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Insights into the formation mechanism of chloropropanol fatty acid esters under laboratory-scale deodorization conditions

Katsuhito Hori,[1,3] Natsuko Hori-Koriyama,[3] Kazunobu Tsumura,[3] Eiichiro Fukusaki,[1] and Takeshi Bamba[1,2,*]

Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan,[1] Medical Institute of Bioregulation, Kyusyu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan,[2] and Fuji Oil Co., Ltd., 1 Sumiyoshi-Cho, Izumisano, Osaka 598-8540, Japan[3]

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Chloropropanol fatty acid esters (CPFAEs) are well-known contaminants in refined oils and fats, and several research groups have studied their formation. However, the results obtained in these studies were not satisfactory because the CPFAEs were not analyzed comprehensively. Thus, in the present study, a comprehensive analysis was performed to obtain new details about CPFAE formation. Each lipid (monopalmitin, dipalmitin, tripalmitin, monoolein, diolein, triolein, and crude palm oil) was heated at 250°C for 90 min, and the CPFAEs were analyzed using supercritical fluid chromatography/tandem mass spectrometry. It was found that CP fatty acid monoesters were formed from monoacylglycerols and diacylglycerols after heating in the presence of a chlorine compound. In addition, CP fatty acid diesters were formed from diacylglycerols and triacylglycerols under the same conditions. In the case of crude palm oil, only CP fatty acid diesters were formed. Therefore, these results indicated that CPFAEs in refined palm oil were formed mainly from triacylglycerols.

[**Key words:** Chloropropanol fatty acid esters; Acylglycerols; Heating; Supercritical fluid chromatography/mass spectrometry]

Chloropropanols (CPs) are compounds consisting of one or two chlorine atoms attached to a glycerol backbone. Recently, 3-chloro-1,2-propanediol (3-MCPD) and 2-chloro-1,3-propanediol (2-MCPD) fatty acid esters were detected in several edible oils, especially in refined palm oil (1–3). *In vitro* experiments revealed that 3-MCPD fatty acid esters were hydrolyzed by lipase to form 3-MCPD (4). Current toxicological assessments assume that these compounds are released in humans. Therefore, it is important to consider the limit of daily intake of free 3-MCPD for humans. Due to its adverse effect on the kidneys, the maximum tolerable daily intake of 3-MCPD is 2 μg/kg of body weight per day (5).

Several research groups have investigated methods for the reduction of CP fatty acid esters (CPFAEs). Typically, crude palm oil is washed with water and/or ethanol to remove the chlorine compounds. This approach reduces 3-MCPD fatty acid esters by 30–50% (6,7). To achieve further reductions, additional studies are required to elucidate the CPFAE formation pathway, identify the CPFAE precursors, and comprehensively profile the CPFAEs in refined palm oil.

Franke et al. (8) showed that CPFAEs were formed predominantly during the refining processes for oils and fats, especially during the deodorization step. Moreover, it was found that CPFAEs were formed at high temperatures (9–14). Shimizu et al. (10) reported the results of heating tests on pure glycerides with the addition of a chloride source. Destaillats et al. (9) analyzed several types of MCPD ester molecular species formed in a model experiment using acylglycerols in the presence of chlorine-containing compounds. Despite these significant studies, more data are required to determine the formation mechanism of CPFAEs.

In the present study, we investigate the reaction of lipids with chlorine compounds at the deodorization temperature to identify the CPFAEs produced. CPFAE profiles are used to provide detailed information on the CPFAE formation mechanism. In addition, liquid chromatography/time-of-flight mass spectrometry (LC/TOF MS) is used to determine the CPFAE molecular species. Quantitative analysis of the CPFAEs is performed through supercritical fluid chromatography/hybrid triple-quadruple mass spectrometry (SFC/QqQ MS), which is generally considered to be suitable for the separation analysis of lipophilic compounds (15–19). Furthermore, this method effectively discriminates the different 3-MCPD fatty acid esters (20). Therefore, it is also considered suitable for CPFAE profiling in lipids.

In the present study, the relationship between the molecular species of precursor lipids and the respective CPFAEs is discussed by analyzing the reaction products that are formed under laboratory-scale deodorization conditions.

* Corresponding author at: Medical Institute of Bioregulation, Kyusyu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel.: +81 92 642 6170; fax: +81 92 642 6172.

E-mail addresses: hori.katsuhito@so.fujioil.co.jp (K. Hori), koriyama.natsuko@so.fujioil.co.jp (N. Hori-Koriyama), tsumura.kazunobu@so.fujioil.co.jp (K. Tsumura), fukusaki@bio.eng.osaka-u.ne.jp (E. Fukusaki), bamba@bioreg.kyushu-u.ac.jp (T. Bamba).

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TABLE 1. Molecular species of CPFAEs screened in this study.

Side chains of glycerol backbone			M.W.	Screening adduct ions		
				H^+	NH_4^+	Na^+
C16:0/	C16:0/	Cl	586.46	587.46	604.46	609.46
C16:0/	C18:0/	Cl	614.49	615.49	632.49	637.49
C16:0/	C18:1/	Cl	612.48	613.48	630.48	635.48
C16:0/	C18:2/	Cl	610.46	611.46	628.46	633.46
C18:0/	C18:0/	Cl	642.52	643.52	660.52	665.52
C18:0/	C18:1/	Cl	640.51	641.51	658.51	663.51
C18:0/	C18:2/	Cl	638.49	639.49	656.49	661.49
C18:1/	C18:1/	Cl	638.50	639.50	656.50	661.50
C18:1/	C18:2/	Cl	636.48	637.48	654.48	659.48
C18:2/	C18:2/	Cl	634.46	635.46	652.46	657.46
C16:0/	OH/	Cl	348.23	349.23	366.23	371.23
C18:0/	OH/	Cl	376.26	377.26	394.26	399.26
C18:1/	OH/	Cl	374.25	375.25	392.25	397.25
C18:2/	OH/	Cl	372.23	373.23	390.23	395.23
C16:0/	Cl/	Cl	366.20	367.20	384.20	389.20
C18:0/	Cl/	Cl	394.23	395.23	412.23	417.23
C18:1/	Cl/	Cl	392.22	393.22	410.22	415.22
C18:2/	Cl/	Cl	390.20	391.20	408.20	413.20

M.W., molecular weight.

MATERIALS AND METHODS

Chemicals The *rac* 1-palmitoyl-3-chloropropanediol (POHCl), *rac* 2-palmitoyl-3-chloropropanediol (OHPCl), *rac* 1-palmitoyl-2-chloropropanediol (PClOH), *rac* 1-oleoyl-3-chloropropanediol (OOHCl), *rac* 2-oleoyl-3-chloropropanediol (OHOCl), *rac* 1-oleoyl-2-chloropropanediol (OClOH), *rac* 1,2-bis-palmitoyl-3-chloropropanediol (PPCl), *rac* 1,3-bis-palmitoyl-2-chloropropanediol (PClP), *rac* 1,2-bis-oleoyl-3-chloropropanediol (OOCl), *rac* 1,3-bis-oleoyl-2-chloropropanediol (OClO), *rac* 1-palmitoyl-2-linoleoyl-3-chloropropanediol (PLCl), *rac* 1-palmitoyl-2-stearoyl-3-chloropropanediol (PSCl), *rac* 1-oleoyl-2-linoleoyl-3-chloropropanediol (OLCl), and *rac* 1,2-bis-palmitoyl-3-chloropropanediol-d_5 (PPCl-d_5) were purchased from Toronto Research Chemicals Inc. (ON, Canada). The *rac* 1-palmitoyl-2-oleoyl-3-chloropropanediol (POCl) and *rac* 1-stearoyl-2-oleoyl-3-chloropropanediol (SOCl) were purchased from Tsukishima Food Industry Co., Ltd. (Tokyo, Japan). Methanol, acetonitrile, isopropanol, hexane, distilled water, chloroform, formic acid, ammonium formate, triolein, and tripalmitin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Monoolein, diolein, monopalmitin, and dipalmitin were purchased from Sigma–Aldrich Corporation (MD, USA). $FeCl_2$ was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Oil samples Crude and refined palm oil were obtained from Fuji Oil Co., Ltd. The monoacylglycerol, diacylglycerol, and triacylglycerol contents in the crude palm oil were <1%, 6.9%, and 93.1%, respectively. The monoacylglycerol, diacylglycerol, and triacylglycerol contents were obtained using reverse-phase liquid chromatography with a refractive index detector, with acetone/acetonitrile (8/2) as the mobile phase. The palm oil used in this study was refined in a process consisting of a bleaching step and a deodorization step. The bleaching step was performed as follows. White clay (7.5 g) was added to 500 g of crude palm oil, after which the mixture was heated at 110°C for 10 min with gentle stirring. During the bleaching process, the pressure in the bleaching system was kept below 20 mmHg. The white clay was removed from the oil using filter paper after completion of the process. Next, deodorization was performed. Deodorization is a refining process used during edible oil production. Deodorization of fats and oils is necessary in order to remove disagreeable flavors and odors that are naturally present or are created during processing. This is a high-temperature (230–260°C), high-vacuum (2–5 mmHg) steam distillation process that removes free fatty acids and volatile compounds from edible fats and oils. In the present study, deodorization was performed in the laboratory using 500 g of bleached oil. In the deodorization process, the oil was first heated at 250°C using a mantle heater. Steam was then injected to an amount of 25 g/500 g oil. Following 90 min of deodorization at a pressure of 3 mmHg, the oil was cooled to 160°C and the steam flow was stopped.

Lipid heat processing Heat processing using $FeCl_2$ was performed as follows. $FeCl_2$ was used as a chlorine-containing compound and dissolved in MeOH. 10 μL of the $FeCl_2$ solution (100 μg/mL) was added to a Pyrex glass ampoule, and the methanol was removed by drying. Next, 10 mg of lipid was added to the ampoule. After flushing with N_2 gas, the ampoule was sealed using a gas burner (Style Index Co., Ltd., Tokyo, Japan). The glass ampoule was heated at 250°C for 90 min in a muffle furnace (Nitto Kagaku Co., Ltd., Aichi, Japan). Heat processing without $FeCl_2$ was performed as follows. 10 mg of lipid was added to a Pyrex glass ampoule. After flushing with N_2 gas, the glass ampoule was sealed with a gas burner and heated at 250°C for 90 min in a muffle furnace.

TABLE 2. Target compounds and analytical conditions for SFC/MS analysis.

sn-1 or *sn-3*	*sn-2*	*sn-1* or *sn-3*	Symbol	Q1 ion (*m/z*)	Q3 ion (*m/z*)	Cone voltage (V)	Collision energy (V)
C16:0	OH	Cl	POHCl	366.1	239.2	10	12
				366.1	349.1	10	8
OH	C16:0	Cl	OHPCl	366.1	239.2	12	8
				366.1	349.1	10	12
C16:0	Cl	OH	PClOH	366.1	239.2	12	12
				366.1	349.1	12	8
C18:1	OH	Cl	OOHCl	392.1	265.1	14	8
				392.1	375.1	10	12
OH	C18:1	Cl	OHOCl	392.1	265.1	12	8
				392.1	375.1	10	12
C18:1	Cl	OH	OClOH	392.1	265.1	10	10
				392.1	375.1	14	14
C16:0	C16:0	Cl	PPCl	604.4	331.1	22	20
				–	–	–	–
C16:0	Cl	C16:0	PClP	604.4	331.1	24	18
				–	–	–	–
C18:1	C18:1	Cl	OOCl	656.4	357.1	24	20
				–	–	–	–
C18:1	Cl	C18:1	OClO	656.4	357.1	22	18
				–	–	–	–
C16:0	C18:2	Cl	PLCl	628.5	331.4	10	12
				628.5	355.3	10	8
C18:1	C18:2	Cl	OLCl	654.5	355.2	12	8
				654.5	357.2	10	12
C16:0	C18:1	Cl	POCl	630.5	331.2	12	12
				630.5	357.2	12	8
C18:0	C18:1	Cl	SOCl	658.5	357.2	14	8
				658.5	359.5	10	12
C16:0	C18:0	Cl	PSCl	632.5	331.2	12	8
				632.5	359.3	10	12

Sample preparation For the LC/TOF MS analysis, the lipid sample was dissolved in 0.5 mL of chloroform. This chloroform solution was diluted two times with hexane. The final lipid concentration in the sample solution was 10 mg/mL.

For the SFC/QqQ MS analysis, the lipid sample following heat processing was dissolved in 1 mL of chloroform. This chloroform solution was diluted 10 times with hexane. The final lipid concentration in the sample solution was 1 mg/mL. Deuterium-labeled PPCl, PPCl-d_5, was then added as an internal standard, at a concentration of 100 ng/mL in the sample solution.

LC/TOF MS analysis An Ultimate 3000 (Dionex Corporation, CA, USA) was used for the LC/TOF analysis, with an L-column ODS (Chemicals Evaluation Research Institute, Tokyo, Japan) with an inner diameter of 4.6 mm, a length of 150 mm, and a particle size of 5 μm. The column temperature was set at 40°C. Mobile phase A was methanol/acetonitrile/water (19/19/2, v/v/v) and mobile phase B was isopropanol. Ammonium formate and formic acid in molar concentrations of 20 mM and 5 mM, respectively, were used as additives to the mobile phases, with a flow rate of 0.5 mL/min. An increasing mobile phase B concentration gradient was used: 10% from 0 to 3 min, 10–100% from 3 to 19 min, 100% from 19 to 39 min, 100–10% from 39 to 40 min, and 10% from 40 to 45 min. The analyzed sample volume was 10 μL. The MS analysis was conducted using a MicrOTOF II mass spectrometer (Bruker Daltonics Inc., MA, USA) with electrospray ionization (ESI). The ESI/MS analysis was performed in positive-ion mode with a capillary voltage of 4.5 kV, a dry temperature of 200°C, and a dry gas flow rate of 8.0 L/min. The scan acquisition rate was 20 spectra/s, and the mass ranged from 50 to 1200. All data were analyzed using the Data Analysis software (Bruker Daltonics Inc.). The screening ions are shown in Table 1.

SFC/QqQ MS analysis An analytical method station SFC system was used (Waters, MA, USA), with two YMC Carotenoid columns (YMC Co., Ltd., Kyoto, Japan) combined in tandem. The columns had an inner diameter of 4.6 mm, a length of 250 mm, and a particle size of 5 μm. The column temperature was set at 40°C and the backpressure was 10 MPa. The mobile phase was carbon dioxide, and the modifier was methanol. Ammonium formate was used as an additive to the modifier at 0.1% concentration. The flow rate was 2 mL/min from 0 to 7 min, 3 mL/min from 7 to 22 min, and 2 mL/min from 22 to 25 min. An increasing modifier concentration gradient was used: 6% from 0 to 1 min, 6–10% from 1 to 5 min, 10% from 5 to 7 min, 10–30% from 7 to 11 min, 30% from 11 to 22 min, and 6% from 22 to 25 min. A sample volume of 5 μL was injected.

The MS analysis was conducted in positive-ion mode using a Xevo TQ mass spectrometer (Waters). The MS parameters were a capillary voltage of 3.0 kV, a cone voltage of 30 V, a desolvation temperature of 600°C, a desolvation gas flow rate of 800 L/h, and a cone gas flow rate of 60 L/h. All data were analyzed using the Mass Lynx software (Waters).