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

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Abstract: We developed a novel, 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. Using this method, the ester analytes were rapidly cleaved by *Candida rugosa* lipase at room temperature for 0.5 h. As a result of the simultaneous hydrolysis and bromination steps, 3-MCPD esters, 2-MCPD esters, and glycidyl esters were converted to free 3-MCPD, 2-MCPD, and 3-monobromo-1,2-propanediol (3-MBPD), respectively. After the addition of internal standards, the mixtures were washed with hexane, derivatized with phenylboronic acid, and analyzed by gas chromatography-mass spectrometer (GC-MS). The analytical method was evaluated in preliminary and feasibility studies performed by 13 laboratories. The preliminary study from 4 laboratories showed the reproducibility (RSD_R) of < 10% and recoveries in the range of 102–111% for the spiked 3-MCPD and 2-MCPD in extra virgin olive (EVO) oil, semi-solid palm oil, and solid palm oil. However, the RSD_R and recoveries of Gly in the palm oil samples were not satisfactory. The Gly content of refrigerated palm oil samples decreased whereas the samples at room temperature were stable for three months, and this may be due to the depletion of Gly during cold storage. The feasibility studies performed by all 13 laboratories were conducted based on modifications of the shaking conditions for ester cleavage, the conditions of Gly bromination, and the removal of gel formed by residual lipase. Satisfactory RSD_R were obtained for EVO oil samples spiked with standard esters (4.4% for 3-MCPD, 11.2% for 2-MCPD, and 6.6% for Gly).

Key words: indirect enzymatic method, lipase, 3-MCPD, 2-MCPD, glycidol

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1 INTRODUCTION

In the 1970s, the presence of free 3-monochloro-1,2-propanediol (3-MCPD) was reported in food containing acid-hydrolyzed vegetable protein and in the 1980s, 3-MCPD fatty acid esters (3-MCPD-Es) were found in food, albeit in trace amounts. The reports of Weisshaar (Germany) on the presence of 3-MCPD-Es (2007) and glycidol fatty acid esters (Gly-Es) (2009) in edible oils raised much concern in Europe^{1, 2)} because the hydrolysis of 3-MCPD-Es and Gly-Es in the body could release free 3-MCPD and glycidol (Gly). A number of studies on the dietary exposure to 3-MCPD-Es and Gly-Es, along with their analytical methods, have been conducted^{3, 4)} but their toxicological consequences are not fully understood.

Direct and indirect methods for the analysis of 3-MCPD-Es, 2-monochloro-1,3-propanediol fatty acid esters (2-MCPD-Es), and Gly-Es in edible oils have been developed. In a direct analytical method, the sample is purified by solid-phase extraction and the individual analytes are then measured by liquid chromatography-mass spectrometer (LC-MS). Through a joint study by Japan Oil Chemists' Society (JOCS) and American Oil Chemists' Society (AOCS), a direct analytical method for Gly-Es was registered as the JOCS Standard Method for the Analysis of Fats, Oils, and Related Materials 2.4.13₂₀₁₃^{5, 6)}. Meanwhile, a direct method for the analysis of 3-MCPD-Es has been reported by Yamazaki *et al.*⁷⁾ and Hori *et al.*⁸⁾. These direct analytical techniques are suitable for the toxicological assessment of individual compounds and the elucidation of their formation mechanism; however, the preparation of numerous standard reagents is required.

Using indirect analytical methods, an individual ester analyte is cleaved, the glycerol skeleton is derivatized, and then analyzed by gas chromatography-mass spectrometer (GC-MS). These methods have the advantage of providing the equivalent concentrations of 3-MCPD, 2-MCPD, and Gly by using fewer standard reagents. In 2013, AOCS registered Cd 29a, Cd 29b, and Cd 29c as indirect analytical methods, which use different ester cleavage conditions^{9–11)}. Cd 29a involves ester hydrolysis under acidic conditions, whereas in Cd 29b, the hydrolysis is performed under basic conditions. In both cases, however, long reaction times are required. In Cd 29c, the ester cleavage is carried out under alkaline conditions with short reaction times; however, this technique is unable to quantify 2-MCPD. Thus, there is a need for a rapid indirect analytical method that can quantify 3-MCPD, 2-MCPD, and Gly simultaneously.

Miyazaki *et al.* developed an analytical method based on the use of lipase for the cleavage of ester analytes in 0.5 h¹²⁾. To validate this method, we organized an MCPD subcommittee consisting of 13 laboratories in JOCS, with 4 of these laboratories conducting a preliminary study to identify possible rectifications in sample preparation and analysis procedures. As discussed in this report, we obtained

good reproducibility from the subsequent feasibility study that was conducted by all 13 laboratories, based on an improved method resulting from the preliminary study.

2 EXPERIMENTAL PROCEDURES

2.1 Reagents

For the ester standard reagents added to the oil samples, which were distributed to the participating laboratories, glycidyl oleate was purchased from Wako Pure Chemical (Japan), whereas 3-MCPD dioleate and 2-MCPD dilinoleate were purchased from Toronto Research Chemicals (Canada). As for the free standard reagents, 3-MCPD and 3-MBPD were purchased from Wako Pure Chemical, 3-MCPD-*d*₅ from CDN Isotopes (Canada), 2-MCPD, 2-MCPD-*d*₅, and 3-MBPD-*d*₅ from Toronto Research Chemicals, and Lipase AYS Amano (derived from *Candida rugosa*) from Wako Pure Chemical. They were distributed to all the laboratories in the subcommittee. Phenylboronic acid (PBA) (purity ≥ 97%) as well as special grade organic solvents and salts were purchased by the respective participating laboratories.

A 30% (w/v) sodium bromide solution was adjusted to pH 5.0 with citric acid and disodium hydrogen phosphate. A sodium bromide solution containing 90 U/mL lipase was prepared by dissolving 3,600 U of *Candida rugosa* lipase in the 30% sodium bromide solution. A PBA solution was prepared by dissolving 1.25 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 2-MCPD, 3-MCPD, and 3-MBPD, whereas 200 µg/mL ethanol solutions were prepared for 2-MCPD-*d*₅, 3-MCPD-*d*₅, and 3-MBPD-*d*₅ as internal standard stock solutions. On the day of the study, each standard stock solution was diluted with 30% sodium bromide aqueous solution to prepare 15 µg/mL standard mixed solutions I and 1.5 µg/mL standard mixed solutions II. Additionally, 2.0 µg/mL internal standard mixed solutions were prepared by diluting each of the internal standard stock solutions with ethanol.

2.2 Sample preparation

The oil samples were commercially available extra virgin olive (EVO) oil and palm oil (semi-solid and solid) at room temperature. Each oil was melted and added to the ester standard reagents. After undergoing sufficient agitation, they were dispensed into glass vials to obtain the homogeneously distributed oil samples (Table 1). The homogeneity of the distributed samples was confirmed by the coordinator (corresponding author). Oil samples Nos. 1–4 used in the preliminary study were preserved in the dark and refrigerated. Oil sample No. 5 used in the feasibility study was preserved in the dark at room temperature.

Table 1 Test samples of preliminary and feasibility studies. Samples Nos. 2–5 were spiked with 3–4 mg/kg standards as free form equivalent concentration.

No	Study	Oil	Spiked standards		Storage temp.
			Indication	Compound	
1	Preliminary	Extra virgin olive ^{a)}	Unindicated	Blank	Refrigeration (5°C)
2		Extra virgin olive ^{a)}		3-MCPD dioleate, 2-MCPD dilinoleate, and Gly oleate	
3		Palm (Semi-solid) ^{b)}		Gly oleate	
4		Palm (Solid) ^{c)}		3-MCPD dioleate and 2-MCPD dilinoleate	
5	Feasibility	Extra virgin olive ^{a)}	Indicated	3-MCPD dioleate, 2-MCPD dilinoleate, and Gly oleate	Room (25°C)

Iodine values (g/100 g) : a) 81.5, b) 51.8, and c) 37.5.

2.3 Equipment

A temperature-controlled water bath, a vortex mixer, a high-speed shaker, a centrifuge, a nitrogen gas sprayer, and a GC-MS were used for each laboratory. A typical running conditions were as follow: water bath, DH-12 (Taitec, Japan); vortex mixer, HM-10H (AS ONE, Japan); high-speed shaker, CM-1000 (EYELA, Japan); centrifuge, Model 4000 (Kubota, Japan); nitrogen gas sprayer, EN-10/DTU-1BN (Taitec, Japan); GC-MS, 5975 series GC/MSD (Agilent, USA).

2.4 Procedure of oil sample and calibration curve

The method developed by Miyazaki *et al.*¹²⁾ was modified and illustrated in Fig. 1. In a 10 mL test tube, 0.1 g of oil sample was dissolved in 200–500 μ L of isooctane. After adding 3 mL of 30% NaBr aqueous solution containing 90 U/mL lipase, the mixture was shaken by a high-speed shaker for 30 min at room temperature (25°C, ester cleavage). The test tube was heated for 10 min in an 80°C water bath (bromination from Gly to 3-MBPD; in the preliminary study, heating was performed during PBA derivatization). The test tube was cooled to room temperature, and 50 μ L of 2.0 μ g/mL internal standard mixed solution was added. Next, 3 mL of hexane was added and shaken for 10 min. The resulting aqueous layer was transferred to a new test tube. Another 3 mL of hexane was added and the tube was shaken for 10 sec; the resulting hexane layer was subsequently removed. A 20 μ L PBA solution was added to the aqueous layer, and the mixture was agitated with a vortex mixer for 10 sec. Next, 3 mL of hexane was added and shaken for 10 sec. The resulting organic layer was transferred to a new test tube and concentrated to approximately 0.5–0.8 mL using a stream of nitrogen. After filtration through a 0.2 μ m membrane filter, the samples were subjected to GC-MS analysis.

To prepare the calibration curve solutions, the 1.5 μ g/mL

standard mixed solutions II were transferred in quantities of 5, 20, 50, and 100 μ L, whereas the 15 μ g/mL standard mixed solutions I were transferred in quantities of 20, 35, 50, and 70 μ L, to 10 mL test tubes (resulting in 0.0075, 0.030, 0.075, 0.15, 0.30, 0.53, 0.75, and 1.1 μ g of material in the respective test tubes). A mixture of 50 μ L of 2.0 μ g/mL internal standard mixed solution and 3 mL of 30% NaBr solution were added to these test tubes. PBA derivatization was performed as described above.

2.5 GC-MS Measurement

A GC capillary column with length 30 m, internal diameter 0.25 mm, and film thickness 0.25 μ m was used. Each laboratory individually decided on their preferred liquid phase of film from VF-5 or VF-17. The optional use of a guard column and a programmed temperature vaporizer (PTV) function in the inlet depended on the participating laboratory. Helium served as a carrier gas at a fixed flow rate of 1.2 mL/min. The sample injection was performed in the splitless mode at a temperature of 250°C. The column oven temperature was held at 60°C for 1 min, increased at 10°C/min to 60–150°C, followed by 3°C/min to 150–180°C, and subsequently 30°C/min to 180–300°C, and finally held at 300°C for 8 min (total 32 min). MS was performed in the positive electron ionization mode with an ion source temperature of 230°C. 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, along with 150 and 245 for the 3-MBPD- d_5 derivative.

2.6 Collaborative Studies

The preliminary study was conducted to identify possible rectifications in sample preparation and analysis proce-

Fig. 1 Procedure flow of this analytical method. Heating procedure: (i) in modified procedure (feasibility study), (ii) original procedure (preliminary study).

dures. Sample Nos. 1–4 in Table 1 were distributed to 4 laboratories and analyzed twice by each laboratory. The problems observed in the preliminary study were corrected prior to the feasibility study. Sample No. 5 in Table 1 was distributed to 13 laboratories and analyzed twice by each laboratory.

Table 2 lists the high-speed shakers used by the participating laboratories as described in the procedures of Section 2.4: (i) horizontal eccentric shaker, (ii) vertical shaker with vertically placed test tubes, and (iii) horizontal shaker with test tubes tilted 60° from a horizontal plane.

3 RESULTS AND DISCUSSION

3.1 Preliminary Study

3-MCPD, 2-MCPD, and Gly of EVO oil and palm oil samples were determined using the original procedure (Fig. 1(ii)). Table 3 lists the results and the statistical parameters obtained by 4 laboratories. Blank EVO oil (sample No.

1) was omitted because no 3-MCPD, 2-MCPD, or Gly was detected. The relative standard deviations for inter-laboratory reproducibility (RSD_R) of 3-MCPD, 2-MCPD, and Gly in EVO (sample No. 2), and of 3-MCPD and 2-MCPD in semi-solid palm oil (sample No. 3), as well as solid palm oil (sample No. 4) were less than 10%. After excluding the aberrant values, the amount of data for samples Nos. 3 and 4 was limited. This is because sample No. 4 from laboratory C was eliminated by Cochran's test, while laboratory D did not report the results of samples Nos. 3 and 4. RSD_R value of Gly in semi-solid palm oil and solid palm oil were 79% and 13%, respectively. We speculated that the wide range of reproducibility value was caused by the instability of samples during storage and by sensitive procedures such as the shaking conditions for ester cleavage.

Recoveries for all analytes are shown in Table 4. The recoveries of 3-MCPD and 2-MCPD were found to be in the range of 102–111% for EVO oil, semi-solid palm oil, and solid palm oil. The indirect method is based on the cleavage of ester analytes into their corresponding free analytes.

Table 2 Conditions selected by 13 laboratories regarding of high-speed shaker of oil samples, isooctane, and the lipase solution.

	Shaking condition		Mounting angle (°) ^{a)}	Participant lab. code	
	Direction	Speed (rpm)		Preliminary study	Feasibility study
i	Eccentric orbit	1,800	90	A, B	A, B, E, G, J, L
ii	Vertical reciprocating	200	90	D	D, F, I, K, M
iii	Horizontal reciprocating	200 – 400	60	C	C, H

^{a)} Test tube angle from horizontal on shaker.

There were concerns about the poor dispersibility of solid oil samples in an aqueous solution at room temperature and consequently, an incomplete ester cleavage in such a system. Additionally, 3-MCPD diester is the most difficult substrate to cleave among all the ester analytes studied¹²⁾. These results showed that this indirect method can also be used in an oil/water system at room temperature for solid oils.

3.2 Optimization of Method

The four procedures cited in the preliminary study were modified. Firstly, the test tubes were shaken during the ester cleavage reaction, and it was necessary to specify the conditions used for each type of high-speed shaker. Since the liposoluble ester analyte was catalytically reacted with a lipase aqueous solution, vigorous shaking of the 10 mL test tubes was required. However, there was a concern regarding the possibility of insufficient agitation depending on the high-speed shakers' mechanism of action. For example, during horizontal reciprocating shaking (Table 2 (iii)), which was the slowest among the three mechanisms of action, the mixing of the test tube solutions was insufficient when the test tubes were tilted at 0°, 30°, and 90° (measured from a horizontal plane) whereas tilting at 60° gave a good result. Before performing the experiments, the participating laboratories were instructed to choose one of the three conditions shown in Table 2. In a multiple comparison test by Tukey's method, 3-MCPD exhibited a higher content by (iii) horizontal shaking (tilting at 60°) than by (i) horizontal eccentric shaking and (ii) vertical shaking, whereas 2-MCPD and Gly showed no difference in mean value with different shaking conditions.

Secondly, in order to accelerate the conversion of Gly to 3-MBPD, the reaction mixture was heated immediately after the ester cleavage in the presence of NaBr (Fig. 1 (i)), whereas the heating step was originally performed after the addition of PBA. In indirect methods, there is a common side reaction that occurs during the hydrolysis and bromination of 3-MCPD-Es; resulting in the formation of 3-MBPD (as an artifact) and the overestimation of Gly-Es¹³⁾. However, the formation of the side reaction was suppressed <1% using our modified method, consisting of

fewer reaction steps without removing the bromination step.

Thirdly, after the ester cleavage and bromination, unnecessary matter originating from the oil sample was removed together with the organic layer during hexane washing. In the preliminary study, the organic layer was removed by pipette, together with the gel originating from lipase, but many analysts considered this procedure cumbersome. Laboratory D could not analyze sample Nos. 3 and 4 (Table 3), because the GC-MS measurement sequence was discontinued due to protein precipitation (presumably lipase) in the glass liner of the injector. In order to solve this problem, the aqueous layer containing the free analyte was transferred to a separate test tube and this step was able to minimize the build-up of residual gel during PBA derivatization.

Fourthly, it was observed in all 4 laboratories that the baseline of the GC-MS spectrum extended beyond a retention time of 15 min after elution of the 3-MCPD derivative at m/z 147. However, although the standard deviation increased slightly, there was no difference in mean value according to a t-test. According to a separate total ion current chromatogram (TICC), these findings could be attributed to the formation of glycerol, a PBA derivative product, in the aqueous layer, which could decrease the functionality of the GC-MS system. This phenomenon is common among indirect analytical methods. We plan to include an additional dehydration step to reduce the formation of PBA derivative products in future studies.

3.3 Glycidol Depletion during the Storage of Oil Samples

In the preliminary study, large RSD_R of Gly were observed in palm oils (Table 3, Nos. 3 and 4). In a separate study conducted by a coordinator, the Gly content in refrigerated oil sample No. 3 decreased by 44% after 72 days, and by 36% in sample No. 4 after 70 days. We speculated that the wide range of reproducibility was caused by the cold storage of the palm oil samples as the storage period was different among the 4 laboratories and thus, conducted an additional low-temperature storage test of the palm oil samples.

Figure 2 shows the time course during which a solid

Table 3 Results and statistical parameters by preliminary study obtained from 4 laboratories; sample No. 1 was blank extra virgin olive oil, and was omitted because it was less than the limit of determination.

Statistical parameters	Lab. code	Repetition	Equiv. conc. (mg/kg)								
			No.2, Extra virgin olive			No.3, Palm (Semi-solid)			No.4, Palm (Solid)		
			3-MCPD	2-MCPD	Gly	3-MCPD	2-MCPD	Gly	3-MCPD	2-MCPD	Gly
	A	1	4.92	3.97	4.86	2.56	1.26	3.46	5.15	4.09	0.99
		2	4.92	3.98	4.83	2.66	1.18	3.59	5.15	4.06	1.01
	B	1	4.80	3.60	4.91	2.37	1.22	2.58	4.57	3.63	0.85
		2	4.47	3.62	4.76	2.29	1.20	2.59	4.43	3.63	0.82
	C	1	4.78	3.78	4.40	2.52	1.30	0.29	3.35	3.18	0.59
		2	4.86	3.82	4.54	2.71	1.25	0.23	5.05	4.32	0.93
	D	1	4.08	3.79	4.54	2.09	1.33	2.45	— ^{a)}	— ^{a)}	— ^{a)}
		2	3.85	3.70	4.83	— ^{a)}	— ^{a)}	— ^{a)}	— ^{a)}	— ^{a)}	— ^{a)}
Total number of laboratories in given assay			4	4	4	3	3	3	2	2	2
Total number of values in given assay			8	8	8	6	6	6	4	4	4
Mean			4.59	3.78	4.71	2.52	1.24	2.13	4.83	3.86	0.92
Repeatability standard deviation (SD _r)			0.14	0.03	0.13	0.09	0.04	0.06	0.07	0.01	0.02
Repeatability relative standard deviation (RSD _r)			3.14	0.89	2.66	3.70	3.11	2.67	1.39	0.31	2.13
Reproducibility standard deviation (SD _R)			0.44	0.15	0.20	0.18	0.04	1.68	0.46	0.31	0.12
Reproducibility relative standard deviation (RSD _R)			9.64	4.07	4.15	7.05	3.53	79.1	9.61	8.12	12.7
Repeatability value (r)			0.41	0.10	0.35	0.26	0.11	0.16	0.19	0.03	0.06
Reproducibility value (R)			1.25	0.44	0.55	0.50	0.12	4.76	1.31	0.89	0.33

^{a)} Not reported.**Table 4** Recovery obtained by the preliminary and feasibility studies; calculated from mean value of all laboratories relative to added quantity.

No	Study	Oil	Recovery (%)		
			3-MCPD	2-MCPD	Gly
2	Preliminary	Extra virgin olive	109	107	103
3		Palm (Semi-solid)	108	103	51
4		Palm (Solid)	111	102	64
5	Feasibility	Extra virgin olive	97	95	107

palm oil sample was preserved by a coordinator in the dark at frozen temperature (-18°C), refrigeration temperature (5°C), and room temperature (25°C). The Gly content under refrigeration decreased by 33% during 84 days, but did not decrease at frozen or room temperature during the same period. Refrigeration may have damaged the Gly-Es in the solid palm oil sample although the mechanism is currently unknown. Although not depicted in the diagram, 3-MCPD and 2-MCPD contents in the solid palm oil sample did not show a notable decrease at any preservation temperature. In the case of rapeseed oil, soybean oil, rice bran oil, and semi-solid palm oil, preservation temperatures did not affect the contents of Gly as well as 3-MCPD, and 2-MCPD. It was concluded that the oil samples should be delivered and preserved at room temperature in the dark.

3.4 Feasibility Study

3-MCPD, 2-MCPD, and Gly of an EVO oil sample were determined using the modified procedure. The results and the statistical parameters obtained from the feasibility study conducted across 13 laboratories are shown in **Tables 5** and **6**, respectively. RSD_R were 4.4% for 3-MCPD, 11.2% for 2-MCPD, and 6.6% for Gly. The result for Gly from laboratory G was eliminated by Cochran's test, whereas the data for 2-MCPD (though doubtful according to Dixon's test) was included in the overall results to give a high RSD_R. Although different conditions were used, these results were considerably better than the RSD_R values (9–30%) in the palm oil sample, with the same content measured by AOCS in a collaborative study using three indirect analytical methods¹⁴⁾. As shown in **Table 4**, sample

Fig. 2 Time course of Gly equivalent concentration in solid palm oil. Preservation temperature: ○ Frozen (-18°C), ◆ Refrigerated (5°C), and △ Room temperature (25°C). Error bars represent the SD of twice analyses.

No.5, the recoveries of 3-MCPD, 2-MCPD, and Gly were found to be in the range of 97–107% for an EVO oil. In this feasibility study, we can confirm that each participant had learned the procedure carefully, and previous problems with sample storage and analysis procedures had been rectified.

A multiple comparison test of GC-MS equipment manufacturers between Agilent, Shimadzu, and Thermo Scientific showed that only the mean value of 2-MCPD measured by an Agilent instrument was found to be lower than that measured by other instruments, whereas no difference was found in 3-MCPD or Gly levels.

The limit of quantification (LOQ) was defined as $\text{LOQ} = 7.2 \sigma/S$, where σ is the standard deviation from five analyses of a 7 ng analyte, and S is the slope of eight data points on the calibration curve. In a separate study performed by a coordinator, the LOQ were <2.1 ng for 3-MCPD, 2-MCPD, as well as Gly (<21 $\mu\text{g}/\text{kg}$ in the case of 0.1 g of oil samples). The LOQ obtained by this method, depending on the GC-MS's sensitivity, were inferentially the same as that in reported by AOCS^{9–11,14} and European Food Safety Authority (EFSA)¹⁵.

4 CONCLUSIONS

In this report, the preliminary and feasibility studies of an analytical protocol were conducted by a committee of 4 and 13 laboratories, respectively. We aim to verify the efficiency of an indirect, lipase-catalyzed ester cleavage

Table 5 Feasibility study results obtained from 13 laboratories.

Lab. code	Repetition	Equiv. conc. (mg/kg)		
		No.5, Extra virgin olive		
		3-MCPD	2-MCPD	Gly
A	1	3.84	3.74	4.80
	2	3.90	3.54	4.62
B	1	4.23	4.27	5.03
	2	3.88	4.01	4.91
C	1	4.56	3.74	4.92
	2	4.30	4.20	4.79
D	1	4.28	3.69	4.28
	2	4.21	3.65	4.32
E	1	4.20	3.46	4.47
	2	4.18	3.54	4.44
F	1	3.83	3.59	4.39
	2	3.96	3.61	4.66
G	1	3.94	2.65	— ^{a)}
	2	4.02	2.69	— ^{a)}
H	1	4.27	3.60	4.60
	2	4.18	3.54	4.51
I	1	3.99	3.67	4.49
	2	3.90	3.63	4.45
J	1	4.32	3.47	4.63
	2	4.00	3.27	4.36
K	1	4.09	3.75	4.76
	2	4.03	3.70	4.67
L	1	4.20	3.90	4.36
	2	4.22	3.80	4.27
M	1	4.05	4.23	5.15
	2	4.16	4.45	5.50

^{a)} Eliminated by Cochran's test.

method for the simultaneous analysis of 3-MCPD-Es, 2-MCPD-Es, and Gly-Es. In the preliminary study, the free equivalent concentrations of 3-MCPD and 2-MCPD in extra virgin olive oil, semi-solid palm oil, and solid palm oil were quantified. It was found that the cold storage of oil samples could affect the accuracy of quantification due to the instability of Gly in palm oil at refrigeration temperature (5°C). After rectifying the problems related to sample storage and analysis procedures, the feasibility study carried out by 13 laboratories presented excellent RSD_R values in extra virgin olive oil: 4.4%, 11.2%, and 6.6% for 3-MCPD, 2-MCPD, and Gly, respectively. We plan to apply this newly developed method to the determination of 3-MCPD, 2-MCPD, and Gly in various oil samples in future studies.

Table 6 Statistical parameters by feasibility study obtained from 13 laboratories.

Statistical parameters	No.5, Extra virgin olive		
	3-MCPD	2-MCPD	Gly
Total number of laboratories in given assay	13	13	12
Total number of values in given assay	26	26	24
Mean	4.11	3.67	4.64
Repeatability standard deviation (SD _r)	0.12	0.13	0.12
Repeatability relative standard deviation (RSD _r)	2.87	3.53	2.66
Reproducibility standard deviation (SD _R)	0.18	0.41	0.31
Reproducibility relative standard deviation (RSD _R)	4.37	11.2	6.62
Repeatability value (r)	0.33	0.37	0.35
Reproducibility value (R)	0.51	1.16	0.87

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