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Fatty Acid Esters of 3-Monochloropropanediol: A Review

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Abstract

Fatty acid esters of 3-monochloropropane-1,2-diol (3-MCPD esters) are a new group of processing-induced chemical toxicants with possible nephrotoxicity and testicular toxicity. 3-MCPD esters have been detected in many food categories, including refined edible oils, bread, coffee, and infant formula. 3-MCPD esters have also been detected in human breast milk, indicating their possible absorption and distribution in human organs and tissues. 3-MCPD esters have become a food safety concern, and in 2013 the European Food Safety Authority estimated a tolerable daily value (TDI) of 2 μg/kg body weight (BW) for the amount of free 3-MCPD. This review summarizes the available information on 3-MCPD ester research, including the analytical methods, exposure biomarkers, absorption and metabolism, toxicities, formation mechanisms, and mitigation strategies as well as the occurrence of 3-MCPD esters in human foods. This review may serve as a scientific foundation for advancing our understanding of 3-MCPD esters and their food safety concerns.

INTRODUCTION

Fatty acid esters of 3-monochloropropane-1,2-diol (3-MCPD esters) are a new group of chemical compounds formed during thermal processing, e.g., the oil-refining process. These chemical compounds have been shown to have nephrotoxicity and testicular toxicity as well as other toxicities. More and more researchers have realized that 3-MCPD esters are a food safety concern. The *European Journal of Lipid Science and Technology* published a special issue focused on the fatty acid esters of chloropropanols and glycidol in March 2011. In this issue, 17 reviews and research articles were published, including articles on the chemical formation mechanism, analytical method development, and toxic effects of 3-MCPD and glycidol esters. In 2013, the European Food Safety Authority estimated a tolerable daily value (TDI) of 2 μg/kg body weight (BW) of free 3-MCPD (EFSA 2013). Since then, much research has been carried out to investigate their toxicity, formation, detection, and mitigation. In this review, the occurrence, analytical approaches, biomarkers, absorption and metabolism, toxicities, formation mechanisms, and mitigation approaches of 3-MCPD esters are summarized and discussed.

OCCURRENCE OF 3-MCPD ESTERS IN FOODS

Fatty acid mono- and diesters of chloropropanediols were isolated and identified from cooking oils in 1983 (Gardner et al. 1983). In 2004, 3-MCPD esters were first reported together with free 3-MCPD in 17 of the 20 selected retail food products, with the greatest level at 36.8 mg/kg fat or 6.1 mg/kg food in French fries, followed by 36.5 mg/kg fat or 0.58 mg/kg in dark malt (**Table 1**) (Svejkovská et al. 2004). Also in 2004, Hamlet & Sadd (2004) reported the presence of MCPD mono- and diesters in the white flour, white bread, and toasted white bread and its crumb and crust. Greater concentrations of 3-MCPD esters were found in crust and toasted white bread at levels of 547 and 160 μg/kg, respectively, which were 82 and 24 times that of the 6.7 μg/kg found in the nontreatment white bread, leading to a consideration that more thermal treatment might be associated with a greater amount of 3-MCPD ester formation (Hamlet & Sadd 2004). In addition, 3-MCPD esters have also been detected in many other food categories, including processed meats, dairy, ready-to-eat cereal, nuts and seeds, coffee, fishery products, chopped onion and garlic, and infant formulas as well as in human breast milk (**Table 1**) (Arisseto et al. 2015, 2017; Becalski et al. 2015; Chung et al. 2013a; EFSA 2013; Karl et al. 2016; Leigh & MacMahon 2017; Svejkovská et al. 2004; Wong et al. 2017; Wöhrlin et al. 2015; Zelinková et al. 2008, 2009; Zwagerman & Overman 2016). In general, mono- and diesters of 3-MCPD esters exist together with mono- and diesters of 2-MCPD and glycidyl esters. Depending on the study, the total amount of MCPD esters, the ratio between 3- and 2-MCPD esters, the ratio between mono- and diesters, and the ratio of MCPD and glycidyl esters may differ because of the variation of their levels in different samples and the analytical method used. It needs to be pointed out that 2-MCPD esters have also been detected in many fats/oils and food products due to the recent advancement in MCPD ester analysis approaches, with a range from not detectable to 930 μg/kg food (Becalski et al. 2015, EFSA 2016, Wöhrlin et al. 2015). It also needs to be pointed out that the liquid chromatographic separation of 3-MCPD esters and 2-MCPD esters was achieved recently, and earlier data for 3-MCPD ester contents might include 2-MCPD esters. In addition, little is known about the toxic effects of 2-MCPD esters compared to those of 3-MCPD esters, and it is unclear whether there are any relationships between the ratios of 2- and 3-MCPD esters.

Several studies have examined different foods for their MCPD and glycidyl esters. For instance, the levels of 3-MCPD esters were detected in ham, salami, and beef powder/paste at concentrations of 0–2,940 μg/kg, <930–1,490 μg/kg, and 1,120–2,310 μg/kg, respectively (**Table 1**).

Table 1 Concentrations of 3-MCPD esters and free 3-MCPDs in different foodstuffs

Foodstuffs	3-MCPD esters (μg/kg)	Free 3-MCPDs (μg/kg)	Reference
Processed meat products	ND–2,940	ND–47.63	Chung et al. 2013a,b; Fu et al. 2007; Svejkovská et al. 2004
Ham	ND–2940	23.18–26.24	Svejkovská et al. 2004, Chung et al. 2013b
Salami	<930–1,490	14.03–47.63	Svejkovská et al. 2004
Beef powder/paste	1,120–2,310	NA	Fu et al. 2007
Fats and oils	ND–4,500	<3–31	Chung et al. 2013a,b; Ermacora & Hrncirik 2013; Weißhaar 2008, 2011; Zelinková et al. 2006
Refined olive oil	<300–2,462	<9	Chung et al. 2013a, Weißhaar 2008, Zelinková et al. 2006
Virgin olive oil	<100–300	<3–9	Zelinková et al. 2006
Refined rapeseed oil	381–670	<9	Ermacora & Hrncirik 2013, Zelinková et al. 2006
Virgin rapeseed oil	<100	<9	Zelinková et al. 2006
Refined sunflower seed oil	<300	<9	Ermacora & Hrncirik 2013, Zelinková et al. 2006
Virgin sunflower seed oil	<100	<9	Ermacora & Hrncirik 2013, Zelinková et al. 2006
Refined soybean oil	1234	<9	Zelinková et al. 2006
Virgin soybean oil	<100	12	Zelinková et al. 2006
Refined safflower oil	2,355–3,218	NA	Weißhaar 2008
Virgin safflower oil	<100–2,461	NA	Weißhaar 2008
Deep-fat-fried foods	22–6,100	14.48–15.41	Arisseto et al. 2015, Chung et al. 2013b, Svejkovská et al. 2004, Zelinková et al. 2009
French fries	35–6,100	15.41	Chung et al. 2013b, Svejkovská et al. 2004, Zelinková et al. 2009
Dark malt	580	NA	Svejkovská et al. 2004
Bakery products	ND–1,200	<9–81.2	Chung et al. 2013a,b; Hamlet et al. 2002; Hamlet & Sadd 2004; Starski et al. 2013; Svejkovská et al. 2004
White flour	<0.5	<0.5	Hamlet & Sadd 2004
White bread	6.7	6.1	Hamlet & Sadd 2004
Toast white bread	160	93	Hamlet & Sadd 2004
Crumb	4.9	<0.5	Hamlet & Sadd 2004
Crust	547	477	Hamlet & Sadd 2004
Dairy	ND–1,280	12.98–82.67	Chung et al. 2013a,b
Ready-to-eat cereal	<2–43	NA	Chung et al. 2013a,b; Hamlet & Sadd 2004
Nuts and seeds	ND–500	22.10	Chung et al. 2013a,b; Svejkovská et al. 2004
Coffee	1–390	16.15	Chung et al. 2013a, Svejkovská et al. 2004
Fishery products	ND–1080	ND–388	Chung et al. 2013b, Karl et al. 2016, Svejkovská et al. 2004
Chopped onion and garlic	<80–990	NA	Arisseto et al. 2015
Infant formulas	<6–920	ND	Arisseto et al. 2017, Becalski et al. 2015, Leigh & MacMahon 2017, Wöhrlin et al. 2015, Zelinková et al. 2009
Human breast milk	6–76	ND	Wöhrlin et al. 2015, Zelinková et al. 2008

Abbreviations: MCPD, monochloropropane-1,2-diol; NA, not available; ND, not detectable.

Recently, Chung et al. (2013b), Karl et al. (2016), and Svejkovská et al (2004) found 3-MCPD esters at nondetectable levels, 150–280 μg/kg, and <530–1,080 μg/kg in raw fish, deep fried fish, and smoked/pickled fish products, respectively. The wide range of 3-MCPD esters in foods might be explained by the different ingredients, processing conditions, and analytical methods.

The available data have strongly suggested that processing affects the overall MCPD ester levels in oils and foods. Refined commercial olive, rapeseed, sunflower, soybean, and safflower oils have relatively greater (3–32 times) concentrations of 3-MCPD esters than their nonrefined counterparts. For example, refined safflower and olive oils had 3-MCPD ester levels of 2,355–3,218 μg/kg and 300–2,462 μg/kg, respectively, which were 1–32 and 1–24 times that of their unrefined counterparts, which had 3-MCPD ester levels of 100–2,461 μg/kg and 100–300 μg/kg, respectively. Refined soybean, rapeseed, and sunflower oils had 3-MCPD ester levels of 1,234 μg/kg, 381–670 μg/kg, and <300 μg/kg, respectively, which were 12, 4–7, and 3 times that of their unrefined counterparts (all with 3-MCPD ester levels under 100 μg/kg) (**Table 1**). In addition, Wong and others found that frying duration, temperature, and concentration of sodium chloride might significantly affect the amounts of 3-MCPD esters in palm oil during deep-frying (Wong et al. 2017), which may partially explain the greater level of 3-MCPD esters (6,100 μg/kg) in French fries (Svejkovská et al. 2004).

In 2008, Zelinková et al. (2008) reported the presence of 3-MCPD esters in human breast milk at a concentration range of 300–2,195 μg/kg fat and a mean value of 1,014 μg/kg fat, which is approximately 35.5 μg/kg milk. The study also observed that six breast-milk samples collected from a nursing mother between 14 and 76 days after labor had 3-MCPD levels ranging from 328 to 2,078 μg/kg fat, or 6 to 19 μg/kg milk (Zelinková et al. 2008). These data indicate that MCPD esters can be absorbed into the human body and may be distributed to different organs and tissues.

3-MCPD esters, together with 2-MCPD and glycidyl esters, have been reported in infant formulas collected from several countries, possibly because of the use of refined vegetable oils containing these contaminants (Arisseto et al. 2017, Becalski et al. 2015). The concentration of 3-MCPD esters in the tested infant formula varied significantly depending on the product brand and sample collection time (Arisseto et al. 2017, Becalski et al. 2015, Leigh & MacMahon 2017, Wöhrlin et al. 2015, Zelinková et al. 2009). For example, 3-MCPD ester concentrations ranged from not detectable to 600 μg/kg (Arisseto et al. 2017). Interestingly, an infant formula made with palm and/or palmolein does not necessarily contain a greater level of 3-MCPD esters (Leigh & MacMahon 2017). In general, different age groups may have a different intake of MCPD and glycidyl esters from infant formula. For example, Arisseto et al. (2017) found that 0–5-month-old infants had a possible average intake of 3-MCPD esters at 2.49 μg/kg BW per day from infant formula, whereas the 6–11-month-olds had a possible average daily intake of 1.05 μg/kg BW per day. These intake values have led to comments from the Joint FAO/WHO (Food and Agriculture Organization/World Health Organization) Expert Committee on Food Additives (JECFA) and the FAO/WHO that the existence of 3-MCPD esters in foods need to be scrutinized and controlled, especially in infant formula and infant foods (Arisseto et al. 2017, JECFA 2016).

ANALYTICAL METHODS FOR 3-MCPD ESTERS

Accurate, rapid, and high-sensitivity chemical analytical methods are needed for detecting the presence and levels of 3-MCPD esters in different types of food and biological samples. To date, the reported analytical methods for 3-MCPD esters can be divided into indirect and direct detection approaches. Generally, an indirect method involves releasing the free 3-MCPD from all the fatty acid esters of 3-MCPD in the sample, preparing derivatives for analysis, and quantifying the amount of free 3-MCPD, with the total amount of 3-MCPD esters to be reported as the

equimolar of free 3-MCPD contents. A direct approach characterizes and quantifies each individual 3-MCPD ester in food samples directly, which is straightforward and easy to understand but much more difficult in sample purification and method development during practical utilizations compared to that for the indirect approach. The current status of both approaches is summarized and discussed below.

Indirect Approaches in Detecting 3-MCPD Esters

The representative indirect approach for 3-MCPD ester estimation is the American Oil Chemists' Society (AOCS) method (AOCS 2017). In brief, 350 μL of methanolic sodium hydroxide solution was added to the approximately 100-mg oil or melted-fat sample containing 50 μL of isotope-labeled 3-MCPD standard working solutions with a concentration of 10 μg/ml in diethyl ether, and the mixture reacts for at least 16 hours at −22°C to −25°C to complete the 3-MCPD ester cleavage. The reaction is stopped with 600 μL of acidified sodium bromide solution. After removal of the organic phase, the aqueous layer containing free 3-MCPD is reacted with 20 μL of saturated phenylboronic acid (PBA) solution in diethyl ether to finish the derivatization. The solvent is removed, and the residue is redissolved in approximately 300–500 μL of *iso*-octane for gas chromatography–mass spectrometry (GC-MS) analysis. 3-MCPD/2-MCPD esters were identified and quantified based on the precursor and product ions. The AOCS method is similar to the indirect analytical method published and revised by the German Society for Fat Science (DGF) (DGF 2012). Both methods release free 3-MCPD with alkaline-catalyzed transesterification/hydrolysis reactions in methanolic sodium hydroxide and prepare the volatile 3-MCPD derivatives using PBA for GC-MS analyses (**Table 2**). The minor difference is that DGF protocol uses sodium chloride, whereas the AOCS method uses acidified sodium bromide solution to enhance the separation of MCPD and fatty acids after stopping the ester cleavage reaction. The NaCl was considered as a penitential reactant and may alter the 3-MCPD contents, possibly leading to an overestimation (Haines et al. 2011). The AOCS and DGF methods are widely accepted certified methods for analyzing 3-MCPD esters indirectly and often used as a reference in new analytical method development for 3-MCPD esters.

In addition to the AOCS and DGF protocols, there are a few other indirect protocols using a different transesterification condition and/or a derivation agent and/or a different GC detector. For instance, samples could be hydrolyzed using sulfuric acid and neutralized with saturated $NaHCO_3$ solution, followed by derivatization of free 3-MCPD with PBA for GC-MS analysis. This approach has been used in analyzing 25 oil samples and 12 human breast-milk fat samples in 2006 and 2008, respectively (Zelinková et al. 2006, 2008). This method first released 3-MCPD using an acid-catalyzed hydrolysis reaction and converted 3-MCPD to volatile derivatives using PBA. The acid-catalyzed hydrolysis of ester bonds generally requires thermal treatment, and the reaction is reversible, which means the hydrolysis may not be 100% completed and could result in an underestimation of total 3-MCPD esters in the samples. Also in 2008, Seefelder and others reported enzymatic hydrolysis to convert 3-MCPD esters in 11 different vegetable oils to free 3-MCPD using an intestinal lipase and derivatization with heptafluorobutyrylimidazole (HFBI) for GC-MS analysis (Seefelder et al. 2008). The major advantage of this method is that it is able to distinguish the sn-position of 3-MCPD esters in foods on the basis of the high selectivity of lipases as well as the mild enzyme-catalyzed hydrolysis reaction. In addition, Weißhaar (2008) reported a method to determine the total amount of 3-MCPD esters in edible fats and oil samples by transesterifying 3-MCPD esters with $NaOCH_3$/methanol and derivatizing with PBA for GC-MS determination. In 2010, the effectiveness of heptafluorobutyryl (HFB), boronic acid, and dioxolane in the derivatization of

Table 2 Representative quantification methods for 3-MCPD esters[a,b]

Food category	Analytical method	Transesterification and derivatization reagents	LOD (μg/kg)	LOQ (μg/kg)	Linear range (μg/kg)	Instruments	Reference
Edible oils and fats	I	NaOH, PBA	NA	NA	NA	GC-MS	AOCS 2017
Foodstuffs	I	NaOH, PBA	NA	NA	NA	GC-MS	DGF 2012
Infant formulas	I	H_2SO_4, PBA	80	160	0–2,600	GC-MS	Arisseto et al. 2017
Foodstuffs	I	Lipase, PBA	20	100	0–2,500	GC-MS	Chung et al. 2013a
Vegetable oils	I/D	H_2SO_4, HFBI	ND	100	NA	GC-MS/LC-TOF MS	Dubois et al. 2012
Oils and fats	I	H_2SO_4, PBA	40	140	0–10,000	GC-MS	Ermacora & Hrncirik 2013
Foodstuffs	I	H_2SO_4, PBA	7	13	13–1,850	GC-MS	Samaras et al. 2016
Edible oils	I	Lipase, PBA	ND	140	NA	GC-MS	Koyama et al. 2016
Infant formula	I	H_2SO_4, PBA	60	200	~500–~11,000	GC-MS	Wöhrlin et al. 2015
Edible oils	I	H_2SO_4, PBA	100	300	300–2,462	GC-MS	Zelinková et al. 2006
Human breast milk	I	H_2SO_4, PBA	100	300	100–2,195	GC-MS	Zelinková et al. 2008
Edible oils	D	NA	10	30	50–1,0000	LC-QQQ MS	MacMahon et al. 2013a
Edible oils	D	NA	~0.02–0.93	~0.08–2.02	0.1–1,086	LC-TOF MS	Hori et al. 2012
Vegetable oils	D	NA	4–60	NA	NA	LC-TOF MS	Haines et al. 2011
3-MCPD esters standards[c]	D	NA	0.5–7.5	1.3–17.5	20–2,000	UHPSFC-QTOF MS	Jumaah et al. 2017

[a]The analytical methods for 3-MCPD esters are summarized according to their applied food categories.

[b]The linear ranges were calculated as amount of free 3-MCPD for the indirect method (I and I/D) and expressed as the amount of the representative 3-MCPD esters for direct methods.

[c]This is a methodology study, so the units of LOD, LOQ, and linear range in this study were reported as ng/ml in heptane solution. The linear ranges are obtained from the lower berth and upper berth of 3-MCPD content values from the study.

Abbreviations: D, direct; GC-MS, gas chromatography–mass spectrometry; HFBI, heptafluorobutyrylimidazole; I, indirect; LC-QQQ MS; liquid chromatography–triple quadrupole mass spectrometry; LC-TOF MS, liquid chromatography–time-of-flight mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; PBA, phenylboronic acid; UHPSFC-QTOF MS, ultrahigh-performance supercritical fluid chromatography–quadrupole time-of-flight mass spectrometry, NA, not mentioned in the reference; ND, not detectable.

3-MCPD esters was reviewed (Baer et al. 2010). In the next few years, HFBI (Liu et al. 2013), PBA (Ermacora & Hrncirik 2013, Kusters et al. 2010, 2011), and dioxolane were all utilized in the derivation of free 3-MCPD from edible oils and food products.

In 2016, Samaras and colleagues reported a simultaneous quantification method of 3-MCPD esters, 2-MCPD esters, and glycidol esters in different food samples using a pressurized liquid

extraction, followed by converting glycerol esters to monobromo-propanediol (MBPD) esters with NaBr in acidic aqueous solution and releasing MCPD and MBPD using sulfuric acid–MeOH solution and preparing volatile derivatives using phenyl boronic acid as described in the AOCS method for GC-MS analysis (Samaras et al. 2016). The quantification of 3-MCPD, 2-MCPD, and 3-MBPD derivatives was achieved using a DB-5^{ms} gas chromatography column (30 m × 0.25 μm × 0.25 mm film thickness) from Agilent Technology (Santa Clara, CA) and an MS detector in a selected ion monitoring (SIM) mode with stable-isotope-labeled MCPDs and MBPD standards. The method has a low detection limit and excellent repeatability and is applicable to a wide variety of food samples (Samaras et al. 2016). Also in 2016, another indirect method for the analysis of 3-MCPD esters, 2-MCPD esters, and glycidol esters in edible oils and fats was developed from a collaborative study involving 13 Japanese laboratories (Koyama et al. 2016). The release of free MCPDs and glycidol using *Candida rugosa* lipase-catalyzed enzymatic reaction, as well as the conversion of glycidol to MBPD with sodium bromide was carried out simultaneously in a reaction mixture at ambient temperature for 30 min. The free 3-MCPD, 2-MCPD, and 3-MBPD were derivatized with PBA and analyzed using GC-MS using similar SIM parameters (Koyama et al. 2016, Samaras et al. 2016).

In summary, the indirect approach for determining the concentrations of 3-MCPD esters has been recommended by the AOCS, DGF, and Pharmacopeia of the People's Republic of China. Indirect approaches generally require fewer analytical standards, which are applicable to a wide range of different food categories, including oils and fats, and easy for establishing a verified standard protocol. An indirect approach is also excellent for measuring the total amount of 3-MCPD-related components in foods. However, the indirect methods require more sample preparation steps and the use of toxic chemical reagents.

Furthermore, the indirect method can only estimate the total amount of 3-MCPD, 2-MCPD, and/or glycidyl esters but not individual 3-MCPD ester profiles. In addition, Haines and others have questioned the accuracy of indirect methods in quantifying 3-MCPD esters because some evidence suggested that part (or even all) of the free 3-MCPD measured by the DGF method might be formed from glycidyl esters or other compounds during sample preparation (Haines et al. 2011). Together, the disadvantages promoted the development of a direct analytical method for detecting 3-MCPD esters.

Direct Approaches in Detecting 3-MCPD Esters

Direct approaches analyze 3-MCPD esters without releasing free MCPD and the derivatization. This type of method was not widely utilized before 2010 because 3-MCPD esters are nonpolar compounds for reverse-phase high-performance liquid chromatography (HPLC) and have relatively low volatility for GC separation. However, the advancement of analytical technologies and new types of LC columns have made direct approaches possible (**Table 2**) (Dubois et al. 2012, Haines et al. 2011, MacMahon et al. 2013a). In 2011, a direct determination of MCPD esters and glycidyl esters in vegetable oils was reported using LC combined with time-of-flight mass spectrometry (LC-TOF MS), and the results were comparable to but consistently lower than those obtained using the DGF protocol (Haines et al. 2011). The LC-TOF MS direct method was able to successfully identify and quantify individual 3-MCPD esters using a Phenomenex Luna C18 column (50 × 3 mm, 3 μm; 100 Å pore size) and an Agilent 1200 Series Rapid Resolution gradient LC system connected to an Agilent 6210 Time-of-Flight Mass Spectrometer upgraded to 4 GHz for high-resolution data acquisition (Haines et al. 2011). In 2012, Dubois and colleagues compared a new direct method with an indirect method for their application in the estimation of 3-MCPD esters in vegetable oils (Dubois et al. 2012). The direct method had

double solid phase extractions and a silicone gel column to separate and concentrate 3-MCPD esters, and a Waters Acquity® ultraperformance liquid chromatography (UPLC) HSS T3 normal phase UPLC column with electrospray ionization (ESI) TOF mass spectrometry for detection, whereas the indirect method included an acidic release of free MCPDs and derivatization with HFBI for GC-MS analysis (Dubois et al. 2012). The two methods were comparable in analyzing 2- and 3-MCPD esters in 29 oil samples. The indirect approach needed fewer chemical standards and allowed relatively easier sample preparation, whereas the direct method provided detailed information about the identification and concentration of individual 3-MCPD esters with a longer sample pretreatment time. After 2012, an increasing number of research articles reported a modified direct method in analyzing the types and concentrations of 3-MCPD esters in food and biological samples (Li et al. 2015; MacMahon et al. 2013a,b, 2014). In general, HPLC or UPLC is combined with high-resolution mass spectrometry to directly detect 2- and/or 3-MCPD esters. In 2017, an ultraperformance supercritical fluid chromatography–mass spectrometry system was used to rapidly separate and detect more than 20 mono- and diesters of 2- and/or 3-MCPD with a 2-picolylamine column within 12 minutes (**Table 2**) (Jumaah et al. 2017). The method uses supercritical carbon dioxide as the primary mobile phase and is considered a green protocol.

In summary, direct analytical methods for measuring 3-MCPD esters have obvious advantages, including profiles of individual MCPD esters and less sample preparation. The MCPD ester profile is very important for toxicological investigations. However, direct approaches require more expensive instruments and more complex post-run calculations.

Both indirect and direct analytical approaches are practical methods for estimating 2- and 3-MCPD esters, but only indirect methods can simultaneously analyze glycidyl and 2-MCPD and 3-MCPD esters. Both methods have their own positive and negative aspects. It is important to select an appropriate method based on the specific need.

BIOMARKERS AND PHYSIOLOGICAL FINDINGS

Biomarkers for 3-MCPD ester exposure have been investigated in several studies. In 2013, Li and others fed male Wistar rats with 0, 12.3, and 267 mg/kg BW per day 3-MCPD dipalmitate for 90 days and investigated their urine profiles using UPLC-MS combined with principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) (Li et al. 2013). This study identified five possible urine biomarkers for 3-MCPD dipalmitate exposure: (*a*) indoxyl sulfate, (*b*) xanthurenic acid, (*c*) phenylacetylglycine, (*d*) nonanedioic acid, and (*e*) taurine. Xanthurenic acid and phenylacetylglycine were identified in the positive MS mode, whereas the other three biomarker compounds were identified in the negative mode. Furthermore, the levels of xanthurenic acid and phenylacetylglycine were significantly increased on day 28, along with a significant reduction of taurine levels. The levels of nonanedioic acid and indoxyl sulfate were significantly increased 35 days after treatment. In addition, the PLS-DA score plot showed that UPLC-MS profiles of the 267 mg/kg BW per day 3-MCPD dipalmitate group were separated from the 12.3 mg/kg BW per day and the control groups, and the three treatment groups were separated during week 13 (Li et al. 2013). In 2015 and 2016, there were a series of proteomic analytical studies on free 3-MCPD and 3-MCPD dipalmitate toxicities in rat liver, kidney, and testis (Braeuning et al. 2015; Sawada et al. 2015, 2016). These studies treated male Wistar rats with a daily dose of 53 mg/kg BW of 3-MCPD dipalmitate for 28 days, collected the protein profiles with two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization–mass spectrometry (MALDI-MS), and analyzed data with the ingenuity pathway analysis software (**http://ingenuty.com**). The proteomic changes in the selected organ tissues upon 3-MCPD dipalmitate exposure may serve as a potential indicator(s) for early organ-specific toxicities.

Parkinson protein 7 (protein DJ-1/PARK7), an organ-independent marker redox-sensing protein, was most upregulated in the rat liver, kidney, and testis (Braeuning et al. 2015; Sawada et al. 2015, 2016). And glutathione S-transferase pi 1 (Gstp1) could be recognized as a sensitive biomarker for the early detection of kidney injury induced by 3-MCPD dipalmitate (Sawada et al. 2016). These data also suggest the possible absorption, distribution, and metabolisms of 3-MCPD esters in vivo.

In addition, biomarkers for free 3-MCPD exposure might be used for detecting 3-MCPD ester exposure because 3-MCPD is a possible metabolic intermediate and product of 3-MCPD esters. In 2015, Andreoli and others detected and quantified 2,3-dihydroxypropyl mercapturic acid in human urine samples using an LC-MS/MS analysis (Andreoli et al. 2015). Later in 2016, ^{1}H NMR (proton nuclear magnetic resonance spectroscopy) fingerprints combined with PCA and orthogonal PLS-DA identified seven potential biomarkers for free 3-MCPD exposure using a Wistar rat model (Ji et al. 2016). These biomarkers included ones responsible for the metabolism of glycine, serine, threonine, taurine, and hypotaurine as well as nicotinate and nicotinamide. These biomarkers and analytical approaches are important for early detection and safety assessment of 3-MCPD ester exposures. The biomarkers may allow early detection of MCPD ester exposure and improve food safety. Additional metabolomics studies are needed to identify the practically useful biomarkers for detecting human dietary exposure of MCPD and glycidyl esters.

ABSORPTION, DISTRIBUTION, AND METABOLISM OF 3-MCPD ESTERS

In 2008, Zelinková and colleagues detected 3-MCPD esters in human breast milk at 6–12 μg/kg milk or 328–2,195 μg/kg fat level using GC-MS analysis with a SIM mode (Zelinková et al. 2008). The results suggested the absorption and distribution of 3-MCPD esters in the human body. A few studies have investigated the absorption, distribution, and metabolism of 3-MCPD fatty acid esters (Abraham et al. 2013, Gao et al. 2017).

Recently, 3-MCPD 1-monopalmitate was investigated for its absorption, distribution, and metabolism using male Sprague-Dawley (SD) rats at a single dose of 400 mg/kg 3-MCPD 1-monopalmitate (Gao et al. 2017). 3-MCPD 1-monopalmitate was detected in the plasma samples at the selected time points, with a maximum concentration of 873.72 ng/ml (C_{max}) at 1.67 h (T_{max}) after oral administration. The $t_{1/2}$ was 3.42 h, and the mean resident time was 4 h under the experimental conditions, with the area under the curve (AUC) value of 1,676 h·ng/ml. In addition, eight possible metabolites, including 3-MCPD, sulfonated 3-MCPD, acetylated 3-MCPD, glucuronide 3-MCPD, and 3-MCPD, bonded with different amino acids were tentatively identified in rat liver, kidney, testis, brain, plasma, and urine samples. The authors proposed that 3-MCPD 1-monopalmitate could undergo hydrolysis and lose its fatty acid fragments to form a free 3-MCPD (phase I metabolite), followed by forming conjugated compounds with greater polarity for easy urine excretion (phase II metabolites). Sulfonated 3-MCPD, acetylated 3-MCPD, glucuronide 3-MCPD, and the identified amino acid–conjugated 3-MCPD are the typical phase II metabolites in vivo.

In 2013, Abraham and colleagues orally dosed male Wistar rats with 53.2 mg/kg BW of 3-MCPD dipalmitate (Abraham et al. 2013). No prototype of 3-MCPD dipalmitate was detected in the plasma or other tissue samples, but significant levels of free 3-MCPD were detected in the blood sample, with a C_{max} of 949 μg/ml, a t_{max} of 3.01 h, and an AUC_{24} of 7,760 ng × h/ml. No 3-MCPD dipalmitate was detected in the blood, liver, kidney, or fat 0.5–8 hours after a single treatment dose, whereas it was detected in small and large intestine samples, with 1,158 μg/g detected in the small intestine 1.5 h after treatment and 540 μg/g observed in the large intestine samples 3 and 6 h after 3-MCPD dipalmitate administration. This study did not measure

any possible 3-MCPD dipalmitate metabolites, and the determination of 3-MCPD dipalmitate was carried out using an indirect approach. It needs to be pointed out that commercial 0.5 M NaOMe/MeOH was used to hydrolyze 3-MCPD dipalmitate in methyl tert-butyl ether (MTBE) at ambient temperature in the Abraham et al. (2013) study. This method may underestimate total 3-MCPD esters, as freshly prepared NaOMe/MeOH at ambient temperature for 5 min can act as a nucleophilic agent and convert 3-MCPD dipalmitate to methyl palmitate while releasing free 3-MCPD for derivatization and quantification of total 3-MCPD esters; however, the cleavage reaction may not be 100% complete because of trace amounts of water in the reaction system, which is a water-saturated MTBE solution (Abraham et al. 2013, Weißhaar 2008). Furthermore, a 5-min reaction at ambient temperature may not be adequate for completing the transesterification reaction (Christie et al. 2017). This statement is supported by the greater total 3-MCPD ester contents found when using an indirect approach with H_2SO_4 to release free 3-MCPD as compared to using NaOMe/MeOH at ambient temperature for free 3-MCPD release (Weißhaar 2008). NaCl was also used in the study, which might lead to an overestimation of total free 3-MCPD or its esters because of the possible alkaline-catalyzed Cl^- nucleophilic substitution reactions to form new 3-MCPD esters from tissue triglycerides in the experimental conditions (Abraham et al. 2013, Weißhaar 2008). In addition, Abraham et al. (2013) discussed the bioavailability of 3-MCPD esters and the possibility of complete hydrolysis of 3-MCPD esters to free 3-MCPD in the gastrointestinal tract. The authors assumed 100% hydrolysis of 3-MCPD esters for food safety risk assessment, but no direct experimental data were provided to support this hypothesis (Abraham et al. 2013).

In 2011, Barocelli and colleagues reported a 90-day subchronic toxicological study of 3-MCPD dipalmitate at oral doses of 156.75, 39.19, and 9.78 mg/kg BW per day using male and female Wistar rats (Barocelli et al. 2011). Free 3-MCPD and 3-MCPD mercapturate were detected in the urine samples of rats with 3-MCPD dipalmitate treatment. It was proposed that free 3-MCPD was released from 3-MCPD dipalmitate by lipases in the gastrointestinal tract during the digestion, whereas a glutathione-conjugated 3-MCPD was degraded to a cysteine conjugate, which was acetylated by acetyl-CoA to form a 3-MCPD mercapturate (Barocelli et al. 2011). These data support the absorption and metabolism of 3-MCPD esters in animals.

TOXIC EFFECTS

LD_{50} and Toxicity Classification of 3-MCPD Esters

A few studies have investigated the acute toxicity and reported the median lethal dose (LD_{50}) of 3-MCPD esters (Liu et al. 2012, 2013, 2017). LD_{50} is a very important value for toxicity classification by the Globally Harmonized Hazard Classification and Labeling scheme (GHS) (**https://www.osha.gov/dsg/hazcom/ghsguideoct05.pdf**) and the European Union (EU) classification system. LD_{50} values for 3-MCPD 1-monopalmitate, 3-MCPD 1-monostearate, 3-MCPD 1-monolinoleate, 3-MCPD 1-monooleate, and 3-MCPD 1-palmitate 2-oleate were determined at 2,676.81, 2,973.8, 2,081.4, 2,016.3, and 5,000 mg/kg BW, respectively, in Swiss mice using a LD_{50} test. These five 3-MCPD esters could be classified in the low toxic grade (Category 5) according to the GHS or the EU classification system (Liu et al. 2012, 2017). The LD_{50} value of 3-MCPD dipalmitate was above 5,000 mg/kg BW in Swiss mice using a limit test, which could be recognized as a nontoxic agent (Liu et al. 2012), whereas there is no suitable classification for 3-MCPD 1-oleate 2-palmitate (Liu et al. 2017). These LD_{50} values for mice also indicated that the degree of unsaturation, chain length, number of substitution, and relative substitution locations of fatty acid(s) might alter the toxicity of 3-MCPD esters. The LD_{50} value of 3-MCPD

dipalmitate was also determined at 1,780 mg/kg BW in Wistar rats in another study using 2 rats per group (Li et al. 2013), showing the potential species difference in 3-MCPD ester toxicity. In addition, a 13-week study at 360 μmol/kg BW per day of 3-MCPD esters and 5 days per week using F344 male and female rats obtained no-observed-adverse-effect levels (NOAELs) of 14, 15, and 8 mg/kg BW per day for 3-MCPD dipalmitate, dioleate, and monopalmitate esters, respectively (Onami et al. 2014b). These values are important for risk assessment of MCPD esters. In general, introducing the second fatty acid into a 3-MCPD monoester reduces its toxicity. Other research has investigated the organ-specific toxic effects of 3-MCPD esters (Huang et al. 2018a,b; Liu et al. 2016; Sawada et al. 2015, 2016; Schilter et al. 2011). The reported organ-specific toxic effects of 3-MCPD esters, including nephrotoxicity, testicular toxicity, and other toxic effects, are discussed in the following paragraphs.

Nephrotoxicity of 3-MCPD Esters

Nephrotoxicity is a major toxic effect of 3-MCPD esters and has mostly been investigated in recent years. In 2011, Schilter et al. (2011) reviewed the available knowledge of toxicological effects of 2-, 3-MCPD, and glycidyl esters. They found very limited evidence supporting the hypothesis that the free 3-MCPD released from 3-MCPD esters was responsible for the toxicity of 3-MCPD esters. Schilter et al. (2011) also suggested that MCPD esters might be metabolized in the same way as triglycerides and absorbed into the circulatory system and distributed to tissues and organs where these metabolites and/or their derivatives could act as toxic agents. In addition, Schilter et al. (2011) indicated that the detection of 3-MCPD esters in human breast milk suggested a possible direct absorption of these original MCPD esters. It is reasonable to hypothesize that the presence of 3-MCPD esters in human milk suggests that *sn2*-monoglyceride released by gut lipases could be used as substrates in the biosynthesis of other 3-MCPD esters in human and other animal bodies. To date, there is no experimental evidence to show a 100% conversion of MCPD esters in animal models.

The acute kidney toxicity of 3-MCPD esters has been investigated in Swiss mice (Liu et al. 2012, 2017). Mice fed a single dose of 2,040 mg/kg BW 3-MCPD monopalmitate or 5,000 mg/kg BW 3-MCPD dipalmitate had degenerative changes in different tubular segments of their kidneys. Tubular necrosis, focal degeneration (hydropic degeneration), and protein casts among tubules, along with significantly increased serum urea nitrogen and creatinine levels, were observed in the kidney sections of monopalmitate-treated mice (Liu et al. 2012). This observation was supported by the results from a later study from the same group (Liu et al. 2017). In the study, focal degeneration (hydropic degeneration), tubular dilation, tubular necrosis, and protein casts were detected in the overlying cortex and subjacent medulla of the Swiss mice treated with the three 3-MCPD monoesters and two 3-MCPD diesters in a dose-dependent manner (Liu et al. 2017). Furthermore, a single dose of 3-MCPD 1-palmitate at 1,000 mg/kg BW induced the degeneration, tubular necrosis, and protein casts in time-toxic effects in male SD rats (Liu et al. 2016). These results indicated possible greater kidney toxicity of 3-MCPD monoesters compared to that of the counterpart 3-MCPD diesters.

The molecular mechanisms behind the acute kidney toxicity of 3-MCPD esters have included apoptosis and necroptosis (Huang et al. 2018a, Liu et al. 2016). In 2016, using SD rats, Liu et al. (2016) utilized 3-MCPD 1-palmitate as a probe compound to investigate the possible involvement of the JNK/P53 pathway in the nephrotoxic effect of 3-MCPD esters. 3-MCPD 1-palmitate was able to alter mRNA expression of the genes involved in the mitogen-activated protein kinase (MAPK), p53, and apoptotic signal transduction pathways based on the microarray analysis of the kidney samples. The changes in the mRNA expressions were consistent with the induction of

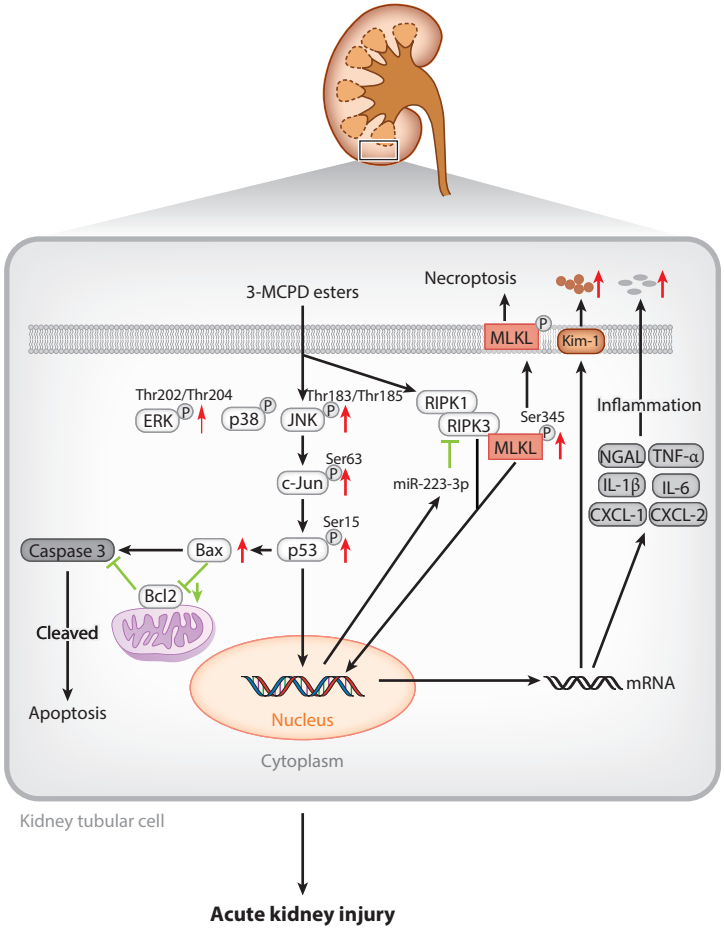

Figure 1

Nephrotoxic mechanisms of 3-MCPD esters. 3-MCPD ester–induced proximal tubular cell apoptosis and necroptosis cause acute kidney injury. 3-MCPD esters activated JNK, which bound and phosphorylated c-Jun on Ser-63 and induced p53 phosphorylation. The activated JNK/p53 signaling pathways induced tubular cell apoptosis by affecting Bax and Bcl-2 expression. However, 3-MCPD esters induced the expression of RIPK1, RIPK3, and MLKL and then induced MLKL phosphorylation. The activated RIPK1/RIPK3/MLKL signaling pathway induced cell necroptosis and inflammation, leading to acute kidney injury. In addition, the miR-223-3p was able to attenuate 3-MCPD ester–induced acute kidney injury by inhibiting RIPK3 expression via binding directly to the 3′ untranslated region of RIPK3 (Huang et al. 2018a, Liu et al. 2016).

tubular cell apoptosis. Furthermore, p53 knockout attenuated the 3-MCPD ester–induced apoptosis and apoptosis-related protein BAX expression and cleaved Caspase-3 activation in the p53 knockout C57BL/6 mice. Furthermore, JNK inhibitor SP600125, but not ERK inhibitor U0126, was able to suppress 3-MCPD ester–induced apoptosis. Together, the results from the Liu et al. (2016) study concluded that JNK/p53 might play an important role in the 3-MCPD ester–induced tubular cell apoptosis (**Figure 1**). A recent study by Huang et al. (2018a) investigated whether and how necroptosis is involved in the nephrotoxic effect of 3-MCPD esters using 3-MCPD dipalmitate as a probe compound in C57 BL/6 mice. 3-MCPD dipalmitate was able to alter mRNA

expressions of the genes involved in the necroptosis pathway and inflammation, including the receptor-interacting protein 1 (RIPK1), RIPK3, and mixed lineage kinase domain-like protein (MLKL). The role of 3-MCPD dipalmitate in necroptosis and inflammatory cytokine production was confirmed with additional experiments involving the deletion of the *RIPK3* or *MLKL* gene and the inhibition of RIPK1, and microRNA analysis shows the suppression of RIPK3 expression by miR-223-3p through binding to the 3′ untranslated region of RIPK3 (**Figure 1**). Generally, necroptosis can be initiated via various signaling pathways. Huang et al. (2018a) investigated only the activated RIPK1/RIPK3/MLKL pathway, and additional research is needed to examine the role of 3-MCPD esters in possible necroptosis pathway activations as well as the potential relationship(s) between necroptosis and apoptosis in 3-MCPD ester–induced acute kidney injury.

In 2011, 3-MCPD dipalmitate at 156.75, 39.19, and 9.78 mg/kg BW per day for 90 days resulted in nephrotoxicity to both male and female Wistar rats, and more tubulotoxicity in the female rats (Barocelli et al. 2011). The benchmark doses (BMD_{10}) and BMDL10 for severe kidney damage in male rats were calculated to be 41.1 and 17.4 mg/kg per day, respectively. In 2013, Li et al. (2013) conducted another 90-day toxicology study using 3-MCPD dipalmitate as the treatment agent in Wistar rats. Li and others observed that 3-MCPD dipalmitate caused a significant increase in blood urea nitrogen and creatinine in the high-dose group (267 mg/kg BW per day) compared to the control rats, along with renal tubular epithelial cell degeneration and hyaline cast accumulation (Li et al. 2013). In 2014, Onami et al. (2014a) observed renal toxicity in F344 *gpt* rats given 3-MCPD dipalmitate, dioleate, and monopalmitate at 220, 240, and 130 mg/kg BW, respectively, via an intragastric tube for 4 weeks (5 times per week). Also in 2014, Onami et al. (2014b) carried out a 13-week study using F344 rats. The absolute and relative kidney weights were significantly increased in the 3-MCPD ester–treated rats at medium and high doses of 55 and 220 mg/kg BW for 3-MCPD dipalmitate, 32 and 130 mg/kg BW for 3-MCPD monopalmitate, and 60 and 240 mg/kg BW for 3-MCPD dioleate. No significant renal lesion was observed in any of the rats treated with 3-MCPD dipalmitate, 3-MCPD 1-palmitate, or 3-MCPD oleate with doses of 220, 130, and 240 mg/kg BW per day, respectively, at the end of the feeding experiment. The results also indicated that these three 3-MCPD esters may still have potential subchronic toxicity to rat kidneys, and the NOAELs of 3-MCPD dipalmitate, monopalmitate, and dioleate were suggested to be 14, 8, and 15 mg/kg per day, respectively, for F344 rats.

In 2016, a repeated-dose 28-day oral toxicity study for 3-MCPD diester in Wistar rats was conducted (Sawada et al. 2016). The results indicated that although no histopathologically visible toxicity was observed, a difference in proteins related to various metabolic pathways, such as carbohydrate, amino acid, and fatty acid metabolism, was detected. These results confirmed the hypothesis that 3-MCPD esters might have potential nephrotoxicity at the protein level. This study did not analyze proteomic changes in different kidney cells.

Taken together, 3-MCPD esters have shown acute and chronic nephrotoxicity. The nephrotoxicity can be reflected in changes in kidney weight, renal function–related chemical indicators, histopathological observation, and changes in molecular biological markers. The possible mechanisms behind the nephrotoxicity may be attributed to the JNK/p53-induced tubular cell apoptosis and RIPK1/RIPK3/MLKL-mediated necroptosis (**Figure 1**).

Testicular Toxicity of 3-MCPD Esters

Testicular toxicity is also a major toxic effect of 3-MCPD esters. 3-MCPD esters had potential toxic effects on the structure and function of testes, such as changes in organ weights and

histopathological appearance (Liu et al. 2012). In 2015, Sawada and colleagues utilized 3-MCPD dipalmitate as a probe compound to investigate the proteomic changes induced by 3-MCPD esters in Wistar rats (Sawada et al. 2015). The Wistar rats were repeatedly dose fed with 53 mg/kg BW 3-MCPD dipalmitate for 28 days. Although there was no statistical difference in testes weight or visual difference, proteome analysis showed that there are 58 proteins in rat testes influenced by 3-MCPD dipalmitate treatment. Furthermore, an important protein, DJ-1, might function as a sensitive molecular marker of 3-MCPD esters, which is related to their testicular toxicity. Compared to the proteomic study in kidney and liver, two important proteins, GSHB and DJ-1, that linked to oxidative stress were affected by 3-MCPD ester treatments in all three organs (Braeuning et al. 2015). In summary, a limited amount of available research data suggests the possible testicular toxicity of 3-MCPD esters.

Other Toxic Effects of 3-MCPD Esters

In addition to major toxicity in kidney and testes, other toxicities such as hepatotoxicity, neurotoxicity, and immunotoxicity have been reported. Growing evidence indicates that 3-MCPD esters may have potential hepatotoxicity, leading to altered liver weight, histopathological changes, including swelling, inflammatory cell infiltration, and liver fat accumulation (Abraham et al. 2013, Braeuning et al. 2015, Liu et al. 2012, Lu et al. 2015, Onami et al. 2014a). In 2015, 18–22-g male C57BL/6J mice were given 3-MCPD dipalmitate, distearate, dioleate, and dilinoleate esters at three levels daily by gavage feeding for four weeks to evaluate their potential effects on lipid metabolism (Lu et al. 2015). At the greatest treatment dose of 16.5 μmol/kg BW per day, all the tested 3-MCPD diesters significantly elevated hepatic total triacylglycerol (TAG), hepatic total cholesterol, serum total cholesterol, and serum low-density-lipid cholesterol and accelerated liver fat accumulation, suggesting liver toxicity. 3-MCPD dipalmitate and dilinoleate at 16.5 μmol/kg BW per day also significantly increased serum aspartate aminotransferase (AST). Furthermore, the dioleate esters were able to increase AST at both 6 and 16.5 μmol/kg BW per day doses. Interestingly, 3-MCPD distearate did not alter AST under the same experimental conditions (Lu et al. 2015). The liver toxicity of 3-MCPD esters has also been demonstrated by a MALDI-MS proteomic analysis using 3-MCPD dipalmitate at 53 and 13.3 mg/kg BW per day as the treatments along with an equimolar 3-MCPD (10 mg/kg BW per day) and a corn oil vehicle control (Braeuning et al. 2015). Both 3-MCPD esters and 3-MCPD altered many rat liver proteins, such as those for DJ-1/PARK7, the urea cycle, ethanol degradation, arginine biosynthesis, fatty acid metabolism, cholesterol biosynthesis, oxidative stress and NRF-2-mediated oxidative stress response, LPS/IL-1-mediated inhibition of RXR function, and dopamine degradation (Braeuning et al. 2015). It needs to be pointed out that these changes at the proteomic level were not accompanied with significant clinical signs of toxicity measured by clinical observations and histopathological examinations, suggesting the potential of these liver proteins, especially those that are organ-independent such as DJ-1/PARK7, as biomarkers for early detection of 3-MCPD esters exposure.

In addition, abnormal behavior (lateral walking) and histopathological difference (small spongiotic lesions with slight gliosis and focal encephalomalacia change) indicated the potential neurotoxicity of 3-MCPD ester in the brain and central nervous system (Barocelli et al. 2011; Liu et al. 2012, 2017; Onami et al. 2014b). In 2017, difficult and labored breathing accompanied with abdominal twitching were also observed in the Swiss mice fed 3-MCPD esters, indicating a possible pulmonary tract problem potentially linked to a central nervous system impairment and pulmonary edema (Liu et al. 2017).

In 2017, Liu et al. (2017) observed apoptotic bodies in the thymus, suggesting possible immune suppression of 3-MCPD esters in Swiss mice. In 2018, Huang et al. (2018b) reported the

toxic effects of 3-MCPD esters in inhibiting the immune system possibly through suppressing T-lymphocyte activation using 3-MCPD 1-palmitate, 2-palmitate, and dipalmitate as the probe compounds in an in vitro model and a female C57 BL/6 mouse model. 3-MCPD 1-palmitate and 2-palmitate at 300 μM, but not dipalmitate, significantly suppressed ConA-induced T-lymphocyte proliferation, cell cycle activity, Th1 and Th2 cytokine secretion, $CD4^+$ T cell populations, and the ratio of $CD4^+/CD8^+$ T cells, reflecting their immune-suppressing toxicity. The activation of ConA-stimulated MAPK and nuclear factor-κ B signaling pathways was also suppressed. In addition, 3-MCPD1-palmitate significantly suppressed 2,4-dinitrofluorobenzene (DNFB)-induced delayed-type hypersensitivity reaction in the mice (**Figure 2**). Additional studies are needed to further investigate the toxic effects of 3-MCPD esters in inhibiting the immune system and the molecular mechanisms behind these effects.

In 2017, lung lesions, including alveolar edema, weight increase, and the hyperemic or hemorrhagic zones, were observed in Swiss mice treated with 3-MCPD 1-stearic, 1-oleic, 1-linoleic,

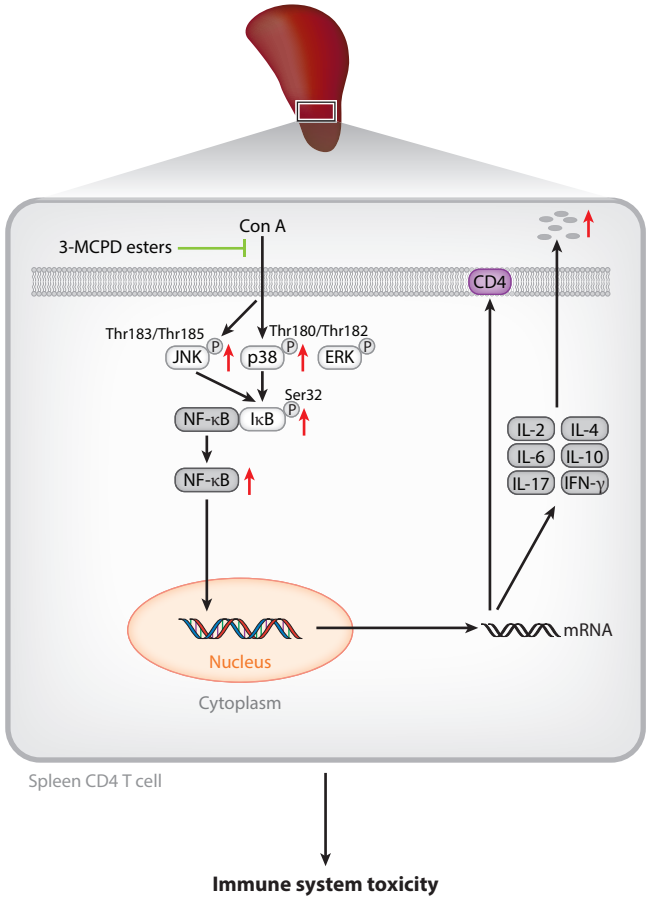

Figure 2

Toxicology mechanisms of 3-MCPD esters in the immune system. 3-MCPD esters suppressed ConA-induced T-lymphocyte proliferation, cell cycle activity, Th1 and Th2 cytokine secretion, $CD4^+$ T cell populations, and the ratio of $CD4^+/CD8^+$ T cells, which clinically presents as immunosuppressive via the MAPK/NF-κB signaling pathways (Huang et al. 2018b, Liu et al. 2017).

1-linoleic-2-palmitic, and 1-palmitic-2-linoleic acid esters (Liu et al. 2017), suggesting potential lung toxic effects of these 3-MCPD esters. These preliminary data suggest potential toxicity of 3-MCPD esters in respiration systems, warranting additional research involving more animal species and cell models to investigate the toxic effects and the molecular mechanism(s) behind them.

In addition, dosing of 3-MCPD monopalmitate, dipalmitate, and dioleate at 130, 240, and 240 mg/kg BW levels, respectively, and 5 days per week for 4 weeks did not result in detectable in vivo genotoxicity in F344 gpt delta rats (Onami et al. 2014a), although kidney toxicity was observed under experimental conditions. Future research is needed involving more animal models and 3-MCPD ester compounds to confirm Onami et al.'s (2014a) conclusion.

In summary, nephrotoxicity and testicular toxicities are the two most researched toxicities of 3-MCPD esters; other toxicities such as hepatotoxicity, neurotoxicity, and immunotoxicity have also been detected in a few studies. The apoptosis and necroptosis in proximal tubular cells have been accepted to be the molecular mechanism of 3-MCPD ester–induced kidney toxicity in a limited number of studies (**Figure 1**). Suppressing T-lymphocyte activation has been suggested to be a molecular mechanism of 3-MCPD ester–induced immunotoxicity (**Figure 2**). Additional research is needed to advance our understanding of the toxic effects of 3-MCPD esters and the molecular mechanisms behind these effects. The major limiting factors for the toxicology research are (*a*) the analytical methods for individual 3-MCPD ester compounds and (*b*) the high purity of 3-MCPD probe or testing compound(s).

FORMATION MECHANISM OF 3-MCPD ESTERS

Understanding the formation mechanisms of 3-MCPD esters is important for developing mitigation strategies for human health. In general, cation-mediated and free radical–mediated molecular mechanisms have been proposed and discussed (Collier et al. 1991; Hamlet et al. 2003; Rahn & Yaylayan 2011a; Svejkovská et al. 2006; Zhang et al. 2013, 2015; Zhao et al. 2016). In 1991, Collier and colleagues investigated the molecular mechanisms behind the formation of chloropropanols and proposed that 3-MCPD diesters might be formed from triglycerides via acid-catalyzed nucleophilic substitution reactions of chlorine anion (Cl^-) attacking in an acidic aqueous solution (**Figure 3**, **path *a***) (Collier et al. 1991). This study also proposed the formation of 2-MCPD and 3-MCPD derivatives through an epoxide three-member ring structure or a five-atom cyclic carbocation intermediate mechanism in an acidic aqueous condition (**Figure 3**, **path *d***). The mechanisms were proposed based on the ratio and type of the MCPD-derivative products (Collier et al. 1991). Later in 2003, Hamlet et al. (2003) observed the influence of composition, water content, acidity, and temperature on the formation and decay of free MCPD compounds as well as the possible conversion between 3- and 2-MCPD. These mechanisms may also be applicable to 3- and 2-MCPD esters in an acidic aqueous condition. The formation of 3-MCPD esters was also investigated in the simulated food models containing NaCl, water, and glyceride compounds, including soybean oil, 1-monopalmitin, and 1,3-dipalmitin or tripalmitin (Svejkovská et al. 2006). The study concluded that 1-monopalmitin, but not dipalmitin, is a preferred precursor for 3-MCPD esters, whereas soybean oil and tripalmitin formed fewer 3-MCPD esters under the same reaction conditions. NaCl, water level, and temperature altered the formation of 3-MCPD esters under the experimental conditions, but acidity was not examined in the study. In the model system, 20% water resulted in the highest level of 3-MCPD esters, whereas the level of Cl^- was proportional to the final amount of 1-monopalmitin (Svejkovská et al. 2006). In addition, the formation of a cyclic acyloxonium cation was investigated using the isotope-labeling technique and by monitoring the infrared (IR)

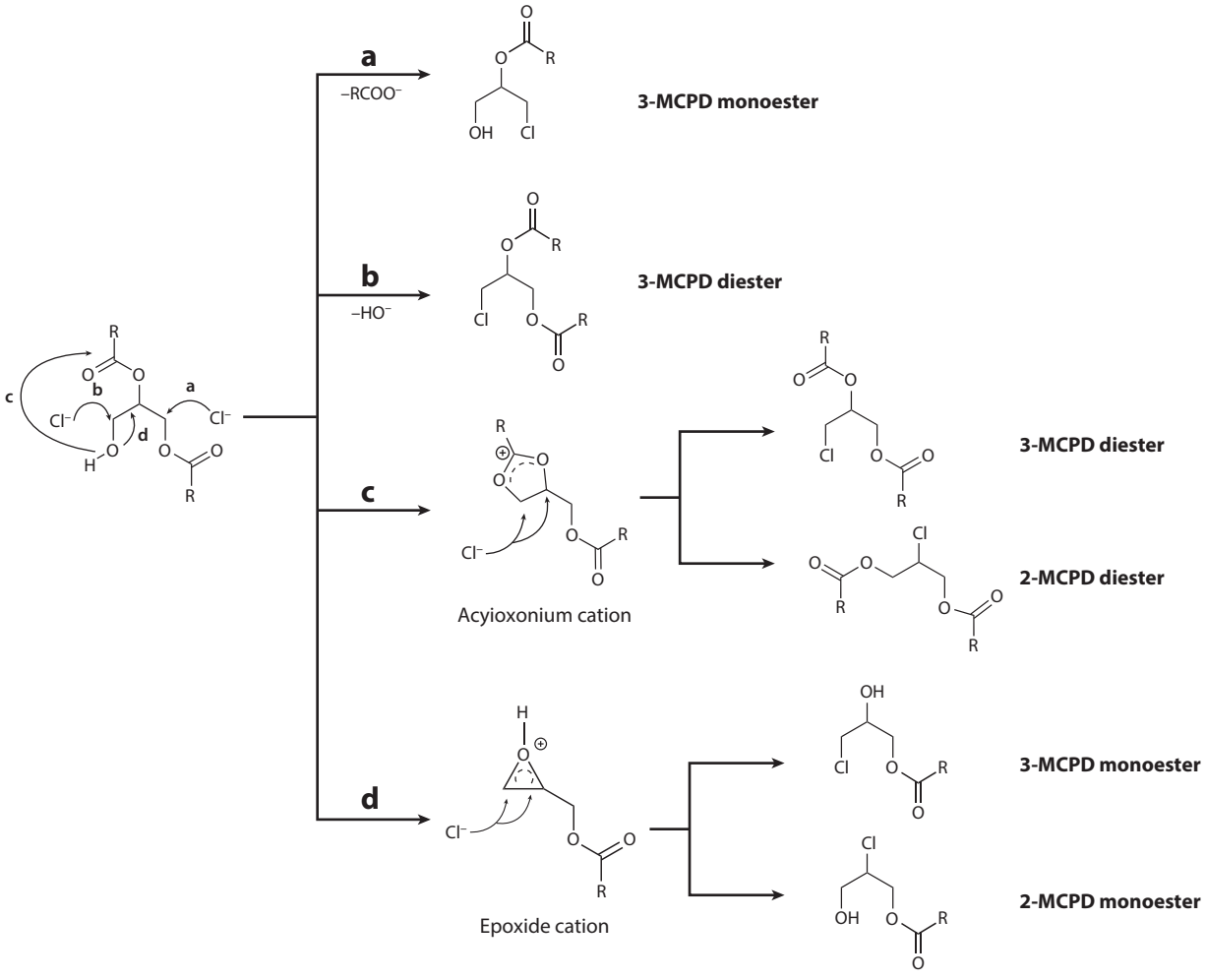

Figure 3

Summary of cation-mediated formation mechanisms of 3-MCPD esters. Path *a* represents a direct nucleophilic substitution by a chloride anion attacking with $RCOO^-$ as a leaving group. Path *b* represents a direct nucleophilic substitution by a chloride anion attacking with HO^- as a leaving group (loss of a molecule of H_2O). Path *c* represents the formation of an acyloxonium cation intermediate followed by a nucleophilic ring opening reaction with a chlorine anion attacking the sn-3 or sn-2 carbon to form 3-MCPD or 2-MCPD diesters, respectively. Path *d* represents the formation of an epoxide intermediate followed by a nucleophilic ring opening reaction by chlorine anion attacking the sn-3 or sn-2 carbon to form 3-MCPD or 2-MCPD monoesters, respectively. R represents the hydrocarbon chain of a fatty acid (Rahn & Yaylayan 2011b), and the asterisk indicates the possible formation of 3- and 2-MCPD esters.

spectroscopy of a heated mixture of $ZnCl_2$ and tripalmitin, 1,2-dipalmitin, or 1-monopalmitin (Rahn & Yaylayan 2011b). The presence of an esterified acyloxonium cation was speculated when heating $ZnCl_2$ and 1,2-dipalmitin together to 100°C, according to the IR data. The IR data confirmed that the chemical environment of the two carbonyl groups in the 1,2-dipalmitin was changed or one of the carbonyl groups was lost under the experimental conditions, supporting the formation of an acyloxonium ring intermediate, but no direct evidence was available to support the formation of a cation intermediate in a dry reaction condition.

Rahn & Yaylayan (2011b) summarized the four possible molecular mechanisms behind the formation of 3-MCPD mono- and di-fatty acid esters (**Figure 3**), including a direct nucleophilic substitution by a chloride anion attacking, with $RCOO^-$ (**Figure 3**, **path *a***) or HO^- (**Figure 3**, **path *b***) as a leaving group, as well as through the formation of an acyloxonium cation intermediate followed by a nucleophilic ring opening reaction by a chlorine anion attacking the sn-3 or sn-2 carbon to form 3-MCPD or 2-MCPD diesters, respectively (**Figure 3**, **path *c***), or the formation of an epoxide ring intermediate followed by a nucleophilic ring opening reaction by chlorine anion attacking the sn-3 or sn-2 carbon to form 3-MCPD or 2-MCPD monoesters, respectively (**Figure 3**, **path *d***).

In 2012, Destaillats et al. (2012) investigated the formation of MCPD fatty acid diester by heating a mixture of triheptadecanoin and lindane, an organochlorine reagent, and observed MCPD diester formation at 200°C and above, in a temperature-dependent manner. No MCPD diester was detected in the temperature range of 100–180°C. The observation could not be explained by any of the reported cation or glycidol mechanisms, suggesting that a new mechanism(s) may exist in 3-MCPD ester formations under low-moisture, high-temperature conditions.

In 2013, a free radical intermediate mechanism was hypothesized and confirmed for 3-MCPD diester formation from 1,2-stearoylglycerol (**Figure 4**) (Zhang et al. 2013). In this study, electron spin resonance spectroscopy signals of the oil heated at 120°C for 20 min were much stronger than those of the oil kept at 80°C for 20 min, suggesting a radical formation in a temperature-dependent matter under a low-moisture experimental condition. Diacylglycerol (DAG) was measured for its Fourier transform–infrared spectroscopy (FT-IR) spectra at 25°C and 120°C to examine whether the ester carbonyl groups were involved in the 3-MCPD ester formation under low-moisture, high-temperature conditions. The FT-IR data indicated that there is a possible involvement of an ester carbonyl group in forming 3-MCPD esters or that the two carbonyl groups were in the same chemical environment. Furthermore, the signals of a cyclic acyloxonium free radical (CAFR) at the carbonyl carbon and the DMPO-trapped CAFR were obtained using a high-resolution ESI-MS/MS analysis approach. These data further confirmed the free radical mechanism for 3-MCPD ester formation under low-moisture, high-temperature conditions. This first report of the free radical mechanism for 3-MCPD diester formation under low-moisture, high-temperature conditions has provided important scientific insight for mitigating 3-MCPD diesters in refined edible oils.

In 2015, Zhang et al. (2015) reported that TAGs are potential precursors to 3-MCPD mono- and diester formation through a cyclic acyloxonium or a glycidyl ester radical intermediate under low-moisture, high-temperature conditions. This study also explained how glycidyl esters, together with 3-MCPD esters, might be formed from TAG. In addition, Fe^{2+} and Fe^{3+} were observed for their potential in catalyzing the formation of both 3-MCPD and glycidyl esters from TAG (**Figure 4**) (Zhang et al. 2015). Fe^{2+} and Fe^{3+} stimulated free radical formation in the reaction mixtures. These results are important for the development of mitigation approaches for the levels of 3-MCPD and glycidyl esters in the refined edible oils and food products. Later in 2016, a free radical intermediated reaction mechanism was confirmed for 3-MCPD ester formation from monoglycerides (Zhao et al. 2016). For the first time, it was proposed by Zhao et al. (2016) that the six-member cyclic acyloxonium structure might be present in the free radical intermediate(s) to explain the coexistence of 3-MCPD and 2-MCPD esters under low-moisture, high-temperature conditions (**Figure 4**). These previous studies also showed the importance of temperature and the type and level of the chlorine compound and catalysts on final MCPD contents in the food products.

In summary, the formation of 3-MCPD esters from mono-, di-, and triglycerides may undergo a cation- or free radical–mediated reaction mechanism. The moisture content, temperature,

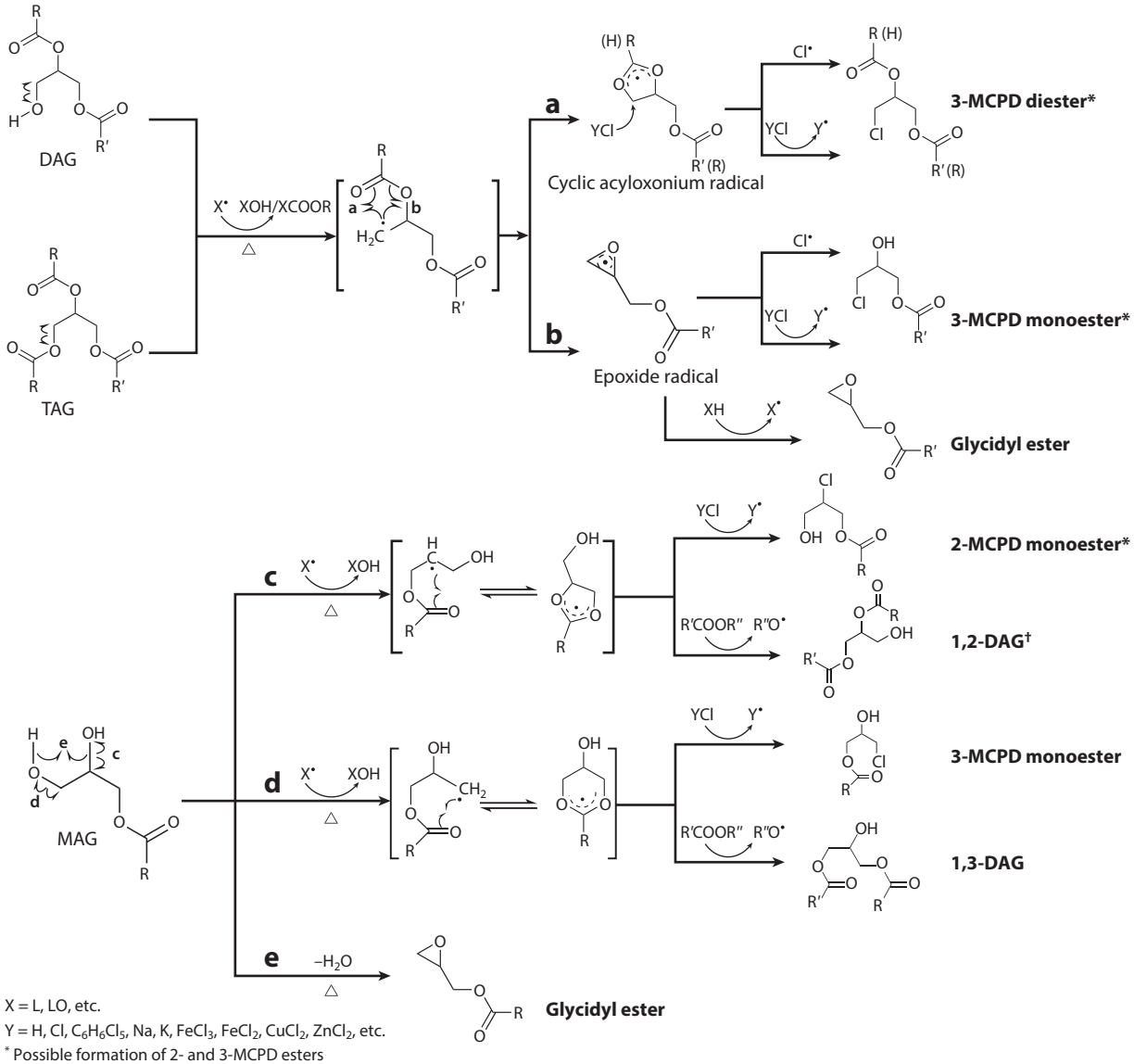

Figure 4

Free radical–mediated formation mechanisms of 3-MCPD esters, 2-MCPD esters, and glycidyl esters. The upper panel represents the putative pathways with a carbon-centered radical and immediate formation of an epoxide (*path a*) or a cyclic acyloxonium free radical intermediate (*path b*) via elimination of an RCOO• or HO• from C-1 or C-3 of triacylglycerol (TAG) and diacylglycerol (DAG). This is followed by free radical ring opening reactions to form 3-MCPD and 2-MCPD mono- and di-esters (*paths a*, *b*), as well as glycidyl esters (*path a*). The lower panel represents the putative pathways (*paths c*, *d*, *e*) to form MCPD esters and coproducts from monoacylglycerol (MAG). Paths *c* and *d* represent cyclic acyloxonium free radical intermediate-mediated reactions, followed by radical ring opening reactions to obtain mono- and diesters of 2- and 3-MCPD esters and 1,2- and 1-3-diacylglycerol. Path *e* represents a free radical–mediated loss of a molecule of H_2O to form glycidyl esters (Zhang et al. 2013, 2015; Zhao et al. 2016). L and LO are lipids and lipid oxides, and the lipids can be TAG, DAG, and MAG.

presence of catalysts, acidity, and type of chlorine compounds are important in determining the reaction mechanism(s). These findings are important for oil-refining and food processing industries to develop mitigation approaches for 3-MCPD esters and coproducts in final food products.

MITIGATION APPROACHES OF 3-MCPD ESTERS

There have been many efforts to mitigate 3-MCPD ester levels in food products (Cheng et al. 2017, Craft & Nagy 2012, Matthäus & Pudel 2013, Ramli et al. 2015). Generally, one may reduce 3-MCPD ester levels by reducing their formation during food and oil processing and storage, and eliminating the existing 3-MCPD esters (Craft & Nagy 2012, Matthäus & Pudel 2013).

Reducing 3-MCPD Ester Formation

Chlorine donors are critical for MCPD ester formation in food and oil products. It has been demonstrated that lower contents of inorganic and organic chlorine agents such as NaCl and lindane may be a practical approach to mitigate 3-MCPD esters in the final oil and food products (Hamlet et al. 2004, Svejkovská et al. 2006). The inorganic chlorines contribute the Cl element when a cation-mediated 3-MCPD ester formation mechanism exists, whereas both inorganic and organic chlorine compounds may be involved in 3-MCPD ester formation through a free radical–mediated reaction mechanism under low moisture, high-temperature conditions (Hamlet et al. 2004; Šmidrkal et al. 2016; Svejkovská et al. 2006; Zhang et al. 2013, 2015, 2016; Zhao et al. 2016). Eliminating and controlling the chlorine compound level in ingredients and processing aids such as washing water through improving agricultural practice, crude oil processing and storage, oil refining, and food preparation and storage are critical to possibly reducing the final 3-MCPD ester level in food and oil products.

Whether DAGs and free fatty acids (FFAs) influence the final 3-MCPD ester contents in foods has also been investigated (Matthäus & Pudel 2013, Matthäus et al. 2011, Pudel et al. 2011, Šmidrkal et al. 2011). Removal of DAGs and/or FFAs has been shown to significantly reduce the overall 3-MCPD formation during the oil-refining process in a few previous studies (Šmidrkal et al. 2011, Svejkovská et al. 2006). However, DAGs and FFAs had no influence on 3-MCPD ester formation during oil processing in other previous studies (Ramli et al. 2015). It has been suggested that 4% DAG is a possible threshold for stimulating 3-MCPD ester formation in oil processing (Matthäus et al. 2011). TAGs, DAGs, and monoacylglycerols (MAGs) are all able to form 3-MCPD esters under both low-moisture, high-temperature and high-moisture, acidic conditions. MAGs formed more 3-MCPD esters than did DAGs or TAGs in an emulsion food model (Svejkovská et al. 2006), but DAGs formed more 3-MCPD esters in another study under simulated deodorization conditions (Šmidrkal et al. 2016). FFAs could also act as an H^+ donor in elevating 3-MCPD ester formation because neutralization of FFAs with bicarbonate or carbonate salts resulted in reduced 3-MCPD ester formation (Šmidrkal et al. 2011). Reduction of acidity of a crude oil has been demonstrated to reduce the 3-MCPD ester level in the final oil product (Ramli et al. 2015, Šmidrkal et al. 2016).

In addition, modification of oil-processing and oil-refining conditions via, e.g., controlling the temperature in the deodorization step, adding alkaline salts before deodorization, washing crude oil with organic solvents, reducing chloride concentration in the strip steam water, washing bleached oil with ethanol before deodorization, adding chelating agents, and changing crude oil processing conditions, agricultural practices, and postharvest treatments may alter the overall 3-MCPD esters in the refined oils (Craft & Nagy 2012, Li et al. 2016, Matthäus & Pudel 2014, Pudel et al. 2011, Šmidrkal et al. 2011, Zhang et al. 2015). Recently, food-grade antioxidants (Li

et al. 2015) and dietary free radical scavengers (Zhao et al. 2016) have been shown to effectively reduce 3-MCPD ester formation under low-moisture, high-temperature conditions, similar to that of the deodorization step of the oil-refining process, possibly through suppressing free radical formation and its availability. Interestingly, a study revealed that food preparation conditions including the prefrying temperature and the thermal treatment period of the prefrying oil, are critical to overall 3-MCPD esters in the finished food products (Merkle et al. 2018). This is one of the few studies that investigated 3-MCPD ester formation and mitigation in processed food products.

Eliminating or Removing 3-MCPD Esters from Oil and Food Products

In 2010, Bornscheuer & Hesseler (2010) reported enzymatic approaches for reducing 3-MCPD esters by converting them into 3-MCPD with *Candida antarctica* lipase A, which was further converted to harmless glycerol in an aqueous system using *Arthrobacter* sp. AD2 halohydrin dehalogenase. Later in 2011, Strijowski et al. (2011) reported a possibility to remove up to 40% glycidyl esters from the refined oils using adsorbing materials, including calcined zeolite and magnesium silicate. Recently, Zhao et al. (2016) reported the degradation of 3-MCPD monoesters upon thermal treatment and a potential catalytic role of Fe^{3+} in the thermal degradation of 3-MCPD monoesters under low-moisture, high-temperature conditions, which supports the possibility of eliminating 3-MCPD esters from the refined oils by thermal treatment with a possible catalyst.

PERSPECTIVES

Significant research has been performed in detecting 3-MCPD esters and related compounds in foods and oils, evaluating their toxic effects and molecular mechanisms, understanding the chemical mechanisms behind their formation during food processing and storage, and discovering the biomarkers for early exposure detection as well as toward possible approaches for reducing their levels in edible oils and food products. These previous studies also revealed many new scientific questions and warrant additional research. Potential future research should advance the analytical methods to better detect individual 2- and 3-MCPD esters with different fatty acids along with glycidyl esters. The advancement of analytical methods is a base for further investigation of absorption, distribution, metabolism, and excretion of MCPD esters as well as the toxicokinetics of individual MCPD ester compounds. A major challenge is the lack of standard compounds for quantification of individual MCPD esters, which requires additional efforts in developing standard compound synthesis and purification. More information is needed for the organ-specific toxic effects and molecular mechanisms of 3- and 2-MCPD esters. This includes the possible different toxic effects and molecular mechanisms of 3- and 2-MCPD esters and the effects of number, chain length, and saturation degree of fatty acids on the toxic effects (the structure–toxicity relationships). The organ-specific toxic effects and molecular mechanisms also may include future omics studies for new biomarkers of their exposure and identification of their new potential toxic metabolites. The information is very important for overall risk assessment and setting up potential regulation guidelines for this group of toxicants. In addition, practical approaches to eliminating or reducing MCPD esters in oils and foods are needed for improving food safety. This may include mechanism-based investigation of individual food formulation and processing conditions and their combinations for possible effects on the final level of MCPD esters in the food and oil products.

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