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Method of Test for 2-MCPDEs, 3-MCPDEs and GEs in Infant Formula

1. Scope

This method is applicable to the determination of 2-monochloropropanediol esters (2-MCPDEs), 3-monochloropropanediol esters (3-MCPDEs) and glycidyl esters (GEs) in infant formula.

2. Method

After fat extraction, using acid catalysis to convert GEs to 3-monobromopropanediol monoesters (3-MBPDEs), and together with 2-MCPDEs and 3-MCPDEs, followed by extraction, transesterification and derivatization, analytes are determined by gas chromatography /tandem mass spectrometry (GC-MS/MS).

2.1. Equipment

2.1.1. Gas chromatograph/tandem mass spectrometer.

2.1.1.1. Ion source: electron ionization, EI.

2.1.1.2. Column: HP-5MS UI capillary column, 0.25 μm, 0.25 mm × 30 m, or an equivalent product.

2.1.2. Centrifuge: centrifugal force ≥ 5000 ×g.

2.1.3. Vortex mixer.

2.1.4. Ultrasonicator: with temperature control.

2.1.5. High speed dispersing device: SPEX SamplePrep 2010 GenoGrinder®, >1000 rpm, or an other machine shaker.

2.1.6. Water bath: with temperature control.

2.1.7. Nitrogen evaporator.

2.2. Chemicals

Tetrahydrofuran, GC grade;
Methanol, GC grade;
Acetone, HPLC grade;
Phenylboronic acid, HPLC grade;
Toluene, AR grade;
Ethyl acetate, AR grade;
n-Hexane, AR grade;
n-Heptane, AR grade;
Sodium bromide, AR grade;
Sodium hydrogen carbonate, AR grade;

Sodium sulfate anhydrous, AR grade;

Sulfuric acid, AR grade;

Deionized water, resistivity ≥ 18 MΩ·cm (at 25°C);

1,3-Dipalmitoyl-2-chloropropanediol (PP-2-MCPD), rac 1,2-bis-palmitoyl-3-chloropropanediol (PP-3-MCPD) and glycidyl palmitate (Gly-P), reference standards;

1,3-Dipalmitoyl-2-chloropropanediol-d_5 (PP-2-MCPD-d_5), rac 1,2-bis-palmitoyl-3-chloropropanediol-d_5 (PP-3-MCPD-d_5) and glycidyl palmitate-d_5 (Gly-P-d_5), isotope-labelled internal standards.

2.3. Apparatus

2.3.1. Centrifuge tube: 15 mL and 50 mL, PP.

2.3.2. Volumetric flask: 5 mL, 10 mL, 20 mL and 100 mL.

2.3.3. Solid phase extraction cartridge: Supelclean™ LC-NH_2, 6 mL, 500 mg, or an equivalent product.

2.4. Internal standard solution

Transfer about 1 mg of PP-2-MCPD-d_5, PP-3-MCPD-d_5 and Gly-P-d_5 isotope-labelled internal standards accurately weighed to each 10-mL volumetric flask, dissolve and dilute to volume with toluene as the internal standard stock solutions. Store in the dark under freezing. When to use, mix appropriate volume of each internal standard stock solution, and dilute with tetrahydrofuran to 10 μg/mL as the internal standard solution.

2.5. Standard solution

Transfer about 10 mg of PP-2-MCPD, PP-3-MCPD and Gly-P reference standards accurately weighed to each 100-mL volumetric flask, dissolve and dilute to volume with toluene as the standard stock solutions. Store in the dark under freezing. When to use, mix appropriate volume of each standard stock solution and the internal standard solution, dilute with tetrahydrofuran to 0-200 ng/mL (containing 50 ng/mL internal standard) as the standard solutions.

2.6. Reagents

2.6.1. 5% Sulfuric acid

Add 2.5 mL of sulfuric acid slowly into 40 mL of deionized water, and dilute with deionized water to 50 mL.

2.6.2. Sulfuric acid containing 0.3% sodium bromide

Dissolve and dilute 150 mg of sodium bromide with 5% sulfuric acid to 50 mL.

2.6.3. 0.6% Sodium hydrogen carbonate

Dissolve and dilute 3 g of sodium hydrogen carbonate with deionized water to 500 mL.

2.6.4. Methanol containing 1.8% sulfuric acid

Add 9 mL of sulfuric acid slowly into 400 mL of methanol, and dilute with methanol to 500 mL.

2.6.5. 20% Sodium sulfate

Dissolve and dilute 50 g of sodium sulfate with deionized water to 250 mL.

2.6.6. Sodium hydrogen carbonate saturated solution

Add 100 mL of deionized water to 15 g of sodium hydrogen carbonate. Stir and heat until sodium hydrogen carbonate is no longer dissolved. After cooling, take the supernatant as sodium hydrogen carbonate saturated solution.

2.6.7. 95% Acetone

Dilute 95 mL of acetone with deionized water to 100 mL.

2.6.8. Acetone containing 25% phenylboronic acid

Dissolve and dilute 5 g of phenylboronic acid with 95% acetone to 20 mL. Prepare freshly before use.

2.6.9. *n*-Hexane: ethyl acetate (85: 15, v/v)

Mix *n*-hexane and ethyl acetate at the ratio of 85: 15 (v/v). Prepare freshly before use.

2.7. Sample solution preparation

Transfer about 3 g of the homogenized liquid sample (fat content 3-5%) or 0.5 g of the homogenized powder sample (fat content 20-30%) accurately weighed into a 50-mL centrifuge tube. Add 10 μL of the internal standard solution, 12 mL of ethyl acetate and 12 mL of deionized water, vortex-mix, and ultrasonicate for 15 min in a water bath at 50°C. Shake at 1500 rpm by the high speed dispersing device for 3 min, and then centrifuge at 4500 ×g for 10 min. Add 10 g of sodium sulfate, shake at 1500 rpm by the high speed dispersing

device for 3 min, centrifuge at 4500 ×g for 10 min, and collect the upper layer. Add 12 mL of ethyl acetate to the lower layer, shake at 1500 rpm by the high speed dispersing device for 3 min, ultrasonicate for 15 min at 50°C, and centrifuge at 4500 ×g for 10 min, collect and combine the upper layers. Evaporate to dryness by gently flushing with a stream of nitrogen at 40°C, and dissolve the fat extracted(Note 1) with 2 mL of tetrahydrofuran. Add 30 μL of sulfuric acid containing 0.3% sodium bromide, vortex-mix, incubate for 15 min in a water bath at 50°C, and add 3 mL of 0.6% sodium hydrogen carbonate to stop the reaction. Add 2 mL of *n*-heptane, vortex-mix, centrifuge at 3500 ×g for 1 min, take the upper layer, and evaporate to dryness by gently flushing with a stream of nitrogen at 40°C. Dissolve the residue with 1 mL of tetrahydrofuran, add 1.8 mL of methanol containing 1.8% sulfuric acid, and incubate for 16 hr in a water bath at 40°C. Add 0.5 mL of sodium hydrogen carbonate saturated solution to stop the reaction, and evaporate the organic solvents of the mixture under gently flushing with a stream of nitrogen at 40°C. Add 2 mL of 20% sodium sulfate and 2 mL of *n*-heptane, vortex-mix, centrifuge at 3500 ×g for 1 min, and discard the upper layer. Add 2 mL of *n*-heptane to the lower layer, vortex-mix, centrifuge at 3500 ×g for 1 min, and discard the upper layer. Add 250 μL of acetone containing 25% phenylboronic acid to the lower layer, vortex-mix, and ultrasonicate for 5 min. Add 1 mL of *n*-heptane, vortex-mix, centrifuge at 3500 ×g for 1 min, and collect the upper layer. Add 1 mL of *n*-heptane to the lower layer, repeat the extraction procedure described above once, and combine the upper layers. Evaporate to dryness by gently flushing with a stream of nitrogen at 40°C, dissolve the residue with 0.4 mL of *n*-heptane, centrifuge at 5000 ×g for 10 min, and take the supernatant as the sample solution. Take an empty 50-mL centrifuge tube, add 10 μL of the internal standard solution, 12 mL of ethyl acetate and 12 mL of deionized water, and perform the same procedure described above as the blank solution(Note 2).

Note: 1. For samples known to contain significant levels of monoacylglycerols (MAGs) and diacylglycerols (DAGs), or

the results for GEs exceeding the regulatory limit, the fat extracted requires a cleanup step by the following procedure. Dissolve the fat extracted in section 2.7 with 1 mL of *n*-hexane: ethyl acetate (85: 15, v/v), transfer into the solid phase extraction cartridge pre-rinsed with 5 mL of *n*-hexane: ethyl acetate (85: 15, v/v), and elute with 10 mL of *n*-hexane: ethyl acetate (85: 15, v/v). Collect the eluent, and evaporate to dryness by gently flushing with a stream of nitrogen at 40°C. Dissolve the residue with 2 mL of tetrahydrofuran, add 30 μL of sulfuric acid containing 0.3% sodium bromide, and prepare the sample solution described in section 2.7.

2. Some consumables, such as glassware, filters and certain plastic materials, may contain free MCPD causing background contamination. The blank solution is used as a reference for quality control of the procedure, but it is not included in the result deduction.

2.8. Calibration standard curve

Separately take 2 mL of the standard solutions, add 30 μL of sulfuric acid containing 0.3% sodium bromide, vortex-mix, and incubate for 15 min in a water bath at 50°C, and follow the procedures described in section 2.7 to obtain the derivatized standard solutions. Operate GC-MS/MS according to the following conditions. Establish the calibration standard curve of each fatty acid ester by the ratios of the peak area of each fatty acid ester to that of the internal standard vs. the added concentrations in the range of 0-1000 ng/mL.

GC-MS/MS operating conditions(Note):

Column: HP-5 MS UI capillary column, 0.25 μm, 0.25 mm × 30 m.

Column temperature:

Initial temperature: 50°C, 1 min;

Temperature rising rate: 10°C/min;

Middle temperature: 210°C;

Temperature rising rate: 30°C/min;

Final temperature: 300°C, 5 min.

Injector temperature: 250°C.
Inject volume: 1 μL.
Carrier gas and flow rate: helium, 1 mL/min.
Interface temperature: 280°C.
Ion source temperature: 230°C.
Ion source: EI, 70 eV.
Injection mode: splitless.
Detection mode: multiple reaction monitoring (MRM). Detection ion pair and collision energy are shown as follows:

Analyte	Ion pair	Collision energy (eV)
	Precursor ion (*m/z*) > product ion (*m/z*)	
2-MCPD derivative	196 > 104*	15
	198 > 104	15
3-MCPD derivative	196 > 147*	5
	198 > 147	5
3-MBPD derivative**	240 > 147*	5
	242 > 147	5
2-MCPD-d_5 derivative (I.S.)	201 > 104	30
3-MCPD-d_5 derivative (I.S.)	201 > 150	5
3-MBPD-d_5 derivative (I.S.)	245 > 150	10

*The quantitative ion.

** 3-MBPD derivative is the reaction compound of GEs after catalysis, transesterification and derivatization.

Note: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2.9. Identification and quantification

Accurately inject 1 μL of the sample solution and the derivatized standard solutions into GC-MS/MS separately, and operate according to the conditions described in section 2.8. Identify each fatty acid ester based on the retention time and the relative ion intensities(Note 1). Calculate the amount of each fatty acid ester in the

sample by the following formula(Note 2):

The amount of each fatty acid ester (expressed as its nonesterified form) in the sample (μg/kg) = $\frac{C \times V}{M} \times F$

Where,

C: the concentration of each fatty acid ester in the sample solution calculated by the calibration standard curve (ng/mL)

V: the final make-up volume of the sample (0.4 mL)

M: the weight of the sample (g)

F: the conversion factor of each fatty acid ester standard to its nonesterified form

PP-2-MCPD and PP-3-MCPD: 0.1882

Gly-P: 0.2371

Note: 1. Relative ion intensities are calculated by peak areas of qualitative ions divided by peak areas of quantitative ions (≤ 100%). Maximum permitted tolerances of relative ion intensities are as follows:

Relative ion intensity (%)	Tolerance (%)
> 50	± 20
> 20~50	± 25
> 10~20	± 30
≤ 10	± 50

2. The amount of 3-MCPDEs, 2-MCPDEs and GEs in the sample are expressed as 3-MCPD, 2-MCPD and glycidol, respectively.

Remark

1. The limit of quantification (LOQ) for each fatty acid ester (expressed as its nonesterified form) is 2 μg/kg in liquid infant formula, and 10 μg/kg in powder infant formula.
2. Further validation should be performed when interfering compounds appear in the samples.

Reference

1. Ermacora, A. and Hrncirik, K. 2013. A novel method for simultaneous monitoring of 2-MCPD, 3-MCPD and glycidyl esters in oils and fats. J.

Am. Oil Chem. Soc. 90: 1-8.

2. Dubois, M., Empl, A. M., Jaudzems, G., Basle, Q. and Konings, E. 2019. Determination of 2- and 3-MCPD as well as 2- and 3-MCPD esters and glycidyl esters (GE) in infant and adult/pediatric nutritional formula by gas chromatography coupled to mass spectrometry method, first action 2018.03. J. AOAC Int. 102: 903-914.

Reference chromatogram

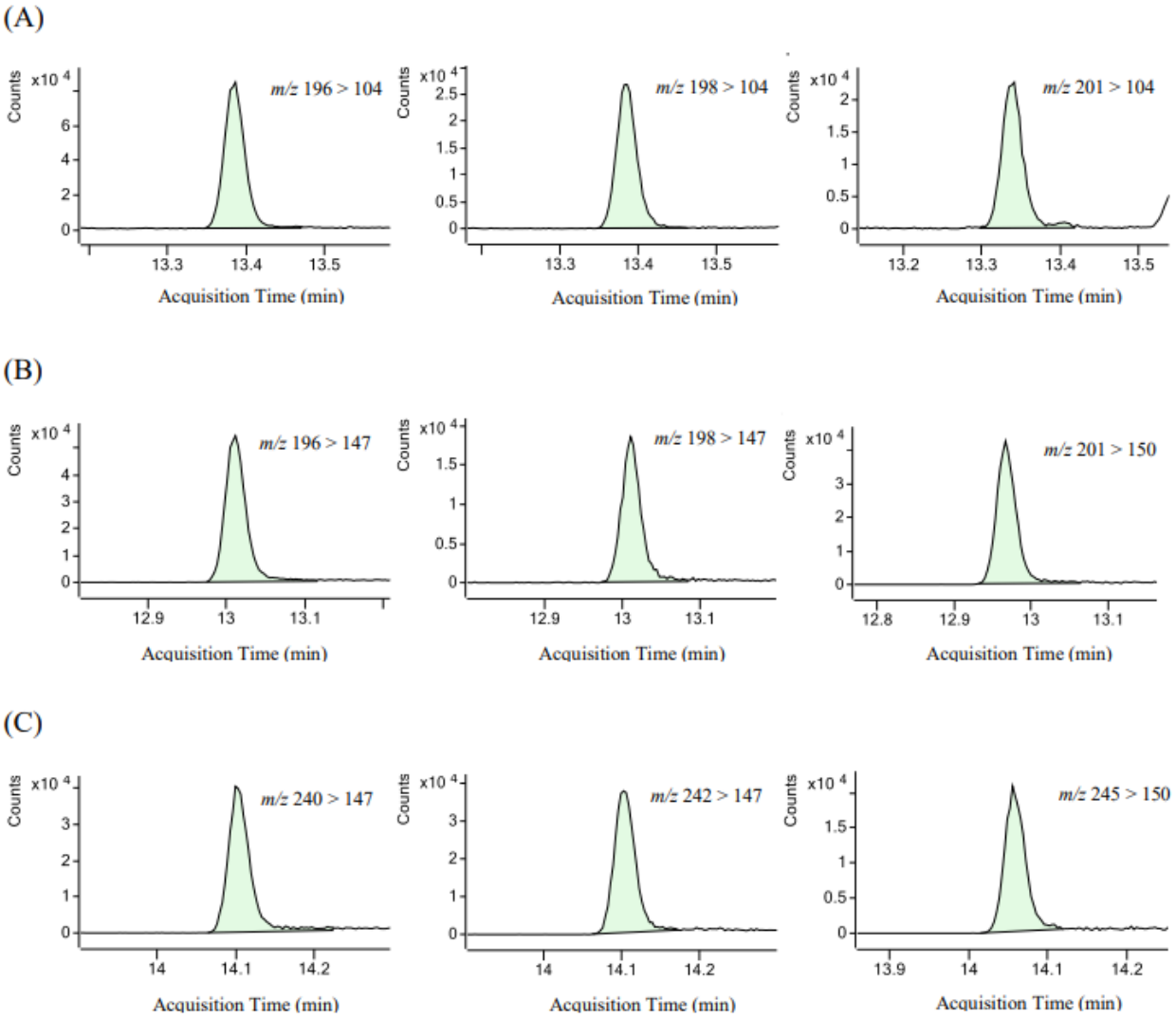

Figure. MRM chromatograms of 2-MCPD derivative (A), 3-MCPD derivative (B) and 3-MBPD derivative (C) of standards and derivatives of their isotope-labelled internal standards analyzed by GC-MS/MS.