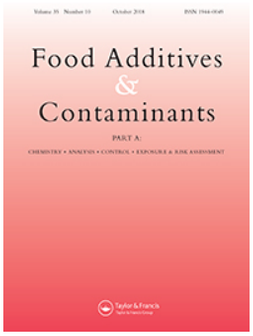

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An investigation of presence of 2- and 3-monochloropropanediol fatty acid esters in Canadian human milk samples

Adam Becalski[a], Tony Zhao[a], Michael Granvogl[b] and Tye Arbuckle[c]

[a]Food Research Division,Bureau of Chemical Safety, Health Products and Food Branch, Health Canada, Ottawa, Canada; [b]Department of Chemistry,Chair for Food Chemistry,Technical University of Munich, Freising, Germany; [c]Population Studies Division,Healthy Environments and Consumer Safety Branch, Health Canada, Ottawa, Canada

ABSTRACT

Occurrence of 2- and 3-monochloropropanediol fatty acid esters (MCPDEs) in 199 human milk samples collected from Canadian women was examined in this study. MCPDEs were determined via indirect analytical approach by using acidic hydrolysis/transesterification and derivatization with cyclohexanone using a fluorinated sulfonic acid resin as a catalyst followed by GC-MS analysis. Analyses were done by a stable isotope dilution assay (SIDA) using 3-monochloropropanediol-d_5 dipalmitate and 2-monochloropropanediol-d_5 distearate as internal standards. In all samples 2-MCPD esters were not detected, at LOD = 1 ng/g for 2-MCPD equivalent. 3-MCPD esters were not detected, at LOD = 2 ng/g for 3-MCPD equivalent, in 193 samples. Six samples had the apparent concentration of 3-MCPD equivalent in the range of 2.0–5.1 ng/g, which was likely caused by accidental contamination during processing of samples. For selected samples, these findings were confirmed by direct analysis via LC-MS/MS analysis based on SIDA for 3-MCPD dipalmitate and 2-MCPD distearate. To our best knowledge, this is the first study to analyze human milk samples for 2-MCPD esters.

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2-monochloropropanediol; 3-monochloropropanediol; monochloropropanediol fatty acid esters; gas chromatography-mass spectrometry (GC-MS); liquid chromatography-tandem mass spectrometry (LC-MS/MS); human milk; bio-monitoring; stable isotope dilution analysis

Introduction

Several recent studies have identified the presence of 2- and 3-monochloropropanediol bound in the form of fatty acid esters – (2- and 3-MCPDEs) – in many commercial baby formulae (Zelinkova et al. 2009; Weisshaar 2011; Becalski et al. 2015; Wöhrlin et al. 2015). These esters of 2- and 3-MCPD are considered to be process-induced food toxicants formed primarily during processing/refining of commercial edible vegetable oils/fats (Zelinkova et al. 2006; Hamlet and Sadd 2009; Velišek 2009; Weisshaar and Perz 2010; Crews 2012; MacMahon et al. 2013) . These contaminants are introduced into baby formulae through their fortification with refined oils/fats to obtain an optimal nutritional profile. Hydrolysis of 2- and 3-MCPDEs *in vivo* gives their respective parent compounds, (Abraham et al. 2013) thus serving as a dietary source of 2- and 3-MCPD (Appel et al., 2013; Frank et al. 2013). 3-MCPD is classified as a non-genotoxic carcinogen and is listed in Group 2B by the International Agency for Research on Cancer (IARC) (European Commission, Scientific Committee on Food 2001; World Health Organization 2002).

In principle, an intake of these contaminants by infants could be avoided by nursing with human milk; however, 3-MCPDE was also detected in human milk samples in one study conducted in the Czech Republic (Zelinkova et al. 2008). The authors calculated an average concentration of 35.5 ng/g of 3-MCPD equivalent, assuming a total hydrolysis of the esters. Zelinkova et al. also hypothesized that dietary intake was the source of 3-MCPDE present in the milk as de-novo synthesis of the 3-MCPD backbone *in vivo* is not likely.

In view of the ubiquity of 2-MCPDE (at levels ~ 50% of the amount of 3-chloro isomer) in processed oils/fats and foods containing such oils/fats (Kuhlmann 2011; Seefelder et al. 2011; Dubois et al. 2012; Becalski et al. 2013), and based on

CONTACT Adam Becalski Adam.Becalski@canada.ca Food Research Division, Bureau of Chemical Safety, Health Products and Food Branch, Health Canada, Locator 2203D, Ottawa K1A 0K9, Canada
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the data published by Zelinkova et al. 2008, the presence of 2-MCPDE in human milk due to dietary intake could also be possible. While adequate risk assessment for 2-MCPD has not been finalized up to now due to the limited toxicological and occurrence data (Schilter et al. 2011; Andres et al. 2013), the precautionary principle would thus warrant a concurrent probe into the presence of the 2-MCPD isomer as well.

The objective of this pilot study was to probe the presence of 2- and 3-MCPDEs in human milk samples collected in Canada as part of the Maternal–Infant Research on Environmental Chemicals (MIREC) program. The MIREC study was one of the initiatives on human biomonitoring under the Chemicals Management Plan (CMP) launched by the government of Canada in 2006. It is a multi-year research study that recruited about 2000 women from 10 cities across Canada (Canada 2010; Arbuckle et al. 2013). One of the goals of this study was to investigate and measure the concentrations of various chemicals, including MCPDEs, in human milk from about 1000 participants.

MCPDEs were determined in this study via an indirect analytical approach by using acidic hydrolysis/transesterification (Divinova et al. 2004) and derivatization with cyclohexanone using a fluorinated sulfonic acid resin (Nafion) as a catalyst followed by GC-MS analysis. (Becalski et al. 2013). In addition, direct analysis via LC-MS/MS analysis based on SIDA for 3-MCPD dipalmitate and 2-MCPD distearate was used to confirm obtained results of selected samples.

Materials and methods

Standards and reagents

3-Monochloropropanediol (98%) was obtained from Alfa Aesar (Ward Hill, MA, USA); 2-monochloropropanediol (98%), 2-monochloropropanediol-d_5 (98%), isotopic purity > 99 atom % D, 3-monochloropropanediol-d_5 dipalmitate (97%), isotopic purity 99 atom % D, 2-monochloropropanediol-d_5 distearate (98%), isotopic purity 98 atom % D were supplied by Toronto Research Chemicals Inc. (Toronto, ON, Canada) and 3-monochloropropanediol-d_5 (97%), isotopic purity 98 atom % D was obtained from CDN Isotopes (Pointe-Claire, QC, Canada). Nafion SAC-13, 10–20% on a silica support, and cyclohexanone (99.8%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sulphuric acid (98%+ trace metal use, A-510–500) was from Fisher Scientific (Ottawa, ON, Canada), while anhydrous sodium sulphate and Extrelut NT was obtained from EMD (Gibbstown, NJ, USA). Sodium sulphate was muffled at 650°C for 12 h before use. All other reagents were of analytical grade. Water was obtained from a Barnstead NANOpure Diamond (Waltham, MA, USA) purification system.

Samples

Human milk samples were collected by the study participants, either by hand or by using the breast pump provided, (Medela, Mississauga, ON, Canada) between the 2nd and 10th weeks after delivery. Approximately 1000 human milk samples were collected from 10 cities (Vancouver, Edmonton, Winnipeg, Toronto, Hamilton, Sudbury, Kingston, Ottawa, Montreal, and Halifax) across Canada over a period of 4 years, 2008–11, and aliquots of 201 of them were analyzed for MCPDEs in a Food Research Division (FRD) laboratory. All sample aliquots of human milk used for the current study were prepared in a FRD laboratory (from original milk samples which were thoroughly homogenized prior to sub-sampling) and stored in 15-mL polypropylene vials (Nalgene, Cat. # 5005–0015) in a freezer at a temperature <-20°C until analysis. Both the breast pump and the 15 mL polypropylene vials were tested for MCPDs migration with methanol/dichloromethane (1:2, v/v) prior to use, and MCPDEs were not detected.

Standard preparation – mcpds and mcpdes

All stock and spiking deuterated standard solutions of 2- and 3-MCPD were prepared in ethyl acetate and stored at −18 °C. Calibration solutions were prepared in 4 mL vials with septa and contained 2, 5, 25, 100, 500, and 1000 ng of 2- and 3-MCPD and 100 ng of 2- MCPD-d_5 and 3-MCPD-d_5 in each vial and 100 µL of cyclohexanone. The total volume of the organic phase was then

adjusted to 1.0 mL using isooctane. Sodium sulphate (0.2 g) and Nafion/silica (10 mg) were added, vials were capped and heated in a heating block at 45°C for 1 h. After cooling to room temperature, the organic phase was pipetted into a 2-mL vial for GC/MS analysis. Internal MCPDE standards used for sample spiking were prepared in ethyl acetate (102.5 µg rac-bispalmitate-3-MCPD-d_5/mL and 112.2 µg 1,3-distearate-2-MCPD-d_5/mL).

Typical sample preparation and indirect analysis of MCPDs

The procedure and conditions described by us earlier for extraction and analysis were generally followed. (Becalski et al. 2012, 2013)

Frozen milk samples were removed from freezer and allowed to stand at room temperature in the dark for 1 h. A whole sample, about 5 g (average 5.0, range 3.8 −8.4 g) of milk was transferred into a 50 mL fluorinated ethylene propylene (FEP) centrifuge tube (Nalgene, Oak Ridge type). Sample container was washed with 25 mL of dichloromethane/methanol mixture (2:1, v/v) into the centrifuge tube and internal standards were added (10 µL of 102.5 µg rac-bispalmitate-3-MCPD-d_5/mL internal standard solution and 10 µL of 112.2 µg 1,3-distearate-2-MCPD-d_5/mL internal standard solution, equivalent to 200 ng of free 2- & 3-MCPD-d_5).

The mixture was shaken for 30 min on a horizontal shaker and centrifuged at 15 000 x g at 4°C for 10 min. Water (6 mL) was added to the tube and the mixture was shaken for 30 min on a horizontal shaker and centrifuged at 15 000 x g at 4°C for 30 min. The upper water-containing phase was removed (together with most of the solids) using a Pasteur pipette. An aliquot of 10 mL water was added to the tube, and the mixture was shaken for an additional 30 min on a horizontal shaker and centrifuged at 15 000 x g at 4°C for 30 min. The upper, water-containing phase, was again removed using a Pasteur pipette, 0.5 g of sodium sulphate was added to the remaining solvent and the tube was let to stand for 15 min. The solvent was transferred into a 100 mL round bottom flask and removed using a rotary evaporator (~ 200 mbar, 35°C). The lipid residue was transferred to a pre-tared 4-mL vial using diethyl ether, ether was removed under a stream of nitrogen and the weight of lipids was measured. Lipids were dissolved in 2.0 mL of tetrahydrofuran (THF) and 1 mL aliquot of the solution was transferred into a 10-mL vial for subsequent hydrolysis of the esters. (The THF was evaporated under a stream of nitrogen from the remaining 1 mL aliquot and the resulted lipid sample was stored in a 2-mL glass vial at −80°C.) To the first aliquot a solution (1.8 mL) of sulphuric acid in methanol, (3.1 g of concentrated acid per 100 mL of methanol) was added and the vial was heated at 40°C in a heating block for 16 h. After cooling to room temperature a saturated sodium hydrogen carbonate solution, 0.5 mL, was added and the vial was vortexed for 2 min. The 10-mL vial was placed in a custom cylindrical glass sleeve (~ 15 cm x 2.5 cm i.d.) with a ground glass joint and organic solvents were removed by a rotary evaporator (~ 25 mbar, 55°C). Hexane, 2 mL, was added to the vial, vortexed for 2 min, phases were allowed to separate and the hexane layer was removed and discarded using a pipette. The extraction with hexane was repeated two more times. Extrelut NT, 0.8 g, was added to the vial, the vial was capped, shaken by hand for 1 min, and let to stand for 30 min.

The extraction column [6 mL blank polypropylene reservoir (Supelco, Bellefonte, PA) with a polypropylene frit] was prepared as follows: a layer of 2 g of a mixture of sodium/magnesium sulphate (1:6, w/w), followed by the sample which had been adsorbed on Extrelut NT, and 0.2 g layer of sodium sulphate. The column was eluted in a SPE manifold (Supelco) with the valve fully open with 6 mL of 10% diethyl ether in hexane mixture (v/v), followed by 100% diethyl ether. The first 5 mL of eluate were discarded, the elution speed was changed to 1 drop per second and elution was continued until 12 mL had been collected.

The ether was removed under a stream of N_2 at 35°C to near dryness, 0.1 mL of cyclohexanone followed by 0.9 mL of isooctane were added to the vial and the sample was derivatized using Nafion resin acidic catalyst as for standards. Analysis was performed using low resolution GC-MS (Agilent 6890/5973N, Mississauga, ON, Canada) on a VF-17MS 30 m × 0.25 mm × 0.25 µm column

(Agilent) and detection of target MCPD derivatives was accomplished in Selected Ion Monitoring Mode with 4 ions for native analytes and 2 ions for internal standards.

Selected derivatized sample extracts were re-analyzed by magnetic sector GC-MS (Agilent 6890/Waters Autospec Ultima, Mississauga, ON, Canada). Pertinent details of GC-HRMS method are given below: Ionization mode: EI^+, 40 eV; Data type: enhanced MRM; Function type: voltage MRM, Acceleration voltage: 7110 V; Mass, Dwell, Delay: 147.0213 (quantification ion), 0.090, 0.020; 149.0183, 0.090, 0.010; 152.0527, 0.090, 0.010; 161.0369, 0.090, 0.010; 190.0761, 0.090, 0.020; 195.1074, 0.090, 0.010, Inter Scan Delay (s): 0.10, Resolution: 3000.

Direct analysis of MCPDs

An aliquot of 100 mg extracted milk lipids (previously stored at −80°C) was dissolved in ethyl acetate, 0.5 mL, and manually injected into a preparative HPLC (Gilson Spot Prep II, Limburg-Offheim, Germany) equipped with a Nucleodur 100–5 C8 ec column (250 x 2 mm ID, 5.0 µm particle size; Macherey-Nagel, Düren, Germany). The flow of the mobile phase was set to 20.0 mL/min using eluent A: 92/8 methanol/water (v/v) acidified with 0.1% of formic acid and eluent B: 98/2 isopropanol/water (v/v) acidified with 0.1% of formic acid. The following gradient was used: 100% A for 2 min, within the next 7.5 min up to 45% B, within the next 14 min up to 70% B, continuing for a further 14.5 min, then within the next 5 min down to 15% B, followed by conditioning to the starting condition. The fraction from 18 to 28 min was collected, which included the target analytes and the corresponding stable isotopically labeled standards, and the solvent was evaporated using a rotary evaporator. The residue was dissolved in 0.5 mL methanol/water (92/8; v/v), and subjected to LC-MS/MS for quantitation. The LC-MS/MS system consisted of a thermostated autosampler (20°C) with a Surveyor HPLC, equipped with a Luna C18(2) 100 Å column (150 × 2.0 mm, particle size 3 µm; Phenomenex, Aschaffenburg, Germany), which was connected to a TSQ Vantage mass spectrometer (both Thermo Finnigan, Dreieich, Germany). The flow of the mobile phase was set to 0.2 mL/min (0–36 min), then to 0.25 mL/min (until end of run) using eluent A: 92/8 methanol/water (v/v) acidified with 0.05% of formic acid and eluent B: 98/2 isopropanol/water (v/v) acidified with 0.05% of formic acid. The following gradient was used: 100% A for 2 min, within the next 18 min up to 50% B, within the next 16 min up to 83% B, within 0.5 min up to 100% B, continuing for a further 6 min, and followed by conditioning to the starting condition.

MS parameters were: capillary temperature: 350°C, vaporizer temperature: 100°C, sheath gas pressure: 40 arbitrary units, spray voltage: positive polarity 4.0 kV, and collision gas pressure: 0.002 mbar.

The following mass transitions were used: *m/z* 605 to *m/z* 331 and *m/z* 605 to *m/z* 95 for 3-MCPD dipalmitate; *m/z* 610 to *m/z* 336 and *m/z* 610 to *m/z* 95 for 3-MCPD dipalmitate-d5; *m/z* 661 to *m/z* 359 and *m/z* 661 to *m/z* 95 for 2-MCPD distearate; and *m/z* 666 to *m/z* 364 and *m/z* 666 to *m/z* 95 for 2-MCPD distearate-d5.

Results and discussion

Samples were processed in sets consisting of up to 10 samples and one or two method blanks per set. Each set also included either a human milk sample (US milk bank sample, Innova Research, cat# IR 100,042, Lot# 22,130-KT, which we have tested to be free of MCPDs) spiked with native 3-MCPD dipalmitate and 2-MCPD distearate at 100 ng per 2.5 g sample of 2- and 3-MCPD equivalents and/or Ready-To-Use (RTU) formula spiked in the same manner. Only one spiking level was used due to expected narrow range of concentrations of analytes in samples. That one lot of RTU milk was collected in October 2010 for method development and was analyzed in triplicate for its

Table 1. Recoveries of milk samples fortified with 3- and 2-MCPD.

	Recovery (%)of 2- and 3-MCPD			
	US Milk Bank *n* = *18*		RTU Milk *n* = *16*	
	3-MCPD	2-MCPD	3-MCPD	2-MCPD
Average	98	100	88	96
St.deviation	2	7	9	17

Spike level 100 ng/per sample of 2.5 g of milk

MCPDEs content. Recoveries for US milk bank and RTU milk samples are listed in Table 1. Multiple containers of RTU samples were kept frozen at −20°C and subsequently used as required. RTU milk had a background concentration of 3-MCPDE of 71 ng/g and 2-MCPDE of 34 ng/g expressed as MCPD equivalents. Additional experiments were conducted by spiking US Milk Bank samples at low levels, i.e. 10 ng/per sample of ~ 2.5 g of milk. Average recoveries of 2- and 3-MCPD for 8 replicates were 112% and 114%, respectively, with corresponding RSDs < 5%. The S/N ratio (peak-to-peak) for the quantifying ion (m/z = 147) exceeded 30 for those spikes, clearly indicating our ability to detect MCPDs at this level, Figure 1. The chromatogram of a typical MIREC sample is depicted in Figure 2.

Each batch set also included quality control sample consisting of 100 µL toluene aliquot of FAPAS oil sample #2628, (Sand Hutton, York, UK) equivalent to the 42.7 mg of neat oil. That control sample was used only at the acidic hydrolysis step. The above FAPAS

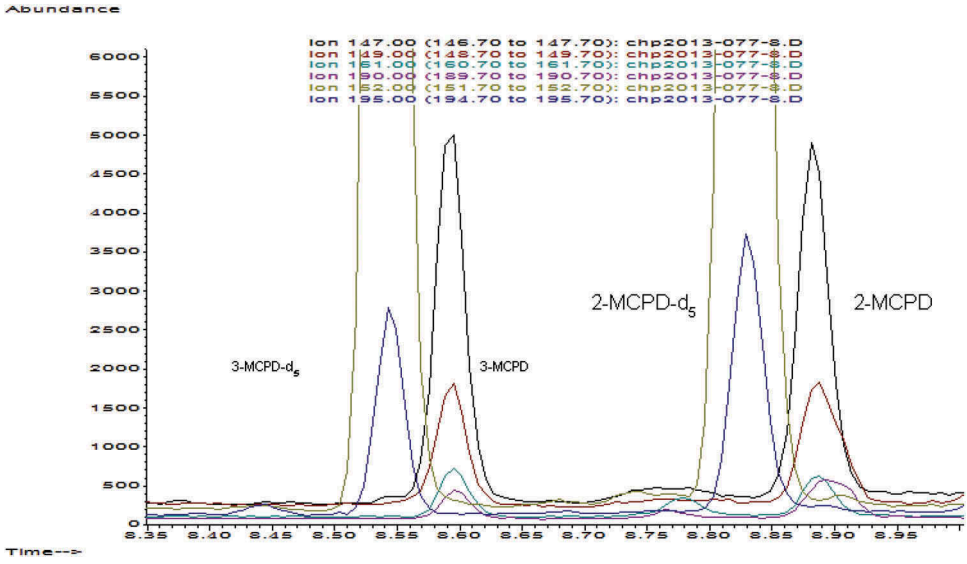

Figure 1. A typical SIM mass chromatogram of a 10 ng equivalent spike of native 2- and 3-MCPDs (in form of distearate or dipalmitate, respectively) into a 2.5 g US human milk bank sample, internal standards, 2-&3-MCPD- d_5 are at 100 ng.

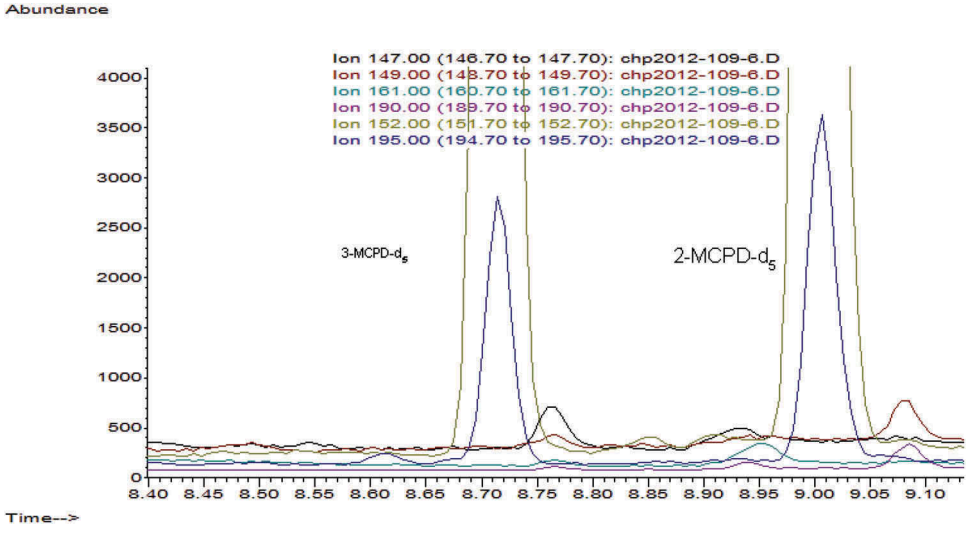

Figure 2. A typical SIM mass chromatogram of MIREC milk sample (07–021) with an 'apparent' concentration (based on intensity of m/z = 147 of 3-MCPD ≈ 0.4 ng/g in a whole milk sample or 9 ng/g in milk lipids (lipid content 3.8%), Internal standards, 2-&3-MCPD- d_5 are at 100 ng.

sample was analyzed by us in February 2011 as part of proficiency testing and had an assigned consensus value of 4.68 mg/kg of 3-MCPDE. Our laboratory reported a value of 4.61 mg/kg for 3-MCPDE (*z*-score = −0.1) and also determined a 2-MCPDE concentration of 1.98 mg/kg. The resulting amount of analytes was then 200 ng of 3-MCPD and 85.5 ng of 2-MCPD equivalents per sample of 42.7 mg of neat oil. For FAPAS #2628, all analyses were within one standard deviation (std. dev.) of 0.936 mg/kg from the assigned value, Table 2. Due to the trace level contamination of reagents and supplies, a tiny residual signal for 3-MCPD was observed in all samples including method blanks. The corresponding signal for 2-MCPD was not found in the blank samples. Altogether, 36 blank samples were processed and the average 3-MCPD concentration was calculated to be 0.4 ng/g with a std. dev. of 0.5 ng/g. The limit of detection (LOD) for 3-MCPD was calculated as 2 ng/g using formula of LOD = average + 3 x (std. dev.). The LOD for 2-MCPD for samples was calculated to be 1 ng/g based on S/N ratios obtained from spiked samples.

In total, 20 analytical batches were processed and 199 results were generated (two samples were lost during extraction procedure and could not be re-analyzed due to the unavailability of a back-up milk); 2-MCPD was not detected in any sample. 3-MCPD was not detected above the LOD of 2 ng/g in blanks and in 17 batches of samples. In three remaining batches, 3-MCPD was found in some blanks and some samples ($n = 8$) at the apparent levels of up to 5.1 ng/g. Since samples were combined into batches in a random manner and contamination was also present in respective method blank samples, it is very likely that those higher concentration values were due to sample contamination within the laboratory. It appears that the source of the contamination was paper products, which came into contact with laboratory equipment, consistent with the presence of 3-MCPD previously reported in some paper items (Boden et al. 1997; Becalski et al. 2016). Those three batches, which we suspected of contamination, were processed at the beginning of the analyses, and after findings of blank contamination, all contact of laboratory equipment with paper products was subsequently excluded. Enough back-up milk samples were available to permit analysis of seven duplicates. For five of those samples, duplicate analyses confirmed the original results of 3-MCPD concentration < LOD. Two samples, for which the initial analysis found an apparent concentration of 3-MCPD to be about 3.5 ng/g were found to contain 3-MCPD < LOD upon re-analysis, thus, supporting our hypothesis about a few samples ($n = 8$) being accidentally contaminated in the laboratory during initial stages of the project.

Table 2. Results of testing FAPAS #2628.

Results of testing FAPAS	Concentration (mg/kg)	
oil sample #2628	3-MCPD	2-MCPD
FAPAS assigned value	4.68	not asigned
Value obtained in this study, *n = 21*	4.91	2.09
Standard deviation of this study	0.39	0.11

After analyses by low resolution GC-MS, the derivatized sample extracts were stored at −80°C and, at a later date, 29 selected samples and 4 blanks were re-analyzed using magnetic sector GC-MS to confirm these findings. Samples from three batches were selected; two batches where some blanks and samples had apparent 3-MCPD concentrations above 2 ng/g and one batch where all blanks/samples were below 2 ng/g. GC-HRMS method was linear ($r^2 > 0.999$) in the range of standards used (1, 5, 10, 25, 100 ng/mL) and was highly sensitive. The S/N ratio of 1480 for 3-MCPD and S/N ratio of 1035 for 2-MCPD ($m/z = 147.0213$) was obtained when a standard containing respective analytes at 1 ng/mL was analyzed. There was a good agreement between datasets obtained using high and low resolution instruments as indicated by linear regression coefficient (R = 0.996) and coefficient of determination ($r^2 = 0.992$), respectively. GC-HRMS analyses also indicated presence of traces of 2-MCPD (at the absolute level of about 0.25 ng per sample or 0.1 ng/g), likely originating from native impurities present in the deuterated internal standard.

Furthermore, a possible conversion of MCPDEs (via ester hydrolysis) into free MCPDs during collection/storage (unlikely considering the storage at temperature <-20°C) was also probed. No free MCPDs were detected (LOD = 2 ng/g for free 2- and 3-MCPD) in any of randomly chosen 11 MIREC samples which indicate lack of conversion of MCPDEs into MCPDs, or a presence of substantial amounts of free MCPDs from the diet in human milk.

To confirm the abovementioned results, randomly selected samples were re-analyzed based on a second quantitation method, also using stable isotope dilution assays. In this way, the analytes and the

corresponding internal standards were isolated from the previously extracted milk lipids by preparative HPLC and the concentrated eluate was analyzed using HPLC-MS/MS. That direct second method, which was different in the sample-work-up as well as in the quantitation aspects from the indirect GC-MS method, confirmed the absence of selected MCPDEs in human milk at LOD of 4 ng/g and 8 ng/g for 3-MCPD dipalmitate and 2-MCPD distearate, respectively. An LC-MS/MS chromatogram of a spiked sample is depicted in Figure 3.

Conclusions

In conclusion, our study of human milk samples (n = *199*) for 2- and 3-MCPD fatty acid esters, the

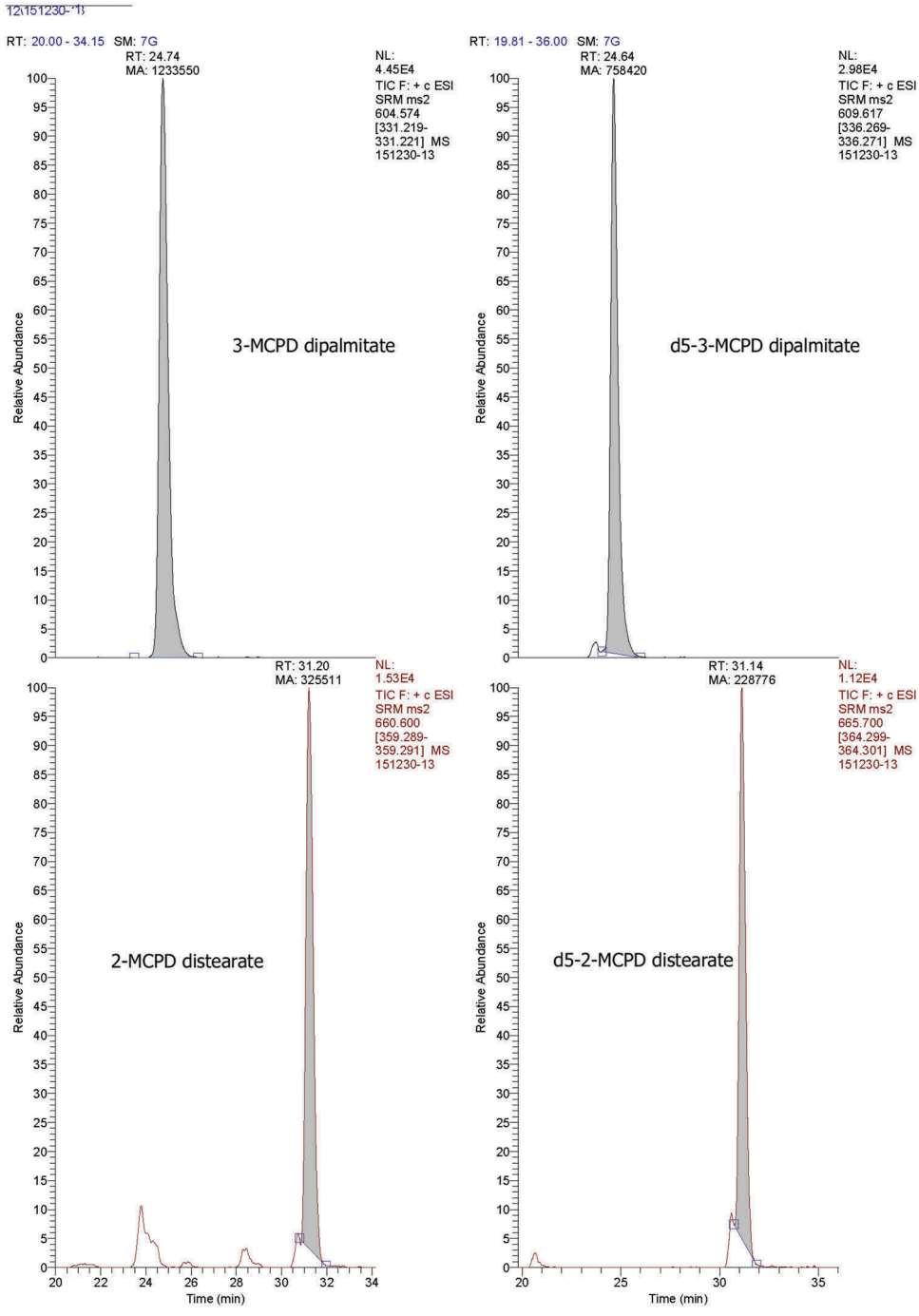

Figure 3. A typical SRM mass chromatogram of a 100 ng equivalent spike of native 2- and 3-MCPDs (in form of distearate or dipalmitate, respectively) into a 2.5 g US human milk bank sample, internal standards, 2-&3-MCPD- d_5 are at 100 ng.

largest and most comprehensive to date, and the first one to analyze human milk samples for 2-MCPDE, did not detect MCPDEs, at LOD = 2 ng/g for 3-MCPD and LOD = 1 ng/g for 2-MCPD, in milk of Canadian nursing mothers using both indirect (GC-MS after derivatization) and direct quantitation (HPLC-MS/MS) methods based on stable isotope dilution assays. Our study also did not detect free 2-MCPD or 3-MCPD (LOD = 2 ng/g) in 11 randomly selected human milk samples.

Levels of MCPDEs in this study were much lower than those found in human milk samples (average of 3-MCPD equivalent 35.5 ng/g, range < 11 – 76, n = 12) in the survey conducted in the Czech Republic. The reason for the discrepancy between this study and that reported by Zelinkova et al. 2008; is unclear, and might be caused by the difference in dietary sources/habits of those two populations. However, from a biological point of view, it is very unlikely that MCPDEs could be transferred to the breast milk as it has been shown in the meantime that MCPDE ingested with food are largely hydrolysed in the gut (Abraham et al. 2013). Moreover, so far, there is no proof that de-*novo* synthesis of MCPDEs occurs *in vivo*.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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