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Determination of Zinc, Copper, Selenium, and Manganese in Human Milk using Acid Digestion by ICP-MS and its Application in Biological Trace Element Monitoring

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ABSTRACT

Introduction: Human milk contains essential trace elements which support healthy development of infants. Previous studies have reported various analytical methods using different instruments to measure trace elements in human milk. This study aimed to determine trace element concentration in human milk using a validated acid digestion method and its application in biomonitoring among postpartum mothers.

Method: Human milk samples were collected from three postpartum mothers and prepared using acid digestion method. All samples were analysed using inductively coupled plasma mass-spectrometry (ICP-MS) and all validation parameters were measured.

Results: Four trace elements which were zinc, copper, manganese and selenium were found to have good linearity ($r^2 > 0.99$), limit of detection in µg/L (0.06, 0.0001, 0.005, 0.00003, respectively) and limit of quantification in µg/L (0.18,0.0003, 0.02, 0.0001, respectively). Good accuracy (83.4 – 112.7%), inter-day, and intra-day repeatability were obtained. Method application on trace element monitoring over postpartum period of three participants showed the median concentration of zinc, copper and selenium in human milk gradually decreased with slight variation, whereas manganese remained stable. Positive significant correlations were observed for most of the elements ($r > 0.40$, $p < 0.001$) except for copper-manganese.

Conclusion: Acid digestion method is sensitive, accurate and precise to analyse and quantify zinc, copper, manganese and selenium concentrations in human milk simultaneously by ICP-MS. It can be applied to monitor trace elements concentration in human milk in clinical and public health settings.

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Introduction

Human milk has been reported to contain essential trace elements such as zinc, copper, iron, iodine, and selenium (Mohd-Taufek et al., 2016). The presence of these trace elements despite only in minute amount is very crucial to support infants' development and prevent trace element deficiencies. Determining the optimum trace elements concentration in human milk will ensure that infants get the most benefits and prevent deficiencies. Trace elements play vital roles in growth and metabolisms, and the concentration have been reported to vary in human milk of different study populations and at different lactational stage according to infant's requirement (Hunt & Nielsen, 2009). It is important to monitor trace elements concentration in human milk and identify reference range at different stages of lactation.

Previously, numerous analytical instruments have been reported to measure trace element analysis in human milk. Some of the reported techniques were using inductively coupled plasma–optical emission spectrometry (ICP-OES) (Durović, et al., 2017), flame atomic absorption spectrometry (FAAS) (Kim et al., 2012), inductively coupled plasma sector field mass spectrometry (ICP-SF-MS) (Ecsedi-Angyal et al., 2019) and graphite furnace atomic absorption spectroscopy (Ait lhaj et al., 2021). Currently, the inductively coupled plasma mass-spectrometry (ICP-MS) has been widely utilised to determine multi-element concentration in human milk due to its capability to detect many elements simultaneously at very low concentrations (Mohd-Taufek et al., 2016; Rovira et al., 2022; Tahboub et al., 2021).

Another advantage of ICP-MS is that it involves simple preparation of samples. For example, acid digestion and alkaline dissolution methods have been used to prepare human milk samples prior to analysis (Jagodic et al., 2020; Levi et al., 2018; Mohd-Taufek et al., 2016). Nitric acid and hydrochloric acid were the most often used acids in acid digestion method, whereas ammonium hydroxide and tetramethylammonium hydroxide (TMAH) were the most frequently used alkali in alkaline dissolution method (Wilschefski & Baxter, 2019). Nitric acid also has been reported to be the most optimum acid media for ICP-MS analysis due to its strong oxidising ability that is able to extract the trace element from the sample by forming soluble nitrate salts (Hu & Qi, 2014). Although there were studies which reported that the alkaline dissolution method was better than the acid digestion method, some elements were reported to be accurately analysed by the acid digestion method and there were also elements that produced the consistent results regardless of using alkaline dissolution or acid digestion method (Levi et al., 2018; Mohd-Taufek et al., 2016).

In human milk studies, there is no certified reference material for human milk available. Therefore, various certified standard reference materials in the form of milk powder such as NIST 1549, CRM 8534, and NIST 8435 and spiked samples have been used as the reference material to validate a developed analytical method (Alves Peixoto et al., 2019; Baranowska-Bosiacka et al., 2016; Jagodic et al., 2020; Mohd-Taufek et al., 2016; Pekou et al., 2022). Currently to our knowledge, there is no study available that has reported about a validated method to measure trace elements concentration in human milk in Malaysia probably due to limited access to analytical instruments and ethical issues about human milk samples. This study aimed to validate a simple acid digestion method to measure simultaneously the concentration of trace elements in human milk by ICP-MS and determine its application in biological monitoring among postpartum mothers.

Methodology

This study received International Islamic University Malaysia (IIUM) Research Ethics Committee (IREC) approval (ID No.: IREC 2021-053).

Study participants

A total of three postpartum women were recruited to provide milk samples. Participant information sheet and informed consent form were provided. The mothers were informed about the study and voluntarily provided human milk samples for analysis at their convenience. Data collection form was filled in by the participants for demographic information, medical and medication history, and their lifestyles. The participants provided expressed breast milk (EBM), which were collected in 2 mL syringes or the EBM plastic container, labelled with date of milk collection. All samples were frozen at -21ºC prior to analysis. Sample analyses were conducted at the Institute of Oceanography and Environment (INOS), University Malaysia Terengganu.

Sample Preparation

The frozen human milk samples were thawed at room temperature and manually shaken until the samples were completely homogenised. For the acid digestion method, 15.4 mL of 65% (v/v) nitric acid (Merck Suprapur) was diluted with deionised water up to 1 L to prepare 1% (v/v) nitric acid. Then, 1 mL of samples was then added to 9 mL of 1% (v/v) nitric acid in a 15 mL polypropylene conical tube (Corning Falcon) and was shaken manually until a homogenous solution was formed. The prepared sample were then analysed using ICP-MS.

Blanks Preparation

A total of 10 sets of blanks were prepared by adding 10 mL of 1% (v/v) nitric acid into a 15 mL polypropylene conical tube. The blanks were used to calculate the limit of detection (LOD) and limit of quantification (LOQ) for

method validation purpose.

Wash solutions

The Milli-Q water was used for wash purposes between samples analysis. Acid washout using 1% (v/v) nitric acid was done after analysing ten to twenty samples.

Standards Preparation

For the preparation of standards, a multi-element standard solution (PerkinElmer Pure VIII) with 100 g/mL of Al, B, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, K, Li, Mg, Mn, Na, Ni, Pb, Se, Sr, Te, Tl, and Zn (Merck Certipur) were used. To make a standard stock solution, 1 mL of multi-element standard solution was added to a polypropylene conical tube that contained 49 mL of 1% (v/v) nitric acid. For calibration purposes, six calibration standard solutions were prepared by mixing 1 mL of milk sample with 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL, and 0.6 mL of multi-element stock solution. All the standard solutions were then further diluted up to 10 mL with 1% (v/v) nitric acid to produce the following final concentrations: 0.02 µg/mL, 0.04 µg/mL, 0.06 µg/mL, 0.08 µg/mL, 0.1 µg/mL and 0.12 µg/mL for all elements.

Quality control

Skimmed milk powder ERM-BD150 was used as a certified reference material for quality control in this study. A total of 0.02 g of ERM-BD150 was weighed and diluted up to 10 mL with 1% (v/v) nitric acid in a polypropylene conical tube. The solution was manually shaken until all milk powder was fully dissolved. Three replicates of certified reference material were prepared daily for method validation purpose. The milk samples were analysed together with the blanks, reference materials, and spiked samples.

Samples Analysis

Sample analysis was conducted using Perkin-Elmer SCIEX ICP-MS model ELAN 9000 connected with DELL PC equipped with ELAN Instrument Control Session software (PerkinElmer Inc., Massachusetts, USA). The working condition for this instrument is presented in Table 1. During analysis, the samples were nebulised with a pneumatic nebuliser and then transported into the plasma for ion production using argon gas. Ions were brought into a quadrupole, where they were separated according to their mass/charge ratio (m/z), after passing through several focusing lenses and a reaction cell. On an electron multiplier detector (SimulScan™), specific isotopes of single-charged ions were identified, and the resulting electrical current was intensified to produce an intensity value in counts per second (cps). The system software was then used to translate the intensities for various isotopes in the tested samples into concentrations and compared them to those obtained from calibration standard solutions. Each sample underwent analysis for a total of one minute and forty seconds.

Table 1: ICP-MS working conditions (ELAN 9000, Perkin-Elmer SCIEX)

Nebulizer gas flow (L min-1)	0.94
RF power (W)	1100
Analog stage voltage (V)	-1700
Lens voltage (V)	6
Pulse stage voltage (V)	900
Ac rod offset (V)	-6
Discriminator threshold (V)	70
Scan mode	Peak hopping
Speed of peristaltic pump (rpm)	26
Detector Pulse Sweeps/Reading	50
Replicates	2
Sampler/Skimmer cones	Nickel
Dwell Time (ms)	2.5
Spray chamber	Ryton® Double-pass Scott-type spray chamber
Nebulizer	Gem-tip Cross-Flow pneumatic nebulizer

Linearity, LOD, LOQ, Accuracy and Repeatability

The validation parameters that were assessed and calculated included linearity, LOD, LOQ, accuracy and repeatability. To assess the linearity of the method, calibration graphs for each element were generated by the system software at final concentrations of 0.02 g/mL, 0.04 g/mL, 0.06 g/mL, 0.08 g/mL, 0.1 g/mL, and 0.12 g/mL. The linearity was assessed using the correlation coefficient value (r^2) derived from the calibration graph given by the system software. Ten series of blank solutions were employed to calculate LOD and LOQ. The ICH Harmonised Tripartite Guideline (2005) formula were used in this study to determine LOD and LOQ based on the slope and the standard deviation of the blank:

$$\mathrm{LOD} = \frac{3.3\ \sigma_{\mathrm{blank}}}{\mathrm{b}}$$

$$LOQ = \frac{10\ \sigma_{blank}}{b}$$

*σblank: standard deviation of 10 series of blanks
*b: slope of calibration curve

Accuracy which was reported in the form of recovery percentage was calculated using Microsoft Excel 2019 by comparing the result obtained from the analysis of ERM-BD150 to the certified reference value provided by the manufacturer.

Repeatability was assessed on the same day (intra-day) and three different days (inter-day). Inter-day repeatability was determined by analysing three ERM-BD150 samples and five human milk samples on three different days, whereas intra-day repeatability was determined by analysing three ERM-BD150 samples and five human milk samples on the same day. Repeatability was reported as %RSD and calculated using the following formula:

$$\%RSD = \frac{\text{Standard deviation}}{\text{mean}} \times 100\%$$

The inter-day repeatability was computed using the pooled relative standard deviation formula derived from the formula provided by Mc Naught & Wilkinson (2012). The inter-day repeatability was calculated as follows:

$$RSD_{pooled} = \sqrt{\frac{(n_1-1)RSD1^2 + (n_2-1)RSD2^2 + \cdots + (n_k-1)RSDk^2}{n_1 + n_2 + \cdots + n_k - k}}$$

k: different series of measurements
n: number of analysed samples

The acceptance range for accuracy and repeatability are 70-120% for recoveries with an RSD% ≤20% based on the 2017 Codex Alimentarius Commission.

Results

Calibration data obtained from this analysis were tabulated in Table 2 that showed good linearity (r^2 > 0.99) calibration curve for four trace elements which were zinc (Zn), copper (Cu), selenium (Se) and manganese (Mn). Zinc had the highest LOD value (0.06 µg/L), whereas Mn had the lowest (0.00003 µg/L).

The concentration of Zn, Cu, Se and Mn were also analysed in certified reference material ERM-BD150 in µg/L. The concentrations in µg/L were multiplied by the dilution ratio for the ERM-BD150 solution, then divided by 1000 to get milligrams per kilogram for the purpose of comparison with the manufacturer's certified value to measure the accuracy. The accuracy values for all four trace elements from the analysed ERM-BD150 were between 83.4% and 112.7%. All trace elements achieved satisfactory percentage recoveries within the acceptable range (Table 3). Except for the inter-day repeatability value of Se (18.37%), the intra-day and inter-day repeatability values for other elements in the ERM-BD150 samples were less than 10%.

Table 4 showed the repeatability values for intra-day and inter-day measurements for human milk samples. It was notable that the very low concentration of Mn and Se found in human milk demonstrated significant variability that lowered the method precision. Nevertheless, this method is considered precise as all repeatability values for all four trace elements were less than 20% which is considered acceptable by the guideline of 2017 Joint FAO/WHO Codex Alimentarius Commissions.

Application in biological trace element monitoring: Case studies

The validated method was then applied to analyse a total of 105 milk samples donated by three postpartum mothers. The data were reported as case studies in this research and demographics of the mothers were presented in Table 5.

Participant A was a 30-year old mother who delivered a male neonate at 41 weeks of gestation with a birth weight (BW) of 3.04 kilograms. She voluntarily donated a total of 64 breast milk samples, collected at different times of the day over six months, at her convenience. The infant was healthy and fully breastfed during the study period.

Participant B was a 27-year old mother who gave birth to a term male infant with a BW of 3.04 kilograms at 38 weeks of gestation. A total of 30 breast milk samples

Table 2: LOD, LOQ, slope and correlation coefficient for four trace elements measured in acid digestion methods.

Trace elements	LOD (µg/L)	LOQ (µg/L)	Slope	Correlation coefficient (r^2)
Zn	0.06	0.18	y= 937x - 5877	0.9976
Cu	0.0001	0.0003	y= 3747x - 21033	0.9992
Se	0.005	0.02	y= 203x - 967	0.9993
Mn	0.00003	0.0001	y= 10617x - 47720	0.9992

Table 3: Accuracy, inter-day (3 days), and intra-day repeatability of ERM-BD150.

ERM-BD150 (n=3)					
Trace elements	Certified value [mg/kg]	Observed value [mg/kg]	Accuracy [%]	Inter-day [%RSD]	Intra-day [%RSD]
Zn	44.8 ± 2.0	50.5 ± 3.7	112.7	4.79	4.39
Cu	1.08 ± 0.06	0.92 ± 0.04	85.2	3.52	2.56
Se	0.188 ± 0.014	0.188 ± 0.086	100	18.37	3.71
Mn	0.289 ± 0.018	0.241 ± 0.042	83.4	9.94	9.48

Table 4: Inter-day (3 days) and intra-day repeatability (n = 5) of human milk samples..

Trace elements	Inter-day [%RSD]	Intra-day [%RSD]
Zn	5.66	5.03
Cu	6.74	3.78
Se	14.10	16.24
Mn	18.10	12.09

Table 5: Demographics of participants (n = 3)

Demographic	A	B	C
Race	Malay	Malay	Malay
Body Mass Index	23.19	24.0	21.83
BW Pre-pregnancy (kg)	55	51.5	56
BW Pre-delivery (kg)	64	62	62
BW Post-delivery (kg)	55	54	58
No. of pregnancies	4	1	5
No. of deliveries	2	1	5
No. of miscarriages	2	0	0
Birth method	Caesarean	Normal	Normal
Dietary intake	RCBV	RCBV	RFCVFr
Medical history	No	No	Gestational diabetes, Asthma
Supplement	No	Yes (Se, Mn, Zn, Cu)	Yes (Zn, Fe)

BW: Body Weight; R: Rice; C: Chicken; B: Beef; F: Fish; V: Vegetable; Fr: Fruit

were collected from 12 months until 15 months postpartum at her convenience. At birth, the infant presented with high fever and jaundice 10 days after birth. He was fully breastfed only until the first six months and then continued with partial breastfeeding and solid food. There were no abnormalities in the growth and development of the infant except he was underweight and had mild eczema.

Participant C was a 27-year old mother who delivered a male infant with a BW of 3.08 kilograms at 37 weeks of gestation. She was able to donate a total of 11 human milk

samples that were expressed at different times of the day in the 21st month postpartum only, at her convenience. The infant showed normal growth and development with no other health problems except for jaundice after birth. He was fully breastfed only until the first six months and then continued with partial breastfeeding and solid food.

Table 6 showed the concentrations of Zn, Cu and Se were found to be the highest at one month postpartum compared to the later months in participant A. However, only Se and Cu gradually decreased wheras Zn showed some fluctuations. On the other hand, Mn exhibited no significant changes in concentration over the time period. The concentration of Zn and Cu also showed relatively higher concentration than Mn and Se. The correlations between trace elements in human milk also were examined with $p < 0.001$ was considered as significant. The Spearman's correlation coefficients and p-value between essential trace elements in human milk of participant A were: Zn-Se ($r = 0.607$, $p = <0.001$), Zn-Cu ($r = 0.615$, $p = <0.001$), Zn-Mn (0.471, $p = <0.001$), Cu-Se ($r=0.735$, $p = <0.001$), Mn-Se ($r=0.467$, $p = <0.001$). All trace elements showed significant positive correlations ($r > 0.40$, $p < 0.001$) except for Cu-Mn.

Table 7 showed the concentrations of Zn, Cu, Se and Mn in participant B at 12 to 15 months postpartum and participant C at 21 month postpartum. For participant B, the median Zn concentrations were slightly decreased from 430.5 µg/L at 12 months to 353 µg/L at 15 months postpartum. For Se, the median concentration was increased from 7.6 µg/L at 12 months to 10.9 µg/L at 13 months and remained around 10 µg/L at 14 and 15 months postpartum. There was a fluctuation in the levels of Cu during 12 to 15 months postpartum. The median concentration of Mn increased from 5.8 µg/L to 7.1 µg/L at 12-13 months and later declined to 4.5 µg/L and 4.7 µg/l at 14-15 months. For participant C, the median values of Zn and Mn were similar but Se and Cu seemed to be higher than participant B despite later lactation period.

Table 6: The concentrations of trace elements in the human milk of participant A over a period of 6 months postpartum (µg/L).

Trace Elements	Mean/ Median	Month postpartum					
		1	2	3	4	5	6
		N = 15	N = 8	N = 10	N = 15	N = 6	N = 10
Zn (µg/l)	Mean±SD	2698±1010	1211±304	1480±355	1296±773	967±221	1254±541
	Median	2640	1110	1495	1190	886	978
	(Range)	(1550-5870)	(907-1810)	(841-1960)	(352-3780)	(716-1250)	(713-2350)
Cu (µg/l)	Mean±SD	403 ± 84	180 ± 20	164 ± 26	116 ± 46	93 ± 29	77 ± 18
	Median (Range)	406 (265-621)	188 (147-200)	166 (114-215)	104 (61-223)	89 (61-143)	75 (50-111)
Se (µg/l)	Mean±SD	24.8 ± 7.2	11.9 ± 4.7	14.8 ± 4.5	12.3 ± 6.8	9.8 ± 4.0	11.3 ± 4.1
	Median (Range)	23.6 (14.6-43.1)	11.9 (2.7-18.3)	14.2 (9.4-22.9)	12.2 (4.4-32.0)	10.7 (4.4-15.0)	10.4 (5.9-19.7)
Mn (µg/l)	Mean±SD	4.7 ± 1.2	3.6 ± 1.1	4.7 ± 1.8	4.7 ± 3.1	3.3 ± 0.5	5.1 ± 3.0
	Median (Range)	4.4 (3.5-7.6)	3.6 (2.2-5.9)	4.3 (1.8-7.7)	3.8 (2.6-14.8)	3.2 (2.9-4.0)	3.5 (2.7-10.5)

Table 7: The concentration of trace elements in human milk of participants B and C at 12 months postpartum and above (µg/L).

Trace Elements	Mean/ Median	B				C
		12	13	14	15	21
		n = 16	n = 4	n = 6	n = 4	n = 11
Zn (µg/l)	Mean±SD	450±117	1178±1557	373±126	390±86	606±532
	Median (Range)	431 (243-642)	454 (292-3510)	354 (203-585)	353 (338-518)	450 (179-2150)
Cu (µg/l)	Mean±SD	8.4±2.3	12.8±5.4	10.1±1.7	9.8±2.0	15.3±4.3
	Median (Range)	7.6 (5.1-14.0)	10.9 (8.9-20.7)	10.3 (7.9-12.6)	10.2 (7.4-11.5)	16.4 (7.2-21.7)
Se (µg/l)	Mean±SD	76.7±23.9	84.2±22.2	82.4±31.2	105.3±40.3	155±33
	Median (Range)	72 (47-136)	84 (57-111)	72 (62-144)	106 (56-154)	166 (83-200)
Mn (µg/l)	Mean±SD	5.7±2.3	7.1±1.7	20.8±34.7	4.6±0.4	3.5±1.3
	Median (Range)	5.8 (3.2-11.2)	7.1 (5.0-9.1)	4.5 (3.8-90.8)	4.7 (4.2-5.0)	3.5 (1.1-6.3)

Discussion

We report a method using acid digestion to measure total elements of Zn, Cu, Se and Mn concentration in human milk. Previous studies that reported acid digestion method have used heat application during sample preparation (Alves Peixoto et al., 2019; Jagodic et al., 2020; Tahboub et al., 2021). Theoretically, by raising the temperature of the samples, the average kinetic energy will rise, thus increase the chances of acid and biological sample collisions and enhance trace element dissolution (Mohammed et al., 2017). Our method did not require heat application since human milk is a biological matrix that contains proteins such as mucins, caseins and whey proteins that can be affected by high temperature. An increase in temperature has been reported to increase the amount of protein adsorbed onto solid surfaces resulting in a lower trace element content in the pasteurized milk solution possibly due to protein precipitation (da Costa et al., 2003; Rabe et al., 2011). Our method was simple and produced good results for the required validation parameters to determine four trace elements simultaneously in 1 mL of human milk for biological monitoring purpose.

Homogenisation of milk samples before the digestion process is also a crucial step during sample preparation prior to the analysis. After being thawed, human milk is known to form two different layers, the fatty and aqueous layers. A study has reported that the concentration of iodine in the fatty layers was significantly different than those in the aqueous layers (Huynh et al., 2015). Therefore, sample homogenisation prior to mixing with nitric acid would ensure that all trace elements in the samples uniformly dispersed. Information about amounts of trace elements in two different layers of human milk have not been available to date. Further studies are needed to investigate the differences in the amount of trace elements in two distinct layers of human milk after thawing.

All validation parameters measured in this study produced good measurements which were within the acceptable range specified by the guideline, indicating that this method performed satisfactorily. The employed method in this study had a lower LOQ value for all four trace elements compared to other studies (Alves Peixoto et al., 2019; Mohd Taufek et al., 2016). The fact that the LOQ values of Zn, Cu, Se and Mn obtained using this method were also significantly lower than the anticipated range concentrations in human milk reported in other countries showed that this method may be used to determine the levels of trace elements in human milk over a wide variety of populations. This method did not utilise any internal standard. It has been demonstrated that the implementation of internal standards that had the mass numbers almost the same as tested trace elements could enhance the precision of the methods used (Vanhaecke et al., 1992). Although still within the acceptable range, Mn and Se in human milk samples had slightly lower precision in the present study. Therefore, the precision of this

method may be further optimised by utilising internal standards in the future.

For the purpose of method application in biological monitoring, we measured the concentration of Zn, Cu, Se and Mn in human milk collected at different postpartum period from three mothers as case studies. The median Zn concentration in participant A was high (2640 µg/L) at the first month postpartum and rapidly decreased (978 µg/L) at 6 months postpartum. This pattern was found to be similar to a previous report that highlighted a rapid decline of zinc concentration in term breast milk from 3000 µg/L at one month to 1200 µg/L at six months postpartum (Hunt & Nielsen, 2009). We found the mean±SD of Zn concentration at 1 month was 2698 ± 1010 µg/L and 1480 ± 355 µg/L at 3 months postpartum. A study by Motoyama et al. (2021) involving 78 Japanese mothers reported higher mean Zn concentration at 1-month and 3-months postpartum with levels of 3000 ± 1300 µg/L and 1680 ± 950 µg/L respectively. Similarly, another study by Dumrongwongsiri et al. (2015) also reported higher Zn levels with a range of 500-3200 µg/L at 4-6 months postpartum than the present case study (352-2350 µg/L). However, lower mean Zn values were found in term milk of 70 mothers in Spain of 1237.76 ± 949 µg/L at 1 month postpartum (Mandiá et al., 2021). In Table 7, the median levels of Zn for both participant B at month 12-15 and participant C at month 21 appeared to be relatively similar. Further monitoring on zinc concentrations in human milk over postpartum period may be needed to understand the relevance and factor behind its variation relative to the needs of infant's growth and development.

In our study, the median Cu values were 406 µg/L in the first month postpartum and 166 µg/L at 3 months, which were lower compared to the values reported by Motoyama et al. (2021) with values of 500 µg/L and 330 µg/L respectively. However, the mean Cu concentration in the first month postpartum in our case study was 403 ± 84 µg/L, which was higher than the reported value by Mandia et al. (2021) of 250.11 ± 163 µg/L. In Iran, a study also reported a higher mean Cu concentration of 1070 ± 1140 µg/L in the breast milk of 160 lactating mothers at 6 months postpartum (Sadeghi et al., 2020). A previous report also highlighted the decline of mean Cu levels from 250 µg/L in the first six months to 100-200 µg/L in seven to 12 months postpartum (Hunt & Nielsen, 2009). Currently, optimal levels of Cu in human milk have not yet been determined at different lactation stages.

The median Se concentration found in the current study was 23.6 µg/L in the first month then decreased to 14.15 µg/L at 3 months and 10.41 µg/L at 6 months postpartum. Another study also found similar level of Se at 1-month which was 22 (17-29) µg/L but higher level at 3 months postpartum, of 21 (16-25) µg/L (Motoyama et al., 2021). However, a previous study by Mandiá et al. (2021) reported lower mean Se values of 8.87 µg/L in the mature milk compared to the current study which was 24.82 µg/L. In a previous study involving 470 lactating women from Slovenia, the mean Se levels found in the human milk at 6 to 8 weeks after delivery was 12.6 µg/L (Snoj Tratnik et al., 2019). Based on the findings by Han et al. (2019), the adequate intake of Se for 0-3 months Chinese infants was 15.29 µg/day, which is almost the same with the levels reported in the current study (14.15 µg/L at 3 month). It is important to monitor selenium status in infants receiving different amounts of selenium from human milk to prevent deficiency or toxicity.

We also observed median Mn concentration that were relatively stable with 4.44 µg/L in the first month postpartum, 4.29 µg/L at three months and 3.54 µg/L at 6 months. These results were lower than those reported by a previous study by Motoyama et al. (2021) which found median Mn levels of 8 µg/L in the first month and 7 µg/L at 3 months postpartum. Similarly, Li et al. (2016) also reported higher Mn levels of 9.33 to 11.53 µg/L in the first 2 months and then declined at 4 to 6 months to a mean value of 7.69 µg/L. Our study suggests that Mn concentrations in human milk are relatively stable over the postpartum period. Human milk was reported to provide an adequate amount of Mn to prevent deficiency at about 3–10 µg/L (Horning et al., 2015). This study observed that trace elements concentration in human milk varied over postpartum period. The variations in the trace elements levels might be due to several factors such as lactation stages, geographical regions, dietary intakes or trace element supplements that were taken by some of the participants. Consistent with the previous reports (Li et al., 2016; Motoyama et al., 2021), we found positive significant correlations for most of the elements ($r > 0.40$, $p < 0.001$) except for Cu-Mn.

The limitation of our study includes the small population size due to challenges in recruiting participants. However, the milk samples provided by these participants were sufficient to validate the method and its application in biomonitoring of trace elements in human milk. Future studies with large sample size would be able to produce a thorough investigation on the factors that may influence the trace element concentrations in human milk. This study did not seek to evaluate the association of factors affecting trace elements in human milk. However, the findings from this study are important since this is the first study reported in Malaysia using a validated acid digestion method that is simple, accurate and sensitive to measure Zn, Cu, Se, and Mn. It is applicable and relevant for clinical and public health settings by using minimal amount of sample for biomonitoring to detect potential deficiency or toxicity relative to the needs of infants. Previous studies reporting data of Malaysian population have examined the concentration of a single trace element (Pb and Fe) in human milk in the past few decades (Huat et al., 1983; Loh & Sinnathuray, 1971). New data can be produced for Malaysian population using a simple and robust method reported in the present study.

Conclusion

Acid digestion method was simple, sensitive, accurate, precise, and robust to quantify zinc, copper, manganese and selenium simultaneously in human milk by ICP-MS. Human milk analysis is challenging due to high variability of trace elements contents between individuals and across postpartum period. There were significant correlations between concentration of Zn-Se, Zn-Cu, Zn-Mn, Cu-Se and Mn-Se. Future studies may utilise this method to determine reference range of trace elements concentration in human milk in larger study population.

Author Contribution

N.H. Mohd-Taufek, A.S. Mohmad Sabere & J. Bidai conceived and designed the experiment, conducted sample and data collection, data analysis, N.B. Amran, U.S. Mohamad Jamahari and A.R. Fata Nahas analysed the sample and data, all authors wrote the manuscript.

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Data Availability

The datasets analysed in the current study are available from the corresponding author upon reasonable request.

Competing Interest

N.H. Mohd-Taufek, A.S. Mohmad Sabere, J.A. Bidai, N.B. Amran, U.S. Mohamad Jamahari and A.R. Fata Nahas declare that there is no competing interest in this research.

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