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Method modified AOAC 2015.01 in the microwave conditions and the addition of HCl during sample digestion for simultaneous Pb, Cd, Hg, and As in infant formula using ICP-MS: a method validation

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Abstract

The presence of heavy metals in infant formula has become a global concern. The most common method to determine heavy metals is AAS. However, as this technique is lacking in several aspects, including the instrument's low sensitivity, a more sensitive instrument such as ICP-MS is necessary. The Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was used in accordance with the standard method AOAC 2015.01 with modifications on the microwave condition and the addition of hydrochloric acid (HCl) during the sample digestion process. The modified standard method requires a validation process. This research aimed to validate the method of analysis for the determination of Pb, Cd, Hg, As in infant formula using ICP-MS and its application in formula milk. This research consists of five stages: 1) instrumental performance; 2) homogeneity test; 3) method orientation; 4) method validation; and 5) the application of the validated method to other products. The findings in the research were: the method linearity was confirmed at working concentration 5-30 µg/kg for all the heavy metals with R^2 value of nearly 1,000; the method limits of detection (LOD) were 0.74 µg/kg (Pb), 0.41 µg/kg (Cd), 0.08 µg/kg (Hg), 0.50 µg/kg (As), while the method's limits of quantification (LOQ) were 2.48 µg/kg (Pb), 1.36 µg/kg (Cd), 0.27 µg/kg (Hg), 1.67 µg/kg (As); the method was found precise with Relative Standard Deviation (RSD) below 2/3 RSD Horwitz and all the recovery values were found to fall within the acceptable range (60–115%); the % RSD intra-lab reproducibility was below RSD Horwitz; and the method was robust, indicating that it was unaffected by small changes in its variables. The validated method can be applied routinely to determine heavy metals in infant formula and formula milk.

1. Introduction

Infant formula is a breast-milk substitute specially formulated to meet infants' nutritional needs during their first few months until they are introduced to suitable complementary foods. It is formulated to contain proteins, fats, carbohydrates, vitamins, minerals, trace elements, etc. (CAC, 2007). To date, a total of 38 microelements and trace elements have been reported found in the fresh milk from cows in the Silesian region (Dobrzański et al., 2005). Microelements such as copper (Cu), iron (Fe), selenium (Se), and zinc (Zn) are essential for human growth. However, heavy metals such as lead (Pb), cadmium (Cd), mercury (Hg), and arsenic (As) are toxic and have cumulative effects (Martino et al., 2000;

Onianwa et al., 1999; Aslam et al., 2011). Children under five years are proven to be more sensitive to Pb and Cd than adults (Tripathi et al., 1999). Due to their lipophilic nature, heavy metals in milk cannot be easily removed (Girma et al., 2014).

Infants are under the age group vulnerable to heavy metal exposure because the consumption of infant formula is relatively high and they have low body weight, making even the smallest heavy metal exposure affects their health. The Indonesian FDA has set regulations on maximum limits for heavy metal contaminations, the Regulation of the Head of Indonesian FDA No. 5/2018. Based on the regulation, the maximum level for Pb, Cd, Hg, and As in infant

formula is 10–20 µg/kg (Indonesian FDA, 2018). The European Commission Regulation (EC) No. 1881/2006 and Commission Regulation (EC) No. 488/2014 recommend the maximum level for Pb and Cd in infant formula are 20 µg/kg and 10 µg/kg (EC, 2006; EC, 2014).

Various testing techniques have been developed to determine the concentration of heavy metals in food products, such as atomic absorption spectrophotometry (AAS). However, the technique is lacking in several aspects, including the instrument's low sensitivity to detect heavy metals in very small concentrations and relatively lengthy testing time (Helaluddin *et al.*, 2016). Therefore, a more sensitive instrument, i.e. inductively coupled plasma mass spectrophotometry (ICP-MS), is necessary to detect heavy metals, particularly in infant formula (Khan *et al.*, 2014).

The ICP-MS was used in accordance with the standard method AOAC 2015.01 with several modifications, i.e. in the microwave conditions and the addition of HCl during sample digestion. In the standard method, Au, whose function is the same as HCl, to stabilize Hg, is to be added during sample digestion (Briscoe *et al.*, 2015). However, the addition of HCl should be taken into careful account for other element analyses, particularly As, as it can lead to polyatomic interference. A suitable analysis mode can minimize such interference. Helium or Hydrogen modes are often used to remove polyatomic interference such as $^{40}Ar^{35}Cl$ and $^{40}Ar^{16}O$, either through collision or reaction mechanism (Thomas, 2013).

The modified standard method requires a validation process to ensure that the method is valid and, therefore, applicable for routine testing in the laboratory with accountable analysis results. The steps of an analysis method validation process are: preparing a validation protocol; testing the instrument's performance; performing validation experiments using several parameters such as method's linearity, method's LOD and LOQ, precision (both repeatability and reproducibility), accuracy, recovery test, and robustness; developing a Standard Operating Procedure (SOP) for executing the method in routine analysis, and documenting the validation experiments and results (Magnusson and Ornemark, 2014). This research aimed to validate the analysis method for the determination of simultaneous Pb, Cd, Hg, and As in infant formula using ICP-MS and its application in formula milk.

2. Materials and methods

2.1 Materials

This research used ERM BD 150 "Skimmed Milk Powder" from European Commission, Belgium; infant formula; follow up a formula for infant (6-12 months); follow up a formula for young children (12-36 months); ultra-pure water (18.2 MΩ) from Milli-Q Ultrapure Water System Merck; high purity HNO_3 (65%, *Suprapur*); 37% HCl *for analysis*; acetic acid (glacial) 100% anhydrous *for analysis*; and H_2O_2 from Merck Co. (Darmstadt, Germany). All the standard solutions used were also from Merck Co. (Darmstadt, Germany), *Certipur* lead, cadmium, mercury and arsenic ICP Standard 1000 mg/L. Other than that, 100 µg/mL mixed internal standard solution (Bi, Ge, In, Li, Lu, Rh, Sc, Tb) from Agilent was also used.

2.2 Instrumentation

The elements were analyzed using ICP-MS (Thermo Scientific iCAP RQ, Germany); sample digestion was carried out using a microwave digestion system (CEM MARS Xpress), and the sample was weighed using an analytical balance from Mettler Toledo. For sample preparation, all the plastic labware were new or had been cleaned by soaking them first for 24 hrs in 10% HNO_3 for analysis and then in ultra-pure water.

2.3 Instrumental performance for heavy metals analysis by ICP-MS

During this step, standard solutions were injected without sample matrices. Several instruments, linearity (n=3), detection and quantification limits (n = 10), and the precision of the intensity ratio between each analyte and the internal standard (n = 6), were evaluated. To determine the instrument's linearity, a calibration curve was made at concentrations 0–5 ng/mL, each in three replications. EURACHEM guideline was used as the reference for analyzing the results of the evaluation (Magnusson and Ornemark, 2014).

2.4 Homogeneity test

Each of the infant formula samples in the same batch was put in a large plastic ware and shaken until homogeneous. The samples were then put in at least 10 small containers and was tested for their moisture content (n=10), each in two replications. The moisture content analysis was carried out following Indonesian National Standard (2015), while the sample homogeneity was analyzed according to Sunanti *et al.* (2013).

2.5 Method orientation

In this step, the test was carried out following the standard method AOAC 2015.01 (Briscoe *et al.*, 2015) with modifications in the microwave conditions and the addition of 37% HCl during sample digestions using a microwave. In addition, during this step, the isotopes for

quantifying the analysis results were also selected.

2.5.1 Sample preparation (Standard method AOAC 2015.01)

A total of 0.25 g sample aliquot was weighed and put into a microwave digestion vessel, and 4 mL concentrated HNO_3, 1 mL H_2O_2, 0.1 mL of the 50 mg/L Au + Lu solution were then added. The solution was then left to stand for a while before the vessel was capped off securely and put into a microwave digester. The sample was digested at a minimum of 180°C for at least 10 mins. Ramp time and cool down time were adjusted in accordance with the type and model of the microwave digestion system. The vessel was let to cool down to room temperature before the cap was removed slowly. The sample solution was then transferred into a centrifuge tube and diluted with ultra-pure water (18.2 MΩ) until the final volume was 20 mL. In addition, an internal standard made in acetic acid solution was then added using the second channel of the peristaltic pump.

2.5.2 Modification method

Approximately 0.5 g sample was weighed precisely and put into a vessel, and 5 mL 65% HNO_3 *Suprapur*, 1 mL 37% HCl, and 1 mL H_2O_2 were then added. The mixture was left to stand until the initial reaction subsided, and the vessels were capped off securely. The vessels were put into a microwave digester chamber and the sample was digested at a minimum of 150°C for at least 5 mins. The vessels were let to cool down and open carefully. The digested solutions were transferred into a 50 mL PP centrifuge tube, and ultra-pure water (18.2 MΩ) was added, followed by 1 mL acetic acid and 0.25 mL mixed internal standard solution in 0.5 μg/mL concentration. The mix was then diluted with ultra-pure water (18.2 MΩ) to the final volume of 50 mL.

2.5.3 Calibration standards

The standard curve for the heavy metals (Pb, Cd, Hg, and As) analysis was prepared using the stock solutions (1000 mg/L). Fresh calibration standards were prepared every day, or as deemed necessary, in 0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, and 5 ng/mL concentrations for each analyte.

2.5.4 The instrumentation and the condition of ICP-MS

Heavy metals in the digested solutions were determined using the ICP-MS instrument (Thermo Scientific iCAP RQ, Germany) with the KED (Kinetic Energy Discrimination) mode using helium as collision gas to decompose ArCl interfering ion at m/z 75. See Table 1 for the analytical conditions.

Table 1. The Conditions of ICP-MS

Parameter	Value
Peristaltic Pump Speed	40 rpm
RF Power	1550 W
Cool Gas Flow	14 L/min
Auxiliary Gas Flow	0.8 L/min
Nebulizer Gas Flow	0.97 L/min
Number of Sweeps	20
Dwell Time	0.001–0.02 ms
Replicate per Analysis	3
Signal Stabilization Time	15 s
Fast Sample Loop	0.5 mL
Fast Uptake Time	3 s
Fast Rinse Time	1+3 s

2.5.5 Determining heavy metals using ICP-MS

The peak intensities of the analyte and internal standard were measured. The concentrations of analyte were calculated based on the intensity ratio between each analyte and the internal standard.

2.6 Method validation

The evaluated parameters were the method's linearity, the method's limit of detection (LOD) and limit of quantification (LOQ), precision and accuracy using CRM, precision and accuracy using recovery testing, intralab reproducibility and robustness (Magnusson and Ornemark, 2014; AOAC, 2016). The method's linearity test was carried out using infant formula matrices spiked in stages with the standard solutions after the weighing and prior to the sample digestion process. The analytes were added in 5, 10, 15, 20, 25, 30 μg/kg concentrations, each in three replications. Determining the linearity value of the method requires a calibration curve that shows the relationship between the analyte concentrations in the spiked samples (μg/kg) and the intensity value (CPS) as the analysis response. From the calibration curve, the slope, the intercept values and the coefficient of determination were obtained. To determine the limit of detection (LOD) value, samples containing no analytes (blank samples) or samples in concentrations near or below the estimated LOD were used. The LOD of the method was determined using non-spiked samples that were analyzed in minimum six replications, and the SD were then calculated. The LOD of the method was calculated using the formula $\frac{3SD}{\sqrt{n}}$, while the LOQ $\frac{10SD}{\sqrt{n}}$.

The precision and accuracy were determined using ERM BD 150 "Skimmed Milk Powder" from the European Commission, Belgium in concentrations known for Pb, Cd, and Hg analytes and the spiked samples at the required limit based on the Regulation of the Head of Indonesian FDA No. 5/2018 (n = 6). Mean, standard deviation (SD), relative standard deviation (RSD), and recovery values were calculated. The intralab reproducibility was determined by evaluating the

analysis results using the same instrument, samples, and personnel at different times. The testing was carried out for 3 consecutive weeks using samples spiked with 20 µg/kg standard solutions (n = 3). The evaluated parameters were the RSD analysis in the 3 different weeks. The last parameter for the method's validation was robustness, and it was determined by making a small change in the 65% HNO_3 *Suprapur* volume for the sample digestion process. In the standard method AOAC Official Method 2015.01, the volume of 65% HNO_3 *Suprapur* is 5 mL, while in this robustness test the volume was changed to 4.5 mL (n=3).

2.7 The application of the validated method in other products with similar matrices

The validated method was applied to analyze heavy metals in four formula milk samples. The results of the analysis were compared with the levels specified in the applicable regulation, the Regulation of the Head of Indonesian FDA No. 5/2018, the Commission Regulation (EC) no 488/2014 and Commission Regulation (EC) no 1881/2006.

2.8 Statistical analysis

Microsoft Excel 2016 Professional Plus was used to calculate the heavy metal contents in infant formula and their descriptive statistics, including t-test for robustness parameter. In addition, the parameter of intralab reproducibility was statistically calculated using the One-Way ANOVA parametric test and Kruskal-Wallis non-parametric test, followed by Tukey HSD and Mann Whitney tests using SPSS version 23. To determine statistical significance, a probability value of 0.05 was used.

3. Results

3.1 Instrumental performance of ICP-MS for heavy metals analysis

To determine the instrument linearity, a calibration curve was made at concentrations 0–5 ng/mL, showing the relation between the analyte concentration (ng/mL) on the x-axis and the intensity value (Counts per Second) as the analysis response on the y-axis. The linear equation was eventually generated with R^2 were

respectively 0.9999, 0.9997, 0.9997, 0.9998 for the analytes Pb, Cd, Hg, and As and based on the results, ICPMS have good linearity indicated by $R^2 \geq 0.990$ (AOAC, 2013).

The other test parameters for the instrument performance were instrument limit of detection (LOD) and limit of quantification (LOQ). The instrument LOD for the analytes Pb, Cd, Hg, and As were respectively 0.014, 0.015, 0.011, and 0.012 ng/mL, while the instrument LOQ were respectively 0.046, 0.048, 0.037, and 0.038 ng/mL.

The next test parameter for the ICP-MS performance was the precision of the analysis response. The results showed that the RSD of the analytes Pb, Cd, Hg, and As at the LOQ concentration (0.03 ng/mL) were respectively 0.19, 1.23, 1.59, and 1.87% and this value was still below 2%.

3.2 Sample homogeneity

The mean moisture content in infant formula was 2.32±0.04% (wet basis). The results of the homogeneity test showed that the mean square within (MSW) and the mean square between (MSB) were respectively 0.0015 and 0.0029. The formula F-statistic = $\frac{MSB}{MSW}$ was used, and the result showed that the F-statistic (1.96) < F-table (3.02), indicating that the infant formula samples in this research were homogeneous.

3.3 Method orientation

3.3.1 Determining the microwave condition

The digestion method using microwave was developed based on the method from AOAC Official Method 2015.01 (Briscoe *et al.*, 2015) and the experiences in our laboratory. See Table 2 for the three microwave conditions selected to validate the method of analysis. The microwave conditions based on the experience in our laboratory (condition 3) were selected for the method validation processes. These conditions were highly dependent on the brand and the condition of the microwave itself when being used for the digestion process.

Table 2. Microwave condition for sample digestion

Condition	Stage	Max. Power	Power (%)	Ramp (min)	Temp (°C)	Hold (min)	Reference
Condition 1	(1)	800 W	100	10:00	180	10:00	AOAC Official Method 2015.01
	(2)	800 W	100	10:00	200	20:00	
Condition 2	(1)	800 W	100	10:00	180	10:00	Experience in our laboratory
	(2)	800 W	100	10:00	200	25:00	
Condition 3	(1)	1600 W	60	15:00	150	5:00	Experience in our laboratory
	(2)	1600 W	60	5:00	180	5:00	
	(3)	1600 W	60	5:00	200	25:00	

3.3.2 Isotopes selection

In this research, several isotopes from the analyzed analytes were screened, except for As which only has one isotope. The selected isotopes were ^{206,208}Pb, ^{110,111}Cd, ^{200,201,202}Hg, and ^{75}As. The samples used were spiked with standard solutions prior to preparation and injection into the ICP-MS instrument. The isotopes for the method validation process were selected based on the recovery values that meet the AOAC requirements (AOAC, 2016). The results showed that the recovery values of all the isotopes were found to meet the requirements. As such, another aspect should also be considered, the abundance in nature. The abundance value is related to the sensitivity where low abundance results in low sensitivity (Wilschefski and Baxter, 2019). Therefore, isotopes with the highest abundance values were selected for the method validation process, i.e. isotopes ^{208}Pb, ^{111}Cd, ^{202}Hg, and ^{75}As with the abundance values of 52.4, 12.8, 29.86, and 100%, respectively.

3.4 Method validation for heavy metals analysis

3.4.1 Method's linearity

The method was found linear for Pb, Cd, Hg, As in working range 5–30 µg/kg with R^2 were respectively 1.000, 1.000, 1.000, 0.998 (Table 3). The results indicated that the analysis method was able to generate responses proportional to the increase in the concentrations of the analyte in the samples.

Table 3. The method linearity of ^{208}Pb, ^{111}Cd, ^{202}Hg, ^{75}As, ^{119}Sn

Parameter	Analyte			
	^{208}Pb	^{111}Cd	^{202}Hg	^{75}As
Method linearity				
Slope	0.0031	0.0003	0.0004	0.0140
Intercept	0.0756	0.0020	0.0033	0.0447
R^2	1.000	1.000	1.000	0.998

3.4.2 Method's LOD and LOQ

Based on the results, the method's LOD for the analytes Pb, Cd, Hg, As were 0.74, 0.41, 0.08, 0.50 µg/kg, while the LOQ 2.48, 1.36, 0.27, 1.67 µg/kg. The values were not confirmed further as the concentrations of the analytes in the samples were already close to the LOD values.

3.4.3 Precision and accuracy using CRM

The CRM was prepared and injected into ICP-MS, the same as the treatment to the test samples. A good precision is confirmed when the RSD analysis <2/3 RSD Horwitz (Magnusson and Ornemark, 2014). In this research, the results of the analysis using CRM (n = 8) revealed that the RSD analysis for the analytes Pb, Cd, and Hg were respectively 10.68, 2.52, and 3.28%. The RSD analysis was below 2/3 RSD Horwitz for the elements, 19.30, 20.90, and 16.49%, respectively (Table 4).

The accuracy is deemed to meet the acceptable requirements when the analysis results of all the eight replications fall within the concentration range in the CoA and the recovery values are also within the required range (AOAC, 2016). The results revealed that the mean concentrations for the analytes Pb, Cd, and Hg were respectively 19.47, 11.37, and 55.30 µg/kg. The concentrations were still within the concentration range for the analytes in the CoA, i.e. respectively 19.0±4, 11.4±2.9, and 60±7 µg/kg. In addition, the recovery values for the analytes Pb, Cd, and Hg were respectively 102.68, 99.75, and 92.17% (Table 4). All the recovery values in this research met the acceptable requirement in AOAC (2016), i.e. to fall within the range of 60–115%.

3.4.4 Precision and accuracy using spiked samples

The samples spiked at concentration 10 µg/kg were prepared and injected into ICP-MS. A good precision is confirmed when the RSD analysis <2/3 RSD Horwitz. Based on the results, the RSD analysis for the analytes Pb, Cd, Hg, and As were respectively 9.81, 2.60, 7.18, and 4.56% (Table 5). The RSD analysis was below 2/3 RSD Horwitz for the elements, i.e. 21.93, 21.50, 21.92, and 21.38%, respectively.

The accuracy value was determined by calculating the recovery value of each replication (at least 6 replications). Based on the results, the mean recovery values for the analytes Pb, Cd, Hg, and As were respectively 83.92, 96.04, 84.44, and 98.99% (Table 5). The values met the acceptable requirement in AOAC (2016), i.e. to fall within the range of 60–115%.

3.4.5 Intra-lab reproducibility

The testing was carried out for 3 consecutive weeks

Table 4. The precision and the accuracy of Pb, Cd, Hg using CRM (n = 8)

Analyte	Concentration (µg/kg)		Range CoA	Recovery (%)		RSD (%)	2/3 RSD Horwitz (%)
	Range	Mean		Range	Mean		
Pb	16.27–21.67	19.47	19.0±4	85.64–114.06	102.47	10.68	19.3
Cd	11.04–11.83	11.37	11.4±2.9	96.37–103.80	99.75	2.52	20.9
Hg	53.76–58.12	55.3	60±7	89.59–96.86	92.17	3.28	16.49

RSD: Relative Standard Deviation, CoA: Certificate of Analysis

Table 5. The precision and the accuracy of the method to analyze Pb, Cd, Hg, As using spiked samples (n = 6)

Analyte	Concentration (µg/kg)		Concentration added (µg/kg)	Recovery (%)		RSD (%)	2/3 RSD Horwitz (%)
	Range	Mean		Range	Mean		
Pb	7.76–9.93	8.33	10	77.85–99.59	83.92	9.81	21.93
Cd	9.23–9.83	9.51	10	92.57–102.33	96.04	2.6	21.5
Hg	7.78–9.33	8.36	10	78.07–97.08	84.44	7.18	21.92
As	9.11–10.39	9.87	10	91.51–104.18	98.99	4.56	21.38

RSD: Relative Standard Deviation.

using samples spiked with 20 µg/kg standard solutions. The evaluated parameters were the RSD analysis in the 3 different weeks. As all the RSD analyses were smaller than RSD Horwitz, the acceptable requirements for precision were met (Table 6).

The differences in the levels of analytes Pb, Cd, Hg, As in weeks 1, 2, and 3 of testing were statistically calculated using One-Way ANOVA parametric analysis for Pb, Cd, As and Kruskal-Wallis non-parametric analysis for Hg, followed by Tukey HSD and Mann Whitney tests. Based on the results of the analysis, there was no significant difference among each data group, indicated by the p-value being greater than the α (0.05) (Table 6). This means, partially there was no difference in the data values of the analytes Pb, Cd, Hg, and As in weeks 1, 2, and 3 of testing.

3.4.6 Robustness

In this research, the parameter robustness was determined by making a small change in the HNO_3 volume for the sample digestion process. In the standard method AOAC Official Method 2015.01, the volume of HNO_3 is 5 mL, while in this robustness test the volume

was changed to 4.5 mL. The results of the analysis were then compared using the t-test parametric method.

The results of the t-test showed that the p-values of all the data groups were greater than 0.05 (Table 7), indicating that the data groups were not significantly different. This means, there was no difference in the results of the analysis on Pb, Cd, Hg, and As between the addition of 5 mL and 4.5 mL HNO_3 *Suprapur*. Therefore, the analysis method was found robust as it was unaffected by the small change in its variable, 65% HNO_3 *Suprapur* volume.

3.5 The application of the validated method to other products with similar matrices

The analysis was carried out using non-spiked samples with three replications each. The results of the analysis were then compared with the heavy metal levels specified in the applicable regulations. Based on the results, all formula milk products contained heavy metals but the concentrations did not exceed the maximum acceptable levels by the Regulation of the Head of Indonesian FDA No. 5/2018, 10–20 µg/kg and the Commission Regulation (EC) no 488/2014, Commission

Table 6. The result of intralab reproducibility testing of heavy metals in infant formula with spiked (n=3)

Week	Concentration±SD (µg/kg)			
	^{208}Pb	^{111}Cd	^{202}Hg	^{75}As
1	19.75 ± 1.97	20.37±0.06	21.41±0.52	20.95±0.70
2	18.12±0.54	21.11±0.68	19.83±0.18	19.13±1.07
3	20.19±0.34	20.49±1.73	20.54±0.25	20.73±1.90
Mean	19.35±1.09	20.66±0.40	20.59±0.79	20.27±0.99
P value	> 0.05			
RSD (%)	5.64	1.91	3.84	4.9
RSD Horwitz (%)	28.97	28.69	28.7	28.77

RSD: Relative Standard Deviation.

Table 7. Comparison of analysis results of heavy metals in the addition of 5 mL and 4.5 mL HNO_3 with spiked (n = 3)

Analyte	Variable	Mean±SD (µg/kg)	RSD (%)	P-value	Conclusion
Pb	HNO_3 5 mL	19.75±1.97	9.99	0.395*	There was no difference
	HNO_3 4.5 mL	19.37±1.11	5.75		
Cd	HNO_3 5 mL	22.10±0.60	2.72	0.265*	There was no difference
	HNO_3 4.5 mL	22.46±0.70	3.11		
Hg	HNO_3 5 mL	21.41±0.52	2.4	0.091*	There was no difference
	HNO_3 4.5 mL	20.66±0.62	2.98		
As	HNO_3 5 mL	20.95±0.70	3.32	0.287*	There was no difference
	HNO_3 4.5 mL	21.47±1.26	5.87		

*t-test

Table 8. Concentration of heavy metals in four formula milk samples analyzed by ICP-MS

Brand	Concentration of analyte (µg/kg)			
	^{208}Pb	^{111}Cd	^{202}Hg	^{75}As
A	ND	1.78±0.16	ND	2.29±0.25
B	ND	2.06±0.30	ND	3.20±0.24
C	Trace (2.38±0.18)	1.54±0.08	ND	3.63±0.20
D	4.12±0.37	8.34±0.61	ND	7.91±0.64
Regulation of the Indonesian FDA (µg/kg)	10	10	10	20
Commission Regulation (EC) no 488/2014, (EC) no 1881/2006	20	10	-	-

ND: Not Detected, Trace: higher than the method's LOD, but still lower than the method's LOQ

Regulation (EC) no 1881/2006, i.e. 10-20 µg/kg for Pb, Cd (Table 8). Pb was found greater than the LOQ in one formula milk only, while analyte Hg was undetected in all tested samples. In addition, varied concentrations were found in analytes Cd and As, where Cd concentrations ranged from 1.54 to 8.34 µg/kg and As concentrations ranged from 2.29 to 7.91 µg/kg.

4. Discussion

Heavy metals analysis was conducted in accordance with the standard method AOAC 2015.01 with some modifications on the microwave condition and the addition of 37% HCl during the digestion process. The digestion was microwave-assisted carried out in closed vessels under pressure where the samples were decomposed using nitric acid and HCl with the addition of H_2O_2 as an oxidizing agent. The sample solution was then transferred to a centrifuge tube, followed by 0,25 mL internal standard solution, 1 mL acetic acid and diluted using ultra-pure water to the final volume of 50 mL.

Condition 3 based on our experience in the laboratory was selected for the method validation process because the recovery of the analysis results was found to meet the requirements under the AOAC (2016), 60–115%. Temperature, time, and volume of strong acids used extremely affect the efficiency of a sample digestion process using a microwave (Momen *et al.*, 2007). A complete digestion process is indicated by the formation of a clear solution. In conditions 1 and 2 (Table 2) (with 800 W pressure), the microwave temperature was failed to be reached. This was presumably why the recoveries in both conditions were not good for some metals, and therefore, in condition 3 the pressure was raised to 1600 W and eventually the temperature was reached. High pressure in vessels increases the boiling point of nitric acid and, therefore, the oxidation power, which eventually accelerates reactions (Solihat *et al.*, 2018).

The sample digestion process generally uses HNO_3 with the addition of H_2O_2 as an oxidizing agent to increase the oxidizing power of HNO_3 during sample destruction and to increase the process of breaking down organic matters in the sample. Some of the times, HNO_3 and H_2O_2 mix is unable to dissolve all the materials in the sample, and therefore the recovery cannot be reached. It is necessary to add and combine HCl with HNO_3 and H_2O_2, particularly for samples that are difficult to digest (Momen *et al.*, 2007).

The HCl was also added particularly for Hg analysis. Several aspects that should be of concern on the Hg analysis are among others: Hg has an extremely high first ionization energy (10.44eV), resulting in its low degree of ionization; has numerous isotopes (7 isotopes) and all their abundances are below 30%, causing Hg to have a low sensitivity for its every isotope, and is volatile, making it easily lost when the vessels are opened after the sample digestion using a microwave. Therefore, it has a relatively lower recovery. One of the aspects being considered to increase the sensitivity of Hg was the addition of chemicals to stabilize Hg. In this study, the addition of HCl in a low concentration was used to form $HgCl_4^{2-}$ which is more stable (Korn *et al.*, 2008; Casey *et al.*, 2018). On the contrary, the standard method AOAC 2015.01 uses Au whose function is the same as HCl, i.e. to stabilize Hg and Lu to avoid analytes from being lost during the sample digestion process (Briscoe *et al.*, 2015).

The addition of HCl must be observed closely for the analysis of the other metals because the process may cause polyatomic interferences. Polyatomic ions are formed in the plasma due to the imperfect atomization process. They can be from sample matrices, reagents for sample preparation, and/or argon gasses. For example, $^{35}Cl^-$ and $^{40}Ar^+$ form $^{40}Ar^{35}Cl^+$ that has m/z 75 and therefore may overlap with isotope As that also has m/z 75 (Wilschefski and Baxter, 2019). This will cause the response of As analysis to be greater than it should be due to the addition from the polyatomic ions that have the same mass as As. To minimize the interference, this research used KED (Kinetic Energy Discrimination) mode in the ICP-MS system. This mode uses Helium as the collision gas that will remove polyatomic

interferences before going to the mass analyzer (Thomas, 2013).

Carbon elements in sample solutions from the CO_2 decomposition during the sample digestion or from the remaining insoluble organic compounds can also become a problem in analysis using ICP-MS, they make the analyte response higher. The most affected element is As due to its low degree of ionization. The carbon will increase the effectivity of As ionization in the plasma, and without the addition of carbon-source elements into the standard solutions, the analyte level will be unnecessarily higher. To avoid such a response, in this research, acetic acid was added as the carbon source after the sample digestion process into the sample, standard, and blank solutions in the same volume. Acetic acid was added to increase As sensitivity because based on the previous studies, the addition is able to increase As intensity up to 2.8 times (Nishimura *et al.*, 2010; Briscoe *et al.*, 2015).

Sample matrices greatly influence the reading of the analysis responses. Using internal standards is effective to correct the signal's declining responses due to the matrix effect and the instrument drift (Vanhaecke *et al.*, 1992; Evans and Giglio, 1993; Wilschefski and Baxter, 2019). The most suitable internal standard should also be taken into account. The mass and the potential ionization of the internal standards should be as close as possible to the target analyte's so that the treatments in the plasma will be the same; stable; non-reactive to the sample matrix and analyte and should not be in the samples to be analyzed (Evans and Giglio, 1993; Martino *et al.*, 2000; Martino *et al.*, 2001; Nishimura *et al.*, 2010; Thomas, 2013; Wilschefski and Baxter, 2019). Using an internal standard whose mass number is close to the target analyte's will increase precision (Vanhaecke *et al.*, 1992). The internal standards should be added into the sample, standard, and blank solutions at the same concentrations (Wilschefski and Baxter, 2019). This research used internal standards ^{209}Bi, ^{103}Rh, and ^{73}Ge. Bismuth was used to correct the analytes Pb and Hg, Rhodium for Cd, and Germanium for As.

The instrument ICP-MS was found to have a great performance in analyzing heavy metal contents in infant formula because it was able to generate intensity values that were proportional with the increase in the analyte concentration as its analysis responses. This step aimed to ensure that all the systems in the ICP-MS performed well before they were used for the validation process. Well-performing instruments affect the results of analysis method validations. All the parameters of the method validation, i.e. the method's linearity, the method's LOD and LOQ, precision and accuracy using

CRM and recovery testing, intralab reproducibility, and robustness, were found to meet the acceptable requirements with the method's LOD for the analytes Pb, Cd, Hg, and As respectively 0.74, 0.41, 0.08, and 0.50 µg/kg, and the LOQ 2.48, 1.36, 0.27, and 1.67 µg/kg, respectively.

Another research using the same instrument and matrices was found to have similar to higher LOD and LOQ, 2, 0.8, and 1 µg/kg (LOD) and 6, 1, and 2 µg/kg (LOQ) for Pb, Cd, and As, respectively. The accuracy of the heavy metal analysis method differed slightly, i.e. 83 –98% in this research and 97–102% in the other research (Lo Dico *et al.*, 2015). A slight difference in LOQ was also found in another research i.e. 4, 1, 3, and 2 µg/kg for Pb, Cd, Hg, and As (Briscoe *et al.*, 2015). Such differences were due to different sample preparation procedures. In this study, the sample digestion process used 5 mL HNO_3, 1 mL HCl, and 1 mL H_2O_2, while Lo Dico *et al.* (2015) used 3 mL HNO_3 and 5 mL ultrapure water and Briscoe *et al.* (2015) used 4 mL HNO_3, 1 mL H_2O_2, and 0.1 mL Au 50 mg/L + Lu solution.

The validated analysis method was then used to analyse heavy metals in formula milk. The analysis was carried out on a non-spiked sample, each in 3 replications. Several differences were found in the results of analysis on heavy metals in formula milk carried out in another research, presumably because the researchers used different analysis mode. This research used KED (Kinetic Energy Discrimination) mode with Helium as the collision gas for all analytes, while the other research used Dynamic Reaction Cell (DRC) mode for As with Hydrogen as reaction gas and standard mode for Pb and Cd. In addition, the other research used isotope ^{114}Cd, while this research used isotope ^{111}Cd. The internal standard used for analyte Pb and As were also different, i.e. Bi and Ge in this research and Lu and Rh in the other research (Sorbo *et al.*, 2014).

5. Conclusion

All the parameters of the analysis method for simultaneous determination of the levels of Pb, Cd, Hg, and As in infant formula using ICP-MS in accordance with the AOAC Official method 2015.01 with several modifications were found to meet the acceptable requirements. The ICP-MS was found to have acceptable linearity for all the tested analytes (Pb, Cd, Hg, and As) with R^2 respectively nearly 1.0000. The method's LOD for the analytes Pb, Cd, Hg, and As were respectively 0.74, 0.41, 0.08, and 0.50 µg/kg, while the LOQ 2.48, 1.36, 0.27, and 1.67 µg/kg, respectively. This analytical method can be applied to analyze heavy metals in formula milk with the analysis results meet the acceptance requirements based on the regulation of the

Indonesian FDA.

Conflict of interest

The authors of the manuscript declare that there is no conflict of interest regarding the publication of this research.

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References

AOAC. (2013). Appendix K : Guidelines for Dietary Supplements and Botanicals. Retrieved from http://www.eoma.aoac.org/.

AOAC. (2016). Appendix F : Guidelines for Standard Method Performance Requirements. Retrieved from http://www.eoma.aoac.org/.

Aslam, B., Javed, I., Khan, F.H. and Rahman, Z. (2011). Uptake of heavy metal residues from sewerage sludge in the milk of goat and cattle during summer season. *Pakistan Veterinary Journal,* 31(1), 75-77.

Briscoe, M., Sullivan, D., Alewijn, M., Austad, J., Boison, J., Christiansen, S., Cook, J.M., Vries, J.D., Indyk, H., Joseph, G., Konings, E., Krynitsky, A., Phillips, T., Popping, B., Reddy, M. and Wong, J. (2015). Determination of heavy metals in food by inductively coupled plasma–mass spectrometry first action 2015.01. *Journal of AOAC International,* 98 (4), 1113-1120. https://doi.org/10.5740/jaoac.int.2015.01

Casey, J.F., Gao, Y., Yang, W. and Thomas, R. (2018). Spectroscopy eBook : Revolutionizing Sample Preparation with Single- Reaction Chamber Microwave Digestion. Retreived from Milestone website: https://alfresco-static-files.s3.amazonaws.com/alfresco_images/pharma/2018/06/27/c29ab419-14c9-4d21-9a71-c2c50c5b84e8/June14Ebook-%20Spectroscopy%20Milestone%20June%202018_v2.pdf

Codex Alimentarius Commission. (2007). Standard for infant formula and formulas for special medical purposes intended for infants CXS 72-1981, Revised in 2007. Retrieved from http://www.fao.org/fao-who-codexalimentarius.

Dobrzański, Z., Kolacz, R., Górecka, H., Chojnacka, K. and Bartkowiak, A. (2005). The content of microelements and trace elements in raw milk from cows in the Silesian region. *Polish Journal of Environmental Studies,* 14(5), 685-689.

EC. (2006). Commission regulation (EC) no. 1881/2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of European Union*, 364, 5-24.

EC. (2014). Commission Regulation (EU) No 488/2014 of 12 May 2014 amending Regulation (EC) No 1881/2006 as regards maximum levels of cadmium in foodstuffs. *Official Journal of the European Union*, 138, 75-79.

Evans, E.H. and Gigglio, J.J. (1993). Interferences in inductively coupled plasma mass spectrometry, a review. *Journal of Analytical Atomic Spectrometry,* 8(1), 1-18. https://doi.org/10.1039/ja9930800001

Girma, K., Tilahun, Z. and Haimanot, D. (2014). Review on milk safety with emphasis on its public health. *World Journal of Dairy and Food Sciences,* 9(2), 166-183.

Helaluddin, A.B.M., Khalid, R.S., Alaama, M. and Abbas, S.A. (2016). Main analytical techniques used for elemental analysis in various matrices. *Tropical Journal of Pharmaceutical Research*, 15(2), 427-434. https://doi.org/10.4314/tjpr.v15i2.29

Indonesian FDA. (2018). Regulation of the Head of Indonesian FDA No. 5/2018 on maximum limits for heavy metal contaminations in food products. Indonesia: FDA.

Indonesian National Standard. (2015). SNI 2970:2015 Milk Powder. National Standardization Agency of Indonesia.

Khan, N., Jeong, I.S., Hwang, I.M., Kim, J.S., Choi, S.H., Nho, E.Y., Choi, J.Y., Park, K.S. and Kim, K.S. (2014). Analysis of minor and trace elements in milk and yoghurt by inductively coupled plasma - mass spectrometry (ICP-MS). *Food Chemistry*, 147, 220-224. https://doi.org/10.1016/j.foodchem.2013.09.147

Korn, M.G.A., Morte, E.S.B., Santos, D.C.M.B., Castro, J.T., Barbosa, J.T.P., Teixeira, A.P., Fernandes, A.P., Welz, B., Santos, W.P.C., Santos E.B.G.N. and Korn, M. (2008). Sample preparation for the determination of metals in food samples using spectroanalytical methods - A Review. *Applied Spectroscopy Reviews*, 43(2), 67-92. https://doi.org/10.1080/05704920701723980

Lo Dico, G.M., Cammilleri, G., Macaluso, A., Vella, A., Giangrosso, G., Vazzana, M. and Ferrantelli, V. (2015). Simultaneous determination of As, Cu, Cr, Se, Sn, Cd, Sb and Pb levels in infant formulas by ICP-MS after microwave-assisted digestion: method validation. *Journal of Environmental and Analytical Toxicology*, 5(6), 328. https://doi.org/10.4172/2161-0525.1000328

Magnusson, B. and Ornemark, U. (2014). The Fitness for Purpose of Analytical Methods - a Laboratory Guide to Method Validation and Related Topics. 2nd ed. Retrieved from Eurachem website: https://www.eurachem.org/index.php/publications/guides/mv

Martino, F.A.R., Sánchez, M.L.F and Medel, A.S. (2000). Total determination of essential and toxic elements in milk whey by double focusing ICP-MS. *Journal of Analytical Atomic Spectrometry*, 15(2), 163–168. https://doi.org/10.1039/A907925D

Martino, F.A.R., Sanchez, M.L.F. and Medel, A.S. (2001). The potential of double focusing-ICP-MS for studying elemental distribution patterns in whole milk, skimmed milk and milk whey of different milks. *Analytica Chimica Acta*, 442(2), 191-200. ttps://doi.org/10.1016/S0003-2670(01)01170-9

Momen, A.A., Zachariadis, G.A., Anthemidis, A.N. and Stratis, J.A. (2007). Use of fractional factorial design for optimization of digestion procedures followed by multi-element determination of essential and non-essential elements in nuts using ICP-OES technique. *Talanta*, 71(1), 443– 451. https://doi.org/10.1016/j.talanta.2006.04.018

Nishimura, T., Nagaoka, M.H., Sakakibara, N., Abe, T., Maekawa, Y. and Maitani, T. (2010). Determination method for total arsenic and partial digestion method with nitric acid for inorganica arsenic speciation in several varieties of rice. *Journal of Food Hygine and Safety Science*, 51(4), 178-181. https://doi.org/10.3358/shokueishi.51.178

Onianwa, P.C., Adetola, I.G., Iwegbue, C.M.A., Ojo, M.F. and Tella, O.O. (1999). Trace heavy metals composition of some Nigerian beverages and food drinks. *Journal of Food Chemistry*, 66(3), 275-279. https://doi.org/10.1016/S0308-8146(98)00257-X

Solihat, I., Faridah, D.N. and Yuliana, N.D. (2018). Validation method of flame-AAS with microwave digestion for mineral analysis in carbohydrate-rich samples. *Molekul*, 13(2), 133-140. https://doi.org/10.20884/1.jm.2018.13.2.436

Sorbo, A., Turco, A.C., Gregorio, M.D. and Ciaralli, L. (2014). Development and validation of an analytical method for the determination of arsenic, cadmium and lead content in powdered infant formula by means of quadrupole inductively coupled plasma mass spectrometry. *Journal of Food Control*, 44, 159-165. https://doi.org/10.1016/j.foodcont.2014.03.049

Sunanti, S.T., Samin and C. Supriyanto. (2013). Determination of the candidate value of in house reference material (RM) ZrO_2 *Indonesian Journal of Nuclear Science and Technology*, 14(1), 23- 36.

Thomas, R. (2013). Practical Guide to ICP-MS A Tutorial for Beginner. 3rd ed. New York, USA: CRC Press. https://doi.org/10.1201/b14923

Tripathi, R.M., Raghunath, R., Sastry, V.N. and Krishnamoorthy, T.M. (1999). Daily intake of heavy metals by infants through milk and milk products. *The Science of the Total Environment*, 227(2-3), 229–235. https://doi.org/10.1016/S0048-9697(99)00018-2

Vanhaecke, F., Vanhoe, H., Dams, R. and Vandecasteele, C. (1992). The use of internal standards in ICP-MS. *Talanta,* 39(7), 737-742. https://doi.org/10.1016/0039-9140(92)80088-U

Wilschefski, S. and Baxter, M.R. (2019). Inductively coupled plasma mass spectrometry: introduction to analytical aspects. *The Clinical Biochemist Reviews,* 40(3), 115-133. https:/doi.org/10.33176/AACB-19-00024.