एफएसएसएआई fssai भारतीय खाद्य सुरक्षा और मानक प्राधिकरण Food Safety and Standards Authority of India स्वास्थ्य और परिवार कल्याण मंत्रालय Ministry of Health and Family Welfare	**Method for determination of 2-and 3-MCPD fatty acid esters and glycidol fatty acid esters in edible oils and fats by acid transesterification**		
Method No.	FSSAI.OM.MCPD & GLY. 002.2024	**Revision No. & Date**	0.0
Scope	This method is for the determination of fatty acid esters of 2-chloropropane-1,3-diol (2-MCPD), 3-chloropropane-1,2-diol (3-MCPD) and glycidol in vegetable oils and fats by GC-MS.		
Caution	Handle the reagents carefully.		
Principle	Glycidyl esters are converted to 3-monobromopropanediol (3-MBPD) mono esters in an acid solution containing a bromide salt. 3-MBPD together with 2- and 3-MCPD esters are then converted into the free form (non-esterified) in an acid methanolic solutions, which was subsequently derivatized with phenylboronic acid prior to GC-MS analysis.		
Apparatus/Instruments	Apparatus: 1) Vortex mixer 2) Oven 3) Ultrasonic bath 4) Nitrogen evaporator 5) Centrifuge 6) GC-MS Equipment consisting of: (a) Capillary GC coupled with a quadrupole mass selective detector and data processing system; (b) Bonded capillary column, poly(dimethylsiloxane) [Supelco Equity 1 or HP-1; 30 m x 0.25 mm i.d. x 1.0 µm film thickness]		
Reagents	1) Tetrahydrofuran, anhudrous 2) Methanol, analytical grade 3) n-Heptane, analytical grade 4) Acetone, analytical grade 5) Toluene, analytical grade 6) Water, Ultra-pure (Milli-Q) 7) Sulfuric acid (purity≥95%) 8) Sodium hydrogen carbonate (purity≥99%) 9) Sodium sulfate (purity≥99%) 10) Phenylboronic acid (purity≥97%) 11) Sodium bromide (purity≥99.5%)		
Solutions: Reagents	1) Acidified aqueous solution of sodium bromide: Dissolve 1 g of sodium bromide in 10 ml of ultra-pure water. Take 180 µl of this concentrated solution into an empty conical flask followed by the addition of 300 µl of sulfuric acid and 5.5 ml of water 2) Sodium hydrogen carbonate solution (0.6% w/v): Accurately weigh 0.6 g of Sodium hydrogen carbonate in a 100 ml volumetric flask and fill up to the mark with water. 3) Sulfuric acid/Methanol solution (1.8% v/v): Pipette 1.8 ml of sulfuric acid in a 100 ml volumetric flask and fill up to the mark with methanol. 4) Sodium hydrogen carbonate solution (saturated): Weigh 9.6 g of Sodium hydrogen carbonate in a 100 ml volumetric flask and fill up to the mark with water. 5) Sodium sulfate solution (20% w/v): Weigh 20 g of Sodium sulfate in a 100 ml volumetric flask and fill up to the mark with water.		

	6) Phenylboronic acid solution (saturated): Weigh 3 g of phenylboronic acid and add 12 ml of acetone:water (19:1, v/v) and shake vigorously.
Standards	1) 1,2-Dipalmitoyl-3-chloroprpanediol (PP-3-MCPD), purity ≥95% 2) 1,3-Dipalmitoyl-2-chloroprpanediol (PP-2-MCPD), purity ≥95% 3) Pentadeuterated 1,2-Dipalmitoyl-3-chloroprpanediol (PP-3-MCPD-d5), purity ≥95% 4) Glycidyl palmitate (Gly-P), purity ≥98% 5) Pentadeuterated Glycidyl palmitate (Gly-P-d5), purity ≥98%
Solutions: Standards	1. Stock Solutions: (a) Weigh 10 mg of PP-3-MCPD (Standard 1) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF (b) Weigh 10 mg of PP-2-MCPD (Standard 2) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF (c) Weigh 10 mg of PP-3-MCPD-d5 (Standard 3) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF (d) Weigh 10 mg of Gly-P (Standard 4) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF (e) Weigh 10 mg of Gly-P-d5 (Standard 5) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF 2. Working Solutions (a) Calibration I (PP-3-MCPD, 55 µg/ml): Pipette 550 µl of stock solution (Standard 1) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF. (b) Calibration II (PP-3-MCPD, 5.5 µg/ml): Pipette 1 ml of Calibration I solution in a 10 ml volumetric flask and fill up to the mark with either toluene or THF. (c) Calibration III (PP-2-MCPD, 55 µg/ml): Pipette 550 µl of stock solution (Standard 2) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF. (d) Calibration IV (PP-2-MCPD, 5.5 µg/ml): Pipette 1 ml of Calibration III solution in a 10 ml volumetric flask and fill up to the mark with either toluene or THF. (e) Calibration V (Gly-P, 100 µg/ml): Pipette 1 ml of stock solution (Standard 4) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF. (f) Calibration VI (Gly-P, 10 µg/ml): Pipette 1 ml of the Calibration solution V in a 10 ml volumetric flask and fill up to the mark with either toluene or THF. (g) Internal Standard I (PP-3-MCPD-d5, 40 µg/ml): Pipette 400 µl of stock solution (Standard 3) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF. (h) Internal Standard II (Gly-P-d5, 50 µg/ml): Pipette 500 µl of stock solution (Standard 5) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF.

Procedure: Test Sample Preparation	1. Accurately weigh ca 100-110 mg (±0.01 mg) of vegetable oil or fats in a screw cap test tube. Add 50 µl of Internal Standard I, 50 µl of Internal Standard II and 2 ml of THF. Mix for 15 s using vortex mixer. 2. Add 30 µl of acidified aqueous solution of sodium bromide (Reagent Solution 1) to the sample, mix vigorously using vortex and incubated at 50 ˚C for 15 min. Reaction was stopped by the addition of 3 ml of 0.6% aqueous solution of sodium hydrogen carbonate (Reagent Solution 2). Add 2 ml of n-heptane, mix for 15 s using vortex mixer and allowed to stand for phase separation. The upper layer was transferred to an empty glass tube and evaporated to dryness under a stream of nitrogen (35-40 ˚C). Dissolve the residue in 1 ml of THF. [if there is no proper phase separation, it is suggested to centrifuge the sample for efficient pfase separation]. *3.* Add 1.8 ml of sulfuric acid/Methanol solution (1.8% v/v) (Reagent Solution 3) to the sample, mix vigorously using vortex, close the cap of the glass tube tightly and incubate the mixture at 40 ˚C for 16 h (Overnight). 4. After the incubation period, stop the reaction by the addition of 0.5 ml of saturated sodium hydrogen carbonate (Reagent Solution 4), vortexed for 15 s and organic solvent was evaporated from the mixture under a stream of nitrogen. 5. Add 2 mL of sodium sulfate solution (Reagent Solution 5) and 2 mL of n-heptane and vortex the content for 2-3 min. After clear phase separation, discard the upper phase (containing FAME) and repeat the extraction of aqueous phase with n-heptane and discard the upper phase. 6. Add 250 µl of phenylboronic acid (Reagent Solution 5) into the lower aqueous phase and vortex the content for 10 s and incubate the mixture for 5 min in an ultrasonic bath (room temperature). 7. Extract the phenylboronic derivatives of 2- and 3- MCPD as well as MBPD by adding 1 ml of n-heptane, vortexed for 15 s and the upper phase was transferred to an empty glass tube. Repeat the extraction of the aqueous phase with 1 ml of n-heptane and combine the two organic extracts. Evaporate the organic phase to dryness under a stream of nitrogen. Dissolve the residue in 400 µl of n-heptane by shaking the mixture for 10 s and transfer the clear supernatant to an empty GC vial.
Preparation of the Calibration Curve:	1. Prepare 9 calibration solutions by pipetting 50 µl of both Internal Standard working solution (Internal Standard I and II) and the varied volume of calibration solutions (Calibration Solutions I to VI) as indicated in Table 1 (**Annexure I**). Add 2 ml of THF and mixed using vortex mixer. 2. Treat the calibration samples according to the procedure of test sample preparation (Steps 2 to 7)
Equipment	**1. GC condition** (a) Injection volume: 1.0 µl (b) Injection mode: Pulsed splitless (c) Injection temperature: 250 ˚C (d) Carrier gas: He (e) Flow rate: 0.8 ml/min (f) Oven temperature programming: 80 ˚C (1 min), raised to 170 ˚C at

	10 ˚C/min, raised from 170 ˚C to 200 ˚C at 3 ˚C/min, raised further from 200 ˚C to 300 ˚C at 15 ˚C/min and finally hold for 15 min at 300 ˚C. **2. MS Condition** (a) Transfer line temperature: 300 ˚C (b) Ion source temperature: 230 ˚C (c) Quadruple temperature: 150 ˚C (d) Ionization mode: EI, SIM mode (e) Parameters for SIM mode: (i) Phenylboronic acid derivative of 3-MCPD (m/z) 147 (quantifier ion); 196, 198 (qualifier ion) (ii) Phenylboronic acid derivative of 2-MCPD (m/z) 196 (quantifier ion); 198 (qualifier ion) (iii) Phenylboronic acid derivative of 3-MCPD-d5 (m/z) 150 (quantifier ion for 3-MCPD); 201 (quantifier ion for 2-MCPD); 203 (qualifier ion) (iv) Phenylboronic acid derivative of 3-MBPD (m/z) 147 (quantifier ion); 240 (qualifier ion) (v) Phenylboronic acid derivative of 3-MBPD-d5 (m/z) 150 (quantifier ion); 245 (qualifier ion) (f) Acquisition time window: 5 - 20 min
Calculation	***Quantification of 3-MCPD esters*** 1. Prepare a calibration curve by plotting the ratio of the amount of standard (expressed as free 3-MCPD equivalent) to amount of Internal Standard (expressed as free 3-MCPD-d5 equivalent) on the X-axis against the ratio of the corresponding peak areas on the Y-axis [A(m/z 147)/A(m/z 150) vs 3-MCPD (µg)/3-MCPD-d5 (µg)]. Ions at m/z 147 (3-MCPD) and 150 (3-MCPD-d5) are used for quantification. Calculate the regression analysis as follows: $y = ax + b$; where a = slope and b = Intercept 2. Determine the concentration of 3-MCPD esters in the test sample (mg/Kg) using the formula: $c = [(A_{147}/A_{150}) - b] \times IS \times 1/a \times 1/W$ Where, c = concentration of 3-MCPD esters in the test sample (mg/Kg oil); A_{147} = Area of the peak corresponding to 3-MCPD derivative (m/z 147); A_{150} = Area of the peak corresponding to 3-MCPD-d5 derivative (m/z 150); IS = Absolute amount (in µg) of internal standard added to the test sample; a = slope of the calibration curve; b = Intercept of the calibration curve; W = Weight of the test sample (in g) ***Quantification of 2-MCPD esters*** 1. Prepare a calibration curve by plotting the ratio of the amount of standard (expressed as free 2-MCPD equivalent) to amount of Internal Standard (expressed as free 3-MCPD-d5 equivalent) on the X-axis against the ratio of the corresponding peak areas on the Y-axis [A(m/z 196)/A(m/z 201) vs 2-MCPD (µg)/3-MCPD-d5 (µg)]. Ions at m/z 196 (2-MCPD) and 201 (3-MCPD-d5) are used for quantification. Calculate the regression analysis as

	follows: y = ax + b; where a = slope and b = Intercept 2. Determine the concentration of 2-MCPD esters in the test sample (mg/Kg) using the formula: $c = [(A_{196}/A_{201}) - b] \times IS \times 1/a \times 1/W$ Where, c = concentration of 2-MCPD esters in the test sample (mg/Kg oil); A_{196} = Area of the peak corresponding to 2-MCPD derivative (m/z 196); A_{201} = Area of the peak corresponding to 3-MCPD-d5 derivative (m/z 201); IS = Absolute amount (in µg) of internal standard added to the test sample; a = slope of the calibration curve; b = Intercept of the calibration curve; W = Weight of the test sample (in g) ***Quantification of glycidyl esters*** 1. Prepare a calibration curve by plotting the ratio of the amount of standard (expressed as glycidol equivalent) to the amount of Internal Standard (expressed as glycidol-d5 equivalent) on the X-axis against the ratio of the corresponding peak areas on the Y-axis [A(m/z 147)/A(m/z 150) vs Glycidol (µg)/Glycidol-d5 (µg)]. Ions at m/z 147 (3-MBPD) and 150 (3-MBPD-d5) are used for quantification. Calculate the regression analysis as follows: y = ax + b; where a = slope and b = Intercept 2. Determine the concentration of glycidyl esters in the test sample (mg/Kg) using the formula: $c = [(A_{147}/A_{150}) - b] \times IS \times 1/a \times 1/W$ Where, c = concentration of glycidyl esters in the test sample (mg/Kg of oil); A_{147} = Area of the peak corresponding to 3-MBPD derivative (m/z 147); A_{150} = Area of the peak corresponding to 3-MBPD-d5 derivative (m/z 150); IS = Absolute amount (in µg) of internal standard added to the test sample; a = slope of the calibration curve; b = Intercept of the calibration curve; W = Weight of the test sample (in g)
Reference	AOCS Official Method Cd 29a-13
Approved by	Scientific Panel on Methods of Sampling and Analysis

Note: The test methods given in the manual are standardised/ validated/ taken from national or international methods or recognised specifications, however it would be the responsibility of the respective testing laboratory to verify the performance of these methods onsite and ensure that it gives proper results before putting these methods in to use".

Table 1 **ANNEXURE**

Cal Sol No.	Cal Sol. (2a-2f)	2-MCPD [µg]	3-MCPD [µg]	Gly [µg]	Internal std sol. (2g-2h)	3-MCPD-d5 [µg]	Gly-d5 [µg]
Cal 0	0	0	0	0	50 µl 2g + 50 µl 2h	0.39	0.62
Cal 1	25 µl each of 2b, 2d, 2f	0.03	0.03	0.06	50 µl 2g + 50 µl 2h	0.39	0.62
Cal 2	50 µl each of 2b, 2d, 2f	0.05	0.05	0.12	50 µl 2g + 50 µl 2h	0.39	0.62
Cal 3	100 µl each of 2b, 2d, 2f	0.10	0.10	0.24	50 µl 2g + 50 µl 2h	0.39	0.62
Cal 4	20 µl each of 2a, 2c, 2e	0.21	0.21	0.47	50 µl 2g + 50 µl 2h	0.39	0.62
Cal 5	30 µl each of 2a, 2c, 2e	0.31	0.31	0.71	50 µl 2g + 50 µl 2h	0.39	0.62
Cal 6	50 µl each of 2a, 2c, 2e	0.52	0.52	1.19	50 µl 2g + 50 µl 2h	0.39	0.62
Cal 7	70 µl each of 2a, 2c, 2e	0.72	0.72	1.66	50 µl 2g + 50 µl 2h	0.39	0.62
Cal 8	90 µl each of 2a, 2c, 2e	0.93	0.93	2.13	50 µl 2g + 50 µl 2h	0.39	0.62

2a	PP-3-MCPD, 55 µg/ml			
2b	PP-3-MCPD, 5.5 µg/ml			
2c	PP-2-MCPD, 55 µg/ml			
2d	PP-2-MCPD, 5.5 µg/ml			
2e	Gly-P, 100 µg/ml			
2f	Gly-P, 10 µg/ml			
2g	PP-3-MCPD-d5, 40 µg/ml	50 µl = 2 µg		
2h	Gly-P-d5, 50 µg/ml	50 µl = 2.5 µg		

3. Working Solutions
   (j) Calibration I (PP-3-MCPD, 55 µg/ml): Pipette 550 µl of stock solution (Standard 1) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF. 2a
   (k) Calibration II (PP-3-MCPD, 5.5 µg/ml): Pipette 1 ml of Calibration I solution in a 10 ml volumetric flask and fill up to the mark with either toluene or THF. 2b
   (l) Calibration III (PP-2-MCPD, 55 µg/ml): Pipette 550 µl of stock solution (Standard 2) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF.
   (m) Calibration IV (PP-2-MCPD, 5.5 µg/ml): Pipette 1 ml of Calibration III solution in a 10 ml volumetric flask and fill up to the mark with either toluene or THF.

(n) Calibration V (Gly-P, 100 µg/ml): Pipette 1 ml of stock solution (Standard 4) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF.
(o) Calibration VI (Gly-P, 10 µg/ml): Pipette 1 ml of the Calibration solution V in a 10 ml volumetric flask and fill up to the mark with either toluene or THF.
(p) Internal Standard I (PP-3-MCPD-d5, 40 µg/ml): Pipette 400 µl of stock solution (Standard 3) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF.

Internal Standard II (Gly-P-d5, 50 µg/ml): Pipette 500 µl of stock solution (Standard 5) in a 10 ml volumetric flask and fill up to the mark with either toluene or THF.