Journal of Oleo Science
Copyright ©2016 by Japan Oil Chemists' Society
doi : 10.5650/jos.ess16021
J. Oleo Sci. **65**, *(7) 557-568 (2016)*

Collaborative Study of an Indirect Enzymatic Method for the Simultaneous Analysis of 3-MCPD, 2-MCPD, and Glycidyl Esters in Edible Oils

Kazuo Koyama[1]*, Kinuko Miyazaki[1], Kousuke Abe[2], Yoshitsugu Egawa[3], Hirotsugu Kido[4], Tadashi Kitta[5], Takashi Miyashita[6], Toru Nezu[7], Hidenori Nohara[8], Takashi Sano[9], Yukinari Takahashi[10], Hideji Taniguchi[11], Hiroshi Yada[12], Kumiko Yamazaki[13] and Yomi Watanabe[14]

[1] *Central Research & Development Institute, House Foods Group Inc. (1-4, Takanodai, Yotsukaido, Chiba 284-0033, JAPAN)*
[2] *Analysis & Assessment, Central Research Laboratory, The Nisshin OilliO Group, Ltd.. (1, Shinmei-cho,Yokosuka 239-0832, JAPAN)*
[3] *Analytical Center for Food Safety, Quality Assurance Department, Fuji Oil Co., Ltd. (1, Sumiyoshi-cho, Izumisano, Osaka 598-8540, JAPAN)*
[4] *Japan Association for Inspection and Investigation of Foods Including Fats and Oils (3-27-8, Nihonbashi-hamacho, Chuo-ku, Tokyo 103-0007, JAPAN)*
[5] *Japan Frozen Foods Inspection Corp. (2-13-45, Fukuura, Kanazawa-ku,Yokohama 236-0004, JAPAN)*
[6] *Quality Assurance Div., Kewpie Corp. (2-5-7, Senkawa-cho,Chohu, Tokyo 182-0002, JAPAN)*
[7] *Food Development Laboratory, ADEKA Corp. (7-2-34, Higashiogu, Arakawa-ku, Tokyo 116-8553, JAPAN)*
[8] *Health Care Food Research Laboratories, Kao Corp. (2-1-3, Bunka, Sumida-ku, Tokyo 131-8501, JAPAN)*
[9] *Fundamental Research Laboratory, J-Oil Mills Inc. (7-41, Dikoku-cho, Tsurumi-ku, Yokohama 230-0053, JAPAN)*
[10] *Analytical and Technological Solution Service, House Food Analytical Laboratory Inc. (1-4, Takanodai, Yotsukaido, Chiba 284-0033, JAPAN)*
[11] *General Testing Research Institute, Japan Oilstuff Inspectors Corp. (1-2-15, Mikagetsuka-machi, Higashinada-ku, Kobe 658-0044, JAPAN)*
[12] *Food Research Institute, National Agriculture and Food Research Organization (NARO) (2-1-12, Kannon-dai, Tsukuba, Ibaraki 305-8642, JAPAN)*
[13] *Japan Food Research Laboratories, Saito Laboratory (7-4-41, Saitoasagi, Ibaraki, Osaka 567-0085, JAPAN)*
[14] *Osaka Municipal Technical Research Institute (1-6-50, Morinomiya, Joto-ku, Osaka 536-8553, JAPAN)*

Abstract: A collaborative study was conducted to evaluate an indirect enzymatic method for the analysis of fatty acid esters of 3-monochloro-1,2-propanediol (3-MCPD), 2-monochloro-1,3-propanediol (2-MCPD), and glycidol (Gly) in edible oils and fats. The method is characterized by the use of *Candida rugosa* lipase, which hydrolyzes the esters at room temperature in 30 min. Hydrolysis and bromination steps convert esters of 3-MCPD, 2-MCPD, and glycidol to free 3-MCPD, 2-MCPD, and 3-monobromo-1,2-propanediol, respectively, which are then derivatized with phenylboronic acid, and analyzed by gas chromatography-mass spectrometry. In a collaborative study involving 13 laboratories, liquid palm, solid palm, rapeseed, and rice bran oils spiked with 0.5–4.4 mg/kg of esters of 3-MCPD, 2-MCPD, and Gly were analyzed in duplicate. The repeatability (RSD_r) were < 5% for five liquid oil samples and 8% for a solid oil sample. The reproducibility (RSD_R) ranged from 5% to 18% for all oil samples. These RSD_R values were considered satisfactory because the Horwitz ratios were ≤ 1.3% for all three analytes in all oil samples. This method is applicable to the quantification of 3-MCPD, 2-MCPD, and Gly esters in edible oils.

Key words: collaborative study, indirect method, lipase, MCPD, glycidol

1 INTRODUCTION

Fatty acid esters of 3-monochloro-1,2-propanediol (3-MCPD) and glycidol (Gly) are known as contaminants in foods and edible oils. In 1978, free chlorinated propanols, especially 3-MCPD, were detected in acid-hydrolyzed vegetable protein (acid-HVP) and in soy sauce[1]. Codex established the maximum level of 3-MCPD in liquid seasonings containing acid-HVP based on an international risk evaluation[2]. In the 1980s, 3-MCPD fatty acid esters (3-MCPD-Es) were found in trace amounts in heat-processed foods[3, 4].

*Correspondence to: Kazuo Koyama, Central Research & Development Institute, House Foods Group Inc., 1-4, Takanodai, Yotsukaido, Chiba 284-0033, JAPAN
E-mail: k-koyama@housefoods.co.jp
Accepted April 1, 2016 (received for review January 30, 2015)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manusriptcentral.com/jjocs

The reports of Weißhaar on the presence of 3-MCPD-Es (2008)[5] and glycidol fatty acid esters (Gly-Es) (2009)[6,7] in edible oils raised much concern in Europe. Gly-Es were also found in diacylglycerol (DAG)-enriched oils marketed in Japan[8], which attracted public attention.

It is possible that 3-MCPD-Es and Gly-Es in foods and oils are hydrolyzed *in vivo* to the free-form carcinogens 3-MCPD and Gly. The Japanese Food Safety Commission reported that the toxicity of Gly-Es in DAG-enriched oil was unconfirmed and denied that they showed carcinogenesis promoting activity[9]. However, the commission did not deny the possibility that Gly-Es might be a genotoxic carcinogen, recommending that Gly-Es levels should be kept as low as possible. The commission also suggested that 3-MCPD-Es would not be genotoxic *in vivo*. International organizations, such as FAO/WHO Joint Expert Committee on Food Additives (JECFA) and European Food Safety Authority (EFSA), have conducted monitoring surveys and toxicity studies of 3-MCPD-Es and Gly-Es. From 2007 to 2009, German Bundesinstitut für Risikobewertung (BfR) conducted a risk assessment under the assumption that the human body absorbed all Gly resulting from the complete hydrolysis of Gly-Es consumed at concentrations found in foods determined using non-validated methods.

In recent years, the concentrations of 3-MCPD-Es and Gly-Es in edible oils and foods have been surveyed in Japan[10] and other countries[11]. The results of these surveys require continual updating using more precise validated methods. Food manufacturers are voluntarily maintaining or lowering the amounts of 3-MCPD-Es and Gly-Es in their products, and using analysis to monitor their achievements.

Direct and indirect methods have been developed for the analysis of 3-MCPD-Es, 2-monochloro-1,3-propanediol fatty acid esters (2-MCPD-Es), and Gly-Es in edible oils[12]. In the direct methods, the sample is purified by solid-phase extraction and then individual esters are analyzed by liquid chromatography-mass spectrometry (LC-MS). A direct analytical method for Gly-Es, reported by Shiro *et al.*, was validated by a joint study by Japan Oil Chemists' Society (JOCS) and American Oil Chemists' Society (AOCS), and was registered as the JOCS Standard Method for the Analysis of Fats, Oils, and Related Materials 2.4.13-2013[13,14]. Two direct methods for the analysis of 3-MCPD-Es have been reported by Yamazaki *et al.*[15] and Hori *et al.*[16,17]. As 3-MCPD can form mono- and diesters with various fatty acids, the direct methods required numerous standard materials and various procedures for sample purification. These direct methods are suitable for the toxicological assessment of individual compounds and elucidation of their formation mechanisms.

In the indirect methods, the esters are hydrolyzed and the resulting glycerol skeletons analyzed by gas chromatography-mass spectrometry (GC-MS). The indirect methods have the advantage over direct methods of requiring only a few standard reagents. In 2013, AOCS registered three indirect methods, Cd 29a, Cd 29b, and Cd 29c, differentiated by their ester cleavage conditions[18–20]. Method Cd 29a performs ester hydrolysis under acidic conditions, whereas method Cd 29b uses alkaline conditions. Both methods require more than 10 hours for hydrolysis. Method Cd 29c performs ester hydrolysis under alkaline conditions with shorter reaction times, but cannot be used for the quantification of 2-MCPD-Es. These AOCS methods require both reference standards and deuterated internal standards in ester form to correct for ester cleavage efficiencies and unintended side reactions between analytes. Using reference standards in the ester form necessitates long hydrolysis times, as needed for the sample being analyzed, to construct calibration curves.

An indirect method using a lipase for ester cleavage was developed by Miyazaki *et al.*[21] and was improved through preliminary and feasibility studies conducted by the MCPD subcommittee organized within JOCS for method validation[22]. In this method, ester hydrolysis is completed in 0.5 hour at room temperature. In addition, the method has the advantage that reference standards need not be in ester form, which eliminates the need for hydrolysis to construct calibration curves. Here, we report the results of a full collaborative study conducted by 13 participating laboratories to validate the improved method.

2 EXPERIMENTAL

2.1 Reagents

Of the ester standard reagents, 3-MCPD dioleate and glycidyl oleate were purchased from Wako Pure Chemical (Japan), and 2-MCPD dipalmitate was purchased from Toronto Research Chemicals (Canada). Of the free standard reagents, 3-MCPD and 3-monobromo-1,2-propanediol (3-MBPD) were purchased from Wako Pure Chemical, 3-MCPD-d_5 from CDN Isotopes (Canada), and 2-MCPD, 2-MCPD-d_5, and 3-MBPD-d_5 from Toronto Research Chemicals. 3-MBPD and 3-MBPD-d_5 were used for the determinations of Gly-Es. Lipase AYS Amano (derived from *Candida rugosa*) was purchased from Wako Pure Chemical. These chemical reagents were distributed to all the laboratories participating in the collaborative study. The participating laboratories separately purchased phenylboronic acid (PBA; purity ≥ 97%), special grade organic solvents, and salts.

A 30% (*w/v*) sodium bromide aqueous solution was prepared and adjusted to pH 5.0 with citric acid and disodium hydrogen phosphate aqueous solutions. This solution was used to prepare a sodium bromide aqueous solution containing 90 U/mL lipase by dissolving *Candida rugosa* lipase. A PBA solution was prepared by dissolving 0.25 g

PBA in 10 mL water/acetone mixed solution (1:19, *v/v*).

For the standard stock solutions, 300 μg/mL ethanol solutions were prepared for 3-MCPD, 2-MCPD, and 3-MBPD, and 200 μg/mL ethanol solutions were prepared for 3-MCPD-d_5, 2-MCPD-d_5, and 3-MBPD-d_5 as internal standard stock solutions. These stock solutions could be stored at ≤ 20℃ for at least three months. On the day of the study, each standard stock solution was diluted with the 30% sodium bromide aqueous solution to prepare 15 μg/mL standard mix I and 1.5 μg/mL standard mix II. Additionally, 2.0 μg/mL internal standard mix was prepared by combining each of the internal standard stock solutions and diluting it with the 30% sodium bromide aqueous solution.

2.2 Oil samples

Commercially available rapeseed, palm (liquid and solid), and rice bran oils were used as samples in the collaborative study. Extra virgin olive oil was chosen as a practice sample. Samples were spiked with ester standards, as shown in Table 1, dispensed into glass vials, and stored in the dark at room temperature. Each laboratory received one vial each of the five spiked samples (rapeseed No. 1, 4, and 5, palm (solid) No. 2, and rice bran No. 6) and one sample without spiking (palm (liquid) No. 3) under blind conditions, along with a practice sample (spiked extra virgin olive oil).

2.3 Equipment

A temperature-controlled water bath (DH-12; TAITEC, Japan), a vortex mixer (HM-10H; AS ONE, Japan), a centrifuge (Model 4000; Kubota, Japan), a nitrogen gas sprayer (EN-10/DTU-1BN; TAITEC), and a GC-MS (Quantum GC and TRACE GC Ultra; Thermo Fisher Scientific, USA), or equivalent apparatuses were used by each laboratory. For high-speed shaking, participants were asked to use one of the following shakers: I, one that shakes vertically mounted test tubes in an eccentric orbital motion at 1,800 rpm (e.g., CM-1000; Tokyo Rikakikai, Japan); II, one that shakes vertical mounted test tubes in a vertical reciprocating motion at 200 rpm (e.g., BF-40LF; TAITEC); or III, one that shakes test tubes mounted at 60° from the axis in a horizontal reciprocating motion at 200–400 rpm (e.g., MW-1; AS ONE) (see Table 2).

2.4 Procedures for analysis of oil samples and construction of calibration curves

The method developed by Miyazaki *et al.*[21] and modified following the feasibility study by JOCS[22] was used in this study (Fig. 1). From each vial, 0.1 g of oil sample was weighed into separate 10 mL test tube, in duplicate. The oil sample was dissolved in 200 μL of isooctane, with an exception of the solid oil sample, which was dissolved in 500 μL of isooctane and heated to 45–60℃ for complete dissolution. After adding 3 mL of the 30% sodium bromide aqueous solution containing 90 U/mL lipase, the mixture was shaken by a high-speed shaker for 30 min at room temperature to hydrolyze the esters. The mixture was then heated in a water bath at 80℃ for 10 min to achieve the bromination of Gly to 3-MBPD. The mixture was cooled to room temperature, and 50 μL of 2.0 μg/mL internal standard mix was added. The mixture was washed twice with 3 mL of hexane by vortexing for 10 sec, and to the resulting aqueous layer, a 100 μL PBA solution was added. After vortexing for a further 10 sec, 3 mL of hexane was added, followed by high-speed shaking for 5–10 min. The resulting organic layer was collected, dehydrated with sodium sulfate, and concentrated to approximately 0.5–0.8 mL under a stream of nitrogen. After filtration through a 0.2

Table 1 Test samples prepared for collaborative study.

No	Oil		Iodine values	Equiv. conc. (mg/kg)[a]					
				3-MCPD		2-MCPD		Gly	
				blank[b]	amount spiked[c-1]	blank[b]	amount spiked[c-2]	blank[b]	amount spiked[c-3]
1,4	Rapeseed	(High)	111	0.08	3.50	0.05	3.68	0.20	4.44
2	Palm	(Solid)	43	2.55	0.85	1.31	0.45	0.93	1.08
3	Palm	(Liquid)	66	3.33	– [d]	1.82	– [d]	2.32	– [d]
5	Rapeseed	(Low)	111	0.08	0.54	0.05	0.56	0.20	0.61
6	Rice bran		104	0.28	1.09	0.14	1.15	0.78	2.77
7	Extra virgin olive	(for practice)	82	0.04	4.17	– [e]	4.11	0.03	4.35

a) Free form equivalent concentration.
b) Concentration measured by the coordinator.
c) Free form equivalent concentration spiked c-1) 3-MCPD dioleate, c-2) 2-MCPD dipalmitate, and c-3) Gly oleate.
d) Not spiked.
e) Not detected. The limit of detection for 2-MCPD corresponds to 0.007 mg/kg in 0.1 g oil samples.

Table 2 Equipment used for the collaborative study.

Lab. code	High-speed Shaker[a)]	Column		GC-MS		
		Separation	Guard	Injection mode		MS
A	I	VF-5ms	EZ-guard	Splitless	Agilent	5975C TAD
B	I	DB-5ms	Non	Splitless	Thermo	TSQ Quantum XLS
C	III	DB-5ms	DuraGuard	Splitless, PVT	Shimadzu	GCMS-QP2010
D	II	HP-5ms	Non	Splitless	Agilent	5975C inertXL
E	I	VF-5ms	EZ-guard	Splitless	Agilent	5975C
F	II	VF-5ms	EZ-guard	Splitless	Thermo	TRACE1310, ISQLT
G	I	SLB-5ms	Non	Splitless	Agilent	5975C inertXL
H	III	DB-5ms	Non	Splitless	Agilent	5975C
I	II	HP-5ms	Non	Splitless	Shimadzu	GCMS-QP2010 Plus
J	I	VF-5ms	Non	Splitless	Varian	320-MS
K	II	DB-5ms	Non	Splitless	Agilent	5973 Network
L	I	SLB-5ms	Non	Splitless	Shimadzu	GCMS-QP5050A
M	II	VF-5ms	EZ-guard	Splitless	Shimadzu	GCMS-QP2010

a) Shaking conditions are described in Chapter 2.3.

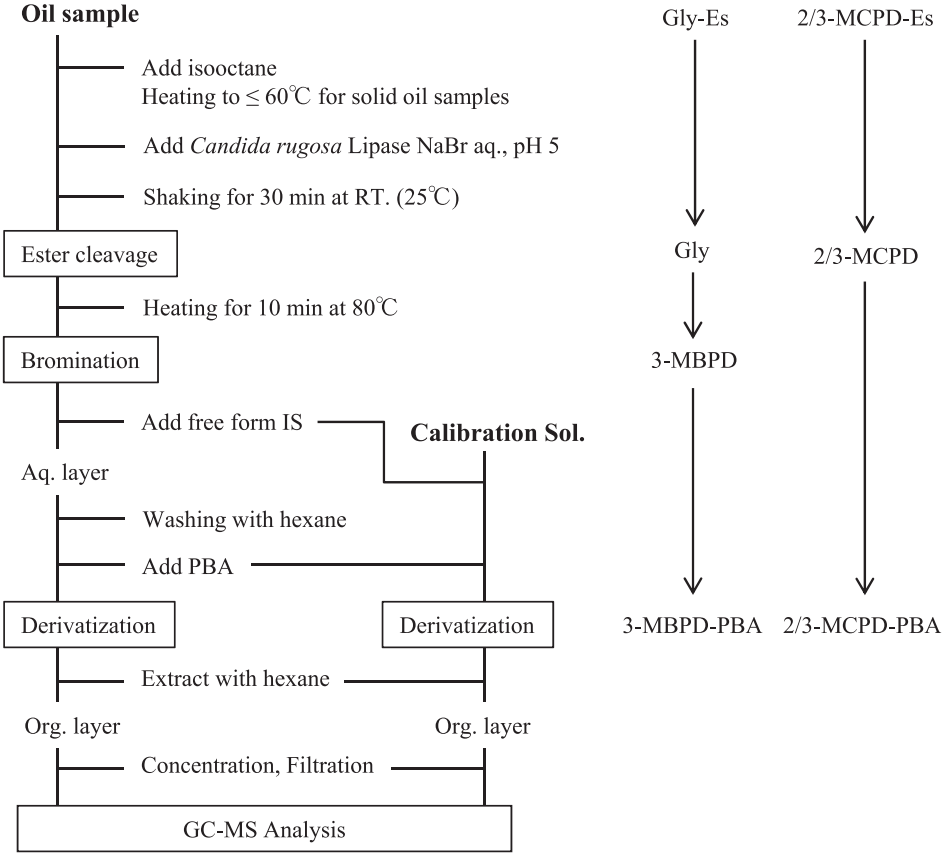

Fig. 1 Procedure flow of this analytical method.

μm membrane filter, the samples were subjected to GC-MS analysis.

For the construction of calibration curves, the 1.5 μg/mL standard mix II in quantities of 5, 20, 50, and 100 μL, and the 15 μg/mL standard mix I in quantities of 20, 35, 50, and 80 μL, were added into different 10 mL test tubes. To each test tube containing 0.0075, 0.030, 0.075, 0.15, 0.30, 0.53, 0.75, or 1.2 μg of each standard, a mixture of 50 μL of the 2.0 μg/mL internal standard mix and 3 mL of the 30% sodium bromide aqueous solution was added, and PBA derivatization was performed as described above.

2.5 GC-MS measurements

A GC capillary column was used with a (5%-phenyl)-methylpolysiloxane liquid phase, 30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness. Some laboratories used a guard column, and one laboratory used a programmed temperature vaporizer (PTV) function in the inlet, at their own discretion (see Table 2). The carrier gas was helium at a fixed flow rate of 1.2 mL/min. The sample was injected in splitless mode at 250℃. The column oven temperature was held at 60℃ for 1 min, then raised to 150℃ at 10℃/min, 180℃ at 3℃/min, and finally 300℃ at 30℃/min, before being held at 300℃ for 8 min (total run = 32 min). MS was performed in positive electron ionization mode with an ion source temperature of 230℃. Quantitative and qualitative analyses were performed by selective ion monitoring (SIM) using ions at *m/z* 147 and 196 for the 3-MCPD derivative, 150 and 201 for the 3-MCPD-d_5 derivative, 196 and 198 for the 2-MCPD derivative, 201 and 203 for the 2-MCPD-d_5 derivative, 147 and 240 for the 3-MBPD derivative, and 150 and 245 for the 3-MBPD-d_5 derivative.

2.6 Collaborative Study

This collaborative study was performed by thirteen laboratories in Japan. The participants analyzed the practice sample first, followed by the six blind samples (Nos. 1–6 in Table 1) in duplicate. The free-form equivalent concentrations of 3-MCPD, 2-MCPD, and Gly were calculated using the internal standards and the calibration curves, and were reported to the coordinator (corresponding author). The coordinator analyzed the distributed samples and the corresponding blank oils (not spiked) in duplicate, and confirmed the homogeneity of the oil samples by duplicate analysis of six vials selected randomly from the distributed samples of rapeseed oil (No. 1) and rice bran oil (No. 6), respectively.

3 RESULTS AND DISCUSSION

3.1 Preliminary performance evaluation of the analytical method by the coordinator

Typical GC-MS chromatograms of the PBA derivatives of 3-MCPD, 2-MCPD, and 3-MBPD (brominated Gly) obtained by the coordinator are shown in Figs. 2 and 3. The analytes in the standard and the oil sample were separated without interruption. The distributed oil samples were spiked with ester standards to blank oils. As shown in Table 3, the homogeneities of the two oil samples were confirmed by one-way analysis of variance. The homogeneities of the other oil samples were considered guaranteed because they were prepared in the same manner. As shown in Table 4, satisfactory recovery rates, in the range 95.1–113.7%, were obtained for all spiked samples. These results suggested that the method was ready for the collaborative study.

3.2 Collaborative study

The results for the free-form equivalent concentrations (mg/kg) of all analytes in all oil samples analyzed by all participant laboratories are shown in Table 5a-c, along with the statistical parameters. Outlier data determined by Cochran's test are indicated with asterisks. Dixon's test showed no outlier or straggler data. After removal of the outlier data, the repeatability and reproducibility were calculated for each analyte in each oil sample.

The repeatability relative standard deviations (RSD_r) for the liquid oils, *i.e.*, rapeseed oils (No. 1, 4, and 5), liquid palm oil (No. 3), and rice bran oil (No. 6), were $<4.2\%$ for 3-MCPD, $<3.4\%$ for 2-MCPD, and $<3.4\%$ for Gly. The RSD_r values for solid palm oil (No.2) were $<8.4\%$ for 3-MCPD, $<1.8\%$ for 2-MCPD, and $<2.8\%$ for Gly. The RSD_r for 3-MCPD was slightly larger in solid palm oil. The reproducibility relative standard deviations (RSD_R) for liquid oils were $<18.0\%$ for 3-MCPD, $<11.7\%$ for 2-MCPD, and $<7.5\%$ for Gly. The large RSD_R for 3-MCPD in rapeseed oil (No. 5) was considered to be due to the low analyte concentration. The RSD_R values for solid palm oil (No.2) were $<18.0\%$ for 3-MCPD, $<8.6\%$ for 2-MCPD, and $<7.7\%$ for Gly. These RSD_r and RSD_R values were similar or smaller than RSD_r (3.5–9.9%) and RSD_R (6.4–31.8%) reported for palm oil samples containing 1–11 mg/kg in the collaborative study conducted by AOAC in 2013 for three indirect methods[23]. Horwitz ratios (HORRAT) for all oils were <1.33 for 3-MCPD, <0.80 for 2-MCPD, and <0.53 for Gly. The Horwitz ratio of <2 means that the current method meets one of the acceptability criteria for recently adopted chemical methods for analysis of AOAC INTERNATIONAL[24, 25].

Two identical rapeseed oil samples (No. 1 and 4) were analyzed under blind conditions to check for possible bias in the analysis potentially caused by an unconscious will for data adjustment, even when taking samples from the same unlabeled vial. When calculated using only the first data of the duplicate analyses of No. 1 and 4, respectively, as a blind duplicate, the RSD_r values were 3.5% for 3-MCPD, 2.0% for 2-MCPD, and 2.0% for Gly. As these

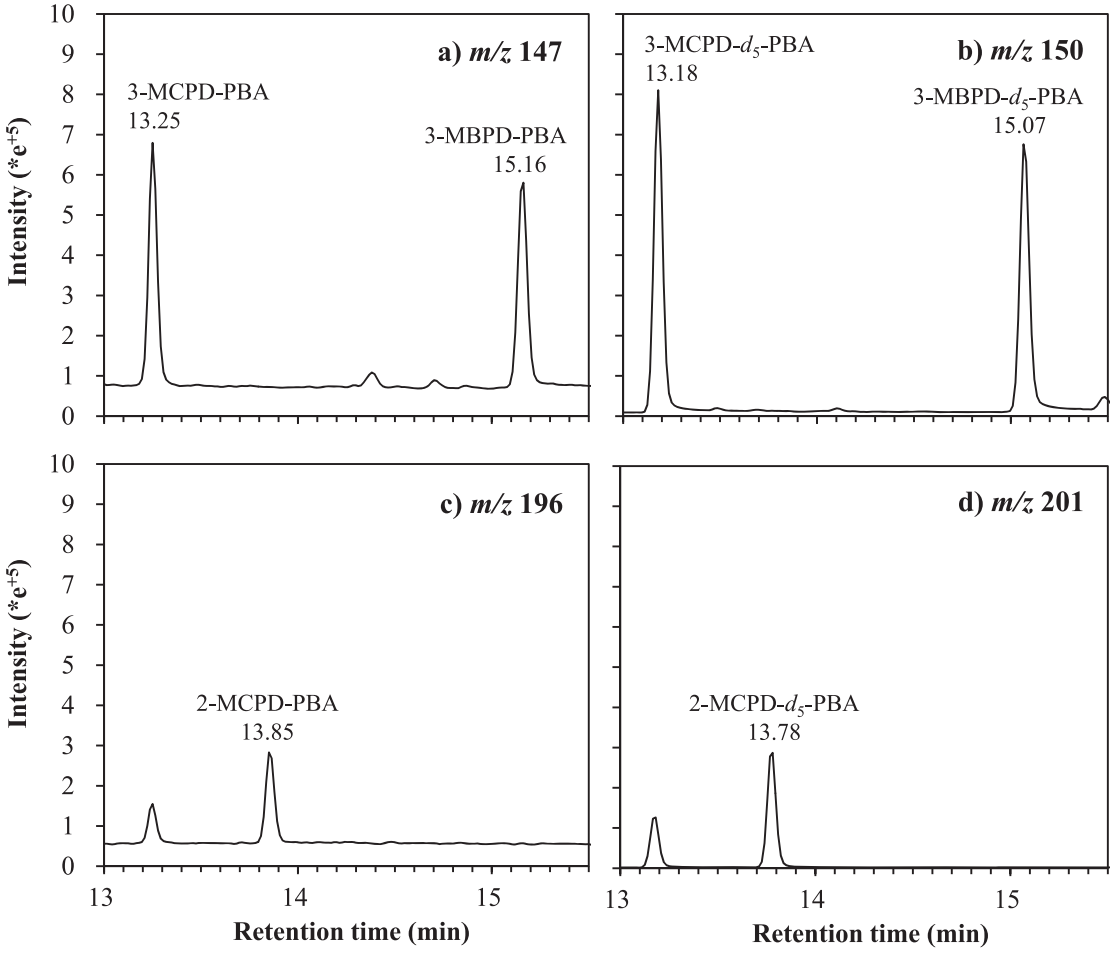

Fig. 2 GC-MS profiles of phenylboronic acid (PBA) derivatives resulting from free type standards with 0.075 μg; a) 3-MCPD and 3-MBPD, b) 3-MCPD-d_5 and 3-MBPD-d_5, c) 2-MCPD, and d) 2-MCPD-d_5.

values were in the same range as those calculated for No. 1 and 4 independently (Table 5a and 5b), it was considered that no bias was observed.

The solid palm oil sample (No. 2) had an iodine value of 43 (Table 1) and a rising melting point of 50℃. The RSD_R for 3-MCPD in the solid palm oil was 18.0%, which was slightly higher than those of other oil samples containing similar analyte amounts (No. 1, 3, 4, and 6). As detailed in the experimental section, participating laboratories were instructed to dissolve the solid oil sample in isooctane by heating the sample to 45–60℃. Three laboratories (C, F, and M), in which the sample was heated to 50℃ or lower, reported lower 3-MCPD concentrations than the other laboratories, in which the sample was heated to 60℃. These lower concentration results for 3-MCPD-Es in the solid oil sample were suspected to be due to incomplete dissolution. Thus, for complete dissolution, a temperature at 60℃ would be recommended. As this indirect method utilizes enzymatic activity, the reaction solvent contained more water than those in the three indirect AOCS official methods. While the water content might hinder the dissolution of oils with higher melting points, it was found that this could be overcome by heating and vigorous shaking. Thus, the current method is expected to be applicable to a wide range of oils and fats.

The concentrations of all three analytes in the rapeseed oil sample (No. 5) were in the range 0.61–0.87 mg/kg (Table 5c), which was within the range of concentrations normally found in many commercial oils. The RSD_r values for the rapeseed oil sample were 2.6% for 3-MCPD, <3.4% for 2-MCPD, and 1.9% for Gly, which were considered sufficiently small. The RSD_R values were 18.0% for 3-MCPD, 9.7% for 2-MCPD, and 7.5% for Gly. The larger RSD_R value of 3-MCPD compared with those of 2-MCPD and Gly was attributed to the selected-ion monitoring chromatogram of the 3-MCPD derivative, which was probably influenced by general background contaminants such as column bleed. The influence appeared to be more pronounced in samples containing low levels of analytes. The Horwitz ratios were also less than 2, calculated as 1.05 for 3-MCPD, 0.57 for 2-MCPD, and 0.46 for Gly, indicating that the current method could be suitable for the determination of 3-MCPD,

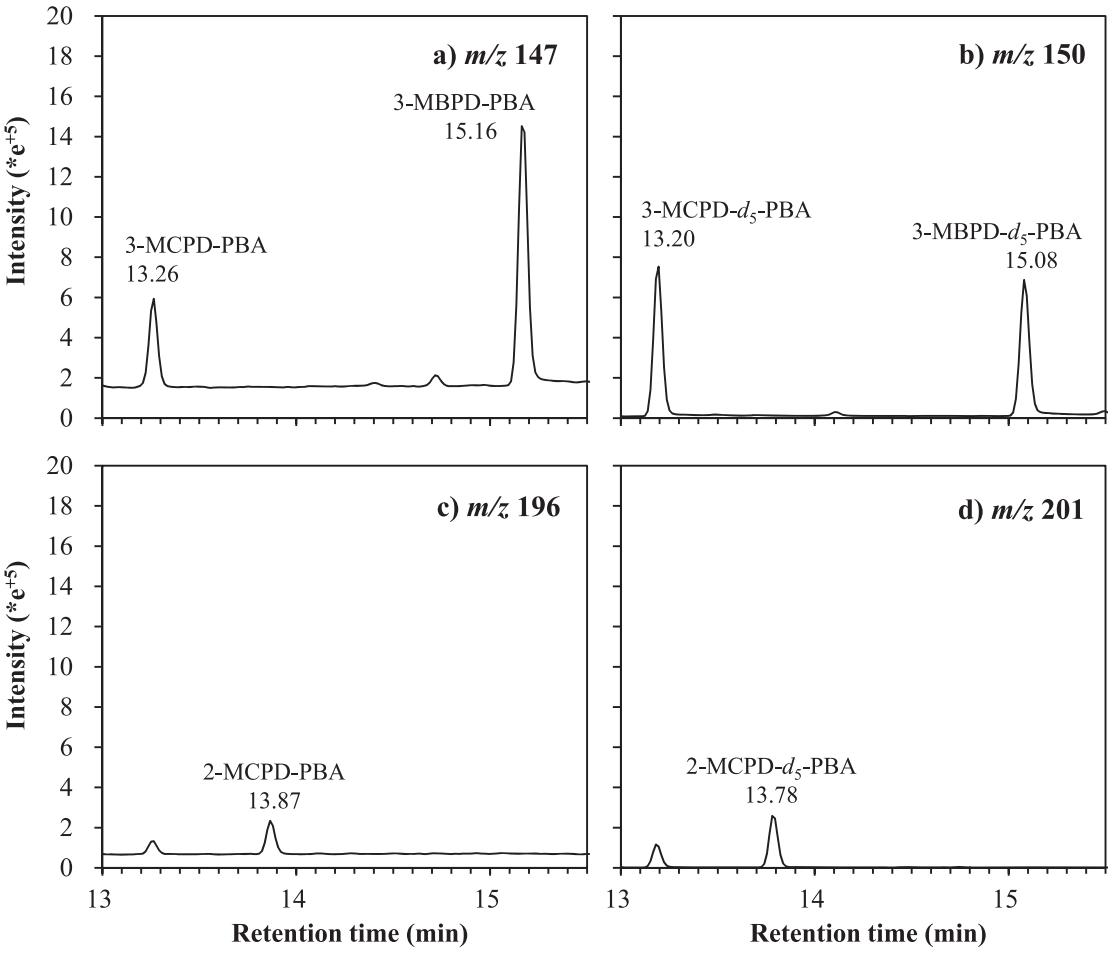

Fig. 3 GC-MS profiles of phenylboronic acid (PBA) derivatives resulting from solid palm oil sample (No. 2); a) 3-MCPD and 3-MBPD, b) 3-MCPD-d_5 and 3-MBPD-d_5, c) 2-MCPD, and d) 2-MCPD-d_5.

Table 3 Homogeneity of oil samples by one-way analysis of variance.

No	Oil		Parameter	3-MCPD	2-MCPD	Gly
1, 4	Rapeseed	(High)	Measured mean (mg/kg)[a)]	3.31	3.62	4.92
			P-value	0.61	0.22	0.27
6	Rice bran		Measured mean (mg/kg)[a)]	1.48	1.41	3.64
			P-value	0.15	0.19	0.37

a) A coordinator analyzed randomly taken six vials of samples in duplicate.

Table 4 Recovery from test sample shown in Table 1.

No	Oil		Recovery (%)		
			3-MCPD	2-MCPD	Gly
1,4	Rapeseed	(High)	102.6, 105.8	102.1, 106.5	106.5, 107.3
2	Palm	(Solid)	101.2, 103.9	105.4, 107.0	106.7, 104.7
5	Rapeseed	(Low)	102.6, 104.0	100.7, 100.0	104.0, 102.4
6	Rice bran		103.6, 113.7	103.4, 105.1	106.4, 105.0
7	Extra virgin olive	(for practice)	106.9, 108.2	95.1, 95.1	109.8, 109.2

Table 5a Statistical parameters by collaborative study obtained from 13 laboratories.

Laboratory	Entry	Equiv. conc. (mg/kg)					
		No.1, Rapeseed (High)			No.2, Palm (Solid)		
		3-MCPD	2-MCPD	Gly	3-MCPD	2-MCPD	Gly
A	1	3.28	3.94	5.11	3.28	1.82	2.13
	2	3.46	4.01	5.10	3.23	1.87	2.19
B	1	3.28	3.77	4.99	3.31	1.83	2.13
	2	3.43	3.84	5.07	3.42	1.80	2.11
C	1	1.89*	3.07	4.75	1.34	1.35*	1.81
	2	2.52*	3.35	4.65	2.00	1.59*	1.78
D	1	3.96	4.04	5.05	3.04	1.91	2.18
	2	3.78	4.05	5.39	2.91	1.88	2.21
E	1	3.56	4.10	4.70	3.04	1.88	2.00
	2	3.57	4.28	4.77	3.35	1.97	1.87
F	1	3.42	3.85	5.34	2.70	1.80	2.04
	2	3.50	4.02	5.63	2.63	1.78	2.20
G	1	3.63	4.09	5.29	3.64	2.08	2.29
	2	3.80	3.99	5.39	3.93	2.10	2.36
H	1	3.51	3.77	4.94	3.15	1.74	1.95
	2	3.51	3.73	4.92	3.18	1.74	1.95
I	1	3.55	3.91	4.99	3.25	1.80	2.06
	2	3.50	3.90	4.90	3.24	1.85	2.03
J	1	3.54*	3.69	4.79	3.36	2.38*	2.90*
	2	3.02*	3.59	5.30	2.45	1.64*	2.12*
K	1	3.53	3.77	4.89	3.06	1.70	1.85
	2	3.53	3.90	4.65	3.18	1.69	1.83
L	1	3.14	3.54	4.99	3.73	1.69	2.04
	2	3.26	3.66	5.43	3.41	1.75	2.20
M	1	3.12	4.78	5.01	2.54	2.18	2.05
	2	3.13	4.74	4.84	2.87	2.24	2.05
Number of laboratories retained after elimination of outliers		11	13	13	13	11	12
Mean		3.48	3.90	5.03	3.05	1.87	2.06
Repeatability							
Standard deviation (SD_r)		0.08	0.09	0.17	0.26	0.03	0.06
Relative standard deviation (RSD_r)		2.31	2.32	3.44	8.38	1.77	2.77
Reproducibility							
Standard deviation (SD_R)		0.22	0.36	0.27	0.55	0.16	0.16
Relative standard deviation (RSD_R)		6.35	9.31	5.28	18.03	8.55	7.65
Horwitz ratio		0.48	0.71	0.42	1.33	0.59	0.53

*) Outlier data as determined by the Cochran test. Dixon test revealed no outlier data.

Table 5b Statistical parameters by collaborative study obtained from 13 laboratories.

Laboratory	Entry	Equiv. conc. (mg/kg)					
		No.3, Palm (Liquid)			No.4, Rapeseed (High)		
		3-MCPD	2-MCPD	Gly	3-MCPD	2-MCPD	Gly
A	1	2.92	1.80	2.25	3.17	3.76	5.05
	2	3.04	1.82	2.31	3.00	3.67	4.85
B	1	3.12	1.79	2.33	3.41	3.71	4.99
	2	2.97	1.66	2.30	3.47	3.71	5.05
C	1	1.91*	1.44*	1.99	2.34	3.13	4.92
	2	3.34*	1.79*	1.86	2.19	3.27	4.78
D	1	3.46	1.90	2.58	3.52	3.95	5.13
	2	3.46	1.81	2.49	3.57	3.98	5.31
E	1	3.30	1.94	2.04	3.59	4.29	4.64
	2	3.24	1.93	2.07	3.56	4.18	4.68
F	1	2.90	1.70	2.24	3.31	3.96	4.97
	2	3.28	1.84	2.35	3.36	3.89	5.12
G	1	3.53	1.96	2.45	3.70	4.00	5.32
	2	3.37	1.97	2.40	3.87	4.10	5.39
H	1	3.04	1.64	2.12	3.46	3.73	5.06
	2	3.16	1.68	2.22	3.47	3.79	5.06
I	1	3.17	1.75	2.25	3.45	3.87	4.89
	2	3.19	1.75	2.21	3.58	3.80	4.96
J	1	3.21*	1.76*	1.91*	3.51	3.72	4.82
	2	2.39*	1.28*	1.58*	3.57	3.76	4.41
K	1	3.25	1.66	2.21	3.55	3.76	4.71
	2	3.33	1.70	2.24	3.40	3.74	4.60
L	1	3.27	1.38	2.29	3.44	3.46	5.64
	2	3.45	1.46	2.29	3.45	3.22	5.62
M	1	2.98	2.20	2.29	3.14	4.60	4.95
	2	2.98	2.27	2.27	2.83	4.44	4.89
Number of laboratories retained after elimination of outliers		11	11	12	13	13	13
Mean		3.20	1.80	2.25	3.34	3.83	4.99
Repeatability							
Standard deviation (SD_r)		0.11	0.05	0.05	0.09	0.08	0.11
Relative standard deviation (RSD_r)		3.42	2.93	2.21	2.79	2.01	2.20
Reproducibility							
Standard deviation (SD_R)		0.19	0.21	0.16	0.39	0.35	0.30
Relative standard deviation (RSD_R)		6.03	11.66	7.18	11.68	9.02	5.98
Horwitz ratio		0.45	0.80	0.51	0.88	0.69	0.48

*) Outlier data as determined by the Cochran test. Dixon test revealed no outlier data.

Table 5c Statistical parameters by collaborative study obtained from 13 laboratories.

Laboratory	Entry	Equiv. conc. (mg/kg)					
		No.5, Rapeseed (Low)			No.6, Rice bran		
		3-MCPD	2-MCPD	Gly	3-MCPD	2-MCPD	Gly
A	1	0.57	0.67	0.90	1.36	1.42	3.69
	2	0.56	0.68	0.91	1.34	1.44	3.86
B	1	0.58	0.57	0.89	1.38	1.38	3.85
	2	0.56	0.57	0.91	1.32	1.32	3.88
C	1	0.24*	0.46*	0.77	0.82*	1.22	3.50
	2	0.94*	0.70*	0.75	1.80*	1.36	3.54
D	1	0.63	0.65	1.01	1.29	1.36	3.86
	2	0.61	0.66	0.97	1.42	1.44	3.98
E	1	0.58	0.66	0.83	1.36	1.52	3.53
	2	0.55	0.58	0.78	1.34	1.56	3.29
F	1	0.56	0.67	0.74*	1.36	1.37	3.71
	2	0.55	0.68	0.91*	1.32	1.36	3.83
G	1	0.83	0.75	0.93	1.40	1.27	3.45
	2	0.84	0.73	0.96	1.58	1.36	3.70
H	1	0.62	0.61	0.84	1.36	1.32	3.83
	2	0.62	0.59	0.83	1.36	1.29	3.59
I	1	0.59	0.59	0.83	1.33	1.30	3.73
	2	0.58	0.57	0.83	1.35	1.31	3.69
J	1	0.60*	0.57	0.81*	1.40	1.25	3.34
	2	1.48*	0.61	0.95*	1.38	1.27	3.42
K	1	0.56	0.56	0.85	1.36	1.27	3.69
	2	0.54	0.57	0.85	1.35	1.30	3.51
L	1	0.76	0.56	0.89	1.61	1.24	3.56
	2	0.81	0.51	0.86	1.77	1.36	3.86
M	1	0.45	0.62	0.86	1.23	1.62	3.76
	2	0.46	0.63	0.85	1.22	1.58	3.77
Number of laboratories retained after elimination of outliers		11	12	11	12	13	13
Mean		0.61	0.62	0.87	1.38	1.37	3.67
Repeatability							
Standard deviation (SD_r)		0.02	0.02	0.02	0.06	0.05	0.12
Relative standard deviation (RSD_r)		2.62	3.41	1.89	4.23	3.41	3.23
Reproducibility							
Standard deviation (SD_R)		0.11	0.06	0.07	0.12	0.11	0.18
Relative standard deviation (RSD_R)		18.03	9.73	7.49	8.74	7.93	5.02
Horwitz ratio		1.05	0.57	0.46	0.57	0.52	0.38

*) Outlier data as determined by the Cochran test. Dixon test revealed no outlier data.

2-MCPD, and Gly at low concentrations in various oil samples. The limit of quantification (LOQ) was calculated as LOQ = 10σ/S, where σ is the residual standard deviation and S is the slope of the calibration curve. The LOQ values from participating laboratories were calculated as 1–46 ng (14 ng average) for 3-MCPD, 3–59 ng (14 ng average) for 2-MCPD, and 1–41 ng (6 ng average) for Gly. The highest LOQ for 2-MCPD, 59 ng, corresponded with 0.59 mg/kg in a 0.1 g oil sample. The lowest concentration found in all oil samples was 0.62 mg/kg for 2-MCPD in the rapeseed oil sample (No. 5). Therefore, all three analytes in all oil samples were considered to be within the LOQ.

Participating laboratories used their own equipment and instruments for the analysis. In a previous report, we described the importance in the conditions of the high-speed shaker used for ester cleavage and solvent extraction. Participating laboratories were asked to choose one of the three sets of conditions regarding the mode and speed of shaking, and the test tube mounting angle, as shown in Table 2. Although there were considerable variations in high-speed shakers and GC-MS systems used, including the capillary columns, the RSD_R values were all within the acceptable range. Thus, the current method can be used for rapid quantitative analysis of esters of 3-MCPD, 2-MCPD, and Gly in edible oils and fats by laboratories equipped with standard analytical instruments.

CONCLUSION

A collaborative study was conducted to evaluate an indirect enzymatic method for the analysis of fatty acid esters of 3-monochloro-1,2-propanediol (3-MCPD), 2-monochloro-1,3-propanediol (2-MCPD), and glycidol (Gly) in edible oils and fats. Thirteen laboratories analyzed liquid palm, solid palm, rapeseed, and rice bran oils containing 3-MCPD, 2-MCPD, and Gly at 0.6–5.0 mg/kg levels as free-form equivalents. The repeatability (RSD_r) were < 5% for the five liquid oil samples and 8% for the solid oil sample. The reproducibility (RSD_R) ranged from 5% to 18% for all oil samples, which were considered satisfactory because the Horwitz ratios were ≤ 1.3% for all three analytes in all oil samples. This method is applicable to the quantification of 3-MCPD, 2-MCPD, and Gly esters in edible oils.

ACKNOWLEDGEMENT

This study was carried out under the guidance of the committee of the of JOCS standard methods. We also thank all the participants in this study for acquiring data.

References

1) Velišek, J.; Davidek, J.; Hajšlová, J.; Kubelka, V.; Janiček, G.; Mánková, B. Chlorohydrins in protein hydrolysates.Z. Lebensm. *Unters Forsch.* **167**, 241-244 (1978).
2) Cordex, Code of Practice for the Reduction of 3-Monochloropropane-1,2-diol (3-MCPD) during the Production of Acid-Hydrolyzed Vegetable Protein (Acid-HVPs) and Products that Contain Acid- HVPs. CAC/RCP, **64** (2008).
3) Svejkovská, B.; Novotný, O.; Divinová, V.; Réblová, Z.; Doležal, M.; Velíšek, J. Esters of 3-chloropropane-1,2-diol in foodstuffs. *Czech J. Food Sci.* **22**, 190-196 (2004).
4) Zelinková, Z.; Svejkovská, B.; Velíšek, J.; Doležal, M. Fatty acid esters of 3-chloropropane-1,2-diol in edible oils. *Food Addit. Contam.* **23**, 1290-1298 (2006).
5) Weißhaar, R. Determination of total 3-chloropropane-1,2-diol (3-MCPD) in edible oils by cleavage of MCPD esters with sodium methoxide. *Eur. J. Lipid Sci. Technol.* **110**, 183-186 (2008).
6) Weißhaar, R. ILSI Europe Workshop (2009). http://www.ilsi.org/Europe/Documents/E2009MCPD-7.pdf.
7) Weißhaar, R. Fatty acid esters of glycidol in refined fats and oils. *Eur. J. Lipid Sci. Technol.* **112**, 158-165 (2010).
8) Masukawa, Y.; Shiro, H.; Nakamura, S.; Kondo, N.; Ooi, N.; Kudo, N. A new analytical method for the quantification of glycidol fatty acid esters in edible oils. *J. Oleo Sci.* **59**, 81-83 (2010).
9) The Japanese Food Safety Commission (2015). http://www.fsc.go.jp/sonota/dag/dag_index.html.
10) Ministry of Agriculture, Forestry and Fisheries of Japan. http://www.maff.go.jp/j/press/syouan/seisaku/pdf/141217-02.pdf.
11) MacMahon, S.; Begleya, T. H.; Diachenko, G. W. Occurrence of 3-MCPD and glycidyl esters in edible oils in the United States. *Food Addit. Contam. A* **30**, 2081-2092 (2013).
12) Crews, C.; Chiodini, A.; Granvogl, M.; Hamlet, C.; Hrnčiřík, K.; Kuhlmann, J.; Lampen, A.; Scholz, G.; Weißhaar, R.; Wenzl, T.; Jasti, P. R.; Seefelder, W. Analytical approaches for MCPD esters and glycidyl esters in food and biological samples: a review and future perspectives. *Food Addit. Contam. A* **30**, 11-45 (2012).
13) *JOCS Standard Method for the Analysis of Fats, Oils and Related Materials* (Japan Oil Chem. Soc. ed.), 2.4.13-2013 (2013).
14) *Joint AOCS/JOCS Official Method* (Am. Oil Chem. Soc. ed.), Cd 28-10 (2013).
15) Yamazaki, K.; Ogiso, M.; Isagawa, S.; Urushiyama, T.; Ukena, T.; Kibune, N. A new, direct analytical method using LC-MS/MS for fatty acid esters of 3-chloro-1,2-

propanediol(3-MCPD esters)in edible oils. *Food Addit. Contam. A* **30**, 52-68(2013).
16) Hori, K.; Koriyama, N.; Omori, H.; Kuriyama, M.; Arishima, T.; Tsumura, K. Simultaneous determination of 3-MCPD fatty acid esters and glycidol fatty acid esters in edible oils using liquid chromatography time-of-flight mass spectrometry. *LWT Food Sci. Technol.* **48**, 204-208(2012).
17) Hori, K.; Matsubara, A.; Uchikata, T.; Tsumura, K.; Fukusaki, E.; Bamb T. High-throughput and sensitive analysis of 3-monochloropropane-1,2-diol fatty acid esters in edible oils by supercritical fluid chromatography/tandem mass spectrometry. *J. Chrom. A.* **1250**, 99-104(2012).
18) *AOCS Official Method*(Am. Oil Chem. Soc. ed.), Cd 29a(2013).
19) *AOCS Official Method*(Am. Oil Chem. Soc. ed.), Cd 29b(2013).
20) *AOCS Official Method*(Am. Oil Chem. Soc. ed.), Cd 29c(2013).
21) Miyazaki, K.; Koyama, K.; Sasako, H.; Hirao, T. Indirect Method for Simultaneous Determinations of 3-Chloro-1,2-Propanediol Fatty Acid Esters and Glycidyl Fatty Acid Esters. *J. Am. Oil Chem. Soc.* **89**, 1403-1407(2012).
22) Koyama, K.; Miyazaki, K.; Abe, K.; Ikuta, K.; Egawa, Y.; Kitta, T.; Kido, H.; Sano, T.; Takahashi, Y.; Nezu, T.; Nohara, H.; Miyashita, T.; Yada, H.; Yamazaki, K.; Watanabe, Y. Optimization of an Indirect Enzymatic Method for the Simultaneous Analysis of 3-MCPD, 2-MCPD, and Glycidyl Esters in Edible Oils. *J. Oleo Sci.* **64**, 1057-1064(2015).
23) Collison, M. W. Current status of MCPD and Glycidol Analysis in Oils. in *104th AOCS Annual Meeting*, Montreal, Canada, Presentation(2013).
24) AOAC Int., Appendix D. Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis. in *Official Methods of Analysis of AOAC Int. 18 ed*. Gaithersburg, USA(2005).
25) Thompson, M. Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing. *Analyst* **125**, 385-386(2000).