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Development of analytical and reduction methods of 2- and 3-monochloropropanediol esters in oil products and estimation of their digestion and intestinal absorption

Naoki Kaze
2016
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Abbreviations

1,3-DCP  1,3-Dichloro-2-propanol
MCPD  Monochloropropanediol
3-MCPD  3-Monochloropropane-1,2-diol
2-MCPD  2-Monochloropropane-1,3-diol
JECFA  Joint FAO/WHO Expert Committee on Food Additives
IARC  International Agency for Research on Cancer
DAG  Diacylglycerol
LCMS  Lipid chromatography mass spectrometry
GCMS  Gas chromatography mass spectrometry
DGF  Deutsche Gesellschaft fur Fettwissenschaft
MAG  Monoacylglycerol
3-MCPD-$d_5$  3-Monochloropropanediol-deuterated
2-MCPD-$d_5$  2-Monochloropropanediol-deuterated
NMR  Nuclear magnetic resonance
D$_2$O  Deuterium oxide
CDCl$_3$  Deuterated chloroform
DMSO  Dimethyl sulfoxide
HPLC  High-performance lipid chromatography
t-BME  tert-Butyl methyl ether
Tris  Tris-(hydroxymethyl)-aminomethane
DMEM  Dulbecco’s modified Eagle’s medium
EDTA  Ethylenediaminetetraacetic acid
TEER  Transepithelial electrical resistance
HBSS  Hank’s balanced salt solution
CAD  Corona charged aerosol detection
TAG  Triacylglycerol
FFA  Free fatty acid
MCPD-FS  MCPD-forming substances
Chapter 1 : General introduction

Ensuring the safety of a nation’s food supply is an important part of protecting the health of its citizens. In recent years, there have been increasing concerns about the safety of food imported into Japan, for example, bovine spongiform encephalopathy (BSE) in beef, avian influenza, food poisoning by *Escherichia coli* O157, melamine and other toxins, and pesticides. Furthermore, food-born contaminants such as acryl amides, furans, heterocyclic amines, and chloropropanols, which are generated in food processing, have been detected, and this is a cause for concern owing to their potential toxicity and negative effects on human health.

**Chloropropanols, the food contaminants**

1,3-Dichloro-2-propanol (1,3-DCP) and 3-monochloropropane-1,2-diol (3-MCPD) are in the class of compounds called chloropropanols (Fig. 1), and have been predicted to be toxic to humans. In the latter half of the 1970s, 3-MCPD has been detected in acid hydrolyzed vegetable protein (HVP), which is used widely as an ingredient for seasoning and for related products, such as soy sauce [1].

![Structures of chloropropanols and glycidol](image)

**Figure 1.** Structures of chloropropanols and glycidol.
Risk assessment and the development of reduction methods of 3-MCPD in foods began immediately in Japan, because this harmful compound was found in common foods that are consumed by many citizens on a daily basis. In the seasoning-related industry, manufacturing methods were improved to reduce MCPDs. Now, very little 3-MCPD can be detected in commercial seasoning products used in Japan.

**Toxicological profile of 3-MCPD**

*In vitro* assays and animal bioassays were carried out to investigate the toxicological profile of 3-MCPD [2, 3]. *In vitro* assays for mutagenicity in bacteria and in mammalian cells were reported to be positive only in high concentrations of 3-MCPD, and negative results were obtained in the presence of an exogenous metabolic activation system from mammalian tissue. The results of *in vivo* assays, including a test for micronucleus formation in mouse bone marrow and an assay for unscheduled DNA synthesis in rats, were negative. Taken together, the data indicated that 3-MCPD is not genotoxic *in vivo*.

The median lethal dose of 3-MCPD in rats after oral administration was reported to be 150 mg/kg of body weight. In several short-term studies of rats and mice, the kidney was shown to be the target organ for toxicity. 3-MCPD was reported to increase the weight of the kidneys relative to body weight, in a 4-week study in rats treated by gavage at 30 mg/kg of body weight per day, and in a 13-week study in rats given an oral dose of 9 mg/kg of body weight per day.

In addition to the above studies, the weight of the kidney was reported to be significantly increased by the administration of 3-MCPD in drinking water, demonstrating a dose-response relationship, at all doses tested in the pivotal long-term study in Fischer 344 rats. Overt nephrotoxicity was seen at higher doses, 5.2 and 28 mg/kg of body weight per day.

In contrast to kidneys, there was no statistically significant increase in
malignant tumors, which would have indicated carcinogenicity of 3-MCPD. There has been no report either that 3-MCPD in food has directly harmed human health. Based on the above survey, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) set the maximum tolerable intake of 3-MCPD at 2 µg/kg body weight per day [2].

International Agency for Research on Cancer (IARC) further evaluated the risk of carcinogenicity of 3-MCPD in 2012 based on the publications reported after the assessment of JECFA, and concluded that there has not been sufficient evidence to prove the carcinogenicity of 3-MCPD in humans, though there has in vivo assays using animals. Thus, IARC categorized 3-MCPD as ‘probably carcinogenic to humans (Group 2A)’ [4].

3-MCPD in foods
Contamination of food with 3-MCPD has been investigated in cereals, marine products, meats, dairy products, oils and fats, and confectionaries, in addition to seasoning products [5, 6, 7]. 3-MCPD exists as a free form in seasoning products, but exists as the ester form bound to fatty acids in oils and fats. It has also been reported that the amount of 3-MCPD detected by assays tended to increase after cooking, particularly flying in oil.

In Japan in 2009, testing of a commercial edible oil that consisted mostly of diacylglycerols (DAG-oil) resulted in the detection of a relatively high amount of 3-MCPD. Later, it was revealed that the standard method established by the Deutsche Gesellschaft für Fettwissenschaft (DGF), namely the only standard method to quantify 3-MCPD that time, had in fact detected a fatty acid ester of glycidol as 3-MCPD. As 3-MCPD and glycidol esters might cause adverse effects on health, production of DAG-oil was discontinued. This change took place, even though the toxicity of 3-MCPD and glycidol in fatty acid ester form had not been specifically demonstrated. In vitro assays revealed that 3-MCPD and glycidol esters were hydrolyzed by pancreatic lipase to produce free 3-MCPD and glycidol. Thus, it has been
reasonable to presume that 3-MCPD and glycidol esters would be hydrolyzed to their free forms after intake and would have toxicity similar to free 3-MCPD and glycidol.

The incident in 2009 sparked an explosion of the investigations and risk assessments of 3-MCPD and glycidol contained in fat and oil products. The oil and fat industry started to develop methods to reduce the concentration of these compounds in processed oils and fat products.

The compounds 3-MCPD and glycidol esters are unintentionally generated in the manufacturing processes of oils and fats, and fat products. It can be assumed that we have been consuming these compounds in our foods for many years, including before they were detected in DAG-oil incident. Ironically, the major contributor to the finding of these compounds may have been the development of microanalytical instruments and methods that have a higher sensitivity for detection of trace amounts of food contaminants.

**Table 1** Properties of direct and indirect detection methods for 3-MCPD esters and glycidyl esters.

<table>
<thead>
<tr>
<th>Property</th>
<th>direct</th>
<th>indirect</th>
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<tr>
<td>instrument</td>
<td>LC/MS</td>
<td>GC/MS</td>
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<tr>
<td>analytes</td>
<td>ester form</td>
<td>free form</td>
</tr>
<tr>
<td>composition of MCPD mono/di- ester</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>fatty acid composition in ester form</td>
<td>+</td>
<td>–</td>
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<tr>
<td>internal standard</td>
<td>multiple</td>
<td>single</td>
</tr>
<tr>
<td>analytical operation</td>
<td>complicated</td>
<td>easy</td>
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<tr>
<td>purpose</td>
<td>compositional analysis</td>
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Analytical methods of MCPD and their problems

In the early days of MCPD study, there were two analytical methods for measuring 3-MCPD esters and glycidyl esters in oils and fats, which were called ‘the direct method’ and ‘the indirect method’ (Table 1). With the direct method, MCPD-monoesters or diesters and their isomers with a different fatty acid are separated and detected by LCMS [8, 9]. This method is time-consuming and laborious, because it requires preparing the calibration curve for each fatty acid ester, and the analytical operations are complicated.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Flow diagram of DGF standard method C-III 18 (09).}
\end{figure}

With the indirect method, MCPD esters and glycidyl esters are converted to free form MCPD, and then derivatized by phenyl boric acid [10]. The resulting MCPD phenyl borates were measured by GCMS, using only one internal standard. This method is suitable for routine measurements of total amount of MCPD and glycidyl esters, because its analytical operations are
simple and less time-consuming than the direct method.

The DGF standard method C-III 18 (09) was the first and most widely used indirect method. It is comprised of two analyses: option A, which measures the total quantity of 3-MCPD esters and glycidyl esters irrespective of the bound fatty acid types, and option B, which measures only the quantity of 3-MCPD esters (Fig. 2). The glycidol content is calculated by subtracting option B from option A. However, some problems have been reported with this method; the sensitivity is extremely low, and the quantification of 3-MCPD is inaccurate due to the residual glycidyl esters in option B [11].

**Aim of this study**

In the work presented in chapter 2, we try to resolve the problems in DGF method C-III 18 (09) to improve the sensitivity and the accuracy of quantification. It was reported that 2-monochloropropane-1,3-diol (2-MCPD), which is an isomer of 3-MCPD, could also be detected simultaneously with 3-MCPD by the DGF method when oils and fats were examined [12]. As of 2011, however, the reference compound of 2-MCPD was not commercially available. Thus, a quantification method specific to 2-MCPD was not established. In this study, the synthesis of pure 2-MCPD was successfully achieved by a novel method. Furthermore, we advanced a new method for measuring 2-MCPD, using only 3-MCPD-\textit{d}_5 as an internal standard. Our new method is advantageous that it does not require expensive 2-MCPD-\textit{d}_5 as an internal standard or the preparation of a 2-MCPD calibration curve for every analysis.

2-MCPD was not commercially available until several years ago and is still extremely expensive. Thus, there have been few reports on the toxicological profile of 2-MCPD. Due to the lack of epidemiological and experimental evidence concerning its carcinogenicity, 2-MCPD has not been classified in the IARC monographs even in 2016.
Chapter 3 presents the results of our experiments on the hydrolysis of synthesized 2-MCPD-oleates by pancreatic lipase and pancreatin, which has not been reported before in the scientific literature. Based on the positional specificity of pancreatic lipase, it has been presumed that 3-MCPD-2-acyl-monoester is in the main product of hydrolysis of 3-MCPD diester. Nevertheless, 3-MCPD-1-acyl-monoester has been evaluated as the substrate in hydrolysis experiments and animal bioassays [13, 14, 15]. This study confirmed that the hydrolysis of 3-MCPD diester produces 3-MCPD-2-acyl-monoester mainly by analyzing the lipase hydrolysates with HPLC-CAD that achieve separation of the two positional isomers of the 3-MCPD monoester. In addition, synthesized 3-MCPD-2-acyl-monoester was subjected to an in vitro absorption study using a Caco-2 cell monolayer which is the widely used human epithelial cell model, to estimate its absorption property in the small intestine.

Chapter 4 describes a method to decrease the amounts of the fatty acid esters of 3-MCPD and glycidol produced during the oil refining process. Unprocessed oils contain very low amounts of the fatty acid esters of 3-MCPD and glycidol, but they are detected in the refined oils. Thus, it appears that they are produced during the refining steps applied to edible oils, especially at the deodorization step [16, 17]. Among the refined vegetable oils, there are oils in which very little MCPD is detected, such as soy and rapeseed oils, and others that contain relatively high amounts of MCPD, such as palm and corn oils [7, 12, 18, 19]. A common feature of palm and corn oils is that they contain relatively high amounts of MAG and DAG in their crude oil state. Thus, these partial acyl glycerols, MAG and DAG, have been proposed to contribute the generation of MCPD and glycidol, probably at the deodorization step. It was also hypothesized that chloride in the crude oil contributes to the formation of MCPD [16]. However, there has been no direct proof that partial acyl glycerols or
chloride, are reagents for unintentional generation of MCPD and glycidol.

In this study, therefore, refined oils were spiked with MAG and DAG and subjected to the deodorization step to determine whether MCPD would be generated in proportion to the added partial acylglycerols. Moreover, it was investigated whether the removal of the partial acylglycerols from the crude oil prior to the deodorization step could suppress the generation of these undesired compounds at the deodorization step.

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Chapter 2 : Development of analytical methods of 2- and 3-monochloropropanediol esters in oil products

Chapter 2-1 : Bidirectional conversion between 3-monochloro-1,2-propanediol and glycidol in course of the procedure of DGF standard method

Introduction
3-Monochloropropane-1,2-diol (3-MCPD) has recently been a big issue due to the concerns to the human health [1,2]. The recommended guideline for its intake is 2 µg/kg bodyweight per day. In order to estimate the daily intake from the diet, the quantification method is essential. The amount of 3-MCPD and its fatty acid esters (referred to as esters hereafter) in fat and oil products is currently measured by the standard method established by the German Society for Fat Science (DGF standard methods C-III 18(9) [3]). In the method, it is stated that the method is not specific to 3-MCPD (esters) and that glycidol and its esters are known to be detected as 3-MCPD. It has thus been revised in 2009 to remove them by the acid treatment (option B) prior to the conventional procedure (option A). The values obtained by option B are defined as the true amount of 3-MCPD, whereas the difference between the values obtained by option A and B is defined as the amount of glycidyl esters, since glycidol is considered to be negligible in fats and oils.

Perplexingly, the amounts of glycidyl esters determined in the revised standard method were not consistent to the amounts of those determined directly by LC-MS method [4] when sample oils spiked with known amount of glycidyl esters were analyzed in our laboratory. The revised standard method is based on the assumption that glycidyl esters were completely detected as 3-MCPD in option A and that the removal of glycidyl esters were complete in option B. However, there was a possibility that the assumption might not be true. Moreover, the mechanism of incorrect
detection of glycidyl esters as 3-MCPD by the standard method is not clearly understood. This chapter reveals that bidirectional conversion between 3-MCPD and glycidol was observed in the course of the analytical procedure of DGF standard methods C-III 18 (09), and that the method was not suitable for fats and oils which include glycidyl esters.

**Experimental procedures**

**Materials**

3-MCPD, 3-MCPD-\(d_5\), glycidol, phenylboronic acid, sodium methoxide/methanol solution, starting materials for syntheses, and solvents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NaCl and acetic acid were purchased from Nakalai Tesque Co. Ltd. (Kyoto, Japan). Soybean oil was the product of Ueda Oils and Fats MFG Co. Ltd. (Kobe, Japan). \(D_2\)O (99.96 atom% D) and CDCl\(_3\) (99.8 atom% D) were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Other chemicals were of the analytical grade.

**Synthesis of glycidyl esters**

**Glycidyl palmitate.**

**Procedure A.** Glycidol (0.27 g, 3.64 mmol) was dissolved in dry pyridine (20 mL) at 0 °C in a three-necked flask equipped with a drying tube. Palmitoyl chloride (1.0 g, 3.64 mmol) was added into the solution by four potions at the intervals of 10 min. The reaction mixture was stirred overnight at room temperature, and the solvent was removed by evaporation. The residue was extracted with chloroform (20 mL), and washed with water (20 mL), 0.1M hydrogen chloride solution (20 mL), sat. sodium hydrogen carbonate solution (20 mL), and finally brine (20 mL). The solvent was evaporated to dryness. The residue was purified by flash chromatography on silica gel (ethyl acetate/hexane = 1/1, vol/vol) to give glycidyl palmitate (0.80 g, 70%) as a white solid and 3-chloro-2-hydroxypropyl palmitate
Glycidol: IR (neat) 2912, 2844, 1737, 1471, 1456, 846; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 4.40 (dd, \(J = 3.1, 12.2\) Hz, 1H), 3.91 (dd, \(J = 6.3, 12.2\) Hz, 1H), 3.26 (m, 1H), 2.84 (t, \(J = 4.6\) Hz, 1H), 2.65 (dd, \(J = 2.6, 4.8\) Hz, 1H), 2.35 (t, \(J = 7.6\) Hz, 2H), 1.63 (m, 2H), 1.26 (m, 24H), 0.88 (t, \(J = 6.5\) Hz, 3H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 173.5, 64.7, 49.3, 44.6, 30.0, 31.9, 29.6-29.1 (10 \(\times\) CH\(_2\)), 24.8, 22.6, 14.0; MS (ESI) \(m/z\) 335.4 [M + Na]+; Anal. Calcd for C\(_{19}\)H\(_{36}\)O\(_3\): C, 73.03; H, 11.61. Found: C, 73.06; H, 11.51. 3-Chloro-2-hydroxypropyl palmitate (3-MCPD palmitate): IR (neat) 3436, 2931, 1737, 1465, 1180, 719; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 4.22 (d, \(J = 5.2\) Hz, 2H), 3.91 (quint, \(J = 5.2\) Hz, 1H), 3.62 (dd, \(J = 5.82, 11.3\) Hz, 1H), 2.65 (bs, 1H), 2.35 (t, \(J = 7.5\) Hz, 2H), 1.63 (quint, \(J = 7.4\) Hz, 2H), 1.26 (m, 24H), 0.88 (t, \(J = 6.8\) Hz, 3H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 173.1, 68.8, 68.4, 47.8, 33.9, 31.9, 29.6-29.1 (10 \(\times\) CH\(_2\)), 25.1, 22.8, 14.1; MS (ESI) \(m/z\) 371.4 [M + Na]+; Anal. Calcd for C\(_{19}\)H\(_{37}\)O\(_3\)Cl: C, 65.40; H, 10.69. Found: C, 65.44; H, 10.52.

**Procedure B.** Palmitic acid (1.0 g, 3.90 mmol) was dissolved in dry methylene chloride (20 mL) at 0 °C under argon atmosphere. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC•HCl) (0.75 g, 3.90 mmol), \(N,N\)-diisopropylethylamine (DIEA) (0.50 g, 3.90 mmol), and \(N,N\)-dimethylaminopyridine (DMAP) (0.048 g, 0.39 mmol) were added to the solution. The reaction mixture was stirred for 5 min, and then cooled down. Glycidol (0.26 mL, 3.90 mmol) was added to the mixture, and stirred for 10 min, then allowed to warm up to room temperature. After the reaction was completed, the methylene chloride solution was washed with water (20 mL), 0.1 M hydrogen chloride solution (20 mL), sat. sodium hydrogen carbonate solution (20 mL), and finally brine (20 mL). The solvent was evaporated to dryness, and the residue was purified by silica gel column chromatography (ethyl acetate/hexane = 1/1, vol/vol) to give glycidyl palmitate (0.96 g, 79%) as a white solid.
3-Chloro-2-hydroxypropyl palmitate (3-MCPD palmitate)

3-Chloro-2-hydroxy propanol (0.40 g, 3.64 mmol) was dissolved in dry pyridine (20 mL) at 0 °C in a three-necked flask equipped with a drying tube. Palmitoyl chloride (1.0 g, 3.64 mmol) was added into the solution by four portions at the intervals of 10 min. The reaction mixture was stirred for 2 h at room temperature. The solvent was then removed by evaporation. The residue was extracted with chloroform (20 mL), washed with water (20 mL), 0.1M hydrogen chloride solution (20 mL), sat. sodium hydrogen carbonate solution (20 mL), and finally brine (20 mL). The solvent was evaporated to dryness. The residue was purified by flash chromatography on silica gel (ethyl acetate/hexane = 1/2, vol/vol) to give 3-chloro-2-hydroxypropyl palmitate (0.82 g, 64%) as a white solid, 3-chloropropyl 1,2-dipalmitate (0.040 g, 2%) as a white solid, and 3-chloro-1-hydroxypropyl palmitate (0.062 g, 5%) as a white solid. 3-Chloropropyl 1,2-dipalmitate: IR (neat) 2913, 2844, 1728, 1471, 1253; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 5.22 (m, 1H), 4.28 (dd, \(J\) = 4.4, 11.8 Hz, 2H), 3.66 (dd, \(J\) = 5.4, 11.8 Hz, 2H), 2.33 (m, 4H), 1.63 (m, 4H), 1.26 (m, 48H), 0.88 (t, \(J\) = 6.8 Hz, 6H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 173.2, 172.8, 70.25, 62.25, 42.2, 34.2, 34.0, 31.9, 29.7-29.1 (20 \(\times\) CH\(_2\)), 24.9, 22.7, 14.1; MS (ESI) \(m/z\) 609.2 [M + Na]\(^+\); Anal. Calcd for C\(_{35}\)H\(_{67}\)O\(_4\)Cl: C, 71.57; H, 11.50. Found: C, 71.66; H, 11.45.

3-Chloro-1-hydroxypropyl palmitate: IR (neat) 3434, 2922, 1737, 1460, 1177; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 5.06 (m, 1H), 3.83 (d, \(J\) = 4.8 Hz, 2H), 3.70 (dd, \(J\) = 5.5, 11.5 Hz, 2H), 2.37 (m, 2H), 1.64 (quint, \(J\) = 7.5 Hz, 2H), 1.26 (m, 24H), 0.88 (t, \(J\) = 6.6 Hz, 3H); \(^{13}\)C NMR \(\delta\) 173.1, 81.3, 64.2, 44.3, 34.2, 31.9, 29.6-29.1 (10 \(\times\) CH\(_2\)), 25.1, 22.8, 14.1; MS (ESI) \(m/z\) 371.4 [M + Na]\(^+\); Anal. Calcd for C\(_{19}\)H\(_{37}\)O\(_3\)Cl: C, 65.40; H, 10.69. Found: C, 65.47; H, 10.47.

3-Propoxypropane-1,2-diol (1-glyceryl 1-propyl ether)

Glycerol (9.7 g, 105 mmol) was dissolved in dry dimethylformamide (100
mL) at 0 °C in a three-necked flask with a condenser. Sodium hydride (60% in oil, washed by n-hexane, 0.49 g, 12.2 mmol) was slowly added to the solution. After hydrogen gas was ceased, propyl bromide (1.0 g, 8.13 mmol) was added to the reaction mixture and was stirred for 18 h at 80 °C. After the reaction mixture cooled to the ambient temperature, ethyl acetate (100 mL) was added and was washed with water (100 mL). The aqueous phase was extracted twice with ethyl acetate (50 mL). The organic phase was combined, and was evaporated to concentrate. The resulting residue was purified by open column chromatography (hexane/ethyl acetate/methanol = 4.5:4.5:1, vol/vol/vol) to give 1-glyceryl 1-propyl ether (0.090 g, 8.3%). $^1$H NMR (300 MHz, D$_2$O) δ 3.84 (m, 1H), 3.69-3.40 (m, 7H), 1.55 (sext, $J$ = 7.2 Hz, 2H), 0.85 (t, $J$ = 7.2 Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 73.8, 71.8, 71.0, 63.4, 23.1, 10.3; MS (ESI) $m/z$ 135.4 [M + H]$^+$; HRMS (ESI) 135.1023 (C$_6$H$_{15}$O$_3$ requires 135.1021).

**Determination of 3-MCPD forming substances by DGF standard methods C-III 18 (09)**

The contents of 3-MCPD forming substances were determined as described in DGF standard methods C–III 18(09) with a slight modification. Option A: soybean oil (0.1 g) mixed with 3-MCPD was dissolved in 0.5 mL solvent consisted of t-buthyl methyl ether and ethyl acetate (=4:1, vol/vol). To the sample, 3-MCPD-$d_5$ (2 µg) and 0.5 M sodium methoxide/methanol solution (1 mL) was added and left for 10 min at room temperature (step 2). The mixture was extracted using n-hexane (3 mL) and water containing 16.7% NaCl and 3.3% acetic acid (3 mL). The aqueous phase was rinsed with n-hexane (3 mL, step 3). The aqueous phase was mixed with derivatization reagent (0.125 g/mL phenylboronic acid solution, 0.5 mL) and left at 80 °C for 20 min (step 4). Then, the extraction was conducted using n-hexane (3 mL, step 5). The organic phase was collected, evaporated to dryness, and was dissolved again to 2,2,4-trimethylpentane (2 mL). The sample was
filtered by paper before it was brought to GC-MS analysis (step 6).

Option B: soybean oils (0.1 g) spiked with glycidyl esters were treated with 0.5% sulfuric acid/propanol solution (0.5 mL) at 45 °C for 15 min in the ultrasonic bath (step 1). The samples were brought to the above-mentioned procedure, steps 2-6.

**GC-MS**

GC-MS was conducted using GCMS QP 2010 (Shimadzu, Kyoto, Japan) connected to DB-5 capillary column (30 m, 0.25 µm, Agilent Technologies, Tokyo, Japan). The column temperature was controlled as follows; it was kept at 60 °C for 1 min, raised at 6 °C /min to 190 °C, further raised at 20 °C /min to 280 °C, and kept at 280 °C for 6 min. The temperature of programmed-temperature vaporizer (PTV) injector was controlled as follows; it was kept at 60 °C for 1 min, raised at 10 °C /min to 180 °C and kept at 180 °C for 20 min. The temperatures of the interface and the ion source were set at 250 °C and 200 °C. Other conditions for GC-MS were the same with those described in DGF standard methods C–III 18(09).

**LC-MS**

Glycidyl esters were treated with 0.5% sulfuric acid/propanol solution (0.5 mL) at 45 °C for 15 min in the ultrasonic bath. To the sample, hexane (3 mL) and water (3 mL) was added and mixed by vortex. The organic phase was collected and dried over sodium sulphate. It was evaporated to dryness and dissolved to acetonitrile (1.5 mL). The resulting sample was then analyzed by API 2000 LC/MS/MS system (Life Technologies Japan, Tokyo, Japan) connected to YMC-Triart C18 column (2.0 x 50 mm, S-3 µm, 12 nm, YMC Co. Ltd., Kyoto, Japan). The column temperature was set at 40 °C. Elution was conducted at the flow rate of 0.2 mL/min, using mobile phase A consisted of acetonitrile/methanol/water (=17:17:6, vol/vol/vol) and mobile phase B consisted of 2-propanol. The binary gradient program was as
follows; mobile phase A, 98% and B, 2% at 0.0 min; a linear gradient elution to A, 85% and B, 15% from 0.0 to 15.0 min; an isocratic elution with A, 5% and B, 95% from 15.1 to 25.0 min. Mass chromatograms were recorded by a triple stage quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) ionizer. The ion spray voltage was +4500 V.

**NMR**

$^1$H and $^{13}$C NMR spectra were recorded on a JEOL AL-300 spectrometer (Tokyo, Japan) at 300 and 75 MHz, respectively and are referenced to internal tetramethylsilane (CDCl$_3$) or to the residual protonated solvent (for D$_2$O and methanol-$d_4$).

**Results**

DGF standard methods C-III 18 (09) describes that glycidol and its fatty acid esters are detected as 3-MCPD in the conventional procedure (option A) and that they should be removed by acid treatment prior to the conventional procedure (option B). As diagrammatically described in Fig. 1, it consists of the following steps; 1) treatment of oil samples with 0.5% sulfuric acid/propanol to open epoxide ring, 2) transesterification of glycerides and other esters with sodium methoxide/methanol, 3) fractionation of fatty acid esters from 3-MCPD using $n$-hexane/water containing 16.7% NaCl and 3.3% acetic acid, 4) derivatization of 3-MCPD in the aqueous phase with phenylboronic acid, 5) extraction of resulting derivatives with $n$-hexane, and 6) GC-MS analysis. In order to verify the effectiveness of the acid treatment (step 1), glycidyl esters were prepared first.
Synthesis of glycidyl fatty acid esters

Schotten-Baumann reaction, which is the one between acid chlorides and alcohols under the basic conditions, is generally applicable for fatty acid ester synthesis. Palmitoyl chloride was reacted with glycidol to give not only the corresponding glycidyl ester (70%) but also the unexpected 3-MCPD ester (9%). The epoxide ring of the ester was nucleophilically substituted by the chloride ion which generated near the epoxide in the reaction. On the other hand, esters are also obtained by the reaction between free fatty acids and alcohols using condensation agents such as...
carbodiimides under the basic conditions. The corresponding glycidyl ester was afforded as the unique product (79%) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC•HCl) as the condensation agent in spite that chloride ion existed in the reaction system. Therefore, the 3-MCPD ester formation by Schotten-Baumann reaction progresses concertedly with the glycidyl ester formation.

Palmitoyl chloride was reacted with 3-MCPD to give the monoester of primary alcohol (3-MCPD ester, 64%), the one of the secondary alcohol (3-MCPD ester isomer, 5%), and the diester (3-MCPD diester, 2%). This result suggested that the first esterification underwent at the less hindered 1-position hydroxyl group. Interestingly, no glycidyl ester production was observed in the EDC•HCl system. The glycidyl ester did not form due to too low basicity of pyridine to deprotonate from the 2-position hydroxide. Thus, the deprotonation may cause the conversion of 3-MCPD ester to glycidyl ester, and the nucleophilicity of the chloride ion may cause the conversion of glycidyl ester to 3-MCPD ester.

**Evaluation of acid treatment**

The resulting glycidyl esters were used to verify the effectiveness of the acid treatment (step 1). Soybean oil spiked with glycidol or glycidyl esters were treated according to the standard method, option B, which included the treatment with 0.5% sulfuric acid/propanol solution (Table 1). 3-MCPD was detected by GC-MS in the oil samples spiked with ≥10 ppm of glycidols but was not in that with 5 ppm. Similar results were obtained with oils spiked with glycidyl palmitate and oleate. These results indicated that the removal of glycidol in addition to its esters were incomplete. The glycidyl esters of ≤ 5 ppm might be reduced to undetectable amount, but should remain still in the samples after the acid treatment. The observation was consistent with the observation of Shimizu *et. al* [5], though they did not directly quantify the residual glycidyl esters.
In order to confirm the incomplete ring-opening of epoxides, the residual glycidyl esters after the acid treatment were directly measured by LC-MS. As shown in Table 2, 0.11, 0.42, and 1.72 ppm of glycidyl palmitate were remained in the oils spiked with 1, 5, and 20 ppm, even after the acid treatment. Likewise, removal of glycidyl oleate was not completed by the acid treatment. The residual ratio was 10% approximately. Therefore, the epoxide ring-opening of glycidyl esters was confirmed to be ca. 90% by the acid treatment.

Table 1 Detection of 3-MCPD in oils spiked with glycidol or glycidyl esters by DGF standard methods C-III 18(09), option B a).

<table>
<thead>
<tr>
<th>Spiked compound</th>
<th>spiked amount (ppm)</th>
<th>detected amount as 3-MCPD b) (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycidol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>n.d. c)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.17±0.44</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.68±0.65</td>
</tr>
<tr>
<td>Glycidyl palmitate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>n.d. c)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.64±0.23</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.04±0.11</td>
</tr>
<tr>
<td>Glycidyl oleate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>n.d. c)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.63±0.17</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.31±0.30</td>
</tr>
</tbody>
</table>

a) Soybean oil was spiked with glycidol or its esters. The oil samples were treated as described in DGF standard method C-III 18(09), option B.
b) The amount in soybean oil without any spike was 0.32 ppm. The amount was subtracted from those detected in the spiked oil samples. All analyses were conducted 3-4 times. The mean values and the standard deviations were presented.
c) Not detected. The minimum limit of detection was 0.2 ppm.
Table 2 Residual amount of glycidyl esters after treatment with 0.5% sulfuric acid/propanol.

<table>
<thead>
<tr>
<th>Compound</th>
<th>amount (ppm)</th>
<th>detected amount$^b$) (ppm)</th>
<th>residual ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycidyl palmitate</td>
<td>1.0</td>
<td>0.11</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.42</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>1.72</td>
<td>8.6</td>
</tr>
<tr>
<td>Glycidyl oleate</td>
<td>1.0</td>
<td>0.11</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.52</td>
<td>10.3</td>
</tr>
</tbody>
</table>

a) Glycidyl esters were treated with 0.5% sulfuric acid/propanol solution at 45 °C for 15 min in the ultrasonic bath as described in DGF standard method C-III 18(09), option B.

b) Glycidyl esters were analyzed by LC-MS.

Possibility of 1-glyceryl 1-propyl ether to cause the incorrect detection of 3-MCPD

The treatment of glycidyl esters by sulfuric acid/propanol gives the corresponding 1-acyloxy-3-propyloxypropane-2-ol. Here, 1-acyloxypropane-2,3-diol (monoacylglycerol) is not expected, because sulfuric acid/propanol does not contain considerable amount of water. Based on the result above, 90% of glycidyl esters should be converted to 1-acyloxy-3-propyloxypropane-2-ol. Its deacylation by the following treatment with sodium methoxide (step 2) gives 3-propyloxypropane-1,2-diol (1-glyceryl 1-propyl ether). In general, 1-glyceryl 1-propyl ether is stable under basic conditions. However, glycidol, which is responsible for the incorrect detection as 3-MCPD, could be generated from the ether by the attack of neighboring 2-position alkoxide to the epoxide-carbon if the alkoxide generated under the basic conditions. In order to investigate the possibility of 1-glyceryl 1-propyl ether to cause
the incorrect detection of 3-MCPD, the compound was synthesized, and its behavior under the treatment with sodium methoxide was monitored by NMR. $^{13}$C NMR spectra were identical before and after the treatment (Fig. 2), since the peak at 49 ppm belonged to methanol. It was thus clarified that glycidol was never generated by the sodium methoxide treatment of 1-glyceryl 1-propyl ether. Consequently, the acid treatment of 1-acyloxy-3-propyloxypropane-2-ol (step 1) did not cause the incorrect detection of 3-MCPD by the standard procedure, option B.

**Figure 2.** $^{13}$C NMR spectra of 3-propoxypropane-1,2-diol.
A. 3-propoxypropane-1,2-diol (standard) dissolved in D$_2$O  
B. 3-propoxypropane-1,2-diol (0.01 g) was dissolved in 0.5 M sodium methoxide/methanol solution (0.2 mL) and kept at room temperature for 10 min. Then, D$_2$O (0.6 mL) was added to the solution. All reactions were conducted in NMR tubes.

**Bidirectional conversion of 3-MCPD and glycidol**

When soybean oil (0.1 g) spiked with 3-MCPD (1 µg) was treated according to the DGF standard method, option A (without acid treatment, steps 2-6),
the peak area of phenylboronic acid derivatives of 3-MCPD detected by GC-MS was 600. On the other hand, it was 2600 when the sample was treated in the same procedure without transesterification step. The peak areas of 3-MCPD-\(d_5\), the internal standard, were 1100 and 4800 with or without transesterification. It was therefore indicated that 77\% of 3-MCPD was lost somehow due to the step. Weisshaar estimated that sodium methoxide might decompose 3-MCPD [6]. However, the details of the decomposition have not yet been understood.

Thus, the behavior of 3-MCPD under the transesterification step was directly monitored by \(^1\)H and \(^{13}\)C NMR. 3-MCPD was dissolved in sodium methoxide/methanol solution, kept at room temperature for 10 min. After the addition of D\(_2\)O, the sample was analyzed by NMR measurement. The peaks derived from 3-MCPD (71.1, 62.5, 45.9 ppm, Fig. 3A) were the only peaks observed before the treatment. After the treatment, the peaks derived from glycidol (61.5, 53.0, 45.0 ppm) appeared on \(^{13}\)C NMR chart (Fig. 3B).

![Figure 3. \(^{13}\)C NMR spectra of 3-monochloropropane-1,2-diol (3-MCPD, ▼), glycidol (●), and their products. A, 3-MCPD (standard) dissolved in D\(_2\)O; B, 3-MCPD (0.13 g) dissolved in 0.5 M sodium methoxide/methanol solution (1 mL) was kept at room temperature for 10 min. Then, D\(_2\)O (1 mL) was added to the solution. C, glycidol (standard) dissolved in D\(_2\)O; D, glycidol (0.01 g) was dissolved in 0.5 M sodium methoxide/methanol solution (0.2 mL) and kept at room temperature for 10 min. Then, D\(_2\)O containing 16.7\% NaCl and 3.3\% acetic acid (0.6 mL), was added to the mixture, and stood at 80 °C for 20 min.; All reactions were conducted in NMR tubes.]
The conversion ratio was 37% calculated on their proton numbers obtained by $^1$H NMR. The behavior of glycidol in the standard method option A was also monitored by NMR. Glycidol was treated in the similar way to the standard method steps 2-4 (transesterification to derivatization steps), except $n$-hexane and phenylboronic acid was not added and D$_2$O was used instead of water (details of reaction conditions were given in the legend Fig. 3D). pH of the reaction mixture was 4.2. In addition to the peaks derived from glycidol, those from 3-MCPD were newly observed by NMR after the treatment (Fig. 3C, D). The conversion ratio from glycidol to 3-MCPD was 70%, when it was calculated on their proton numbers. Interestingly, the conversion was not detected when the derivatization step (step 4) was conducted at room temperature instead of 80 °C. On the other hand, the conversion increased to nearly 100% when glycidol was directly dissolved in D$_2$O containing 16.7% NaCl and 3.3% acetic acid (pH 1.9), and stood at 80 °C for 20 min. It was thus indicated that the heating under the acidic conditions at the derivatization step accelerated the conversion of glycidol to 3-MCPD greatly.

**Production of 3-MCPD derivatives at the derivatization step**

As shown Fig. 3D, treatment of glycidol with steps 2-4 without phenylboronic acid generated 3-MCPD with 70% of conversion. The treatment was then conducted with phenylboronic acid. The reaction conditions were the same with the standard method steps 2-4 except $n$-hexane was not added and D$_2$O was used instead of water. The conversion of glycidol to 3-MCPD was again observed by $^{13}$C NMR (Fig. 4B). The degree of conversion was 74% calculated on the proton numbers observed by $^1$H NMR. Suppose that the errors of integration value measured in $^1$H NMR were ±5%, the conversion ratio were nearly the same with or without phenylboronic acid at the derivatization step. On the other hand, the 3-MCPD phenylborate was not detectable (Fig. 4B). Association constant of
phenylboronic acid and diols was reported to drastically change at pH ~7.5, and the phenylborate was hardly formed below pH 6.5 [7]. It was therefore speculated that the 3-MCPD phenylborate were produced only in a small amount in the experimental conditions of pH 4.2, and thus were undetectable by NMR.

The reaction mixture was further extracted by \( n \)-hexane as the procedure step 5. After the removal of the hexane phase, the aqueous phase was analyzed by NMR again. Both of glycidol and 3-MCPD were observed by \(^{13}\)C NMR (Fig. 4C), with the ratio of 13:87, calculated from the proton numbers obtained by \(^1\)H NMR. These results indicated that the extraction of 3-MCPD phenylborate, which was more hydrophobic than 3-MCPD, to the organic phase was shifted the equilibrium of the three compounds, namely 3-MCPD.

**Figure 4.** The conversion of glycidol to 3-MCPD under the condition of option A. The circle and triangle signs represent the peaks of glycidol and 3-MCPD, respectively. A, glycidol (standard) dissolved in D\(_2\)O; B, glycidol (0.01 g) was dissolved 0.5 M sodium methoxide/methanol solution (0.2 mL) and kept at room temperature for 10 min. Then, D\(_2\)O containing 16.7% NaCl and 3.3% acetic acid (0.6 mL) and of acetone-d\(_6\) containing 12.5% (w/v) phenylboronic acid (0.133 mL) were added to the mixture, and stood at 80 °C for 20 min.; C, After treatments described in B, the reaction mixture was washed by hexane (1 mL). The resulting aqueous phase was analyzed; all reactions were conducted in NMR tubes. The reaction mixture was further extracted by \( n \)-hexane as the procedure step 5. After the removal of the hexane phase, the aqueous phase was analyzed by NMR again. Both of glycidol and 3-MCPD were observed by \(^{13}\)C NMR (Fig. 4C), with the ratio of 13:87, calculated from the proton numbers obtained by \(^1\)H NMR. These results indicated that the extraction of 3-MCPD phenylborate, which was more hydrophobic than 3-MCPD, to the organic phase was shifted the equilibrium of the three compounds, namely 3-MCPD.
phenylborate, 3-MCPD, and glycidol in the aqueous phase. As a result, the ratio of 3-MCPD against glycidol increased from 74% to 87%. It should be noted that glycidol and 3-MCPD remained in the aqueous phase even after the hexane extraction at step 5 of the standard method. The low efficiency of the 3-MCPD phenylborate formation and extraction might explain the relatively high standard deviations given in Table 1.

Discussion

It has been described that the epoxide ring-opening in glycidol and its esters was incomplete by the acid treatment described in the DGF standard methods C-III 18 (09), option B, and that the bidirectional conversion between 3-MCPD and glycidol was observed by NMR in the course of the method. The behaviors of 3-MCPD esters and glycidyl esters, which were supposed to be in fats and oils, in the course of the standard method were schematically shown in Fig. 5. 3-MCPD produced by the transesterification using sodium methoxide was partly converted to glycidol in the step (37%). There also is a possibility that 2-MCPD were converted to glycidol, though it should be proven. The resulting glycidol, in addition to glycidol derived from glycidyl esters, were partly converted to 3-MCPD in the following steps, which were conducted in the presence of saturated NaCl under acidic conditions at 80 °C (74%). Glycerol was not observed from glycidol under the conditions, which could be explained that there were abandoned chloride ions, which are highly nucleophylic, and little hydroxyl ions in the solution. What important was that the degree of conversion from glycidol to 3-MCPD depended on the conditions of the procedure steps 2-5 (transesterification, derivatization, and extraction), and was not 100%. This observation contradicted to that of Kuhlmann cited in ref. 8 that the conversion was nearly complete. Based on our observation, the standard method, option A, did not give combined amount of 3-MCPD esters and glycidyl esters correctly. The removal of epoxides by the acid treatment
described in option B was not complete either, as shown in Table 2. Therefore, the difference of the values obtained by options A and B did not correspond to the amount of glycidyl esters.

The conversion of 3-MCPD to glycidol at the transesterification step was estimated to be 37% by NMR, whereas that of glycidol to 3-MCPD at the following steps was 74%. The loss of 3-MCPD in total was thus ca. 10%, which was not consistent with the observation described in the section ‘bidirectional conversion of 3-MCPD and glycidol’, where the loss was estimated to be 77% under the influence of the transesterification step. In another report of ours, it was clarified that the loss was also caused by the

![Figure 5](image_url)

**Figure 5.** Behaviors of 3-MCPD esters and glycidyl esters under the procedure of DGF standard method C-III 18(09).

R represents fatty acyl group. Arrows with solid lines represent the conventionally known/believed routes, whereas those with dotted lines represent routes newly confirmed in this study. Reagents and conditions: a) Sodium methoxide/methanol, rt, 10 min. b) Acetic acid, NaCl, water. c) Phenylboronic acid, 80 °C, 20 min. d) Hexane extraction.
low extraction capability of \( n \)-hexane used in the extraction step (step 5) [9]. The substitution of \( n \)-hexane to more polar solvent such as chloroform increased the recovery of 3-MCPD derivatives. In the mean time, the derivatives of 3-MCPD with phenylboronic acid were not observed in NMR analysis in the aqueous phase at the derivatization step (step 4), although they were detected by GC-MS in the hexane phase obtained in step 5. It was thus estimated that the derivatives were rather formed in the course of hexane extraction (Fig. 5), than at the derivatization step, and the polarity of the solvent might affect to the production as well as the recovery of the derivatives.

**Conclusion**

This chapter reports that bidirectional conversion was confirmed between 3-MCPD and glycidol in the course of the analytical procedure of DGF standard methods C-III 18 (09), option A; 3-MCPD was partly converted to glycidol at the transesterification step, and glycidol was converted partly to 3-MCPD at the derivatization step conducted at 80 °C under acidic condition in the presence of NaCl. In addition, epoxide ring-opening of glycidol and its esters was shown to be incomplete by the acid treatment described in the method, option B. Thus, the standard method, option A, did not give combined amount of 3-MCPD esters and glycidyl esters correctly, and the difference of the values obtained by options A and B did not correspond to the amount of glycidyl esters, either. The restricted application of the standard method, option A, to glycidyl ester-free samples is recommended. In addition, the conversion of 3-MCPD to phenylboronic acid was not observed by NMR at the derivatization step. The derivatization was estimated to rather occur in the following hexane extraction step. The observations presented in this chapter are important for our understanding to the standard method, and for the interpretation of the values so far given by the standard method.
References


Chapter 2-2: Improvement of accuracy in quantification of 3-monochloropropane-1,2-diol and its esters by DGF Standard Methods C-III 18

Introduction
3-Monochloropropane-1,2-diol (3-MCPD) has recently been of the great attention due to the concerns to the human health [1,2]. It is currently quantitated by the standard method established by the German Society for Fat Science (DGF standard methods C-III 18[3]). The methods also described its occurrence in various vegetable oils varied from 0.24 to 8.28 mg/kg. Since a maximum intake recommended in the guideline is 2 µg/kg bodyweight, correct determination of 3-MCPD, especially at the low concentration, is important.

By conducting the standard method, however, we have encountered a problem that the determination of 3-MCPD content below 1 ppm in natural oils was hard due to the small peak areas of 3-MCPD phenylborate and 3-MCPD-\(d_5\) phenyl borate, the internal standard, detected by GC-MS. In this chapter, modification is proposed in order to improve the accuracy and the sensitivity of the standard method.

Experimental procedures
Materials
3-MCPD, 3-MCPD-\(d_5\), phenylboronic acid, sodium methoxide/methanol solution, \(t\)-buthyl methyl ether, methanol, \(n\)-hexane, \(n\)-butanol, chloroform, and ethyl acetate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NaCl and acetic acid were purchased from Nakalai Tesque Co. Ltd. (Kyoto, Japan). Soybean oil was the product of Ueda Oils and Fats MFG Co. Ltd. Other chemicals were of the analytical grade.
Quantification of 3-MCPD

The contents of 3-MCPD forming substances were determined based on DGF standard methods C–III 18(09) with a slight modification. Typical procedure for preparing samples was as follows; 0.1 g soybean oil mixed with 3-MCPD was dissolved in 0.5 mL solvent consisted of t-buthyl methyl ether and ethyl acetate (=4:1, vol/vol). To the sample, 2 µg of 3-MCPD-$d_5$ and 1 mL of 0.5 M sodium methoxide/methanol solution was added and left for 10 min at room temperature (step 1). The mixture was extracted using 3 mL n-hexane and 3 mL water containing 16.7% NaCl and 3.3% acetic acid. The aqueous phase was rinsed with 3 mL n-hexane (step 2). It was then mixed with 0.5 mL derivatization reagent (ca. 0.125 g/mL phenylboronic acid solution) and left at 80 °C for 20 min (step 3). Then, the extraction of 3-MCPD phenylborate was conducted using 3 mL n-hexane, or other solvents (step 4). Organic phase was collected, dried by evaporation and was dissolved again to 2 mL 2,2,4-trimethylpentane and filtered by paper before it was brought to GC-MS analysis (step 5). Samples for calibration were prepared as follows; 2 µg of 3-MCPD-$d_5$ dissolved in t-buthyl methyl ether was added to a test tube and dried under N2 gas stream. Required amount of 3-MCPD and water containing 16.7% NaCl were added to make the final volume of 3 mL. The samples were subjected to steps 3-5 as described above. Analyses were conducted 3 times independently and the mean values were presented.

GC-MS

GC-MS was conducted using GCMS QP 2010 (Shimadzu, Kyoto, Japan) connected to DB-5 capillary column (30 m, 0.25 µm, Agilent Technologies, Tokyo, Japan). The column temperature was controlled as follows; it was kept at 60 °C for 1 min, then raised at 6 °C /min to 190 °C, further raised at 20 °C /min to 280 °C, and kept at 280 °C for 6 min. The temperature of programmed-temperature vaporizer (PTV) injector was controlled as follows; it was kept at 60 °C for 1 min, raised at 10 °C /min to 180 °C and
kept at 180 °C for 20 min. The temperatures of the interface and the ion source were set at 250 °C and 200 °C. Other conditions for GC-MS were same with those described in DGF standard methods C–III 18(09).

Results and Discussion

Comparison of sample preparation procedures by GC-MS

The analytical procedures for determination of 3-MCPD forming substances by DGF standard methods C–III 18(09), option A consist of the following 5 steps; 1) transesterification of acyl glycerols and other esters by sodium methoxide in the presence of 3-MCPD-$d_5$, 2) removal of resulting fatty acid methyl esters by hexane extraction, 3) derivatization of 3-MCPD in the aqueous phase by phenylboronic acid, 4) extraction of 3-MCPD phenylborate by $n$-hexane from aqueous phase, 5) GC-MS analyses of organic phase (for experimental details, see ‘materiars and methods quantificaton of 3-MCPD’). By conducting the analyses according to the standard method, however, it was suspected that the detection limit of the method might be different between the actual oil samples and the standard samples for calibration.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Peak area (A, arbitrary unit)</th>
<th>Ratio</th>
<th>Corrected amount (µg)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$m/z$ 196</td>
<td>$m/z$ 201</td>
<td>$(A_{m/z196}/A_{m/z201})$</td>
</tr>
<tr>
<td>Oil sample$^b$</td>
<td>090</td>
<td>1240</td>
<td>0.07</td>
</tr>
<tr>
<td>Calibration</td>
<td>260</td>
<td>4310</td>
<td>0.06</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>sample$^c$</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>

$^a$) Correction was conducted based on the calibration curve given in Fig. 1.
$^b$) 0.1 g soybean oil mixed with 0.1 µg of 3-MCPD
$^c$) 1 µg of 3-MCPD

In order to confirm this, 0.1 µg 3-MCPD added to 0.1 g soybean oil was analyzed by the standard method. As shown in Table 1, the peak areas of $m/z$
196 and 201, corresponding to 3-MCPD phenylborate and 3-MCPD-\(d_5\) phenylborate, were 90 and 1240, respectively. The peak areas were 260 and 4310, respectively, when the same amount of 3-MCPD was analyzed according to the procedure to prepare samples for calibration, which consisted of the aforementioned steps 3, 4, and 5. The ratio of the two peaks were 0.07 (=90/1240) and 0.06 (=260/4310) and gave similar results (0.11 and 0.10 µg 3-MCPD) when they were corrected by the factor obtained by the calibration (Fig. 1). It was thus confirmed that the peak areas in GC-MS analyses of oil samples are 3-4 times smaller than those of the samples for calibration.

![Figure 1. Calibration curves using \(n\)-hexane and chloroform as extraction media. Analyses were conducted as described in section 2.2, using chloroform (●) instead of \(n\)-hexane (○) at step 4.](image)

**Effect of solvent in the recovery of 3-MCPD derivatives**

The smaller peak areas obtained by the procedure of the standard method (steps 1-5) implied the low recoveries of 3-MCPD phenylborate compared to the procedure preparing samples for calibration. Here, we focused on step 4, in which 3-MCPD phenylborate were extracted from the aqueous phase by \(n\)-hexane. The recoveries might improve by substituting the conventional solvent with more polar solvents (Table 2). As expected, \(n\)-butanol, chloroform, and ethyl acetate, which have higher solvent polarity scale [4] than \(n\)-hexane, successfully increased the recovery of 3-MCPD derivatives to
the organic phase to the relative extent of 5.6, 4.7, and 3.8, respectively. Interestingly, when 0.1 g soybean oil containing 0.1 µg 3-MCPD was treated by the standard method without transesterification step (step 1), the relative recovery by n-hexane reached 4.9. The pH of the aqueous phase at step 4 was 4.2 and 1.9 with and without transesterification step, respectively. This indicated that pH of the aqueous phase might affect the recovery of 3-MCPD phenylborate. Weisshaar considered the low recovery of 3-MCPD-\textit{d}_5 (49-72%; coefficient of variation, 13.4%) by the standard method was caused by the decomposition of 3-MCPD by sodium methoxide [5]. In another report of ours, it was shown that the conversion of 3-MCPD to glycidol actually observed by NMR [6]. However, it was shown in Table 2 that there should be another reason; the low extraction capability of the organic phase caused the low recovery. It might also explain the relatively large value of coefficient of variation, 13.4%, described in ref. 5.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure2.png}
\caption{Analyses of oils spiked with 3-MCPD. Soybean oil spiked with 3-MCPD was conducted as described in section 2.2. (A) \textit{n}-hexane; (B) chloroform was used as extraction solvent at step 4. Expressions of the linear curve fittings were (A) \(y = 0.0457x + 0.0203\), and (B) \(y = 0.0526x + 0.0118\).}
\end{figure}

\textit{n}-Butanol achieved the highest recovery. However, it co-extracted more impurities, which had higher vaporization temperature and thus showed
longer retention times than 3-MCPD phenylborate by GC analyses. The impurities were undesirable for MS detector, though they would not disturb the quantification of 3-MCPD phenylborate. In addition, $n$-butanol has the highest boiling point (108 °C) among the four solvents tested, and was hardest to be dried out before bringing the samples to GC-MS analyses. From the practical point of view, chloroform was chosen for further study.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solvent polarity scale</th>
<th>Relative peak area of 3-MCPD derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$-Hexane</td>
<td>0.519</td>
<td>1.0</td>
</tr>
<tr>
<td>$n$-Butanol</td>
<td>0.837</td>
<td>5.6</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0.786</td>
<td>4.7</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.795</td>
<td>3.8</td>
</tr>
<tr>
<td>$n$-Hexane</td>
<td>(without transesterification step)</td>
<td>0.519</td>
</tr>
</tbody>
</table>

a) Ref. 3  
b) 0.1 g soybean oil containing 1 µg of 3-MCPD was subjected to the procedure described in section 2.2. All analyses were conducted 3 times independently and the mean values were presented.  
c) The identical sample as a) was subjected to the procedure described in section 2.2, except that step 1 was omitted.

The calibration curve using chloroform as extraction solvent showed that the solvent was as good as $n$-hexane for quantification of 3-MCPD phenylborate (Fig. 1). In addition, analyses of oils spiked with 3-MCPD (0.25~1.0 ppm) were conducted using the two solvents (Fig. 2). It was obvious that the variations of data were smaller when chloroform was used. In addition, the correlation coefficients (R-squared values) of the linear curve fittings were 0.906 and 0.988, for $n$-hexane and chloroform, respectively. The absolute values of x-intercepts (Fig. 2A, B) give the estimated contents of 3-MCPD forming substances contained in the...
un-spiked oil, which were calculated to be 0.44 and 0.22 ppm, respectively. The latter value obtained using chloroform as solvents had higher reliability. In conclusion, substitution of \textit{n}-hexane with chloroform contributed to increase the accuracy of DGF standard method, especially at the low concentration.

**Conclusion**

By DGF standard methods C-III 18 for the determination of 3-MCPD, the minimum limit of detection was lower in the case of actual oil samples compared to the calibration samples. The problem was found to be lied in the low recovery of 3-MCPD derivatives from the aqueous phase to the organic phase at the extraction step of the standard procedure. The substitution of the conventional solvent, \textit{n}-hexane, with \textit{n}-butanol, chloroform, and ethyl acetate increased the recovery to the relative extent of 5.6, 4.7, and 3.9, respectively. The modification contributed to improve the accuracy of the method, especially at lower concentration (<1 ppm) of 3-MCPD.

This chapter provides the modification of DGF standard methods C-III 18(09) in order to improve the accuracy to quantify 3-MCPD at lower concentration. It might be important for estimation and control of our daily intake of 3-MCPD, and for the product control in the fat and oil processing.

**References**


the United Nations/World Health Organization).


Chapter 2-3 : 2-Monochloro-1,3-propanediol (2-MCPD) Dynamics in DGF Standard Methods and Quantification of 2-MCPD

Introduction

3-Monochloropropane-1,2-diol (3-MCPD) and glycidyl fatty acid esters are undesired contaminants in food processing. It has been known since 2006 that 3-MCPD fatty acid esters occur in refined oils and they have recently been a major issue because of concerns regarding human health [1]. The amounts of 3-MCPD and its fatty acid esters in fat and oil products were previously measured using the standard method established by the Deutsche Gesellschaft für Fettwissenschaft (DGF), standard method C-III 18 (09) [2]. The determination method consisted of alkali-catalyzed transesterification, followed by derivatization of the released 3-MCPD and quantification via GC/MS. In this method, overestimation of 3-MCPD was observed because reactive glycidyl-ester-derived glycidol was converted to 3-MCPD in the presence of chloride ions during the experimental procedure. The method was therefore revised in 2009 to eliminate glycidyl esters by acidic treatment (option B) prior to the conventional procedure (option A). The value obtained using option B was defined as the true amount of 3-MCPD, and the difference between the values obtained using options A and B was defined as the amount of glycidyl esters, because glycidol is considered to be negligible in fats and oils. However, the revision only took account of glycidol conversion to 3-MCPD in the extraction step, not 3-MCPD conversion to glycidol in the transesterification step. We reported that 3-MCPD and glycidol were interconverted under basic or acidic conditions during the transesterification and extraction steps, as directly measured by NMR [3]. Furthermore, the incomplete elimination of glycidyl esters at high concentrations was responsible for the incorrect quantification. These problems were resolved in DGF standard method C-VI 18 (10) by
eliminating the step for removal of glycidyl esters and using NaBr (in assay B) instead of NaCl (in assay A) in the extraction step to avoid formation of 3-MCPD from glycidol [2].

Kuhlmann reported that 2-monochloropropane-1,3-diol (2-MCPD), which is the positional isomer of 3-MCPD, could also be detected simultaneously [4]. The amount of 2-MCPD was estimated based on the calculated ionization efficiency of 2-MCPD phenylborate, which was deduced from the FID intensity compared with that of 3-MCPD phenylborate. The hypothesis that the two compounds had similar FID intensities was based on the assumption that 2-MCPD has similar physical and chemical properties to those of 3-MCPD. However, this assumption had not been proven since a reference compound had not been available at that period. Moreover, the correct quantification has not achieved.

We found two synthetic methods for pure 2-MCPD in a literature search. Ilczuk et al. synthesized 2-MCPD from glycerol 1,3-diacetate [5], and Tsatsas et al. synthesized it from glycerol 1,3-dibenzyl ether [6]. However, all attempts ended in failure in our laboratory, although the methods were followed faithfully; not 2-MCPD but 3-MCPD was obtained as the main product in the former method, and the final acidic debenzylation was unsuccessful in the latter.

This chapter reports the synthesis of pure 2-MCPD and the conversion factor for its quantification using DGF standard methods C-VI 18 (10). In addition, the dynamics of 2-MCPD, 3-MCPD, and glycidol under the conditions of the standard methods were investigated directly by NMR and GC/MS.

**Experimental Procedures**

**Materials and methods**

3-MCPD, 3-MCPD-$d_5$, glycidol, sodium methoxide/methanol solution, and other starting materials for syntheses, and solvents were purchased from
Wako Pure Chemical Industries Ltd. (Osaka, Japan). NaCl, sulfuric acid, and acetic acid were purchased from Nakalai Tesque Co., Ltd. (Kyoto, Japan). D$_2$O (99.96 atom% D), DMSO-$d_6$ (99.9 atom% D), methanol-$d_4$ (99.8% atom% D) and CDCl$_3$ (99.8 atom% D) were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, USA). Open column chromatography was performed on Silica Gel 60 N (spherical, neutral, 63-210 µm) which was purchased from Kanto Kagaku Co., Inc. (Tokyo, Japan). Other chemicals were of analytical grade. All chemicals were used without purification.

$^1$H and $^{13}$C NMR spectra were recorded on a JEOL EX-270 or JEOL AL-300 spectrometer. Infrared spectra were recorded on a HORIBA FT-730. Elemental analysis was conducted on a CE Instruments EA1110. ESIMS were recorded on a ThermoQuest Finnigan LCQ$^{DECA}$ spectrometer.

**Determination of MCPDs and glycidyl-ester-forming substances by DGF standard method C-VI 18 (10)**

The contents of glycidyl-ester-forming substances were determined as described in DGF standard method C-VI 18 (10), with slight modifications using free-form 3-MCPD-$d_5$ instead of palmitic acid ester-form as an internal standard. Assay A: soybean oil (100 mg) mixed with a spiking component was dissolved in 100 µL of tert-butyl methyl ether. To the sample, a toluene solution of 3-MCPD-$d_5$ (5.0 µg/mL) and 0.5 M sodium methoxide/methanol solution (200 µL) were added, and the mixture was left for 3.5–4.5 min at room temperature. 3-MCPD-$d_5$ was used instead of the esters in this work in order to neglect the transesterification efficiency. Sodium chloride/sulfuric acid solution (200 g/L with 0.9% H$_2$SO$_4$, 600 µL) was added to the solution, and then iso-hexane (600 µL) was added to the solution and the mixture was left for 5 min. The aqueous phase was washed twice with iso-hexane, and the products formed were extracted three times with a mixed solvent (600 µL, ether/ethyl acetate = 6/4, v/v). The organic
phase was dried over sodium sulfate. A phenylboronic acid/ether solution (10–100 µL) was added to the solution, and solvent was removed using a gentle nitrogen stream. Isooctane (1.5 mL) was added to the residue and filtered using paper with sodium sulfate. The solution was analyzed by GC/MS.

**GC/MS quantification**

GC/MS was carried out using a SHIMADZU GCMS QP-2010 instrument equipped with an AOC-20i auto-sampler or a Thermo Scientific ITQ 1100, connected to a DB-5ms capillary column (30 m, 0.25 mm, 0.25 µm, Agilent Technologies). The column temperature was controlled as follows. It was maintained at 85 °C for 0.5 min, raised at rates of 6 °C/min to 150 °C, 12 °C/min to 180 °C, and 25 °C/min to 280 °C, and maintained at 280 °C for 7 min. The carrier gas was high-purity helium at a constant flow of 40 cm/s. Two microliters of sample were injected in pulsed splitless mode. The temperature of the programmed-temperature vaporizer injector for the SHIMADZU GCMS QP-2010 instrument was controlled as follows. It was maintained at 85 °C, raised at a rate of 250 °C/min to 160 °C, maintained at 160 °C for 9.9 min, and further raised at a rate of 250 °C/min to 350 °C. The injector temperature of the Thermo Scientific ITQ 1100 instrument was controlled to be isothermal at 160 °C. The temperatures of the interface and the ion source were set at 250 and 200 °C, respectively. Other conditions for GC/MS were the same as those described in DGF standard method C-VI 18 (10). A mass-selective detector was used for selected ion monitoring, focusing on ions with m/z values of 147, 150, 196, and 201.

**Dynamics observation by NMR**

$^1$H and $^{13}$C NMR spectra were recorded on a JEOL EX-270 spectrometer at 270 MHz and 67.5 MHz, respectively, and were referenced to the residual protonated solvent. $^1$H NMR measurement was conducted by NON pulse
sequence, which consists of a single pulse followed by acquisition of FID. \(^{13}\)C-NMR measurement was conducted by BCM pulse sequence, which uses broadband proton decoupling. Accumulation times were 1 min (8 times) and 20 min (400 times) for \(^1\)H and \(^{13}\)C NMR, respectively.

**Synthesis**

**Glycerol 1,3-dibenzyl ether**

Benzyl alcohol (23.4 g, 216.16 mmol) was dissolved in dry THF (100 mL) at 0 °C under an argon atmosphere. NaH (60% in oil, 6.48 g, 162.12 mmol), which was washed with hexane, was slowly added to the solution. After hydrogen gas evolution stopped, 2-chloromethyloxirane (5.00 g, 54.04 mmol) was slowly added to the reaction mixture, and it was stirred for 30 min. The reaction mixture was heated to reflux by stirring in an oil bath for 2 h. After cooling, ether was added to the mixture, and the mixture was washed with water and brine. The solvent was evaporated to dryness, and the residue was purified by Kugelrohr distillation (185 °C, 40 Pa) to give 1,3-dibenzyl ether as a liquid (13.0 g, 47.59 mmol, 88%): IR (neat) \(v_{\text{max}}/\text{cm}^{-1}\): 3502, 2871, 1718, 1454, 1349, 1276; \(^1\)H NMR (270 MHz, CDCl\(_3\)) \(\delta/\text{ppm}\): 7.32 (s, 10H), 4.54 (s, 4H), 4.02 (m, 1H), 3.55 (m, 4H), 2.47 (bs, 1H); \(^{13}\)C NMR (67.5 MHz, CDCl\(_3\)) \(\delta/\text{ppm}\): 137.9, 128.4, 127.7, 73.4, 71.3, 69.6; MS (ESI) \(m/z\): 295.4 [M + Na\(^+\)]; Anal. Calcd for C\(_{17}\)H\(_{20}\)O\(_3\): C, 74.97; H, 7.40. Found: C, 74.88; H, 7.39.

**2-Chloro-1,3-propanediol dibenzyl ether**

Glycerol 1,3-dibenzyl ether (8.00 g, 29.4 mmol) was dissolved in dry pyridine (2.09 g, 26.5 mmol) at 0 °C, and cooling thionyl chloride (3.15 g, 26.5 mmol) was added to the mixture in small quantities, and then the mixture was stirred for 30 min. The reaction mixture was warmed to 60 °C and stirred for 12 h. After the reaction was complete, the product was extracted with ether, and washed with water, 0.1 M hydrogen chloride
solution, saturated sodium hydrogen carbonate solution, and finally brine. The solution was evaporated to dryness and purified by Kugelrohr distillation (70 °C, 2.3 × 10³ Pa) to give the chloride as a liquid (5.30 g, 18.2 mmol, 62%): IR (neat) ν max/cm⁻¹ 3064, 3031, 2863, 1722, 1496, 1454, 1272, 1205; ¹H NMR (270 MHz, CDCl₃) δ/ppm 7.30 (s, 10H), 4.56 (s, 4H), 4.16 (m, 1H), 3.75 (m, 4H); ¹³C NMR (67.5 MHz, CDCl₃) δ/ppm 137.7, 128.4, 127.7, 127.6, 71.0, 57.8, 21.4; MS (ESI) m/z 313.1 [M + Na]+; Anal. Calcd for C₁₇H₁₉ClO₂: C, 70.22; H, 6.59. Found: C, 70.11; H, 6.33.

2-Chloro-1,3-propanediol (2-MCPD)

2-Chloro-1,3-propanediol dibenzyl ether (5.0 g, 17.2 mmol) and formic acid (2 g, 43.4 mmol) were dissolved in ethanol (20 mL). Pd/C (10%, 100 mg) was added to the stirred solution. The resulting reaction mixture was stirred vigorously under a hydrogen atmosphere for 24 h. The resulting reaction mixture was filtered through a membrane filter, and then washed with ethanol. The filtrate was evaporated to dryness, and the residue was purified by open-column silicagel chromatography (hexane/EtOAc/methanol = 45/45/10, v/v/v) to give 2-MCPD (950 mg, 8.6 mmol) as a clear liquid: IR (neat) ν max/cm⁻¹ 3350, 3938, 3879, 1078, 1035; ¹H NMR (270 MHz, D₂O) δ/ppm 4.11 (m, 1H), 3.81 (m, 4H); ¹³C NMR (67.5 MHz, CDCl₃) δ/ppm 62.3, 61.8; Anal. Calcd for C₃H₇ClO₂: C, 32.60; H, 6.37. Found: C, 32.45; H, 6.13. 2-MCPD was acetylated for further characterization. 2-MCPD (150 mg, 1.36 mmol) was dissolved in dry pyridine (1 mL), and acetic anhydride (255 mg, 2.50 mmol) was added to the solution at 0 °C, and the mixture was then stirred at room temperature for 4 h. The reaction mixture was quenched with methanol at 0 °C, extracted with ethyl acetate, and washed with water, 0.1 M hydrogen chloride solution, saturated sodium hydrogen carbonate solution, and finally brine. The organic layer was evaporated to dryness to give diacetylated 2-MCPD (230 mg, 1.20 mmol, 88%) as a clear liquid: ¹H NMR (270 MHz, CDCl₃) δ/ppm 4.32 (m, 4H), 4.24 (m, 1H), 2.11 (s, 6H);
Results and Discussion

Synthesis of 2-MCPD

Two synthetic methods for 2-MCPD have been reported, as far as we know, although the evidential spectra were not assigned in either report. Ilczuk et al. described the synthesis of 2-MCPD via glycerol 1,3-diacetate as a synthetic intermediate from 1,3-dichloro-2-propanol. In this method [5], however, 2,3-diacetoxy-1-propanol, the migrated isomer of the desired product, was afforded as the main product by treatment with sodium acetate. In contrast, Tsatsas et al. reported the preparation of 2-MCPD via 2-chloro-1,3-dibenzyloxypropane by aqueous or alcoholic hydrogen chloride acidolysis [6]. 2-Chloro-1,3-dibenzyloxypropane was successfully prepared, but no reaction was observed in the following deprotection step of the benzyl ether and the original compound was recovered in good yield. Other acidic treatments, for example, with trifluoroacetic acid, were not effective. Hydrogenation using Pd/C was therefore used for deprotection. The complete removal of thionyl chloride and the addition of formic acid improved the deprotection by 50%. The NMR spectra and elemental analysis of the resulting 2-MCPD (purity >99%) are described in the Experimental Procedures section.

2- and 3-MCPD conversion to glycidol under basic conditions in the course of DGF standard method C-VI 18 (10)

The dynamics of 3-MCPD and glycidol during DGF standard method C-III 18 (09) were monitored by $^1$H and $^{13}$C NMR in our previous research [3]. The results conclusively indicated that 3-MCPD and glycidol were bidirectionally interconverted under different conditions: 3-MCPD to
glycidol under basic conditions, and glycidol to 3-MCPD under acidic conditions. The dynamics of 2-MCPD has been assumed to be similar to that of 3-MCPD, and 2-MCPD has been naturally believed to be converted to glycidol under basic conditions, as in the case of 3-MCPD. However, this has not yet been verified. The behavior of 2-MCPD in the transesterification step was therefore monitored directly using $^1$H and $^{13}$C NMR.

![Figure 1](image)

**Figure 1.** $^{13}$C NMR spectra of 2-MCPD, 3-MCPD, glycidol, and their products. Diamonds, peaks derived from 2-MCPD; circles, glycidol; inverted triangles, 3-MCPD. (a) 2-MCPD (10 mg) dissolved in D$_2$O (1 mL); (b) 2-MCPD (10 mg) dissolved in 0.5 M sodium methoxide/methanol solution (1 mL) was kept at room temperature for 4 min. Then, D$_2$O (1 mL) was added to the solution. (c) 2-MCPD (10 mg) dissolved in 0.5 M sodium methoxide/methanol solution (0.2 mL) was kept at room temperature for 4 min. Then, D$_2$O containing 20% sodium chloride and 0.9% sulfuric acid (0.6 mL) was added to the mixture, and it was allowed to stand for 5 min. (d) 3-MCPD (10 mg) dissolved in D$_2$O (1 mL). (e) Method similar to (b), except using 3-MCPD (10 mg). (f) Method similar to (c), except using 3-MCPD (10 mg). All reactions were conducted in NMR tubes. The accumulation time for $^{13}$C NMR measurement took 20 min described in the section “Dynamics observation by NMR”, the signals served as an average for accumulation.

When 2-MCPD (10 mg) was dissolved in D$_2$O, the $^{13}$C peaks derived from 2-MCPD (63.4, 62.9 ppm) were the only peaks (Fig. 1a). 2-MCPD (10 mg) was dissolved in sodium methoxide/methanol solution, and kept at room temperature for 5 min (pH 10.8), the same as in the transesterification step. After the addition of D$_2$O, the sample was examined using NMR. After the treatment, peaks derived from glycidol (61.5, 53.0, 45.0 ppm) appeared (Fig. 1b) in addition to those derived from 2-MCPD. The conversion was 3
mole% after 5 min treatment, calculated from the proton numbers obtained by $^1$H NMR (Fig. 2b). When 3-MCPD (10 mg) was treated similarly, the peaks of 3-MCPD (71.2, 62.6, 45.9 ppm) and glycidol (61.5, 53.0, 45.0 ppm) were observed (Fig. 1e). The conversion was 22 mole% after 5 min treatment (Fig. 2b). When the sample concentration decreased to 2 mg, both of their conversions increased to 4 mole% for 2-MCPD and 34 mole% for 3-MCPD (Fig. 2a). The conversions might differ depending on the sample concentration. The treatment time (3.5-5.5 min) described in DGF standard method C-VI 18 (10) is appropriate in order to minimize the generation of glycidol.

**Bidirectional conversion between 2- and 3-MCPD and glycidol under standard method conditions**

The samples in sodium methoxide/methanol solution were subsequently treated with 20% NaCl and 0.9% sulfuric acid, and then allowed to stand for
5 min, the same as in the extraction step of standard method C-VI 18 (10), except that iso-hexane and phenylboronic acid were not added and D$_2$O was used instead of water. The pH of the reaction mixture was 3.9. In the case of 3-MCPD, only 3-MCPD (71.2, 62.6, 45.9 ppm) was generated from glycidol, as reported in our previous research (Fig. 1f, ref. 3). In the case of 2-MCPD, only peaks derived from 2-MCPD (65.8, 65.3 ppm) were observed (Fig. 1c), though glycidol must be converted to only 3-MCPD. These results indicated that both 2- and 3-MCPD were converted to glycidol under basic conditions, and the generated glycidol was reconverted to the original MCPD under acidic conditions.

In order to clarify the strange phenomenon, the memory effect, the same treatment was conducted using a mixed system of 2-MCPD, 3-MCPD, and glycidol. Equimolar amounts of 2-MCPD and glycidol treated under basic conditions gave 2-MCPD (46 mole%) and glycidol (54 mole%), and the subsequent acidic treatment below pH 4.2 afforded 2-MCPD (50 mole%) and 3-MCPD (50 mole%), and trace amount of glycidol (Fig. 3a, 3b). Equimolar amounts of 3-MCPD and glycidol treated under basic conditions gave 3-MCPD (25 mole%) and glycidol (75 mole%), and the subsequent acidic treatment below pH 4.2 afforded mainly 3-MCPD and trace amount of glycidol (Fig. 3c, 3d). These results indicated that the purchased glycidol was converted to 3-MCPD under acidic conditions. Equimolar amounts of 2-MCPD and 3-MCPD treated under basic conditions gave 2-MCPD (47 mole%), 3-MCPD (32 mole%), and glycidol (21 mole%), and the subsequent acidic treatment below pH 4.2 afforded 2-MCPD (50 mole%) and 3-MCPD (50 mole%) (Fig. 3e, 3f). This result indicated that both 2-MCPD and 3-MCPD were converted to glycidol, and the glycidol was reconverted to the original 2- and 3-MCPD, respectively. Low pH treatment is essential for the complete reconversion of glycidol to MCPD, as glycidol was clearly observed in the samples that the acid treatment was conducted above pH 4.2 (eg. pH 5.5).
Overall, the above observations suggested that glycidol derived from 2- or 3-MCPD was chlorinated at the original position to mainly generate the original MCPD by an unknown steric effect. Eliminated chloride anion from MCPD might form a glycidol-chloride anion complex and recombined to

Figure 3. $^{13}$C NMR spectra of 2-MCPD, 3-MCPD, glycidol, and their products. Diamonds, peaks derived from 2-MCPD; circles, glycidol; inverted triangles, 3-MCPD; triangles, glycerol 2-methyl ether. (a) 2-MCPD (13 mg) and glycidol (13 mg) dissolved in 0.5 M sodium methoxide/methanol solution (1 mL) were kept at room temperature for 4 min. Then, D$_2$O (1 mL) was added to the solution. (b) 2-MCPD (13 mg) and glycidol (13 mg) dissolved in 0.5 M sodium methoxide/methanol solution (0.2 mL) were kept at room temperature for 4 min. Then, D$_2$O containing 20% sodium chloride and 0.9% acetic acid (0.6 mL) was added to the mixture, and it was allowed to stand for 5 min. (c) Method similar to (a), except using 3-MCPD (13 mg) and glycidol (13 mg). (d) Method similar to (b), except using 3-MCPD (13 mg) and glycidol (13 mg). (e) Method similar to (a), except using 2-MCPD (13 mg) and 3-MCPD (13 mg). (f) Method similar to (b), except using 2-MCPD (13 mg) and 3-MCPD (13 mg). The accumulation time for $^{13}$C NMR measurement took 20 min described in the section “Dynamics observation by NMR”, the signals served as average for accumulation.
glycidol to generate original MCPD. Unfortunately, the NMR observations did not provide structural evidence of the glycidol-chloride anion complex.

Dynamics of MCPDs and glycidol in soybean oil
Soybean oil spiked with MCPDs was treated according to standard method C-VI 18 (10), assay A. A small amount (< 2 mole%) of 3-MCPD was observed in the sample of soybean oil spiked with 2-MCPD (Fig. 4a).

![Figure 4](image)

**Figure 4.** Analyses of oils spiked with (a) 2-MCPD and (b) 3-MCPD. Soybean oil spiked with MCPDs was analyzed using DGF standard method C-VI 18 (10). Circles and triangles represent the concentration of detected 3- and 2-MCPD, respectively. (a) Linear curve-fitting equations are $y = 0.02x + 0.064$ for 3-MCPD, and $y = 0.99x + 0.076$ for 2-MCPD. (b) Linear curve-fitting equations are $y = 1.37x - 0.085$ for 3-MCPD and $y = 0.06x - 0.053$ for 2-MCPD. All analyses were carried out in duplicate.

The correlation coefficients ($R^2$ values) were >0.999 for 2-MCPD phenylborate and 0.997 for 3-MCPD phenylborate. 3-MCPD was not detected from 2-MCPD by NMR as described in the previous section, but was detected by GC/MS, probably because of better sensitivity, although the initial concentration was sufficiently high in the case of NMR. Similarly, a small amount (<4 mole%) of 2-MCPD was observed in the sample spiked with 3-MCPD (Fig. 4b). The interconversions of 2- and 3-MCPDs differed by only a few per cent after treatments of the standard method. This could
be explained by supposing that the conversion of MCPDs to glycidol in the transesterification step was approximately equal to that of glycidol to MCPDs in the following extraction step.

Soybean oil spiked with glycidyl stearate and glycicyl oleate, respectively, was then similarly treated. The concentrations of the products were plotted as the ordinate and the initial concentration of glycidyl fatty acids as the abscissa (Fig. 5).

![Figure 5. Analyses of oils spiked with glycidyl ester.](image)

Soybean oil spiked with (a) glycidyl stearate and (b) glycidyl oleate were analyzed using DGF standard method C-VI 18 (10). Circles and triangles represent the concentrations of detected amount of 3- and 2-MCPD, respectively. Linear curve-fitting equations are (a) \( y = 1.00x + 0.623 \) \((R^2 = 0.99998)\) for 3-MCPD and \( y = 0.06x + 0.278 \) for 2-MCPD \((R^2 = 0.9998)\) and (b) \( y = 0.82x + 0.682 \) for 3-MCPD \((R^2 = 0.99995)\) and \( y = 0.048x + 0.210 \) for 2-MCPD \((R^2 = 0.9997)\). All analyses were carried out in duplicate.

The values for 2-MCPD phenylborate and 3-MCPD phenylborate increased linearly according to the initial concentration of glycidyl fatty acids. The ratio of 2-MCPD phenylborate/3-MCPD phenylborate was 5.7/94.3 for glycidyl stearate and 5.5/94.5 for glycidyl oleate, calculated from the slopes. This indicated that glycidol derived from glycidyl ester afforded both 2-MCPD and 3-MCPD, with a preference for the latter. The amount of 3-MCPD generated from the glycidyl ester was corrected using the
transformation factor \((t)\) described in section 8.1.3 of standard method C-VI 18 (10), but the generation of 2-MCPD is currently neglected. The factor relevant to 2-MCPD should be determined for accurate estimation of the amount of glycidol ester because the value depends on the individual experimental environment. Otherwise, removal of glycidol esters using an adsorbent prior to the transesterification step [7] is recommended.

Quantification by GC/MS of 2-MCPD and its sensitivity compared with 3-MCPD

2-MCPD phenylborate, which forms a 1,3-cyclic ester, might have a different sensitivity from that of 3-MCPD phenylborate because of the different fragmentation patterns. Kuhlmann claimed that the sensitivity of 2-MCPD phenylborate calculated from FID intensities was approximately twice that of 3-MCPD phenylborate. However, the true sensitivity should be determined using a pure 2-MCPD standard. The quantification of 2-MCPD phenylborate and its sensitivity compared with that of 3-MCPD phenylborate were therefore investigated using two GC/MS systems, SHIMADZU GCMS QP-2010 and Thermo Scientific ITQ 1100, in different institutes. The mass unit of QP-2010 was composed of an electron impact (EI) ionizer and a standard quadrupole (Q) detector, and that of ITQ 1100 was composed of an EI ionizer and an ion-trap unit before a Q detector (Table 1).

Table 1. Peak area ratios of 2- and 3-MCPD phenylborates after treatment of MCPD spiked soybean oil (20 ng/mL) using DGF standard method C-VI 18 (10), assay A.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Peak area ratio of (m/z) 196 to (m/z) 201</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-MCPD</td>
</tr>
<tr>
<td>QP-2010</td>
<td>13.8 ± 0.1</td>
</tr>
<tr>
<td>ITQ1100</td>
<td>08.8 ± 0.0</td>
</tr>
</tbody>
</table>

(a) A theoretical value is 4.0.
When soybean oil samples containing various concentrations of pure 2-MCPD and 3-MCPD-$d_5$ were treated using standard method C-VI 18 (10), the peak area ratio of $m/z$ 196 (the precursor ion peak of 2-MCPD phenylborate) to $m/z$ 201 (the precursor ion peak of 3-MCPD-$d_5$ phenylborate) was plotted against the spiked 2-MCPD concentrations. The calibration curves were linear (Fig. 6). Although the slopes of the two lines were different, both had correlation coefficients ($R^2$ values) greater than 0.999.

![Figure 6. Calibration curves of 2-MCPD obtained using two GC/MS systems.](image)

Circles and triangles represent the area ratio of 2-MCPD ($m/z$ 196) phenylborate to 3-MCPD-$d_5$ ($m/z$ 201) phenylborate, detected using QP-2010 and ITQ1100, respectively. Linear curve-fitting equations are $y = 3.26x + 0.306$ for QP-2010 and $y = 2.16x + 0.571$ for ITQ1100. All analyses were carried out in duplicate.

From the slopes of the lines, the sensitivity of 2-MCPD phenylborate compared with that of 3-MCPD phenylborate under the respective machine conditions were found to be 3.26-fold (QP-2010) and 2.16-fold (ITQ1100), as shown in Table 2. The peak area ratio of the fragment ion of 3-MCPD-$d_5$ phenyl borate ($m/z$ 150) to the precursor ion of 3-MCPD-$d_5$ phenyl borate ($m/z$ 201) gave constant values of $5.11 \pm 0.20 \ (n = 9)$ and $3.30 \pm 0.11 \ (n = 9)$,
measured by QP-2010 and ITQ 1100, respectively. The sensitivity of 2-MCPD phenylborate is referenced by the precursor ion of 3-MCPD-\textsubscript{d5} phenylborate. Though 3-MCPD-\textsubscript{d5} phenylborate fragments at different efficiency for individual equipment due to the complex fragmentation, the amount of 2-MCPD phenylborate ion is constant due to the stable precursor ion.

Table 2. Ion ratio of the fragment to the precursor of 3-MCPD-\textsubscript{d5} phenylborate and the sensitivity ratio of 2-MCPD phenylborate to 3-MCPD phenylborate obtained from GC/MS measurements.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Ion-ratio of fragment ((m/z \text{ 150})) to precursor ((m/z\text{ 201})) of 3-MCPD-\textsubscript{d5}</th>
<th>Sensitivity ratio of 2-MCPD phenylborate to 3-MCPD-\textsubscript{d5} phenylborate</th>
<th>Correction constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>QP-2010</td>
<td>5.11±0.20</td>
<td>3.26±0.02</td>
<td>0.64</td>
</tr>
<tr>
<td>QP-2010\textsuperscript{a}</td>
<td>5.42±0.49</td>
<td>3.45±0.01</td>
<td>0.64</td>
</tr>
<tr>
<td>ITQ 1100</td>
<td>3.30±0.11</td>
<td>2.16±0.01</td>
<td>0.66</td>
</tr>
</tbody>
</table>

\(\textsuperscript{a}\) The measurement conducted on a different day.

Thus, the sensitivity ratio of 2-MCPD phenylborate depends on the ion ratio of the fragment \((m/z \text{ 150})\) to the precursor \((m/z\text{ 201})\) of 3-MCPD-\textsubscript{d5} phenylborate. The ratio of the sensitivity of 2-MCPD to the ion-ratio of the fragment to the precursor \(201\) of 3-MCPD-\textsubscript{d5} is constant (0.65±0.01, Table 2). A measurement by QP-2010 in another day gave same results.

Since the ion-ratio of 3-MCPD-\textsubscript{d5} can be adjusted as mentioned above, the quantification of 2-MCPD without a pure 2-MCPD standard might be achieved by using the sensitivity ratio (peak intensity ratio of 2-MCPD phenylborate \((m/z \text{ 196})\) to 3-MCPD-\textsubscript{d5} phenylborate \((m/z\text{ 201})\)) and the ion-ratio of the fragment to the precursor (peak intensity ratio of the fragment \((m/z \text{ 150})\) to the precursor \((m/z\text{ 201})\) of 3-MCPD-\textsubscript{d5} phenylborate) obtained using any instrument. The suggested formula is
The term $SF_{1150}/SF_{1201}$ in Eq. 1 is the observed fragmentation efficiency. So,

$$w_{2-MCPD} = \frac{SF_2 \times w_{3-MCPD-d5}}{SF_{1201}} \times \frac{SR \times SF_{1150}}{SF_{1201}}$$

(1)

The term $SF_{1150}/SF_{1201}$ in Eq. 1 is the observed fragmentation efficiency.

So,

$$w_{2-MCPD} = \frac{CC \times SF_2 \times SF_{1150} \times w_{3-MCPD-d5}}{SF_{1201}^2}$$

(2)

where

$w_{2-MCPD}$ is the mass fraction, in mg/kg, of 2-MCPD;

$w_{3-MCPD-d5}$ is the mass fraction, in mg/kg, of 3-MCPD-$d_5$;

$SF_{1201}$ is the area of 3-MCPD-$d_5$ (m/z 201);

$SF_{1150}$ is the area of 3-MCPD-$d_5$ (m/z 150);

$SF_2$ is the area of 2-MCPD (m/z 196);

$SR$ in Eq. 1 is the sensitivity ratio of 2-MCPD phenylborate (m/z 196) to 3-MCPD-$d_5$ phenylborate (m/z 201) obtained using our GC/MS instrument (see Table 2);

$IR$ in Eq. 1 is the ion-ratio of the fragment to the precursor of 3-MCPD-$d_5$ phenylborate obtained using our GC/MS instrument (see Table 2); and

$CC$ in Eq 2 is a correction constant (0.65 ± 0.01).

Suggested dynamics of MCPDs and glycidol in reported assays

The suggested dynamics of MCPDs and glycidol in previously reported assays are discussed based on our NMR and GC/MS results in this section. In the DGF standard method C-VI 18 (10), assay A (Fig. 7), 2-MCPD and 3-MCPD were partly converted to glycidol by basic treatment with methanolic sodium methoxide, and the generated glycidols were mostly (>96 mole%) converted to the original 2-MCPD and 3-MCPD by subsequent acidic treatment with aqueous sulfuric acid and sodium chloride. The glycidol was unchanged by the basic treatment, whereas the glycidol was
converted to 2-MCPD (5.6 mole%) and 3-MCPD (94.4 mole%) by the acidic treatment. In the standard method, the true amount of 3-MCPD is calibrated using the transformation factor (\( t \)). However, as the total amount of 2-MCPD and 3-MCPD is not consistent with the initial amount of glycidyl

![Diagram of MCPD and glycidol dynamics](image)

**Figure 7.** Suggested dynamics of MCPDs and glycidol during proposed methods. Arrows and broken arrows represent the path observed using NMR and the minor path observed only in GC/MS, respectively. DGF standard method C-VI 18 (10), assay A: the first arrows represent basic treatment with methanolic sodium methoxide, and the second arrows represent acidic treatment with sulfuric acid/sodium chloride. DGF standard method C-VI 18 (10), assay B: the first arrows represent basic treatment with methanolic sodium methoxide, and the second arrows represent acidic treatment with sulfuric acid/sodium bromide. Ermacora method: the first arrows represent acidic treatment with sulfuric acid/methanol, and the second arrows represent neutral treatment with sodium sulfate.
ester (Fig. 5), the transformation factor needs to be further corrected, considering the conversion to 2-MCPD, for accurate calibration. In the course of the method using acidified sodium bromide solution at the extraction step in assay B, glycidol was claimed to be converted to both 2-monobromopropnanediol (2-MBPD) and 3-MBPD [4]. There is a possibility that MBPDs could also be generated by sodium bromide treatment of glycidols, generated from MCPDs under the basic conditions in the previous transesterification step. To investigate the possibility that 2-MCPD-derived glycidol is converted to 3-MBPD, NMR spectra were measured after treatments with basic sodium methoxide and then acidic sodium bromide. Peaks similar to 2-MCPD were obtained in the $^{13}$C-NMR (Fig. 8) and $^1$H NMR spectra (data not shown). These peaks were assigned to 2-MCPD, based on the chemical shifts, as unconverted 2-MCPD remained from the previous step (Fig. 8b) and one chemical species was observed after the treatment with sodium bromide. These results suggested that glycidol generated from 2-MCPD might be reconverted to the same 2-MCPD by the recombination of the eliminated chloride ion under the conditions of standard method C-VI 18 (10), assay B, even in the presence of an excess amount of bromide ions. This means that the amounts of MCPDs do not change before and after the treatment with sodium bromide, and that the MCPDs can be quantified correctly. However, the amount of glycidyl esters should be estimated based on both 2- and 3-MBPD phenylborate because glycidyl-ester-derived glycidol is converted to both 2- and 3-MBPD. If the peaks shown in Fig. 8c were assigned to 2-MBPD, although the possibility is low, the amount of glycidyl esters cannot be quantified by standard method C-VI 18 (10), assay B, but 3-MCPD can be correctly quantified because the possibility of conversion of 3-MCPD to 3-MBPD should be equal to that of conversion of the reference 3-MCPD-$d_5$. 2-MCPD can be quantified correctly for the same reason, but glycidol cannot be quantified from the increase in MBPDs from MCPDs.
In the course of the method using methanolic sulfuric acid as an acidic transesterification reagent, proposed by Ermacora et al. [8], 2-MCPD and 3-MCPD remain unconverted in the transesterification step. Complete demineralization of chloride ions by a pre-cleaning step and elimination of glycidol by acidic treatment of an oil sample enable the accurate and precise quantification of MCPDs.

**Conclusion**

2-MCPD was synthesized and first quantitatively analyzed by DGF standard method C-VI 18 (10). Preparation of a calibration curve using a pure 2-MCPD standard is not required for the quantification. The amount can be determined by a calculation to correct the ion-ratio of the fragment to the precursor of 3-MCPD-$d_5$ phenylborate of the individual GC/MS in any

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**Figure 8.** $^{13}$C NMR spectra of 2-MCPD and their products. (a) 2-MCPD (10 mg) dissolved in $D_2O$. (b) 2-MCPD (10 mg) dissolved in 0.5 M sodium methoxide/methanol solution (1 mL) was kept at room temperature for 10 min. Then, $D_2O$ (1 mL) was added to the solution. (c) 2-MCPD (10 mg) dissolved in 0.5 M sodium methoxide/methanol solution (0.2 mL) was kept at room temperature for 10 min. Then, $D_2O$ containing 20% sodium bromide and 0.9% acetic acid (0.6 mL) was added to the mixture, and it was allowed to stand at 80 °C for 20 min. Peaks with same chemical shifts as those of 2-MCPD were obtained; all reactions were conducted in NMR tubes. Diamonds, peaks derived from 2-MCPD; circles, glycidol.
laboratory. In addition, the amounts of 2-MCPD in previous samples or previous data could be re-analyzable using the peak intensity of 2-MCPD and the peak area of 3-MCPD-$d_5$ fragment ($m/z$ 150).

The comprehensive dynamics and analyses of MCPDs and glycidol in the course of the treatments in DGF standard method C-VI 18 (10) have been presented in this chapter. The direct observation of MCPDs and glycidol by NMR in the course of the analytical procedures of the standard methods and indirect observations by GC/MS revealed the following dynamics: MCPDs were partly converted to glycidol in the basic transesterification step, and the glycidol was mainly converted to the original MCPD isomers in the extraction step conducted under acidic conditions in the presence of NaCl (or NaBr). Isomerization between 2- and 3-MCPDs was imperceptible in the less-sensitive NMR observations. However, GC/MS analyses indicated that 2- and 3-MCPD spiked in soybean oil respectively were converted to 2-MCPD (98 mole%) and 3-MCPD (2 mole%), and 2-MCPD (4 mole%) and 3-MCPD (96 mole%), in the course of standard method C-VI 18 (10), assay A. In addition, glycidyl ester spiked in soybean oil was converted to 2-MCPD (5.5-5.7 mole%) and 3-MCPD (94.3-94.5 mole%) in this method. The amount of converted 2-MCPD should therefore be considered in the quantification of glycidyl esters, or they should be removed prior to the alkaline transesterification. Acidic transesterification is also recommended for the correct quantification of MCPDs.

References


2. Deutsche Gesellschaft für Fettwissenschaft, DGF Standard Methods Section C-Fats C-VI 18 (10) (2011) Fatty-acid-bound
3-chloropropane-1,2-diol (3-MCPD) and 2,3-epoxipropene-1-ol (glycidol) Determination in oils and fats by GC/MS (Differential measurement)


Chapter 3 : Estimation of the intestinal absorption and metabolism behaviors of 2- and 3-monochloropropanediol esters

Introduction
3-Monochloropropanediol (MCPD) is classified as being potentially carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (IARC) monographs [1], with a maximum tolerable intake of 2 µg/kg body weight per day set by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [2]. However, the regioisomer of this compound, 2-MCPD, is not currently classified in this list because of a lack of epidemiological and experimental evidence concerning its carcinogenicity [3, 4]. Yet, food contamination by 2-MCPD is generally discouraged.

2- and 3-MCPDs have been detected as the corresponding fatty acid esters in a variety of different natural and processed oils and fats [5,6]. The levels of 2-MCPD esters detected in palm oil were in the range of 20–50% of the 3-MCPD esters [7]. It has been suggested that the MCPD esters present in materials are mainly generated during the distillation steps (i.e., deodorization and deacidification) of the oil purification process, especially in the treatment of oils with partial acylglycerols and chloride-containing substances [8, 9].

Although the toxicities of 3-MCPD esters have been estimated to be mild compared with the toxicity of free 3-MCPD, they can be hydrolyzed following their ingestion to release free 3-MCPD, and therefore represent a
risk to human health. The internal kinetics associated with the metabolism and breakdown of 3-MCPD esters have therefore been evaluated in detail [10, 11]. Notably, the amount of 3-MCPD detected in the blood of rats after the oral administration of 3-MCPD-dipalmitate was 86% of the amount achieved following the administration of the same dose of 3-MCPD [10]. It has also been reported that the administration of 3-MCPD-dipalmitate resulted in a 30% reduction in the level of 3-MCPD urinary metabolites compared with the administration of 3-MCPD in a study pertaining to the bioavailability of MCPD esters [11].

With regard to the *in vitro* evaluation of the metabolism of MCPD following its ingestion, it has been reported that 3-MCPD esters can be hydrolyzed by pancreatic lipases (EC 3.1.1.3) to release 3-MCPD [12]. Although the primary metabolites of 3-MCPD diesters are generally believed to be the corresponding 3-MCPD-2-monoesters, the impact of the accumulation and metabolic fate of these substances has not been monitored. There have also been no studies pertaining to the differences in the hydrolysis behaviors of the two regioisomers of the 3-MCPD monoesters. In addition, to the best of our knowledge, the hydrolysis of 2-MCPD esters by pancreatic lipase has not yet been investigated in detail.

Caco-2 cells, which are human colon adenocarcinoma cells, exhibit enterocyte-like characteristics and have been widely used as an *in vitro* model of intestinal absorption in epithelial cells [13, 14]. The absorption behaviors of 2- and 3-MCPDs, as well as their esters, have been evaluated using a Caco-2 cell model [15, 16]. The results of these studies revealed
that the free MCPD transferred from the apical side of the Caco-2 monolayer to the basolateral side with time to reach equilibrium. In contrast, the MCPD esters were only detected on the apical side of the monolayer. Interestingly, free 2-MCPD was detected on the basolateral side of the monolayer when 2-MCPD-dipalmitate was applied to the apical side, suggesting that the 2-MCPD-dipalmitate was being hydrolyzed in the presence of the Caco-2 cells. In contrast, the application of 3-MCPD-dipalmitate to the apical side of a Caco-2 monolayer did not result in the detection of free 3-MCPD on the apical or basolateral side of the monolayer, even though the amount of 3-MCPD-dipalmitate on the apical side of the monolayer decreased with time. The mechanism responsible for this observed decrease in the level of 3-MCPD-dipalmitate has not yet been fully explained, because the authors of these reports did not quantify all the hydrolysates that could be generated from the MCPD diesters (i.e., the two isomers of the MCPD monoesters or the free MCPD). It is noteworthy that the absorption and metabolism behaviors of 3-MCPD-2-monoesters, which represent the most plausible products of the pancreatic lipase-mediated hydrolysis of 3-MCPD diesters, have not been evaluated.

In this study, we have synthesized 2-MCPD-dioleate and evaluated its hydrolysis in the presence of porcine pancreatic lipase and pancreatin to estimate the internal kinetics of 2-MCPD esters compared with those of the corresponding 3-MCPD esters. 2- and 3-MCPD dioleates were selected as a model system because it exists as a liquid at 37 °C, and were therefore considered to be more suitable than a solid material for the in vitro
evaluation of the absorption and metabolism of MCPD esters. In contrast to MCPD dioleates, MCPD dipalmitates, which has been used in several previous reports, is a solid at 37 °C, making its application to these studies more challenging. In addition, the intestinal absorption of 2-MCPD-dioleate was estimated in vitro using Caco-2 cells as an epithelial cell model. The regioisomers of the MCPD monoesters were successfully separated by HPLC analysis using a corona charged aerosol detection system, which allowed for the detection of the different compounds in the lipase hydrolysates and the solutions recovered from the Caco-2 cell monolayer system.

The results of this study showed that 2-MCPD-dioleate is mainly hydrolyzed to give free 2-MCPD by pancreatic lipase and pancreatin, whereas 3-MCPD-dioleate is mainly hydrolyzed to produce 3-MCPD-2-oleate. Furthermore, we have synthesized 3-MCPD-2-oleate and evaluated its digestion and absorption behaviors in vitro.

Experimental procedures

Chemicals and materials

3-MCPD was purchased from Kanto Chemical Co. (Tokyo, Japan), and 3-MCPD-\(d_5\) was purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-MCPD and the mono- and di-oleates of MCPD were synthesized as described previously [17, 18]. Pancreatic lipase and myristic acid (14:0) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Pancreatin from porcine pancreas was purchased from Sigma-Aldrich Japan (Tokyo,
Japan).

Chloroform, n-hexane, isoctane, methanol, tert-butyl methyl ether (t-BME), tris-(hydroxymethyl)-aminomethane (Tris), deoxycholic acid sodium salt, CaCl$_2$, anhydrous sodium sulfate and all of the other reagents used in the current study were purchased from Kanto Chemical Co, Wako Pure Chemical Industries (Osaka, Japan) and Nakalai Tesque Inc. (Kyoto, Japan). All of these materials were purchased as the analytical grades unless otherwise specified.

**Hydrolysis of the MCPD esters by pancreatic lipase and pancreatin, and the following sample preparation for HPLC-CAD and GC-MS**

An MCPD ester (0.02 mmol) was added to 1 mL of solution A (2.7 mM deoxycholic acid, 1 mM CaCl$_2$, 50 mM Tris-HCl, pH 8.0), and the resulting mixture was sonicated for 5 min. The mixture (1 mL) was then treated with 0.2 mL of pancreatic lipase solution (10 mg/mL of 50 mM Tris-HCl, pH 8.0), and the resulting mixture was stirred at 37 °C for 0–3 h. The reaction was stopped by the addition of 5 mL of chloroform/methanol (4:1, by vol). 3-MCPD-$d_5$ (0.15 mg) and myristic acid (2 mg) were then added to the mixture as internal standards, followed by 1 mL of brine, and the resulting mixture was vigorously agitated for 1 min. The mixture was then centrifuged for 5 min at 1,600 ×g. The bottom phase was subsequently dried over anhydrous sodium sulfate, sampled (1 mL) and filtered through paper before being subjected to HPLC analysis to measure the MCPD ester and free fatty acid contents of the sample. The remainder of the dried bottom phase was
treated with 0.025 mL of a saturated phenyl boric acid solution in diethyl ether to measure the content of free MCPD, based on DGF standard method C-VI 18 (10) [19]. The resulting mixture was dried under a gentle stream of nitrogen gas to give a residue. The residue was then dissolved in 2 mL of iso-octane, and the resulting mixture was filtered through paper to remove any insoluble material (e.g., anhydrous sodium sulfate). The filtrate was then subjected to GC-MS analysis to quantify the amount of free MCPD. The hydrolysis reactions of the MCPD esters with pancreatin were conducted in a similar manner, except the concentration of the pancreatin solution was 2 mg/mL. All of the reactions and analyses were conducted in triplicate and the results reported as the mean values ± SD.

**Cell culture**

Caco-2 cells (human colon adenocarcinoma cells) were obtained from Riken BioResource Center (Tsukuba, Japan). The culture media and supplements used in these experiments were purchased from Nakalai Tesque Inc. Caco-2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 150 mL/L fetal calf serum, 10 mL/L MEM Non-Essential Amino Acids Solution and 10 mL/L antibiotics (10,000 U/mL penicillin and 10 mg/mL streptomycin) in a humidified atmosphere of 5% CO$_2$ at 37 °C. The cells were passaged at 70–80% confluence every 5–7 days by the treatment of the cells with 0.25% trypsin and a 1 mM EDTA solution. The cells were then seeded in new plates at a density of $1.6 \times 10^4$ cells/cm$^2$. 
Transport analysis

For transport analysis, the Caco-2 cells were cultured on Transwell® inserts (1.12 cm² growth surface area, 0.4 µm pore size, polycarbonate membranes; Corning Inc., Lowell, MA) for 3 weeks to allow for cellular differentiation and the formation of a monolayer. The culture medium was substituted with DMEM supplemented with 100 U/mL penicillin and 0.1 mg/mL streptomycin, and the cells were then incubated overnight in a humidified atmosphere of 5% CO₂ at 37 °C. The integrity of the Caco-2 cell monolayer was checked by measuring the transepithelial electrical resistance (TEER) using an epithelial volt-ohm meter (Millicell ERS-2, Millipore Corp., Billerica, MA). Inserts with TEER > 560 ohm × cm² were used for the subsequent analysis. The medium in each chamber was changed to HBSS buffer and the plates were allowed to stand for 1 h in a humidified atmosphere of 5% CO₂ at 37 °C. Five hundred microliter samples of HBSS buffer containing the test substances were added to the apical chamber. Each ester was added as a solution in solution A to give a final concentration of 1 or 10 mM, and the resulting mixture was sonicated for 5 min. The mixture was then diluted 10-fold in HBSS. HBSS buffer (1.5 mL) was added to the basolateral chamber and the plate was incubated in a humidified atmosphere of 5% CO₂ at 37 °C. The solutions in the apical and basolateral chambers were subsequently recovered. Two hundred microliters of HBSS buffer containing 0.05% TritonX-100 was then added to the apical chamber, and the cells were allowed to stand for 5 min at room temperature. The Caco-2 cells were then scraped out from the membrane, and recovered together with the
solution. The samples were stored at –20 °C for further analysis. MCPD and its esters were extracted from each sample as described in section ‘Hydrolysis of MCPD esters by pancreas lipase and pancreatin, and the following sample preparation for HPLC-CAD and GC-MS’ using 0.01 mg of 3-MCPD-\(d_5\) and 0.2 mg of myristic acid as internal standards. All of the sample preparations and analyses were conducted in triplicate and the results reported as the mean values ± SD.

**HPLC-CAD**

HPLC analysis was conducted on an LC-2000 system (JASCO Analytical Instruments, Tokyo, Japan), equipped with a corona charged aerosol detector (CAD, Thermo Fisher Scientific K.K., Yokohama, Japan) and two consecutive YMC-Pack PVA-Sil-NP columns (250 × 4.6 mm, 5 µm, 12 nm, YMC Co., Kyoto, Japan). The column temperature was controlled at 20 °C. The mobile phase used for the elution consisted of \(n\)-hexane/ \(t\)-BME/ methanol (88:10:2, by vol). The HPLC system was run at a flow rate of 1.0 ml/min. A standard curve was prepared on each day of analysis. The extraction rate was corrected based on the response of the internal standard, myristic acid, as it was a pure reagent, and did not overlap with other peaks derived from the samples on the HPLC chromatogram.

**Quantification of free MCPDs by GC-MS**

Samples for GC-MS analyses were prepared as described in the previous sections. Conditions for GC-MS analyses were set based on DGF standard
method C-VI 18 (10, section 7.5) [19]. A Shimadzu QP-2010 GC-MS instrument (Shimadzu, Kyoto, Japan) was equipped with an AOC-20i auto-sampler and a DB-5ms capillary column (30 m × 0.25 mm, 0.25 µm; Agilent Technologies, Santa Clara, CA). The column temperature was controlled as follows: 85 °C for 0.5 min; 85 to 150 °C at a rate of 6 °C/min; 150 to 180 °C at a rate of 12 °C/min; 180 to 280 °C at a rate of 25 °C/min; and 280 °C for 7 min. High-purity helium was used as the carrier gas at a constant flow rate of 40 cm/s. Two microliters of sample were injected into the system in the pulsed splitless mode. The temperature of the programmed-temperature vaporizer injector for the Shimadzu GCMS QP-2010 instrument was controlled as follows: maintained at 85 °C; 85 to 160 °C at a rate of 250 °C/min; maintained at 160 °C for 9.9 min; and 160 to 350 °C at a rate of 250 °C. The temperatures of the interface and the ion source were set at 250 and 200 °C, respectively. A mass-selective detector was used to monitor selected ions with m/z values of 147, 150, 196 and 201. 3-MCPD and 2-MCPD was quantified using 3-MCPD-\textit{d}_{5} as an internal standard by the following equation (1) [19] and equation (2) [18], respectively;

\[
W_{3-MCPD} = \frac{SF_{3} \times W_{3-MCPD-d_{5}}}{SF_{i150}}
\]  

(1)

\[
W_{2-MCPD} = \frac{SF_{2} \times W_{3-MCPD-d_{5}}}{CC \times SF_{i150}}
\]

(2)

Where:

\( W_{3-MCPD} \) is the mass fraction, in mg/kg, of 3-MCPD;
$W_{2\text{-MCPD}}$ is the mass fraction, in mg/kg, of 2-MCPD;

$W_{3\text{-MCPD-d5}}$ is the mass fraction, in mg/kg, of 3-MCPD-d5;

$SF_{150}$ is the area of 3-MCPD-d5 ($m/z$ 150);

$SF_3$ is the area of 3-MCPD ($m/z$ 196);

$SF_2$ is the area of 2-MCPD ($m/z$ 196);

$CC$ is a correction constant ($0.65 \pm 0.01$).

**Results**

**Hydrolysis of MCPD dioleate by pancreatic lipase**

2-MCPD esters are regioisomers of the corresponding 3-MCPD esters, and these compounds have also been detected in fats and oils. However, to the best of our knowledge, the metabolism of these compounds by pancreatic lipase has not yet been studied in detail. With this in mind, we synthesized 2-MCPD-1,3-dioleate, which has been reported to be one of the most common FA species found in oils and fats [6], and evaluated its hydrolysis with porcine pancreatic lipase. This investigation was conducted to develop a deeper understanding of the hydrolytic profiles of MCPD esters following their ingestion, using 3-MCPD-1,2-dioleate as a control (Fig. 1). The products of the hydrolysis reactions were analyzed by HPLC-CAD, which allowed for the separation and detection of the MCPD esters, as well as the different regioisomers of the MCPD mono- and di-esters, which were produced as intermediates during the hydrolysis (Fig. 2).

The results revealed a significant decrease in the detected amount of 2-MCPD-1,3-dioleate from 18.0 to 8.4 µmol after 3 h of incubation with
pancreatic lipase at 37 °C, as well as the formation of free 2-MCPD (6.1 µmol) and free oleic acid (11.8 µmol) (Fig. 1a). The results also revealed that the amount of 2-MCPD-1-oleate present in the mixture increased for the first 0.5 h to reach approximately 3 µmol, where it reached a plateau.

The results for 3-MCPD-1,2-dioleate revealed a similar trend to the other isomer, with the amount of substrate decreasing from 18.9 to 7.7 µmol after 3 h of incubation with pancreatic lipase at 37 °C. In contrast to the 2-isomer, the hydrolysis of 3-MCPD-1,2-dioleate resulted in the formation of a large amount of the monoester 3-MCPD-2-oleate (9.8 µmol), whereas the amount

![Figure 1. Hydrolysis reactions of 2- and 3-MCPD dioleate with pancreatic lipase. Twenty micromoles of MCPD dioleate and 2 mg of pancreatic lipase in 1.2 mL of 50 mM Tris-HCl buffer (pH 8.0) containing 2.7 mM deoxycholic acid and 1 mM CaCl₂ was stirred at 37 °C. Free MCPD was measured by GC-MS following the extraction of MCPD and the corresponding esters into a chloroform/methanol solution, whilst the MCPD esters were measured by HPLC-CAD. a, filled square, 2-MCPD-1,3-dioleate; open triangle, 2-MCPD-1-oleate; filled circle, 2-MCPD; diamond, oleic acid. b, filled square, 3-MCPD-1,2-dioleate; filled triangle, 3-MCPD-1-oleate; open triangle, 3-MCPD-2-oleate; filled circle, 3-MCPD; diamond, oleic acid. All of the reactions and analyses were conducted in triplicate and the results reported as the mean values ± SD.](image-url)
of oleic acid (10.8 µmol) was similar in both cases (Fig. 1b). Notably, the hydrolysis of 3-MCPD-1,2-dioleate resulted in the accumulation of a small amount of the monoester 3-MCPD-1-oleate (0.7 µmol). The accumulation of free 3-MCPD was also small (2.2 µmol) compared to that of free 2-MCPD from 2-MCPD-1,3-dioleate.

These results indicated that the two acyl groups of 2-MCPD-1,3-dioleate were being readily hydrolyzed by pancreatic lipase under these conditions to produce 2-MCPD, and that the acyl group at the primary position of 3-MCPD-1,2-dioleate was being hydrolyzed preferentially to produce 3-MCPD-2-oleate.

**Figure 2.** Standard HPLC-CAD chromatogram for MCPD ester analysis. For HPLC conditions, see Materials and Methods. a, Tri-palmitate; b, 2-MCPD-dioleate; c, 3-MCPD-dioleate; d, 3-MCPD-dilinolenate; e, glycidyl-oleate; f, glycidyl-linoleate; g, 1,3-dioleate; h, oleic acid; i, 1,2-dioleate; j, 3-MCPD-1-oleate; k, 2-MCPD-1-oleate; l, 3-MCPD-2-oleate; m, 3-MCPD-1-linolenate
Hydrolysis of MCPD monooleate by pancreatic lipase

To further investigate the hydrolytic characteristics of pancreatic lipase, we synthesized the three different regioisomers of MCPD monooleate and subjected them to the reaction with pancreatic lipase (Fig. 3).

The results revealed that the amount of 2-MCPD-1-oleate decreased with time, whereas almost identical amounts of 2-MCPD and oleic acid were produced (Fig. 3a). The amount of 3-MCPD-1-oleate decreased in a similar manner to that of 2-MCPD-1-oleate, with similar amounts of 3-MCPD and oleic acid being produced at the same time (Fig. 3b). The hydrolysis reactions of 2-MCPD-1-oleate and 3-MCPD-1-oleate almost reached completion in 3 h under the reaction conditions used in the current study. In contrast, the hydrolysis of 3-MCPD-2-oleate occurred at a much slower rate.

Figure 3. Hydrolysis reactions of 2- and 3-MCPD monooleate by pancreatic lipase.

The conditions for the lipase reactions were the same as those described in Fig. 1, except MCPD monooleate was used instead for MCPD dioleate. a, filled square, 2-MCPD-1,3-dioleate; open triangle, 2-MCPD -1-oleate; filled circle, 2-MCPD; diamond, oleic acid. b and c, filled square, 3-MCPD-1,2-dioleate; open triangle, 3-MCPD-1-oleate; filled triangle, 3-MCPD-2-oleate; filled circle, 3-MCPD; diamond, oleic acid.
under these conditions, with only 4 µmol of the substrate being consumed after 3 h (Fig. 3c), representing only 1/5 of the consumed amount of 2- and 3-MCPD-1-oleate. Furthermore, only 4 µmol of 3-MCPD was released from 3-MCPD-2-oleate after 3 h, and the amount of 3-MCPD-1-oleate was less than 1.5 µmol after 3 h of hydrolysis. These results therefore suggested that the hydrolysis of an acyl group at the \( sn\)-2 position of MCPD or the transfer of an acyl group from the \( sn\)-2 position to the primary position of MCPD was a slow process. To the best of our knowledge, this study represents the first detailed evaluation of the hydrolytic behaviors of synthetic 2-MCPD-1-oleate and 3-MCPD-2-oleate in the presence of porcine pancreatic lipase, as well as their reaction products.

**Hydrolysis of MCPD dioleate by pancreatin**

The hydrolysis reactions of the MCPD mono- and di-esters were further studied using pancreatin from porcine pancreas (Fig. 4). The composition of the products resulting from this reaction was expressed as the relative molar concentration of each MCPD-containing compound to the sum of free MCPD, MCPD monooleate and MCPD dioleate. The hydrolysis of 2-MCPD-1,3-dioleate under these conditions gave free 2-MCPD, which reached 26 mol% after 3 h of hydrolysis, while the levels of 2-MCPD-1-oleate after 1 and 3 h were 17–18 mol% (Fig. 4a). In contrast, 3-MCPD-1,2-dioleate was mainly converted to 3-MCPD-2-oleate, with the formation of only small amounts of 3-MCPD-1-oleate and 3-MCPD (Fig. 4b). This observation was therefore consistent with the above results.
obtained using pancreatic lipase (Fig. 1).

![Diagram of hydrolysis reactions](image)

**Figure 4.** Hydrolysis reactions of 2- and 3-MCPD dioleate by pancreatin. The conditions used for the pancreatin reactions were the same as those described in Fig. 1, except the amount of pancreatin was 0.4 mg. The compositions have been expressed as the relative molar contents of each MCPD-containing compound compared with the sum of free MCPD, MCPD monooleate and MCPD dioleate. a, gray box, 2-MCPD-1,3-dioleate; mesh-like pattern box, 2-MCPD-1-oleate; dotted box, 2-MCPD. b, gray box, 3-MCPD-1,2-dioleate; mesh-like pattern box, 3-MCPD-1-oleate; filled box, 3-MCPD-2-oleate; dotted box, 3-MCPD. All of the reactions and analyses were conducted in triplicate and the results reported as the mean values.

**Hydrolysis of MCPD monooleate by pancreatin**

The three different regioisomers of MCPD monooleate were also hydrolyzed with pancreatin (Fig. 5). 2-MCPD-1-oleate and 3-MCPD-1-oleate were hydrolyzed by approximately 80% after 3 h of incubation to mainly afford 2- and 3-MCPD, respectively (Fig. 5a and b). In contrast, 3-MCPD-2-oleate was hydrolyzed by only 15% after 3 h (Fig. 5c). This observation was therefore consistent with the previous result described above using pancreatic lipase (Fig. 3).
Notably, this reaction also led to the formation of around 5 mol% of both 3-MCPD-1-oleate and 3-MCPD-dioleate, which indicated the occurrence of intra- and/or intermolecular acyl transfer processes from the $sn$-2-position to the primary of MCPD under the experimental conditions.

**Figure 5.** Hydrolysis reactions of 2- and 3-MCPD monooleate by pancreatin. The conditions used for the pancreatin reactions were identical to those described in Fig. 4, except MCPD monooleate was used as a substrate instead of MCPD dioleate. The compositions have been expressed as the relative molar contents of each MCPD-containing compound compared with the sum of free MCPD, MCPD monooleate and MCPD dioleate. a, gray box, 2-MCPD-1,3-dioleate; mesh-like pattern box, 2-MCPD-1-oleate; dotted box, 2-MCPD. b and c, gray box, 3-MCPD-1,2-dioleate; mesh-like pattern box, 3-MCPD-1-oleate; filled box, 3-MCPD-2-oleate; dotted box, 3-MCPD.

**Penetration of 2- and 3-MCPD by Caco-2 cell monolayer**

Caco-2 cells were cultured on Transwell® inserts under standard conditions for 3 weeks to allow for the differentiation of the cells and the formation of an epithelial cell like-monolayer. To the apical chamber was added a 0.1 or 1.0 mM (Fig. 6a or 6b) solution of 2- or 3- MCPD. The results of our preliminary trials involving the addition of 2- or 3-MCPDs to the apical chamber revealed that these substrates remained unchanged. The theoretical concentrations of the MCPDs at equilibrium were 2.75 and 27.5 µg/mL for A and B respectively, because the ratio of the volumes in apical and basolateral
chambers was 1:3. The results also revealed simultaneous decreases in the levels of 2- and 3-MCPDs in the apical chamber, which were accompanied by increases in the levels of these compounds in the basolateral chamber. This result therefore indicated that MCPD had translocated from the apical chamber to the basolateral chamber to reach equilibrium (Fig. 6a).

Minor differences were observed between the 2- and 3-MCPD, which were consistent with those of previous reports [15, 16]. It is noteworthy that the translocation profiles observed in the current study for the 2- and 3-MCPD were similar when the concentration of the MCPD solution placed in the

![Figure 6. Permeabilities of 2- and 3-MCPD by the Caco-2 cell monolayer method.](image)

a, 0.1 mM; b, 1.0 mM 2- or 3-MCPD solution in HBSS buffer (0.5 mL) was added to the apical chamber. After incubation at 37 °C, the solutions in the apical and basolateral chambers were collected, together with the Caco-2 cells. After the extraction of MCPD into a chloroform/methanol solution, the amount of free MCPD was measured by GC-MS. The theoretical concentration of each MCPD at equilibrium (a, 2.75 µg/mL; b, 27.5 µg/mL) was expressed by the dotted line. a and b, filled circle, 2-MCPD-apical; filled triangle, 3-MCPD-apical; open circle, 2-MCPD-basolateral; open triangle, 3-MCPD-basolateral. All of the sample preparations and analyses were conducted in triplicate and the results reported as the mean values ± SD.
apical chamber was increased from 0.1 to 1 mM (Fig. 6b). These results therefore suggested that MCPD were passing through the Caco-2 cell monolayer via a simple diffusion mechanism rather than an active transportation system.

Permeability of the 2- and 3-MCPD monoester through a Caco-2 cell monolayer

The results described above for the hydrolysis reactions of MCPD diesters with pancreatic lipase and pancreatin suggested that 2-MCPD-1-oleate and 3-MCPD-2-oleate were the primary hydrolytic products to reach small intestine (Fig. 1, 3-5). In the previous study, 3-MCPD-1-monoester but not 3-MCPD-2-monoester was subjected to the permeability study [15]. Thus, 3-MCPD-2-oleate as well as 2-MCPD-1-oleate and 3-MCPD-1-oleate were subjected to a permeability study using a Caco-2 cell monolayer (Fig. 7a-c). The total amount of 3-MCPD-2-oleate in the system decreased by half after 6 h (Fig. 7c), but it was not detected in the basolateral chamber and the cells. On the other hand, the amount of 3-MCPD in the apical chamber increased with time, and reached its maximum value of 15 nmol after 2 h, before decreasing slightly. The amount of 3-MCPD in the basolateral chamber increased after 2 h, reaching its maximum value of approximately 10 nmol after 6 h. Similar trends were also observed for 2-MCPD-1-oleate (Fig. 7a) and 3-MCPD-1-oleate (Fig. 7b).
In contrast that the hydrolysis of 3-MCPD-2-oleate by pancreatic lipase or pancreatin was not observed (Fig. 3, 5), MCPD-monoesters, regardless of their regioisomeric forms, were suggested to be hydrolyzed in the presence of Caco-2 cell monolayer.

Even the amount of 2-MCPD-1-oleate or 3-MCPD-2-oleate added to the apical chamber was increased 10-fold, and even though the detected amount of MCPD monooleate decreased 170–190 nmol after 6 h (Supplementary Fig. 1a, c), they were still not detected in the basolateral chamber. The amount of

Figure 7. Permeabilities of the 2- and 3-MCPD-monooleates as determined using Caco-2 cells.
The conditions for the transport experiments were the same as those described in Fig. 6, except 0.1 mM MCPD monoester solution were used instead for free MCPD after 5 min sonication. After the extraction of MCPD and its esters into a chloroform/methanol solution, the free MCPD was measured by GC-MS, and the MCPD esters were measured by HPLC-CAD. a, filled triangle, 2-MCPD-1-oleate-apical; filled circle, 2-MCPD-apical; open triangle, 2-MCPD-basolateral; open diamond, 2-MCPD-cell. b, filled triangle, 3-MCPD-1-oleate-apical; filled circle, 3-MCPD-apical; open triangle, 3-MCPD-basolateral; open diamond, 3-MCPD-cell. c, filled triangle, 3-MCPD-2-oleate-apical; filled circle, 3-MCPD-apical; open triangle, 3-MCPD-basolateral; open diamond, 3-MCPD-cell.
the corresponding 2- or 3-MCPD accumulated in the apical chamber was around 30 nmol. When the experiments were conducted using Transwell inserts in the absence of a Caco-2 cell monolayer, the amounts of 2-MCPD-1-oleate and 3-MCPD-2-oleate decreased similarly without accumulation of free MCPD (Supplementary Fig. 1d-f). The lack of mass balance in this experiments (Supplementary Fig. 1a-c) can thus be explained by the incomplete recovery of the MCPD monoesters from the Transwell chambers under the current experimental conditions. MCPD dioleates were also subjected to the permeability study (data not shown). Although the detected amount of 2-MCPD-1,3-dioleate or 3-MCPD-1,2-dioleate in the apical chamber decreased by half, they were not detected in the basolateral chamber. 2- or 3-MCPD monooleate was not detected in any of the fractions recovered from the Caco-2 cell monolayer system, either. In addition, the detected amount of free MCPD was negligible, approximately 0.5 mol% of the original amount of dioleate. In addition, the detected amount of free MCPD was negligible, approximately 0.5 mol% of the original amount of dioleate. Thus, it was suggested that MCPD diesters remained unchanged in the presence of Caco-2 cell monolayer, and that they were not penetrated the monolayer. The results were partly consistent with the previous reports [15, 16]; free MCPD was detected from 2-MCPD-dipalmilate, but not from 3-MCPD-dipalmitate under similar transport conditions using a Caco-2 cell monolayer.
Supplementary Figure 1.
Permeabilities of the 2- and 3-MCPD-monooleates as determined using Caco-2 cells.
The conditions for the transport experiments were the same as those described in Fig. 7, except the concentration of 2- and 3-MCPD monooleate was increased from 0.1 to 1.0 mM, and using Transwell inserts in the presence or in the absence of Caco-2 cell monolayer.
a, filled triangle, 2-MCPD-1-oleate-apical; filled circle, 2-MCPD-apical; open triangle, 2-MCPD-basolateral; open diamond, 2-MCPD-cell. b and c, filled triangle, 3-MCPD-1-oleate-apical; filled diamond, 3-MCPD-2-oleate-apical; filled circle, 3-MCPD-apical; open triangle, 3-MCPD-basolateral; open diamond, 3-MCPD-cell.
d, 2-MCPD-1-oleate-apical in the presence (filled triangle) and in the absence (open square) of Caco-2 cell monolayer. e, 3-MCPD-1-oleate-apical in the presence (filled triangle) and in the absence (open square) of Caco-2 cell monolayer. f, 3-MCPD-2-oleate-apical in the presence (filled diamond) and in the absence (open square) of Caco-2 cell monolayer.
Discussion

The European Food Safety Authority (EFSA) reported in 2013 that the major contributors of the human exposure to 3-MCPD are processed oil and fats in their fatty acid ester form [20]. EFSA also reported in 2011 that toxicity of 3-MCPD-dipalmitate was lower than free 3-MCPD in a 90-day toxicological study on rats [11]. Recently, Onami et al. reported that 3-MCPD ester was not \textit{in vivo} genotoxin but had potential to be subchronically toxic to the similar degree to their free form, with the no-observed-adverse-effect levels of 3-MCPD ester to be 8-15 mg/kg body weight per day in rats [21, 22].

The toxicity of 3-MCPD-diester and 3-MCPD-1-monoester has been evaluated \textit{in vitro} and \textit{in vivo}, previously [11, 16, 21, 22]. However, the metabolism of 3-MCPD-diester after ingestion had not been monitored in detail, let alone 2-MCPD-diester, which are also detected in fats and oils. Moreover, 3-MCPD-1-monoester is not a natural metabolite of diester as we presented in this study; we showed that pancreatic lipase mainly hydrolyzed 3-MCPD-dioleate to produce 3-MCPD-2-oleate, and that further hydrolysis of 3-MCPD-2-oleate to produce 3-MCPD was slow. The hydrolysis of 3-MCPD-2-oleate was not significant by pancreatin, either. Pancreatin prepared from pancreas is an enzyme mixture and known to contain proteases, amylases and others in addition to lipases. Our results suggested that pancreatin might not contain enzymes with esterase activity to hydrolyze 3-MCPD-2-monoester, and that 3-MCPD-diester after injection should reach to small intestine mainly as 3-MCPD-2-monoester in addition to the remaining diester. In this report, it was presented that
3-MCPD-2-oleate was hydrolyzed to produce free 3-MCPD in the presence of Caco-2 cells, implying the possibility of \textit{in vivo} hydrolysis of 3-MCPD-2-monoester in the presence of epithelial cells of small intestine, followed by the intestinal absorption as free 3-MCPD form (Fig. 8). Our observation is consistent with the previous one that 3-MCPD-1-monoester was hydrolyzed to produce 3-MCPD by the supernatant of Caco-2 cell culture [15].

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{mcpd_disposition.png}
\caption{Estimated disposition of MCPD and their esters. \textit{FFA} free fatty acid}
\end{figure}

Abraham \textit{et al.} reported that the oral administration of 3-MCPD to rats increased free 3-MCPD levels in blood to the maximum within 10 min, whereas the administration of 3-MCPD-dipalmitate increased free 3-MCPD levels in blood to the maximum after 2 h [10]. Here, the maximum level was 1/5 compared to the administration of the free 3-MCPD. Orally administered 3-MCPD-dipalmitate was mainly recovered in small intestine first, then in
large intestine with the maximum recovery of 70% in total. This could be interpreted that roughly 20-30% of 3-MCPD-dipalmitate was digested and absorbed as free form, whereas the rest remain in the intestine as ester form. MCPD ester was not detected in any organs or blood, other than intestine. In the report, however, it was not exactly identified if the remaining MCPD ester was in di- or mono-ester form. Our in vitro observation were consistent the above study [10], and provided additional implication that the MCPD ester detected in the intestine after the oral administration to rat might be a mixture of 3-MCPD diester and 3-MCPD-2-monoester mainly.

In conclusion, it was observed that the two acyl groups of 2-MCPD-1,3-dioleate were readily hydrolyzed by pancreatic lipase and pancreatin to produce 2-MCPD. In contrast, the hydrolysis of 3-MCPD-1,2-dioleate occurred preferentially at the primary acyl position to produce 3-MCPD-2-oleate. 2-MCPD-1-oleate and 3-MCPD-1-oleate were further hydrolyzed to 2- and 3-MCPD by pancreatic lipase and pancreatin. Meanwhile, the hydrolysis of 3-MCPD-2-oleate was approximately 80% slower than that of 3-MCPD-1-oleate. The in vitro evaluation of the intestinal absorption of these compounds using a Caco-2 cell monolayer model revealed that the MCPD-monooleates were hydrolyzed to give the free MCPD in the presence of Caco-2 cells, whereas the MCPD-dioleates remained unchanged. The free MCPD permeated the Caco-2 monolayer, most probably via a diffusion mechanism, because the permeation profile was independent of the MCPD dose. Minor differences were observed
between the permeation profiles of 2- and 3-MCPD.

Overall, it was estimated that 2- and 3-MCPD-diester would be digested to free or monoester form by pancreatic lipase, and the resulting monoesters would further be hydrolyzed, though not completely, to free form in the presence of epithelial cells of small intestine to be absorbed. Absorption may not occur in mono or di-ester form.

**Conclusion**

The regioisomers of the di- and mono-oleate of MCPD have been synthesized and subsequently hydrolyzed with pancreatic lipase and pancreatin to estimate the intestinal digestion and absorption of these compounds after their intake. The hydrolysates were analyzed by HPLC using a corona charged aerosol detection system, which allowed for the separation and detection of the different regioisomers of the MCPD esters. The hydrolysates were also analyzed by GC-MS to monitor the free MCPD. The results indicated that the two acyl groups of 2-MCPD-1,3-dioleate were smoothly hydrolyzed by pancreatic lipase and pancreatin to give free 2-MCPD. In contrast, the hydrolysis of 3-MCPD-1,2-dioleate proceeded predominantly at the primary position to produce 3-MCPD-2-oleate. 2-MCPD-1-oleate and 3-MCPD-1-oleate were further hydrolyzed to free 2- and 3-MCPD by pancreatic lipase and pancreatin, although the hydrolysis of 3-MCPD-2-oleate was 80% slower than that of 3-MCPD-1-oleate. The intestinal absorption characteristics of these compounds were evaluated in vitro using a Caco-2 cell monolayer. The results revealed that the
MCPD-monooleates, but not the MCPD-dioleates, were hydrolyzed to produce the free MCPD in the presence of the Caco-2 cells. The resulting free MCPD permeated the Caco-2 monolayer most likely via a diffusion mechanism because their permeation profiles were independent of the dose. Similar permeation profiles were obtained for 2- and 3-MCPDs.

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Chapter 4: Estimation of the cause for fatty acid esters of 3-MCPD and glycidol formation and development of method to reduce their presence in refined oils

Introduction

Palm oil is produced at a rate of about 60 million tons per year and is one of the most used edible oils in the world [1]. It accounted for 36% of the world vegetable oil production in 2014 (Fig. 1). Palm oil is 40-50% palmitic acid. The melting point of saturated fatty acids, such as palmitic acid, is higher than unsaturated fatty acids, and palm oil is solid at room temperature. On the other hand, a high content of saturated fatty acids, which do not contain double bonds that are easily oxidized, contributes to the superior oxidative stability of palm oil. Furthermore, palm oil is produced at a lower cost than other oils, partly because palm trees require 10 times less land than other oil-producing crops. For these properties, palm oil is widely used in foods, for example, in margarine and shortening, and for the frying oil of instant noodles and potato chips. Thus, palm oil is important for the oils and fats industry.

Indonesia and Malaysia are the main producers of palm oil, and together account for 85% of the world palm oil production. In these countries, crude palm oil (CPO) is first pressed from palm fruits similarly to other oil crops, and is subjected to the refining steps of degumming, bleaching, and deodorization. These steps remove free fatty acids and colorants and the end product can be qualified as edible oil.

The resulting oil is called Refined Bleached Deodorized Palm Oil (RBDPO), and is exported to Japan, where it is further refined to remove remaining free fatty acids and odors by deodorization (Fig. 2).
MCPD esters and glycidyl esters, which are undesirable compounds in edible oils, are detected at very low concentrations in CPO, but at about 10 ppm in RBDPO, a much higher level. Thus, it has been deduced that the majority of MCPD esters and glycidyl esters are generated during the deodorization step [2, 3]. The industrial process for deodorization is conducted at a high temperature (230-250°C) by reduced pressure with steaming and removes impurities from the oil. Furthermore, palm oil refined by ‘chemical refining’, in which free fatty acids are removed by neutralization, contains very little MCPD esters and glycidyl esters, compared to oil produced by ‘physical refining’ (Fig. 2)[2, 3]. This observation is consistent with the conclusion that MCPD esters and glycidyl esters are generated during deodorization, and it is the high temperature that affects their formation [4, 5, 6].

In addition, analyses of MCPD esters in various edible oils have been carried out both domestically and internationally. Palm oil as well as corn oil was reported to contain relatively high amounts of 3-MCPD esters and glycidyl esters, whereas soy and canola oils contained much less [7, 8, 9].
Palm and corn oils contain relatively high levels of monoacylglycerols (MAG) and diacylglycerols (DAG), whereas soy and canola oils do not. Therefore, it has been presumed that the amount of partial acylglycerols in crude oils might be related to MCPD ester formation during deodorization [6, 11].

![Diagram of oil refining process](image)

**Figure 2.** Physical and Chemical refining of palm oil

However, there has not been direct proof that these partial acylglycerols are the source of MCPD/glycidyl esters in refined oil. In this study, refined oil spiked with MAG or DAG was subjected to the deodorization test, to evaluate the correlation between partial acylglycerols and the formation of MCPD/glycidyl esters in the deodorization step. It was confirmed that MCPD/glycidyl esters were generated in the deodorization process in proportion to the MAG contents.

Furthermore, these results suggested that removal of MAG from oils might be effective at preventing the formation of MCPD/glycidyl esters in the
deodorization process. To evaluate this effect, MAG was partially removed from the samples by short path distillation. The amounts of MCPD/glycidyl esters in oils were measured by the DGF standard method after deodorization.

**Experimental Procedures**

**Materials and Methods**

3-MCPD was purchased from Kanto Chemical Co. (Tokyo, Japan). 3-MCPD-\(d_5\), glycidol, phenyl boronic acid, sodium methoxide/methanol solution were purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-MCPD was synthesized as described previously \[12\]. Canola oil and crude palm oil were the products of Ueda Oils and Fats MFG Co. Ltd. (Kobe, Japan) and Pasir Gudang Industrial Estate (Johor, Malaysia). EMALGEE P-100 was purchased from RIKEN VITAMIN CO. LTD (Tokyo, Japan). GALLEON EARTH was purchased from MIZUSAWA INDUSTRIAL CHEMICALS, LTD, (Tokyo, Japan). Chloroform, n-hexane, isooctane, methanol, tert-butyl methyl ether (t-BME), anhydrous sodium sulfate, glycerol and all of the other reagents used in the current study were purchased from Kanto Chemical Co, Wako Pure Chemical Industries and Nakalai Tesque Inc. (Kyoto, Japan). All of these materials were purchased as the analytical grades unless otherwise specified.

**High DAG Oil Production**

High DAG oil was prepared by transesterification of canola oil (600 g) with glycerol (27 g) using 14% sodium hydroxide solution (0.4 g). The mixture was heated to 180 °C, at a pressure below 1.3 kPa for 30 min. The resulting oil was washed with water, and bleached with activated clay (GALLEON EARTH). High DAG oil contained 5.1% MAG, 41.5% DAG and 52.6% triacylglycerol (TAG).
Oil Bleaching
Oil samples were heated to 110°C under ordinary atmospheric pressure with 3 vol % of activated clay. The mixture was stirred at 200 rpm for 5 min at 110°C, and filtered through paper to remove activated clay from the oil. Bleached oil was subjected to short path distillation and deodorization.

Short Path Distillation
Short path distillation was carried out with Type2-03 WIPRENE (KOBELCO ECO-SOLUTIONS Co. Ltd, Kobe, Japan). Oil was heated to 240°C and passed the distillator at a flow rate of 10 mL/min. The pressure was controlled and kept below 2.7 Pa by a vacuum pump (PVD-180 pump, ULVAC Inc., Kanagawa, Japan).

Deodorization
MAG and DAG were added to canola oil, which did not contain MCPD-forming substances (MCPD-FS) (< 0.2 ppm), to give a final content of 0.5, 1, 2, 5, and 10 wt %. The mixed oil (300 g) was subjected to the deodorization test, which was carried out in a 1 kg scale deodorizer made of glass. Oil was heated to the designated temperature (230/240/250 °C), and deodorization was run for 60 min, at a pressure below 10 mmHg with steaming.

Contents of Partial Acylglycerols
An oil sample (200 mg) was dissolved in hexane or hexane/acetone (1:1, by vol). The contents of partial acylglycerols were determined using GC-2014A device (Shimadzu, Kyoto, Japan) connected to a DB-1ht capillary column (30 m, 0.25 mm, 0.25 μm, Agilent Technologies, Tokyo, Japan). The column temperature was controlled as follows: it was kept at 140°C for 1 min, raised at 10°C/min to 250°C, kept at 250°C for 5 min, further raised at 5°C/min to 300°C, and kept at 300°C for 19 min. The
carrier gas was high-purity helium at a constant flow of 25.5 cm/s. One microliter of sample were injected in pulsed split mode. The temperature of the injector and the detector were 350°C and 380°C, respectively. Peak area % was considered to be equal to wt %.

**Determination of MCPD-forming substances by DGF standard method C-VI 18 (10)**

The amounts of MCPD-forming substances (MCPD-FS) in oil samples were determined as described in DGF standard method C-VI 18 (10) [13], which expressed as the total amount of MCPD esters and glycidyl esters. The protocol was slightly modified by using free-form 3-MCPD-\(d_5\) instead of the ester-form of palmitic acid as an internal standard. Assay A: soybean oil (100 mg) mixed with a spiking component was dissolved in 100 µL of tert-butyl methyl ether. To the sample, a toluene solution of 3-MCPD-\(d_5\) (5.0 µg/mL) and 0.5 M sodium methoxide/methanol solution (200 µL) were added, and the mixture was left for 3.5–4.5 min at room temperature. 3-MCPD-\(d_5\) was used instead of the esters in this work in order to neglect the transesterification efficiency. Sodium chloride/sulfuric acid solution (200 g/L with 0.9% \(\text{H}_2\text{SO}_4\), 600 µL) was added to the solution, and then iso-hexane (600 µL) was added and the mixture was left for 5 min. The aqueous phase was washed twice with iso-hexane, and the products formed were extracted three times with a mixed solvent (600 µL, ether/ethyl acetate = 6/4, \(\text{v}/\text{v}\)). The organic phase was dried over sodium sulfate. A phenylboronic acid/ether solution (10–100 µL) was added to the solution, and solvent was removed using a gentle nitrogen stream. Isooctane (1.5 mL) was added to the residue and filtered using paper with sodium sulfate. The solution was analyzed by GC/MS.
**GC/MS quantification**
GC/MS was carried out using a SHIMADZU GCMS QP-2010 instrument equipped with an AOC-20i auto-sampler, connected to a DB-5ms capillary column (30 m, 0.25 mm, 0.25 μm, Agilent Technologies). The column temperature was controlled as follows. It was maintained at 85 °C for 0.5 min, raised at rates of 6 °C/min to 150 °C, 12 °C/min to 180 °C, and 25 °C/min to 280 °C, and maintained at 280 °C for 7 min. The carrier gas was high-purity helium at a constant flow of 40 cm/s. Two microliters of sample were injected in pulsed splitless mode. The temperature of the programmed-temperature vaporizer injector for the SHIMADZU GCMS QP-2010 instrument was controlled as follows. It was maintained at 85 °C, raised at a rate of 250 °C/min to 160 °C, maintained at 160 °C for 9.9 min, and further raised at a rate of 250 °C/min to 350 °C. The temperatures of the interface and the ion source were set at 250 °C and 200 °C, respectively. Other conditions for GC/MS were the same as those described in DGF standard method C-VI 18 (10). A mass-selective detector was used for selected ion monitoring, focusing on ions with m/z values of 147, 150, 196, and 201.

**Results and Discussion**

**Deodorization of canola oil with added MAG or DAG**
MCPD-FS were detected in processed oil made from crude oil contained relatively high amounts of MAG and DAG. Thus, it has been suggested that MAG and DAG were precursors of MCPD or glycidyl esters that formed during the deodorization process. However, to the best of our knowledge, there has not been direct proof that these partial acylglycerols are the sources of MCPD/glycidyl esters in refined oil.

In this study, refined canola oil spiked with MAG or DAG was subjected to the deodorization test, to evaluate the correlation between partial acylglycerols and the formation of MCPD/glycidyl esters in the
deodorization step. First, it was confirmed that canola oil, MAG, and DAG did not contain 3-MCPD-FS. The amounts of 3-MCPD-FS in oil samples were measured by DGF standard method C-VI 18 (10), which expressed as the total amount of MCPD esters and glycidyl esters.

**Figure 3.** Deodorization of canola oil with MAG or DAG addition
MAG (a) or DAG (b) was added to canola oil to give a final content of 0.5, 1, 2, 5, 10 wt%. Oil was deodorized at 230 or 250 °C for 60 min, at the pressure below 10 mmHg. The contents of MCPD-forming substances (MCPD-FS) in oil samples were measured by DGF standard method C-VI 18 (10), which expressed as the total amount of MCPD esters and glycidyl esters. a, b Open circle, distillation at 250 °C; filled triangle, distillation at 230 °C. All of the tests and analyses were conducted once.

When oil with MAG added to 0.5% was subjected to the deodorization process conducted at 250 °C for 60 min, which is the standard condition of oil refining, the detected amount of 3-MCPD-FS was 0.7 ppm. Furthermore, the detected amounts of 3-MCPD-FS increased as more MAG was added, and reached to 56.6 ppm when 10.0 % MAG was added to the oil (Fig. 3). Thus, it was estimated that 3-MCPD-FS were generated in the deodorization process in proportion to the MAG concentration. When the temperature of deodorization was reduced to 230 °C, the amounts of 3-MCPD-FS generated were 1.8 and 9.6 ppm in oil added with 5.0 % and 10.0 % MAG, respectively. These results indicated that the generation of 3-MCPD-MS was dependent both on the MAG content and on the temperature of the deodorization process.
In contrast, the detected amounts of 3-MCPD-FS were only 3.0 ppm when oil with DAG added to 10 % was subjected to the deodorization test at 250 °C for 60 min. Thus, the contents of DAG in oil affected the generation of 3-MCPD-FS only weakly compared with MAG.

These results suggested that pre-processing to reduce MAG from oils and fats might be effective at preventing 3-MCPD-FS formation during the deodorization process.

Mitigation of 3-MCPD/glycidol esters formation during the deodorization process, by removing MAG from oil

To evaluate the effect of MAG reduction on 3-MCPD/glycidol esters formation during the deodorization process, the concentration of MAG was reduced in the oil sample by short path distillation. The resulting oil was subjected to the deodorization test, conducted at 240 °C for 60 min (Table 1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>MAG (%)</th>
<th>DAG (%)</th>
<th>TAG (%)</th>
<th>MCPD-FS (ppm)</th>
<th>3-MCPD</th>
<th>2-MCPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPO</td>
<td>0.2</td>
<td>4.9</td>
<td>89.7</td>
<td>1.8</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>MAG reduced CPO</td>
<td>0.1</td>
<td>2.1</td>
<td>94.0</td>
<td>0.8</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>DAG40</td>
<td>6.1</td>
<td>41.5</td>
<td>52.6</td>
<td>25.0</td>
<td></td>
<td>1.7</td>
</tr>
<tr>
<td>MAG reduced DAG40</td>
<td>0.0</td>
<td>42.4</td>
<td>54.0</td>
<td>11.7</td>
<td></td>
<td>1.1</td>
</tr>
</tbody>
</table>

After the deodorization test of CPO, which contained 0.2 % MAG and 4.9 % DAG, 1.8 ppm of 3-MCPD-FS was detected in CPO. When the content of MAG and DAG was reduced to 0.1 % and 2.0 % by short path
distillation of CPO prior to the deodorization test, the quantity of 3-MCPD-FS produced was reduced from 1.8 ppm to 0.8 ppm.

Next, high DAG oil (DAG 40), which contained 5.1 % MAG and 41.5 % DAG, was subjected to the deodorization test. Even though the temperature of the test was set low at 230 °C, 25.9 ppm of 3-MCPD-FS was generated. Then, the content of MAG was reduced to below 0.1 % from high DAG oil by short path distillation. The quantity of 3-MCPD-FS produced was reduced to 11.7 ppm, after the deodorization test conducted under the same condition.

These results indicated that preprocessing to reduce MAG was effective in suppressing the generation of 3-MCPD-FS during the deodorization step.

**Conclusion**

In this study, refined canola oil spiked with MAG and DAG was subjected to the deodorization process. The amount of MCPD esters and glycidyl esters produced during deodorization increased depending on the added quantity of MAG. The effect was smaller with DAG addition compared to that with MAG addition. These results suggested that preprocessing to reduce MAG in oils and fats might be effective at suppressing the generation of those esters during the deodorization process. In fact, after reducing the amount of MAG in crude palm oil and high DAG oil using short path distillation, the production of MCPD esters and glycidyl esters in the deodorization step was successfully reduced. This study, for the first time, provides direct proof that MAG is the main cause of the undesirable esters produced during the deodorization process.

**REFERENCES**

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13. Deutsche Gesellschaft für Fettwissenschaft, DGF Standard Methods Section C-Fats C-VI 18 (10) (2011) Fatty-acid-bound 3-chloropropane-1,2-diol (3-MCPD) and 2,3-epoxipropane-1-ol (glycidol) Determination in oils and fats by GC/MS (Differential measurement)
Chapter 5 : Summary

The compound 3-monochloropropanediol (3-MCPD) is a cause for concern in terms of its adverse effect on human health. Since the latter half of the 1970s, it has been detected mainly in acid hydrolyzed vegetable proteins, which are widely used as seasoning ingredients in condiments like soy sauce. 3-MCPD has been classified as being potentially carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (IARC) monographs, with a maximum tolerable intake of 2 µg/kg body weight per day set by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). However, the regioisomer of this compound, 2-MCPD, has not been currently classified in the list because of a lack of epidemiological and experimental evidence concerning its carcinogenicity.

2- and 3-MCPDs have been detected as the corresponding fatty acid esters in a variety of natural and processed oils and fats. Also, glycidol, an analogous compound to MCPD, has been classified as probably carcinogenic to humans (Group 2A) by IARC, and this compound has been detected as glycidyl ester in oils and fats and in processed oil and fat products.

MCPD esters and glycidyl esters are likely to be hydrolyzed in the body after they are ingested, and are assumed to be converted to free forms. Thus, their adverse health effects are cause for concern. Therefore, it is desirable to establish methods to quantify and to reduce the content of MCPD esters and glycidyl esters contained in oils and fats and processed oil and fat products.

In this study, we have tried to establish a method to quantify MCPD and glycidol compounds in oils and fats, more sensitive and convenient than conventional methods, as well as to estimate the digestion and absorption kinetics of MCPD esters ingested into the body. We also investigated methods to reduce these compounds in oils and fats.
Chapter 1 reported the bidirectional conversion of 3-MCPD and glycidol under the analytical condition the DGF (German Society for Fat Science) standard method. As of 2009, the indirect analysis method using GCMS and the direct analysis method using LCMS have been advocated as major methods for measuring the 3-MCPD esters and glycidyl esters contained in oils and fats. With the indirect analysis method, the 3-MCPD esters and glycidyl esters contained in oils and fats are converted to free forms through transesterification, derivatized by phenylboric acid, and analyzed using GCMS. The analysis technique is comparatively simple, and this method is advantageous to quantify the entire amount of 3-MCPD esters and glycidyl esters in oils and fats irrespective of the bound fatty acid types.

The first standard method, or the DGF standard method C-III 18 (9), was comprised of two analyses: option A, which measures the total quantity of 3-MCPD and glycidol, and option B, which measures only the 3-MCPD quantity after removal of glycidol with acid treatment. The glycidol content was calculated by subtracting option B from option A.

When reference material was analyzed using the DGF standard method to analyze oils and fats spiked with known quantities of MCPD, two problems became apparent: firstly, the 3-MCPD quantity was overestimated. Secondly, the recovery rate of 3-MCPD was extremely low resulting in the low sensitivity.

First, the cause of the overestimation of the 3-MCPD quantity was thought to be the residual glycidyl ester in option B, so we investigated this further. Our study clarified that the glycidyl ester reference product added to refined soybean oil was not completely removed by hydrolysis using sulfuric acid/propanol (option B), leaving approximately 10% remaining, which produced a false positive for 3-MCPD.

When the substance kinetics in each process of the DGF standard method was measured directly using $^{13}$C-NMR, the mutual conversion of 3-MCPD and glycidol was observed. That is, 37% of the 3-MCPD was converted to
glycidol in the transesterification process under alkaline conditions, while
74% of the glycidol was converted to 3-MCPD in the phenylboric acid
derivatization process under acidic conditions. Furthermore, in the
derivatization process, most of 3-MCPD did not undergo phenylboric acid
derivatization, which was thought to be the cause of the low recovery rate
of the spiked 3-MCPD.

In Chapter 2 we reported on improving the accuracy of 3-MCPD
quantification using the DGF standard method. The lower limit of the
quantitative analysis of 3-MCPD and glycidol in oils and fats using the DGF
standard method is approximately 1 ppm. However, the quantity of 3-MCPD
contained in natural oils and fats is often less than 1 ppm, so it was vital to
improve the detection sensitivity.

It was clarified in Chapter 1 that almost no phenylborate is formed in the
DGF standard method derivatization process. Furthermore, the majority of
the 3-MCPD that did not undergo derivatization remained in the aqueous
layer during hexane extraction, and this was thought to be the cause of the
low recovery rate of the added 3-MCPD. Hence, the 3-MCPD extraction
solvent was substituted from the conventional hexane. We checked whether
the recovery rate of 3-MCPD phenylborate improved, and found that the
recovery rate of 3-MCPD increased in ethyl acetate, chloroform and
1-butanol. As a result of choosing ethyl acetate based on the ease of
handling during analysis, the extraction efficiency of the modified DGF
standard method increased approximately four-fold, and the detection
sensitivity improved from the previous method’s 1ppm to approximately 0.2
ppm.

In Chapter 3 we described the kinetics of 2-MCPD using the DGF standard
method and the respective determination. As of 2011, 2-MCPD was
indicated to be detectable by the DGF standard method, but the reference
material of 2-MCPD was not commercially available. Thus, the identification and quantification were not possible by the DGF method.

The 2-MCPD synthesis method was reported over 40 years ago. The final product actually obtained by the method is a mixture with 3-MCPD, and adequate quantities of pure 2-MCPD could not be obtained. In this study, we used a novel method for pure 2-MCPD synthesis.

Furthermore, using the synthesized 2-MCPD reference product, we attempted simultaneous determination with 3-MCPD using the DGF standard method. The stable isotope labeling compound 2-MCPD-\(d_5\) was not commercially available at that time to be used as an internal standard, so the available 3-MCPD-\(d_5\) was used to determine 2-MCPD. With GCMS analysis using the DGF standard method, 2-MCPD can be detected for a longer retention time than 3-MCPD. We decided it was quantifiable, as added quantities of 2-MCPD demonstrated linearity to the internal standard 3-MCPD-\(d_5\). It was also found that the apparent detection sensitivity of 2-MCPD for the internal standard was higher than 3-MCPD, as 2-MCPD phenylborate did not produce detectable fragment ions, and that the 2-MCPD detection sensitivity for 3-MCPD is fixed and independent on the GCMS device. A correction factor was worked out based on this information, and a 2-MCPD determination method was successfully established. Using only 3-MCPD-\(d_5\), which is the conventional internal standard, the method is advantageous in terms that it does not require expensive 2-MCPD-\(d_5\) and preparation of a 2-MCPD calibration line for each analysis.

In Chapter 4, we reported the estimation of the 2- and 3-MCPD ester metabolism behaviors and intestinal absorption. 3-MCPD was found to be carcinogenic when administered in high concentrations in animal experiments, but there was no commercially available product of the isomer 2-MCPD until recently, so its toxicity and effect on the living body was unknown. However, as traces of 2-MCPD esters have been detected in some
oils and fats and processed oil and fat products, it is urgent to ascertain the behavior of 2-MCPD in the body. In this study, 2- and 3-MCPD-dioleate were synthesized, and their hydrolytic products created with pancreatic lipase and pancreatin were monitored. Their intestinal absorption was estimated based on an *in vitro* permeation test using Caco-2 cells which are widely used as epithelial cell models.

The hydrolytic products and the Caco-2 permeation test solutions were analyzed by HPLC connected to corona charged aerosol detection (CAD), which can isolate the positional isomers of MCPD monoester. The hydrolytic products of 3-MCPD-dioleate by pancreatic lipase and pancreatin was mainly 3-MCPD-2-oleate, and little free 3-MCPD was detected. On the other hand, a small amount of 2-MCPD-1-oleate was detected from 2-MCPD-dioleate, and free 2-MCPD increased over time. Therefore, we concluded that the 3-MCPD diesters after ingestion were converted mainly to 3-MCPD-2-monoester, and 2-MCPD diesters were rapidly hydrolyzed and reached the small intestine predominantly as free 2-MCPD.

Then, the permeability through Caco-2 cell monolayer using free 2- and 3-MCPD was evaluated. They appeared on the subepithelial side of the solution at roughly the same speed. Thus, we concluded that the transportation mechanism was permeation through simple diffusion. Conversely, the MCPD esters added to the intestinal tract side did not permeate the Caco-2 cells and were not detected from the subepithelial side of the solution. Also, some of the MCPD monoesters were hydrolyzed to free forms and detected from the subepithelial side of the solution. Therefore, we assumed that the MCPD diesters ingested into the body would be hydrolyzed into free MCPDs and monoesters by pancreatic lipase, and some of the produced monoesters would be further hydrolyzed into free MCPDs in epithelial cells and absorbed, but absorption of MCPD esters would not occur.
In Chapter 5, we described a method to reduce MCPD esters and glycidyl esters in edible oils and fats. Generally, crude oils and fats go through purification processes such as deoxidation, bleaching and deodorization to become the final product. MCPD esters and glycidyl esters have been known to increase during the oil and fat deodorization process, and it was assumed that the partial glycerides such as mono and diacylglycerol (MAG, DAG), and chlorine compounds are the origin of those esters. However, the effect of removing partial acylglycerols on reducing MCPD esters and glycidyl esters had not been verified.

In this study, refined canola oil spiked with MAG and DAG was subjected to the deodorization process. It was confirmed that the produced amount of MCPD/glycidol increased in line with the added quantity of MAG. In the deodorization process, 60 ppm MCPD/glycidol was produced, with 10% MAG added oil. Conversely, the effect was smaller with DAG addition compared to that with MAG addition, and only 3 ppm was produced even with 20% DAG added oil. These results suggested that preprocessing to reduce MAG in oils and fats might be effective at suppressing the generation of MCPD/glycidol in the deodorization process. In fact, after reducing MAG to 0.1% and DAG to 2.0% in crude palm oil through short path distillation, the production of MCPD/glycidol was successfully reduced from 1.8 ppm to 0.8 ppm in the deodorization process (230 – 250°C, 1 hour).
**List of publication**


**Other publications**

1. 風直樹「グリシドール脂肪酸エステル及び3-MCPD脂肪酸エステル含有量を低減させた油脂の製造方法、およびこれらを低減した油脂」
公開技法 (2014) 技法番号：2014-502808

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