ELSEVIER

Food Chemistry 68 (2000) 95–99

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods Section

Copper, iron and zinc determinations in human milk using FAAS with microwave digestion

M.D. Silvestre [b], M.J. Lagarda [a], R. Farré [a,*], C. Martínez-Costa [c], J. Brines [c]

[a] *Nutrición y Bromatología, Facultad de Farmacia, Universidad de Valencia, Avda. Vicente Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain*
[b] *Area de Veterinaria. C.E.U San Pablo, Valencia, Spain*
[c] *Departamento Pediatría, Obstetricia y Ginecología. Facultad de Medicina. Universidad de Valencia, Valencia, Spain*

Received 5 January 1999; received in revised form 28 May 1999; accepted 28 May 1999

Abstract

A method for determining copper, iron and zinc in human milk was optimized and validated. It includes microwave mineralization of the sample and measuring the elements by flame atomic absorption spectroscopy (FAAS). Only 2 ml of milk is needed, and the method is free of matrix interferences. The values obtained for the detection limits (0.07; 0.07; 0.11 μg/ml milk, for copper, iron and zinc) precision of the method, intra-assay (2.9; 5.2; 6.1%RSD for copper, iron and zinc) and accuracy, evaluated using recovery assays (98.8; 100.4; 95.9% for copper, iron and zinc) show that the method is useful for the purpose mentioned. Moreover, the method is rapid and simple, and the determinations are carried out by FAAS.

Keywords: Copper; Zinc; Iron; Human milk; FAAS; Microwave digestion

1. Introduction

The role of the trace elements copper, iron and zinc in different biological functions makes them important in nutrition and especially in infant nutrition. In recent years this has led scientists to examine the trace element content of human milk, the ideal infant food during the first months of life, in order to estimate infant requirements and establish reference values for use in manufacturing infant formulas.

The choice of an analytical method for determining these trace elements in human milk offers certain difficulties: the contents of the elements are low (g/ml); there is a risk of contamination during the sample treatment; the matrix is complex, and in practically all cases the amount of sample available is limited. In addition, a technique for analysing trace elements has to be highly sensitive, free of interferences, must include a simple sample treatment involving minimum handling and reagent use and should be fast and permit several elements to be measured.

The analytical techniques usually applied to the determination of Cu, Fe and Zn in human milk are: flame (FAAS) (Benemariya, Robberecht & Deelstra, 1995; Fransson & Lonnerdal, 1984; Picciano & Guthrie, 1976; Robberecht, Benemariya & Deelstra, 1995) or electrothermal atomic absorption spectroscopy (ET-AAS) (Arnaud, Bouillet, Alary & Favier, 1992; Casey, Neville & Hambidge, 1989) and ICP emission spectroscopy (Anderson, 1993; Silva, Dorea & Boaventura, 1997). When there is a sufficient volume of sample and the analyte content is high enough FAAS is the technique of choice. However, the composition of human milk makes it necessary to do a previous sample oxidation, which is often cumbersome and involves the risk of contamination and of analyte loss by adherence to the walls of the container or volatilization. Besides the classic dry mineralization (Feeley, Eitenmiller, Benton & Barnhart, 1983; Gunshin, Yoshikawa, Doudou & Kato, 1985; Lamounier, Danelluzi, Vannucchi, 1989; Siimes, Vuori & Kuitunen, 1979) and wet digestion (Benemariya et al., 1995; Casey, Hambidge & Neville, 1989; Fransson & Lonnerdal, 1984; Neville, Keller, Seacat, Casey, Allen & Archer, 1984; Robberecht et al., 1995; Silva et al., 1997) a system of wet digestion in closed Teflon vessels and microwave heating is also used. This favours organic matter destruction, shortens the time needed for the analysis and offers the advantage of simple, fast organic matter destruction, minimum reagent volume, reduction of possible analyte losses by volatilization or

* Corresponding author.

0308-8146/99/$ - see front matter
PII: S0308-8146(99)00160-0

retention and elimination of the environmental contamination risks (Amaro-Lopez, Moreno-Rojas, Sanchez Segarra & Zurera Cosano, 1996).

The preference for microwave over other digestion methods for biological samples is evident in the literature (Kingston & Jassie, 1986; Mingorance & Lachica, 1985; Schelkoph & Milne, 1988; Stripp & Bogen, 1989), and in recent years it has also been used in human milk (Alegria, Bnarbera, Farre, Lagarda & Torres, 1996; Alkanani, Friel, Jackson & Longerich, 1994; Coni, 1990; Frkovick, Kras & Alebic-Juretic, 1997; Krachler, Shu, Rossipal & Irgolic, 1998). The conditions i.e. the type and amount of added acid, the possible use of coadjutants, the time-power program and the need for additional steps must be adapted to the characteristics of the elements and to the specific study.

The aim of this study was to optimize and validate a method for determining copper, iron and zinc in human milk that includes microwave mineralization of the sample and determination of the elements by FAAS. The method was also applied to determine these elements in milk provided by healthy women of the Comunidad Valenciana (Spain).

2. Methods and materials

2.1. Instrumentation

The following apparatuses were used: a Perkin–Elmer model 2380 atomic absorption spectrophotometer; hollow cathode lamps for zinc, iron and copper; Milestone MLS 1200 microwave system with MV 100 medium pressure vessels with burst discs; Relpa PL-3920 thermal plate. Milk was obtained using an electric breast pump fitted with a vacuum regulator (Mamilat S.M. 122).

2.2. Reagents

All reagents were of analytical reagent grade. Nitric acid 65% (sp.gr. 1.40, Merck); hydrogen peroxide 33% (Panreac). Copper, iron and zinc stock solutions (Titrisol Merck) 1000 ± 0.002 mg/l. Standard working solutions were prepared from stock solution immediately before use. Deionized water was used (Milli-Q System, Millipore). All glassware was soaked in nitric acid for 15 min and rinsed with deionized water before use. In order to avoid iron contamination from the metalic components in the work place, a PTFE Teflon spray ref. OKS 571 was used.

2.3. Samples

Human milk samples were provided by volunteers from the maternal unit of the Hospital Clínico de Valencia. The proposed method was applied to 10 samples (approximately 5 ml) collected at each of the following lactation steps: colostrum, transitional and 30-, 60- and 90-day mature milk. Samples were kept frozen at −18°C until analysis.

2.4. Procedure

Copper, iron and zinc were measured by flame atomic absorption spectrometry (FAAS) after a wet microwave destruction of the organic matter. The applied method is an adaptation of the one used in our laboratory to measure selenium in human milk by ET-AAS (Alegria et al., 1996). Two millilitres of thawed sample was introduced into the Teflon vessel and 1 ml of concentrated nitric acid and 0.25 ml of H_2O_2 (33% w:w) were added. The following heating program was applied: 300 W/4 min; 600 W/4 min and ventilation 5 min. Then the digested solution was transferred to a tube with 10 ml of deionized water and allowed to evaporate on a heating plate at 70–80°C (2.30 h), while avoiding complete dryness. The residue was dissolved with deionized water and the volume completed to 10 ml. The digestion solution obtained showed particles in suspension that made it difficult to measure by flame atomic absorption spectroscopy (FAAS). In order to obtain a digestion solution free of interfering particles three approaches were assayed: (a) filtration of the digested solution; (b) modification of the microwave heating program (power/time) and (c) modification of the evaporation/drying step. The FAAS instrumental conditions are reported in Table 1.

3. Results

3.1. Optimization of the microwave digestion method

3.1.1. Filtration step

Filtration of the digestion solution through a paper filter (Schleicher-Schüll ref. 334543) was assayed. To check the risk of element contamination, the zinc content was measured in the digested solution before and after filtration. The results were an increase in the zinc content as a consequence of filtration, probably due to

Table 1
Instrumental conditions for copper, iron and zinc determinations by flame AAS

	Copper	Iron	Zinc
Wavelength (nm)	324.8	248.3	213.9
Slit width (nm)	0.7	0.2	0.7
Lamp intensity (mA)	15	30	15
Air flow (l/m)	17.5	17.5	17.5
Acetylene flow (l/m)	2.0	2.0	2.0
Nebulizer	Impact ball	Impact ball	Impact ball
Background	No	No	No
Expansion	3	3	No

contamination. The filtration step, was therefore, not included in the procedure.

3.1.2. Microwave heating program

An increase in the permanence times at the powers of the program were assayed. For this purpose eight aliquots of a sample of human milk were taken. To four of them (A) the initial heating program (300 W/4 min and 600 W/4 min) was applied, while the other four (B) were subjected to the same power but the times were increased (300 W/5 min and 600 W/5 min). The digested solutions were evaporated and the residues dissolved with 10 ml of deionized water. The values obtained for copper, iron and zinc contents are reported in Table 2. Since the increased heating times give a digestion solution free of particles in suspension and absorbance values with a lower variability than the initial program, they were applied.

3.1.3. Drying step

To verify that there is no loss of elements from volatilization and/or risk of contamination during the evaporation of the sample in open vessels, the following assay was carried out: the microwave mineralization was applied to five samples of human milk. After digestion two aliquots of each sample were taken, and one was subjected to evaporation in a covered vessel and the other in an open one. No differences were observed between the contents of the two vessels (see Table 3). Therefore, the samples were dried in an open vessel because less time (about an hour) is needed than when the vessels are covered.

Table 3
Copper, iron and zinc determination. Comparison between dried uncovered (UC) and covered (C) samples with a watch glass (μg/ml of milk)

Samples	1	2	3	4	5
Copper					
C	0.33	0.32	0.49	0.44	0.25
UC	0.33	0.32	0.48	0.43	0.25
Iron					
C	0.51	0.25	0.30	0.22	0.34
UC	0.51	0.25	0.26	0.22	0.28
Zinc					
C	0.45	4.16	0.99	3.20	0.98
UC	0.35	4.20	0.96	3.09	0.97

3.2. Analytical parameters

To check the quality and usefulness of the optimized method for determining the copper, iron and zinc contents of human milk, the absence of interferences was checked and the analytical parameters of the method (linearity, detection limit, precision and accuracy) were determined.

3.2.1. Matrix interferences

Two calibration curves were constructed: (a) aqueous standards and (b) aqueous standards with matrix added (5 ml of digested samples at a final volume of 10 ml). A comparative slope test (covariance test) of the two regression equations was applied. The absence of significant differences between the slopes of the two curves indicates that there are no matrix interferences, and that the aqueous standards can be used to determine the elements. Since the residual variances are homogeneous, the following equations must be true.

(Mean square between regression coefficients/mean square within regression) $\leqslant F_{1,4}$

The obtained F values were: Cu = 2.0450; Fe = 0.3006; Zn = 3.308×10^{-5}

The tabulated F value at a 95% probability level was 7.7086.

Therefore no interferences were present.

Table 2
Copper, iron and zinc determination. Comparison of two microwave digestion programs applied to milk samples. (μg/ml of milk)

Samples	1	2	3	4	$\bar{x}$[c]	S_{n-1}[d]
Copper: $y = 0.2818\ x - 0.0003$;	$r = 0.9999$					
A[a]	0.61	0.61	0.71	0.66	0.65	0.048
B[b]	0.63	0.61	0.59	0.61	0.61	0.016
Iron: $y = 0.1740\ x - 0.0070$;	$r = 0.9994$					
A	0.95	0.69	1.01	1.09	0.94	0.173
B	0.84	0.89	0.98	0.81	0.88	0.074
Zinc: $y = 0.4781\ x + 0.00490$;	$r = 0.9990$					
A	3.43	3.22	3.76	3.45	3.47	0.222
B	3.33	3.31	3.34	3.35	3.33	0.017

[a] A : 4 min—300 W and 4 min—600 W.
[b] B : 5 min—300 W and 5 min—600 W.
[c] $\bar{x}$ = average.
[d] S_{n-1} = standard deviation.

3.2.2. Linearity

The linearity of the response was verified using standards ranging from 0.025 to 1 μg/ml for Cu and Fe and 0.1–1 μg/ml for Zn. The adjusted linear equations and correlation coefficients were:

$$\text{Copper}: \quad y = 0.2888x + 0.0034 \quad r = 0.9999$$
$$\text{Iron}: \quad y = 0.2474x + 0.0014 \quad r = 0.9998$$
$$\text{Zinc}: \quad y = 0.4246x + 0.0078 \quad r = 0.9999$$

Therefore, when measuring zinc in colostrum or transitional milk, a 1-to-2 dilution of the digested solution with deionized water is needed.

3.2.3. Detection limit (American Chemical Society [ACS], 1983)

The detection limit was defined as the concentration corresponding to 3 times the standard deviation of ten blanks. The values obtained are reported in Table 4.

3.2.4. Precision

The instrumental precision was estimated from 10 consecutive determinations of a single dilution of a digested sample. The precision of the method was estimated by analyzing eight homogeneous aliquots of a sample of milk. Intra- and inter-assay precision was estimated. The results obtained expressed as relative standard deviation are reported in Table 4.

3.2.5. Accuracy

Appropriate reference material was not available. Because the copper, iron and zinc contents of the available reference material, BCR dry milk, were very low. This meant that the amount of sample that had to be used was so large that it could not be digested in our digestion vessels. Recovery assays were carried out to check the accuracy of the method. Adequate amounts of the elements were added to milk before the digestion of the sample. The recovery percentages are reported in Table 4.

3.3. Copper, iron and zinc contents of the analyzed human milk samples

The values obtained are reported in Table 5.

Table 4
Copper, iron and zinc determination in human milk. Analytical parameters

	Copper	Iron	Zinc
Detection limit			
(ng/ml assay)	15	14	22
(mg/ml milk)	0.07	0.07	0.11
Instrumental precision (%RSD)	1.46	1.22	0.53
Intra-assay precision (%RSD)	2.88	5.21	6.13
Inter-assay precision (%RSD)	7.25	6.84	8.90
Accuracy (%recovery)	98.8 ± 11.04	100.4 ± 6.78	95.9 ± 6.73

Table 5
Copper, iron and zinc contents in human milk. Confidence levels (μg/ml milk)

	Copper	Iron	Zinc
Colostrum	0.32 ± 0.15	0.52 ± 0.14	8.60 ± 1.82
Transitional	0.45 ± 0.06	0.48 ± 0.13	3.45 ± 0.58
Mature 30 days	0.34 ± 0.05	0.43 ± 0.13	1.97 ± 0.25
Mature 60 days	0.24 ± 0.05	0.45 ± 0.12	1.24 ± 0.33
Mature 90 days	0.17 ± 0.06	0.38 ± 0.12	0.89 ± 0.27

4. Discussion

The analytical parameters obtained in the recovery assays (nearly 100%), the precision (lower than 9% in all cases) and the detection limit (far below the concentration observed for the three elements during the lactation period studied) show that the optimized method is useful for determining copper, iron and zinc at the usual levels in human milk.

A drying stage to remove the nitric acid is needed after applying the selected power/time microwave program and prior to the FAAS determination. This constitutes a difference with respect to other analytical techniques: ICP (Alkanani et al., 1994; Coni et al., 1990; Krachler et al., 1998), ETAAS (Frkovic et al., 1997) or spectrofluorometry (Alegria et al., 1996).

With respect to other methods proposed for the determination of copper, iron and zinc in human milk our method offers the advantages of simplicity and the short time necessary: <2 h versus 5 h (Fransson & Lonnnerdal, 1980) and 48 h (Siimes et al., 1979) for dry digestion, and from >4 h (Picciano & Guthrie, 1976) to 12 h (Casey et al., 1985) for wet digestion. Another advantage is the small volume needed (2 ml) in relation to the amount required in other studies, 5 ml (Krebs, Hambidge, Jacobs & Mlyer, 1985; Picciano & Guthrie, 1976) 10 ml (Vuori & Kuitunen, 1979); 7 g (Vaughan, Weber & Kemberling, 1979). The dilution applied in the assay, 2 ml of milk to 10 ml of digested solution, is adequate for measuring the usual contents of copper, iron and zinc in human milk. When measuring zinc in colostrum and transitional milk, a 1-to-2 dilution of the digested solution with deionized water is needed.

However, microwave digestion has certain limitations. The number of samples that can be simultaneously digested is restricted to the number of places (eight) for digestion vessels in the microwave oven. Moreover, precautions must be adopted to prevent alterations of the inner surface of the digestion vessels that can be responsible for the adsorption of the elements, and, therefore, aggressive conditions like a large proportion of acid, high temperatures or long times should be avoided.

The copper, iron and zinc contents of the analysed human milk were within the ranges reported by other

authors: copper from 0.78 mg/ml (Carrion, Itriago, Murillo, Elju & Fernandez, 1994) to 0.08 mg/ml (Robberecht et al., 1995); iron from 1.6 μg/ml to < 0.1 mg/ml (Picciano & Guthrie, 1976); zinc from 11.96 μg/ml (Arnaud & Favier, 1985) to 0.14 μg/mL (Picciano & Guthrie) and gradually decreased during the lactation period (Carrión et al., 1994; Feeley et al., 1983; Rajalakshmi & Srikantia, 1980).

5. Conclusions

The optimized method, which includes microwave digestion of the sample and determination of copper, iron and zinc by FAAS, is rapid and simple. The time and small volume needed together with the values of the analytical parameters ensure the suitability of the proposed method for determining copper, iron and zinc in human milk during the usual lactation period (from birth to the third month after birth).

References

American Chemical Society (1983). Limit of detection. A closer look at the IUPAC definition. *Anal. Chem., 55*, 712–724.

Alegría, A., Barberá, R., Farré, R., Lagarda, M. J., & Torres, A. (1996). Effectiveness of microwave based digestion procedures for human milk and infant formulas prior to fluorimetric determination of selenium. *Nahrung, 40*, 92–95.

Alkanani, T., Friel, J. K., Jackson, S. E., & Longerich, H. P. (1994). Comparison between digestion procedures for the multielemental analysis of milk by inductively coupled plasma mass spectrometry. *J. Agric. Food Chem., 42*, 1965–1970.

Amaro-López, M. A,. Moreno-Rojas, R., Sánchez Segarra, P.J. & Zurera Cosano, G. (1996). Tratamiento de las matrices lácteas para la determinación de trazas metálicas por absorción atómica. *Alimentaria*, April, 71–78.

Anderson, R. R. (1993). Longitudinal changes of trace elements in human milk during the first 5 months of lactation. *Nut. Res., 13*, 499–510.

Arnaud, J., Bouillet, M. C., Alary, J., & Favier, A. (1992). Zinc determination in human milk by flameless atomic absorption spectrometry after dry ashing. *Food Chem., 44*, 213–219.

Arnaud, J., & Favier, A. (1985). Copper, iron, manganese and zinc contents in human colostrum and transitory milk of French women. *Sci. Total Environ., 159*, 9–15.

Benemariya, H., Robberecht, H., & Deelstra, H. (1995). Copper, zinc and selenium concentrations in milk from middle-class women in Burundi (Africa) throughout the first 10 months of lactation. *Sci. Total Environ., 164*, 161–174.

Carrión, N., Itriago, A., Murillo, M., Elju, E., & Fernández, A. (1994). Determination of calcium, potassium, magnesium, iron, copper and zinc in maternal milk by inductively coupled plasma atomic-emission spectrometry. *J. Anal. At. Spectrom., 9*, 205–207.

Casey, C. E., Hambidge, K. M., & Neville, M. C. (1985). Studies in human lactation: zinc, copper, manganese and chromium in human milk in the first month of lactation. *Am. J. Clin. Nutr., 41*, 1193–1200.

Casey, C. E., Neville, M. C., & Hambidge, K. M. (1989). Studies in human lactation: secretion of zinc, copper and manganese in human milk. *Am. J. Clin. Nutr., 49*, 773–785.

Coni, E., Falconieri, P., Ferrante, E., Semeraro, P., Beccaloni, E., Stacchini, A., & Caroli, S. (1990). Reference values for essential and toxic elements in human milk. *Ann. Ist. Super. Sanita., 26*(2), 119–130.

Feeley, R. M., Eitenmiller, R. R., Benton, J. B., & Barnhart, H. (1983). Copper, iron and zinc contents of human milk at early stages of lactation. *Am. J. Clin. Nutr., 37*, 443–448.

Fransson, G. B., & Lönnerdal, B. (1980). Iron in human milk. *J. Pediatr., 96*, 380–384.

Fransson, G. B., & Lönnerdal, B. (1984). Iron, copper, zinc, calcium and magnesium in human milk fat. *Am. J. Clin. Nutr., 39*, 185–189.

Frkovic, A., Kras, M., & Alebic-Juretic, A. (1997). Lead and cadmium content in human milk from the Northern Adriatic area of Croatia. *Bull. Environ. Contam. Toxicol., 58*, 16–21.

Gunshin, H., Yoshikawa, M., Doudou, T., & Kato, N. (1985). Trace elements in human milk, cow's milk and infant formula. *Agric. Biol. Chem., 49*, 21–26.

Kingston, H. M., & Jassie, L. B. (1986). Microwave energy for acid decomposition at elevated temperatures and pressures using biological and botanical samples. *Anal. Chem., 58*, 2534–2541.

Krachler, M., Shi Li, F., Rossipal, E., & Irgolic, K. J. (1998). Changes in the concentrations of trace elements in human milk during lactation. *J. Trace Elem. Med. Biol., 12*, 159–176.

Krebs, N. F., Hambidge, M., Jacobs, M. A., & Myler, S. (1985). Zinc in human milk: diurnal and within-feed patterns. *J. Pediatr. Gastroenterol. Nutr., 4*, 227–229.

Lamounier, J. A., Danelluzi, J. C., & Vannucchi, H. (1989). Zinc concentrations in human milk during lactation: a 6-month longitudinal study in southern Brazil. *J. Trp. Pediat., 35*, 31–34.

Mingorance, M. D., & Lachica, M. (1985). Direct determination of some trace elements in milk by electrothermal atomic absorption spectrometry. *Anal. Lett., 18*, 1519–1531.

Neville, M. C., Keller, R., Seacat, J., Casey, C. E., Allen, J. C., & Archer, P. (1984). Studies on human lactation. I. Within-feed and between-breast variation in selected components of human milk. *Am. J. Clin. Nutr., 40*, 635–646.

Picciano, M. F., & Guthrie, Ph.D. (1976). Copper, iron and zinc contents of mature human milk. *Am. J. Clin. Nutr., 29*, 242–254.

Rajalakshmi, K., & Srikantia, S. G. (1980). Copper, zinc and magnesium content of breast milk of Indian women. *Am. J. Clin. Nutr., 33*, 664–669.

Robberecht, H., Benemariya, H., & Deelstra, H. (1995). Daily dietary intake of copper, zinc and selenium of exclusively breast-fed infants of middle-class women in Burundi, Africa. *Biol. Trace Elem. Res., 49*, 151–159.

Schelkoph, G. M., & Milne, D. B. (1988). Wet microwave digestion of diet and fecal samples for inductively coupled plasma analysis. *Anal. Chem., 60*, 2060–2062.

Siimes, M. A., Vuori, E., & Kuitunen, P. (1979). Breast milk iron. A declining concentration during the course of lactation. *Acta Paediatr. Scand., 68*, 29–31.

Silva, P. R., Dorea, J. G., & Boaventura, G. R. (1997). Multielement determination in small samples of human milk by inductively coupled plasma atomic emission spectrometry. *Biol. Trace Elem. Res., 59*, 57–62.

Stripp, R. A., & Bogen, D. C. (1989). The rapid descomposition of biological materials by using a microwave acid digestion bomb. *J. Anal. Toxicol., 13*, 57–59.

Vaughan, L. A., Weber, C. W., & Kemberling, S. R. (1979). Longitudinal changes in the mineral content of human milk. *Am. J. Clin. Nutr., 32*, 2301–2306.

Vuori, E., & Kuitunen, P. (1979). The concentrations of copper and zinc in human milk. *Acta Paediatr. Scand., 68*, 33–37.