Development of Indirect Quantitative Methods for 3-MCPD Fatty Acid Esters (3-MCPDEs) and Glycidyl Fatty Acid Esters (GEs) in Thermally Processed Foodstuffs

Kinuko Miyazaki1*, Yoshiyuki Takagishi2, Kazuhiro Sakamoto2, Yukiko Yamada2, and Kazuo Koyama1

1 Research & Development Headquarters, House Foods Group Inc., 1-4, Takanodai, Yotsukaido, Chiba 284-0033, JAPAN
2 Ministry of Agriculture, Forestry and Fisheries, 1-2-1, Kasumigaseki, Chiyoda-ku, Tokyo 100-8950, JAPAN

Abstract: For indirect determination of 3-chloro-1,2-propanediol fatty acid esters (3-MCPDEs) and glycidyl fatty acid esters (GEs) in thermally processed foodstuffs distributed in Japan, we modified two published methods, an enzymatic method (later approved as JOCS Standard Method for the Analysis of Fats, Oils, and Related Materials 2.4.14-2016 and Joint JOCS/AOCS Official Method Cd 29d-19) and EFSA method developed by the Joint Research Centre of the European Commission. The performance of these methods was demonstrated to be satisfactory. The partially modified enzymatic method showed mean recoveries of 93.7–98.5% for 3-MCPDEs, 94.4–98.4% for GEs, and HorRat (r) values of 0.06–0.78 in analyses of 6 types of foods including Japanese specific foods (fried rice cracker, fried instant noodle, biscuit, karinto, vegetable tempura, and frozen deep-fried chicken) spiked with 3-MCPD dioleate and glycidyl oleate at 0.02–0.04 mg/kg or 0.2–0.4 mg/kg. The partially modified EFSA method showed mean recoveries of 96.6–99.4% for 3-MCPDEs, 95.7–100.1% for GEs, and HorRat (r) values of 0.14–1.05 in analyses of 5 types of foods (not including karinto) spiked simultaneously with 3-MCPD dioleate and glycidyl oleate at either 0.02–0.04 mg/kg or 0.2–0.4 mg/kg. The results of analyses of 9 samples (fried rice cracker, biscuit, 2 potato crisps, fried potato snack, baked cracker, cracker dough, seafood tempura, and frozen deep-fried chicken) using these 2 methods were comparable. The 95% confidence intervals determined with weighted Deming regression analysis between the results of 3-MCPDEs or GEs in the same samples analyzed by the 2 methods showed: the slope around 1 (3-MCPDEs, 0.982–1.025; GEs, 0.887–1.078); and intercept close to 0 (3-MCPDEs, –0.002–0.003; GEs, –0.011–0.015). These data confirmed that the concentrations of 3-MCPDEs and GEs in food samples determined by 2 independent analytical methods were equivalent.

Key words: 3-MCPDEs, GEs, lipase, enzymatic method, EFSA method

1 Introduction

3-Chloro-1,2-propanediol fatty acid esters (3-MCPDEs) and glycidyl fatty acid esters (GEs) occur unintentionally during the refining process of edible fats and oils1–2). 3-MCPDEs and GEs have been reported to be hydrolyzed in the gastrointestinal tract to form 3-chloro-1,3-propanediol (3-MCPD) and glycidol, respectively1–5). The free form of 3-MCPD was first identified in acid-hydrolyzed vegetable protein6) and showed adverse effects on kidneys and male reproductive organs of rats7). The free form of glycidol was classified in Group 2A, probable human carcinogen, by the International Agency for Research on Cancer (IARC)8). At its 83rd meeting in November 2016, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated 3-MCPDEs, 3-MCPD, GEs and glycidol and recommended that efforts to reduce 3-MCPDEs and 3-MCPD in infant formula be implemented and that measures to reduce GEs and glycidol in fats and oils continue, particularly when used in infant formula9). In 2018, the European Commission started the enforcement of maximum levels for GEs in edible vegetable fats and oils, infant formula, follow-on formula, and foods for special medical purposes intended for infants and young children10).

Concerns over the potential health risks of orally ingested 3-MCPDEs and GEs have prompted the development of quantitative methods for these compounds in edible fats
and oils. The analytical methods may be divided into direct and indirect methods. The direct methods determine each of individual fatty acid esters of 3-MCPD and glycidol separately using liquid chromatography mass spectrometry (LC-MS). The Japan Oil Chemists’ Society (JOCS) and the American Oil Chemists’ Society (AOCS) approved some direct methods for GEs as JOCS Standard Method for the Analysis of Fats, Oils, and Related Materials 2.4.13-2013 and the Joint AOCS/JOCS Official Method Cd 28-10. The Joint AOCS/JOCS Standard Method for the Analysis of Fats, Oils, and EDs, 2-MCPDEs and GEs, respectively, by hydrolysis with water or methanol. In order to analyze glycidol simultaneously with 3-MCPD and 2-MCPD, glycidol is brominated to cleave the epoxy ring and convert glycidol to 3-bromo-1,2-propanediol (3-MBPD). After derivatization with phenylboronic acid, resulting 3-MCPD, 2-MCPD and 3-MBPD derivatives are quantified using gas chromatography mass spectrometry (GC-MS). As the indirect methods are simpler and easier to perform and require only a limited number of expensive reference standards, they are well-suited for screening or routine analyses for quality control purposes. To date, numerous indirect methods have been approved as official methods: AOCS Official Methods Cd 29a-13, Cd 29b-13, and Cd 29c-13 for analyzing edible fats and oils, in which methanolysis of the ester forms to the free forms are achieved under the acid or alkaline conditions. Miyazaki et al. developed an indirect method employing lipase from Candida cylindracea (previously referred to as C. rugosa) for hydrolysis of the ester forms to the free forms. Although methanolysis in AOCS Cd 29a-13 and Cd 29b-13 took 16 hours, a new enzymatic hydrolysis can be completed within 0.5 hour. Following a collaborative study performed by JOCS, this enzymatic method for determining 3-MCPDEs and GEs in edible fats and oils (except for fish oils) was approved as JOCS Standard Method for the Analysis of Fats, Oils, and Related Materials 2.4.14-2016. Since then, the enzymatic method was improved to be also applicable to the analysis of fish oils and approved as JOCS Recommended Method for the Analysis of Fats, Oils, and Related Materials R7-2017. In 2019, these enzymatic methods were improved to be also applicable to the analysis of 3-MCPDEs and GEs in fats and oils in order to help the MAFF to determine whether measures need to be taken to reduce 3-MCPDEs and/or GEs in foods.

The authors evaluated whether the enzymatic hydrolysis method without modification is applicable to the analysis of 6 foods commonly distributed in Japan, by comparing the performance of the 2 methods: the method developed by Miyazaki et al. and quoted by JOCS Standard Method 2.4.14-2016 (an enzymatic method), and the method reported by the European Food Safety Authority (EFSA) in 2015 and 2017 (the 2015 EFSA method and the 2017 EFSA method). Where the methods were found not applicable to one of the foods, we investigated the reason for non-applicability and, as appropriate, the analytical procedures were modified. Then the modified methods were validated for the commodity concerned.

2 Experimental Procedures

2.1 Reagents and preparation of reagents

2.1.1 Reagents

The ester-form standard reagents, 3-MCPD dioleate, 3-MCPD dipalmitate, glycidyl oleate, glycidyl palmitate and 3-MCPD dipalmitate-d5, were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan); and glycidyl oleate-d5 was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). The free-form standard reagents, 3-MCPD and 3-MBPD, were purchased from FUJIFILM Wako Pure Chemical Corporation; 3-MCPD-d5 was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada), and 3-MBPD-d5 was purchased from FUJIFILM Wako Pure Chemical Corporation and Tokyo Research Chemicals. Phenylboronic acid (PBA) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Sulphuric acid and citric acid were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Sodium bromide, disodium hydrogen phosphate dodecahydrate, tert-butyl methyl ether, ethanol, isooctane (2,2,4-trimethyl pentane), hexane, sodium sulfate, ethyl acetate, anhydrous tetrahydrofuran, methanol, toluene, diethyl ether, sodium hydrogen carbonate, ammonium sulfate and Lipase AYS Amano from Candida cylindracea (C. rugosa) were purchased from FUJIFILM Wako Pure Chemical Corporation. One unit of lipase was defined as generating 1 μmol of fatty acid in one minute at 37°C and pH 7.0 under the conditions of the fat digestion test described in the section ‘General Tests’ of the Japanese Pharmacopoeia.

2.1.2 Preparation of reagents for the enzymatic method

Sodium bromide solution (30%, w/v) [reagent 2.1.2.1]
was prepared by dissolving 30 g sodium bromide in 80 mL ultrapure water, and then diluting with ultrapure water to a final volume of 100 mL after adjusting the pH to 5.0 with 1 mM citric acid solution and 1 mM disodium hydrogen phosphate solution. Sodium bromide solution containing C. cylindracea lipase [reagent 2.1.2.1] was prepared by dissolving 3,600 U of C. cylindracea lipase (Lipase AYS amano) in 40 mL of 30% (w/v) sodium bromide solution [reagent 2.1.2.1], sufficient amount for the analysis of 10 test samples. For the analysis of fats and oils containing short- or and medium-chain fatty acids, 7,200 U of the lipase was used. PBA solution [reagent 2.1.2.2] for the enzymatic method was prepared by dissolving 0.25 g PBA in 10 mL water/acetone (1:19, v/v). For preparing the internal standards, 200 μg/mL stock solutions were prepared by dissolving either 3-MCPD-d₅ or 3-MBPD-d₅ in ethanol. On each day of analysis, 2 μg/mL internal standard mix [reagent 2.1.2.4] was prepared by mixing an equal volume of each internal standard stock solution and diluting the mixture with 30% (w/v) sodium bromide solution. For the standards, 300 μg/mL stock solutions were prepared by dissolving either 3-MCPD or 3-MBPD in ethanol. On each day of analysis, 15 μg/mL standard mix I [reagent 2.1.2.5] and 1.5 μg/mL standard mix II [reagent 2.1.2.6] were prepared by mixing an equal volume of each of 300 μg/mL standard stock solutions and diluting the mixture with 30% (w/v) sodium bromide solution.

2.1.3 Preparation of reagents for the EFSA method

On each day of analysis, 3 mg/mL acidic aqueous solution of sodium bromide [reagent 2.1.3.1] was prepared as follows: (1) a concentrated aqueous solution was prepared by dissolving 1 g sodium bromide in 10 mL of ultrapure water; and (2) 0.18 mL of the concentrated solution, 5.5 mL of ultrapure water, and 0.3 mL of sulfuric acid were mixed in a glass vessel, and the vessel was capped and shaken vigorously to thoroughly mix. Sodium hydrogen carbonate solution 0.6% (w/v) [reagent 2.1.3.2] was prepared by dissolving 0.6 g sodium hydrogen carbonate in 100 mL ultrapure water. On each day of analysis, 1.8% (v/v) sulfuric acid/methanol solution [reagent 2.1.3.3] was prepared by dissolving 1.8 mL sulfuric acid in 100 mL methanol. Sodium hydrogen carbonate solution [reagent 2.1.3.4] for terminating methanolysis was prepared by dissolving 4.8 g sodium hydrogen carbonate in 50 mL ultrapure water. Ammonium sulfate solution [reagent 2.1.3.5] was prepared by dissolving 20 g ammonium sulfate in 50 mL ultrapure water. The PBA solution [reagent 2.1.3.6] described in the EFSA method was prepared by dissolving 0.4 g PBA in 10 mL diethyl ether. As the internal standards, 1,000 μg/mL stock solution was prepared by dissolving 3-MCPD dipalmitate-d₅ or glycidyl oleate-d₅ in toluene. On each day of analysis, an internal standard mix [reagent 2.1.3.7] was prepared by adding 0.5 mL each of the 3-MCPD dipalmitate-d₅ and glycidyl oleate-d₅ stock solutions in a 20 mL amber glass volumetric flask, followed by the addition of toluene to a final volume of 20 mL. As the standards, 1,000 μg/mL stock solutions were prepared by dissolving 3-MCPD dipalmitate [reagent 2.1.3.8] or glycidyl palmitate [reagent 2.1.3.9] in toluene, respectively.

2.2 Analytical samples

Various food samples were purchased locally for single-laboratory validation of the 2 methods: fried rice cracker, fried instant noodle, biscuit, crunchy deep fried wheat flour dough coated with melted brown sugar and refined sugar (karinto), pieces of vegetables battered with wheat flour and then deep-fried (vegetable tempura) and frozen deep-fried chicken. The selection criteria for the foods for validation include (i) fats and oils reported to contain relatively high concentrations of 3-MCPDEs and/or GEs and used as raw material or oil for frying; (ii) baked or fried at a high temperature; (iii) their 3-MCPDEs and GEs levels not reported in Japan or other countries; and/or (iv) consumed in large amounts in Japan. Baked rice cracker and 3 types of karinto were used to check the efficacy of extraction of fats and oils in the EFSA method. The samples used for comparing the analytical results of 3-MCPDEs and GEs concentrations from the 2 methods were also purchased locally: fried rice cracker, biscuit, 2 types of potato crisps, seafood tempura, and frozen deep-fried chicken. Baked rice cracker, cracker dough, and potato snack were prepared in the laboratory of the House Foods Group Inc., and these foods were heated using a household oven or industrial fryer.

Food samples were ground in a 1 kg batch using Cuisinart DLC-10PRP food processor and/or Waring 7011HBC bottle blender. For vegetable tempura, seafood tempura or frozen deep-fried chicken, the entire foods including the batter and the foods were homogenized. The ground samples were then divided into 80 to 90 g subsamples sufficient for analysis and placed in aluminum pouches. The vegetable tempura, seafood tempura, and 2 frozen deep-fried chicken samples were stored in a freezer at −20°C until analysis, whereas all the other samples were kept at room temperature (approximately 25°C).

2.3 Enzymatic method for foods containing fats and oils

2.3.1 Extraction of fats and oils from food samples

On a basis of the nutritional labelling of each food item, the amount of sample necessary for obtaining 100–110 mg of fats and/or oils was determined. For example, if a food contains 50% of fats or oils, then 200–220 mg of sample is necessary. The amount of food sample thus calculated was weighed into a screw-capped 10 mL glass test tube. To each test tube, 3 mL of isooctane/tert-butyl methyl ether (1:2, v/v) and 1 mL of ethanol were added. After sealing with the screw cap and vortexing for 10 s, the tube was kept at 60°C in a water bath for 5 min. The tube was
removed from the water bath and, without allowing it to cool, shaken on a shaker at room temperature for 10 min. To the mixture, 4 mL of 30% (w/v) sodium bromide solution [reagent 2.1.2.1] was added, vortexed for 10 s, and centrifuged at 3,500 rpm \((2,270 \times g)\) for 1–5 min. The organic layer was transferred to a new tube, after which 3 mL of isoctane/\text{tert}-butyl methyl ether \((1:2, \text{v/v})\) was added to the remaining aqueous layer. The tube was vortexed for 10 s and centrifuged at 3,500 rpm \((2,270 \times g)\) for 5–10 min. The organic layer from this second tube was combined with the first one. As a small amount of remaining aqueous extract would not interfere with the subsequent hydrolysis, as much organic layer was transferred as possible. The organic layer was evaporated at 40°C under a flow of nitrogen. Because the organic solvent will significantly interfere with the hydrolysis, the organic solvent needs to be evaporated completely.

### 2.3.2 Enzymatic method

The residue in the test tube was dissolved in 0.5 mL of isoctane by vortexing for 10 s. For residues containing fats of high melting point, 0.5 mL of isoctane was added, and the mixture was heated to 60°C to ensure complete dissolution. To the solution, 3 mL of sodium bromide solution containing *C. cylindracea* lipase [reagent 2.1.2.2] was added. Hydrolysis of esters was achieved at room temperature \((\text{approximately 25°C})\) by shaking the tube on an orbital motion high speed shaker \((\text{exp. Cute Mixer CM-1000, EYELA, Tokyo, Japan})\) at 1,800 rpm for 30 min. The mixture in a tube was centrifuged at 3,000 rpm \((1,670 \times g)\) for 10 s and heated in a water bath at 80°C for 10 min for bromination of glycidol. After cooling the tube to room temperature, 50 μL of 2 μg/mL internal standard mix \((3\text{-MCPD}-d_5\text{ and } 3\text{-MBPD}-d_5)\) [reagent 2.1.2.4] was added. For samples containing medium-chain fatty acid such as milk fat or coconut oil \((\text{e.g., biscuit in this study})\), since medium-chain fatty acids after hydrolysis could not be fully removed by hexane washing, 3 mL of diethyl ether was added to the aqueous layer, and the tube was vortexed for 10 s. After centrifugation at 3,000 rpm \((1,670 \times g)\) for 10 s, the organic layer was removed. For samples not containing medium-chain fatty acids, the washing step using diethyl ether is not necessary. To the tube, 3 mL of hexane was added, and the tube was vortexed for 10 s. If organic and aqueous layers did not separate, the tube was centrifuged at 3,000 rpm \((1,670 \times g)\) for 10 s. The aqueous layer was transferred to a new test tube. To the aqueous layer, 3 mL of hexane was added again, and the tube was vortexed for 10 s, after which the organic layer was removed. To this tube, 0.1 mL of PBA solution [reagent 2.1.2.3] and 3 mL of hexane were added, and the tube was shaken at room temperature for 10 min. After centrifugation at 3,000–3,500 rpm \((1,670–2,270 \times g)\) for 5–10 min, the organic layer was transferred to a new test tube containing a small amount of sodium sulfate and concentrated to approximately 0.5 mL under a flow of nitrogen. After filtration through a 0.2 μm membrane filter, the sample was subjected to GC-MS analysis.

### 2.3.3 Preparation of samples for construction of standard curves

For developing calibration curves, 0, 5, 20, 50, and 100 μL of 1.5 μg/mL standard mix II [reagent 2.1.2.6], and 20, 35, 50, and 80 μL of 15 μg/mL standard mix I [reagent 2.1.2.5] were placed separately into 9 test tubes. To each tube, 50 μL of 2.0 μg/mL internal standard mix [reagent 2.1.2.4] and 3 mL of 30% sodium bromide solution [reagent 2.1.2.1] were added. Each tube was then vortexed for 10 s. PBA derivatization was carried out in the same manner as described in Section 2.3.2. Calibration curves were drawn by plotting the peak area ratios (3-MCPD/3-MCPD-\(d_5\) and 3-MBPD/3-MBPD-\(d_5\)) against the concentration ratios of standards and internal standards, and the simple linear regression equations were estimated.

### 2.3.4 GC-MS analysis

GC-MS analysis was carried out on TRACE GC Ultra system equipped with TSQ Quantum GC \((\text{Thermo Fisher Scientific, Waltham, MA, USA})\). Chromatography was performed on Factor Four Capillary Column VF-5 ms \((250 \times 0.25 \text{ mm inner diameter, } 0.25 \mu \text{m film thickness; Agilent Technologies, Santa Clara, CA, USA})\). The carrier gas was helium at a constant flow of 1.2 mL/min. The GC oven temperature was kept at 60°C for 1 min, raised to 150°C at 10°C/min, then to 180°C at 3°C/min, to 300°C at 30°C/min, and kept at 300°C for 8 min. The whole process took a total of 32 min. The other temperature settings were as follows: inlet, 250°C; transfer line, 280°C; and ion source, 230°C. The MS system was operated in selected ion monitoring (SIM) mode. Quantitative analyses were carried out by monitoring ions at: \(m/z\ 147\) and 196 for the 3-MCPD derivative; \(m/z\ 150\) and 201 for the 3-MCPD-\(d_5\) derivative; \(m/z\ 147\) and 240 for the 3-MBPD derivative; and \(m/z\ 150\) and 245 for the 3-MBPD-\(d_5\) derivative. In this study, 3-MCPD and GEs concentrations are expressed as 3-MCPD and glycidol concentration equivalents, respectively.

### 2.3.5 Approximating fats and/or oils content in food samples

If the content of fats and/or oils in a food sample could not be determined from the labeling information, the following method was used to estimate the approximate content for determining sampling amounts as in Section 2.3.1. A ground food sample \((300–500 \text{ mg})\) was placed in a screw-capped 10 mL glass test tube, after which 3 mL of isoctane/\text{tert}-butyl methyl ether \((1:2, \text{v/v})\) and 1 mL of ethanol were added to the tube. After sealing the screw cap, the tube was vortexed for 10 s and heated in a 60°C water bath for 5 min. Immediately after removing from the water bath, without allowing the tube to cool, the tube was placed on a shaker at room temperature for 10 min. To the mixture, 4 mL of 30% (w/v) sodium bromide solution...
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[reagent 2.1.2.1] was added, and the tube was vortexed for 10 s and centrifuged at 3,500 rpm (2,270 × g) for 1–5 min, after which 2 mL of the organic layer was transferred to a weighed new tube. The organic layer was then evaporated at 40°C under a flow of nitrogen. The content of crude fats and/or oils in the food sample was calculated using the following formula: \[
\frac{\text{(weight of the extract evaporated to dryness) + weight of the glass tube} - \text{(weight of the glass tube)}}{\text{(weight of the food sample)}} \times 3/2 \times 100.
\]

2.4 Modified EFSA method

2.4.1 Extraction of fats and oils from food samples

Pressurized liquid extraction (PLE) was carried out using SpeedExtractor E-916 (Nihon BUCHI K. K., Tokyo, Japan). A total of 4.9–5.1 g of ground food sample was placed in a vessel. Because water might remain in PLE extracts of rice cracker, “a tall vessel”, such as a 30 mL or 50 mL centrifuge tube was used instead of “a horizontally long tube vessel”. Next, 5 g of sodium polycarbonate and 15 g of sand were added to the tube and mixed well, especially for samples of rice cracker. The mixture was transferred into a 40 mL cell for PLE using a micro spatula and/or spatula made of silicone, and the cell was placed in the SpeedExtractor E-916. The PLE extraction conditions were as follows: pressure, 50 bar (50 MPa, the minimum of the instrument); temperature, 40°C; heat time, 5 min; static time, 5 min; purge time, 3 min; static cycles, 2; and solvent, tert-butyl methyl ether (100%). During PLE, the extract was collected into a solvent collection bottle and transferred into a weighed new glass tube. The tert-butyl methyl ether extract was evaporated to dryness under a flow of nitrogen at 40°C. The content of fats and/or oils in the food sample was calculated using the following formula:

\[
\left(\text{weight of the extract evaporated to dryness + weight of the glass tube}\right) - \left(\text{weight of the glass tube}\right) / \text{weight of the food sample} \times 100.
\]

2.4.2 Preparation of extracted fat or oil

In this study, while fried instant noodle was analyzed using the 2015 EFSA method, the remaining food samples were analyzed using the 2017 EFSA method, to which modifications, such as the addition of a clean-up step with sodium carbonate solution, were incorporated.

2.4.2.1 2015 EFSA method

Approximately 100 mg of extracted fats and/or oils from each food sample was weighed into a screw-capped 10 mL amber glass test tube, to which 50 μL of internal standard mix (3-MCPD dipalmitate-d₅ and glycidyl oleate-d₅) [reagent 2.1.3.7] was added.

2.4.2.2 2017 EFSA method

Approximately 100 mg of extracted fats and/or oils from each food sample was weighed into a 1.5 mL glass vial, to which 50 μL of internal standard mix (3-MCPD dipalmitate-d₅ and glycidyl oleate-d₅) [reagent 2.1.3.7] and 0.5 mL of hexane/ethyl acetate (85:15, v/v) were added. The vial was vortexed for 15 s. An SPE cartridge containing 500 mg of aminopropyl (HyperSep™, Thermo Fisher Scientific) was preconditioned with 2 mL of hexane/ethyl acetate (85:15, v/v), and the mixture in the vial was loaded on to the cartridge. Subsequently, 10 mL of hexane/ethyl acetate (85:15, v/v) was passed through the cartridge under gravity. The eluate from the cartridge was collected, and evaporated to dryness under a flow of nitrogen at 40°C.

2.4.2.3 Preparation of samples

The sample obtained from either Section 2.4.2.1 or 2.4.2.2 was subject to the following procedure. To the test tube, 2 mL of tetrahydrofuran and 30 μL of 3 mg/mL acidic aqueous solution of sodium bromide [reagent 2.1.3.1] were added. The tube was vortexed for 15 s, and incubated at 50°C for 15 min, after which 3 mL of 0.6% (w/v) sodium hydrogen carbonate solution [reagent 2.1.3.2] was added to stop bromination of the GEs. To the mixture, 2 mL of hexane was added, and the tube was vortexed for 15 s. The organic layer was transferred to a new test tube, and evaporated to dryness under a flow of nitrogen at 40°C. After dissolving the residue in 1 mL of anhydrous tetrahydrofuran, 1.8 mL of 1.8% (v/v) sulfuric acid/methanol solution [reagent 2.1.3.3], Methanolation of 3-MCPDEs and GEs was achieved by incubating at 40°C for 16 h. The reaction was stopped by the addition of 0.5 mL of sodium hydrogen carbonate solution [reagent 2.1.3.4], and the organic layer was evaporated under a flow of nitrogen at 40°C until reduced to approximately 1 mL. In case of analysis of food samples made from fats and oils containing medium chain fatty acids, the organic layer was evaporated until reduced to approximately 0.5–0.8 mL. To the residue in test tube, 1.3 mL of ammonium sulfate solution [reagent 2.1.3.5] and 1 mL of hexane were added, and the tube was vortexed for 10 s. The organic layer was removed, and 1 mL of hexane was added to the tube and vortexed for 10 s. After removal of the organic layer, 0.6 mL of ethyl acetate was added to the remaining mixture and vortexed for 10 s. The organic layer was then transferred to a new tube containing a small amount of sodium sulfate. To the remaining aqueous layer, 0.6 mL of ethyl acetate was added. The tube was vortexed for 10 s, and the organic layers were combined by transferring the second organic layer to the first. Furthermore, 0.6 mL of ethyl acetate was added to the remaining aqueous layer. The tube was vortexed for 10 s, and the resulting organic layer was combined with the previous organic layers. To the combined organic extract, 0.15 mL of PBA solution [reagent 2.1.3.6] was added and the mixture was incubated at room temperature for 5 min in an ultrasonic bath. The organic extract was evaporated to dryness under a flow of nitrogen at 40°C. The residue was dissolved in 0.3 mL of isooctane by vortexing for 10 s, and centrifuged at 3,500 rpm (2,270 × g) for 20–30 s. The supernatant was transferred to a GC vial for GC-MS analysis. The EFSA

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method reports 3-MCPD and glycidol equivalent concentrations in the extracted fats and oils from food sample. In this study, 3-MCPD and glycidol equivalent concentrations in food samples were calculated using the content of fats and oils in food samples, calculated as described in Section 2.4.1.

2.4.3 Preparation of samples for construction of standard curves

The 2015 EFSA method includes preparation of standard mix I on the day of analysis using the 1,000 μg/mL stock solutions by adding 2.5 mL of the 3-MCPD dipalmitate stock solution\[reagent 2.1.3.8\]and 1.25 mL of the glycidyl palmitate\[reagent 2.1.3.9\]stock solution to a 50 mL amber glass volumetric flask, and dilution with toluene to a final volume of 50 mL. Standard mix II was prepared by adding 1 mL of standard mix I to a 10 mL amber glass volumetric flask, diluted by toluene to a final volume of 10 mL. To prepare the calibration curves, 20 and 60 μL of standard mix II and 20, 40, 60, 80, 100, 120, 150, and 200 μL of standard mix I were placed separately in 10 test tubes. To each tube, 50 μL of internal standard mix\[reagent 2.1.3.7\]was added, and the calibration standard samples were prepared in the same manner after the SPE step described in Section 2.4.2.

The 2017 EFSA method describes the preparation of the standard mix on the day of analysis using the 1,000 μg/mL stock solutions by adding 2.5 mL of the 3-MCPD dipalmitate stock solution and 1.25 mL of glycidyl palmitate stock solution to a 50 mL amber glass volumetric flask, and diluted with toluene to a final volume of 50 mL. Calibration solutions 1, 2, 3, 4, and 5 were prepared by adding 0.1, 1, 2, 3, and 4 μL of standard mix to separate 10 mL amber glass volumetric flasks, respectively, and diluted with toluene to a final volume of 10 mL. Into 6 screw-capped 10 mL amber glass test tubes, 100 mg of fat or oil not containing 3-MCPDEs and GEs\[linseed oil in this study\]was weighed and placed separately. To each tube containing linseed oil, 0.1 mL of calibration solutions 1–5 or 0.1 mL of toluene was added. Calibration standard samples were prepared in the same manner as described in Section 2.4.2. Standard curves were drawn by plotting the peak area ratios (3-MCPD/3-MCPD-\(d_5\) and 3-MBPD/3-MBPD-\(d_5\)) against the concentration ratios of the standards and internal standards, and the simple linear regression equations were calculated. In this study, the concentrations of 3-MCPDEs and GEs are expressed as the concentrations of 3-MCPD and glycidol equivalents, respectively.

2.4.4 GC-MS analysis

GC-MS analyses were carried using TRACE GC Ultra equipped with TSQ Quantum GC. Chromatography was performed using Factor Four Capillary Column VF-5 ms (30 m × 0.25 mm inner diameter, 0.25 μm film thickness). The carrier gas was helium at a constant flow of 1.2 mL/min. The GC oven temperature was kept at 60°C for 1 min, raised to 150°C at 6°C/min, and kept for 2 min, and subsequently raised to 300°C at 10°C/min (total 33 min). The other temperature settings were as follows: inlet, 250°C; transfer line, 300°C; ion source, 230°C (the 2015 EFSA method) or 250°C (the 2017 EFSA method). The MS system was operated in SIM mode. Quantitative analyses were carried out by monitoring ions at: \(m/z\) 147 and 196 for the 3-MCPD derivative; \(m/z\) 150 and 201 for the 3-MCPD-\(d_5\) derivative; \(m/z\) 240 and 146 for the 3-MBPD derivative; and \(m/z\) 150 and 245 for the 3-MBPD-\(d_5\) derivative. In this study, the peak of 3-MBPD derivative\((m/z 146)\) was overlapped by an interfering peak\(\)data not shown\). Since the interfering peak was not detected in the enzymatic methods, it was considered to be derived from extraction and purification steps of the EFSA method. The EFSA method also states that either the quantitative ion or the qualitative ion may be used as the selective ion, it would be better to monitor 3-MBPD derivative with \(m/z\) 146, 147, 240, or 245 prior to the analysis, and the ions should be selected, which are not detected as food processing contaminants overlapping with the holding time of 3-MBPD and which can obtain stable sensitivities for each analysis institute.

2.5 Recovery tests

Prior to extraction of fats and/or oils, 3-MCPD dioleate and glycidyl oleate were added to the food samples in the test tubes\(\)the enzymatic method\) or the PLE cell\(\)the EFSA method\), and these spiked food samples were left standing for 15 min. 3-MCPD dioleate and glycidyl oleate were spiked at 2 different concentrations corresponding to the free forms, 3-MCPD and glycidol, as follows: (i) 0.02–0.04 mg/kg as the target concentration for the limit of quantification\(\)LOQ\) or (ii) 0.2–0.4 mg/kg as the intermediate concentration of the calibration curve. Each food sample was analyzed in quintuplicate on the same day, with analyses run on 4 different days\(n = 5/day, 4 days\). A sample without spiking was also run in duplicate in each analysis. The Codex Alimentarius Commission Procedural Manual requires recoveries in a range of 80% to 110% for 3-MCPD and glycidol at 100 μg/kg–1 mg/kg\(29\). Analytical precision was assessed according to the HorRat\(i\) value, which was calculated as follows: (repeatability relative standard deviation\(\)RSD\)\)/\(\)\(\)predicted reproducibility relative standard deviation\(\)RSD\)\) determined using the Thompson equation \(× 0.67\), with HorRat\(i\) < 2 considered acceptable, which is equivalent to HorRat\(i\) < 1.3 calculated according to the Guidelines on Analytical Terminology\(\)CXG 72-2009\) by the Codex Alimentarius Commission\(30\).

2.6 LOQ

The target LOQ concentration for both 3-MCPD and glycidol in foods was set at 0.025 mg/kg. The food sample
used to estimate the LOQ was frozen deep-fried chicken, which contained the lowest concentrations of 3-MCPD and glycidol among the 6 food samples examined. The frozen deep-fried chicken samples without spiking were analyzed on the same day ($n = 6$), and the analysis was conducted on 2 different days. LOQ concentrations of 3-MCPD and glycidol were calculated according to the “EFSA supporting publication 2015: EN-779”

3 Results and Discussion

3.1 Enzymatic method modifications

Modifications were introduced into the enzymatic method to overcome 2 major issues affecting the analysis of fats and oils containing short- and/or medium-chain fatty acids (e.g., milk fat, coconut oil). The low levels of hydrolysis of 3-MCPD diesters in sample containing ≥ 20% of milk fats can be addressed by doubling the amount of $C. cylindracea$ lipase in the sodium bromide solution for hydrolysis [reagent 2.1.2.2] [17, 25]. For foods containing coconut oil (e.g., biscuit in this study), an interfering peak associated with lauric acid was detected at $m/z$ 150 in the chromatogram (Fig. 1A) due to the remaining medium-chain fatty acids in aqueous layer after hexane washing of hydrolysis/bromination mixture. A diethyl ether washing step was incorporated before the hexane washing. As shown in Fig. 1B, by incorporating a diethyl ether washing step in the procedure, the lauric acid interfering peak at $m/z$ 150 became undetectable.

3.2 EFSA method modifications

3.2.1 Dehydration of food samples during PLE

In the EFSA method, fats and oils are extracted from food samples by PLE. Sodium polyacrylate and sand are thoroughly mixed with food samples for dehydration prior to placing the food samples in the PLE cell. In this study, when analyzing these reagents with food samples using mortars or flat aluminum dishes, water or solid remained in the PLE extracts of some food samples (Fig. 2). However, whether the water content of the food sample was high or low had no effect on the presence of water in the PLE extracts. Visual inspection of the PLE extracts revealed that fried rice cracker had the highest residual water content. Similarly, water remained in the PLE extracts of other rice cracker products when they were mixed with sodium polyacrylate and sand using a mortar (Fig. 2). These results suggested that water tends to remain in rice cracker products after PLE and evaporation. In order to eliminate residual water in the PLE extracts, the following modifications were examined: changing the type of dehydrating reagent (e.g., magnesium sulfate, sodium sulfate, and diatomaceous earth); removal of residual water in the PLE extract after evaporation by dehydration using sodium sulfate; and alteration of the vessel used for mixing (data not shown). The simplest and most effective modification

![Fig. 1](image_url) GC-MS chromatograms ($m/z$ 150) of biscuit produced with coconut oil and analyzed with (A) the enzymatic method or (B) a partially modified enzymatic method, in which a diethyl ether washing step was added.

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was the alteration of the vessel used for mixing the food sample and dehydrating reagents prior to PLE. By using a tall vessel such as a centrifuge tube or a Falcon tube rather than a horizontally long vessel such as the mortar or flat dish, followed by vigorous mixing using a slim spatula or stirring rod, residual water in PLE extracts was removed. In addition, by changing the type of vessel, residual solid in the PLE extracts of frozen deep-fried chicken was removed. As a result, in this study, a 30 or 50 mL glass centrifuge tube or 50 mL Falcon tube was used as the mixing vessel for food samples and dehydrating reagents. Because water may remain in PLE extracts of rice cracker after insufficient mixing even in a vertical vessel, careful mixing is essential for the analysis of this type of samples.

3.2.2 PLE of fats and oils

In the EFSA method, “the fats and oils content in the food” are calculated from the amount of food sampled and the amount of fats and oils extracted by PLE. After analysis of the concentrations of 3-MCPDEs and GEs in the extracted fats and oils, the concentrations of 3-MCPDEs and GEs in each food sample were calculated as follows: (concentrations of 3-MCPDEs and GEs in the extracted fats and oils) × (content of fats and oils in the food). Table 1 shows the content of fats and oils in the food samples analyzed in this study, as obtained using either Soxhlet extraction with diethyl ether or PLE. For fried rice cracker, fried instant noodle, biscuit, vegetable tempura and frozen deep-fried chicken, the content of fats and oils in the food samples were comparable between Soxhlet extraction with diethyl ether and PLE. In contrast, for a karinto product from one company, the fats and oils obtained from PLE were lower than those from Soxhlet extraction. Similarly, for 3 karinto products from another companies the content of fats and oils obtained by PLE using the EFSA method were 2–3% lower than those obtained by Soxhlet extraction with diethyl ether, and the differences were statistically significant (Fig. 3). Karinto is coated with melted brown sugar and refined sugar after frying in fats or oils. As a reason for low extraction efficiency of fats and oils for karinto, we considered that the melted sugars coating on the fats and oils of karinto impeded the PLE step in the EFSA method when using organic solvents (under anhydrous conditions). Validation of the EFSA method on karinto was not further pursued.

3.2.3 Analysis of foods made from fats or oils containing medium-chain fatty acid

In the EFSA method, after methanolation of the ester-forms of 3-MCPDEs and 3-MBPDEs to the free forms, the
Table 1  Extraction efficiency of fats and oils in food samples by Soxhlet extraction and Pressurized Liquid Extraction (PLE).

| Extraction method | Fats and oils (%) (w/w) |
|-------------------|-------------------------|
| Soxhlet extraction⁰ | Fried rice cracker | Fried instant noodle | Biscuit | Karinto | Vegetable tempura | Frozen deep-fried chicken |
|                   | 27.7, 27.7            | 17.6, 17.6          | 26.4, 26.4 | 30.9, 31.8 | 20.8, 20.4       | 10.7, 11.0                |
| Pressurized Liquid Extraction (PLE) of EFSA method⁰ | Day 1         | 26.4 ± 1.0 | 18.0 ± 0.8 | 26.3 ± 0.2 | 23.1 ± 0.7 | 21.9 ± 1.0 | 12.0 ± 0.8 |
|                   | Day 2         | 25.6 ± 0.4 | 19.0 ± 0.7 | 26.4 ± 0.3 | 23.3 ± 0.9 | 19.8 ± 0.3 | 10.8 ± 0.5 |
|                   | Day 3         | 24.9 ± 0.8 | 17.4 ± 0.5 | 25.7 ± 0.4 | -        | 22.0 ± 0.2 | 11.2 ± 0.5 |
|                   | Day 4         | 25.3 ± 0.4 | 18.0 ± 0.4 | 26.7 ± 0.1 | -        | 21.9 ± 0.5 | 9.7 ± 0.2 |

⁰ extracted with diethyl ether at 65°C for 16 h, replicate extraction (n = 2).

Mean ± Standard deviation of 12 replicate extractions (n = 12) conducted on each of 4 different days.

* for karinto, extraction test was not conducted on day 3 and day 4 due to low extraction efficiency.

Fig. 3  Extraction efficiency of fats and oils in 3 karinto products by Soxhlet extraction with diethyl ether at 65°C for 16 h and by PLE used in the EFSA method (black columns). Soxhlet extraction with diethyl ether showed significantly higher efficiency of fat extraction than PLE (triplicate extraction. *: p < 0.05, **: p < 0.01). Karinto products used for testing extraction efficiency here are different from the karinto product shown in Table 1.

organic solvent in the test tube is evaporated under a flow of nitrogen to approximately 1 mL. If the organic solvent is not adequately evaporated, fatty acid methyl esters produced by methanolysis will remain in the tube. If these are long-chain fatty acid methyl esters, they can be removed by hexane washing in the next step. However, medium-chain fatty acid methyl esters produced from food samples made from fats or oils containing medium-chain fatty acid-binding oils and fats, such as coconut oil and milk fat, cannot be completely removed by hexane washing. In our study, for foods containing coconut oil (e.g., a biscuit product), an interfering peak associated with lauric acid methyl ester and myristic acid methyl ester was detected at m/z 150 in the chromatogram (Fig. 4A). In order to ensure complete removal of organic solvent, a test sample was prepared by evaporating the organic solvent to 0.6 mL, which was found successful (Fig. 4B). Therefore, for food samples containing fats and oils containing medium-chain fatty acids, it was found necessary to evaporate the solvent after methanolysis to a final volume of 0.5–0.8 mL.

3.3 Single laboratory validation

3.3.1 Recovery tests and HorRatᵣ values

To verify the applicability of the enzymatic method and the EFSA method to the analysis of food samples, recovery tests were performed with 5–6 food samples. Among samples, biscuit sample was analyzed using a partially modified enzymatic method. While fried instant noodle sample was analyzed using the 2015 EFSA method, the samples of other 4 types of foods were analyzed using the 2017 EFSA method. All food samples were spiked with 3-MCPD dioleate and glycidyl oleate at 0.02–0.04 mg/kg or 0.2–0.4 mg/kg, respectively. Recoveries in a range of 80%–110%, deemed acceptable for 3-MCPD and glycidol at 100 μg/kg–1 mg/kg as required in the Codex Alimentarius Commission Procedural Manual. As shown in Table 2, analysis of the 5 spiked food samples using the enzymatic method gave high recoveries for 3-MCPDEs (93.7–98.5%) and GEs (94.4–98.4%), with HorRatᵣ values of 0.06–0.53 and 0.09–0.78 for 3-MCPDEs and GEs, respectively. As shown in Table 3, analysis of the 6 spiked food samples using the EFSA method gave high recoveries for 3-MCPDEs (96.6–99.4%) and GEs (95.7–100.1%), with HorRatᵣ values of 0.14–0.49 and 0.16–1.05 for 3-MCPDEs and GEs, respectively.

3.3.2 LOQ

The proposed LOQs for both 3-MCPDEs and GEs in foods were 0.025 mg/kg. Of the 6 types of foods used for recovery tests, frozen deep-fried chicken had the lowest concentrations of 3-MCPDEs and GEs (in 3-MCPD equivalents and glycidol equivalents) and were thus used for LOQ.
determination. As expected, 3-MCPDEs and GEs in frozen deep-fried chicken resulted in the lowest concentration among all the samples because of the thin batter that would absorb frying oil. In order to investigate if there was any influence of GC column condition and MS ion source contamination on the peak resolution and sensitivity, LOQ were determined on 2 different days.

The samples using the enzymatic method were analyzed both after GC-MS cleaning and after a total of 107 sample solutions extracted and purified according to the enzymatic method. As shown in Table 4 left, the LOQ values for 3-MCPDEs and GEs in frozen deep-fried chicken were lower than the target concentration of 0.025 mg/kg. The samples using the 2017 EFSA method were analyzed both after GC-MS cleaning and after a total of 18 sample solutions extracted and purified according to the 2017 EFSA method were analyzed. As shown in Table 4 right, the LOQ for 3-MCPDEs met the target concentration but
Table 3  Recoveries, RSD, and HorRat values from 5 food samples spiked simultaneously with 3-MCPD dioleate and glycidyle olate and analyzed with the EFSA method. While the fried instant noodle sample was analyzed according to the 2015 EFSA method, other samples were analyzed according to the 2017 EFSA method (n = 5/day, 4 days).

| Sample          | 3-MCPDEs Spiked level (mg/kg)
|-----------------|--------------------------------|
| Fried rice cracker | 0.3 0.03 0.2 0.02 0.3 0.03 |
| Fried instant noodle | 97.6 98.9 96.8 97.1 98.0 97.7 |
| Biscuit          | 4.4 6.8 4.2 8.7 4.5 3.9 |
| Karinto          | 0.19 0.14 0.29 0.30 0.32 0.19 |
| Vegetable tempura | — — — — — — |
| Frozen deep-fried chicken | 0.2 0.02 0.2 0.02 |

| Sample          | GEs Spiked level (mg/kg)
|-----------------|------------------|
| Fried rice cracker | 0.4 0.04 0.2 0.02 0.3 0.03 |
| Fried instant noodle | 97.0 95.7 95.8 97.1 97.0 97.6 |
| Biscuit          | 4.9 5.2 8.0 5.9 3.8 4.6 |
| Karinto          | 0.27 0.30 0.76 1.05 0.60 0.16 |
| Vegetable tempura | — — — — — — |
| Frozen deep-fried chicken | 0.3 0.03 0.2 0.02 |

| Sample          | Mean recovery (%) |
|-----------------|-------------------|
| Fried rice cracker | 99.6 98.9 96.8 97.1 98.0 97.7 |
| Fried instant noodle | 97.0 95.7 95.8 97.1 97.0 97.6 |
| Biscuit          | 4.9 5.2 8.0 5.9 3.8 4.6 |
| Karinto          | 0.27 0.30 0.76 1.05 0.60 0.16 |
| Vegetable tempura | — — — — — — |
| Frozen deep-fried chicken | 0.3 0.03 0.2 0.02 |

| Sample          | RSD (%) |
|-----------------|---------|
| Fried rice cracker | 4.4 6.8 4.2 8.7 4.5 3.9 |
| Fried instant noodle | 97.6 98.9 96.8 97.1 98.0 97.7 |
| Biscuit          | 4.9 5.2 8.0 5.9 3.8 4.6 |
| Karinto          | 0.19 0.14 0.29 0.30 0.32 0.19 |
| Vegetable tempura | — — — — — — |
| Frozen deep-fried chicken | 0.2 0.02 0.2 0.02 |

| Sample          | HorRat (r) |
|-----------------|------------|
| Fried rice cracker | 0.19 0.14 0.29 0.30 0.32 0.19 |
| Fried instant noodle | 97.6 98.9 96.8 97.1 98.0 97.7 |
| Biscuit          | 4.9 5.2 8.0 5.9 3.8 4.6 |
| Karinto          | 0.27 0.30 0.76 1.05 0.60 0.16 |
| Vegetable tempura | — — — — — — |
| Frozen deep-fried chicken | 0.3 0.03 0.2 0.02 |

The LOQ for GEs was 0.027 mg/kg, higher than the target concentration. GC-MS cleaning were as follows: cutting off the insert side of GC column, replacing the insert, and cleaning the ion source parts with alumina powder.

These results suggest that GC-MS conditions such as the presence of PBA and any other components in the sample extracts in the injection port, ion source, and MS system greatly affect the LOQ in the EFSA method. A factor that significantly affects the potential for contamination is PBA, which is used for derivatization. The enzymatic method used hexane for extracting PBA derivatives in order to prevent PBA from contaminating the GC-MS samples and the GC-MS instrument itself. For the enzymatic method, the peak shapes of the target compounds remained unchanged after 5 consecutive days of analyses. For the EFSA method, by contrast, the quantity of PBA added to the sample was about 2.5 times that in the enzymatic method, which resulted in deposits of PBA remaining in the test tubes after derivatization and solvent evaporation, in addition to PBA in some of the GC vials. A large amount of PBA adsorbed to the GC insert wall and GC columns after analysis of 24 samples prepared using the EFSA method. For samples prepared using the EFSA method, the peak shapes of the target compound, particularly that of the 3-MBPD derivative, started to show tailing and significantly lower height after 3 consecutive days of analysis. It is thus recommended that GC-MS cleaning be performed after each analysis of samples prepared using the EFSA method and that the volume of PBA used for derivatization be reduced for obtaining sufficient sensitivity.

3.4 Comparison of 3-MCPDEs and GEs concentrations in food samples determined using 2 methods

We compared the 3-MCPDEs and GEs concentrations for 14 samples without spiking determined using the enzymatic method and the EFSA method. In the enzymatic method, biscuits were analyzed using a partially modified method, in which an ether washing step was added before the hexane washing. In the EFSA methods, while only fried instant noodle was analyzed using the 2015 EFSA method, the remaining 13 samples were analyzed using the 2017 EFSA method. For method validation, 5 samples were ana-
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analyzed in duplicate, with analyses run on 4 different days \((n = 2/\text{day} \times 4 \text{ days})\). The remaining 9 samples were analyzed in triplicate \((n = 3 \times 1 \text{ day})\). Average concentrations quantified using the enzymatic method were plotted against those quantified with the EFSA method in Fig. 5.

Tables 5 and 6 showed the results of evaluations of the equivalence of 3-MCPDEs and GEs concentrations quantified using both methods for 14 samples using \(t\) tests and JIS Z 8402-6 \(^\ast\)Comparison of mean values of Method A and Method B \(^\ast\). Results were considered statistically insignificant for \(P\) values \(>0.05\), and JIS Z 8402-6 \(\frac{[\text{mean value of the EFSA method} - \text{ (mean value of the enzymatic method)}]}{\sqrt{[\text{standard deviation of the EFSA method}}^2 + \text{ (standard deviation of the enzymatic method)}^2]} \leq 2\). For the 13 samples excepting fried instant noodle, there were no statistically significant differences between the enzymatic method and the 2017 EFSA method.

The 3-MCPDEs concentrations of fried instant noodle

Table 5

|                        | Fried rice cracker (Product A) | Fried instant noodle\(^\ast\) | Biscuit (Product A) | Vegetable tempura | Frozen deep-fried chicken (Product A) |
|------------------------|--------------------------------|-------------------------------|--------------------|------------------|--------------------------------------|
| 3-MCPDEs \(t\) test, \(P\) value (two side) | 0.89                           | 0.04\(^\ast\)                | 0.22               | 0.18             | 0.91                                 |
| JIS Z 8402-6           | 0.07                           | 1.05                         | 0.48               | 0.48             | 0.04                                 |
| GEs \(t\) test, \(P\) value (two side)    | 0.79                           | 0.26                         | 0.27               | 0.93             | 0.14                                 |
| JIS Z 8402-6           | 0.10                           | 0.35                         | 0.38               | 0.02             | 0.67                                 |

\(^\ast\) while the fried instant noodle sample was analyzed according to the 2015 EFSA method, other samples were analyzed according to the 2017 EFSA method.

Table 6

|                        | Fried rice cracker (Product B) | Biscuit (Product B) | Potato crisp (Product A) | Potato crisp (Product B) | Fried potato snack | Baked cracker | Cracker dough | Seafood tempura | Frozen deep-fried chicken (Product B) |
|------------------------|--------------------------------|---------------------|--------------------------|--------------------------|-------------------|---------------|---------------|----------------|--------------------------------------|
| 3-MCPDEs \(t\) test, \(P\) value (two side) | 0.69                           | 0.72               | 0.26                     | 0.91                     | 0.45              | 0.56          | 0.97          | 0.98           | 0.56                                 |
| JIS Z 8402-6           | 0.30                           | 0.31               | 0.36                     | 0.04                     | 0.66              | 0.44          | 0.02          | 0.01           | 0.26                                 |
| GEs \(t\) test, \(P\) value (two side)    | 0.69                           | 0.26               | 0.59                     | 0.19                     | 0.62              | 0.85          | 0.19          | 0.88           | 0.29                                 |
| JIS Z 8402-6           | 0.25                           | 0.94               | 0.38                     | 0.49                     | 0.29              | 0.12          | 1.16          | 0.10           | 0.79                                 |

Fig. 5 Comparisons of the mean analytical results of 3-MCPDEs (solid circles) and GEs (open diamonds) in 14 different food samples by the enzymatic method and the EFSA method.

Table 5 Statistical analysis for equivalence between 3-MCPDEs or GEs concentrations in 5 food samples from the enzymatic method and the EFSA method (replicate analyses, with analyses run on 4 different days \((n = 2/\text{day}, 4 \text{ days})\). *: \(p < 0.05\)).

Table 6 Statistical analysis for equivalence between 3-MCPDEs or GEs concentrations in 9 food samples from the enzymatic method and the EFSA method (triplicate analyses on the same day \((n = 3/\text{day}, 1 \text{ day})\)).

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Table 7  Weighted Deming regression analyses for 3-MCPDEs or GEs in 5 food samples from the enzymatic method and the EFSA method. The analytical results in Table 5 on fried rice cracker, fried instant noodles, biscuit, vegetable tempura and frozen deep-fried chicken (replicate analyses, with analyses run on 4 different days, \( n = 2/\text{day}, 4 \text{ days} \)) were statistically analyzed.

| Coefficient | Standard error | 95% confidence limit |
|-------------|----------------|---------------------|
|             |                | Lower | Upper    |
| 3-MCPDEs    | Intercept     | 0.000 | 0.004 | -0.013 | 0.013 |
|             | Slope         | 1.042 | 0.044 | 0.902  | 1.181 |
| GEs         | Intercept     | -0.004 | 0.003 | -0.013 | 0.005 |
|             | Slope         | 1.069 | 0.058 | 0.885  | 1.254 |

\(^a\) Standard errors were calculated by the jackknife method.

Table 8  Weighted Deming regression analyses for 3-MCPDEs and GEs in 9 food samples from the enzymatic method and the EFSA method. The analytical results in Table 6 on fried rice cracker, biscuit, 2 potato crisps, fried potato snack, baked cracker, cracker dough, seafood tempura, and frozen deep-fried chicken (triplicate analyses on the same day, \( n = 3/\text{day, 1 day} \)) were statistically analyzed.

| Coefficient | Standard error | 95% confidence limit |
|-------------|----------------|---------------------|
|             |                | Lower | Upper    |
| 3-MCPDEs    | Intercept     | 0.001 | 0.001 | -0.002 | 0.003 |
|             | Slope         | 1.004 | 0.009 | 0.982  | 1.025 |
| GEs         | Intercept     | 0.002 | 0.006 | -0.011 | 0.015 |
|             | Slope         | 0.982 | 0.040 | 0.887  | 1.078 |

\(^a\) Standard errors were calculated by the jackknife method.

quantified using the enzymatic method and the 2015 EFSA method were statistically significant by the \( t \) test but not by JIS Z 8402-6. In the 2015 EFSA method, only the Hor\( \text{Rate}_\text{Adj} \) value of fried instant noodle spiked with GEs at a low concentration exceeded 1.0 (actual value = 1.05). At the beginning of this study as a regulatory research project for food safety, animal health and plant protection by the Ministry of Agriculture, Forestry and Fisheries of Japan in 2017, the 2015 EFSA method was published\(^{26}\). However, as this 2015 EFSA method was found to have several problems\(^{20}\), the 2017 EFSA method was reported with modifications such as adding the SPE step prior to the bromination/methanolysis steps\(^{27}\). Since the 2017 EFSA method was reported during the implementation of this study, fried instant noodle, for which performance validation was completed at that time, were analyzed using the 2015 EFSA method. In order to determine whether the significant differences by \( t \) test in the analytical value of 3-MCPDEs in fried instant noodle was due to food-related factors (specific to fried instant noodle) or the 2015 EFSA method, it would be necessary to compare the analytical values for fried instant noodle analyzed using the enzymatic method and the 2017 EFSA method. We assume that the Hor\( \text{Rate}_\text{Adj} \) value of fried instant noodle would be even lower for samples analyzed using the 2017 EFSA method. At the time of preparation of this paper, a new 2018 EFSA method was published\(^{32}\).

The results of weighted Deming regression analyses for method comparison\(^{33}\) were shown in Tables 7 and 8. The analytical standard deviations were estimated from duplicate measurements, and the coefficients of variation were assumed to be constant over the range of concentrations analyzed. The standard errors for the slope and the intercept were determined using the jackknife method and used for confidence interval calculations. The analyses were performed in R software\(^{34}\) using the “mcr” package\(^{35}\). Table 7 showed the results for 5 samples used for recovery test (\( n = 2/\text{day} \times 4 \text{ days} \)). For both 3-MCPDEs and GEs, the 95% confidence intervals for the slope and the intercept showed the slope around 1 (3-MCPDEs, 0.902–1.181; GEs, 0.885–1.254) and intercept close to 0 (3-MCPDEs, −0.013–0.013; GEs, −0.013–0.005). Table 8 showed the results for 9 samples used for recovery test (\( n = 3/\text{day} \times 1 \text{ day} \)). The 95% confidence intervals for the slope and the intercept...
showed the slope around 1 (3-MCPDEs, 0.982–1.025; GEs, 0.887–1.078) and intercept close to 0 (3-MCPDEs, −0.002–0.003; GEs, −0.011–0.015). Therefore, it was concluded that the results quantified using the enzymatic method were not significantly different from those quantified using the EFSA method.

4 Conclusion

The enzymatic method and the EFSA method were partially modified for application to the analysis of thermally processed food products widely distributed in Japan containing fats and oils and subjected to single-laboratory validation. The enzymatic method showed mean recoveries of 93.7–98.5% for 3-MCPDEs, 94.4–98.4% for GEs, and HorRat\(\alpha\) values of 0.06–0.78 in analyses of foods spiked 3-MCPD diol and glycidyl diolate at 0.02–0.04 mg/kg or 0.2–0.4 mg/kg. The EFSA method showed mean recoveries of 96.6–99.4% for 3-MCPDEs, 95.7–100.1% for GEs, and HorRat\(\alpha\) values of 0.14–1.05 in analyses of foods spiked 3-MCPD diol and glycidyl diolate at 0.02–0.04 mg/kg or 0.2–0.4 mg/kg. These validation results met the requirements of the Codex Alimentarius Commission (recoveries ranging from 80% to 110% at 100 μg/kg–1 mg/kg, and HorRat\(\alpha\) values < 1.3). The concentrations of 3-MCPDEs and GEs in food samples determined using the enzymatic method and the 2017 EFSA method were equivalent.

Even considering the extracting step for fats and oils, the total time for the enzymatic method needed for preparing a GC-MS test sample was half a day to 1 day, reduction to one-third compared with the time necessary for the EFSA method. The enzymatic method also decreased the need for frequency of GC-MS instrument cleaning.

The above results suggested some advantages of the enzymatic method in routine analysis of 3-MCPDEs and GEs in fats and oils and foods containing them. Another study investigating the effects of heating of refined oils on the formation of 3-MCPDEs and GEs in foodstuffs in 2017 used the enzymatic method\(^{40}\).

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