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Melamine in Infant Formula Sold in Canada: Occurrence and Risk Assessment

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An analytical method incorporating simple liquid extraction followed by mixed mode cation exchange/reversed phase solid phase extraction and liquid chromatography−tandem mass spectrometry was developed and validated for the analysis of melamine (MEL) in liquid and powdered infant formula. The method used two different MEL stable isotope labeled internal standards to monitor analyte recoveries and to account for matrix effects. The method is sensitive (limit of quantitation of 4 ng/g), accurate, and precise (during validation, recoveries corrected by internal recovery standard averaged between 92 and 104% for all fortification levels and matrices). The method was used to analyze 94 samples of infant formula purchased from major retailers in Ottawa, ON, Canada, to examine whether or not Canadian infants are exposed to background levels of MEL. MEL was detected in 71 of the 94 products analyzed at concentrations ranging from 4.31 to 346 ng/g (median = 16 ng/g). A comparison of estimated dietary exposures to the recently recommended World Health Organization toxicological reference value for melamine suggests that the presence of low levels of MEL in infant formula purchased in Canada does not represent a health risk.

KEYWORDS: Diet; food; exposure; contaminant; triazine; LC-MS/MS; risk assessment; melamine; infant formula

INTRODUCTION

In September 2008 reports began appearing in the media that some infant formula manufactured in China was contaminated with melamine (MEL; 1,3,5-triazine-2,4,6-triamine). Various recalls of Chinese products containing milk powder were prompted by more than 54000 infants and young children seeking treatment for kidney stones in China. Six deaths have been attributed to consumption of the contaminated infant formula and related dairy products (*1*).

This adulteration of infant formula mirrors a previous incident with tainted pet food in 2007. Chinese-sourced vegetable proteins contaminated with MEL and cyanuric acid, a related triazine compound, were incorporated into North American pet food and resulted in over a hundred pet deaths, and more reports of kidney failure (*2*). In both of the pet food and infant formula incidents, it is thought that MEL was intentionally added in order to increase the apparent protein content of the foods.

Subsequent to the initial reports of tainted infant formula, MEL was found at levels of concern in a number of food items that contain Chinese-sourced milk powder. Contaminated food items included various candies, beverages, and cookies. Recalls have been initiated for these imported items in Canada (*3*). However, infant formula produced in China is not approved for sale in Canada and no ingredients used in the manufacture of infant formula sold in Canada are reported to be sourced from China.

The purpose of this study was to examine whether or not Canadian infants are exposed to baseline levels of MEL − that is, concentrations lower than those observed during adulteration incidents. A sensitive analytical method incorporating simple liquid extraction followed by mixed mode cation exchange/reversed phase solid phase extraction (SPE), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed and validated. The method was then used to analyze 94 samples of infant formula purchased from major retailers in Ottawa, Canada.

MATERIALS AND METHODS

Samples. Ninety-four infant formula products were purchased from major retailers in Ottawa, ON, Canada. Products were selected on the basis of their immediate availability, and those purchased included liquid (n = 31) and powdered (n = 63) formulas. Both milk- and soy-based formula were selected for analysis. Items were purchased between September 22 and October 6, 2008. All items aside from one had expiration dates on, or later than, November 1, 2008. Further details on the infant formula samples analyzed are provided in the Supporting Information. All infant formula products were analyzed as received without dilution.

Chemicals and Materials. Melamine (MEL; 98% purity) and $^{15}N_3$,$^{13}C_3$-melamine ($^{15}N_3$,$^{13}C_3$-MEL;$^{15}N_3$ at 98% purity; $^{13}C_3$ at 99% purity) were obtained from Cambridge Isotope Laboratories, Andover,

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Table 1. Multiple Reaction Monitoring Conditions Used for the Analysis of Melamine

compound	transition (*m*/*z*)	transition type	dwell time (s)	cone voltage (V)	collision energy (eV)
melamine	127 → 43	secondary confirmation	0.06	30	24
	127 → 68	primary confirmation	0.06	30	25
	127 → 85	quantitation	0.06	30	17
$^{13}C_3$-melamine	130 → 44	primary confirmation	0.06	30	25
	130 → 87	quantitation	0.06	30	18
$^{13}C_3$,$^{15}N_3$-melamine	133 → 45	primary confirmation	0.06	30	25
	133 → 89	quantitation	0.06	30	18

MA. The dually labeled $^{15}N_3$,$^{13}C_3$-MEL was used as an internal performance standard. This internal standard was used to account for matrix effects on MEL ionization, because it was not feasible to prepare matrix-matched standard calibration curves for all different infant formula products. $^{13}C_3$-Melamine ($^{13}C_3$-MEL; $^{13}C_3$ at 99% purity) was synthesized according to the method of Varelis and Jeskelis (*4*) and used as an internal recovery standard. Melamine (≥99% purity) from another source was obtained from Sigma-Aldrich, Madison, WI, for use as a quality control check standard. All standards were prepared in a 90:10 (v/v) acetonitrile/water solution.

All water used in sample and standard preparation was Milli-Q purified (Millipore, Billerica, MA). The other solvents and reagents used in the method were used without extra purification: hydrochloric acid (HCl; 37.4%, ACS reagent grade, Sigma-Aldrich), dichloromethane (DCM; OmniSolv grade, EMD Chemicals, Darmstadt, Germany), methanol (MeOH; OmniSolv grade, EMD Chemicals), ammonium hydroxide (NH_4OH; 30%, J. T. Baker Chemicals), acetonitrile (ACN; OmniSolv grade, EMD Chemicals), water (LC-MS grade, OmniSolv, EMD Chemicals), concentrated formic acid (>96% reagent grade, Sigma-Aldrich), ammonium formate (Sigma-Aldrich), and nitrogen gas (high purity).

Sample Preparation. Both liquid and powdered infant formula samples were prepared in the same manner. Samples were analyzed as purchased; that is, powdered and concentrated liquid formulas were not prepared as for consumption prior to analysis. Approximately 5 g of sample was placed in a 50 mL polypropylene centrifuge tube and fortified with $^{13}C_3$-MEL internal recovery standard (25.0 μL of a 100 μg/mL solution). Powdered samples were left to stand at room temperature for 1 h; liquid samples were left to stand for 30 min. Extraction solvent (24 mL H_2O and 1 mL of 1.0 N HCl) was then added to all samples. Samples were shaken briefly by hand, on a mechanical vortex mixer for 5 s, and then on a rotary mixer for 10 min. Samples were then centrifuged for 10 min at 3200 to 5500*g* at 4 °C. A 5.00 mL aliquot of the aqueous supernatant was taken and mixed with 10.0 mL of DCM (mechanical vortex mixer for 5 s, rotary mixer for 10 min, centrifuged for 10 min at 3200−5500*g* at 4 °C). The entire aqueous top layer was removed and placed in a glass culture tube. The remaining DCM was extracted with 2.50 mL of water as described above. The aqueous layer was removed and combined with the first aqueous layer obtained.

Mixed mode cation exchange/reversed phase SPE cartridges (Oasis MCX, 150 mg, 30 μm, 6 cm^3, Waters Corp., Milford, MA) were conditioned with 5.0 mL each of MeOH and H_2O. The entire sample extract was then loaded onto the SPE cartridge and passed through under the force of gravity. Next, the SPE cartridge was washed with 5.0 mL of HCl (0.1 N) followed by 2.0 mL of MeOH. The SPE cartridge was dried by pushing air through the cartridge using an empty disposable syringe and adaptor. MEL was eluted from the SPE cartridge using 5.0 mL of NH_4OH (5% v/v in MeOH). The eluate was dried under N_2 in a water bath held at 50 °C. The extract was reconstituted in 1.00 mL of 90:10 (v/v) ACN/H_2O and mixed well. The reconstituted extract was filtered through a 0.2 μm nylon syringe filter using a disposable polypropylene syringe. The extract was diluted by a factor of 10 by adding $^{15}N_3$,$^{13}C_3$-MEL internal performance standard (10 μL of a 500 ng/mL solution) to 100 μL of filtered extract and 890 μL of 90:10 (v/v) ACN/H_2O. Samples were mixed well and stored at room temperature until instrumental analysis.

Quality Control Procedures. A variety of blanks, quality control (QC) samples, and replicate measurements were used to track the performance of the method during the infant formula analyses. Reagent blanks consisting of 5 g of H_2O were processed along with every 10 samples to monitor the background concentration of MEL. The consistency of the data generated by the method was monitored using two in-house reference materials. Aliquots of one liquid and one powdered infant formula, each known to contain approximately 25 ng/g MEL, were processed along with every 40 samples as QC samples. Duplicates were run on a sample randomly selected from every group of 20 processed. In addition, solvent blanks of 90:10 (v/v) ACN/H_2O were run throughout the instrumental analysis to monitor for carry-over. Finally, a solution of MEL (50 ng/mL), prepared using a source independent of the calibration standards, was also run during instrumental analysis to monitor the stability of the standard calibration on a day-to-day basis.

Liquid Chromatography−Tandem Mass Spectrometry Analysis of Sample Extracts. All samples, blanks, and standards were analyzed for MEL using a Waters Acquity ultrahigh-pressure liquid chromatograph coupled to a Waters Premier triple-quadrupole tandem mass spectrometer (UPLC-MS/MS; Manchester, U.K.). Samples (5.0 μL injection) were chromatographed at 30 °C on a hydrophilic interaction column (2.1 × 100 mm Acquity UPLC BEH HILIC column, 1.7 μm, Waters Corp.) using a binary mobile phase of (A) aqueous solution of 0.5 mM ammonium formate and 0.01% (v/v) formic acid and (B) 0.01% formic acid (v/v) in ACN. A gradient program was used, with an initial flow rate of 0.17 mL/min and mobile phase composition of 10% A and 90% B. These initial conditions were held for 2 min, increased to 40% A at 4 min, and then held for 6 min. At 10.5 min the flow rate was increased to 0.25 mL/min to speed the conditioning of the column back to the initial mobile phase of 10% A and 90% B. At 12.6 min the flow rate was decreased to the initial flow rate of 0.17 mL/min. Under these conditions, MEL eluted at a retention time of 5.1 min.

The analysis of MEL was performed using multiple reaction monitoring in the positive ion electrospray mode. The capillary voltage was held at 3.5 kV, the source temperature at 120 °C, and the desolvation temperature at 400 °C. The cone and desolvation gas (N_2) flows were 50 and 900 L/h, respectively. The collision gas (argon) pressure was maintained at 9.8×10^3 mbar. The multiplier voltage was held at 650 V. Both quadrupole mass analyzers were run at baseline unit resolution. Details of the transitions monitored and their associated conditions are provided in **Table 1**.

Data Analysis. Data analysis was performed using the Masslynx 4.1 Datasystem (Waters, Manchester, U.K.) on the UPLC-MS/MS system. Analyte peaks were considered to be identified if the retention times of analytes in samples and blanks were within 0.3 min of the average retention time in calibration standards, the peak had a signal-to-noise ratio of greater than 9:1, and the ratio of the peak height of the quantitation transition to the peak height of the primary confirmation transition (*m*/*z* 127 → *m*/*z* 68) was within 20% of the average ratio in calibration standards.

MEL was quantitated using a seven-point calibration curve (ranging from 0.10 to 100 ng/mL) of standards prepared in 90:10 (v/v) ACN/H_2O. A $1/X$ weighted linear curve was used to plot the MEL response factor versus the MEL concentration in the calibration standards. The MEL response factor was calculated as the ratio of the peak height of the MEL quantitation transition to the peak height of the $^{15}N_3$,$^{13}C_3$-MEL quantitation transition. Concentrations of MEL in samples were blank and recovery corrected by subtracting the concentration of MEL in the associated reagent blank and dividing by the percentage of $^{13}C_3$-MEL internal recovery standard present in each sample.

Table 2. Average ± Standard Deviation Percent Absolute Recovery of Melamine (MEL), Internal Recovery Standard (^{13}C-MEL), and Corrected Percent Recovery of MEL from Fortified Powdered and Liquid Infant Formula[a]

		fortification level		
		10 ng/g (n = 4)	50 ng/g (n = 4)	250 ng/g (n = 4)
powdered formula	absolute MEL % recovery	91 ± 6	78 ± 7	79 ± 6
	^{13}C-MEL % recovery	94 ± 4	84 ± 7	79 ± 5
	corrected MEL % recovery	97 ± 8	92 ± 2	100 ± 5
liquid formula A	absolute MEL % recovery	96 ± 6[b]	90 ± 5	86 ± 6
	^{13}C-MEL % recovery	105 ± 1[b]	86 ± 6	90 ± 7
	corrected MEL % recovery	92 ± 7[b]	105 ± 6	95 ± 6
liquid formula B	absolute MEL % recovery	61 ± 15	66 ± 7	53 ± 2
	^{13}C-MEL % recovery	63 ± 4	65 ± 5	50 ± 1
	corrected MEL % recovery	97 ± 18	101 ± 5	104 ± 3

[a] The corrected percent recovery of MEL was calculated as the absolute MEL % recovery divided by the ^{13}C-MEL % recovery. [b] n = 3.

Dietary Exposure Calculations. The estimated dietary exposure of infants to MEL via consumption of infant formula is summarized in **Table 4**. Probable daily intakes were estimated using the average and maximum MEL concentrations in all formula indicated for a particular age group. The MEL concentrations used in the exposure estimates were calculated using the data generated in this study and took into account the necessary dilutions of powdered and concentrated liquid formula indicated by the manufacturers of the various products. The maximum amount of formula consumed per day by infants for a specific growth period (*5*) was multiplied by the average and maximum MEL concentrations in the formula to obtain the amount of MEL consumed per day; division by the average body weights of infants in different age groups (*6*) gave the MEL probable daily intake on a micrograms per kilogram of body weight per day basis.

RESULTS

Method Development. The analytical procedure used in this study was adapted from the method of Andersen et al. (*7*). Some minor changes were made to increase the ease of use of the method for infant formula. The use of diluted HCl as an extraction solvent helped to separate the phases during the initial centrifugation step. In addition, peak height as opposed to peak area was used during quantitation. Infrequently, a small interfering peak was observed near the retention time of MEL in the m/z 127 → m/z 68 primary confirmation transition. Although this small peak would not affect the quantitation of MEL, its area could distort the relative peak area ratio between the m/z 127 → m/z 85 and m/z 127 → m/z 68 transitions, which was used as one of the confirmation criteria. Because no variation in the MEL peak shape was otherwise observed during the analysis of various samples, peak height was chosen as the metric for MEL quantitation. No interference was observed for any of the quantitation transitions of MEL and the two internal standards.

Method Limit of Quantitation. The absolute instrumental detection limit was approximately 0.1−0.2 pg per injection. Trace amounts of MEL were detected in reagent blanks. The analytical method limit of quantitation was 4 ng/g. It was calculated as the average concentration of MEL plus 3 times the standard deviation quantitated in reagent blanks (n = 22).

Method Validation. The accuracy and precision of the method were examined by fortifying three different infant formula products containing milk or milk proteins (two liquid and one powdered) with MEL and analyzing the fortified samples. Each formula was fortified at 10, 50, and 250 ng/g (n = 4 replicates). Recovery data for the method validation exercise are presented in **Table 2**. The recoveries of MEL varied according to the infant formula analyzed, indicating the presence of considerable matrix effects. The absolute recovery of MEL (i.e., uncorrected by the recovery of $^{13}C_3$-MEL internal standard) from liquid formula A was greater than the absolute recovery from liquid formula B at all three fortification levels (Student's t test, $p < 0.025$). This clearly demonstrates that MEL recoveries are sample specific and that an appropriate internal recovery standard is necessary. Otherwise, MEL recoveries can be substantially over- or underestimated if method validation is performed using only one sample matrix. The correction of MEL by the $^{13}C_3$-MEL internal standard accounted for the sample matrix effects and demonstrated that the method can accurately quantify MEL at the three fortification levels; MEL recoveries corrected by internal recovery standard averaged between 92 and 105% for all fortification levels and matrices.

The precision of the validation data was also very good. The coefficient of variation of the $^{13}C_3$-MEL-corrected MEL recoveries was < 10% in all but one instance, which occurred at the low fortification level for liquid formula B.

Analysis of Infant Formula for MEL. Recoveries of the internal standard $^{13}C_3$-MEL averaged 77 ± 20% for the 94 samples analyzed. Because there was a relatively large variation in the $^{13}C_3$-MEL recovery, final concentrations of MEL were recovery corrected as described under Data Analysis.

MEL concentrations measured in various QC samples were consistent. Measured MEL concentrations in the QC check standard deviated by < 14% from the expected concentration over the course of the study (12 days of analyses). As well, MEL concentrations observed in the replicate analyses of the QC liquid and powdered formula samples varied by < 7 and < 12%, respectively. Only three sets of duplicates analyzed had detectable levels of MEL. The percent difference between the three pairs of duplicate measurements averaged 5%.

A summary of the infant formula analyses is presented in **Table 3**. MEL concentrations listed in the table are blank and recovery corrected. Results on the individual 94 samples analyzed are provided in the Supporting Information. MEL was detected in 71 of the 94 infant formula products analyzed. Concentrations observed ranged from 4.31 to 346 ng/g. A histogram of detected melamine levels in these samples is shown in **Figure 1**.

Additional items from the same and different production lots were purchased and analyzed when available for six selected products, including those that contained MEL at concentrations of > 100 ng/g. MEL concentrations observed in separate items from the same production lot appeared to be more consistent than concentrations measured in items from different production lots of a particular brand. The percent variation of MEL concentrations in items within the same lot ranged from 3 to 30%,

Table 3. Summary of the Results of the Measurement of Melamine in Infant Formula Samples Sold in Canada

formula type	formula subtype	formula base	*N*	% > LOQ	geometric mean MEL (ng/g)	max MEL (ng/g)	min MEL (ng/g)
liquid	ready to use	milk	6	83	27.4	68.9	15.2
		other[a]	1	100	16.9		
	concentrate	milk	17	100	16.8	34.5	6.77
		soy	7	100	11.1	31.1	5.50
powder		milk	50	68	29.4	183	4.31
		soy	12	58	78.9	346	32.0
		other[b]	1	0			

[a] Contained a casein derivative and soy oil. [b] Contained both milk and soy.

Table 4. Estimated Probable Daily Intakes of Melamine from Infant Formula for Different Infant Age Groups

			concn of melamine in consumed infant formula		probable daily intake		% WHO TDI[a]
age group	av body wt (kg)	max formula intake (g/day)	av (ng/g)	max (ng/g)	av (ng/kg of body wt/day)	max (ng/kg of body wt/day)	max[b]
premature infants	1.5	100	8.5	68.9	0.57	4.6	2.3
0−1 month	3.9	1080	8.5	68.9	2.4	19	9.5
2−3 months	5.5	1470	8.5	68.9	2.3	18	9.0
4−7 months	7.2	1440	7.8	68.9	1.6	14	7.0
8−12 months	9.0	960	7.9	68.9	0.84	7.3	3.7
12−18 months	10.6	900	8.7	68.9	0.74	5.8	2.9

[a] World Health Organization tolerable daily intake = 200 μg/kg of body wt/day. [b] Calculated using the maximum probable daily intake estimate.

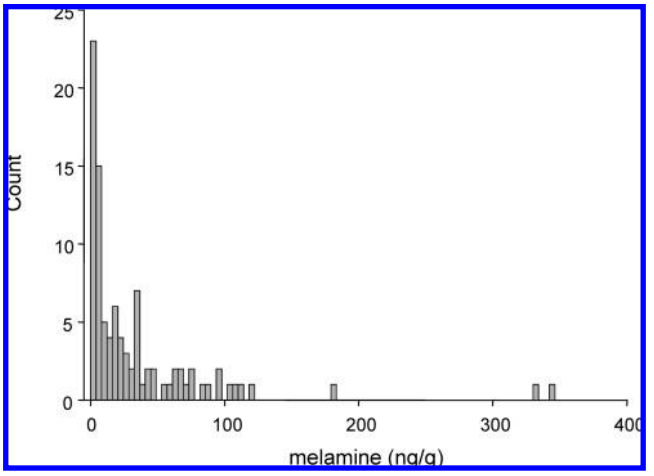

Figure 1. Histogram of measured concentrations of melamine in infant formula purchased in Canada.

whereas the variation of concentrations in items from different lots ranged from 15 to 99%.

DISCUSSION

Method Performance. LC-MS/MS was selected as the method of detection for the analysis of MEL for two main reasons. First, no derivatization is required for the small and relatively polar MEL molecule as opposed to gas chromatographic methods. Second, MS/MS is very selective and sensitive and could thus achieve the low limits of quantitation desired to measure MEL at very low levels in infant formula.

The method validation exercise and the various QC data generated during the analysis of the infant formula demonstrated that the method used produced accurate and precise MEL data. As observed with other analytical methods that use LC-MS/MS (*8*, *9*), there were signs of considerable matrix effects occurring during the analysis of some samples. However, the use of the stable isotope labeled $^{13}C_3$-MEL internal recovery standard compensated for any effects on MEL quantitation driven by variations in matrices.

Low levels of background MEL contamination were observed in approximately 70% of the reagent blanks, ranging from 0.24 to 1.44 ng/g. The source of the MEL is unknown and was not further investigated because the concentrations observed in the blanks were fairly stable and did not substantially affect the sensitivity of the analytical method.

Presence of MEL in Infant Formula Sold in Canada. MEL was detected in 71 of the 94 infant formulas analyzed. In all instances in which MEL was detected, concentrations observed were below the standard of 0.5 μg/g set by Health Canada for infant formula and sole source nutrition products (*10*). The maximum concentration observed was 346 ng/g in a powdered infant formula. Most concentrations measured were low (as illustrated in **Figure 1**); the majority were < 20 ng/g.

In addition, there was no apparent relationship between concentrations of MEL and the various forms of infant formula analyzed. MEL concentrations did not differ significantly between soy- and milk-based products, liquid and powdered products, and concentrated and ready-to-use liquid formula products (Mann−Whitney rank sum test, $p > 0.2$).

The concentrations observed in this study are generally lower than those that have been previously reported. There are no peer-reviewed published data on MEL in infant formula. However, recent reports from the State Council of China state that MEL was found in powdered infant formula at concentrations ranging from 0.09 to 2563 μg/g (*11*). The U.S. Food and Drug Administration has reported levels of 140 ng/g in one infant formula (*12*). Other current information on MEL in food products is mainly in the context of exceeding permissible levels in the low micrograms per gram range and, therefore, does not provide any details on MEL concentrations that may be unrelated to intentional adulteration.

Source of MEL in Infant Formula Sold in Canada. The source of MEL in the infant formula sold in Canada is not currently known. The relatively lower concentrations than those reported in Chinese products, plus the confirmation from the four major manufacturers of infant formula sold in Canada that they do not use any milk ingredients sourced from China, imply that intentional adulteration is not occurring in the Canadian products.

It does not appear that the MEL in the products analyzed in this study originated from the packaging. MEL has been shown to migrate into test solutions and real food samples from melamine−formaldehyde plasticware. Ishiwata et al. (*13*) demonstrated that MEL migrates from melamine−formaldehyde resin cups into different types of hot beverages, such as coffee and juice, at concentrations ranging from 0.45 to 3.24 μg/g. Bradley et al. (*14*) observed a similar migration of MEL into a dilute aqueous acid food simulant. Epstein et al. (*15*) proposed that 1 μg/g MEL found in canned beef muscle tissue from both control animals and those dosed with the insecticide cyromazine was due to melamine−formaldehyde resin linings in the cans. However, our pilot studies in which cans from the infant formula containing the highest levels of MEL were extracted with various solvent mixtures did not provide any indication that MEL was present in the packaging (data not shown).

Other possible sources of MEL arise from the use of it, or structurally related compounds, in various products ranging from construction materials to pesticides. For example, MEL is a metabolite of the insecticide cyromazine, which is approved for use in Canada on various vegetables. MEL has been detected in cyromazine-dosed cattle at concentrations of 10 to 170 ng/g of muscle tissue (*15*). The levels of MEL observed were related to the amount of cyromazine administered to the cattle. The MEL metabolite of cyromazine has not been studied in cow's milk, but it has been reported that MEL in milk from ^{14}C-cyromazine-dosed goats represented between 5 and 9% of the total radioactive residue (*16*, *17*). It appears plausible that milk from cattle exposed to cyromazine may contain MEL; if such milk is used to prepare infant formula, the MEL may be incorporated into the final product.

Melamine Infant Exposure and Risk Assessment. The average MEL probable daily intakes (PDIs) for infants consuming infant formula purchased in Canada ranged from 0.57 to 2.4 μg/kg of body wt/day (**Table 4**). The estimated maximum PDIs are approximately 10 times greater than the average PDIs. The maximum PDIs are $<10\%$ of the tolerable daily intake of 200 μg/kg of body wt/day recently recommended by the World Health Organization. This toxicological reference value was derived as the lower limit of a one-sided 95% confidence interval on the benchmark dose for the occurrence of urolithiasis in Fischer 344 male rats following dietary MEL exposure for 13 weeks (*18*). Overall, the relatively low concentrations observed suggest that the presence of MEL in infant formula purchased in Canada does not represent a health risk.

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Supporting Information Available: Details of infant formulas tested. This material is available free of charge via the Internet at http://pubs.acs.org.

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