Application Note

Food Testing and Agriculture

Determination of 2-MCPD and 3-MCPD Fatty Acid Esters in Infant Formula Using an Agilent 8890 GC System with an Agilent 5977B GC/MSD

Authors

Youjuan Zhang, Xia Yang, and Shuai Wang
Agilent Technologies (Shanghai) Co. Ltd. Shanghai 200131 P. R. China

Abstract

This application note describes a reliable analytical method for determining the fatty acid esters of 3-monochloropropane-1,2-diol (3-MCPD) and 2-monochloropropane-1,3-diol (2-MCPD) in infant formula. Two different derivatization reagents, heptafluorobutyrylimidazole (HFBI) and phenylboronic acid (PBA), were evaluated for sample preparation. An Agilent 8890 GC system coupled with an Agilent 5977B GC/MSD was used for qualitative and quantitative analyses. Results demonstrated the benefits of the workflow solution for the analysis of monochloropropanediols in infant formula. Great peak shape and resolution were obtained. Satisfactory recoveries were achieved, ranging from 86.9 to 106.7%. Precision was also good, with the relative standard deviations less than 15%.

Introduction

Monochloropropanediols (MCPDs) are commonly monitored in edible fats and oils. MCPDs are food contaminants that are generated during food processing. Studies have found that many of the MCPDs found in food exist in the form of chloropropanediol fatty acid esters (MCPDEs). Free forms of MCPDs are released from their esterified forms during digestion. The chemical structures of 2- and 3-MCPD and their esters are shown in Figure 1.

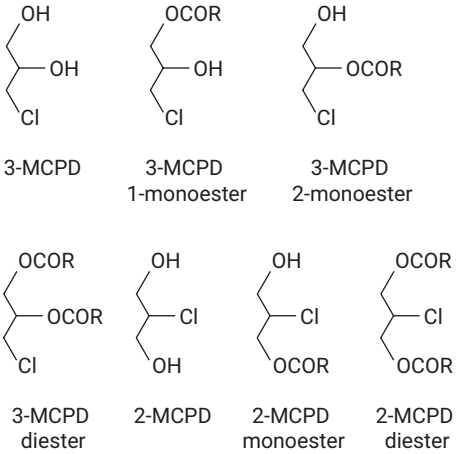

Figure 1. Chemical structures of 2- and 3-MCPD and their esters.

In general, analytical methods for the determination of MCPDEs follow two distinct routes: direct and indirect determination. The direct analysis of MCPDEs by LC/MS does not destroy the molecular structure. Due to the variety of MCPDEs and the difference of MCPDEs in different matrices, the corresponding standards and internal standards are needed for quantitative analysis using the direct LC/MS approach. The indirect determination of MCPDEs entails the cleavage of the MCPD from its esterified form, and therefore fewer standards are required. Several analytical methods have been standardized for the indirect analysis of MCPDEs in foods, such as: AOCS 2013a[1], AOCS 2013b[2], AOCS 2013c[3], ISO 18363-1[4], ISO 18363-2[5], ISO 18363-3[6], GB 5009.191-2016[7], and SN/T 5220-2019[8]. Those indirect methods all follow a similar protocol: cleavage of MCPD, sample cleanup, derivatization, and GC/MS analysis. The challenging steps are the cleavage of the MCPD from its esterified form (transesterification) and the derivatization reaction. The cleavage of MCPDs is carried out under acidic or alkaline conditions to form fatty acid methyl esters and MCPDs. Samples must be derivatized before GC/MS analysis due to the low volatility and high polarity of MCPDs. Despite the challenges and complex sample preparation, the indirect method is more popular than the direct method. The indirect determination is more desirable because of the low cost for standards and method versatility.

MCPDEs can potentially be found in various types of processed food. Many investigations have been reported about MCPDs analysis in oils.[9] However, analysis in milk matrices has been seldom reported. This application note specifies a procedure for the simultaneous determination of 2-MCPD and 3-MCPD fatty acid esters in infant formula in a single assay. The method is based on acid catalyzed ester cleavage and derivatization of the cleaved (free) analytes with HFBI or PBA prior to GC/MS analysis. Both the sample preparation and GC/MS conditions were optimized, and the performance of the analytical method was evaluated.

Experimental

Chemicals and reagents

All reagents and solvents were HPLC or analytical grade. Water was ultrapure, which was obtained using a purification system. *n*-Hexane, tetrahydrofuran (THF), heptane, sulfuric acid (purity ≥95%), methanol, acetone, heptafluorobutyrylimidazole (HFBI, purity ≥99%), rac 1,2-*bis*-palmitoyl-3-chloropropanediol (purity ≥98%), rac 1,2-*bis*-palmitoyl-3-chloropropanediol-d_5 (purity ≥99%), 1,3-distearoyl-2-chloropropanediol (purity ≥98%), 1,3-distearoyl-2-chloropropanediol-d_5 (purity ≥98%), and phenylboronic acid (PBA, purity ≥98%) were purchased from ANPEL Laboratory Technologies (Shanghai) Inc. Sodium hydrogen carbonate (purity ≥99.5%) was purchased from J&K Scientific Ltd. Sodium sulfate (purity ≥99%) was purchased from Sinopharm Chemical Reagent Co. Ltd.

Solutions and standards

- The stock solution of MCPD esters was prepared using hexane as the solvent.
- The sulfuric acid/methanol solution (1.8%, volume fraction) was prepared by pipetting 1.8 mL of sulfuric acid into a 100 mL volumetric flask and filling it up to the mark with methanol.
- The sodium hydrogen carbonate solution (9.6%, mass concentration) was prepared by weighing 9.6 g of sodium hydrogen carbonate into a 100 mL volumetric flask and filling it up to the mark with ultrapure water. An ultrasonic bath was used to ensure the complete dissolution of the reagent.
- The phenylboronic acid solution (PBA solution) was prepared by weighing 2.5 g of phenylboronic acid and adding 20 mL of an acetone/ultrapure water mixture (19/1, volume fraction). The mixture was then shaken vigorously.
- The sodium sulfate solution (20%, mass concentration) was prepared by weighing 20 g of sodium sulfate into a 100 mL volumetric flask and filling it up to the mark with ultrapure water. An ultrasonic bath was used to ensure the complete dissolution of the reagent.

Samples and calibration standard preparation

The infant formula samples were purchased from a local grocery store. The entire sample preparation workflow is shown in Figure 2. Four major parts are included in the sample preparation process: sample extraction, acid transesterification, sample cleanup, and derivatization of cleaved (free) analytes with HFBI or PBA. The first two steps, sample extraction and acid transesterification, are the same for all samples. The final two steps, cleanup and derivatization, are different based on which derivatization reagent is used. Users can choose one of the two processes for sample preparation according to their preference and laboratory situation.

Matrix blanks were created by taking clean infant formula samples through the entire sample preparation procedure. Matrix matched calibration standards were prepared by spiking a standard solution into the matrix after sample extraction and before acid transesterification. The calibration standards correspond to 10, 20, 50, 100, 200, 500, and 1,000 µg/kg (equivalent to free form). The concentration levels mentioned in this study are all equivalent to MCPD free form. Rac 1,2-*bis*-palmitoyl-3-chloropropanediol-d_5 and 1,3- distearoyl-2-chloropropanediol-d_5 were used as the internal standards for 3-MCPD and 2-MCPD esters respectively. Internal standards were prepared with a concentration of 100 µg/kg. Prespiked quality control (QC) samples were made by spiking the appropriate standard working solution into infant formula,

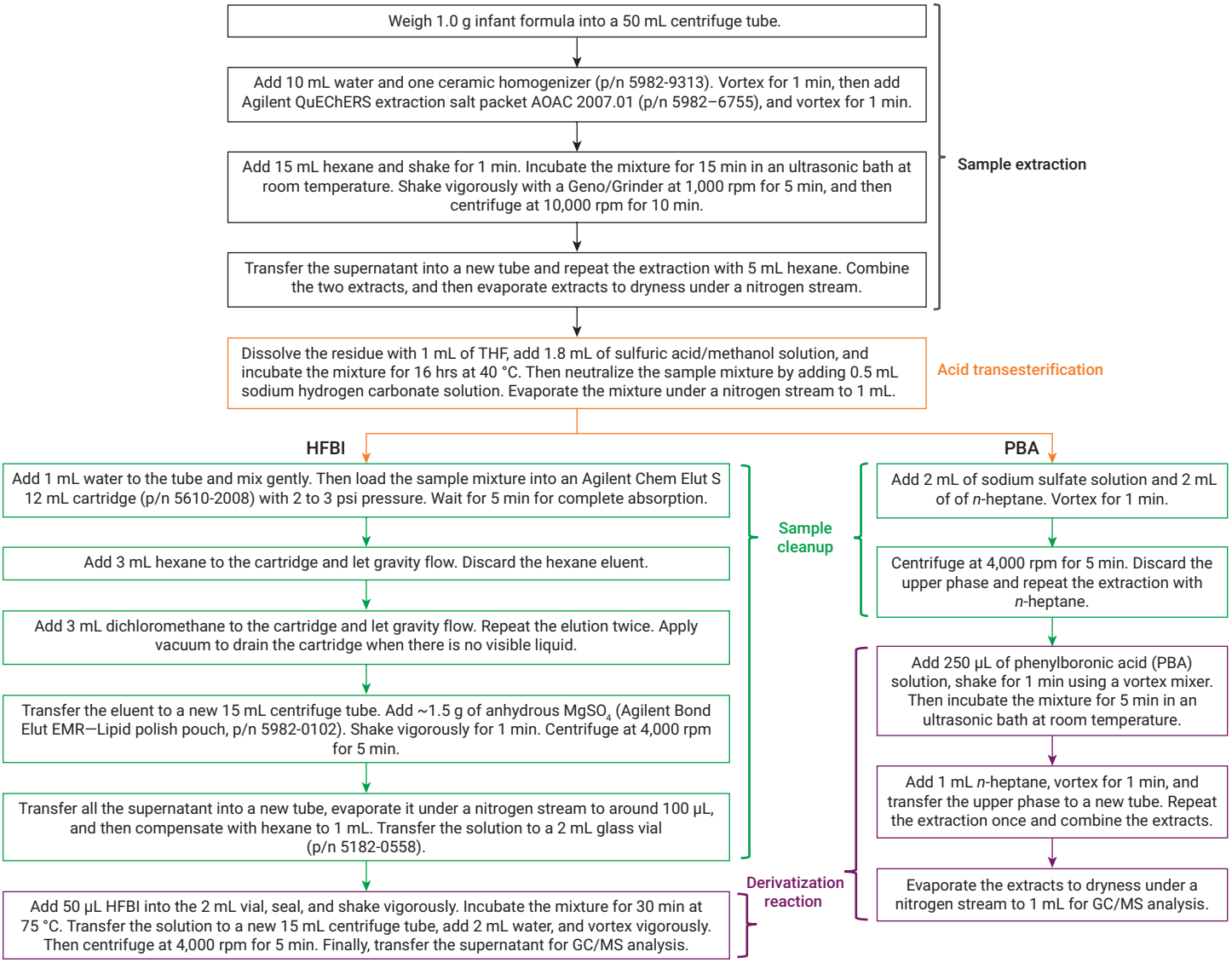

Figure 2. The step-by-step procedure for sample preparation using HFBI or PBA as the derivatization reagent.

vortexing for 1 minute, then allowing them to settle for 5 minutes to achieve equilibrium for the sample extraction process. QC samples were quantified against calibration curves at the level of 200 µg/kg with five replicates.

Equipment and material

Equipment and material used for sample preparation include:

- SPEX SamplePrep 2010 Geno/Grinder (Metuchen, NJ, USA)
- Eppendorf Centrifuge (Hamburg, Germany)
- Agilent Vac Elut 20 manifold (part number 12234101)
- Agilent QuEChERS extraction salt packets, AOAC 2007.01 method (part number 5982-6755)
- Agilent Bond Elut EMR—Lipid polish pouch, 3.5 g anhydrous $MgSO_4$ (part number 5982-0102)
- Agilent ceramic homogenizer for 50 mL (part number 5982-9313)
- Agilent Chem Elut S, 3 mL sample, 12 mL tube (part number 5610-2008)

Instrument conditions

Analyses were performed on an Agilent 8890 GC system with an Agilent 5977B GC/MSD. The instrument conditions are listed in Table 1.

Table 1. Conditions for MCPD analysis.

Parameter	Value
Injection Volume	1 µL
Inlet	Split/splitless; temperature: 280 °C; splitless mode, purge flow 60 mL/min at 0.75 min
Inlet Liner	Agilent Ultra Inert, splitless, single taper, glass wool (p/n 5190-2293)
Column	Agilent J&W DB-5ms Ultra Inert GC column, 30 m × 0.25 mm, 1 µm (p/n 122-5533UI)
Carrier Gas	Helium, 1 mL/min, constant flow
Oven Program	**HFBI derivatives:** 50 °C (1 min), 20 °C/min to 90 °C, 2 °C/min to 100 °C (4 min), then 30 °C/min to 300 °C; post run temperature: 310 °C, post run flow: 3 mL/min, post run time: 5 min **PBA derivatives:** 50 °C (1 min), 25 °C/min to 180 °C (2 min), 2 °C/min to 190 °C (2 min), then 30 °C/min to 230 °C (10 min) post run temperature: 310 °C, post run flow: 3 mL/min, post run time: 5 min
Transfer Line Temperature	280 °C
Source Temperature	230 °C
Quadrupole Temperature	150 °C
Acquisition Mode	SIM
EM Voltage Gain Mode	2
Solvent Delay	5 min
Tune File	Etune.u

Results and discussion

Chromatogram

This study was performed on an 8890 GC system equipped with a split/splitless inlet and a 5977B GC/MSD with an electron ionization (EI) source. The MSD data were collected in selected-ion monitoring (SIM) mode and analyzed by Agilent MassHunter software. The quantification of 2- and 3-MCPD is based on the 2- and 3-MCPD-d5 signal ratios, respectively. Figures 3 and 4 show the extracted-ion chromatogram (EIC) traces for target compounds in infant formula at the spiking level of 200 µg/kg with HFBI derivatization. The system shows great resolution. The target compounds, including the internal standards, are baseline separated. Figure 5 shows the EIC traces at the spiking level of 200 µg/kg with PBA derivatization.

Compared to HFBI, the internal standards and target compounds are not well separated when using PBA. The coeluting compounds do not share common MSD fragments, so the ions that are unique to each compound can be extracted and processed separately. Many nontarget peaks can be seen eluting before and after the target compounds in the chromatograms, which are caused by the multistep reaction in the sample preparation. The postrun function of MassHunter software was used with higher oven temperature and column flow. This postrun function can reduce the analysis time, increase the sample throughput, and also reduce the contamination for the MSD. Although the post column backflush device was not used in this study, it is still recommended because it effectively protects and reduces the contamination for the column and MSD.

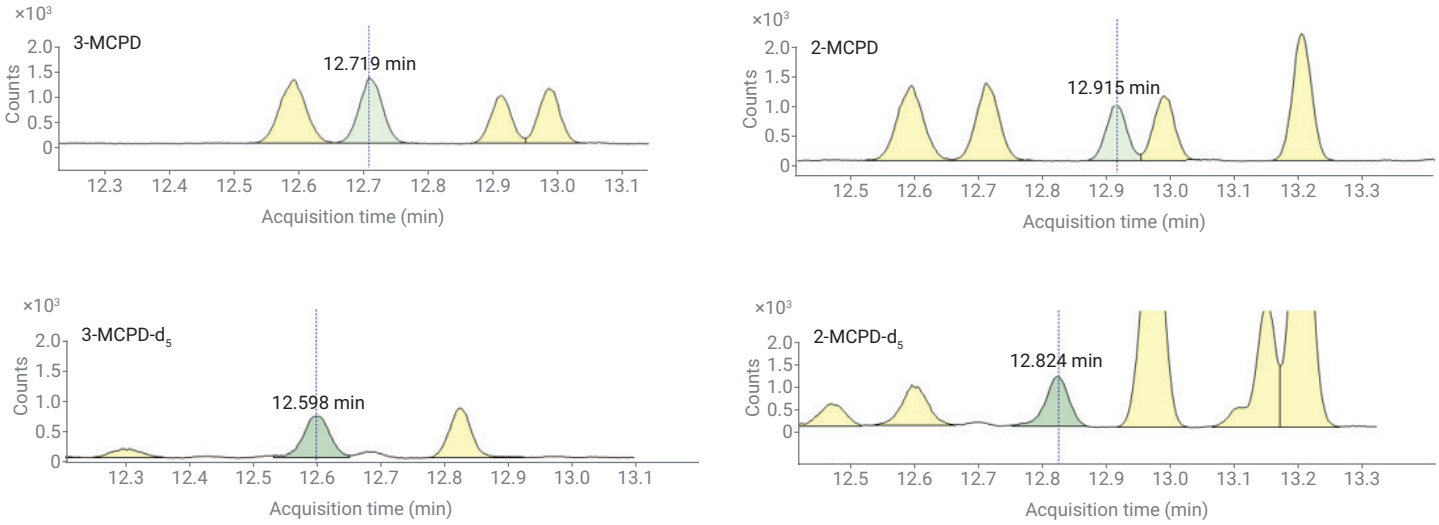

Figure 3. GC/MSD EIC trace of target compounds with HFBI derivatization at the spiking level of 200 µg/kg in infant formula.

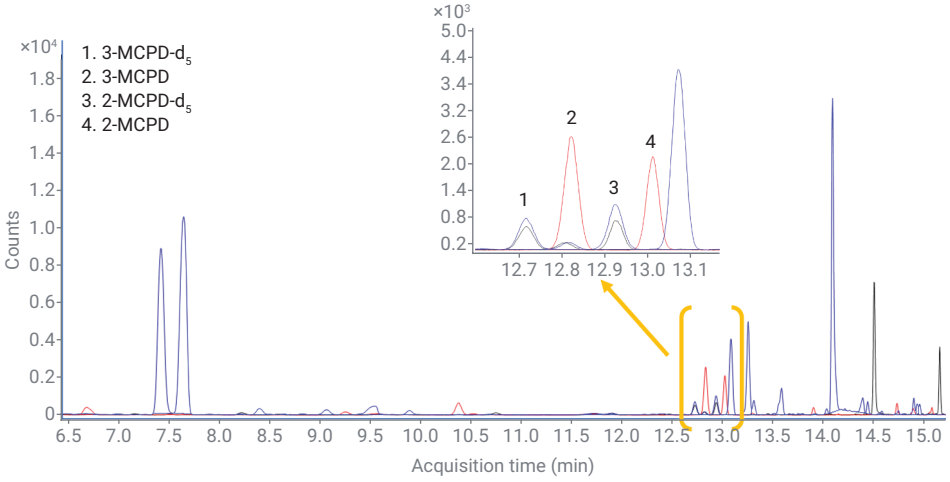

Figure 4. GC/MSD EIC trace of HFBI derivatives at the spiking level of 200 µg/kg in infant formula.

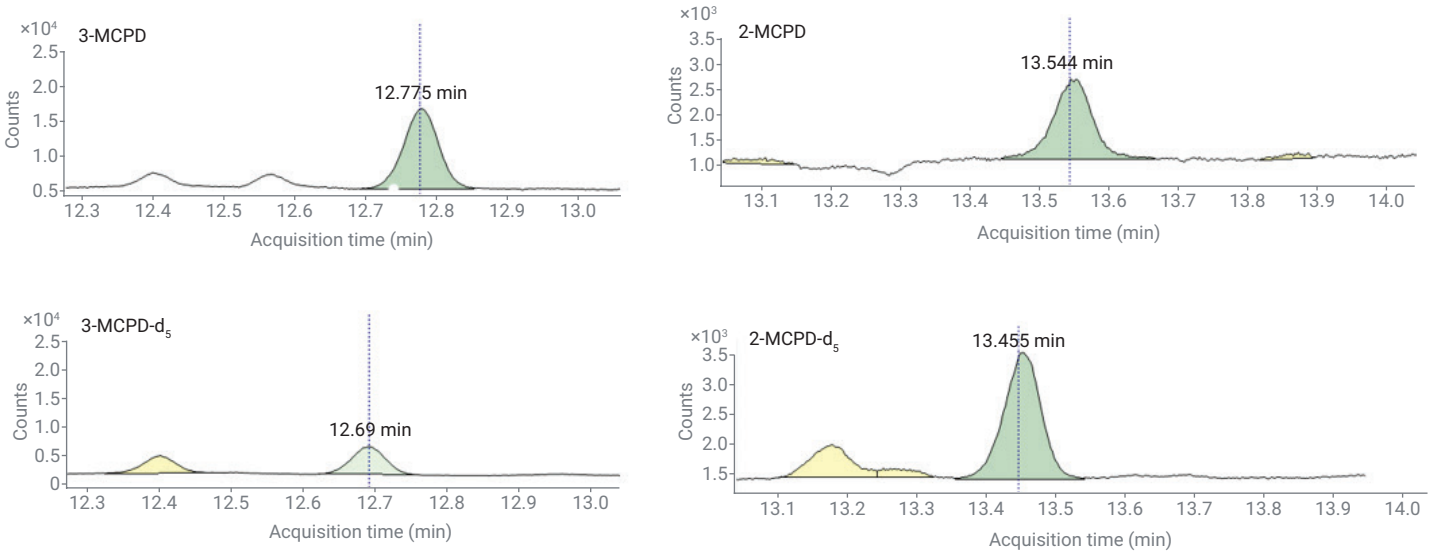

Figure 5. GC/MSD EIC trace of target compounds with PBA derivatization at the spiking level of 200 µg/kg.

Method optimization

Column evaluation: Stationary phase and dimensions are the most important factors in choosing the best capillary GC column. A low polarity column with dimensions of 30 m × 0.25 mm, 0.25 µm was recommended in methods GB5009.191-2016 and SN/T5220-2019. However, when using HFBI as the derivatization reagent, 3-MCPD and 2-MCPD could not be baseline separated on an Agilent J&W HP-5ms Ultra Inert, 30 m × 0.25 mm, 0.25 µm column, and 3-MCPD and 2-MCPD shared a quantitative ion, *m/z* 289 for high sensitivity. In this study, a column with thicker film, the Agilent J&W DB-5ms Ultra Inert, 30 m × 0.25 mm, 1 µm column, was used to improve resolution.

Salt packet evaluation: In the sample extraction process, 10 mL water and one ceramic homogenizer were added to 1 g of infant formula, and then 15 mL hexane was added for extraction. Emulsification appeared during the hexane extraction. However, a clear separation was observed between the aqueous and organic phases when salt was added to the sample. The addition of salt increases the ionic strength of the aqueous phase, which helps the lipids distribute into the organic phase, effectively reducing emulsification. In this study, the QuEChERS extraction salt packet (AOAC 2007.01 method) was used, which showed clear layers of separation, consistent extraction efficiency, and good recoveries.

Cleanup evaluation with HFBI derivatization: There are different processes available for sample cleanup. The Agilent Chem Elut S 12 mL supported liquid extraction (SLE) cartridge and a traditional liquid/liquid extraction (LLE) were compared. The step-by-step operation using the Chem Elut S is detailed in Figure 2. LLE was conducted in a centrifuge tube, with dichloromethane as the elution solvent. To improve extraction efficiency, a vortex mixer was used to mix the samples thoroughly. After centrifugation, the dichloromethane stayed at the lower layer for collection. The upper water layer was transferred to a new tube to repeat the extraction, which needs extra caution. This process was conducted three times to extract the target analytes efficiently, and the dichloromethane layers were combined. The response results from the two cleanup processes were similar, but LLE was more time-consuming and labor-intensive. Compared to LLE, SLE showed a walk-away extraction workflow without the need for special precautions. The less labor-intensive SLE method made it easier to extract the samples, and it also allowed for significant improvements to productivity and throughput. Thus, SLE was used in this study to provide a simplified workflow.

Solvent evaluation with HFBI derivatization: Dichloromethane was used as the elution solvent during the cleanup process, prior to derivatization. Two different solvents, hexane and dichloromethane, were investigated for the derivatization reaction and for use as the final sample solvent for the MCPD derivatives. As demonstrated in Figure 6, a higher response was obtained with hexane as the sample solvent for both 3-MCPD and 2-MCPD, which means improved sensitivity. Therefore, after SLE sample cleanup and water removal, the collected eluent (dichloromethane) was evaporated under a nitrogen stream to around 100 µL. The sample was then reconstituted with hexane to 1 mL before the derivatization reaction.

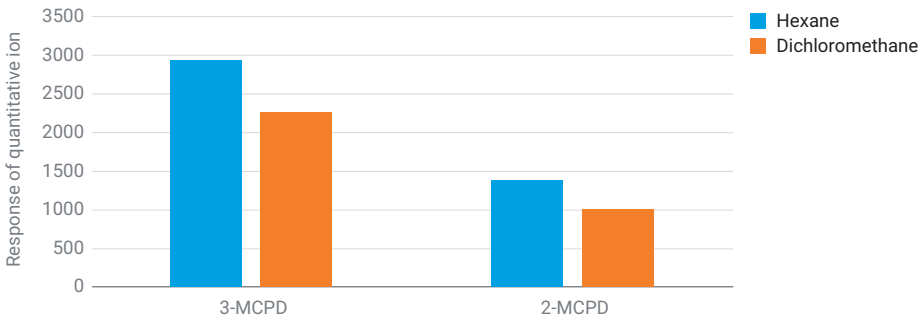

Figure 6. Comparison of different sample solvents with HFBI derivatization.

Method validation

MCPD compounds are highly polar and have high boiling points, which can cause peak shape issues. To improve the peak shape and sensitivity, MCPD compounds are usually derivatized before GC analysis. The selection of the best derivatization reagent is critical to the method success. The reaction speed, ease of operation, production of potentially interfering by-products, and selectivity are all factors that need to be considered. HFBI is recommended as the derivatization reagent in GB5009.191-2016 method, while PBA is used in ISO 18363-3, AOCS Cd 29a and SN/T5220-2019 methods. For HFBI methods, the derivatization reagent is sensitive to water, and the derivatization reaction will fail in the presence of water. Therefore, it is a critical step to remove water completely before HFBI derivatization. Also, attention should be paid to the storage of HFBI to prevent failure due to moisture absorption. The advantage of the HFBI method over the PBA method is that PBA only reacts with diols to form nonpolar cyclic products, while HFBI can react with all nucleophilic molecules. So, PBA is often selected as the derivatization reagent in the determination of 2-MCPD and 3-MCPD. However, HFBI is more suitable for the simultaneous determination of MCPD and dichloropropanol. When mass spectrometry is used for detection, high fragment ions could be chosen for HFBI derivatives to avoid interference with low molecular weight compounds. The advantage of the PBA method is that it is simpler and less time consuming than the HFBI method. For PBA, the derivatization reaction can occur in the presence of water and needs less reaction time. Both HFBI and PBA are evaluated in this study, and satisfactory performance is obtained with both.

HFBI method: Linearity, repeatability, detection limit, and recovery were tested in this study to evaluate the quantitative method with HFBI derivatization. Matrix matched calibration standards prepared at concentration levels from 10 to 1,000 µg/kg yielded correlation coefficient values (R^2) ≥ 0.997 for the two analytes. Calibration curves are shown in Figure 7. The repeatability measurements (concentration % RSD) were evaluated using eight injections of spiked samples at 10, 100, and 1,000 µg/kg. Table 2 lists the concentration RSDs, which were in the range of 0.5 to 3.1%, showing excellent performance. Eight repeat injections of matrix matched calibration standard with a low concentration level of 10 µg/kg were analyzed to calculate the detection limit. The detection limit for 3-MCPD and 2-MCPD were 1.35 and 1.30 µg/kg, respectively. Recoveries and precisions were determined at the spiking level of 200 µg/kg with five replicates. Mean recoveries were 86.9% for 3-MCPD and 106.7% for 2-MCPD with RSD <15%, as shown in Table 3.

PBA method: For the PBA method, the same spiking level of 200 µg/kg was analyzed as a QC sample for method validation. The detailed quantitation results are shown in Table 3. Acceptable recoveries of 80 to 120% were achieved for the two analytes with RSD <10%.

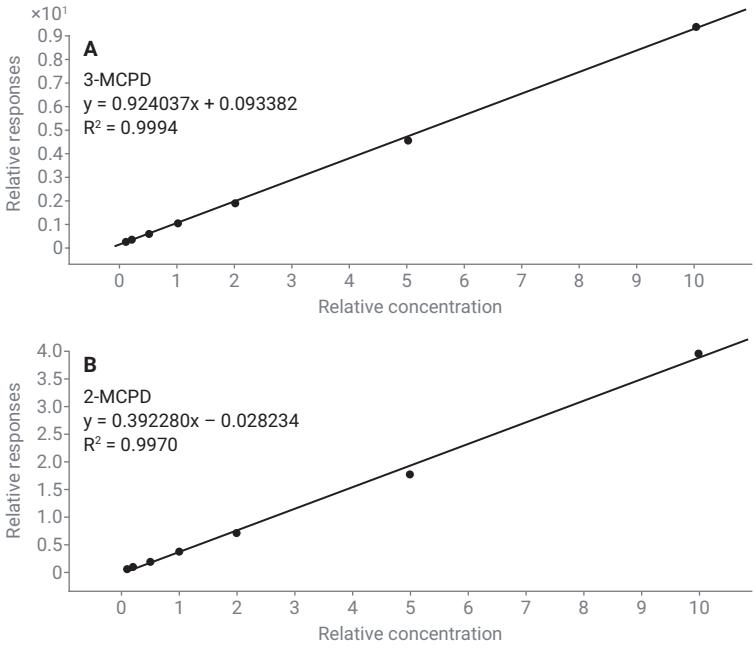

Figure 7. Calibration curve from 10 to 1,000 µg/kg for A) 3-MCPD HFBI derivative, and B) 2-MCPD HFBI derivative.

Table 2. Method quantitation results for HFBI derivatives.

Compound Name	Retention Time (min)	Concentration % RSD (n = 8)			Detection Limit (µg/kg)
		10 µg/kg	100 µg/kg	1,000 µg/kg	
3-MCPD	12.820	3.1	1.3	0.5	1.35
2-MCPD	13.012	2.1	1.6	1.3	1.30

Table 3. Recovery percentages and precision results for QC samples at 200 µg/kg (n = 5).

	HFBI			PBA		
Compound Name	Quant Ion	Mean Recovery %	% RSD	Quant Ion	Mean Recovery %	% RSD
3-MCPD	289	86.9	14.3	147	103.4	9.2
2-MCPD	289	106.7	5.8	198	100.9	4.2

Conclusion

This application note describes a solution for the simultaneous determination of 2-MCPD and 3-MCPD fatty acid esters in infant formula. Two reliable and robust workflows using different derivatization reagents for sample preparation were developed. The Agilent 8890 GC system with the Agilent 5977B GC/MSD was used for data acquisition and analysis. The method was validated by linearity, detection limit, recovery, and precision for HFBI derivatives. Recovery and precision results were also evaluated for PBA derivatives. The satisfactory results obtained from two sample preparation processes serve as a useful reference for MCPD esters analysis in infant formula.

References

1. 2- and 3-MCPD Fatty Acid Esters and Glycidol Fatty Acid Esters in Edible Oils and Fats by Acid Transesterification. *American Oil Chemist Society (AOCS)* **2013**.
2. Determination of Bound Monochloropropanediol-(MCPD-) and Bound 2,3-Epoxy-1-Propanol (Glycidol-) by Gas Chromatography/ Mass Spectrometry (GC/MS) (2013). *American Oil Chemist Society (AOCS)* **2013**.
3. Fatty-Acid-Bound 3-Chloropropane-1,2-Diol (3-MCPD) and 2,3-Epoxi-Propane-1-ol (Glycidol), Determination In Oils and Fats by GC/MS (Differential Measurement). *American Oil Chemist Society (AOCS)* **2013**.
4. Animal and Vegetable Fats and Oils—Determination of Fatty-Acid-Bound Chloropropanediols (MCPDs) and Glycidol by GC/MS—Part 1: Method Using Fast Alkaline Transesterification and Measurement for 3-MCPD and Differential Measurement for Glycidol. *International Organization for Standardization* **2015**, ISO 18363-1:2015.
5. Animal and Vegetable Fats and oils—Determination of Fatty-Acid-Bound Chloropropanediols (MCPDs) and Glycidol by GC/MS—Part 2: Method Using Slow Alkaline Transesterification and Measurement for 2-MCPD, 3-MCPD, and Glycidol. *International Organization for Standardization* **2018**, ISO 18363-2:2018.
6. Animal and Vegetable Fats and Oils—Determination Of Fatty-Acid-Bound Chloropropanediols (MCPDs) and Glycidol by GC/MS—Part 3: Method Using Acid Transesterification and Measurement for 2-MCPD, 3-MCPD, and glycidol. *International Organization for Standardization* **2017**, ISO 18363-3:2017.
7. Determination of Chloropropanol And its Fatty Acid Esters in Food. *National Food Safety Standard* **2016**, GB 5009.191-2016.
8. Determination of 3-Chloropropanol Esters (3-MCPDE) and Glycidol Esters GE in Food Products for Export—Gas Chromatography Mass Spectrometry Method. **2019**, SN/T 5220-2019.
9. Arisseto, A.; Marcolino, P. Determination of 3-MCPD Diesters and Monoesters in Vegetable Oils and Fats. *Agilent Technologies application note*, publication number 5991-3406EN, **2013**.

www.agilent.com/chem

DE44301.716712963

Printed in the USA, April 16, 2021
5994-3233EN

Agilent
Trusted Answers