American Journal of Analytical Chemistry, 2023, 14, 421-433
https://www.scirp.org/journal/ajac
ISSN Online: 2156-8278
ISSN Print: 2156-8251

Simultaneous Determination of 2- and 3-MCPD Esters in Infant Formula Milk Powder and Edible Vegetable Oils by Gas Chromatography-Mass Spectrometry

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How to cite this paper: Yao, H., Zeng, S.Q., Wang, X.H., Li, H. and Du, B. (2023) Simultaneous Determination of 2- and 3-MCPD Esters in Infant Formula Milk Powder and Edible Vegetable Oils by Gas Chromatography-Mass Spectrometry. *American Journal of Analytical Chemistry*, **14**, 421-433.
https://doi.org/10.4236/ajac.2023.1410024

Received: September 2, 2023
Accepted: October 15, 2023
Published: October 18, 2023

Abstract

Esters of 2- and 3-monochloropropane-1,2-diol (MCPD) are significative contaminants of processed edible oils used as foods or food ingredients. The aim of this study was to develop and validate a new method by GC-MS for the simultaneous quantification of 2 and 3-MCPD esters in infant milk powder and edible vegetable oils. The developed protocol included fat fraction in infant milk powder and edible vegetable oils samples was extracted and treated with sodium methylate-methanol to cleave the ester bonds of the 2- and 3-MCPD esters, moreover, standard samples of deuterium isotope-labeled 2- and 3-MCPD palmitic acid double esters and stearic acid double esters were used as the internal standards. Furthermore, this method was validated when it was applied to food products, concrete manifestation in its good accuracy (the recovery of MCPD esters ranged from 86% to 114%), high sensitivity (the LOD of 3-MCPD and 2-MCPD esters were 0.025 and 0.020 mg/kg, LOQ were 0.075, 0.060 mg/kg, respectively) and satisfactory repeatability (RSD below 6.8%) for all analytes. In the 150 commercial edible vegetable oils and infant formula milk powder samples, we obtained a preliminary profile of MCPD ester contamination.

Keywords

3-MCPD, 2-MCPD, GC-MS, Infant Milk Powder, Edible Vegetable Oils

1. Introduction

Fatty acid esters of chloropropanols (including 2-monochloropropane-1,3-diol

(2-MCPD) ester and 3-monochloropropane-1,2-diol (3-MCPD) ester) are known as contaminants in infant milk powder and edible vegetable oils [1]. As shown in **Figure 1**, MCPDs are chlorinated analogues of glycerol having a chlorine atom in positions 3 or 2 [2]. MCPD esters can take many forms, being either mono- or di-esters [3] [4]. Lately, toxicological and risk assessments of 3-MCPD, 2-MCPD and their fatty acid ester have been reviewed in detail in the literature and the 2016 European Food Safety Authority (EFSA) journal [5] [6]. So far, it can come to a conclusion that free 3-MCPD is a nongenotoxic carginogen in rats, and the Joint Expert Committee on Food Additives (JECFA) recommended a maximum tolerable daily intake for 3-MCPD from its esters of 4 μg/kg body weight per day [6]. While 2-MCPD is not nearly as well studied from a toxicological perspective, it has been shown to have heart and muscle affects [7]. Besides, 3-MCPD ester and 2-MCPD ester both have been identified as suspected genotoxic carcinogen by the European Union's Scientific Committee for Food (SCF) [8]. Therefore, serious attention has turned to these two MCPD and their easters in the past few years, in addition, efforts must be made to control the level of these contaminants present in the food products.

So far, there are two common approaches (*i.e.* the direct and indirect methods) to determine the content of MCPD ester [9]. The direct method involves liquid chromatography with minimum sample preparation [10] [11]. However, the determination of individual MCPD ester is extremely difficult due to the large amount of possible combination contributed by MCPD mono and di-esters structures [12] [13]. Currently, several indirect methods for the routine analysis of MCPD esters in infant milk powder and edible vegetable oils are currently available and have already been standardized by international organizations [13] [14] [15] [16] [17]. But all these methods have some common shortcomings which limit their abilities. In several Labs, the absolute recovery rate was in the range of 0.3% to 7% 2- and 3-MCPD esters and the derivatives of the internal standards, the data are found to significant changes among difference

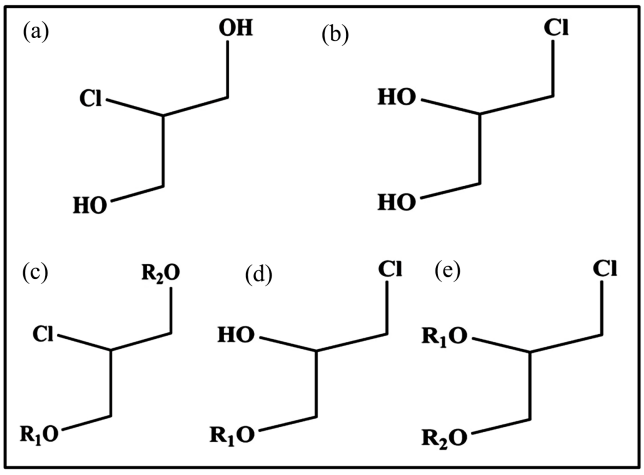

Figure 1. Structures of MCPD and their esters: (a) 2-MCPD; (b) 3-MCPD; (c) 2-MCPD di-ester; (d) 3-MCPD mono-ester; (e) 3-MCPD di-ester. R1, R2-acyl of fatty acid [2].

laboratory. However, these methods have been improved to some extent. For instance, a modified method has been reported by Kinuko Miyazaki *et al.* [18]. The results showed that the recovery was in the 80% - 110% range, and the absolute recovery rates were lower than the internal standards. Consequently, further improvements to these methods are urgently needed.

In this paper, we had developed a method for the determination of 3-MCPD ester (3-MCPDE) and 2-MCPD ester (3-MCPDE) in infant milk powder and edible vegetable oils, which based on the Chinese national standard GB 5009.191-2016. For the quantitation of the 3-MCPD ester and 2-MCPD ester, this study critically evaluated the effects of *n*-hexane, reaction time of methanolic sodium hydroxide solution and standard solutions, a remarkable result has been achieved by optimization of the reaction time of methanolic sodium hydroxide solution and selection of the standard solutions. This method employs an indirect approach using validation data to indicate good linearity, recovery, accuracy for 3-MCPD ester and 2-MCPD ester.

2. Materials and Methods

2.1. Chemicals and Reagents

3-MCPD palmitic acid double ester (3-MCPD ester, purity ≥ 98%), 2-MCPD stearic acid double ester (2-MCPD ester, purity ≥ 95%), 3-MCPD-d5 palmitic acid double ester, (3-MCPD-d5 ester, purity ≥ 98%, 98% atom), 2-MCPD-d5 stearic acid double ester, (3-MCPD-d5 ester, purity ≥ 98%, 98% atom), 3-Chloro-1,2-propanediol (3-MCPD, purity ≥ 98%), 2-Chloro-1,3-propanediol (3-MCPD, purity ≥ 98%),3-MCPD-d5, (purity ≥ 98%, 98% atom), 2-MCPD-d5, (purity ≥ 98%, 98% atom) were bought from Sigma, Saint Quentin Fallavier, France. Glacial acetic acid, Sodium bromide, sodium chloride, methyl tertiary butyl ether (MTBE) and anhydrous sodium sulfate (analytical grade) were purchased from Kelong, Chengdu, China. And sodium methoxide of analytical grade was supplied by Shanghai Macklin Biochemical Co, shanghai, china. Acetic ether, *n*-hexane, methanol were bought from Fisher Scientific, Fair Lawn, USA. N-Heptafluorobutyrylimidazole (HFBI) of analytical grade was manufactured by Tokyo Chemical Industry Co, Tokyo, Japan.

2.2. Standard Solutions of MCPD and MCPD Esters

Precisely 25.00 mg each of 3-MCPD palmitic acid double ester, 2-MCPD stearic acid double ester, 3-MCPD-d5 palmitic acid double ester, 2-MCPD-d5 stearic acid double ester, 3-Chloro-1,2-propanediol, 2-Chloro-1,3-propanediol, 3-MCPD-d5 and 2-MCPD-d5 was weighed and dissolved in acetic ether to prepare 1 mg/mL, and further diluted with *n*-hexane into working solutions at the concentration needed (10 μg/mL). Standard solutions were kept at −20˚C until needed.

2.3. Materials

All the food products were purchased from local supermarkets chosen by ran-

dom selection. The selected food products included three edible vegetable oils (rapeseed oil, sesame oil and peanut oil), three infant milk powder, three soy sauce.

2.4. Sample Preparation Procedure

The sample preparation procedure was described in the Chinese National Standard GB 5009.191-2016.

For soy sauce: a 4 g portion of the soy sauce was weighed precisely, to which 30 μL mixed internal standard working solution (3-MCPD-d5, 2-MCPD-d5, 10 μg/mL) was added and mixed thoroughly with a alkaline diatomite SPE colum (12 mL, 4 g; Agela technologies, USA). The sample was allowed to adsorb onto the column for 10 min, a washing step was done using 10 mL n-hexane to remove any interferences. The retained analytes were eluted using 2 × 10 mL ethyl acetate, which was then concentrated to approximately 0.5 mL in a rotavap under stream of nitrogen at 35˚C and the residue was re-dissolved in 2 mL *n*-hexane and measured. All the analysis was carried out in triplicate. Followed by addition of 0.040 mL HFBI, the mixture was then heated in a water bath at 70˚C for 20 min. And the mixture was cooled to room temperature, and 2 mL of 20% sodium chloride solutions was added, after vortexing for 10 sec. The organic layer was collected, dehydrated with anhydrous sodium sulfate. After filtration through a 0.22 μm membrane filter, the sample was subjected to GC-MS analysis.

For edible vegetable oils: 0.2 g portion of the edible vegetable oils was weighed precisely, to which 180 μL mixed internal standard working solution (3-MCPD-d5 ester, 2-MCPD-d5 ester, 10 μg/mL) was added, followed by addition of 0.5 mL methyl tert-butyl ether-acetic ether (8 + 2, v/v) and 1.0 mL of 0.5 mol/L methanol solution of sodium methoxide. After the mixture was mixed using a MS-3 vortex mixer (IKA, Germany) to allow hydrolysis for 3.0 min, 100 μL glacial acetic acid was quickly added to neutralize the remaining alkaline solution. 3 mL of 20% sodium bromide solutions was added, 3 mL n-Hexane was then added into the reaction system, and the clear aqueous fraction at the bottom of the test tube was collected. The aqueous fraction that contained the extracted MCPD esters was loaded onto an alkaline diatomite SPE colum (12 mL, 4 g; Agela technologies, USA), The sample was allowed to adsorb onto the column for 10 min, a washing step was done using 10 mL n-hexane to remove any interferences. The retained analytes were eluted using 2 × 10 mL ethyl acetate, which was then concentrated to approximately 0.5 mL in a rotavap under stream of nitrogen at 35˚C and the residue was re-dissolved in 2 mL *n*-hexane and measured. All the analysis was carried out in triplicate. Followed by addition of 0.040 mL HFBI, the mixture was then heated in a water bath at 70˚C for 20 min. And the mixture was cooled to room temperature, and 2 mL of 20% sodium chloride solutions was added, after vortexing for 10 sec. The organic layer was collected, dehydrated with anhydrous sodium sulfate. After filtration through a 0.22 μm membrane filter, the sample was subjected to GC-MS analysis.

For infant milk powder: a 2 g portion of the infant milk powder was weighed precisely, to which 180 μL mixed internal standard working solution (3-MCPD-d5 ester, 2-MCPD-d5 ester,10 μg/mL) was added, followed by addition of 3 mL water, the mixture was shaken by a high-speed shaker for 30 min to extracte the lipid fraction. The extracted lipid fractions were then concentrated to approximately 1 mL in a rotavap under stream of nitrogen at 35˚C. Followed by addition of 0.5 mL methyl tert-butyl ether-acetic ether (8 + 2, v/v) and 1.0 mL of 0.5 mol/L methanol solution of sodium methoxide. After the mixture was mixed using a MS-3 vortex mixer (IKA, Germany) to allow hydrolysis for 3.0 min, 100 μL glacial acetic acid was quickly added to neutralize the remaining alkaline solution. 3 mL of 20% sodium bromide solutions was added, 3 mL *n*-Hexane was then added into the reaction system, and the clear aqueous fraction at the bottom of the test tube was collected. The aqueous fraction that contained the extracted MCPD esters was loaded onto a alkaline diatomite SPE column (12 mL, 4 g; Agela technologies, USA), The sample was allowed to adsorb onto the column for 10 min, a washing step was done using 10 mL n-hexane to remove any interferences. The retained analytes were eluted using 2 × 10 mL ethyl acetate, which was then concentrated to approximately 0.5 mL in a rotavap under stream of nitrogen at 35˚C and the residue was re-dissolved in 2 mL *n*-hexane and measured. All the analysis was carried out in triplicate. Followed by addition of 0.040 mL HFBI, the mixture was then heated in a water bath at 70˚C for 20 min. And the mixture was cooled to room temperature, and 2 mL of 20% sodium chloride solutions was added, after vortexing for 10 sec. The organic layer was collected, dehydrated with anhydrous sodium sulfate. After filtration through a 0.22 μm membrane filter, the sample was subjected to GC-MS analysis.

2.5. GC Parameters

GC-MS analysis was carried out by Agilent Technologies Gas Chromatograph 7890B coupled with Agilent Technologies Mass Spectrometer 5977B with the separation of analytes on Agilent Technologies chromatographic column HP5-MS (30 m; id: 0.25 mm; film thickness: 0.25 μm, stationary phase: 95% PDMS, 5% phenyl groups) (Agilent Technologies, Santa Clara, CA, USA). For GC analysis, the injector was held at 250˚C, high-purity helium was used as the carrier gas at the flow rate of 0.8 mL/min., and the column temperature was programmed to maintain 50˚C for 1 min, followed by heating to 70˚C at the rate of 4˚C/min, after by heating to 76˚C at the rate of 1˚C/min, and then heating further to 300˚C at the rate of 40˚C/min, (and held for 4 min). Two microliter of the sample was injected in a splitless mode.

2.6. MS Parameters

MS was performed using a mass spectrometer (Model No. 5977B; Agilent) with electrospray ionization at 70 eV and an electron multiplier voltage of 1057 V. The temperature settings were ion source 230˚C, quadrupole 150˚C, and transfer

line 280˚C, with a solvent delay time of 6 min. Qualitative and quantitative analysis by mass spectrometer was carried out by monitoring target and qualifier ions in selected-ion monitoring (SIM) mode. The detection parameters are listed in Table 1.

2.7. Method Validation

In order to verify the reliability of the method and the accuracy of the experimental results, we chose to collaborate with each of three laboratories (Lab A, Hubei Institute for Food and Drug Control; Lab B, Shenzhen Academy of Metrology & Quality Inspection; Lab C, Henan Provincial Institue of Food and Drug Control). Specifically, we randomly gave them a same sample of edible vegetable oil. And then, they used the same method as we did to determine 2- and 3-MCPD Esters of the identical sample. Lastly, the results of these experiments would be compared.

3. Results and Discussion

Alkaline hydrolysis (methanol solution of sodium methoxide) of the 3-MCPD esters, 2-MCPD esters and deuterated isotope internal standards generated the corresponding MCPDs and their deuterated counterparts to allow GC-MS analysis after derivatization. Figure 2 present the SIM of the 2-MCPD, 3-MCPD,

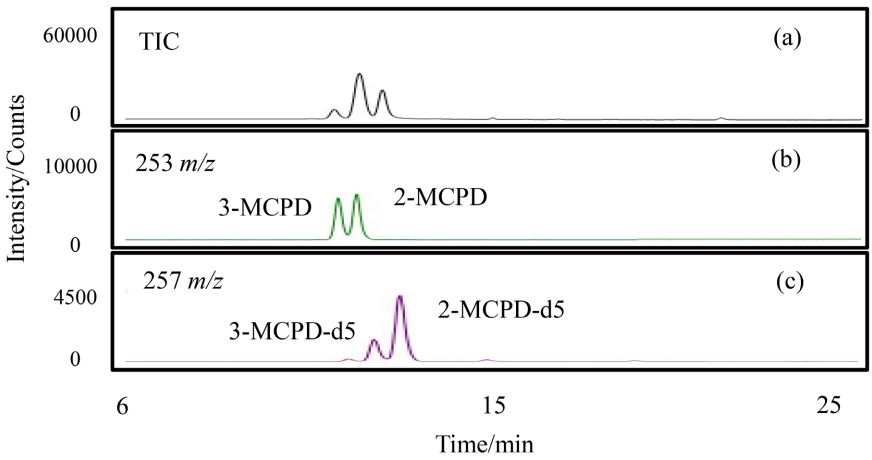

Figure 2. SIM of the MCPDs and deuterated MCPDs. The concentrations of samples and internal standard were 0.5425 and 0.1500 μg/ml, respectively.

Table 1. SIM detection mass spectrometry parameters.

Compounds	Retention time, min	Target ion, *m/z*	Qualifiers ion 1, *m/z*	Qualifiers ion 2, *m/z*	Qualifiers ion 3, *m/z*
2-MCPD	13.043	75	253	289	291
2-MCPD-d5	13.241	79	257	294	296
3-MCPD	12.851	253	275	289	291
3-MCPD-d5	13.082	257	278	294	296

2-MCPD-d5 and 3-MCPD-d5. However, the results have showed that the signal intensity of 3-MCPD-d5 was considerably less than 2-MCPD-d5 in the same conditions. For this phenomenon, we speculate that it may be due to their different chemical structures, which lead to different ionic efficiency, and thus produce significantly different responses eventually.

3.1. Optimal Alkalinity for Hydrolysis

In the study, we used methanol solution of sodium methoxide for ester hydrolysis based on the method described by Liu *et al.* [19] The results obtained in collaboration with other laboratories have showed that the absolute recovery rates was in the range of 0.3% to 7% of 2- and 3-MCPD esters and the derivatives of the internal standards, and the results of each laboratory had great difference (**Figure 3**).

The Chinese National Standard GB 5009.191-2016 compared the sample preparation procedure of three methods. For soy sauce, the method did not rely on alkalinity for Hydrolysis. In order to confirm and verify that absolute recovery rate was impacted by the alkalinity for Hydrolysis, we establish a fair comparison between with the methods, three concentrations of 3-MCPD-d5 ester, 2-MCPD-d5 ester, 2-MCPD-d5 and 3-MCPD-d5 were prepared in matrix-matched derivatization. The results showed that for soy sauce, absolute recovery rate was in the range of 60% to 70% of 2-MCPD-d5 and 3-MCPD-d5. These results demonstrate that absolute recovery rates were significant impacted by the alkalinity for Hydrolysis.

We establish a fair comparison between methanol solution of sodium methoxide and methanol solution of sulfuric acid for ester hydrolysis based on the methods described by GB 5009.191-2006 and Ermacora *et al.* [20] **Table 2** showed

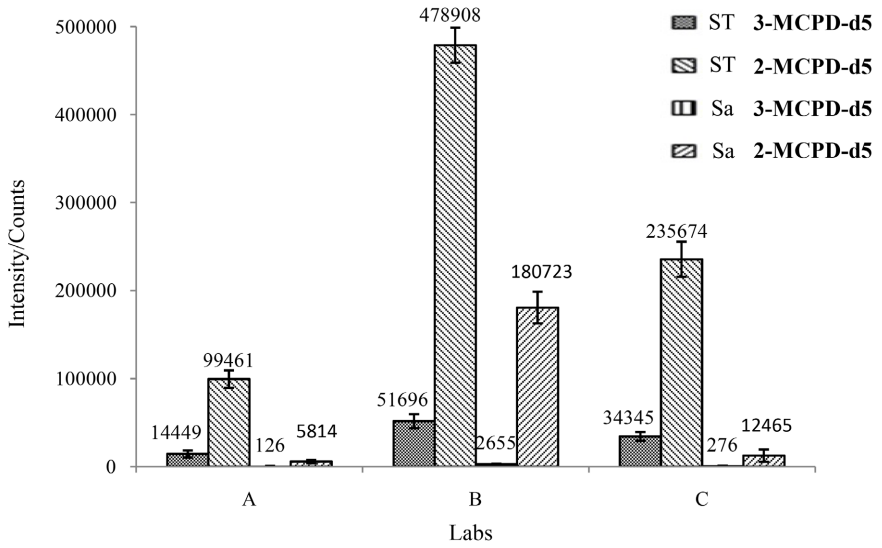

Figure 3. The absolute recovery of the deuterated MCPDs. ST, standards; Sa, samples. Lab A, Hubei Institue for Food and Drug Control; Lab B, Shenzhen Academy of Metrology & Quality Inspection; Lab C, Henan Provincial Institue of Food and Drug Control.

Table 2. Comparison of intensity between methanol solution of sodium methoxide and methanol solution of sulfuric acid.

Compounds	edible vegetable oils (methanol solution of sodium methoxide)		infant milk powder (methanol solution of sodium methoxide)		Edible vegetable oils (methanol solution of Sulfuric acid)		infant milk powder (methanol solution of Sulfuric acid)	
	intensity	Concentration, mg/L	intensity	Concentration, mg/L	intensity	Concentration, mg/L	intensity	Concentration, mg/L
3-MCPDE	1580	1.5178	132	0.1347	53,031	2.4305	6994	0.2177
2-MCPDE	56,000	0.5553	4713	0.0945	122,400	1.3451	6588	0.1126
3-MCPDE-d5	132	/	106	/	2784	/	3702	/
2-MCPDE-d5	22,689	/	9533	/	20,917	/	11,519	/

that, the intensity of 2-MCPD-d5 was found to no significant changes among identical samples, and acid for hydrolysis achieved the higher intensity of 3-MCPD-d5. During the experiment, we found a higher concentration of 3-MCPD and 2-MCPD among different samples, and it was consistent with the situdation described by Liu *et al.* [19]. Furthermore, it is speculated that a high concentration of Sulfuric acid in the reaction system tended to result in excessive hydrolysis of the esters, giving rise to undesired substances.

In a subsequent experiment, it has already shown that a high concentration of sodium methoxide in the reaction system tended to result in excessive hydrolysis of the esters, giving rise to undesired substances, whereas a low concentration was not sufficient to allow full ester hydrolysis [11].

We optimized sodium methoxide concentration by dissolving 2.7 g sodium methoxide in 100 mL methanol to prepare a 0.5 mol/L methanol solution of sodium methoxide, which achieved the highest hydrolytic efficiency [15].

3.2. Optimized Duration of Hydrolysis

We optimized the duration of hydrolysis and found that hydrolysis for 3.0 min achieved the highest intensity of 2- and 3-MCPD esters and the derivatives of the internal standards (**Table 3**).

3.3. Selection of the Standards

In this paper, the standard used was based on Chinese National Standard GB 5009.191-2016, AOCS, AOAC and Noor Asma Shaari *et al.* [21]. For the construction of calibration curves, all the working solutions were prepared from their standard stock solutions for 3-MCPD and 2-MCPD, respectively. Internal standards were also prepared from their stock solutions 3-MCPD-d5 and 2-MCPD-d5. Whereas, for infant formula milk powder and edible vegetable oils samples, we used 3-MCPDE-d5 palmitic acid double ester and 2-MCPDE-d5 stearic acid double ester as the internal standards. As shown in **Table 4**, for the 3-MCPDE, compared with the other two Labs, Lab C enhanced the mean content

Table 3. Impact of transesterification time of 2- and 3-MCPD esters and the derivatives of the internal standards on the intensity.

Compounds	intensity				
	transesterification time, 2 min	transesterification time, 3 min	transesterification time, 4 min	transesterification time, 8 min	transesterification time, 12 min
3-MCPDE	1256	2139	1007	153	40
2-MCPDE	16,567	21,089	12,084	5763	2797
3-MCPDE-d5	1097	1978	836	242	15
2-MCPDE-d5	5367	7743	4861	2257	859

Table 4. The results of the identical sample in 3 laboratories.

Compounds	Lab A Content, mg/kg	Lab B Content, mg/kg	Lab C Content, mg/kg
3-MCPDE	48.2	49.4	78.5
2-MCPDE	34.7	17.8	35.1

Lab A, Hubei Institute for Food and Drug Control; Lab B, Shenzhen Academy of Metrology & Quality Inspection; Lab C, Henan Provincial Institute of Food and Drug Control.

by over 58%. Besides, for the 2-MCPDE, compared with Lab B, Lab A and Lab C enhanced the mean content by over 94%. These results demonstrate that the content was impacted by the standards of calibration curves. If 3-MCPDE-d5 palmitic acid double ester and 2-MCPDE-d5 stearic acid double ester as the internal standards shall be used, 3-MCPDE, 2-MCPDE, 3-MCPDE-d5 and 2-MCPDE-d5 appears to be the better option.

3.4. Method Validation

3.4.1. Specificity

Figure 2 showed that there were no interfering peaks at the retention times of the targeted MCPD esters.

3.4.2. Linearity

The calibration curves of the 2 target MCPD esters were linear in the range of 0.025 - 20.0 mg/L and the internal standards. All the standard working solutions contained 0.150 mg/L of the internal standards. Two microliters of each standard working solution underwent hydrolysis, extraction with the alkaline diatomite SPE colum, and derivatization with HFBI for GC-MS analysis. The standard curve was drawn, with the MCPD-to-internal standard ratio of chromatographic peak area as the ordinate and MCPD and internal standard concentration as the abscissa with the correlation coefficient not less than 0.9992.

3.4.3. Limits of Detection (LODs) and Quantification (LOQs)

As shown in Table 5, the LODs for 3-MCPDE and 2-MCPDE were 0.025 mg/kg and 0.020 mg/kg in edible vegetable oils and infant milk powder, and the LOQs were 0.075 mg/kg and 0.060 mg/kg, respectively.

Table 5. The linear equations, LOD, and LOQ.

Compounds	Linear equation	Linear range, mg/L	LOD, mg/kg	LOQ, mg/kg
3-MCPDE	$y = 1.063x - 0.021$	0.025 - 20.0	0.025	0.075
2-MCPDE	$y = 0.697x + 0.021$	0.025 - 20.0	0.020	0.060
3-MCPDE-d5	-	-	-	-
2-MCPDE-d5	-	-	-	-

3.4.4. Accuracy and Precision

A spiked recovery experiment was performed to test the accuracy and precision of the method, blank infant formula milk powder and edible vegetable oils were spiked with 0.075, 0.150, and 0.300 mg/kg 2-MCPD stearic acid double ester and 3-MCPD palmitic acid double ester, respectively. The recovery of MCPD esters ranged between 86% and 114%. The RSD value was in the range 0.6% - 6.8% for 2-MCPD stearic acid double ester and 3-MCPD palmitic acid double ester.

3.4.5. Application to Real Samples

A total of 150 samples (50 edible vegetable oils samples and 100 infant formula milk powder samples) available from local markets were analyzed using the established method. Of these 100 infant formula milk powder samples, 100 (100%) were found positive for 3-MCPD ester, with contents ranging from 0.0451 to 2.184 mg/kg (mean, 0.382 mg/kg), and 8 (8.0%) were found positive for 2-MCPD ester, with contents ranging from 0 to 0.0231 mg/kg (mean, 0.0393 mg/kg). Of these 50 edible vegetable oils samples, 32 (64.0%) were found positive for 3-MCPD ester, with contents ranging from 0 to 48.2 mg/kg (mean, 3.420 mg/kg), and 29 (58.0%) were found positive for 2-MCPD ester, with contents ranging from 0 to 34.7 mg/kg (mean, 1.947 mg/kg). The levels of 3-MCPD esters and 2-MCPD esters derived from the 150 samples are consistent with that of Kazua koyama [22]. Haines [23] reported that 3-MCPD esters were not detected in several edible vegetable oils with the LOD of 1 mg/kg and 3-MCPD esters were present at concentrations of 3.7 - 6.2 mg/kg. Additionally, Wang [11] reported that 3-MCPD esters were not detected in 17.1% infant formula milk powder with the LOD of 0.03 mg/kg, and 2-MCPD esters were detected in 3.2% infant formula milk powderpresent with the LOD of 0.03 mg/kg.

4. Conclusion

In the present study, we established a GC-MS method for simultaneous detection of 3- and 2-MCPD esters in edible vegetable oils and infant formula milk powder. Compared with the Chinese National Standard GB 5009.191-2016 using MCPDs and deuterated MCPDs for the construction of calibration curves, the method is accurate and reliable. The method validation data including calibration, LOD/LOQ, accuracy and repeatability and specificity indicated that the new method could be successfully applied to the determination of 3- and 2-MCPD esters. By the method, we obtained a preliminary profile of MCPD ester conta-

mination in edible vegetable oils and infant formula milk powder products.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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