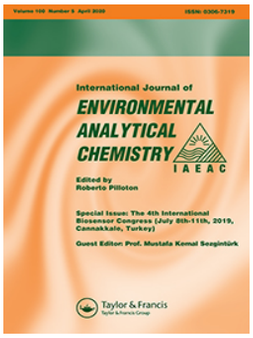

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ARTICLE

Determination of low-level arsenic, lead, cadmium and mercury concentration in breast milk of Hungarian women

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ABSTRACT

Breast milk was taken at different stages of lactation from 27 healthy lactating women living in Budapest and its metropolitan area with ages comprising between 25 and 41 years who exclusively breastfed their full-term newborns. Samples were subjected to microwave-assisted (MW) acid digestion in PTFE vessels containing quartz microvials. After fine-tuning of the MW-assisted acid digestion method by determination of total organic carbon content of the digested samples, the volume ratio for sample: 67% by weight HNO_3 : 30% by weight H_2O_2 solutions = 1 : 2 : 1 proved to be suitable for determination of As, Cd and Pb by inductively coupled plasma sector field mass spectrometer. Mercury concentration was <0.4 µg/L in all samples. The order of Cd, As and Pb mean concentration in the samples were 0.188 ± 0.071 µg/L < 0.41 ± 0.20 µg/L < 1.74 ± 0.77 µg/L, respectively. Mean and median concentration of As and Pb data generally grouped into two-week intervals of lactation decreased by about 25–35% after two months. Moreover, statistically significant increase was observed for Pb concentration with the age of the mothers according to paired sample *t*-test almost 20 years after the prohibition of the marketing of leaded gasoline in the European Union and about 40 years after the ban on Pb pipes for drinking water delivery in Hungary (Pearson's correlation coefficient 0.8285; $|t| < t_p$ at $p < 0.05$). However, according to our dietary intake estimations, no other threat for newborns fed by exclusive breastfeeding was detected.

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Breastfeeding; human milk; infant; ICP-MS; lactation

1. Introduction

During the first few days after child delivery, the mother produces colostrum, rich in protein and antibodies that provide passive immunity to the baby. This will gradually change to become mature milk. Potential inorganic contaminants in the breast milk (e.g. As, Cd, Hg, Pb) are passed along to newborns through breastfeeding, which may affect their growth and nervous system [1]. Toxic elements are absorbed at a larger extent by

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newborn infants than adults and bile secretion in the first weeks of life is poor [2]. Nevertheless, exclusive breastfeeding is recommended for the first six months after newborn delivery because, normally, it provides the optimal conditions for nutritional, immunological and psychological development.

Fish and seafood consumption is the main source for the occurrence of As in breast milk [3]. The As concentration in breast milk in the regions affected by endemic As contamination (e.g. aquifers) resulted to be lower than expected [4]. Therefore, breastfeeding is recommended instead of using As-contaminated water for preparation of infant formulae in As-contaminated areas [5]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established 3.0 μg/kg body weight (BW) per day as the lower limit on the benchmark dose (BMDL) for a 0.5% increased incidence of lung cancer ($BMDL_{0.5}$) [6].

For the provisional total monthly intake of Cd, JECFA established a 25 μg/kg body weight (BW)/month value in 2011 [7]. At the same time, the European Food Safety Authority (EFSA) set a 2.5 μg/kg BW tolerable weekly Cd intake [8].

The Hg concentration in human breast milk is mainly attributed to fish consumption [3,9]. Thus, total Hg mean concentration in Japan and in the Mediterranean basin countries were 0.81 μg/kg [10] and 0.2–0.6 μg/kg [3,9,11,12], respectively. The proportion of $MeHg^+$ in the samples collected in Japan was about 50–60% of the total Hg [10].

The Pb concentration in human breast milk of nonsmokers decreased more than by half of the concentration in colostrum [13–16]. Lead pooled in the skeleton [13] as well as Cd [17] and Hg [14,18] temporarily stored in the kidney may be mobilized during pregnancy and lactation. The Ca intake during lactation decreases the Pb content of blood and breast milk [13,19]. Larger Pb concentrations in colostrum are typical for elder mothers [13,17]. Moreover, Pb concentration in human breast milk is still larger in urban areas [15]. Since Pb exposure results in severe physiological damage at any Pb concentration, a BMDL of daily 0.5 μg lead/BW kg in blood determined at the 95th percentile lower confidence for 1% extra risk ($BMDL_{01}$) causing 1 point reduction on the IQ scale for children was established [20].

The composition of the breast milk not only changes over the lactation period but also with the time elapsed from the last breastfeeding from the given mammary gland. Ideally, 24-h pooled samples taken from the same mother [21] should be suitable for further analyses. Since this is not a realistic scenario, the whole amount of milk from one mammary gland not used for breastfeeding in the 2–3 h prior to sampling [21], is usually collected. The most common sample preparation is the microwave-assisted (MW) digestion with a mixture of cc. HNO_3 and cc. H_2O_2. Samples can also be freeze-dried [15,21,22]. In this latter case, the sample demand is lower [22]. Finally, the digested samples can be analyzed by inductively coupled plasma mass spectrometry (ICP-MS) due to its excellent limits of detection required (LODs) [23–27] compared to the worse ones offered by ICP-optical emission spectrometry especially for As. Being commonly considered as a mono-elemental technique, graphite furnace atomic absorption spectrometry is neither suitable for the determination of As due to the worse LOD for this element.

The aim of this study was to determine the toxic element content in human breast milk originating from breastfeeding women in Hungary taken at early stages of lactation with special emphasis on arsenic, cadmium, lead and mercury also due to the

corresponding local data scarcity, the last peer-reviewed relevant information on the elemental concentration of breast milk in Hungary dating back to 1991 [28].

2. Experimental

2.1. Study design and sampling

Breast milk samples were taken between September 1st, 2017 and January 1st, 2018 from Budapest and its metropolitan area (n = 27) under paediatrician guidance and according to research ethics approval issued by the Hungarian Health Research Council through No. 45773–2/2017/EKU. Personal and dietary habit data of the volunteers (n = 27) have been compiled in Table 1. Sampling was performed after the consent form was signed by each volunteer. Besides personal and infant data, a questionnaire on the living standards and diet was filled in by each mother. All samples and questionnaires were provided with identification codes and the data contained in them were confidentially treated.

Table 1. Characteristics of the mothers and infants included in the present study.

Mothers (n = 27)	
Age (y ± SD)	**32.9 ± 4.4**
Parity (n ± SD)	**2.3 ± 1.3**
primipara (%)	**40.7**
Education level	
Vocational school (%)	**33.3**
Bachelor degree (%)	**33.3**
Master or higher degree (%)	**33.3**
Dwelling built after 1970 (%)	**40.7**
Exposure to passive smoking (%)	**18.5**
Dental amalgam (%)	**11.1**
Dietary habits of mothers	
Water consumption	
Tap water exclusively (%)	**44.4**
Bottled mineral water exclusively (%)	**22.2**
Well water (%)	**11.1**
Milk consumption	
Not at all (%)	**14.8**
UHT milk exclusively (%)	**14.8**
Fresh milk in carton exclusively (%)	**44.4**
Farm milk (%)	**14.8**
Marine fish consumption	**1.0 ± 1.0**
Monthly (portions of 150–200 g ± SD)	
Broiler chicken consumption	
Monthly (portions of 160–180 g ± SD)	**7.6 ± 6.1**
Rice consumption	
Monthly (portions of 120–150 g ± SD)	**6.6 ± 3.7**
Mushroom consumption	
Monthly (portions of 100–120 g ± SD)	**1.6 ± 1.3**
Fetus protection vitamin consumption (%)	**70.4**
Mineral supplement consumption (%)	**70.4**
Infants (n = 27)	
Female/male	**14/13**
Gestational age (wk ± SD)	**39.3 ± 1.1**
BW at birth (g ± SD)	**3395 ± 426**
BW after 30 d (g ± SD)	**4360 ± 586**
BW after 60 d (g ± SD)	**5476 ± 821**

Abbreviations: BW = body weight; wk = week; y = year.

Prior to sampling, mothers washed their hands and breast with tap water. Sampling was performed at the dwelling place by manual expression in the morning into 50-mL PP centrifuge tubes previously soaked in 10% HNO_3 solution for 24 h, then rinsed with deionized water (DW). The resulted volume of each sample varied between 20 and 50 mL. Samples were placed into a cooler bag and transported immediately to the laboratory, where they were stored at −18°C prior to analysis. Breast milk samples were collected at different stages of lactation from 5 days to 2 months. Moreover, a 31-year-old mother provided samples weekly for 8 weeks.

2.2. Sample preparation

2.2.1. Materials and reagents

Throughout the experiments, DW (with a resistivity of 18 MΩ cm) was taken from an ELGA Purelab Option-R7 ultra-pure water unit (ELGA LabWater/VWS Ltd., High Wycombe, UK). Concentrated (cc., i.e. 67% by weight) HNO_3 and 30% by weight H_2O_2 were of Suprapur® (Merck, Darmstadt, Germany) quality. For external calibration, 1 g/L acidic As(V), Cd, Hg and Pb stock solutions (Merck) were used after appropriate dilutions. All standard solutions were prepared daily from stock solutions *via* appropriate dilutions with deionized water in PP Falcon® centrifuge tubes (Fisher Scientific, Waltham, MA, USA). Before use, the 50-mL centrifuge tubes were soaked in 20% (v/v) HNO_3 for 24 h and then rinsed with DW. The final HNO_3 concentration of each sample solution was set to 10% (v/v). Mercury was determined separately. In this case, an intermediate stock solution of 1 mg/L containing HCl in 20% by weight to prevent Hg loss was used for the preparation of the calibration solutions on a daily basis. For quality assurance measurements, NIST rice flour SRM 1568a and skim milk powder SRM No. 150 (Sigma Aldrich) were used.

2.2.2. Microwave (MW)-assisted acid digestion

Prior to elemental analysis, breast milk samples were subjected to MW-assisted acid digestion. For this, samples were thawed at room temperature, then placed into a water bath at 40°C to enhance solubilization of the fatty content and finally, homogenized on a Vortex shaker. Aliquots of 1 mL were taken with an automatic pipette and placed into quartz microvials. Then, 2 mL of 67% by weight HNO_3 and 1 mL of high purity H_2O_2 of 30% by weight were also added. Each quartz vial closed with its cap was transferred into a PTFE digestion vessel (n = 10). After this step, 2 mL of 30% H_2O_2 and 8 mL of DW were added in the outer space of the PTFE vessels. The MW-assisted digestion steps performed in an Ethos Plus 1 equipment (Milestone S.r.l. Sorisole, Italy) were chosen in accordance with the guidelines of the manufacturer for such type of matrices. The detailed MW-assisted digestion program can be seen in Table 2. After completing the digestion and cooling down of the vessels, the digested samples were transferred into PP tubes and their volume was made up to 8 mL with DW. For each sample, digestion was performed in triplicates. Prior to elemental analysis, Ge and In as internal standards were added to the digested samples in a concentration of 20 µg/L and 10 µg/L, respectively.

2.3. Total organic carbon (TOC) and trace element analyses

The total organic carbon (TOC) content of the digested samples was checked by a Multi N/C 2100 Analyzer (Analytik Jena, Germany). Briefly, 100 µL of the properly diluted digested samples was injected into the equipment heated to 800°C. The flow rate of the oxygen stream was 160 mL/min. Trace element analysis was performed by inductively coupled plasma sector field mass spectrometry (ICP-SF-MS) on an Element 2 equipment (Thermo-Fisher Scientific, Germany). The optimized operating conditions and the monitored isotopes are listed in Table 3.

2.4. Statistical data evaluation

Descriptive statistical data analysis was applied for elemental concentration generally grouped in data sets according to biweekly lactation time periods. Statistical data evaluation on data as a function of the age of mothers was done by paired sample *t*-tests. Normality assumption was tested by histogram plotting. Outliers were checked by using a boxplot. Thus, all data sets proved to have a normal distribution. The graphs plotted based on the obtained data were provided with the equation of the linear fitting, the value of the Pearson's correlation coefficient (r), its square, coefficient of determination (R^2), test statistic (t) and critical value of a *t*-distribution (t_p).

Table 2. Optimized microwave-assisted digestion steps applied for digestion of human breast milk samples in PTFE vessels (n = 10) containing quartz microvials.

N°.	Time (min)	Nominal power (W)	Temperature limit (°C)
1	0–1	250	85
2	1–3	0	85
3	3–8	200	140
4	8–13	350	140
5	13–18	550	180
6	18–23	250	180
7	23–33	0	-

Table 3. Optimized operating conditions of the ICP-SF-MS instrument and monitored isotopes.

Parameter	
RF power (W)	1200
Carrier gas (Ar) flow rate (L/min)	14
Outer gas (Ar) flow rate (L/min)	0.8
Nebulizer gas (Ar) flow rate (L/min)	1.1
Nebulizer	Meinhard (equipped with Scott spray chamber)
Sampler cone material and orifice diameter (mm)	Ni and 1.0
Skimmer cone material and orifice diameter (mm)	Ni and 0.8
Detector	Electron multiplier
Resolution (R)	
Low (for Cd, Hg & Pb)	R = 300
High (for As)	R = 10000
Monitored isotopes	
Arsenic	^{75}As
Cadmium	^{114}Cd
Mercury	^{202}Hg
Lead	$^{206}Pb + {}^{207}Pb + {}^{208}Pb$
Germanium as internal standard (IS) for As	^{74}Ge
Indium as IS for Cd, Hg and Pb	^{115}In

3. Results and discussion

3.1. Evaluation of the questionnaires on personal data and dietary habits of volunteers

Only healthy (i.e. not suffering from chronic diseases, not under medication), nonsmoker women with adequate body mass indexes were considered to be included in the study that would have a normal pregnancy with no complications living in dwellings situated in non-industrial areas. Exclusively those mothers were included in the study who were available for providing breast milk within the 1–8 weeks of delivery. Thus, 27 healthy, nonsmoker volunteers with an age comprising 25 to 41 years living in residential areas of Budapest and its metropolitan areas participated in this study who delivered full-term newborns with a gestational age from 38 to 41 weeks between August and December 2017 (Table 1). None of the women reported having been exposed to specific sources of toxic metals, whether at home or at work. Their mean age and parity were 32.9 ± 4.4 y and 2.3 ± 1.3, respectively. None of the mothers included in the study gave birth to twins. Their educational level was very similar. Thus, one third graduated from vocational schools, one third had a bachelor degree and another one third had a university master degree. All infants were healthy, had normal birth weight (Table 1) and they were exclusively breastfed. The BW of the infants and BW gain during the first two months after gestation can be seen in Table 1.

About 15% of the mothers included in the present study did not drink any type of milk. Only six mothers drank bottled mineral water solely. The rest of them also consumed tap water. However, each mother used tap water for cooking. Drinking water quality in Budapest and in its metropolitan area is adequate for dwellings built after the 1970s. For example, the mean As, Cd, Hg and Pb concentration of drinking water in Budapest in 2017 was 1.6 μg/L, <0.2 μg/L, <0.05 μg/L and 1.3 μg/L [29], respectively. The health limit values in force in Hungary for As, Cd, Hg and Pb in drinking water are 10 μg/L, 5 μg/L, 1 μg/L and 10 μg/L, respectively. About half of the mothers lived in dwellings built after 1970 (Table 1). That year, the use of pipes made of Pb for drinking water delivery was banned for construction of new dwellings [29]. However, it should be emphasized that Pb-based antiknock fuel additives were still used by the birth time of the volunteers. This meant a certain exposure to Pb as a function of the age of the volunteers. Moreover, about 20% of the milk donators were exposed to passive smoking (Table 1). Among dietary risk factors responsible for elevated As levels in breast milk, rice consumption was similar in Budapest and in the metropolitan area. Nevertheless, marine fish, broiler chicken and mushroom consumption were larger in the metropolitan area than in Budapest by 113%, 42% and 50%, respectively. It is well known that these food items contain mainly organic As species, which are less toxic. According to the responses given to our questionnaire, exposure of the mothers to Hg was also low, as none of the mothers from Budapest had dental amalgam fillings, while this percent for those living in its metropolitan area was less than 20%. Moreover, marine fish consumption – as a dietary source mainly for Hg – was also very low (Table 1). This is not surprising as fish consumption in Hungary is about 4.6 kg/capita/year according to the report of the European market observatory for fisheries and aquaculture products for 2014 [30]. Moreover, freshwater fish (e.g. carp) is the favourite

type of fish consumed in Hungary, the Hg concentration of which is low (e.g. 0.1–0.3 mg/kg) [31].

The processed data of this questionnaire pointed out that the elemental analysis of the samples by ICP-SF-MS would be a challenge as the maternal exposure during pregnancy and breastfeeding to As, Cd, Hg and Pb would be low. Therefore, a proper selection of sample preparation protocol should precede the ICP-MS measurements.

3.2. Analytical capabilities of the proposed method

In order to avoid plasma overload, the TOC value of a sample analyzed by ICP-MS should ideally be less than 0.1%. Therefore, the volume of HNO_3 added to 1 mL of a composite breast milk sample was varied between 2 and 5 mL, while the volume of H_2O_2 added was left unchanged. From the percent values of the digested samples further diluted with DW to decrease the HNO_3 concentration of the samples to 10% by volume shown in Figure 1, it can be seen that the TOC was less than 0.05% in all cases. Finally, the volume ratio for nem kell sample : cc. HNO_3 : cc. H_2O_2 = 1 : 2 : 1 corresponding to the largest TOC value was chosen for the MW-assisted digestion of the samples due to the expected low concentration of toxic elements. By applying the optimized MW-assisted digestion parameters, the limit of quantitation (LOQ) determined as 10σ of background for As, Cd, Hg and Pb in human breast milk was 0.2 µg/L, 0.01 µg/L, 0.4 µg/L and 0.02 µg/L, respectively. For the quantitative

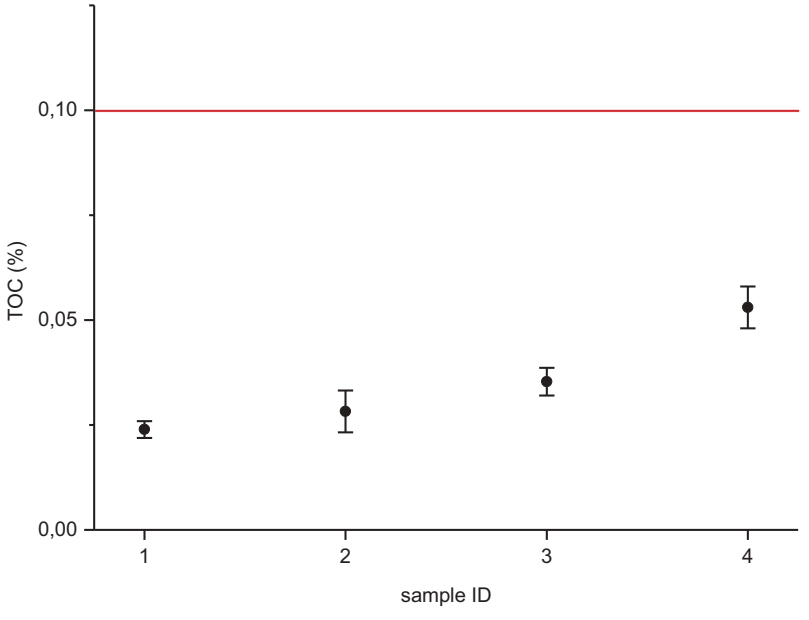

Figure 1. Total organic carbon (TOC ± RSD) content (%) in breast milk subjected to microwave-assisted 67% (w/w) HNO_3 and 30% (w/w) H_2O_2 digestion as a function of the acid volume added to 1 mL sample.

Meaning of sample IDs on the X axis: **1** = 1 mL breast milk + 5 mL HNO_3 + 1 mL H_2O_2 and further diluted five times; **2** = 1 mL breast milk + 4 mL HNO_3 + 1 mL H_2O_2 and further diluted four times; **3** = 1 mL breast milk + 3 mL HNO_3 + 1 mL H_2O_2 and further diluted three times; **4** = 1 mL breast milk + 2 mL HNO_3 + 1 mL H_2O_2 and further diluted twice after digestion; Red line indicates the maximum TOC value recommended for ICP-MS measurements.

determination of the elements of interest, external calibration was used. The concentration range of As in the calibration solutions was between 0.1 µg/L and 5 µg/L, while for Cd and Pb between 0.1 µg/L and 10 µg/L. In the case of Hg, its concentration range in the calibration solutions was between 0.1 µg/L and 2 µg/L. For the elements of interest, sensitivity defined as the slope (s) of the linear fittings for the 10% (v/v) HNO_3 containing calibration solutions was excellent and the regression coefficient was always greater than 0.999. Typical *s* values for As, Cd, Hg and Pb were 0.00654 L/µg, 0.00657 L/µg, 0.01292 L/µg and 0.09860 L/µg, respectively. Matrix effects caused by organic residues remained in the solution after the oxidative digestion of milk can influence the aerosol formation during the nebulization resulting changes in material transport to the plasma. On the hand, experimental results showed for our elements of interest such as As and Hg that their sensitivities were always higher for carbon-containing solutions due to changes in the plasma characteristics and ion distribution in the plasma as well an increase in analyte ion population as a result of charge transfer reactions involving carbon-containing charged species in the plasma [32]. Thus, inaccurate results would have been obtained for As and Hg when an external calibration method without matrix matching had been applied for quantitative determination. Therefore, it is very important to keep the carbon content below 0.1% in the samples to be analyzed by ICP-MS. To check this parameter, the TOC analyzer is an ideal tool.

Quality assurance of the analytical data was checked with NIST rice flour SRM 1568a for As and skim milk powder SRM No. 150 for Cd and Pb. Thus, by analyzing appropriate amounts of these standard reference materials with the analytical method proposed in the present manuscript, the recovery values obtained for As, Cd and Pb were 98.8 ± 7.4%, 92.4 ± 10.7%, and 107 ± 8%, respectively.

3.3. Mean As, Cd and Pb concentration in human breast milk of lactating women

In the first step, the mean toxic element concentrations were calculated for each mother. The Hg concentration values in all samples were below 0.4 µg/L corresponding to its LOQ. This outcome was not surprising since no volunteer had occupational exposure to Hg. Moreover, fish did not form part of their regular diet. Also, as it was mentioned, only 18.75% of the mothers had at least one amalgam filling. Non-occupational Hg exposure of humans is attributed to amalgam dental fillings [22,33] and fish and seafood consumption [3,34]. The lack of Hg detection in human breast milk samples of the present study can be explained with the facts that, in Hungary, fish and seafood consumption is very low [30] and there is a general trend to replace all amalgam dental fillings, the latter proved by the very low percentage (i.e. 11.1%) of this type of dental fillings in the case of the volunteers (Table 1). Low median values for Hg concentration (i.e. about 0.2 µg/L) in human breast milk have been reported from Italy [35], Slovenia [34] and Croatia [22], while higher (about 0.5 µg/L) mean values from Spain [11], Japan [36] and Greece [3]. In a recent study conducted in Brazil, the mean Hg concentration in human breast milk samples was 2.56 µg/L, while extremely high values reaching to 614 µg/L have been reported from Pakistan [37].

Concentration of As, Cd and Pb could be determined in all samples with the optimized ICP-MS method. Thus, the following order could be established for the elemental concentration obtained: Cd < As < Pb. The Cd, As and Pb mean concentration in the samples of women included in the present study were 0.188 ± 0.071 µg/L, 0.41 ± 0.20 µg/L and 1.74 ±

0.77 μg/L, respectively. It should be also emphasized that about 40% of the mothers lived in dwellings built after 1970 (corresponding to the date when Pb pipes for drinking water delivery in dwellings was prohibited in Hungary) (Table 1). At the same time, the median values for Cd, As and Pb concentration in all samples were 0.188 μg/L, 0.35 μg/L and 1.61 μg/L, respectively, showing that the concentration distribution of these elements was nearly symmetric. Concerning the elemental composition of human breast milk in Hungary, only the report of Parr et al. from 1991 [28] contains relevant information on such samples. However, these samples were taken after three months of lactation (mature milk), while the samples analyzed in the present study originated from the first two months of the lactation period. Moreover, the previously reported As, Cd and Hg concentration values in Hungary were determined by neutron activation analysis, while Pb by atomic absorption spectrometry [28]. Since our data were obtained exclusively by ICP-MS, characterised by excellent LODs, it is understandable that Cd was not detectable at that time in many of the samples originating from Hungarian mothers living in urban areas having different socio-economic status (i.e. n = 24/33 and 25 well-to-do/poor mothers from urban and rural areas, respectively). Median values for As, Hg and Pb in Hungarian human breast milk samples back in 1991 were 0.24 ± 0.02 μg/L (n = 11), 1.43 ± 0.16 μg/L (n = 71), 14.9 ± 0.9 μg/L (n = 68), respectively [28]. While the median value for As in the present study was slightly higher (cca. 45%) than in the Hungarian mature milk taken in 1991, the biggest difference between our results and those from 1991 is that As was quantifiable in all recent samples. This may be due not only to advances in the instrumental analysis but to sharp changes in the dietary intake patterns of the past 30 years (i.e. consumption of much more poultry meat considered as being healthier and availability of seafood and marine fish in higher quantities nowadays). The As concentration values in breast milk samples originating from Taiwan were also similar to those in the present study: 0.68 ± 1.09 μg/L in transitional milk, 0.27 ± 1.26 μg/L in early mature milk and 0.16 ± 0.24 μg/L in mature milk [13]. The As concentration in mature milk samples taken from Portugal was about 10 times larger (5.8 μg/L) than in the present study, presumably due to the regular consumption of fish and seafood, while the Pb concentration was less [38]. At the same time, the As concentration values obtained in the present study are in good agreement with mean values obtained in similar samples taken from Sweden (0.55 μg/L) [39], and even lower in samples originating from Italy, Croatia and Slovenia. Arsenic concentration values higher than those reported in the present study for Europe were registered in Greece [3]. Thus, the mean values (sample size, n) in Greece, Italy, Croatia and Slovenia were 0.8 μg/kg (n = 39), 0.3 μg/kg (n = 602), 0.2 μg/kg (n = 123) and 0.04 μg/kg (n = 287), respectively [3]. Outside Europe, higher As mean concentration was registered in Pakistan (0.5 μg/L) [37], Iran (0.85 μg/L) [40], Bangladesh (1 μg/L) [41], Japan (1.4 μg/L) [36] and Lebanon (2.3 μg/L) [42]. The main sources for As is due to marine fish and seafood consumption, while in Hungary poultry meat consumption is the major As dietary source as pointed out in the Italian study of Lovreglio et al. [43]. For the elucidation of the role of environmental factors for the As concentration in breast milk, a survey on 187 samples was conducted in three industrialized regions of Germany (i.e. Soltau, Münster and Hamburg). By comparing the concentration data, the geometrical mean in the three regions were comparable (0.2 μg/L). The maximal value of 2.8 μg/L was registered in a region which has been shown to be contaminated with As from chemical weapons [44].

At the same time, the median value of Pb in the present study is drastically lower (by about 90%) than in 1991. This is understandable since occupational exposure to Pb has

been minimized in the past 30 years, use of leaded gasoline was banned in 1999 in Hungary and Pb drinking water pipes have been gradually phased out in Hungary since 1970. Similar trends for the decrease in the Pb concentration of human breast milk samples were registered in Sweden in 2012 [39]. At the same time, higher Pb concentration data were still found in some European countries. In the frame of the BioMadrid project, the relationship was searched between the elemental concentration in breast milk taken from women living in Madrid, Spain and their lifestyle, dietary habits and environmental nuisances [11]. The geometrical mean of Pb concentration in breast milk was 15.6 μg/L and the Pb could be detected in 93% of the samples. The Pb concentration in clinical samples originating from women living close to roads with busy traffic was always larger [45]. Thus, 15 μg/L Pb concentration was registered in Spain [11] (attributed mainly to heavy vehicle traffic), while below 10 μg/L in Poland [46], Italy [35] and Croatia [22]. However, the Pb concentration was unusually low (<0.2 μg/L) in Slovenia [33] and Japan [36]. Nevertheless, Pb concentration in human breast milk is still relatively high in Asia. Thus, mean values about 40 μg/L were registered in China [47], Pakistan [37] and Iran [40], while values around 20 μg/L were characteristic to human breast milk samples originating from Turkey [23], Taiwan [13] and Lebanon [42]. The role of environmental factors for the occurrence of Pb in human breast milk was clearly demonstrated in the aforementioned study conducted in Ankara (Turkey) [23]. According to this study, Pb could be detected (LOD = 0.2 μg/L) in 95% of the 144 investigated samples. The health limit value of 5 μg/L [28] was exceeded in 85% of the samples. The median was 20.6 μg/L, and an extremely large concentration value of 1515 μg/L was also registered in one sample.

The elemental concentration values in mature milk samples taken in Greece showed many similarities to those of the present study, especially for Cd, its mean was 0.142 ± 0.120 μg/L [15]. However, the Cd and Pb concentration in the Taiwanese samples were about three and four times larger than in the present study, respectively [13]. Smoking increased the Cd concentration in breast milk by a factor of 2.5 compared to nonsmokers [14]. The geometrical mean for the Cd concentration in breast milk taken in the frame of the BioMadrid project [11] was 1.31 μg/L, with the concentration range between 0.25 and 2.8 μg/L. Cadmium could be detected in 96% of the investigated samples. The Cd geometric mean concentration of the samples taken from women who exclusively breastfed their newborns was 51% less than for the group combining breast and artificial milk. The Cd concentration in the human breast milk of smokers was 37% larger than those of non-smokers [11]. However, the number of cigarettes smoked a day during pregnancy did not significantly increase the Cd content of the samples [11]. To conclude, taking into consideration that smoking is the main source for occurrence of Cd in human breast milk [11,23,42] and that all the mothers included in the present study were nonsmokers exposed in less than 20% to passive smoking (Table 1), it is understandable that the Cd concentration in the samples investigated in the present work was low. According to recent literature data on Cd concentration in human breast milk samples in Europe, lower than hereby reported Cd concentration (i.e. 0.086 μg/L) was found in samples taken in Sweden [39]. Median Cd concentration values of 0.13 μg/L and 0.14 μg/L have been reported in Bangladesh [41] and Japan [36], respectively. In Europe, higher concentration values than those of the present study have been reported in Poland (2.11 μg/L) [46] and Spain (1.31 μg/L) [11]. Extremely high concentration

Table 4. Risk assessment of infants to arsenic, lead and cadmium through breast milk taken in the present study.

Element	Estimated intake			Health guidance value	Estimated risk
	µg/kg BW/day				MOE
As	Minimum	Median	Maximum	$BMDL_{0.5}$:	48
	0.023	0.062	0.16	3.0	
Pb	Minimum	Median	Maximum	$BMDL_1$:	1.7
	0.092	0.29	0.75	0.5	
	µg/kg BW/week				HI
Cd	Minimum	Median	Maximum	TWI:	0.05
	0.054	0.23	0.40	2.5	
	µg/kg BW/month				HI
	Minimum	Median	Maximum	PTMI:	0.04
	0.23	1.00	1.71	25	

Abbreviations: BMDL = benchmark dose (lower confidence limit); BW = body weight; HI = hazard index expressed as ratio between the estimated average intake and the corresponding PTI value; MOE = margin of exposure expressed as ratio between the BMDL value and estimated average intake; PTMI = provisional tolerable monthly intake; TWI = tolerable weekly intake.

values have been reported from Pakistan (52 µg/L) [37], while values around or just less than 1 µg/L have been reported in samples taken in Turkey [23], China [48], Taiwan [13] and Lebanon [42].

On the basis of the obtained data, the risk posed by the dietary intake of Cd, As and Pb through exclusive breastfeeding could be estimated. Thus, taking the median toxic element concentration values of the present study and assuming that a 1-month-old infant with a BW of about 4.5 kg is fed exclusively every 3 h with approximately 100 mL of breast milk, the dietary intake could be estimated (Table 4). Moreover, risk assessment of infants to As, Pb and Cd through breast milk taken in the present study was performed according to Rebelo and Caldas [17]. Thus, the margin of exposure (MOE) expressed as the ratio between the corresponding BMDL and the estimated average intake was calculated for As and Pb. A MOE should be as high as possible so as not to represent a public health concern [48]. For the estimation of risk exposure of infants to As though breast milk, all MOE values were higher than 1. However, a MOE of 50 or higher for As (typical for the USA), based on the $BMDL_{0.5}$ from a human study, would be of low concern from a public health point of view [48]. In the present study, MOE value was slightly lower than 50 (Table 4). Nevertheless, the MOE value calculated in the present study is typical for Europe [17].

In its evaluation, the EFSA concluded that the risk from Pb exposure for 3-month breastfed infants can be significant when the MOE is lower than 1; risk is likely to be low when the MOE is between 1 and 10; and a MOE of 10 or greater indicates no appreciable risk of a clinically significant effect on IQ [49]. The MOE calculated in the present study for Pb was 1.7, indicating a potential risk to breastfed infants. However, MOE would have been 0.7 for the worst-case intake using the maximum-detected concentration for calculation.

In the case of Cd, hazard index was calculated as the ratio between estimated average intake and the corresponding (provisional) tolerable intake values (both weekly and monthly recommended by EFSA and JECFA in 2012 and 2011, respectively). Risk may exist when the index is greater than 1. Our estimations proved that practically there was no risk by feeding infants with breast milk from the point of view of its Cd content (Table 4).

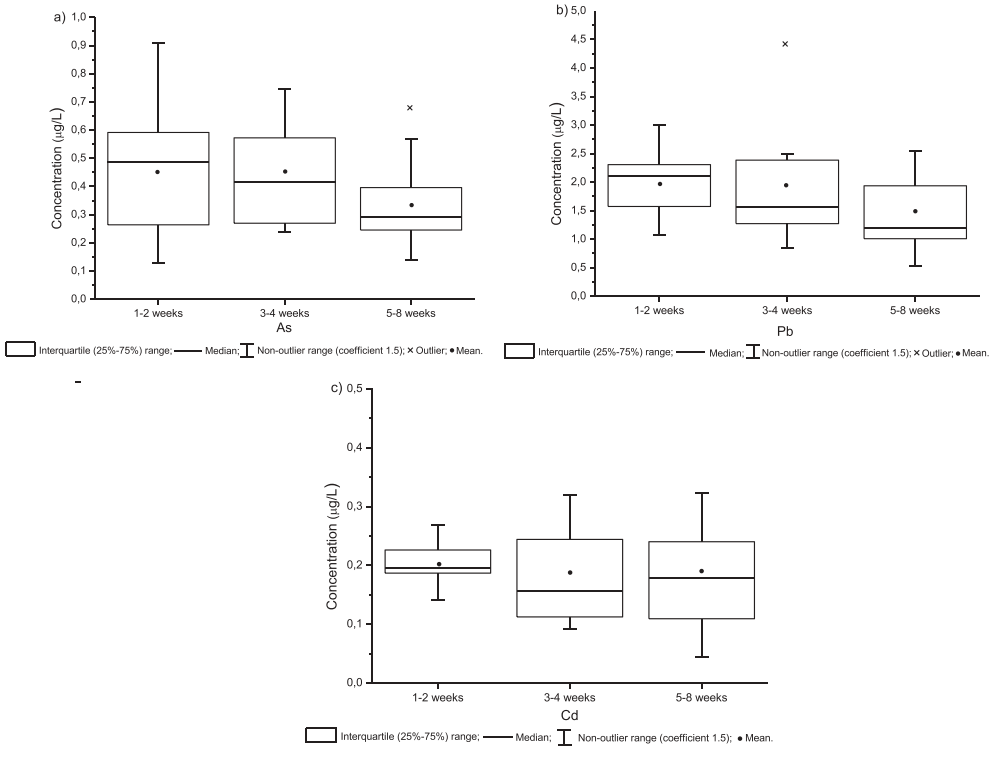

Figure 2. Box and whisker plot displays of arsenic (a), lead (b) and cadmium (c) concentration in human breast milk (n = 34*) in three lactation stages grouped as weeks 1–2; 3–4 and 5–8.

* One out of the 27 mothers provided one sample weekly for 8 weeks.

3.4. Arsenic, cadmium and lead concentration in human breast milk as a function of lactation time

After birth, the concentration of toxic elements in breast milk generally decreases. According to box and whisker plots for concentration of each element belonging to the same fortnight (except for weeks 5–8 for which all data sets were grouped together due to the lower sample size) indicating the minimum and maximum values, data at 25th and 75th percentiles, mean and median values (Figure 2), the mean Pb concentration was very similar for the first 4 weeks of lactation (1.94 ± 0.6 vs. 1.91 ± 0.91 µg/L), then it decreased after two months (Figure 2b) by about 25% compared to the initial data. The decrease registered for the median in the first two fortnights as well as for weeks 5–8 was even sharper: 2.11 µg/L, 1.57 µg/L and 1.20 µg/L, respectively (Figure 2b). At the same time, the As concentration also decreased monotonously after two months similarly to what registered for Pb (Figure 2a). Thus, data for the mean (median) in the case of As in the samples corresponding to the first two fortnights and weeks 5–8 of lactation in the present study were 0.46 ± 0.26 (0.49) µg/L, 0.45 ± 0.19 (0.42) µg/L and 0.34 ± 0.17 (0.29) µg/L, respectively (Figure 2a). Thus, the decrease in the As mean and median data by the end of the two months of lactation period was roughly 25% and 35%, respectively. This latter phenomenon was also observed in breast milk samples taken in

Portugal [38], although the As concentration was much larger in that study as it was already mentioned in Section 3.3. In that study, Zn and Se concentrations decreased at the greatest extent, i.e. by 23% and 44%, respectively [38].

In the present study, the Cd concentration was almost constant in the samples taken during two months of lactation (Figure 2c). Thus, the mean Cd concentration in the samples investigated in the present study for the first two fortnight and weeks 5–8 were 0.206 ± 0.041 μg/L, 0.180 ± 0.075 μg/L and 0.182 ± 0.085 μg/L, respectively, while the corresponding median values were 0.195 μg/L, 0.157 μg/L and 0.178 μg/L, respectively. In the study conducted on 180 breast milk samples in a heavily industrialized urban site of Taiwan, the concentration of Pb, Cd, Al and As in mature milk after two months of lactation decreased by about 80%, 75%, 76% and 90%, respectively [13]. Moreover, a significant correlation could not be established between the consumption of canned products and Cd content in colostrum. However, an unequivocal relationship was observed between Cd content in colostrums, what is not surprising, since Sn, Pb and Zn are the most significant pollutants in this type of packaging and smoking habits. Thus, Cd concentration was almost four times larger than in the samples originating from nonsmokers. In another study conducted in Greece, the concentration of Pb in young mature milk also rapidly decreased by 70% compared to that in colostrum, while that of Cd decreased only by 25% [15]. These authors concluded that the large toxic element content of colostrum is owed to its high protein content [15].

In conclusion, the concentration of Pb always decreased in human breast milk taken in Iran [40], Poland [46] and Taiwan [13] over a time period of 12 months. The decrease in the Pb concentration was accompanied by similar changes of other calcophile elements such as Cu, Zn [46] and Hg [22]. Similar results were obtained by Letinic et al. [22] investigating colostrum (n = 20), transitional (n = 33) and mature milk (n = 51). Apart from data reported from Taiwan [13], similar trends could not be registered neither for Cd [46] nor for As [40].

3.5. Arsenic, cadmium and lead concentration in human breast milk as a function of volunteer age

Evolution of the toxic element concentration as a function of the age of women was also represented graphically (Figure 3). The statistical data evaluation performed on the data set revealed that there was a significant correlation between the Pb concentration in breast milk as a function of the age of volunteers (Table 5). Taking into account that the European Commission prohibited the marketing of leaded petrol within the territory of the member states since 1 January 2000 through Directive 98/70/EC [50], which entered in force in Hungary on the same date, the significant Pb concentration increase indicates mobilization of this element from bones of the volunteers that were about 20 years old at the time when antiknock fuel additives were phased out. Hence, these mothers could be more exposed to leaded gasoline in the urban areas than the much younger ones. Moreover, exposure due to redistribution of the Pb containing aerosol particles as well as Pb pipes for drinking water delivery has been registering a decreasing tendency since the 1970s in Budapest [29,51]. In the frame of an ongoing national survey conducted by the National Public Health and Medical Officer Service, 2700 tap water samples intended for human consumption originating from Budapest have been analyzed [52]. In 20% of

Figure 3.

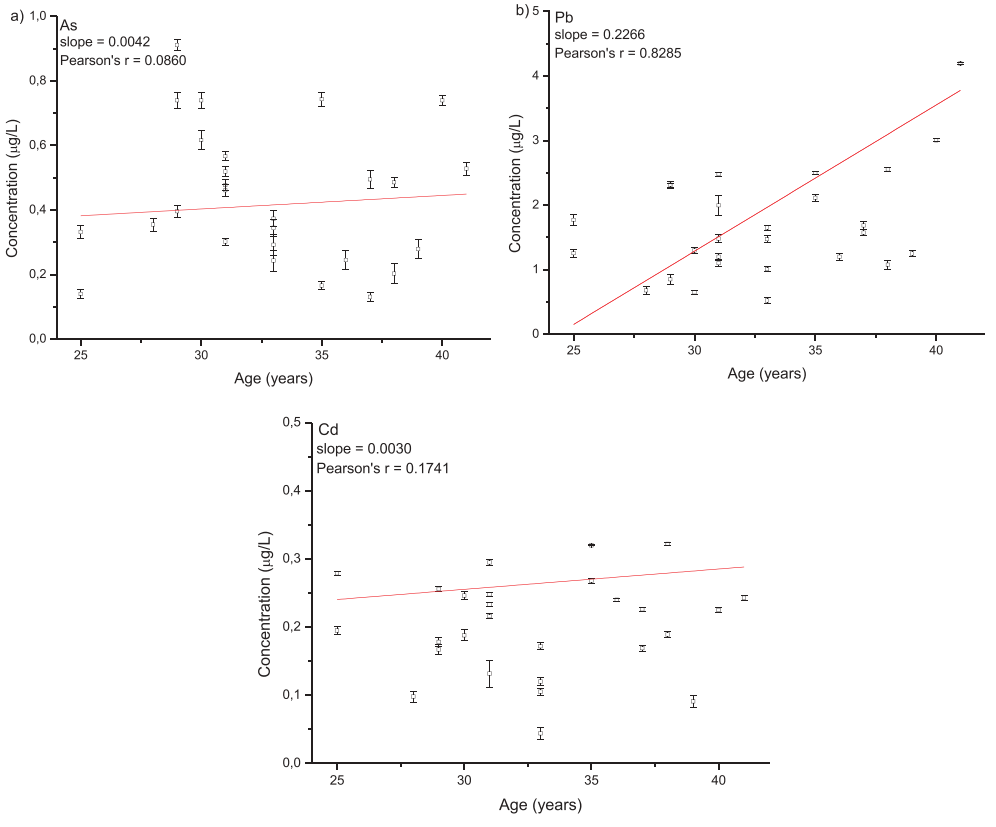

Figure 3. Arsenic (a), lead (b) and cadmium (c) concentration in human breast milk (n = 27*) as a function of the age of mothers.

* One out of the 27 mothers provided one sample weekly for 8 weeks. In this case, only average elemental concentration data of the samples have been considered.

Table 5. Results of the paired sample *t*-test for the changes in the toxic element concentration in breast milk samples ($n = 27$) as a function of the age of mothers at a degree of freedom n-2 and at a significance level, $p < 0.05$.

	Element		
Parameter	Arsenic	Lead	Cadmium
R^2	0.0074	0.6864	0.0303
t	0.4316	7.3975	0.8840
n-2		25	
t_p		2.06	
$\|t\| < t_p$	Yes	No	Yes
Correlation	No	Yes	No

Abbreviations: r = correlation coefficient; R^2 = coefficient of determination; t = test statistics, t_p = critical value of a *t*-distribution. Calculation was made *cf.* $t = \sqrt{n-2}\frac{r}{\sqrt{1-R^2}}$, where r = Pearson's correlation coefficient (see Figure 3 for the specific values).

these samples, the Pb concentration exceeded the 10 µg/L health limit value. In two-thirds of the cases, the Pb concentration could be lowered below the health limit value if water was let run for a while before repeating sampling. The majority of these Pb-contaminated samples were taken from households located in buildings of the

downtown of Budapest built before 1945. Almost two-thirds of the mothers included in the present study were living at the time of the study in relatively modern buildings where water distribution pipes are surely not made of Pb and special attention was paid from our side to get detailed information on their dietary habits. Unfortunately, exposure of (elderly) mothers to Pb in the past either through drinking water or contaminated food or due to the use of leaded gasoline could not be evaluated in the present study; hence, it cannot be either excluded. Similar significant relationships could not be established for As and Cd data sets of the present study, their concentrations proved not to depend on the age of the volunteers (Figure 3). A significant increase (at $p< 0.0.5$) in the Pb concentration in breast milk as a function of the age of the mothers has been reported in Poland [46]. Thus, the mean Pb concentration in those samples generally increased with the age of the mother as follows: 4.76 µg/L, 6.93 µg/L, 6.22 µg/L and 7.41 µg/L for the age groups 20–25 (n = 35), 26–30 (n = 158), 31–35 (n = 70) and 36–40 (n = 60), respectively. At the same time, a similar increase for Cd was shifted towards the younger mothers, namely, belonging to the age group of 20–25 [46]. In the present study, such grouping of the concentration series could not be done for statistical data analysis due to the relatively low sample size. However, paired sample *t*-tests performed on all data are in agreement with the outcomes of the aforementioned study obtained for Pb.

4. Conclusions

Monitoring of toxic contaminants in human breast milk is crucial, since exclusive breastfeeding of newborn infants is recommended due to its beneficial effects during the first six months of their life. In order to achieve this, it is important to develop reliable methods for assessment of toxic element concentration in human breast milk even in the case of low-maternal exposure to toxic elements such as As, Cd, Hg and Pb in order to be able to register trends in the change of their concentration. However, mobilization of the previously accumulated Cd, Hg and Pb in different soft and hard tissues of the human body can occur during pregnancy and lactation. Therefore, the expected low elemental concentrations require adequate sampling strategy planning and careful execution of sample preparation procedures as well as through development of analytical methods suitable for quantification. Up to our knowledge, this is the first report of the last 30 years in Hungary on toxic element investigation in human breast milk performed with modern instrumental analytical techniques. The newborns with mothers of increasing age resulted to be a vulnerable group due to the elevated Pb concentration in the breast milk in spite of the fact that almost 20 years elapsed after the prohibition of leaded gasoline marketing in the EU and about 40 years since Pb pipes for drinking water delivery were phased out. This latter outcome is also important since Pb exposure results in severe physiological damage at any Pb concentration, a daily dose of 0.5 µg lead/BW kg in blood determined at the 95th percentile lower confidence for 1% extra risk ($BMDL_1$) causing 1 point reduction on the IQ scale for children. However, according to our dietary intake estimations, no other threat for newborns fed by exclusive breastfeeding was detected.

As it has been reported, the difference in the breast milk levels may depend on various factors such as time of sampling, lactation period, sampling method (instrumental vs. manual), maternal factors (parity and maternal Pb burden), as well as

environmental factors (dwelling type/location and exposure level/duration) [23]. Considerable advances registered in the instrumental analysis (e.g. wider implementation of high resolution ICP-MS) and certified reference material market have led to the improvement of precision and accuracy of the results.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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