatty acid, phospholipid

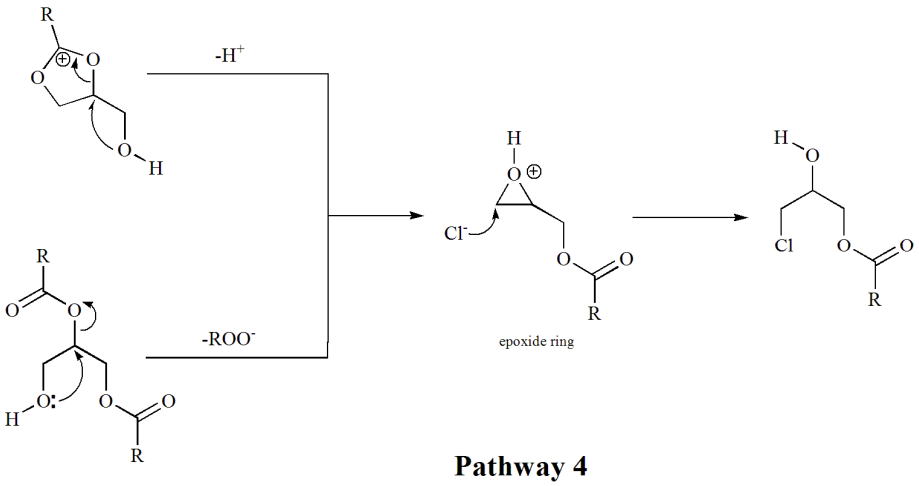
Pathway 1



Pathway 2



Pathway 3



Pathway 4

Pathway 1 represents the acid-catalyzed nucleophilic substitution of the hydroxyl and ester group in glycerol, triolein and 1,2-diacyl-sn-glycerol-3-phosphorylcholine by chlorine anion in 5.5 M hydrochloric acid (Rahn & Yaylayan, 2011b). The reaction solution was heated at 107 °C for 16 hours, and the ratios of 3-MCPD, 2-MCPD, 1,3-dichloropropanol (1,3-DCP) and 2,3-DCP, respectively) were calculated to support a proposed direct nucleophilic substitution, and to propose a possible involvement of the carbonyl group in forming possible cyclic carbocation, a cyclic acyloxonium cation intermediate (Collier et al., 1991). The formation of the acyloxonium cation intermediate suggested the electronic and steric effects of the neighboring ester carbonyl group in formation of MCPD and DCP and their fatty acid esters.8 Svejkovská and colleagues (Svejkovska et al., 2006) evaluated the role of sodium chloride, moisture content and glycerides, including 1-monopalmitin, 1,3-dipalmitin, tripalmitin and soybean oil, in formation of 3-MCPD esters. The study confirmed the formation of 3-MCPD fatty acid esters in model emulsion system in the presence of sodium chloride and water under a thermal treatment condition. The results also supported the presence of possible acyloxonium cations derived from both diacylglycerol and monoacylglycerol. It was also concluded that triacylglycerols were hydrolyzed to diacylglycerol to participate in 3-MCPD ester formations, but not directly participate in 3-MCPD esters formation. The amount of sodium chloride was proportional to the final 3-MCPD esters concentration, supporting a nucleophilic substitution mechanism. In 2011, Rahn and colleagues heated triplamitin, 1,2-dipalmitin, 1-monopalmitin and C^{13} labeled triglyceride and diglyceride at 100 °C with the presence of $ZnCl_2$ and measured the mixtures with FT-IR (Rahn & Yaylayan,

2011a). The FT-IR results from this study confirm involvement of carbonyl group in possible 3-MCPD ester formation, but could not serve as a direct evidence for the existence of the acyloxonium anion intermediate under a low moisture high temperature condition.

In 2010, Weiβhaar and colleagues (Weisshaar & Perz, 2010) proposed that 3-MCPD esters could be formed by chlorine anion attack to the epoxide ring in the glycidol, which might be produced from monoacylglycerol-derived acyloxonium by nucleophilic substitution reaction at the sn-2 carbon. In 2011, Rahn and Yaylayan (Rahn & Yaylayan, 2011b) reviewed previous studies on 3-MCPD ester formation, and proposed a possible formation of glycidol ester intermediate through direct nucleophilic attack of the sn-3 hydroxyl group at the sn-2 carbon with an ester group eliminated from the sn-3 carbon simultaneously. However, no clear evidence is available confirming the mechanism although glycidol ester has been detected in the refined oils that may also contain 3-MCPD esters (Destaillats et al., 2012).

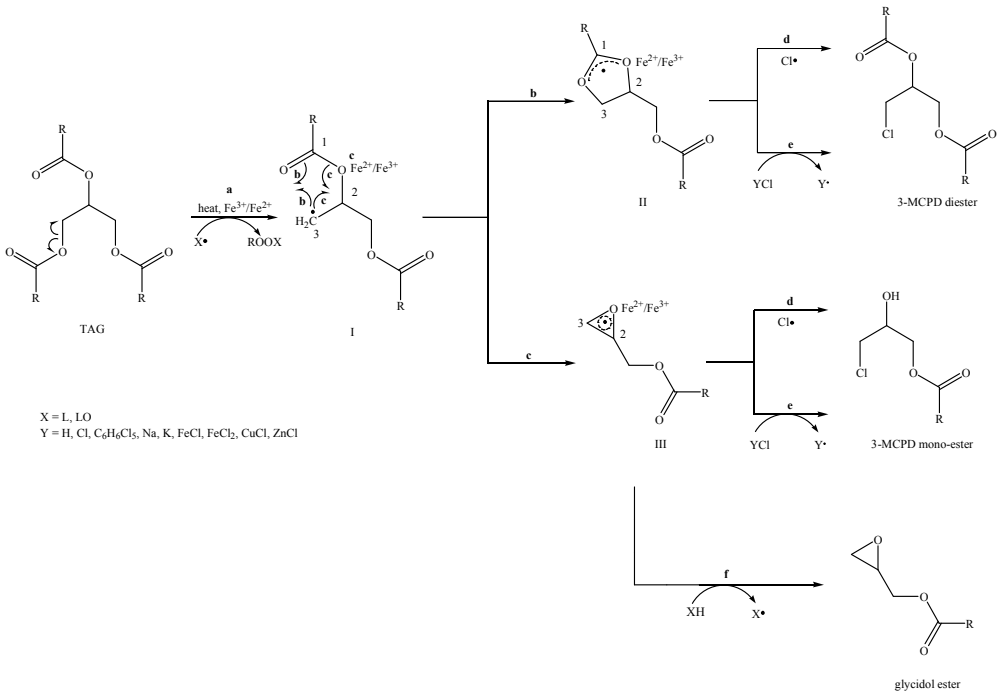
In 2012, Destaillats and others investigated the formation of MCPD fatty acid diester by heating triheptadecanoin in the presence of lindane, an organochlorine compound, and observed a formation of MCPD di-ester formation at 200 °C and above at a temperature dependent matter, whereas no detectable MCPD diester in the temperature range of 100-180 °C (Destaillats et al., 2012). This observation could not be explained by any of the reported cation or glycidol mechanisms, but suggested that

new mechanism(s) may exist in 3-MCPD ester formations under low moisture and high temperature conditions.

In 2013, our group reported a novel research approach about how 3-MCPD ester formed from diacylglycerol (Zhang et al., 2013). This study indicated a free radical mediated reaction mechanism for 3-MCPD diester formation from DSG. In this study, electron spin resonance (ESR) spectroscopy signals in the oil heated at 120 °C for 20 min were much stronger than that kept at 80 °C for 20 min, suggesting a possible temperature dependence of the radical formation under the experimental conditions. Diacylglycerol (DAG) were measured for its FT-IR spectra at 25 and 120 °C to determine the possible involvement of the ester carbonyl groups in the 3-MCPD ester formation under high temperature and low moisture conditions. The absorbance peaks at 1733 and 1711 cm^{-1} were observed for the DAG measured at 25 °C, indicating the presence of two ester groups. The DAG only had a single IR absorbance at 1744 cm^{-1} when heated to 120 °C, indicating the possible involvement of an ester carbonyl group in forming 3-MCPD esters or the two carbonyl groups were in the same chemical environment. Moreover, the signals of a cyclic acyloxonium free radical (CAFR) at the carbonyl carbon and CAFR trapped by a trapping agent DMPO were detected by the high resolution ESI-MS/MS spectrum, which further confirm the free radical mechanism for 3-MCPD ester formation under the low moisture high temperature condition. This is the first report for the free radical mechanism for 3-MCPD diester formation under high-temperature and low-

moisture conditions, and provides important scientific insight for controlling the level of 3-MCPD diesters in refined edible oils.

In 2015, additional research results from our research group were published showing that the possible mechanisms behind the formation of 3-MCPD mono- and diesters from triglycerides might involve either a cyclic acyloxonium or a glycidol ester radical intermediate under a high-temperature and low-moisture condition (Zhang et al., 2015). This study also reported that glycidol esters might be co-products from TAG during the formation of 3-MCPD esters under the experimental conditions. In addition, Fe^{2+} and Fe^{3+} might catalyze both 3-MCPD and glycidol esters' formation from TAG. These results suggested that reducing the availability of Fe^{2+} and Fe^{3+} through controlling ingredients, equipment, and containers and processing agents including water, using chelating agents in selected processing steps, as well as improvement of the thermal processing degree may reduce levels of 3-MCPD esters and related toxicants such as glycidol esters in the refined edible oils and food products.

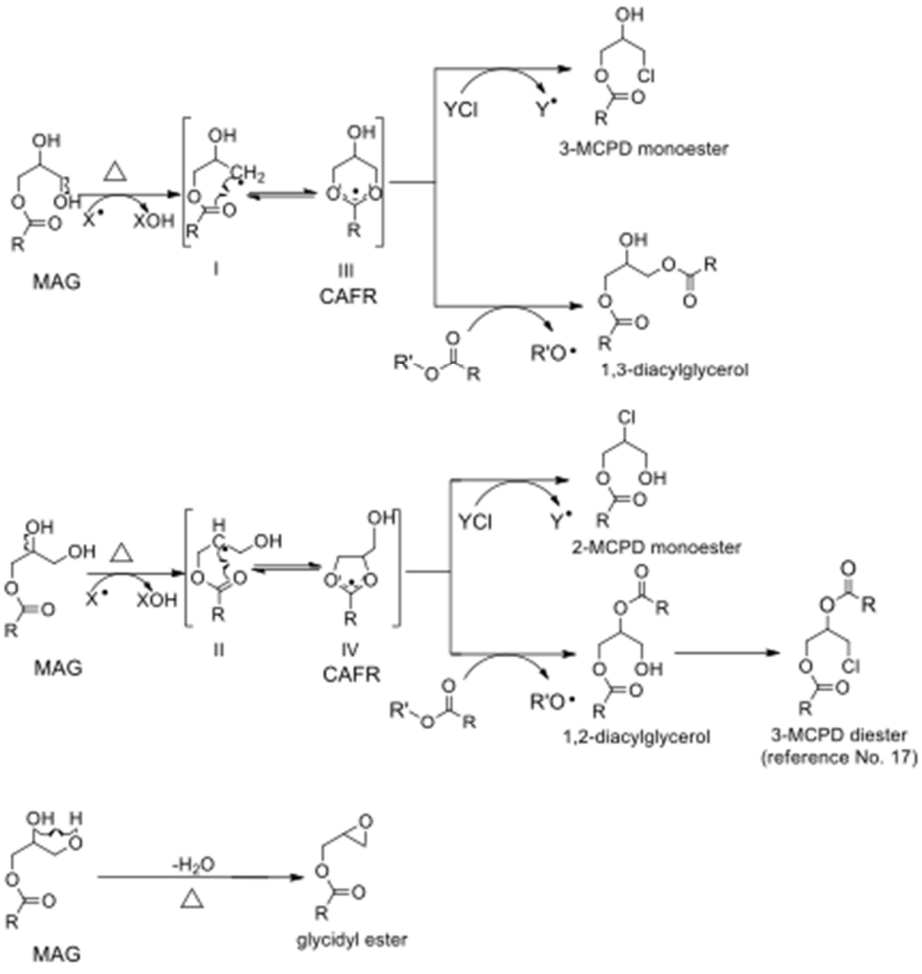


(Zhang, et.al., 2015, J. Agric. Food Chem.)

In 2016, our group reported a further study about the formation of 3-MCPD esters from mono-glycerides. The results in this study further confirmed that a free radical intermediated reaction was the mechanism for the formation of 3-MCPD esters. For the first time, this study suggested that the five or six member cyclic acyloxonium structures might involve in the free radical intermediates, which could explain the coexistence of 3-MCPD and 2-MCPD esters under a high temperature and low moisture condition. Besides, this study also reported that 3-MCPD monoesters may degrade through losing a chlorine or RCO-/RCOO- group, and Fe^{3+} may play a potential role in the thermal degradation of 3-MCPD monoesters.

Besides, the effects of temperature and NaCl in the formation of 3-MCPD esters and glycidyl esters during frying chips with refined edible oils were reported in 2017. As the results, the amounts of 3-MCPD esters trend to decrease with the frying time increased, whereas the amount was increased when frying temperature and concentration of NaCl increased. The concentrations of glycidyl esters increased when the frying temperature, frying time and concentration of NaCl increased. These results indicated that frying duration, temperature and the concentration of NaCl have close relationships with the formation of 3-MCPD esters and glycidyl esters during food processing. Temperature plays the most important role in forming 3-MCPD esters and glycidyl esters, followed with the processing duration and the amounts of NaCl (Wong et al., 2017).

In summary, the possible mechanisms behind the formation of 3-MCPD esters from glycerides might involve a cyclic acyloxonium or a glycidol ester radical intermediate under a high-temperature and low-moisture condition, and Fe^{2+} and Fe^{3+} might catalyze the formation in this condition. All of these findings are important for oil refining and food processing industries to develop mitigation approaches for 3-MCPD esters in the final food products.



X=L, LO
Y=$C_6H_6Cl_5$, MgCl, $AlCl_2$, CuCl, MnCl, SnCl, ZnCl, $FeCl_2$
L represent lipid.

(Zhao, et.al., 2016, J. Agric. Food Chem.)

1.4. Toxic effects of 3-MCPD esters

In early 2000s, 3-MCPD esters were considered as potential food-source toxicants due to their possible degradation to a known toxicant, free 3-MCPD in vivo. In recent years, increasing research results indicated that the toxic effects and their molecular mechanisms of 3-MCPD esters might be much more complecated than that were recognized before (Schilter et al., 2011).

1.4.1. Nephrotoxicity of 3-MCPD esters

Nephrotoxicity is a kind of toxic effect on renal function induced by some chemical toxins or medications. Some medical reagents, including a widely used cancer chemotherapeutic agent cisplatin (DeWoskin & Riviere, 1992; Dobyan et al., 1980; Levi et al., 1982; Miller et al., 2010), ionic contrast media such as diatrizoate (Duan et al., 2013; Morcos, 1998; O'Donnell et al., 2010; Ozgur et al., 2012; Rudnick et al., 1995; Solomon, 2014), and some illegal food additives like melamines (Bhalla et al., 2009; Park et al., 2011; Zhang et al., 2015), are recognized as possible nephrotoxicity components, and their toxic mechanisms were investigated in the past decades. One of the hypotheses about the mechanism of nephrotoxicity is that chronic progressive kidney disease may be mediated by abnormalities of lipid metabolism (Moorhead et al., 1982). In this study, Moorhead and colleagues indicated that glomerular injury might be induced by self-perpetuating secondary events, and the injury of glomerular could result in hyperlipidemia. After the abnormalities of lipid metabolism and s series of in vivo chronic progress, tubule-interstitial disease might initiate or aggravate to represent the kidney failure, and the chemical compounds that induced these changes represent nephrotoxicity. This hypothesis provided a novel

approach to understand the chronic progressive kidney disease by proposing a major pathogenetic role for lipid abnormalities.

To investigate the nephrotoxicity of ionic and nonionic contrast media, a randomized clinical trial was designed in 1196 patients in 1995 (Rudnick et al., 1995). This study aims to demonstrate the potential nephrotoxicity of intravascular iodinated contrast media in causing hospital-acquired acute renal failure. The results indicated that the nonionic contrast agent represented significantly less nephrotoxicity than the ionic contrast agent in the patients with pre-existing renal insufficiency. On the other hand, for the patients with normal renal function, nonionic contrast media showed almost similar nephrotoxic compared to ionic contrast agents.

Nitric oxide (NO) is one type of vasodilator factor, and its level in vivo is closely related with the renal vasoconstriction. Previous studies indicated that NO pathway is one of the major pathways related to nephrotoxicity (Bachhav et al., 2014; Valdivielso et al., 1996; Valdivielso et al., 1997). Increasing markers of oxidative stress and the protective effect of several free radical scavengers and antioxidants strongly support the role of oxidative and nitrosative stress in the effects of nephrotoxicity (Chirino & Pedraza-Chaverri, 2009). Besides, NO/cGMP pathway was reported related with the antioxidant, anti-inflammatory and anti-apoptotic activities, while all these bioactivities could retard the progression of renal injury (O'Donnell et al., 2010). To investigate the possible mechanism of NO deficiency-related nephrotoxicity and protective effect, a 21-day multi-dose rats study modeling with

cyclosporine A treated with/without sildenafil (Abdel-latif et al., 2013). The results indicated that cyclosporine A nephrotoxicity was characterized by intense renal vasoconstriction that often progresses to chronic injury with irreversible structural damage, while the renal vasoconstriction is related to the imbalance in releasing of NO. On the other hand, as a phoshodiestrase-5 (PDE-5) inhibitor, Sildenafil could exhibited nephrotoxicity by significantly decrease the level of serum creatinine and urea, reduce the ratio of spot urine albumin-creatinine, and decrease the levels of renal malondialdehyde and nitric oxide. Taken together, the renoprotective effects against nephrotoxicity in rats might through NO pathway as well as antioxidant, anti-inflammatory and anti-apoptotic activities.

All of these studies suggested that nephrotoxicity is a widely existed side effect caused by many types of chemical compounds. And the existence of nephrotoxic compounds in food might be more urgent because of the wider range and greater amount people might consume.

The nephrotoxicity is one the major toxic effects of 3-MCPD esters reported in these years. The hypothesis about the nephrotoxicity of 3-MCPD esters was started at the known results about the kidney toxicity of free 3-MCPD (Schilter et al., 2011). In 2011, a 90-day toxicology study using 3- MCPD di-palmitic esters at the doses of 9.78, 39.19, and 156.75 mg/kg body weight (BW) per day by daily oral gavage in Wistar rats led to the tubular epithelial degeneration in a dose-dependent manner (Barocelli et al., 2011). Results in histopathological study confirmed that kidney and testes of male rats are the critical organs for free 3-MCPD. 3-MCPD esters treated

groups represented similar trends but milder phenomenon. Rats treated with 3-MCPD esters showed a slower and lower bioavailability and excretion rate while compared with groups treated with free 3-MCPD, but the nephrotoxicity could still be observed.

In 2012, Liu and colleagues reported the acute oral toxicity of 3-MCPD monoester and di-ester in Swiss mice (Liu et al., 2012). The results indicated that 3-MCPD 1-monopalmitate dose-dependently increased serum urea nitrogen and creatinine in the treatment group of mice, and the LD50 value of 3-MCPD 1-monopalmitate was 2676.81 mg/kg body weight. The LD50 value of 3-MCPD dipalmitate was presumed to be greater than 5000 mg/kg BW, which could be recognized as non-toxic compound. No difference was observed on mean body weight, absolute and relative organ weight or serum urea nitrogen and creatinine levels in mice fed 3-MCPD dipalmitate as compared to the control group. However, renal tubular necrosis, protein casts and spermatids decrease were also observed in the dead mice. In addition, MTT and LDH assay results showed the cytotoxicity of 3-MCPD 1-monopalmitate in NRK-52E rat kidney cells in a dose-dependent manner. All these results indicated a possible greater toxicity of 3-MCPD mono-esters compared to the 3-MCPD di-esters containing the same fatty acid.

The semi-long term and long term toxicity studies about 3-MCPD esters were also conducted. In 2013, Li and colleagues reported a 90-day toxicology study feeding Wister rats with 3-MCPD dipalmitate, and the LD50 value of 3-MCPD dipalmitate was determined at 1780 mg/kg body weight, increase serum urea nitrogen

and creatinine were also detected (Li et al., 2013). The results also indicated that 3-MCPD dipalmitate caused a significant increase in blood urea nitrogen and creatinine in the high-dose group (267 mg/kg bw/day) compared to the control rats. Renal tubular epithelium cell degeneration and renal tubular hyaline cast accumulation were the major histopathological changes in rats after oral administion of 3-MCPD dipalmitate. Urine samples were collected after the 90-day feeding test and analyzed by UPLC-MS, and the results indicated that the differences in metabolic profiles between the control and treated rats were clearly distinguished. Five metabolite biomarkers, including indoxyl sulfate, xanthurenic acid, phenylacetylglycine, nonanedioic acid and taurine had been identified and considered as sensitive biomarkers in evaluating the effect of 3-MCPD dipalmitate exposures. The possible mechanism of these biomarkers variation was also elucidated. The combination of histopathological examination, clinical chemistry and metabolomics analyses in rats resulted in a systematic and comprehensive assessment of the long-term toxicity of 3-MCPD dipalmitate.

In 2004, Onami and colleagues reported a 13-week repeated dose study of 3-MCPD 1-monopalmitate, 3-MCPD dipalmitate, 3-MCPD dioleicitate and free 3-MCPD to F344 rats (Onami et al., 2014). As the results, five out of ten females died from acute renal tubular necrosis in the group of free 3-MCPD treated rats, but none of the ester-treated rats. Decreased hemoglobin (HGB) was observed in all high-dose 3-MCPD fatty acid ester-treated rats, except for the 3-MCPD di-oleate ester treated male rats. The absolute and relative kidney weights were significantly increased in the

3-MCPD ester-treated rats at medium and high doses. Relative liver weights were significantly increased in the 3-MCPD esters-treated rats at high dose, except for 3-MCPD palmitate mono-ester treated female rats. Significant increase in apoptotic epithelial cells in the initial segment of the epididymis of high-dose 3-MCPD ester-treated male rats was also observed. The results indicated that although acute renal toxicity was lower than 3-MCPD, these three 3-MCPD esters still have the potential to exert subchronic toxicity to the rat kidneys and epididymis, to a similar degree as 3-MCPD under the experimental conditions. In the same year, Onami and colleagues also reported their additional study of a 4-week comprehensive toxicity study about the genotoxicity of 3-MCPD esters and free 3-MCPD in F344 rats (Onami et al., 2014). The results indicated that the relative kidney weights of the 3-MCPD and all three ester-treated groups were significantly increased compared with the control group. However, there was no difference in the frequency of micro nucleated reticulocytes and Pig-a mutant red blood cells among groups. Moreover, no change was observed in mutant frequencies of gpt and red/gam (Spi-) genes in the kidney and the testis of either 3-MCPD or 3-MCPD esters treated rats. In histopathological analyses, no treatment related changes were observed, except for the decrease of eosinophilic bodies in the kidneys of all treated groups. These results suggested that 3-MCPD and its fatty acid esters are not in vivo genotoxins, although they may exert renal toxicity.

In 2016, Liu and colleagues reported the research results about the molecular mechanisms of the nephrotoxicity both in vivo and in vitro (Liu et al., 2016a). In this study, 3-MCPD 1-palmitate was utilized as a probe component to investigate the

possibility of JNK/P53 pathway in influencing the nephrotoxic effect of 3-MCPD esters in Sprague Dawley (SD) rats. The microarray analysis of the kidney samples of SD rats revealed that mRNA expressions of the genes involved in the mitogen-activated protein kinase (JNK/ERK), p53 and apoptotic signal transduction pathways were altered after treated with 3-MCPD esters. The changes in the mRNA expressions were consistent with the induction of tubular cell apoptosis. Moreover, p53 knockout attenuated the apoptosis, and the apoptosis-related protein bax expression and cleaved caspase-3 activation induced by 3-MCPD 1-palmitate in the p53 knockout C57BL/6 mice, whereas JNK inhibitor SP600125 but not ERK inhibitor U0126 inhibited 3-MCPD ester induced apoptosis, supporting the conclusion that JNK/p53 might play an important role in the tubular cell apoptosis induced by 3-MCPD esters.

Also in 2016, Sawada and colleagues designed and processed a 28-day oral toxicity study using the Wister rats for both free 3-MCPD and its di-ester to treat rats everyday. The results indicated that although no histopathologically visible toxicity was observed, the difference in proteins related to various metabolic pathways, such as carbohydrate, amino acid, and fatty acid metabolism, were detected. These results confirmed the hypothesis that 3-MCPD and its esters might have potential nephrotoxicity at the protein level (Sawada et al., 2016).

All these research results indicated that 3-MCPD esters have the nephrotoxicity both in vivo and in vitro. These previous studies also raised an

additional question: how could 3-MCPD esters distribute in vivo after consumed, and in what form could them practice their nephrotoxicity in vivo?

1.4.2. Hepatotoxicity of 3-MCPD esters

Liver is one of the most important metabolic organs in human body, where most of the bioactive components or toxins were metabolized. These bioactive compounds or toxins might induce liver injury or damage and cause acute or chronic liver diseases. This process is known as hepatotoxicity. Most of the xenobeotics, both bioactivity and toxic components, were metabolized in liver to form various reactive metabolites to have their therapeutic or toxic effects. During this period, toxicity may accrue through accumulation of either parent xenobiotic or formation of reactive metabolites, and target on liver for hepatotoxicity.

In these years, increasing research articles reported possible mechanisms that might cause hepatotoxicity, including bile acid-induced hepatocyte apoptosis, pathophysiological effects of mitochondrial dysfunction, and cell damage induced by oxidant stress (Jaeschke et al., 2002). Bile is important and necessary to the body, and the formation of bile is one of the functions of liver. The failure or reduction of bile formation is one kind of pathophysiologic process called cholestasis, and cholestasis might induce hepatocyte apoptosis due to the accumulation of hydrophobic bile acids in liver (Patel et al., 1998; Rodrigues et al., 1998; Rodrigues et al., 1995). After that, a translocation of cytoplasmic Fas to the membrane of plasma, aggregate the receptors and cause cell death in apoptosis approach (Faubion & Gores, 1999). Mitochondrial dysfunctions, including microvesicular steatosis, nonalcoholic steatohepatitis (NASH) and cytolytic hepatitis, were another major mechanism to induce hepatotoxicity. A

series of metabolic effects might work together to block mitochondrial oxidation and cause microvesicular steatosis (Fromenty & Pessayre, 1995; Pessayre et al., 1999; Tang, 2007; Tang et al., 1995). The nonalcoholic steatohepatitis (NASH) leading to liver cell death, cirrhosis, fibrosis or polynuclear cell infiltrates in the patients with the obesity or insulin resistance. Based on the previous studies, the NASH was major caused by respiratory inhibition induced lipid peroxidation and ROS-induced cytokine release (Pessayre et al., 2001; Pessayre et al., 1999; Sobaniec-Lotowska & Lebensztejn, 2003). Cytolytic hepatitis is also an important type of serious liver injury that most frequently caused by the toxicity or immune reactions induced by the cytochrome P450-dependent formed metabolites. These metabolites might cause DNA damage, glutathione depletion and overexpression of p53, thus cause liver cell death or other damage to the liver (Bradham et al., 1998; Feldmann et al., 2000; Haouzi et al., 2000; Hatano et al., 2000; Pessayre et al., 1999). Another major mechanism of hepatotoxicity is oxidant stress modulated hepatic adhesion molecule and chemokine formation, which could induce necrotic cell death through the release of reactive oxygen and proteases. Alcoholic hepatitis and other drug-induced liver toxicities are belong to this mechanism of hepatotoxicity (Akbay et al., 1999; Aydinoz et al., 2007; Jaeschke, 1990; Jaeschke, Knight & Bajt, 2003; Smith, 1987; Smith & Mitchell, 1985). To sum up, liver damage might be induced by many different mechanisms, including not only the toxic effects of drug and xenobiotic but also some other factors, such as gene expression profile and oxidant stress. All these biological mechanisms about the hepatotoxicity above demonstrated a fact that hepatotoxicity is one of the most important toxic effects that related to the xenobiotics, understanding

these mechanisms could dramatically improve our knowledge in clinical practices and safety concerns.

Free 3-MCPD was recognized as a potential hepatotoxicity component (Branchflower & Pohl, 1981; Smith et al., 1983). The ester form of 3-MCPD was also considered hepatotoxicity, since 3-MCPD esters might be hydrolyzed to free 3-MCPD (Abraham et al., 2013). Recently, the acute hepatotoxicity of 3-MCPD esters obtained more attentions. In 2012, Liu and colleagues reported the acute oral toxicity of 3-MCPD monoester and di-ester in Swiss mice. The results showed that the relative liver weights in survived mice were significantly greater compared with that in the control group, whereas the absolute liver weights in dead mice were significantly lower compared to the control. Histopathology results represented swelling and fatty change in the liver samples of the treated mice (Liu, et al., 2012). Theses results indicated that 3-MCPD esters could significantly increase the weight of liver, although there are no other evidences about their hepatotoxicity. These changes might happen during the metabolism of 3-MCPD esters in vivo. The phenomenon of liver weight change was confirmed by further study.

The semi-long and long term hepatotoxicity studies about 3-MCPD esters were also reported. In 2014, a 13-week repeated dose oral administration of 3-MCPD di-ester to F344 rats was reported, and the results indicated that the relative liver weights were significantly increased in the ester-treated group (Onami et al., 2014).

In 2013, a kinetic study about 3-MCPD esters and their metabolite progress to free 3-MCPD in rats was reported (Abraham, et al., 2013). By comparing the areas under the curves, the relative amount of free 3-MCPD metabolized from 3-MCPD di-ester was 86 % on average while comparing with oral administration of free 3-MCPD directly. The results indicated that most of the 3-MCPD esters were enzymatically hydrolyzed to free 3-MCPD in vivo, and the concentration of free 3-MCPD in liver was as great as in blood and kidney and cannot be ignored. Considering a fact that the ester forms of 3-MCPD had greater concentrations in many processed foods; the hepatotoxicity of 3-MCPD esters should be further investigated.

In 2015, Braeuning and colleagues reported a study that free 3-MCPD and 3-MCPD esters induced proteomic change in rat liver (Braeuning et al., 2015). Histopathological examination of liver revealed a slight focal hepatocellular necrosis in both free 3-MCPD and 3-MCPD di-ester treated rats. The lesion was related with a slight focal inflammatory cell infiltration. Results from proteome analysis indicated that livers from rats treated with free 3-MCPD and 3-MCPD ester showed overlap of proteomic alterations, which also verified that 3-MCPD esters might express their hepatotoxicity via hydrolyzed to free 3-MCPD first. Moreover, protein DJ-1/PARK7 was the strongest up-regulated protein identified by MALDI-TOF/TOF mass spectrometry, while β-actin and tropomyosin were on the list of strongly down-regulated proteins. All these results indicated that free 3-MCPD and 3-MCPD esters could induce a series of protein changes to alter the hepatic functions. And organ-

independent biomarker proteins could be changed with 3-MCPD exposure and showed hepatic toxic effects in vivo.

Also in 2015, a 4-week study of 3-MCPD di-esters on lipid metabolism in C57 mice was reported (Lu et al., 2015). As a result, significant increase in hepatic triglyceride levels was observed in all high dose 3-MCPD di-esters groups while compared with the control group. Meanwhile, significant increase in hepatic cholesterol levels was observed in low and medium dose 3-MCPD oleate di-ester treated rats and all high dose 3-MCPD di-esters treated rats when compared with the control group. The levels of serum aspartate aminotransferase were significantly increased in 6 mg/kg 3-MCPD oleate di-ester treated rats and all high dose treated rats except for 3-MCPD stearate di-ester treated ones, while compared with that in control group rats. All these results indicated that 3-MCPD di-esters could potentially cause hyperlipidemia in C57 mice and cause fat accumulation in liver.

All these research results above indicated the hepatotoxicity of 3-MCPD esters, however their possible mechanisms were still unclear. Additional in vivo studies about the hepatotoxicity of 3-MCPD esters are needed to clarify the possible mechanism of hepatotoxicity effects of 3-MCPD esters, especially the mechanisms related to the metabolism of 3-MCPD esters in liver.

1.4.3. Testicular toxicity of 3-MCPD esters

Testicular toxicity is the toxicity related to the testes; especially the toxicity related with the male reproductive systems induced by xenobiotics. Heavy metals are

recognized as one of the most important reason to induce testicular toxicity in vivo. To clarify the toxic effects and mechanisms of heavy metals in inducing testicular toxicity, a subchronically study that treated male bank voles with dietary cadmium (Cd) was practiced to evaluated the heavy metal accumulation, oxidative stress, metallothionein in determining testicular toxicity (Bonda et al., 2004). The results represented that Cd-induced apoptosis difference closely related with testicular lipid peroxidation and decreased testicular zinc concentration. The Cd-induced histopathological changes were also accompanied by increased testicular lipid peroxidation, decreased testicular Zn concentration and elevated levels of hepatic and renal MT and Zn. These data indicate that dietary Cd produces testicular lesions indirectly in young bank voles, through decreasing testicular Zn. A 90-day animal study was processed using Wistar male rats treated with/without mercury in three different doses to evaluate the testicular toxicity of heavy metals (Boujbiha et al., 2009). The results showed that mercury exposure for 90 days resulted in significantly decreased sperm count/motility and a decline of the reproductive ability while mated with female rats. These results were statistically related with the oxidative stress, represented a fact that the oxidative damage might lead to the functional inactivation of testes and express testicular toxicity. In 2013, a further study got the similar results using mercury induced testicular toxicity in rats, and indicated that the combination of mercury and sodium selenite or vitamin E to treated the rats could reduce the toxic effects. This results might confirm the hypothesis that the oxidative stress might induce testicular toxicity, since both sodium selenite and vitamin E are recognized as typical antioxidants (Kalender et al., 2013). An in vivo study about the adverse trends

of male reproductive health was designed and practiced using modeled mice in 2010 (Wu et al., 2010). The epigenetic changes and impact on gene expression were evaluated, and the results indicated that DNA methylation might plays important role in the abnormal testicular function. All these studies above could improve our understanding about the mechanisms of testicular toxicity, and further improve research about the prevention of testicular toxicity.

Testicular toxicity effect was another major toxic effect reported for 3-MCPD esters. Growing evidences indicated that 3-MCPD esters might represent potential toxic effects to the structures and functions of testis in vivo. In 2015, Sawada and colleagues designed and processed a proteomic approach to analyze the change induced by 3-MCPD esters and free 3-MCPD in the testis during/after 28-days repeated-dose feed rats (Sawada et al., 2015). Although there was no statistical difference in testes weight or visually difference in histopathology study, proteome analysis of the proteins in rats' testes indicated that 42 out of the 58 proteins were influenced by the 3-MCPD ester and showed significantly deregulation. Furthermore, an important protein DJ-1 might function as a sensitive molecular marker of both 3-MCPD esters and free 3-MCPD that related to their testicular toxicity. In the same year, researchers in the same group also reported their research results about the proteomic study in rats liver, kidney and testis induced by 3-MCPD esters and free 3-MCPD, and the results indicated that there are ten proteins down-regulated by 3-MCPD ester in all three organs, two important proteins GSHB and DJ-1/PARK7 that linked to oxidative stress were identified as up regulated by 3-MCPD esters

treatments in all three organs (Braeuning et al., 2015). These results indicated a possible testicular toxicity of 3-MCPD esters. Although the data about testicular toxicity of 3-MCPD esters is limited, the toxic effects of 3-MCPD esters on testes should still be considered.

1.5. Toxicokinetics study of 3-MCPD esters

Without the absorptive and metabolic information about 3-MCPD esters in vivo, it is difficult to fully understand their toxic effects and molecular mechanisms behind. Toxicokinetics, the kinetics study about a chemical toxin, adapted from pharmacokinetics, aim to determine the relationship between the systemic metabolism of a toxic compound in vivo and its toxicity. Toxicokinetics evaluates a chemical compound for its absorption, distribution, metabolism and excretion (ADME) using an animal model. Absorption describes the original form of chemical compound enter the body and absorbed into circulatory system. After this chemical compound was absorbed into body, it can be distributed to other organs/tissues of the body through diffusion or other biological processes. At this point, the chemical may undergo metabolism and be bio-transformed into other chemicals called metabolites, and this process is metabolism. These metabolites usually have a greater polarity and can be less or more toxic than the parent compound. At last, the metabolites may excrete out of the body, be transformed into other compounds, or be kept in the body compartments. The whole progress of ADME is critical to a xenobiotic, since the aims of ADME are to increase the polarity and accelerate the excretion of xenobiotic, and the therapeutic/toxic effects are showed in this period of time. Therefore,

understanding the toxicokinetics of 3-MCPD esters is important and necessary for advanced understanding of the toxic effects and the molecular mechanisms of 3-MCPD esters in vivo.

Traditional toxicokinetics studies shared the similar methodologies as pharmacokinetics, and the major aims of toxicokinetics were to investigate the adverse drug reactions in pharmaceutical researches. Physiologically based pharmacokinetic (PBPK) models, together with the physiological toxicokinetic (PT) models were utilized to analyze compounds that might cause toxic effects (Andersen, 2003). Previous studies reported various applications about the toxicokinetics of drugs or other synthesized bioactive compounds (Kraemer & Maurer, 2002; Maurer et al., 2000). The results showed that the metabolisms of amphetamine-derived designer drugs in rats and human were catalyzed by cytochromes P450. Possible hepatotoxic and nephrotoxic effects were observed due to the demethylation progress.

Another application about the toxicokinetic study of acetaminophen using dried blood spots (DBS) combined with HPLC-MS/MS was reported in 2008 (Barfield et al., 2008). The results suggested that this methodology was applicable in a toxicokinetic (TK) study. And the results detected by this method could be physiologically comparable with results from duplicate blood samples analyzed using identical HPLC-MS/MS conditions. Besides, this is the first reported application of DBS analysis to a toxicokinetic study, and the success of this study has led to the

intent to apply DBS approach for the pharmacokinetics (PK)/toxicokinetic (TK) studies for all oral small molecules.

Recently, toxicokinetic studies were also utilized for food factors with food safety concerns. One typical example is the toxicokinetic study about acrylamide in vivo. Acrylamide was firstly detected in cooked starchy foods and recognized as carcinogenicity chemical compound (Tareke et al., 2002). To elucidate the dosimetry of acrylamide and its most important metabolite, glycidamide in human adult and children, a toxicokinetic study was carried out to figure out investigated the possible physiologically-based toxicokinetic (PBTK) models (Walker et al., 2007). Results indicated that acrylamide could be metabolized to glycidamide by cytochrome P450 (CYP) 2E1 pathway and showed neurotoxic effects in vivo. The PBTK model clarified that the disposition of acrylamide in vivo results from CYP2E1, glutathione conjugation, and epoxide hydrolase. Moreover, the results also provided the range for child/adult dosimetry differences in area under curve (AUC) for both acrylamide and glycidamide even while considering 99^{th} percentile child to 50^{th} percentile adult values. All of these results about the toxicokinetic study of acrylamide made it critical to consider both carcinogenic and non-carcinogenic risk assessments. In 2010, a further study about the PBTK modeling in investigating acrylamide and glycidamide in humans and rats was reported (Sweeney et al., 2010). Based on the results of human volunteers consumed known does of acrylamide, the kinetic parameters for human model were estimated with the study of the urinary metabolites of acrylamide, and the levels of hemoglobin adducts of acrylamide and glycidamide were also

determined. The simulations conducted with the rat and human models indicated that rats and humans ingesting comparable levels of acrylamide (in mg/kg day) would have similar levels of glycidamide in blood and tissues. These studies showed the applications of toxicokinetics and metabolisms approaches in chemical compounds related with food safety concerns, and similar approaches could also be used in the studies about 3-MCPD esters.

Just like acrylamide, 3-MCPD esters are also a group of potential food processing-induced toxic compounds. To elucidate the possible toxic mechanisms in vivo, the kinetics and metabolisms conditions about 3-MCPD esters should also be researched. The fact is that only few studies reported the metabolism and metabonomic of 3-MCPD and its fatty acid esters in vivo and their metabonomic results in the rat urine and the cultured cells. In 2013, a toxicological and metabonomics study that treated Wistar rats with 3-MCPD di-ester for 90 days was reported (Li et al., 2013). Metabonomic results reported five major biomarkers identified from urine samples, including indoxyl sulfate, xanthurenic acid, phenylacetylglycine, nonanedioic acid and taurine. Possible mechanisms of variations about these biomarkers were also elucidated. These metabonomic results were helpful in determining the toxic effects of 3-MCPD esters and their possible toxic mechanisms in vivo. In 2015, a qualitative and quantitative study about free 3-MCPD and its metabolite was reported (Andreoli et al., 2015). The concentration of free 3-MCPD in human urine was determined. The major metabolite of free 3-MCPD, 2,3-dihydroxypropyl mercapturic acid (DHPMA) was also detected and quantified in

human urine. Results confirmed that free 3-MCPD and DHPMA could be detected in urine samples in 67 % and 100 % of healthy subjects after consuming free 3-MCPD, respectively. In 2016, the metabolomic study of free 3-MCPD in Wistar rats was performed (Ji et al., 2016). The results indicated that the metabolism of glycine, serine and threonine, the metabolism of taurine and hypotaurine, as well as the metabolism of nicotinate and nicotinamide were three key pathways related with the metabolism of free 3-MCPD. All of these 7 potential biomarkers having a close relationship with 3-MCPD toxicity, 4 potential biomarkers were up-regulated and 3 were down-regulated, which demonstrated a down-deregulation of the glycine metabolism, taurine and nicotinate metabolism, leading to toxicological effects in rat kidney.

Till now, most of the kinetic and metabolic studies were conducted for free 3-MCPD, and the data is very limited. No systemic research about the ADME of 3-MCPD esters in vivo was reported; the metabolism of 3-MCPD esters was also unknown. Due to this fact, a systematic study about the absorption, distribution, metabolism and excretion of 3-MCPD esters in vivo is needed.

Chapter 2: Absorption, distribution, metabolism and excretion of 3-MCPD 1-monopalmitate after oral administration in rats

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2.1 Abstract

Fatty acid esters of monochloropropane 1,2-diol (3-MCPD) are processing-induced toxicants and have been detected in several food categories. This study investigated the absorption, distribution, metabolism and excretion of 3-MCPD esters in Sprague Dawley (SD) rats using 3-MCPD 1-monopalmitate as the probe compound. The kinetics of 3-MCPD 1-monopalmitate in plasma was investigated using (SD) rats, and the results indicated that 3-MCPD 1-monopalmitate can be absorbed directly in vivo and metabolized. Its primary metabolites in the liver, kidney, testis, brain, plasma and urine were tentatively identified and relatively quantified at 6, 12, 24 and 48 hours after oral administration. Understanding the metabolism of 3-MCPD esters in vivo is critical for assessing their toxicities.

2.2 Introduction

Fatty acid esters of 3-monochloropropane 1,2-diol (3-MCPD esters), a group of chemical toxicants for kidney and testis, could be formed during oil refining process and have been detected in many food categories including infant and baby foods (Svejkovska, Dolezal, Velisek, 2006; Zelinkova et al., 2006; Divinova, Dolezal, Velisek, 2007; Seefelder et al., 2008; Weisshaar, 2008; Zelinkova, Dolezal, Velisek, 2009; Zelinkova et al., 2008). In 2004, 3-MCPD ester was first reported together with free 3-MCPD in the processed foods, with the esters form in a much higher concentration, especially in oils and fats as well as the fried foods (Svejkovska et al., 2004). In 2013, the European Food Safety Authority estimated a tolerable daily value (TDI) of 2 μg/kg body weight for the total amount of free 3-MCPD (EFSA, 2013).

Recent research has investigated the detection methods, the possible formation mechanisms, and the toxicology of 3-MCPD esters in vivo and in vitro (Liu et al., 2012; Zhang et al., 2013; Zhang et al., 2015; Liu et al., 2016). In 2015, Sawada and colleagues reported an overview of proteomic changes caused by 3-MCPD di-esters and free 3-MCPD in rat testis in an early phase of organ impairment (Sawada et al., 2015). Onami and colleagues reported the potential subchronic toxicity of two 3-MCPD di-esters and one 3-MCPD mono-ester to the rat kidneys and epididymis after a repeated dose study for 13 weeks (Onami et al., 2014). Our group also reported the acute toxicity of several 3-MCPD mono- and di-esters in mice and their cytotoxicity in rat kidney cells (Liu et al., 2012). The results indicated that 3-MCPD mono-ester might have stronger toxicity compared with di-esters. To our best knowledge, there is little information about the

absorption, distribution, metabolism and excretion (ADME) of 3-MCPD esters, although these data are important to access the risk of 3-MCPD esters intake.

Absorption, distribution, metabolism and excretion (ADME) may alter the pharmacokinetics of food components, and consequently influence their beneficial or toxicological activities at a selected organ or tissue. Only few studies reported the metabolism and metabonomic of 3-MCPD and its fatty acid esters in vivo and their metabonomic results in the rat urine and the cultured cells (Li et al., 2013; Buhrke et al., 2015; Andreoli, Cirlini, Mutti, 2015; Ji et al., 2016). In 2013, Li and colleagues reported that indoxyl sulfate, xanthurenic acid, phenylacetylglycine, nonanedioic acid and taurine can be used as biomarkers and represent the consumption of 3-MCPD esters in a long-term toxicity study (Li et al., 2013). In 2015, Andreoli and colleagues reported that 2,3-dihydroxypropyl mercapturic acid (DHPMA) was detected and identified as the metabolite of free 3-MCPD in human urine (Andreoli et al., 2015). In 2016, the metabolomic study of free 3-MCPD in Wistar rats was evaluated; the results indicated that the metabolism of glycine, serine and threonine, as well as the metabolism of taurine and hypotaurine, were two key pathways related with the 3-MCPD (Ji et al., 2016). Till now, no systemic research about the ADME situation of 3-MCPD esters in vivo was reported. Due to this fact, a systematic study about the absorption, distribution, metabolism and excretion of 3-MCPD esters in vivo was performed.

In this study, the absorption of 3-MCPD 1-monopalmitate in rat plasma was quantified at different time points after oral administration of 3-MCPD 1-monopalmitate to rats. The relative concentrations of 3-MCPD 1-monopalmitate in different organs and tissues as well as plasma and urine samples were also determined. In addition, major metabolites of

3-MCPD 1-monopalmitate in rat liver, kidney, testis, brain, plasma and urine were tentatively identified and relatively quantified. The results of this study suggested how 3-MCPD 1-monopalmitate might be excreted from rats for the first time. These results could significantly advance our understanding about the physiological and toxicological properties of 3-MCPD esters.

2.3 Materials and Methods

2.3.1 Chemicals and reagents.

3-MCPD 1-monopalmitate was synthesized according to a previously reported protocol (Liu, Huang, Wang, et.al., 2016) and its purity was above 98% tested by UPLC-Q-TOF MS analysis. LC-MS grade isopropanol and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS grade water was obtained from a Milli-Q 10 ultra-pure water system (Billerica, MA, USA). All the other chemical reagents were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemical reagents and solvents were used without further purification.

2.3.2 Animals, treatment and sample collection.

The animal study protocols were approved by the Animal Ethics Committee of the Shanghai Jiao Tong University. Male Sprague-Dawley rats, weighing 160-180 g, were bought from SLAC experimental animal Co. Ltd (Shanghai, China). The rats were maintained in a temperature controlled room with a 12 h light/dark cycle, and were allowed free access to drinking water and diet. The animals were acclimatized to the

facilities for a week, and then fasted with free access to water for 12 h prior to experiment.

Animals were divided into ten groups randomly, and each group had six rats. Two groups were kept in regular cages and treated with 400 mg/kg body weight of 3-MCPD 1-monopalmitate or pure olive oil vehicle, and 300 μL of blood sample was collected in heparinized eppendorf tube via the oculi chorioideae vein before dosing and subsequently at 0, 0.167, 0.33, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, and 24 h after the treatment for the kinetic study. Another four groups were kept in metabolic cages, treated with 400 mg/kg body weight of 3-MCPD 1-monopalmitate, and sacrificed to collect the liver, kidney, testis, brain, plasma and urine samples at 6, 12, 24 and 48 hours after treatment. The four control groups were sacrificed at 6, 12, 24 and 48 hours after oral administration of same volume pure olive oil. Water and food were fed ad libitum 4 hours after treatment during the experiment.

2.3.3 Animal samples.

The blood samples were centrifuged at 4000 rpm for 10 minutes at 4 °C, and 100 μL of supernatant plasma was accurately collected and immediately frozen at -80 °C until further analysis. 300 μL of acetonitrile was added to each 100 μL of the plasma sample. The mixture was vortexed for 30 s, and followed by centrifugation at 10000 rpm for 10 minutes at 4 °C. The supernatant was transferred into a UPLC vial for UPLC-QQQ-MS analysis.

The liver, kidney, testis and brain samples were removed, weighed, flushed in ice-cold saline (0.9% NaCl), and homogenized at 4 °C in 9 times weight ice-cold saline to

make 10% tissue homogenate. The homogenate was stored at -80 °C, and centrifuged at 10000 rpm at 4 °C for 10 min to obtain the supernatant for UPLC-Q-TOF-MS analysis.

The urine samples were collected in metabolic cages at different time points. For example, urine samples at 12 hour indicated the urine collected between 6 hour and 12 hour. 0.2 mL of each urine sample was extracted with 1 mL of water-saturated ethyl acetate by vortexing for 30 s and followed by centrifugation at 10000 rpm for 10 min at ambient temperature. The supernatant was transferred into a clean tube and the solvent was removed using a nitrogen evaporator. The residue was re-dissolved in 0.2 mL of methanol and subjected to UPLC-Q-TOF-MS analysis.

2.3.4 UPLC-MS conditions.

A Waters Acuity UPLC-TQS triple quadrupole MS system (Waters, Milford, MA, USA) was selected for quantitatively analysis of 3-MCPD 1-monopalmitate in rat plasma samples with a Waters Phenyl column (2.1 mm i.d. × 100 mm, 1.7 μm). The mobile phase consisted of A) water/methanol (9:1, v/v) and B) methanol/ isopropanol (4:1, v/v) using the elution gradient started with 0% phase B, changed linearly to 35% in 4 min, increased linearly to 95% B at 8 min and maintained for 2 min, and returned to its initial condition for 2 min to re-equilibrate the column for the next injection. The flow rate was 0.4 mL/min with an injection volume of 2 μL. The MS detector conditions were: capillary voltage 3.50 kV; sampling cone voltage 60 V; extraction cone voltage 4.0 V; source temperature 120 °C; and desolvation temperature 450 °C. The flow rates were 150 L/h for cone gas and were 800 L/h for the desolvation gas. An ion pair with a parent ion at m/z 371.2329 ($[M+Na]^+$) and a daughter ion at m/z 110.0135 was selected to create a multiple

reaction monitoring (MRM) system with the collision energy of 35 eV and a mass range from 50 to 1000 *m/z* in an ESI positive mode.

2.3.5 Method evaluation.

Different mobile phases, including different ratios of methanol/water, acetonitrile/water and methanol/acetonitrile/isopropanol/water, were tested in the preliminary study to select an appropriate mobile phase. Finally, mobile phase with A) water/methanol (9:1, v/v) and B) methanol/isopropanol (4:1, v/v) with gradient elution was utilized in this study. The limit of detection (LOD) and limit of quantification (LOQ) were tested by adding different concentrations of 3-MCPD 1-monopalmitate into blank rat plasma samples. The LOD was determined in the relative standard deviation (RSD) less than 20% and accuracy (average concentration deviated from the formulated concentration) to less than ± 20% of the measured results. Every sample was tested in triplicate. The LOQ was determined in the signal/noise (S/N) = 10, and the LOQ in this study was 2 ng/mL of 3-MCPD 1-monopalmitate. The standard curve showed linear range between 10 to 1000 ng/mL determined by 7 different concentrations of 3-MCPD 1-palmitate with a r^2 value of 0.998.

Recovery was determined by adding low (30 ng/mL), medium (300 ng/mL) and high (1000 ng/mL) concentrations of 3-MCPD 1-monopalmitate into blank rats' plasma samples, and prepared them following the same protocol for quantification. Each concentration was examined in triplicate. The results indicated that the recovery of 3-MCPD 1-monopalmitate in plasma samples was 82.55 to 104.63%.

2.3.6 Kinetic data analysis and statistical evaluation.

Pharmacokinetic analysis was performed using 3P97® software (Practical Pharmacokinetic Program, Beijing, China). Several kinetic parameters such as the maximum concentration (C_{max}), time to maximum concentration (t_{max}), mean residence time (MRT), and area under the concentration–time curve (AUC) was determined. C_{max} and t_{max} were obtained directly from the plasma concentration–time data and AUC was calculated by the trapezoidal rule.

Results were expressed as mean ± standard deviation (S.D.). Statistics were analyzed using the SPSS for Windows (version rel. 10.0.5, 1999, SPSS Inc., Chicago, IL).

2.4 Results and Discussion

2.4.1 Kinetics of 3-MCPD 1-monopalmitate in the plasma.

The kinetics of 3-MCPD 1-monopalmitate in rat plasma after oral administration was evaluated for the first time. The greatest concentration of 3-MCPD 1-monopalmitate in the plasma was 873.72 ng/mL (C_{max}) at about 1.67 hours (T_{max}) after oral administration (**Table 1**), and no free 3-MCPD was detected in the plasma samples at any tested time points under the experimental conditions. A previous study reported the existence of high level of free 3-MCPD in the blood of Wistar rats using indirect GC-MS determination of its heptafluorobutyric acid derivatives (Abraham et al., 2013). Different results between this study and the present study might because of different types and age of rats utilized in these two studies, the amount of blood samples used to prepare the

analytical samples, and the limitation of detecting free 3-MCPD using LC-MS. Plasma 3-MCPD 1-monopalmitate concentration reduced to half after 3.42 hours ($t_{1/2}$). No 3-MCPD 1-monopalmitate could be detected after 4 hours, which was its mean resident time (MRT). The area under curve (AUC) for 3-MCPD 1-monopalmitate in rat plasma was 1676.15 h.ng/mL, which represented the maximum amount of 3-MCPD 1-monopalmitate absorbed into plasma under the testing conditions. These results indicated that 3-MCPD 1-monopalmitate could be absorbed and involved in body circulation in vivo in its ester form, and could be eliminated from the circulation system in 4 hours possibly through metabolism and/or elimination or tissue distribution mechanisms. To the best of our knowledge, this is the first report on 3-MCPD 1-monopalmitate kinetics in an animal model.

2.4.2 3-MCPD 1-monopalmitate concentrations in rats' organs and tissues.

The relative concentrations of 3-MCPD 1-monopalmitate in the liver, kidney, testis and brain of rats were also evaluated at 6, 12, 24 and 48 hours after administration in this study. Unfortunately the matrix effects of different organs and tissues made it difficult to measure the concentration of 3-MCPD 1-monopalmitate in each organs and tissues by UPLC QQQ-MS, and UPLC QTOF-MS was used. 3-MCPD 1-monopalmitate could be detected only in the liver sample collected 6 h after administration, and the average absolute peak area was 90.88. This observation could also be partially due to the limitation in the quantitative resolution of UPLC QTOF-MS. All these data that 3-MCPD 1-monopalmitate was existed in vivo with its original form raised a question whether and how 3-MCPD monoester might be delivered into and be metabolized locally in each rat organ or tissue.

Table 1. Absorption of 3-MCPD 1-monopalmitate in rats

		Animal ID							
		1	2	3	4	5	6	mean	sd
C_{max}	ng/mL	814.74	887.47	680.83	752.69	988.05	1118.55	873.72	160.33
T_{max}	h	2.50	1.50	1.50	1.50	1.50	1.50	1.67	0.41
$t_{1/2}$	h	3.50	3.50	4.00	3.00	3.50	3.00	3.42	0.38
MRT	h	4.00	4.00	4.00	4.00	4.00	4.00	4.00	0.00
AUC 0-∞	h.ng/mL	2119.80	2000.66	1201.04	1433.48	1472.26	1829.66	1676.15	361.08

C_{max} is the maximum concentration of 3-MCPD 1-monopalmitate in rats' plasma; T_{max} is the time of reach the C_{max} after oral administration to rats; t1/2 is the time that the concentration of 3-MCPD 1-monopalmitate reduced to half of C_{max}; MRT is the abbreviation of mean residence time, indicate the average time of 3-MCPD 1-monopalmitate stays in the body; AUC is the abbreviation of area under the curve, represent the total amount of 3-MCPD 1-monopalmitate stay in the body.

2.4.4 Tissue distribution of 3-MCPD 1-monopalmitate metabolites.

After tentatively identified the eight metabolites, the distribution of these metabolites in different tissues and body fluids were examined. Acetylated 3-MCPD was detected in liver, kidney, testis, urine and brain samples, but not in the plasma samples. Glucuronide conjugated 3-MCPD was detected in urine and plasma samples. Free 3-MCPD, sulfate conjugated 3-MCPD and glycine conjugated 3-MCPD was only detected in urine samples. Hydroxylated and glucuronide conjugated 3-MCPD, cysteine conjugated 3-MCPD and taurine conjugated 3-MCPD was only detected in plasma samples. Acetylated 3-MCPD is the most widely distributed metabolite in the rats after oral administration 3-MCPD 1-monopalmitate. It presented in all the four tested tissues, including liver, kidney, testis and brain, suggesting its potential to pass through the blood-brain barrier. It was also detected in the urine samples, indicating that this metabolite might be one excreting form of 3-MCPD 1-monopalmitate and possibly other 3-MCPD esters. Glucuronide conjugated, sulfate conjugated, glycine conjugated and free 3-MCPD was also detected in urine samples, and might be other possible excreting forms for 3-MCPD 1-monopalmitate and other esters. Glucuronide conjugated, hydroxylated and glucuronide conjugated, cysteine conjugated and taurine conjugated 3-MCPD was detected in plasma, suggesting that glucuronide and amino acid addition might be two important ways to distribute 3-MCPD 1-monopalmitate into blood.

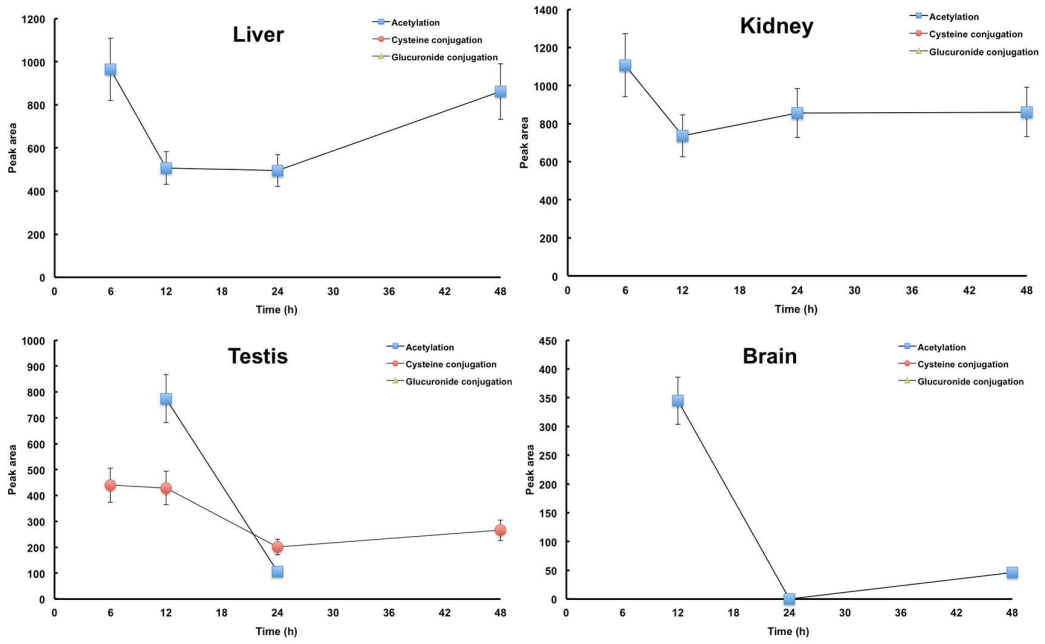

Figure 2.1. Relative concentration of three typical metabolites, acetylated, cysteine conjugated and glucuronide conjugated metabolites in different tissues in different time point.

In addition, the existences of these metabolites in the selected tissue and fluid samples were also evaluated at different time points. Acetylated 3-MCPD was detected at all the four time points (4, 12, 24 and 48 h) in liver and kidney samples, at 12 and 24 hours in the testis and urine samples after oral administration of 3-MCPD 1-monopalmitate, respectively. Free 3-MCPD, glycine conjugated and sulfate conjugated 3-MCPD was only detected at 12-hour urine samples. Glucuronide conjugated, hydroxylated and glucuronide conjugated and taurine conjugated 3-

MCPD was detected in 6-hour plasma samples, whereas cysteine conjugated 3-MCPD presented in the plasma samples at all the four tested time points.

Besides, the relative amounts of three important metabolites were also quantified based on their peak areas in UPLC-MS spectrums. The peak areas of three major metabolites, including acetylation, cysteine conjugation and glucuronide conjugation metabolites, in each tissue samples at each time point were presented in **Figure 2.1**. The data indicated that the peak areas of these metabolites are different in tissue samples at different time points. The acetylated 3-MCPD showed the greatest concentration in liver and kidney at all the four tested time points. It also showed greatest concentration in testis as well as in urine samples at 12 hours and 24 hours after oral administration of 3-MCPD 1-monopalmitate. Besides, it showed greatest concentration in the12-hour brain samples, and a relatively lower amount in the 48-hour brain samples. No acetylated 3-MCPD was detected in plasma samples.

Cysteine conjugated 3-MCPD is detected only in testis samples, and its concentration decreased from 6-hour testis samples to 48-hour samples. Beside those two metabolites we mentioned above, all the other identified metabolites were existed primarily in the urine samples, especially in the 12-hour and 24-hour urine samples. Trace amount of free 3-MCPD was detected only in 24 h urine samples. These results indicated that 3-MCPD 1-monopalmitate could be absorbed and metabolized in vivo.

In summary, this study represents the absorption, distribution, metabolism and excretion of 3-MCPD 1-monopalmitate in rats. The toxicokinetic factors were clarified, 8 chlorine-related metabolites were tentatively identified and relatively quantified based on their peak areas in the major tissues, plasma and urine in four different time points, and the major excretion form of 3-MCPD 1-monopalmitate was also detected as its phase II metabolites. Besides, there are still large amount of metabolites remain in tissues 48 hours after oral administration of 3-MCPD 1-monopalmitate to rats, such as the acetylated 3-MCPD in liver and kidney, and the cysteine conjugated 3-MCPD in testis. Suppose that people consume foods contained 3-MCPD esters everyday, these metabolites might be accumulated in the organs/tissues. Therefore, the toxicity effects of these metabolites should also be investigated. The present study might advance our understanding of the metabolism process of 3-MCPD esters and provide a base for further studies to have a better understanding about the toxicity effects and mechanisms of 3-MCPD esters in vivo.

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University 985-III disciplines platform and talent funds (Grant Nos. TS0414115001; TS0320215001).

Chapter 3: Metabolites identification after oral administration of 3-MCPD 1-monopalmitate to rats.

3.1 Abstract

Esters of mono-chloropropane 1,2-diol (3-MCPD) are a group of potential food source toxins formed during food processing. The toxic effects, especially the nephrotoxicity and hepatotoxicity of 3-MCPD esters were widely reported in the past decades. To improve the understanding about the possible toxicology effects and mechanism of 3-MCPD esters, it is important to clarify their metabolic conditions, including the metabolites and metabolism pathways in vivo. In this study, 3-MCPD 1-monopalmitate was selected as a representative and oral administration to Sprague Dawley (SD) rats. The possible metabolites not through free 3-MCPD mid-metabolite were identified in the liver, kidney, testis, brain, plasma and urine samples at 6, 12, 24, and 48 hours. A total of 39 metabolites were identified, including 8 metabolites related with the free 3-MCPD intermediates, and 31 metabolites not related with that intermediates. The identification of these metabolites could improve our knowledge about the metabolic conditions and toxic effects of 3-MCPD esters in the future.

3.2 Introduction

Fatty acid esters of 3-Monochloropropane 1,2-diol (3-MCPD) are a group of chemical compounds formed during the food processing. They were considered as potential food toxins and being researched in the past decades. In 2004, 3-MCPD esters was firstly reported together with the free 3-MCPD in processed foods, and the esters form of 3-MCPD have a much higher concentrations than their free form, especially in the vegetable oils and fats as well as in fried foods (Hamlet et al., 2004; Svejkovska et al., 2004). These results about 3-MCPD esters made them an important food safety concern, since the esters of 3-MCPD might hydrolyze to a known food toxin, free 3-MCPD. In this condition, even the concentration of free 3-MCPD in the food might be lower than the tolerable level, the esters form of 3-MCPD that people consumed could be metabolized to free 3-MCPD or other toxins in vivo and exceed the tolerable level currently established. To avoid this situation and improve the understanding about 3-MCPD esters, it is necessary and important to clarify the toxicity effect of 3-MCPD esters in vivo.

In these years, increasing studies focus on the toxicology effects of 3-MCPD esters both in vitro and in vivo. Sawada and colleagues reported an overview of proteomic changes induced by 3-MCPD di-esters and free 3-MCPD in rat testis at the early phase of organ impairment (Sawada et al., 2015). In 2015, Onami and colleagues reported the potential subchronic toxicity of two 3-MCPD di-esters and one 3-MCPD mono-ester to the rat kidneys and epididymis after a 13-week repeated dose study (Onami et al., 2015). Our group also reported an acute toxicity study of 3-MCPD mono- and di-esters in mice and their cytotoxicity in rat kidney cells, and the results indicated that 3-MCPD mono-ester represented greater toxicity compared with di-ester (Liu et al., 2012). In 2016, our group reported tubular cell apoptosis in vivo

via JNK/p53 pathway induced by 3-MCPD 1-palmitate (Liu et al., 2016). All these results proved that 3-MCPD esters were potential toxins, and the toxic working mechanisms still need to be investigated in the further study. Based on the biological central dogma and principal of pharmacokinetics, the bioactivity/toxic materials induced a residue-by-residue transfer of sequential information from DNA to RNA, RNA to protein, then from protein to metabolites, and finally the metabolites represent bioactivity/toxic effects in vivo (Nicholson et al., 1999). To better understanding the toxic effects and mechanisms of 3-MCPD esters, it is important to clarify their absorption, distribution, metabolism and excretion conditions in vivo. Recently, our group reported the kinetic study about the absorption of 3-MCPD 1-monopalmitate in SD rats, and identified 8 chloride-related metabolites in different tissues of rats. The results in this study indicated that with free 3-MCPD as the mid-metabolite, 3-MCPD 1-monopalmitate could be metabolized to a group of chloride-related metabolites and excreted out of body. Based on the results above, further questions about the metabolism of 3-MCPD esters raised up: except for the chloride-related metabolites, whether there are some other types of metabolites existed in vivo, and what are the metabolic pathways of these metabolites in vivo. To solve these problems, the further research about the metabolism of 3-MCPD 1-monopalmitate in vivo was designed and processed in this study.

In this study, 3-MCPD 1-monopalmitate was oral administration to SD rats, tissues including liver, kidney, testis, brain, as well as the plasma and urine were collected and analyzed in different time points. All the possible metabolites that not through free 3-MCPD mid-metabolite were identified using UPLC-QTOF MS system. The results in this study can greatly enhance our understanding about the metabolism

situation of 3-MCPD esters in vivo and promote further research about the toxicity study of 3-MCPD esters.

3.3 Materials and Methods

3.3.1 Chemicals and reagents.

3-MCPD 1-monopalmitate was synthesized based on our previous method (Liu, Huang, Wang, Sun, Yu, 2016), and the purity of final product was greater than 98% verified by UPLC-QTOF MS. LC-MS grade isopropanol and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS grade water was purified from a Milli-Q 10 ultra-pure water system (Billerica, MA, USA). All the other chemical reagents were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.3.2 Animals, treatment and sample collection.

The animal studies were approved by the Animal Ethics Committee of Shanghai Jiao Tong University. Male Sprague-Dawley rats were purchased from SLAC experimental animal Co. Ltd (Shanghai, China) with the body weight between 160 and 180 grams. The rats were maintained in a room with controlled temperature and 12-hour light/dark cycle, and were allowed free access to regular diet and water. The animals were acclimatized to the environment for one week, and then fasted with free access to the water for 12 hours before the experiment.

The experimental design was described in our previous study. Briefly, rats were separated into eight groups randomly, and each group has six rats. Four groups were kept in metabolic cages, treated with 400 mg/kg body weight of 3-MCPD 1-monopalmitate, then sacrificed to collected the liver, kidney, testis, brain, plasma and urine at 6, 12, 24 and 48 hours after oral administration. Another four groups were set as control groups, oral administration of pure olive oil, and sacrificed at similar four time points as those four test groups. The water and food were fed ad libitum 4 hours after treatment during the experiment.

3.3.3 Animal samples preparation.

300 μL of blood samples were collected and centrifuged at 4000 rpm for 10 minutes at 4 °C, and 100 μL of supernatant plasma was accurately transferred and immediately frozen at -80 °C until further analysis. 300 μL of acetonitrile was added to each 100 μL of plasma sample, vortex for 30 s, followed by centrifugation at 10000 rpm for 10 minutes at 4 °C. The supernatant was transferred into a UPLC vial for further analysis.

The liver, kidney, testis and brain samples were removed, weighed flushed in ice-cold saline (0.9% NaCl), and homogenized at 4 °C in 9 times weight ice-cold saline to make 10% tissue homogenate and stored at -80 °C before use. The protein in homogenates was precipitated with acetonitrile with the similar method as plasma processing, and the supernatant was collected for further analysis.

The urine samples were collected in metabolic cages at different time points. For example, urine samples at 24 hour represent the urine collected between 12 hour and 24 hours. After collection, 0.2 mL of urine sample was extracted with 1 mL of water-saturated ethyl acetate, vortex for 30 s, centrifuged at 10000 rpm for 10 min at ambient, transfer the supernatant and removes all the solvent using nitrogen evaporator. The residue was dissolved with 0.2 mL of methanol and injected to UPLC-QTOF-MS for further analysis.

3.3.4 UPLC-MS conditions.

A Waters Acuity UPLC-Xevo G2 QTOF MS system (Waters, Milford, MA, USA) was selected for metabolite identification in this study with a Waters Phenyl column (2.1 mm i.d. × 100 mm, 1.7 μm). The mobile phase consisted of A) water/methanol (9:1, v/v) and B) methanol/ isopropanol (4:1, v/v) using the elution gradient started with 0% phase B, changed linearly to 35% in 4 min, increased linearly to 95% B at 8 min and maintained for 2 min, and returned to its initial conditions for 2 min to re-equilibrate the column for the next injection. The flow rate was 0.4 mL/min with an injection volume of 2 μL. For MS detector conditions, capillary voltage 3.00 kV; sampling cone voltage 60 V; extraction cone voltage 4.0 V; source temperature 120 °C; and desolvation temperature 450 °C. The cone gas flow rate was 150 L/h and the desolvation gas was 800 L/h. A MS^E method was used with mass range from 50 to 1200 m/z in both ESI positive and ESI negative mode, scan time was 0.3 s and the ramp collision energy was 20-35 eV.

3.3.5 Metabolynx software conditions.

Metabolic software Metabolynx XS (Waters, Milford, MA, USA) was utilized to analyze the possible metabolites in this study. Both phase I and phase II metabolites were processed and analyzed, with mass defect filter tolerance less than 20 mDa. Mass find mode was most intense mode, with the relative intensity threshold at 5 %. Dealkylation tool was utilized with the mass cut-off molecular weight at 100 Da. Metabolites were identified based on the mass matching with control samples in the threshold of mass window less than 0.05 Da and retention time no more than 0.2 min.

3.4 Results and Discussion

Four important tissues, including liver, kidney, testis and brain, as well as plasma and urine samples were collected after oral administration of 3-MCPD 1-monopalmitate to rats. The metabolic results in these tissues/organs, plasma and urine samples can represent the total metabolism conditions in vivo. Liver is the most important metabolic organ, most of the metabolism reacted in liver catalyzed by numerous enzymes in liver (Aarsaether et al., 1990; Akhtar, 1983; Cheng et al., 2012). Kidney and testis were reported as two major tissues related to the toxicity effects of 3-MCPD esters in previous results (Onami et al., 2015; Onami et al., 2014; Li et al., 2010). Brain was selected to investigate the possibility of original or metabolites of 3-MCPD 1-monopalmitate to transfer through the blood-brain barrier. Blood plasma is the most important body fluid and can represent the whole environment condition in

vivo, while urine is one of the most widely used biological samples in studying the excretion of metabolites. Liver, kidney, testis, brain and blood were collected on sacrificed rats on different time points, while urine samples were collected using the metabolic cage.

3.4.1 Identification of chlorine-related 3-MCPD 1-monopalmitate metabolites in rats' tissues, plasma and urine.

Liver, kidney, testis and brain, as well as plasma and urine of rats were collected at 6, 12, 24 and 48 hours after oral administration of 3-MCPD 1-monopalmitate. Liver is the most important metabolic organ, whereas kidney and testis have been reported as the targets for the toxic effects of 3-MCPD esters (Liu et al., 2012; Onami et al., 2014; Onami et al., 2015). Brain was selected because the possible nerve toxicity of 3-MCPD esters observed in our previous studies (Liu et al., 2012), and it is important to investigate whether original form or metabolites of 3-MCPD 1-monopalmitate could pass through the blood-brain barrier. Blood plasma is the most important body fluid and could reflect in vivo environment, while urine is one of the most widely used biological samples in studying the excretion of metabolites. Liver, kidney, testis, brain and blood were collected from the sacrificed rats at the selected time points, while urine samples were collected using the metabolic cage.

The chlorine-related metabolites were recognized as the most important metabolites, and were firstly identified using UPLC Q-TOF MS and Waters Masslynx 4.1 and Waters MetaboLynx XS softwares. A total of 8 metabolites were tentatively

identified based on their accurate formula masses and fragment ion patterns. Major metabolites including 3-MCPD, sulfonated 3-MCPD, acetylated 3-MCPD, glucuronide 3-MCPD, and 3-MCPD bonded with different amino acids (**Figure 3.1**). Besides, some metabolites were also detected and speculate as multi-hydroxylated 3-MCPD and methylated-hydroxylated 3-MCPD, and their structures were not reported in the **Figure 3.1**.

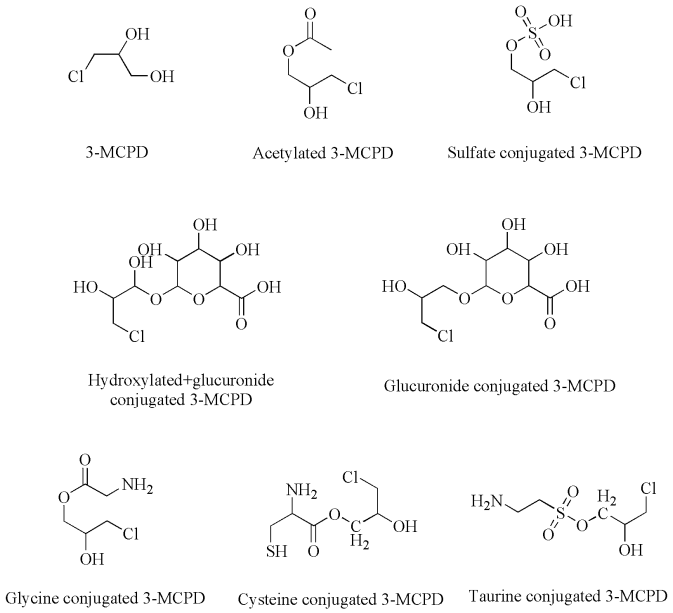


Figure 3.1 Chemical structures of metabolites.

The acetylated metabolite was selected as the example to demonstrate how those metabolites were characterized. The total ion chromatography of a typical LC-MS chromatography of a liver sample and the extracted ion chromatography of acetylated metabolites were showed in **Figure 3.2A** and **3.2B**, respectively. The MS1 (parent ion) and MS2 (fragment ion) mass spectra of the acetylated metabolite with a retention time of 1.13 min (**Figure 3.2B**) were obtained (**Figure 3.3A** and **3.3B**). In

the MS1 spectrum, the molecular ion peak of the metabolite was 151.0258 $[M-H]^-$, so the molecular formula could be calculated as $C_5H_9ClO_3$. In the MS2 spectrum, the major fragment ion peak was 135.0306 [M-OH], combined with the information supplied from MetaboLynx XS, the structure could be identified as the acetylated 3-MCPD, the major peak at m/z 135.0306 in the MS2 could be the structure obtained by eliminating a sn-2 hydroxyl group from 3-MCPD. It is understandable that the acetylation could also be at the sn-2 position. The site for acetylation of 3-MCPD was finally proposed as the sn-1 position due to the relatively lower steric hindrance of this structure.

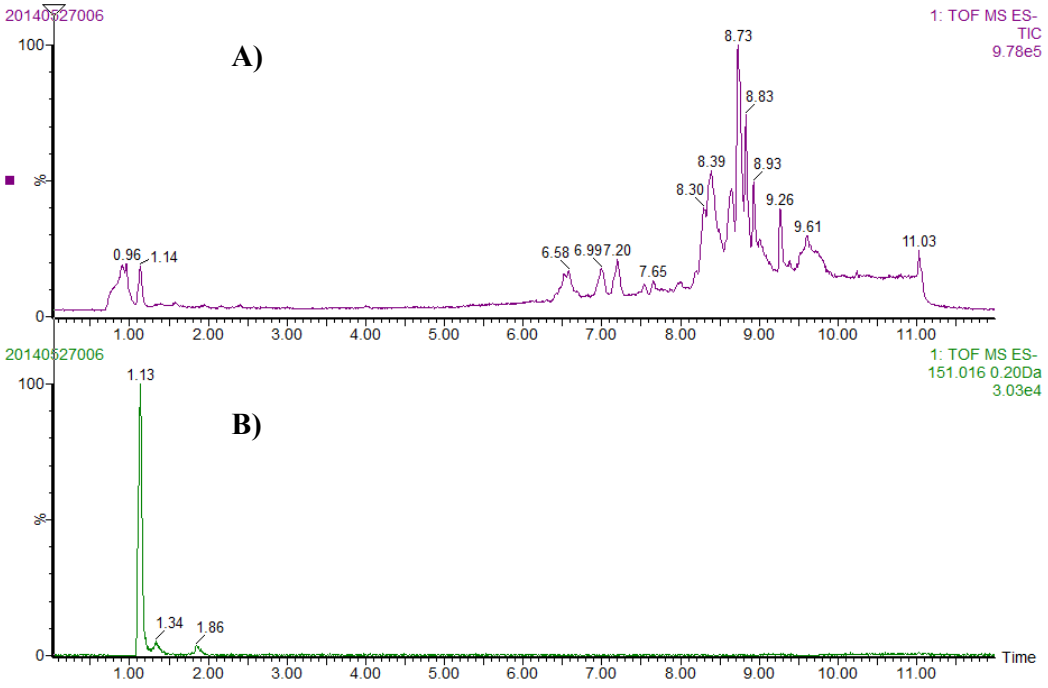


Figure 3.2 Typical UPLC-QTOF-MS A) total ion chromatogram (TIC) of rat liver sample extracts and B) extract ion chromatogram for the acetylated metabolite after oral administration of 3-MCPD 1-monopalmitate to rats.

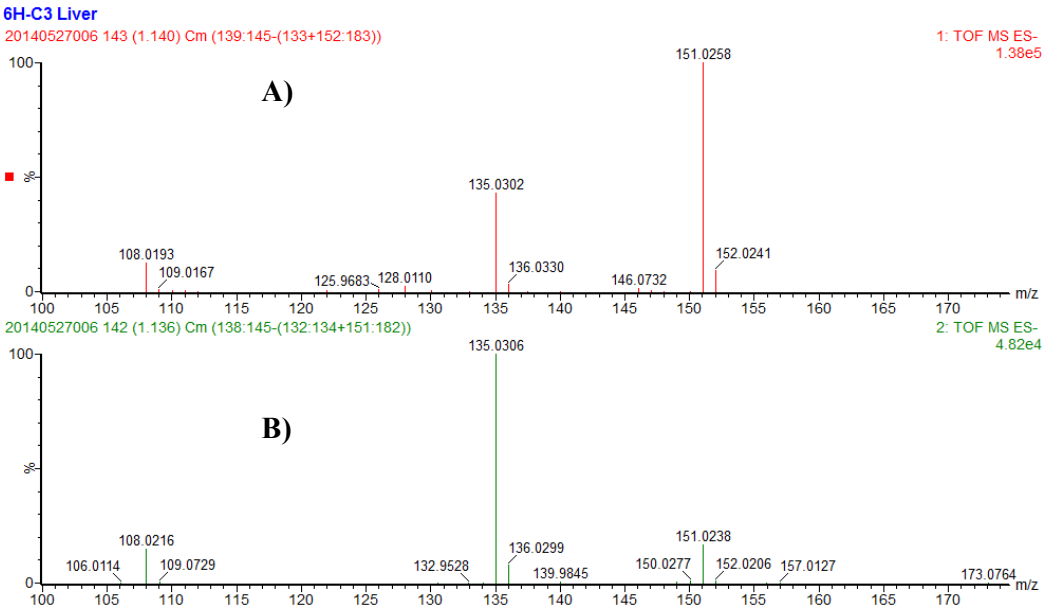


Figure 3.3 MS spectra of the acetylated metabolite. A) MS1 spectrum and B) MS2 spectrum.

A previous study reported that free 3-MCPD could be detected in the serum 30 min after oral administration of 3-MCPD esters to F344 rats (Onami et al., 2015). Glycidol was also detected in rats serum after oral administration of either glycidol or 3-MCPD di-esters. These two metabolites were not detected in plasma samples in this study at all tested time points. Only trace amount of free 3-MCPD was detected in urine samples at 24 hours after oral administration of 3-MPCD 1-monopalmitate to SD rats. No glycidol was detected in any tissue or urine samples in this study. In brief, this study characterized 8 metabolites in rats' tissues and/or plasma and urine samples after oral administration of 3-MCPD 1-monopalmitate for the first time.

3.4.2 Identification of other 3-MCPD 1-monopalmitate metabolites.

All the other metabolites of 3-MCPD 1-monopalmitate after oral administration to rats were identified based on the high resolution mass spectrum results and Waters Metabolynx^{TM} software (Waters, Milford, MA, USA). A total of 31 metabolites not related with the free 3-MCPD intermediates were identified in this study, these metabolites were identified using the accurate mass weight from MS1 and accurate mass fragment from MS2 combined with software. The identification processes of three typical metabolites were conducted as follow to evaluate the derivation of all the metabolites.

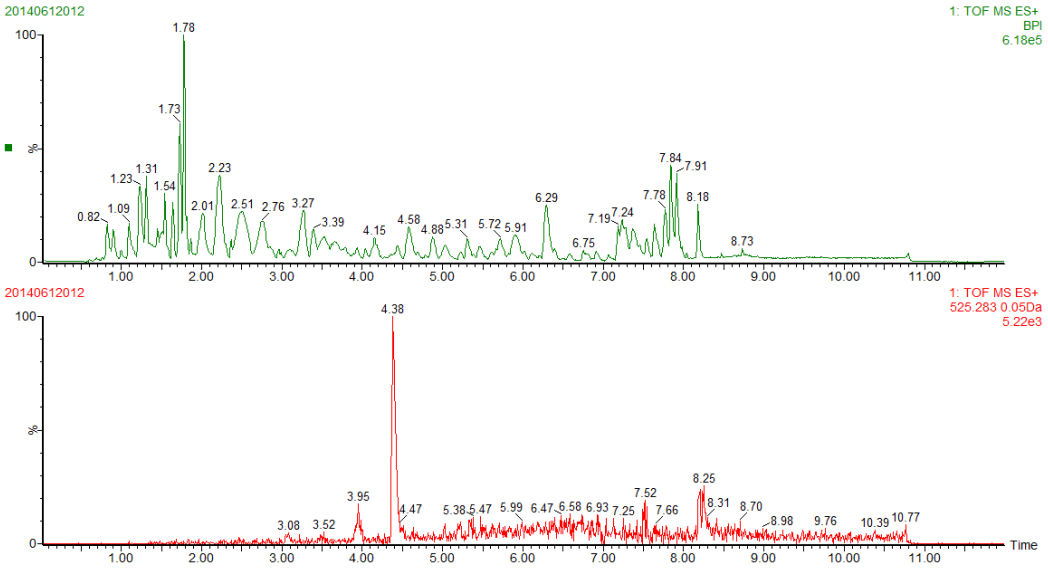


Figure 3.4 Typical UPLC-QTOF-MS A) base peak intensity (BPI) of rat urine sample extracts and B) extract ion chromatogram for the glucuronide conjugated metabolite after oral administration of 3-MCPD 1-monopalmitate to rats.

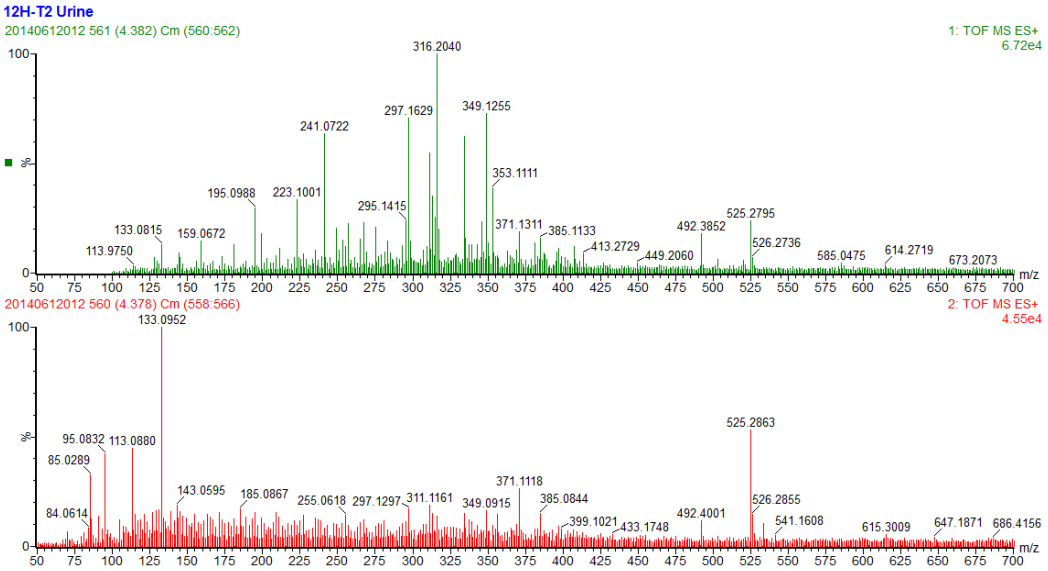
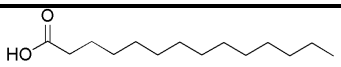
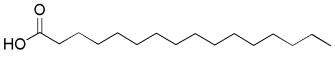
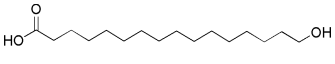
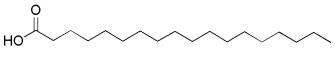
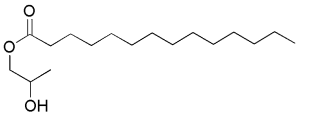
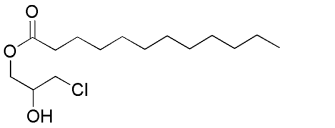
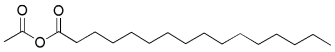
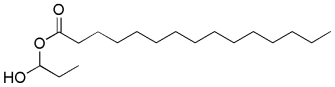
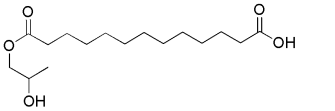
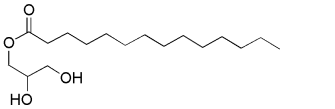
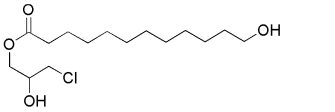
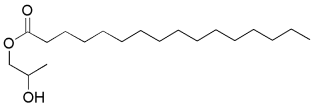
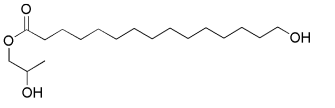
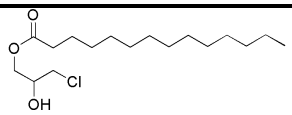
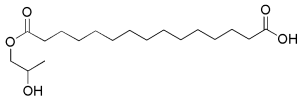
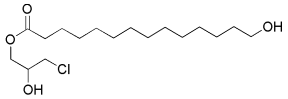
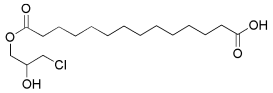
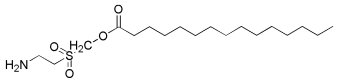
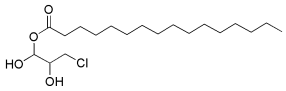
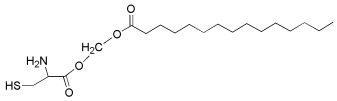
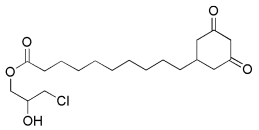
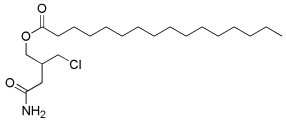
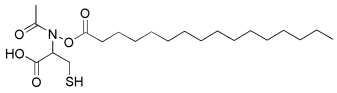
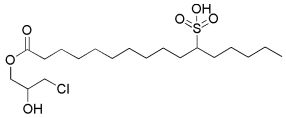
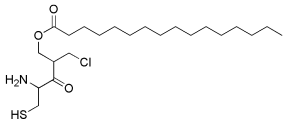
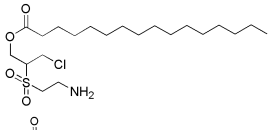
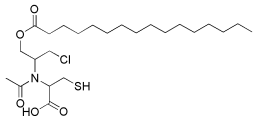
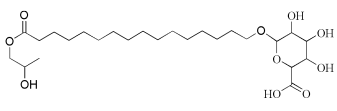


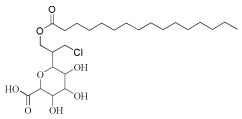
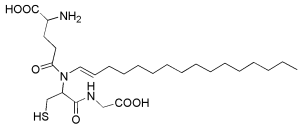
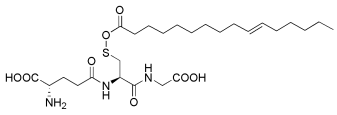
Figure 3.5 MS spectra of the glucuronide conjugated metabolite. A) MS1 spectrum and B) MS2 spectrum.

Metabolite 29 was selected as an example for characterizing a typical metabolite detected from urine sample after oral administration of 3-MCPD 1-monopalmitate. The base peak intensity chromatography and extracted ion chromatography of metabolite 29 was represented in **Figure 3.4**, and the retention time of this metabolite was 6.83 min. High accurate mass weight was the most important information to identify the possible chemical structures of metabolites. For metabolite 29, the high accurate mass spectrum of parent ion (MS1) and fragment ion (MS2) was represented in **Figure 3.5**. The $[M+H]^+$ of metabolite 29 was m/z 525.2795, so the elemental composition of this metabolite was $C_{25}H_{45}ClO_9$. Possible fragment ions, including m/z 223.1001 ([M-glucuronic acid-C_9H_{18}]), m/z 195.0988 (glucuronic acid), were detected in the mass spectrum. Combined with the identification information supplied by Waters Metabolynx^{TM} software, metabolite 29 was identified as glucuronide conjugated 3-MCPD 1-monopalmitate (**Table 2**). Glucuronide conjugation is one of the most important and widely existed metabolites both in animal and in human beings. It is the addition of glucuronic acid to the substrate involved in xenobiotic metabolism (D'Andrea et al., 2005; Hu et al., 2015; Jaggi et al., 2002). The glucuronidation is one of phase II metabolic pathways that plays important role in increasing the polar of substrate and detoxication approach. The glucuronide conjugated 3-MCPD 1-monopalmitate was existed in rats' urine samples in 12 hours after oral administration of 3-MCPD 1-monopalmitate, which indicated that this might be a possible way to excrete 3-MCPD 1-monopalmitate through urine.

Table 2. Metabolites of 3-MCPD 1-monopalmitate in rats.

No.	Metabolite	Mol. weight	Mol. formula	Structure	Existence in tissue/body fluid*
1	Deethylation-C3H5OCl (Pathway 3)	228.2089	$C_{14}H_{28}O_2$		L (6, 12), K (6), P (6), T (12), U (12), B (12, 48)
2	Parent-C3H5OCl (Pathway 3)	256.2402	$C_{16}H_{32}O_2$		L (6, 12), K (6), P (6), T (12), U (12), B (12, 48)
3	Hydroxylation-C3H5OCl (Pathway 3:)	272.2351	$C_{16}H_{32}O_3$		L (6, 12), K (6), P (6), T (12), U (12), B (12, 48)
4	Hydroxymethylene loss-Cl+H (Pathway 2)	284.2715	$C_{18}H_{36}O_2$		L (6, 12), K (6), P (6), T (12), U (12), B (12, 48)
5	Deethylation-Cl+H (Pathway 2)	286.2508	$C_{17}H_{34}O_3$		L (6, 12), K (6), P (6), T (12), U (12)
6	Tert-butyl dealkylation (Pathway 4)	292.1805	$C_{15}H_{29}ClO_3$		L (6,), K (6), P (6), T (12), U (12)
7	Acetylation-C3H5OCl (Pathway 3)	298.2508	$C_{18}H_{34}O_3$		L (6, 12), K (6), P (6), T (12), U (12), B (12)
8	Demethylation-Cl+H / 2)	300.2664	$C_{18}H_{36}O_3$		L (12), K (6), T (12), U (12), B (12)
9	Tert-butyl to acid-Cl+H (Pathway 2)	302.2093	$C_{16}H_{30}O_5$		L (6, 12), K (6), P (6), T (12), U (12)
10	Ethyl to alcohol-Cl+H (Pathway 2)	302.2457	$C_{17}H_{34}O_4$		L (12), K (6), P (6), T (12), B (12)
11	Tert-butyl to alcohol (Pathway 4)	308.1754	$C_{15}H_{29}ClO_4$		K (6), P (6), T (12), U (12)
12	Parent-Cl+H (Pathway 2)	314.2821	$C_{19}H_{38}O_3$		U (12)
13	Demethylation + hydroxylation-Cl+H (Pathway 2)	316.2614	$C_{18}H_{36}O_4$		L (6), U (12)

14	Deethylation (Pathway 4)	320.2118	$C_{17}H_{33}ClO_3$		L (6, 12), K (6), P (6), T (12), U (12), B (12)
15	Ethyl to carboxylic acid-Cl+H (Pathway 2)	330.2406	$C_{18}H_{34}O_5$		L (6, 12), K (6), P (6), T (6)
16	Ethyl to alcohol (Pathway 4)	336.2067	$C_{17}H_{33}ClO_4$		L (6), K (6), P (6), T (12), U (12)
17	Isopropyl to acid (Pathway 4)	350.186	$C_{17}H_{31}ClO_5$		P (6), T (12), U (12), B (12)
18	Taurine conjugation-C3H5OCl (Pathway 3)	363.2443	$C_{18}H_{37}NO_4S$		B (12)
19	Hydroxylation (Pathway 4)	364.2380	$C_{19}H_{37}ClO_4$		L (12), K (6), P (6), T (12), U (12)
20	S-Cysteine conjugation-C3H5OCl (Pathway 3)	375.2443	$C_{19}H_{37}NO_4S$		L (6, 12), K (6), P (6), T (6), B (12, 48)
21	Quinone formation (Pathway 4)	378.2173	$C_{19}H_{35}ClO_5$		L (12), U (12)
22	Glycine conjugation (Pathway 1)	389.2697	$C_{21}H_{40}ClNO_3$		L (6, 12), U (12)
23	N-acetylcysteine conjugation-C3H5OCl (Pathway 3)	417.2549	$C_{21}H_{39}NO_5S$		P (6)
24	Sulfate conjugation (Pathway 4)	428.1999	$C_{19}H_{37}ClO_6S$		U (12)
25	Cysteine conjugation-O (Pathway 1)	435.2574	$C_{22}H_{42}ClNO_3S$		L (6)
26	Taurine conjugation-O (Pathway 1)	439.2523	$C_{21}H_{42}ClNO_4S$		P (6)
27	N-acetylcysteine conjugation-O (Pathway 1)	493.2629	$C_{24}H_{44}ClNO_5S$		U (12)
28	Hydroxylation + glucuronide conjugation-Cl+H	506.3091	$C_{25}H_{46}O_{10}$		L (6, 12)

	(Pathway 2)				
29	Glucuronide conjugation-O (Pathway 1)	508.2803	$C_{25}H_{45}ClO_8$		U (12)
30	Glutathione conjugation-C3H5O2Cl (Pathway 3)	529.3186	$C_{26}H_{47}N_3O_6S$		L (6, 12), P (6), U (12), B (12, 48)
31	Desaturation + S-Glutathione conjugation-C3H5OCl (Pathway 3)	559.2927	$C_{26}H_{45}N_3O_8S$		T (12)

* The existence letters in each column represent the metabolites detected in tissue/body fluids. L, K, P, T, U and B represent liver, kidney, plasma, testis, urine and brain samples, respectively. The numbers after each letter represent the detected time point. For example, L (6, 12) represent that this metabolite was detected in liver samples in 6 hours and 12 hours after oral administration 3-MCPD 1-monopalmitate to rats.

Metabolite 20 was selected as another example for characterizing a typical metabolite detected from brain samples after oral administration of 3-MCPD 1-monopalmitate. The base peak intensity chromatography and extracted ion chromatography of metabolite 20 was represented in **Figure 3.6**, and the retention time of this metabolite was 6.35 min. The high accurate mass spectrum of parent ion (MS1) and daughter ion (MS2) of metabolite 20 was represented in **Figure 3.7**, and the $[M+H]^+$ of metabolite 20 was m/z 376.2522. The chemical formula was $C_{19}H_{37}NO_4S$, and the metabolite was identified as S-Cysteine conjugation substrate-C3H5OCl (Ishizuka et al., 1990). S-Cysteine conjugation is also an important phase II detoxification and belongs to amino acid conjugation. The structure of metabolite 20 was 3-MCPD 1-monopalmitate deduct free 3-MCPD, and the fatty acid fragment combined with S-Cysteine. This metabolite could be detected in rats' brain samples, which indicated that the free fatty acid related metabolite tend to be easier to go through the blood-brain barrier.

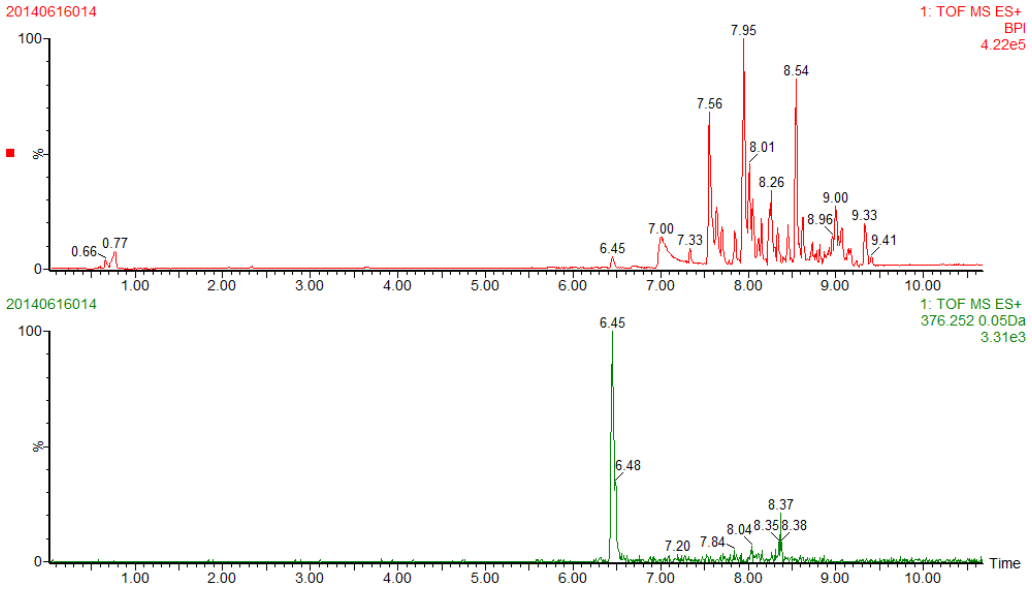


Figure 3.6 Typical UPLC-QTOF-MS A) base peak intensity (BPI) of rat brain sample extracts and B) extract ion chromatogram for the S-Cysteine conjugated metabolite after oral administration of 3-MCPD 1-monopalmitate to rats.

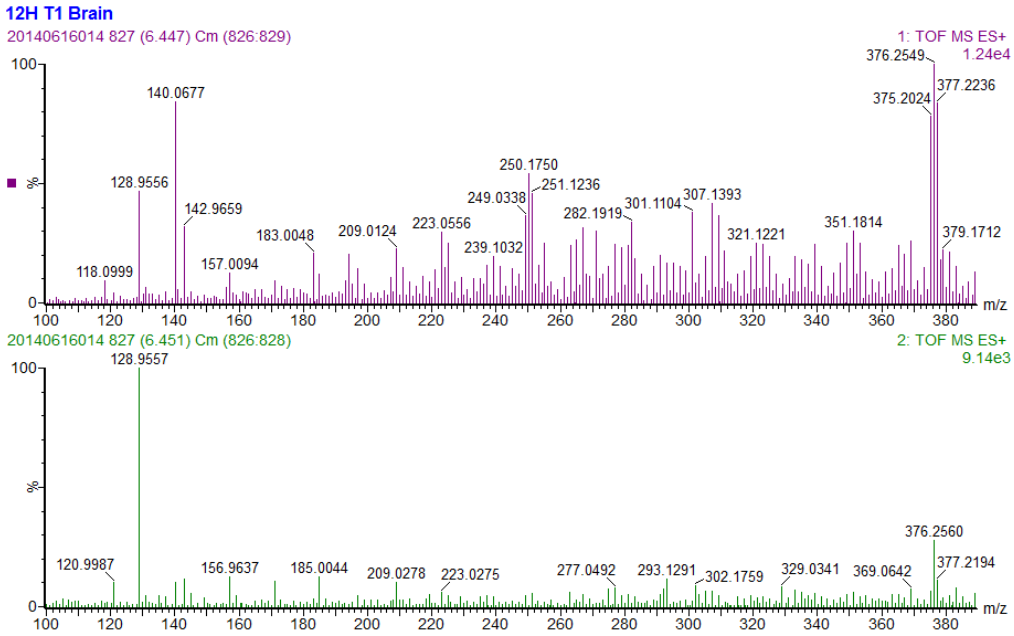


Figure 3.7 MS spectra of the S-Cysteine conjugated metabolite. A) MS1 spectrum and B) MS2 spectrum.

All the 30 metabolites were identified using the similar method above. The chemical structures of all these metabolites can be separated into four different groups, including the basic structure of 3-MCPD 1-monopalmitate deduct the hydroxyl group, 3-MCPD 1-monopalmitate deduct the chloride, 3-MCPD 1-monopalmitate deduct the free 3-MCPD (free fatty acid), and prototype of 3-MCPD 1-monopalmitate. Possible metabolic pathways could be conducted based on these four groups of structures.

To sum up, the present study identified the possible metabolites in rats after oral administration of 3-MCPD 1-monopalmitate in rats. The toxicity effects of these metabolites should also be focused in the coming study. The present study might advance our understanding of the metabolism process of 3-MCPD esters and provide

a base for further studies to have a better understanding about the toxicity effects and mechanisms of 3-MCPD esters in vivo.

Chapter 4: Metabolic pathway derivate after oral administration of 3-MCPD 1-monopalmitate to rats.

4.1 Abstract

Esters of mono-chloropropane 1,2-diol (3-MCPD) are a group of potential food source toxins formed during food processing. The toxic effects, especially the nephrotoxicity and hepatotoxicity of 3-MCPD esters were widely reported in the past decades. To improve the understanding about the possible toxicology effects and mechanism of 3-MCPD esters, it is important to clarify their metabolic pathways in vivo. In this study, 3-MCPD 1-monopalmitate was selected as a representative and oral administration to Sprague Dawley (SD) rats. Five major metabolism pathways can be derivate based on the results of identified metabolites. These five pathways represented the possible metabolism progress of 3-MCPD esters in vivo: 3-MCPD 1-monopalmitate metabolized to four mid-metabolites, including dehydroxylation 3-MCPD 1-monopalmitate, dechlorination 3-MCPD 1-monopalmitate, free 3-MCPD and palmitic acid. These four mid-metabolite, as well as the original form of 3-MCPD 1-monopalmitate, kept on metabolizing to different types of final metabolites. Understanding the metabolism of 3-MCPD esters in vivo is beneficial for the toxicity effects research, and promoting the entire research on 3-MCPD esters both in food science and in food industry.

4.2 Introduction

Fatty acid esters of 3-Monochloropropane 1,2-diol (3-MCPD) are a group of chemical compounds formed during the food processing. They were considered as potential food toxins and being researched in the past decades. In 2004, 3-MCPD esters was firstly reported together with the free 3-MCPD in processed foods, and the esters form of 3-MCPD have a much higher concentrations than their free form, especially in the vegetable oils and fats as well as in fried foods (Hamlet et al., 2004; Svejkovska et al., 2004). These results about 3-MCPD esters made them an important food safety concern, since the esters of 3-MCPD might hydrolyze to a known food toxin, free 3-MCPD. In this condition, even the concentration of free 3-MCPD in the food might be lower than the tolerable level, the esters form of 3-MCPD that people consumed could be metabolized to free 3-MCPD or other toxins in vivo and exceed the tolerable level currently established. To avoid this situation and improve the understanding about 3-MCPD esters, it is necessary and important to clarify the toxicity effect of 3-MCPD esters in vivo.

In these years, increasing studies focus on the toxicology effects of 3-MCPD esters both in vitro and in vivo. Sawada and colleagues reported an overview of proteomic changes induced by 3-MCPD di-esters and free 3-MCPD in rat testis at the early phase of organ impairment (Sawada et al., 2015). In 2015, Onami and colleagues reported the potential subchronic toxicity of two 3-MCPD di-esters and one 3-MCPD mono-ester to the rat kidneys and epididymis after a 13-week repeated dose study (Onami et al., 2015). Our group also reported an acute toxicity study of 3-MCPD mono- and di-esters in mice and their cytotoxicity in rat kidney cells, and the results indicated that 3-MCPD mono-ester represented greater toxicity compared with di-ester (Liu et al., 2012). In 2016, our group reported tubular cell apoptosis in vivo

via JNK/p53 pathway induced by 3-MCPD 1-palmitate (Liu et al., 2016). All these results proved that 3-MCPD esters were potential toxins, and the toxic working mechanisms still need to be investigated in the further study. Based on the biological central dogma and principal of pharmacokinetics, the bioactivity/toxic materials induced a residue-by-residue transfer of sequential information from DNA to RNA, RNA to protein, then from protein to metabolites, and finally the metabolites represent bioactivity/toxic effects in vivo (Nicholson et al., 1999). To better understanding the toxic effects and mechanisms of 3-MCPD esters, it is important to clarify their absorption, distribution, metabolism and excretion conditions in vivo. Recently, our group reported the kinetic study about the absorption of 3-MCPD 1-monopalmitate in SD rats, and identified 8 chloride-related metabolites in different tissues of rats. The results in this study indicated that with free 3-MCPD as the mid-metabolite, 3-MCPD 1-monopalmitate could be metabolized to a group of chloride-related metabolites and excreted out of body. Based on the results above, further questions about the metabolism of 3-MCPD esters raised up: except for the chloride-related metabolites, whether there are some other types of metabolites existed in vivo, and what are the metabolic pathways of these metabolites in vivo. To solve these problems, the further research about the metabolism of 3-MCPD 1-monopalmitate in vivo was designed and processed in this study.

In this study, 3-MCPD 1-monopalmitate was oral administration to SD rats, tissues including liver, kidney, testis, brain, as well as the plasma and urine were collected and analyzed in different time points. Five major metabolic pathways of 3-MCPD esters were derivate to evaluate their metabolic conditions in vivo. The results in this study can greatly enhance our understanding about the metabolism situation of

3-MCPD esters in vivo and promote further research about the toxicity study of 3-MCPD esters.

4.3 Materials and Methods

4.3.1 Chemicals and reagents.

3-MCPD 1-monopalmitate was synthesized based on our previous method (Liu, Huang, Wang, Sun, Yu, 2016), and the purity of final product was greater than 98% verified by UPLC-QTOF MS. LC-MS grade isopropanol and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS grade water was purified from a Milli-Q 10 ultra-pure water system (Billerica, MA, USA). All the other chemical reagents were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.3.2 Animals, treatment and sample collection.

The animal studies were approved by the Animal Ethics Committee of Shanghai Jiao Tong University. Male Sprague-Dawley rats were purchased from SLAC experimental animal Co. Ltd (Shanghai, China) with the body weight between 160 and 180 grams. The rats were maintained in a room with controlled temperature and 12-hour light/dark cycle, and were allowed free access to regular diet and water. The animals were acclimatized to the environment for one week, and then fasted with free access to the water for 12 hours before the experiment.

The experimental design was described in our previous study. Briefly, rats were separated into eight groups randomly, and each group has six rats. Four groups were kept in metabolic cages, treated with 400 mg/kg body weight of 3-MCPD 1-monopalmitate, then sacrificed to collected the liver, kidney, testis, brain, plasma and

urine at 6, 12, 24 and 48 hours after oral administration. Another four groups were set as control groups, oral administration of pure olive oil, and sacrificed at similar four time points as those four test groups. The water and food were fed ad libitum 4 hours after treatment during the experiment.

4.3.3 Animal samples preparation.

300 μL of blood samples were collected and centrifuged at 4000 rpm for 10 minutes at 4 °C, and 100 μL of supernatant plasma was accurately transferred and immediately frozen at -80 °C until further analysis. 300 μL of acetonitrile was added to each 100 μL of plasma sample, vortex for 30 s, followed by centrifugation at 10000 rpm for 10 minutes at 4 °C. The supernatant was transferred into a UPLC vial for further analysis.

The liver, kidney, testis and brain samples were removed, weighed flushed in ice-cold saline (0.9% NaCl), and homogenized at 4 °C in 9 times weight ice-cold saline to make 10% tissue homogenate and stored at -80 °C before use. The protein in homogenates was precipitated with acetonitrile with the similar method as plasma processing, and the supernatant was collected for further analysis.

The urine samples were collected in metabolic cages at different time points. For example, urine samples at 24 hour represent the urine collected between 12 hour and 24 hours. After collection, 0.2 mL of urine sample was extracted with 1 mL of water-saturated ethyl acetate, vortex for 30 s, centrifuged at 10000 rpm for 10 min at ambient, transfer the supernatant and removes all the solvent using nitrogen evaporator. The residue was dissolved with 0.2 mL of methanol and injected to UPLC-QTOF-MS for further analysis.

4.3.4 UPLC-MS conditions.

A Waters Acuity UPLC-Xevo G2 QTOF MS system (Waters, Milford, MA, USA) was selected for metabolite identification in this study with a Waters Phenyl column (2.1 mm i.d. × 100 mm, 1.7 μm). The mobile phase consisted of A) water/methanol (9:1, v/v) and B) methanol/ isopropanol (4:1, v/v) using the elution gradient started with 0% phase B, changed linearly to 35% in 4 min, increased linearly to 95% B at 8 min and maintained for 2 min, and returned to its initial conditions for 2 min to re-equilibrate the column for the next injection. The flow rate was 0.4 mL/min with an injection volume of 2 μL. For MS detector conditions, capillary voltage 3.00 kV; sampling cone voltage 60 V; extraction cone voltage 4.0 V; source temperature 120 °C; and desolvation temperature 450 °C. The cone gas flow rate was 150 L/h and the desolvation gas was 800 L/h. A MS^E method was used with mass range from 50 to 1200 m/z in both ESI positive and ESI negative mode, scan time was 0.3 s and the ramp collision energy was 20-35 eV.

4.4 Results and Discussion

4.4.1 Possible metabolic pathway related with the free 3-MCPD intermediates

Based on the information above, the possible metabolic pathway of 3-MCPD 1-monopalmitate with the free 3-MCPD as the intermediates was proposed using MetaboLynx (**Figure 4.1)**. Briefly, 3-MCPD 1-monopalmitate hydrolyzed and loss its fatty acid fragments to form the free 3-MCPD (phase I metabolite). After that, the free 3-MCPD combined with the endogenous substances in vivo, increased polarity for easy urine excretion (phase II metabolites). Sulfonated 3-MCPD, acetylated 3-MCPD, glucuronide 3-MCPD and all the amino acid conjugated 3-MCPD are the typical

phase II metabolites in vivo. This pathway clearly represent the possible metabolic process of 3-MCPD 1-monopalmitate in vivo, especially the chlorine-contained fragment of 3-MCPD ester distributed and excreted. 3-MCPD 1-monopalmit was same as common glycerides and could be metabolized through acetylation, glucuronide and sulfonation, but was unique to be able to form the three amino acid conjugated 3-MCPD. The existence of these metabolites might because of the potential toxicity of 3-MCPD 1-monopalmitate induced more endogenous metabolic reactions to promote its excretion.

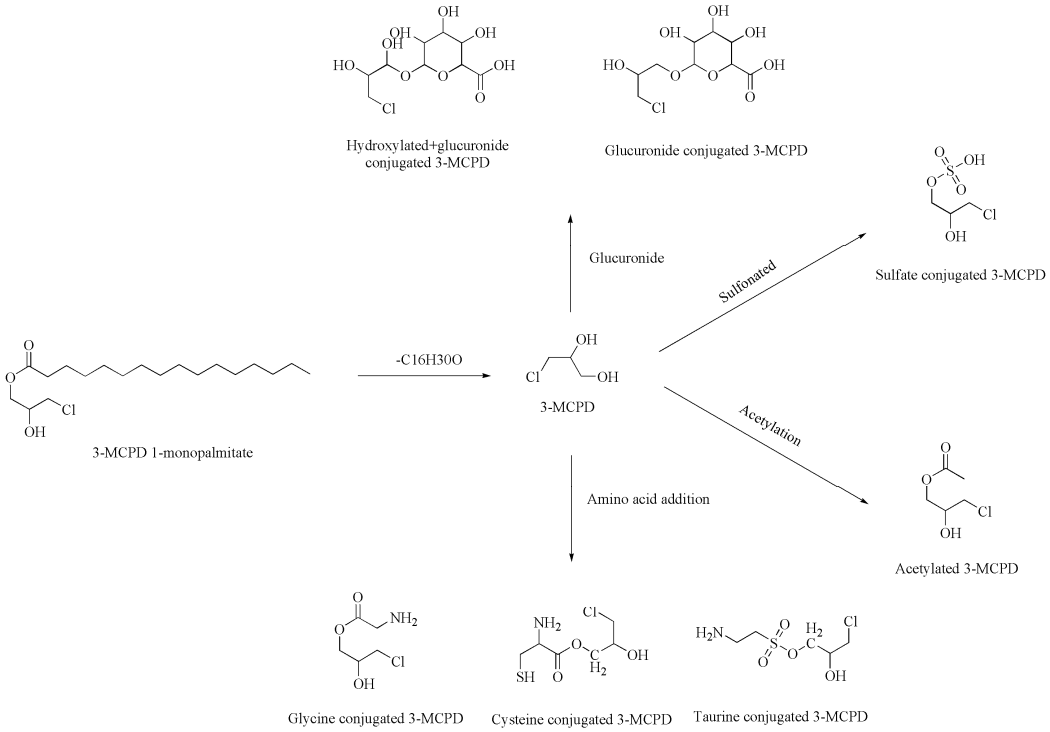


Figure 4.1 Possible metabolic pathway of 3-MCPD 1-monopalmitate in rats.

4.4.2 All the other metabolic pathways of 3-MCPD 1-monopalmitate in rats.

After we identified the possible metabolites in rats after oral administration of 3-MCPD 1-monopalmitate, it is important to clarify the metabolic pathways of 3-MCPD 1-monopalmitate in vivo. In this study, there are totally four major groups of metabolites, and their metabolic pathways were represented in **Figure 4.2**.

The first metabolic pathway is 3-MCPD 1-monopalmitate deduct the hydroxyl group in sn-2 position to form the mid-metabolite, then this mid-metabolite continue the metabolism progress to add some functional groups such as amino acids, N-acetylcysteine or glucuronic acid to form a group of phase II metabolites, including the glycine conjugation (metabolite 22), cysteine conjugation (metabolite 25), taurine conjugation (metabolite 26), N-acetylcysteine conjugation (metabolite 27) and glucuronide conjugation (metabolite 29). These metabolites were major existed in rats' liver, kidney and urine samples, indicated that these conjugation pathways were one of the major approach to increased the polar of 3-MCPD 1-monopalmitate and then helped the excretion (Falany et al., 1994; O'Byrne et al., 2003). Consider that 3-MCPD esters are potential food source toxins, pathway one might be one of the possible detoxification pathways in vivo.

The second metabolic pathway is 3-MCPD 1-monopalmitate deduct the chloride to form a mid-metabolite (metabolite 12), then methylated, hydroxylated, carboxylated or glucuronidated to formed metabolites 4, 5, 8, 9, 10, 13, 15 and 28. These metabolites showed relatively lower toxic effects, and mainly existed in rats' liver and plasma samples.

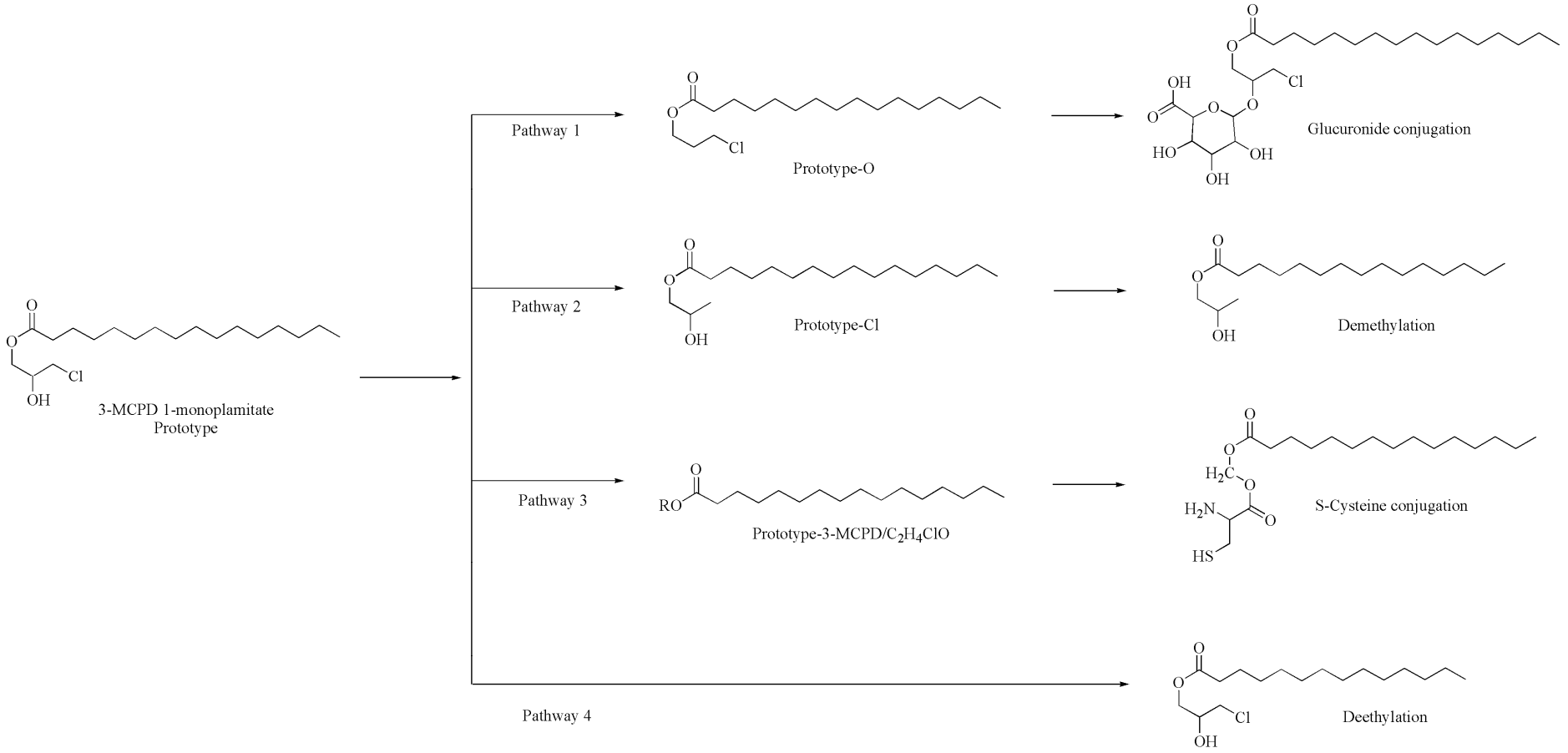


Figure 4.2 Possible metabolic pathway of 3-MCPD 1-monopalmitate in rats.

The third metabolic pathway is 3-MCPD 1-monopalmitate deduct the free 3-MCPD to form palmitic acid (metabolite 2), then deethylated, hydroxylated, acetylated, amino acid conjugated or glutathione conjugated to formed metabolites 1, 3, 7, 8, 20, 23 and 30. In all these substituents, glutathione conjugated metabolites were very important, especially for the metabolism of 3-MCPD esters. Glutathione (GSH) is a tripeptide combined with glutamate, cysteine and glycine. It reacts enzymatically or non-enzymatically with toxic compounds to form GSH conjugates (Anderson, 1998). In 2015, Sawada and colleagues reported their study about the proteomic analysis of both free 3-MCPD and 3-MCPD esters in rat testis, their results indicated that both free 3-MCPD and 3-MCPD esters can affect the expression of glutathione synthetase (GSS) (Sawada et al., 2015). In this study, it is the first time that two glutathione related metabolites were identified in rats' liver samples after oral administration of 3-MCPD 1-monopalmitate. This result represented the fact that glutathione might plays important role in the detoxification progress of 3-MCPD esters in vivo. Combined with our previous research results that 3-MCPD esters can metabolized to free 3-MCPD, metabolic pathway 3 could be explained like this: first, 3-MCPD esters formed free 3-MCPD and free fatty acid in the behavior of enzymes including glutathione, then free 3-MCPD and fatty acid fragment continue their metabolic progress to form different metabolites. Besides, metabolites 1, 2, and 20 can be detected in rats' brain samples, which indicated that these non-polar metabolites might transfer the blood-brain barrier to brain.

The last metabolic pathway is direct metabolism, means that the 3-MCPD 1-monopalmitate add or deduct some chemical groups and metabolized directly. In this pathway, metabolic reactions including dealkylation, hydroxylation, deethylation,

carboxylation, quinone formation and sulfate conjugation result in metabolites 6, 11, 14, 16, 17, 19, 21 and 24. The metabolize progress of sulfate conjugated metabolite related with an important amino acid cysteine, and it can be recognized as an important detoxification phase II metabolites (Han et al., 2016). As a group of chloride related metabolites, pathway 4 were also closely related with the excretion of 3-MCPD esters out of body.

Four metabolic pathways that related to these metabolites were conducted based on the chemical structures of these metabolites. The present study might advance our understanding of the metabolism process of 3-MCPD esters and provide a base for further studies to have a better understanding about the toxicity effects and mechanisms of 3-MCPD esters in vivo.

Appendix

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Publication:	Journal of Agricultural and Food Chemistry
Publisher:	American Chemical Society
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