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Comparison of digestion procedures and methods for quantification of trace lead in breast milk by isotope dilution inductively coupled plasma mass spectrometry

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Abstract

Measurement of lead in breast milk is an important public health consideration and can be technically quite challenging. The reliable and accurate determination of trace lead in human breast milk is difficult for several reasons including: potential for contamination during sample collection, storage, and analysis; complexities related to the high fat content of human milk; and poor analytic sensitivity at low concentrations. Breast milk lead levels from previous published studies should therefore be reviewed with caution. Due to the difficulty in identifying a method that would successfully digest samples with 100% efficiency, we evaluated three different digestion procedures including: (1) dry ashing in a muffle furnace, (2) microwave oven digestion, and (3) digestion in high pressure asher. High temperature, high pressure asher digestion was selected as the procedure of choice for the breast milk samples. Trace lead analysis was performed using isotope dilution (ID) inductively coupled plasma mass spectrometry (ICP-MS). Measured lead concentrations in breast milk samples ($n = 200$) from Mexico ranged from 0.2 to 6.7 ng ml⁻¹. The precision for these measurements ranged from 0.27–7.8% RSD. Use of strict contamination control techniques and of a very powerful digestion procedure, along with an ID-ICP-MS method

for lead determination, enables us to measure trace lead levels as low as 0.2 ng ml^{-1} in milk (instrument detection limit = 0.01 ng ml^{-1}).

Introduction

Detectable levels of numerous contaminants have been documented in breast milk of women with no known environmental or occupational exposures.¹ Yet, there is limited information available on validating measurement of environmental chemicals in human milk and few data exist to quantify the hazards posed to breastfed infants, especially for the U.S. population.² Generally, there is a low potential for transfer of lead through milk when maternal exposure levels are low.³ However, since over ninety percent of lead in the adult human body is stored in bone,^{4,5} the possibility exists for significant redistribution of cumulative lead stores from bone into breast milk during periods of heightened bone turnover (*e.g.*, pregnancy and lactation).

Studies of lead in human breast milk worldwide over the past three decades have found concentrations ranging over three orders of magnitude from <1 to greater than 100 ng ml^{-1} (ppb).^{6–11} These differences are partially attributable to true differences in population exposures across time and geographic location.¹² However, it is also likely that a variety of methodological factors affect the analytic variability and validity of the reported results and makes drawing conclusions from the existing literature difficult.

Breast milk lead levels from previously published studies with extremely high values should be reviewed with caution due to the high potential for contamination during sample collection, storage, and analysis. Documented sources of contamination include: use of lead acetate ointment,¹³ lead in nipple shields,^{13,14} foil from alcohol wipes used in sample collection,¹⁵ and latex laboratory gloves.¹⁶ Pre-treatment of biological materials is subject to unintentional addition of contaminants from chemical reagents, digestion devices, and atmospheric particles.¹⁷ Since the concentrations of lead in breast milk are low, the influence of contamination is high.¹⁸

Additionally, measurement of lead in breast milk is complicated by the high (~4%) fat content of human milk,¹⁹ which changes during feeding and over the course of lactation.²⁰ Precise and accurate analysis is challenging due to the difficulty of identifying a method that will successfully digest samples with 100% efficiency. Any partitioning of lead into the fat layer of milk must be accounted for in the analysis. The detection power of ICP-MS can only be realized if contaminants are kept to a minimum and biological samples are completely digested.^{21,22} Our laboratory has developed techniques to obtain and analyze breast milk samples that minimize the potential for contamination and maximize the percent recovery of lead.

In this study, three different digestion procedures for human milk were evaluated: dry ashing in a muffle furnace, microwave oven (MWO) digestion, and digestion in a high temperature, high pressure asher (HPA). Trace lead analysis was performed using isotope dilution (ID) inductively coupled plasma mass spectrometry (ICP-MS). We compared the ID method with

the addition of the ID spike manually, online, and by external calibration with an internal standard.

Methods

Sample collection

Human milk samples ($n = 200$) were collected from breast-feeding women at one-month postpartum in Mexico City using a strict contamination control protocol.⁷ Prior to expressing milk, hands were washed thoroughly with soap and water and then rinsed with deionized water. The breast was also rinsed with deionized water. Breast rinse water and blank deionized water samples were also collected and analyzed for lead contamination. Breast milk (~10 ml) was directly expressed by hand into pre-cleaned 30 ml polypropylene bottles after discarding the first few milliliters of milk expressed during the nursing session. Care was taken to avoid any hand contact with the nipple or breast milk dripping into the bottle. Samples were frozen and stored at $-30\text{ }^{\circ}\text{C}$ (Fisher IsoTempPlus, Thermo Fisher Scientific Inc., Waltham, MA) until analysis.

Sample pre-treatment

Samples were first thawed to room temperature and then sonicated (Barnson Model 2200, Cleaning Equipment Company, Shelton, CT) for 15 minutes. Even after 15 minutes of sonication at room temperature, some samples still had separated fat floating on the top. To disperse fat homogeneously, we tried adding a few drops of 1% Triton® X-100 (Sigma, St. Louis, MO) solution to the sample before sonication which did not produce the desired results. Finally, sonicating samples at human body temperature ($98\text{ }^{\circ}\text{F}$) and keeping samples in a water bath (maintained at $98\text{ }^{\circ}\text{F}$ until aliquoting) produced the most homogeneous samples. Results for duplicate analysis (percent difference) lead in fat-separated milk samples by this method was $<20\%$ compared to $>30\%$ without warming to body temperature. This effect was more prominent when aliquoting small sample volumes (1–2 ml) compared to large sample volumes (~10 ml).

Sample preparation

All sample handling was performed in a Class 100 clean room. All of the glassware was cleaned by soaking in 50% trace-metal grade HNO_3 (Seastar Chemicals Inc., Sidney, BC, Canada) acid for 24 hours. Sample collection bottles, plastic tubes, and transfer pipettes were pre-cleaned by soaking in 10% trace- metal grade HNO_3 acid for 24 hours and followed by rinsing several times with deionized water and drying under a clean hood. Teflon® liners for PAAR bombs were cleaned by first sonicating with 20% micro wash and then rinsing with deionized water. Secondly, these liners were filled with 10% trace- metal grade HNO_3 acid, sonicated for 30 minutes, and followed by several rinses with deionized water. Quartz crucibles and HPA quartz vessels were cleaned as described by Amarasiriwardena, *et al.*²³

Digestion procedures

During the development of the method to analyze breast milk samples for trace lead, three digestion procedures were evaluated: (i) dry ashing in a muffle furnace; (ii) digestion with

HNO₃ in a MWO using PAAR bombs; and (iii) digestion with HNO₃ in a high temperature, high pressure asher (HPA) (Anton Paar, Graz, Austria). Breast milk samples were mixed thoroughly for 15 minutes in an ultrasonic mixer (sonicator) at 37 °C. It was found that when samples were warmed to body temperature (98 °F/ ~37 °C) floating separated fat will mix easily to form a homogeneous sample.

(i) Dry ashing in muffle furnace—Ten grams of breast milk or 1 gram of standard reference material was treated in a 100 ml quartz crucible with 2.5 ml of a 10 ng ml⁻¹ solution of National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 983 (²⁰⁶Pb- enriched Radiogenic LeadIsotopic Standard) and 5 ml of HNO₃ acid. Samples were pre-treated with HNO₃ to minimize losses during ashing. Samples were covered and left overnight; the samples were then gently heated on a hotplate to evaporate to dryness before being put in the muffle furnace (Fisher Isotemp Model 126, Thermo Fisher Scientific Inc., Waltham, MA). Samples were ashed in the muffle furnace at 450 °C for ten hours using a temperature–time program (Table 1). Resultant ash was dissolved in 10 ml of 5% HNO₃ and transferred into pre-cleaned 15 ml plastic tubes.

(ii) Digestion in microwave oven—Two grams of breast milk or reconstituted QC standard (6.0 g of NIST SRM 1549 (Non-fat Milk Powder) in 30 ml of DI water or 5.0 g of NIST RM 8435 (Whole Milk Powder) in 50 ml of DI water) were measured into the Teflon® liner of the PAAR bomb. Samples were then spiked with 0.5 ml of 10 ng ml⁻¹ NIST SRM 983. After addition of 1 ml of HNO₃ acid, PAAR bombs were sealed and digested in a MWO at 80% power (1100 watt oven). For comparison, samples were digested in the MWO by three methods: two 2 minutes of heating cycles; two 2 minutes heating cycles with 30 minutes cooling in between cycles; and three 2 minutes heating cycles with 30 minutes cooling in between cycles. The digested samples were diluted to 5 ml with deionized water after the addition of 1 ml of H₂O₂.

(iii) Digestion in high pressure asher—One ml breast milk or 0.1 gram of NIST SRM 1549 or NIST SRM 8435 were weighed into a pre-cleaned 35 ml HPA quartz digestion vessel (Anton Paar, Graz, Austria) and then samples were spiked with 0.5 ml of 10 ng ml⁻¹ NIST SRM 983 and 1 ml of HNO₃ acid was added. The vessel was then sealed with a quartz cap and Teflon® (PTFE) tape and secured with a tungsten clip. Seven vessels were inserted in an aluminum heater block and placed in an autoclave unit. Each batch contained one method blank. Two sets of seven samples were digested each day. The unit was pressurized with nitrogen and the samples were digested using a modified temperature–time program described by Amarasiriwardena *et al.*²³ (Table 2). The time given in their method for stages 1–3 was increased to 60 minutes to slow down the pre-digestion step and ramping to the final heating step to prevent sample leaking out of the small (35 ml) digestion vessels. Digestion vessels with 35 ml capacity were chosen to increase sample throughput (since seven 35 ml vessels can be placed in the HPA instead of five 50 ml vessels). The bulk of the dissolution reaction occurred at 230 °C for 90 min. After the digestion, the samples were cooled to room temperature, and the resulting solution was diluted to 5 ml with DI water.

Instrumentation

The inductively coupled plasma mass spectrometer (ICP-MS) used in this study was an Elan 6100 (Perkin-Elmer, Norwalk, CT, USA) with nickel skimmer and sampler cones. A sample introduction system consisted of a Tracey Spray Chamber (cyclonic spray chamber) (Glass Expansion, West Melbourne, Australia) and a Meinhard concentric nebulizer (TQ-30-A3) (J.E. Meinhard Associates, Inc., Santa Ana, CA, USA). All samples measured in the optimization of the digestion protocol utilized the ID analytical protocol. For comparison, samples were also analyzed with an external calibration using indium as the internal standard. The instrument operation parameters are summarized in Table 3.

Reagents

Reagents used in this study were: HNO_3 (Optima, Seastar chemical Co., Pittsburgh, PA), 30% hydrogen peroxide (ULTREX® Ultrapure, Reagents, J.T. Baker, Phillipsburg, NJ), Pb (1000 $\mu\text{g ml}^{-1}$, High Purity Standards, Charleston, SC), NIST SRM 1643d (Trace Elements in Water), NIST SRM 983 (^{206}Pb -enriched Radiogenic Lead Isotopic Standard), and NIST SRM 981 (Common Lead Isotopic Standard).

Quality control

Quality control samples included NIST SRM 1549 (Non-fat Milk Powder) and NIST RM 8435 (Whole Milk Powder) spiked with certified lead concentrations (NIST SRM 983 ^{206}Pb spike solution to give isotopic ratio $^{208}\text{Pb}/^{206}\text{Pb} = 1.0$). Lead standards of known isotopic composition NIST SRM 981 (Common Lead Isotopic Standard) and NIST SRM 983 (^{206}Pb -enriched Radiogenic Lead Isotopic Standard) were used for isotopic ratio measurement and for mass bias correction.

Instrumental analysis

Samples were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (Sciex Elan 6100, Perkin Elmer, Norwalk, CT), with standard instrument operating and data collection parameters, using the ID procedure. Before ICP-MS analysis, the mass spectrometer setting and nebulizer flow rate were optimized to give a maximum peak intensity for lead. Ten percent of the samples were randomly selected and digested with and without the ID spike. The samples digested without the ID spike were later analyzed for their isotopic abundance of lead and compared with the “natural ratio” of lead (*i.e.* the isotopic abundance of lead as it varies in nature).

Samples with enriched ID spike (^{206}Pb added) were analyzed by ID-ICP-MS analysis.^{24–26} Data given are the average of five replicate measurements by the instrument. The instrument detection limit (0.1 ng ml^{-1}) was calculated as three SD from ten replicate measurements of reagent blank (5% HNO_3). NIST SRM 981 was used as a QC standard for isotope ratio measurement and for mass bias correction. Samples which were prepared without addition of the enriched isotope spike were also analyzed for lead concentration with an external calibration using indium (In) as the internal standard (0.2 ng ml^{-1}) and by mixing ID spike (0.5 ng ml^{-1}) on line with the sample using a Y-connector.

Results

Comparison of digestion procedures

Comparison of the three different sample digestion methods is shown in Table 4. During the method development stage, we studied the recovery of both QC standards (NIST SRM 1549 & NIST SRM 8435). Since the whole milk SRM represents very closely the fat content of human breast milk and no additional information was gained by analyzing two QC standards, we only analyzed NIST SRM 8435 to maximize sample throughput when we analyzed study samples. Digestion with HNO_3 in the High Temperature High Pressure Asher (HPA) (Anton Paar, Graz, Austria) was chosen as the preferred method since reproducibility of the lead concentration measurement was better for the samples prepared by the HPA method than by dry ashing in muffle furnace or by MWO digestion methods.

Dry ashing in muffle furnace

Initially, recovery of the QC standards was >90% during the method validation and up to the analysis of the sixth batch of samples. After the sixth batch, the recovery of the QC standards went down, for unexplained reasons, to an average of ~80% ranging from 67% to 88%. Average recovery of the pre-digestion spiked samples was ~83% and recovery of the post digestion spike was ~101% indicating a loss during sample digestion (ashing). This loss was observed even with adding the ID spike before the sample preparation step. We studied the effect on recovery by placing samples on various positions on the hot-plate, in various positions in the muffle furnace, and with varying number of samples placed in the oven at one time. We were unable to determine the cause of this sample loss which occurred suddenly after experience with the previous six batches. Due to this problem, we explored the other (MWO and HPA) digestion procedures for breast milk analysis.

Digestion in microwave oven

Recovery of lead in NIST SRM 1549 and NIST RM 8435 improved with increasing total digestion time. Percent recovery with a total of six minutes digestion time was above 98% for both QC standards. Therefore, two 3 minute heating cycles with 30 minute cooling in between cycles was chosen as the best method for breast milk digestion. Even though we obtained very good recoveries for the QC standards, reproducibility for the analysis of breast milk samples was poor with percent difference in duplicate analyses ranging from 3–36%. We also observed undigested fat in a ring formed around the wall of the Teflon® liner. Adding another heating step did not eliminate the fat ring.

Digestion in high pressure asher

Samples digested in HPA gave clear colorless solutions and recoveries for NIST RM 8435 and NIST SRM 1549 were between 90% and 108%. The percent differences for duplicate analyses were <20%.

Instrumental analysis

The standard method for ID involves determining the isotopic composition of the sample and using that information in the equation for the concentration calculation especially when

the natural isotopic ratio changes. Due to the very low concentration of lead in the sample ($<1 \text{ ng ml}^{-1}$ in the analytical solution), the measurement of the isotope ratio is not very precise (1–7% RSD) or not possible to measure accurately for very low abundant isotopes. Therefore, it would not be reliable to measure the isotopic abundance of each isotope or isotope ratio of each sample and input that information into the ID calculation. Since none of the breast milk samples analyzed had an isotope ratio value significantly different from each other or significantly different from the “natural ratio”, we used the natural ratio as the sample isotope ratio of each sample.

Comparison of analytical methods

We compared the ID method with the addition of the ID spike manually (Manual-ID-ICP-MS) before digestion by HPA to the addition of the ID spike online (Online-ID-ICP-MS) after digestion just before the instrumental analysis. All measurements for Manual-ID are done by weight which is more accurate than by volume. Manual-ID-ICP-MS will also correct for any losses and dilution errors that might happen during sample preparation while addition of Online-ID-ICP-MS and use of the external calibration method do not compensate for sample preparation losses. Ideally, we would expect the same results for Online-ID and external calibration since both methods use the same digestate. However, small differences in calibration curves or mixing during online dilution may have created differences in measured concentrations at these low levels. Precision (% RSD) of Manual-ID-ICP-MS analysis was better than that of Online-ID-ICP-MS method. If the measured concentration of the breast milk sample was unusually high, the sample was reanalyzed and the isotope ratio of the sample was checked to see whether it was out of the expected range. Higher concentrations from Online-ID indicate that there may have a problem with the spike, while lower concentrations observed with external calibration indicate that some losses may have been occurring. The ID method gave more precise analytical results for samples with low lead concentrations such as our breast milk samples (Table 5). Since we made an ID spike suitable for measuring the low concentrations in the breast milk samples, it may not have been suitable for higher concentrations (such as that of NIST RM 8435) thus resulting in poorer performance of Manual-ID for the QC standard.

The limit of detection for lead analysis in breast milk by HPA digestion and ID-ICP-MS is 0.1 ng ml^{-1} milk. The accuracy of the spiked samples and QC standards at this concentration is $>90\%$ and precision of the measurement is $<5\%$ RSD. Each batch contains one method blank and all analytic blanks have performed very well.

Conclusion

The reliable and accurate determination of trace lead in human breast milk can be technically quite challenging and is difficult for several reasons including: potential for contamination during sample collection, storage, and preparation; the high fat content of human milk; and poor analytical sensitivity for ultra trace amounts of lead present in the samples. Due to the difficulty in identifying a method that would successfully digest samples with 100% efficiency, we evaluated three different digestion procedures including: dry ashing in a muffle furnace, MWO digestion, and digestion in HPA. By comparing their

respective performances, it was determined that digestion by the HPA method should be the procedure of choice for breast milk samples. We believe the sensitivity of the dry ashing method is low even when there is no sample dilution, whereas MWO method matrix is diluted 1 : 5 and HPA method matrix is diluted to 1 : 10, due to the analytical measurement being done in high matrix solution. Even though minimizing the analyte dilution is preferable, the high calcium and other components of breast milk may be interfering with the sensitivity of the analytical measurement. Use of a powerful sample preparation procedure, such as HPA, appears to give a complete digestion of the high fat-content breast milk sample and reproducibility of the lead concentration measurement of samples prepared by HPA was better than for the samples prepared by dry ashing or by the MWO digestion methods. Improved homogeneity of the fat-separated samples was achieved by warming samples up to body temperature before aliquoting.

We also evaluated methods for trace lead instrument analysis using ID-ICP-MS and external calibration. The analytic capabilities and the potential interferences affecting them were assessed and a reliable analytical procedure was developed. An additional source of variability or error may be environmental contamination, so we implemented a sample collection, storage and handling protocol to minimize and monitor any potential contamination during this process. In summary, the main sources of error: potential contamination, analyte losses, and incomplete digestion²⁷ can be minimized for the quantification of trace lead in human milk. Use of strict contamination control techniques and of a very powerful digestion procedure, along with an ID-ICP-MS method for lead determination, enabled us to measure trace lead levels as low as 0.2 ng ml⁻¹ in milk. Carefully designed exposure assessment and toxicokinetic studies are needed to elucidate mechanisms and establish relationships between human milk and other biologic matrices.²⁸

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Table 1

Temperature–time program for dry ashing of milk samples in muffle furnace

Temperature (°C)	Ramp/hold time	Process
Ambient-100	1 °C min ⁻¹	Ramp
100	0.5 hours	Hold
100–200	1 °C min ⁻¹	Ramp
200	4.0 hours	Hold
200–450	10 °C min ⁻¹	Ramp
450	10 hours	Hold

Table 2

Temperature –time program for nitric acid digestion of milk samples in high-pressure high temperature asher

<u>Temperature</u>		<u>Temperature</u>
<u>Initial (°C)</u>	<u>Time (minutes)</u>	<u>Final (°C)</u>
40	60	110
110	60	110
110	60	230
230	90	230 ^a

^aDigested samples were allowed to cool naturally to room temperature for about 60 minutes before opening the autoclave.

Table 3

ICP-MS (Elan 6100) instrument operation parameters

ICP system	
RF power	1200 W
Sampler and skimmer	Nickel
Torch	Quartz
Nebulizer	Concentric
Spray chamber	Cyclonic
Gas flow rate/l min⁻¹	
Plasma	15.0
Auxiliary	0.80
Nebulizer	0.9
Data acquisition	
Dwell time/ms	100
Sweeps/reading	10
Reading/replicate	1
Number of replicates	5
Masses analyzed	²⁰⁶ Pb and ²⁰⁸ Pb
Sample uptake^a	
Total	~1 ml min ⁻¹

^aWhen online spike addition and internal standard addition assumed 1 : 1 mixing. Absolute mixing rate was calculated daily using reverse ID using NIST 1643e for ID method.

Table 4

Comparison of Pb recoveries and reproducibility for the three sample digestion methods

	Dry ashing	MWO digestion	HPA digestion
Blank (Pb ng ml ⁻¹)	0.19	0.27	0.05
Detection limit (ng ml ⁻¹ milk)	0.10	0.30	0.10
NIST RM 8435 (% recovery)	67–101	92–114	90–108
NIST SRM 1549 (% recovery)	71–111	85–109	90–108
Duplicate analysis (% difference)	9–30	3–36	<20

Table 5

Comparison of Pb concentrations (ng ml⁻¹) for five samples chosen at random and NIST RM 8435 by the three analytical methods

Sample ID	Isotope dilution manual			Isotope dilution on-line			External calibration in (internal standard)		
	Mean ± SD	% RSD		Mean ± SD	% RSD		Mean ± SD	% RSD	
3024	0.3044 ± 0.0203	6.7		0.3452 ± 0.0339	9.8		0.2162 ± 0.0179	8.3	
3053	0.6874 ± 0.0159	2.3		0.6650 ± 0.0471	7.1		0.5440 ± 0.0147	2.7	
3072	0.4305 ± 0.0248	5.8		0.4577 ± 0.0261	5.7		0.3342 ± 0.0296	8.9	
3089	0.3792 ± 0.0134	3.5		0.6813 ± 0.0598	8.8		0.5392 ± 0.0274	5.1	
3102	0.5770 ± 0.0257	4.5		0.8928 ± 0.0466	5.2		0.7438 ± 0.0217	2.9	
NIST RM 8435 ^a	96.4926 ± 3.57	3.7		113.2931 ± 4.49	4.0		110.338 ± 4.91	4.5	

^a NIST RM 8435 Whole Milk Powder, certified lead concentration: 0.11 ± 0.05 mg kg⁻¹.