International Journal of Food Science and Nutrition Engineering 2012, 2(4): 44-53
DOI: 10.5923/j.food.20120204.01

Methodological Validation for the Determination of Ca, Cr, Mg and Mn in Umbilical Cord and Maternal Blood (City of Vale do Paraíba, São Paulo, Brazil)

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Abstract This investigation focused on the validation of the methodology used in determining the presence of specific elements in human blood. Concentrations of macro- and micronutrients in umbilical blood (UCB) samples from 37 lactating volunteers who gave birth in the city of Taubaté, State of São Paulo, Brazil. The determinations of Ca and Mg were carried out in a flame atomic absorption spectrometer (FAAS) and those of Cr and Mn in a graphite furnace atomic absorption spectrometer (GFAAS). A procedure for preparing samples was optimized and evaluated. Standard addition methods and determinations by ICP OES were used to validate the analytical procedures. An acetylene/air mixture was optimized at 2.0/17.0 L min-1 (Ca and Mg). Pyrolysis and atomization temperatures for Cr were at 1400 ℃ and 2100 ℃, respectively, for Mn at 1300 ℃ and 1700 ℃, respectively. The most efficient chemical modifier was a solution containing 5 g of Pd + 3 g of Mg(NO3)2. Characteristic masses for Cr and Mn were 2.6 and 2.7 pg, respectively. The methods presented high analytical efficiency in the determination of Ca, Cr, Mg and Mn (recovery from 98.68% to 108.22 %). Minimal data variations in repeatability and reproducibility indicated significient precision and accuracy for the proposed methodology. The placenta did not block transport of elements from mother to fetus. The contents of the elements analyzed in the UCB were compared to those detected in maternal blood (MB). Most of the children exhibited normal weight (from 2.5 to 3.0 kg) as per the Brazilian Ministry of Health standards. Such results indicate that the levels of concentrations of the elements in the UCB did not affect the weights of the neonates.

Keywords FAAS, GFAAS, Umbilical Cord Blood, Micro- , Macroelements, Infant Nutrition

1. Introduction

The determination and study of micro - and macroelements in umbilical blood, essential or toxic, is of great interest to public health. Various studies in the literature deal with the biological importance, essential nature, toxicity, deficiency and possible sources of different macro and microelements including: calcium[1-7]; magnesium[2,4,6,7]; manganese[7-9] and chromium[7,10-12].

Children, especially neonates, exhibit high rates of bodily growth. Contrasted with adults, the rapid growth rate is attributed to an elevated metabolism and differences in body composition (total body water), fluid pH, and reflects an elevated energy requirement per body mass unit. Renal activity is also reduced[8,13,14]. These characteristics in neonates facilitate a higher potential for the absorption of elements in contrast with adults.

This greater absorption capacity is also applicable for various toxic elements[15].

A clear understanding of the levels of toxic-element transfer during pregnancy is exceptionally important for pediatricians in the prevention and advance treatment of diseases affecting fetal health. Prior studies exist relating the levels of elements in maternal blood to those found in the umbilical blood. As examples, we can cite evaluations of Hg, Pb, Cd as well as essential elements (Cu, Zn and Se) concentrations in this matrix[16]. Other studies conducted in Saudi Arabia[17] and Pakistan[18] noted relationships between maternal contamination by Pb and subsequent transfer of this element to the umbilical cord. Japanese studies[19] documented the transfer of Hg, Pb, As and Cd found in maternal blood to the umbilical cord.

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Published online at http://journal.sapub.org/food

Unfortunately, studies of metallic elements in maternal and neonate blood are largely lacking for Brazil. Rare exceptions from Brazil are those studies that focused on Fe[20],[21] and Pb[22].

Validating a methodology for chemical analysis involves parameters of precision, selectivity, bias/recovery, ruggedness, sensitivity/linearity/working range, detection limit and quantification limit[23]. The validation process also necessitates a definition of parameters with the greatest importance. Determining metallic elements in biological matrices, among other aspects, both quantification and sensitivity allowing the detection of macro- and micro concentrations. In routine uses, reliability and cost savings are also important factors.

Among various spectrophotometric technologies available, flame atomic absorption spectrometry (FAAS) and electrothermal heating in a graphite furnace atomic absorption spectrometer (GFAAS) possess superior analytic capacities for biological samples. White & Sabbione showed that atomic absorption spectrometry (AAS) presented similar results to more sophisticated techniques, such as inductively coupled argon plasma mass spectrometry (ICP-MS) and neutron activation analysis (NAA)[24].

The primary goal of this study was the validation of methodology for determination of elements in blood by GFAAS and FAAS. Specific evaluation objectives for the validation were: (i) sensitivity; (ii) accuracy in analyte recovery; (iii) precision in comparison with established techniques, and (iv) verification of the transfer of metallic elements from maternal to neonate blood using statistical models.

2. Material and Methods

2.1. Glassware, Reagents and Analytical Standard Solutions Used

Requisite Teflon™ materials and glassware for the preparation of standard solutions and samples were conditioned in an HNO_3 solution at 10% (v v^{-1}) for 24 hours. A *Simplicity* bench-top deionizer from *Milipore*™ was used to obtain deionized water at a resistance of 18.2 MΩ cm^{-1}. All decontamination procedures for hardware and preparation of samples were carried out with water obtained from this system.

Analytical-grade (PA) acid solutions of HNO_3 65% (m m^{-1}), HCl 37% (m m^{-1}) and HF 40% (v v^{-1}) from *Merck*™ were utilized.

Standard analytical solutions used for addition and recovery tests, external calibration, and as a matrix modifier in GFAAS were uniformly prepared with dilutions of *SpecSol*™ containing 1,000 mg g^{-1} of the target element, as per NIST standards.

2.2. Description of the Spectrometer and Operating Conditions

Determinations of the elements in the blood samples were performed in an Atomic Absorption Spectrometer, *PerkinElmer* brand, model *Aanalyst 800*. This equipment has integrated system components for both flame and graphite furnace operations in a single instrument allowing for an automated atomization technique selection. This device also has a dual-band optical system (single-band for graphite furnace operations), optical components coated with anticorrosive and protective materials, motorized Littrow-type monochromator for automatic wavelength selection, and alignment adjustment. The device has a working range between 185 to 870 nm with an 1800-line nm^{-1} diffraction grating and solid state detector, as well as correction for flame-background and structured background (i.e., molecular absorption) for GFAAS by deuterium lamp.

The graphite furnace used had transversal heating providing a uniform temperature profile with longitudinal Zeeman-effect structured background correction. Other functions include automatic software controls facilitated by an analytic program with up to 12 steps of programmable parameters such as temperature (up to 2600 ℃ with 10 ℃ intervals) and heating ramp time (programmable from 1 to 99 seconds). All the experiments utilized the graphite furnace atomic absorption instrument under STPF (Stabilized Temperature Platform Furnace) conditions with specific chemical modifiers.

2.3. Selection of the Lactating Women

Invitations were distributed to women under prenatal care and who gave birth between November, 2004 and January, 2006 at the Taubaté University Hospital (Hospital Universitário de Taubaté), located in the city of Taubaté, Vale do Paraíba Region, State of São Paulo, Brazil. Participants were selected through the SUS (Sistema Único de Saúde) records of the Brazilian health care system. Those participating in the study did so as signatories of an informed-consent form which provided information about the project. The form was previously approved by the Ethics Commission of the Taubaté University Hospital (protocol CEP/UNITAU number 192/04).

2.4. Maternal and Neonate Parameters

Selected parameters related to mothers and children are presented in Table 1. The maternal parameters showed a great range of categorical values. Ages ranged from teenager from adult (15 to 40 years, mean = 24.9 ± 6.63). Previous pregnancies varied from 1 to 5 (mean = 2.4 ± 1.30). Abortions varied from 0 to 3 (calculated individually as the difference in pregnancies and births). The breadth of range in these parameters suggests a generalized population profile is represented. Other parameters relevant to the health of pregnant women were: tabagism (8%), occasional smoking (40%), alcohol consumption (19%), known medical pathology (4%), known pregnancy pathology (50%), the proximity of metals industries (17%), and the use of hair dyes (0%). The consumption of medications and vitamins were factored as were education and occupation (data was

not published).

Table 1. Maternal and Neonate Parameters

	Range	Mean ± S.D. [1]
Maternal Parameters		
Age (yr)	15 – 40	24.9 ± 6.63
Pregnancies	1 - 5	2.4 ± 1.30
Births	1 - 4	2.2 ± 1.01
Abortions	0 - 3	0.3 ± 0.60
Neonate Parameters		
Weight (kg)	2.27 – 4.02	3.23 ± 0.424
Height (cm)	41.5 – 52.0	48.0 ± 2.19
Cephalic perimeter (cm)	32 – 37	34.7 ± 1.36
Thoracic perimeter (cm)	29 - 37	33.3 ± 1.78
Capurro Method (wk)	36.42 – 42.57	38.25 ± 1.36
Apgar Method (1min)	4 – 9	8.04 ± 1.02
Apgar Method (5 min)	8 – 10	9.3 ± 0.69

[1]: S.D.: Standard deviation. For maternal and neonate weight, n = 37. For other specifically neonate parameters, n = 26

The percentage of neonate males (57.7%) was slightly greater than females (42.3%), Neonate weight varied from 2.27 to 4.02 kg (Table 1). This range approaches the World Health Organization (WHO) standard of 2.5 to 4 kg[26]. The average neonate weight (3.24 ± 0.424 kg) is less than that specified by the WHO (3.5 kg for males and 3.25 kg for females)[26]. However, the mean approximates that recommended by the Brazilian Ministry Health (2.5 – 3.0 kg)[27]. The mean height of neonates (48.0 ± 2.19 cm) is close to WHO standards (48 to 52 cm). However, the thoracic perimeter, reflecting the nutritional status of neonates, varied from 29 to 37 cm with a mean of 33.3 ± 1.78 cm. This is under the recommended values from the WHO (34 to 35 cm) for some neonates in this study. The mean cephalic perimeter, another important anthropometric indicator, reflects the brain development. The mean in this study (34.7 ± 1.36 cm) approaches the normative standard (34 – 35 cm)[31].

The Capurro Method indicators are used in determining gestational age[28, 29]. The mean Capurro value (38.25 ± 1.36 weeks) was derived for the gestational period[29]. A pre-term gestational age was noted for some neonates. Considering that the thoracic perimeter, to some extent, reflects the nutritional state of neonates[27], observed values (29 to 37 cm, mean = 33.3 ± 1.78 cm) are below the range recommended by the WHO at 34 to 35 cm[26].

The Apgar score is a simple method to promptly assess the health of postpartum neonates. The score was created to determine optimal anesthesia dosages during childbirth[30, 31]. The obtained scores for Apgar at 1 min and 5 min postpartum (8.04 ± 1.02 and 9.3 ± 0.69) are considered normal[31].

2.5. Collection and Storage of Blood Samples

The 37 pairs of samples, containing approximately 5 mL of both umbilical cord blood (UCB) and maternal blood (MB), were collected during hospitalization within 24 hours after birth using disposable syringes. The collected samples were then transferred to containers previously treated with HNO3 1:1 (v v^{-1}) and deionized water. Collection containers were stored at -20 °C up to the time of analysis[32].

2.6. Digestion of the Blood Samples

A determination of small concentrations of elements present in biological samples requires a pre-treatment which destroys the entire organic matrix without the loss of the target elements or contaminants. As in any analytic procedure, digestion must not introduce appreciable errors. Acid digestion is a commonly used procedure to destroy organic matter samples.

To digest the blood samples in this study, 1.0 mL of the sample was combined (using micropipette) with 3.5 mL of an acid mixture containing HCl, HNO_3 and HF in the proportion in volume of 4:2:1 in *Teflon* capsules of 15.0 mL capacity. The *Teflon* capsule was subsequently covered and inserted into an aluminum capsule. The loaded capsule was closed with a lid and kept sealed under pressure by a spring system. The sample was then placed within a stainless steel oven system that allowed examination under low pressure and controlled temperatures (decomposition pump). This entire assembly was heated with resistive heating maintained by a temperature controller. Figure 1 is a schematic diagram of the digestion pump used. This system provides a more effective decomposition through an optimal reflux of the acid mixture and corresponding isolation of the system with the medium in contrast with block digestion systems[33].

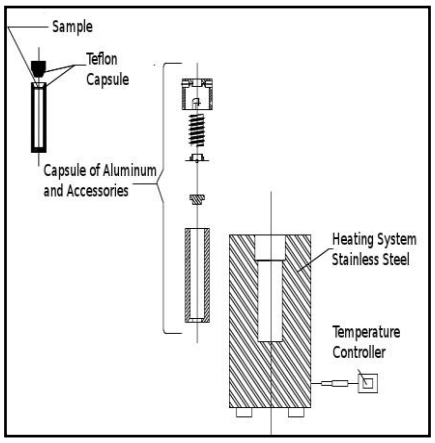


Figure 1. Diagram of the decomposition pump used in the digestion of blood samples

The heating protocol was: (i) 50 °C and time ramp of 30 min, (ii) 80° C and time ramp of 30 min, (iii) 110 °C for 4 h and (iv) cooling at 25 °C for 2 h. Digestion pumps were removed from the heating block in the cooling phase. Finally, the system was disassembled and the digested material was transferred in 25.0 mL units to volumetric polypropylene flasks.

2.7. Analytical Methodology Validation

2.7.1. Optimization of the Pyrolysis , Atomization Parameters and Temperature Program

Pyrolysis and atomization curves were prepared for Cr and Mn in the graphite furnace to ensure a symmetrical and

representative atomization signal of the analyte mass. The temperature variation used was 100 ℃, both for pyrolysis and for atomization. Cr samples were used containing 26 pg of Cr and 10 μL of digested blood diluted 25 times. The curves were prepared both in the presence and absence of a chemical modifier (15 μg of $Mg(NO_3)_2$). Mn samples were used containing 26.25 pg of Cr and 10 μL of digested blood diluted 250 times. A stable signal was established for each element (sample volume and a standard). The atomization temperature was subsequently established and the temperature of pyrolysis varied. The atomization temperature varied for each element under optimized the pyrolysis temperatures. The external calibration curves were prepared in the presence of a chemical modifier (5 μg of Pd + 3 μg of $Mg(NO_3)_2$). The external calibration curves prepared in the absence of the chemical modifier are not presented in this study. Temperature programs were prepared after optimization of the pyrolysis and atomization temperatures for Cr and Mn. For each stage of the heating program (drying, pyrolysis, atomization and cleaning), the temperature, duration, step (stance phase) and the internal discharge of air were defined.

The analytical parameters for Ca and Mg determinations in FAAS (flame) were optimized for variable gas-flow in conjunction with the air/acetylene ratio to provide the optimal sensitivity in atomization.

2.7.2. Evaluation of the Analytic Sensitivity

One parameter expressing the sensitivity of an analytic determination of characteristic mass (m_0) is expressed in Equation 1 below[33]:

$$m_0 = \frac{\text{injected volume} \times \text{analite concentration} \times 0{,}0044}{\text{measured signal (area)}[\text{analite} - \text{blank}]} \quad (1)$$

where m_0 is calculated in pg, the injected volume given in μL, and the analyte concentration in μg L^{-1}.

Another parameter also expressing the sensitivity of an analytic determination of elements in various electrothermal systems is the limit of quantification (LOQ). The LOQ (in pg) may be estimated as of the baseline noise and sensitivity of measurement as per Equation 2 below[34]:

$$LOQ = \frac{3 \times \delta_{Blank}}{a} \quad (2)$$

in which δ_{Blank} is a standard deviation of the signal obtained for the blank and a is the angular coefficient of the calibration curve.

2.7.3. Accuracy Assessment Method

In the absence of both certified validation standards and reference materials approaching the complexity of the matrix sample, an alternative was used to evaluate the accuracy of the method where analyte addition/recovery tests (SAM) of the matrix were contrasted with inter-laboratory tests. In the addition method, the initial concentration of a known element in the matrix is quantified. A known concentration of the element is then added and the element concentration obtained is determined experimentally. In theory, the concentration obtained experimentally in these samples is given by the sum of the initial concentration together with the added known concentration[34,35].

Also, the precision of the methodology for recovering the analyte was evaluated, calculating the relative error (RE) by Equation 3 below:

$$RE = \frac{\text{obtained concentration} - \text{expected concentration}}{\text{expected concentration}} \quad (3)$$

in which the *obtained concentration* is the concentration determined experimentally in the blood sample after the addition of the target element (doping) and the *expected concentration* corresponds to the theoretical concentration, calculated by the sum of the concentration of the target element before the doping and the known concentration added.

The concentrations of Cr and Mn in this test were determined in GFAAS, using doped and undoped digested blood samples in triplicate. The concentration added was approximately half the concentration of the element present in the sample before the addition. This corresponds to one of the concentrations recommended by the National Institute of Metrology, Standardization and Industrial Quality (*Instituto Nacional de Metrologia, Normalização e Qualidade Industrial*)[23].

Large quantities of aluminum in blood samples may interfere in determination of Ca. In previous tests, the methodical insufficiency to quantify Ca was verified through recovery values of around 48%. After optimization of the calibration solutions and samples by an addition of 0.1% of lanthanum, interferences in Ca measurement were eliminated with a value of 108.81%, near that expected by the analyte addition method (100%).

2.7.4. Precision Assessment Method

The performance evaluation procedure for the method-in-question compared results from that method with result derived from a previously validated reference method. The objective was to contrast the degree of proximity between the results obtained from the two methods, that is, evaluate the accuracy of the method in the process of validation with that of reference[23]. In this study, the results of the methods under study (FAAS and GFAAS) were compared with those obtained by inductively coupled plasma optic emission spectrometry (ICP OES). A sequential spectrometer, Model *3410* from *ARL*, was used. Determinations were made in triplicate and the results of the two methods compared by Student's *t*-test.

The experimental parameters and operating conditions adopted in the technique of determination by ICP (used to evaluate precision with the method optimized here) are found in Tables 2 and 3, respectively.

Table 2. Experimental parameters used in determination of the elements by ICP

Element	Emission lines (nm)	Integration time (s)	PMT (1)
Ca	422.673	1.0	8
Mg	285.213	1.0	8
Cr	267.716	1.0	8
Mn	294.920	0.5	10

(1): PMT: photomultiplier tube power

Table 3. ICP OES operating conditions

Operating conditions	Values
Plasma gas flow	0.8 L min^{-1}
Carrier gas flow	0.8 L min^{-1}
Cooling gas flow	8.8 L min^{-1}
Incident Power	650 W
Reflected Power	< 2 W
Breathing rate	2.5 mL min^{-1}

2.7.5. Repeatability and Reproducibility assessment method

Validation of the methodology for detecting metallic elements in blood used a statistical cross-correlated variable analysis measurement system. The validation used three analysts. Each analyst performed three measurements conducted on different days, for three values of predetermined concentrations (a total of 27 measurements). The standard deviation of the process was equal to 1 with 95% confidence. For this assay, we used only one sample (collected in large volume in comparison with the others). The selected sample was analyzed *in natura* and with two different dilutions of the sample (blood) and deionized water at 1:1 and 1:2 (v-v^{1}). Collected data were analyzed by the *Minitab 16*™ software program.

2.8. Evaluation of the Interest Element Concentration Levels in Blood Samples

2.8.1. Transfer of the Maternal Blood Elements to the Neonate Umbilical Cord

Forty pairs of blood samples from the umbilical cord of neonates (UCB) and from maternal blood (MB) were analyzed. The mean values between these two groups were compared using Student's *t*-test for the possibility of placental transport blocking of metallic elements from mother to fetus.

The presence of each selected element in neonate UCB was correlated with the respective MB samples. The aim was the determination of the direction of the flow of these metals as mother-to-fetus or fetus-to-mother. Also, the concentration of each element in the umbilical cord and the weight of the neonate were correlated.

Evidently, neonate weight does not depend solely on the concentration of a determined metal, nor is it possible to affirm that there is a relationship of dependency between these two variables. What was investigated in this study was if possible variations in the concentration of the element in neonate blood would be sufficient to affect the weight of the child, either in a positive or negative way. Lastly, correlations between elements in UBC were analyzed with other neonate parameters: height, cephalic perimeter, thoracic perimeter, Capurro Method values, and 1 and 5 min Apgar values.

2.8.2. Statistical Treatment

Student's *t*-test was used to correlate the mean values of the analyte concentrations with neonate body mass. A 5% significance was adopted in these studies, that is, if the value of *p* found is less than 0.05, it indicates that the means are different at a 5% significance level (Type I error, when the initial hypothesis $H_0 : x_2 = x_1$ is rejected and the alternative hypothesis $H_a : x_2 \neq x_1$ is accepted). The data were analyzed by the software program *Epi.Info* 6.04 (for the *Windows*™ platform).

To evaluate the degree of association among variables, regression variance analysis was carried out, calculating the Pearson product-moment correlation coefficient. The significance of the F of this analysis (*p*) was then calculated. The *p* value represents the probability of obtaining results outside the region of conclusion by the regression variance analysis. For example, if $p < 0.05$, the correlation between the two variables is significant at 5%; if $p < 0.01$, it is significant at 1%. In this estimate, the computing application *Gnumeric* v.1.10.3 (for the *Linux* platform) was used.

3. Results and Discussion

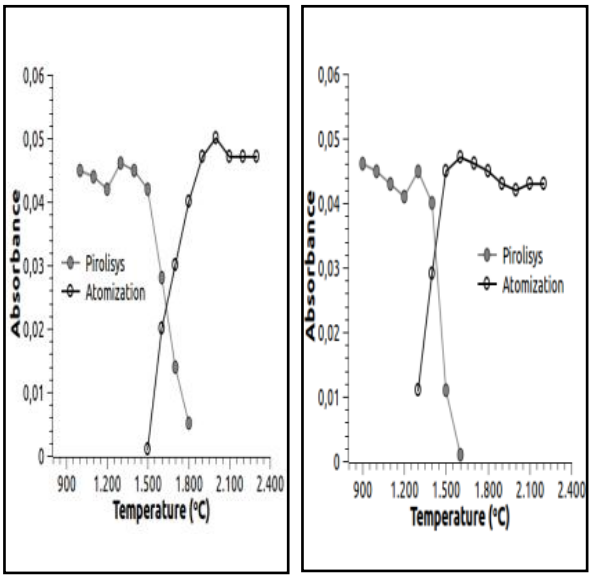


Figure 2. Pyrolysis and atomization profile in the presence of chemical modifier: a) Cr and b) Mn

Table 4. Analytical operating conditions for determination by AAS (atomic absorption spectrophotometry) using two atomization techniques: FAAS (flame) and graphite furnace (GFAAS), and operating conditions of gas flow for determination of Ca and of Mg by FAAS (flame)

Atomiz. Methods	Element	Type of lamp	Operating conditions		Gas Rate	
			λ[1]	Gap opening	Acetylene	Atmosph Air
			(nm)		(L min^{-1})	
FAAS[2]	Ca	HCL[4]	422.7	0.7	2.0	17.0
	Mg	HCL	285.2	0.7	2.0	17.0
GFAAS[3]	Cr	HCL	357.9	0.7	-	-
	Mn	HCL	279.5	0.2	-	-

[1]: wavelength ; [2]: flame; [3]: graphite furnace; [4]: hollow cathode lamp.

Table 5. Temperature programs used for determination of chromium and manganese in GFAAS (graphite furnace)

Stage	Elem.	Temperat.	Ramp time	Level	Internal air discharge
		(°C)	(s)		(mL min^{-1})
1st Drying	Cr	150	40	20	250
	Mn	150	40	20	250
2nd Drying	Cr	250	30	20	250
	Mn	250	30	20	250
Pyrolysis	Cr	1400	30	10	250
	Mn	1300	30	10	250
Atomization	Cr	2100	0	5	0
	Mn	1700	0	5	0
Cleaning	Cr	2300	5	5	250
	Mn	2200	5	5	250

Table 6. Results of the analyte addition/recovery test and quantification limits

Elem.	Concentrations				Relative Error[6]	LOQ[6]
	Blood[1]	Added[2]	Expected[3]	Obtained[4]		
	(mg L^{-1})				(%)	(mg L^{-1})
Ca	190.05	95.025	285.075	310.19	+ 8.81	0.115
Mg	32.125	16.06	48.185	52.5	+ 8.96	0.087
Cr	0.3636	0.1818	0.5454	0.5595	+ 2.59	0.00159
Mn	0.3125	0.1563	0.4688	0.46270	- 1.30	0.00036

[1]: concentration of the quantified element in blood samples before the addition of the analyte; [2]: concentration of the element in the added solution; [3]: theoretically expected concentration, calculated by the sum of the blood concentration before the addition of the analyte + added amount; [4]: quantified concentration in the doped blood sample (after the addition of the analyte); [5]: RE =[(conc. obtained – conc. expected) / (conc. expected)] x 100 ; [6]: limit of quantification. 3.2. Sensitivity Assessment Method

3.1. Instrumental Optimization for FAAS and GFAAS

Table 4 shows the analytical operating conditions used for determination of Cr and Mn by GFAAS and Ca and Mg by FAAS.

The pyrolysis and atomization curves in the presence of the chemical modifier for Cr and Mn are found in Figure 2.

Based on analysis of Figure 2, pyrolysis and atomization temperatures were established at 1400 and 2100 °C for Cr and at 1300 and 1700 °C for Mn respectively. Once these parameters were optimized, a temperature program was constructed for the elements of interest, as presented in Table 5.

Cr, in the presence of chemical modifier, showed an estimated characteristic mass of 2.6 pg and 2.7 pg for Mn. This indicates a heightened sensitivity of the method. Interference from chloride in an HCl solution used for digestion of the samples was possible in the atomization of the elements. Nevertheless, the final concentration of chloride found in the samples after dilution was less than that specified for an interferent chloride concentration[36].

3.3. Accuracy Assessment Method in Analytic Recovery

Table 6 shows the results of the analyte addition/recovery test, as well as the quantification limit for each element.

Considering the calculated RE, the recovery values ranged from 98.68% to 108.22% in relation to those expected theoretically (100%). In other words, since the recovery margin was acceptable (less than 10%), the method is reliable in regards to this parameter of evaluation.

3.4. Precision Assessment Method

According to the results obtained (Table 7), together with the paired Student's *t*-test ($p < 0.05$) it was observed that there was no statistical difference of the results obtained using two distinct spectrometric techniques for the doped blood samples. Sample preparation procedures for determination of Ni in plant tissue samples by inter-laboratory analysis using FAAS, GFAAS and ICP OES were validated in a previous work[30].

3.5. Repeatability and Reproducibility Method

Table 8 shows the application of analytical techniques for measuring cross-variables for the determination of each

element of interest in blood.

Table 7. Determinations of concentrations of Ca, Mg, Cr and Mn in the samples doped by AAS and ICP (with respective standard deviations)

Element	Spectrophotometric techniques	
	EAA	ICP OES
	($mg\,L^{-1}$)	($mg\,L^{-1}$)
Ca	63.21 ±3.16	58.79 ±2.27
Mg	22.15 ±0.84	19.54 ±0.51
Cr	0.110 ±0.0046	0.130 ±0.0039
Mn	0.220 ±0.0078	0.200 ±0.0048

Table 8. Gage Repeatability and Reproducibility (R&R) for Ca, Mg, Cr and Mn.

Source	Statistic	Elements			
		Ca	Mg	Cr	Mn
Total Gage (R&R)	SD[1]	0.5834	0.3835	0.00157	0.00271
	SV(%)[2]	2.78	5.27	4.92	3.88
Repeatability	SD	0.5132	0.1688	0.00154	0.00271
	SV (%)	2.44	2.32	4.84	3.88
Reproducibility	SD	0.2776	0.3443	0.00029	0,00000
	SV (%)	1.32	4.73	0.90	0.00
Operators	SD	0.0000	0.0000	0.00029	0,00000
	SV (%)	0.00	0.00	0.90	0.00
Operators * Parts	SD	0,2776	0.3443	-	-
	SV (%)	1.32	4.73	-	-
Part-To-Part	SD	20.9915	7.2697	0.03179	0.0699
	SV (%)	99.96	99.86	99.88	99.92
Total Variation	SD	20.9996	7.2798	0.03183	0.06995
	SV (%)	100.00	100.00	100.00	100.0

[1] Standard Deviation; [2] Study System Variance (%)

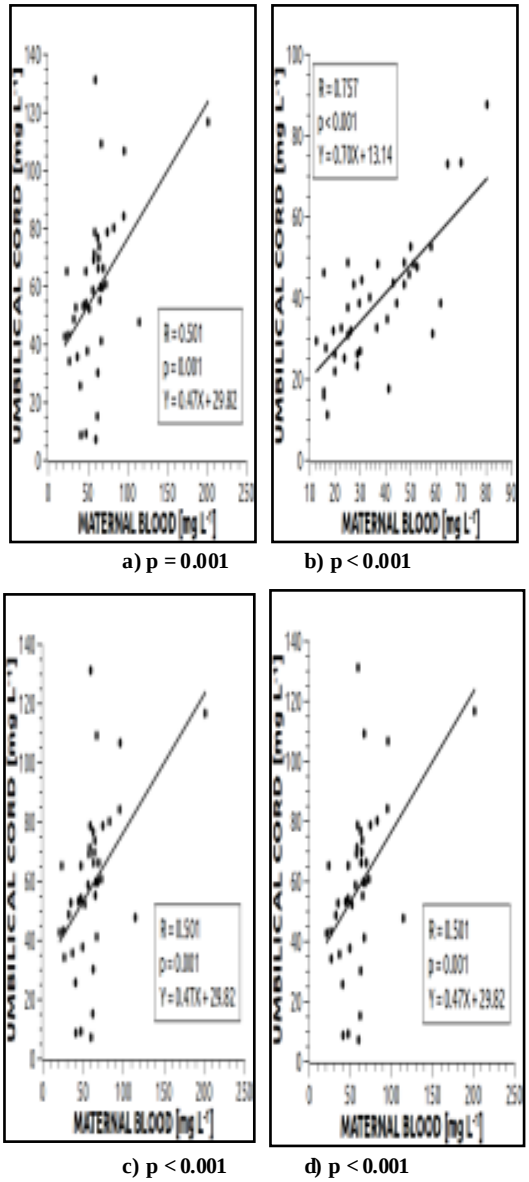
Table 9. Mean and standard deviation values for each element in the umbilical cord blood of the Neonate (UCB) and in maternal blood (MB)

Elements	Sampled Matrixes	
	UCB	MB
	($mg\,L^{-1}$)	($mg\,L^{-1}$)
Ca	58.37 ± 28.00	61.25 ± 30.11
Mg	38.32 ± 15.74	36.08 ± 17.06
Cr	0.624 ± 0.883	0.636 ± 0.843
Mn	0.903 ± 1.47	0.863 ± 1.55

A general analysis shows that the standard deviations of the parameters relative to the elements were lower than 1%. From the analysis of result variability, variance attributed to the sample is probably due to the various preparation steps intrinsic to the experimental procedure given a variation of <1%. From these analyzses, we obtained a maximum percentage of repeatability of 4.84 for Cr and the reproducibility of 4.73 Mg. This demonstrated sufficient significant precision in the method as such results are control-relevant in minimizing systematic and random errors. According to commonly used criteria, it is possible to determine whether the measurement system is acceptable using the following guidelines: if the Total Gage Repeatability and Reproducibility (R&R) contribution in the Study Variation (SV) is less than 10%, then the measurement system is acceptable. In this work, all the Total Gage R&R were < 10% (Table 8). Hence, the measurement system can be considered acceptable.

3.6. Transfer of the Maternal Blood Elements to the Neonate Umbilical Cord Blood

Table 9 shows the mean values determined in UBC and MB samples as well as their standard deviations.



a) p = 0.001 **b) p < 0.001**

c) p < 0.001 **d) p < 0.001**

Figure 3. Correlation between maternal blood (MB) and of the umbilical cord of the newborn (UCB) for the elements: a) Ca; b) Mg; c) Cr; d) Mn

No comparison of statistical means between the two groups was greater than 5% by Student's *t*-test. In other words, if the concentrations of each element did not differ between the two groups, placental blockage of the transport of elements from the mother to the fetus is unlikely. The question of the transfer of elements from mother to fetus requires additional studies since previous work has produced different conclusions. Paiva, et al., were consistent with no

finding of element transfer from maternal to fetal blood[21]. Contrastingly, Santos, et al., did find a transfer of Hg from maternal blood to fetal blood[22]. The transfer of elements from maternal to fetal blood was also found by Sakamoto, et al., (2010) when analyzing for Hg, Pb, As, Cd and Se[19]. Figure 3 shows correlations between maternal blood (MB) and that of the umbilical cord of the neonate (UCB) for the elements Ca, Mg, Cr and Mn.

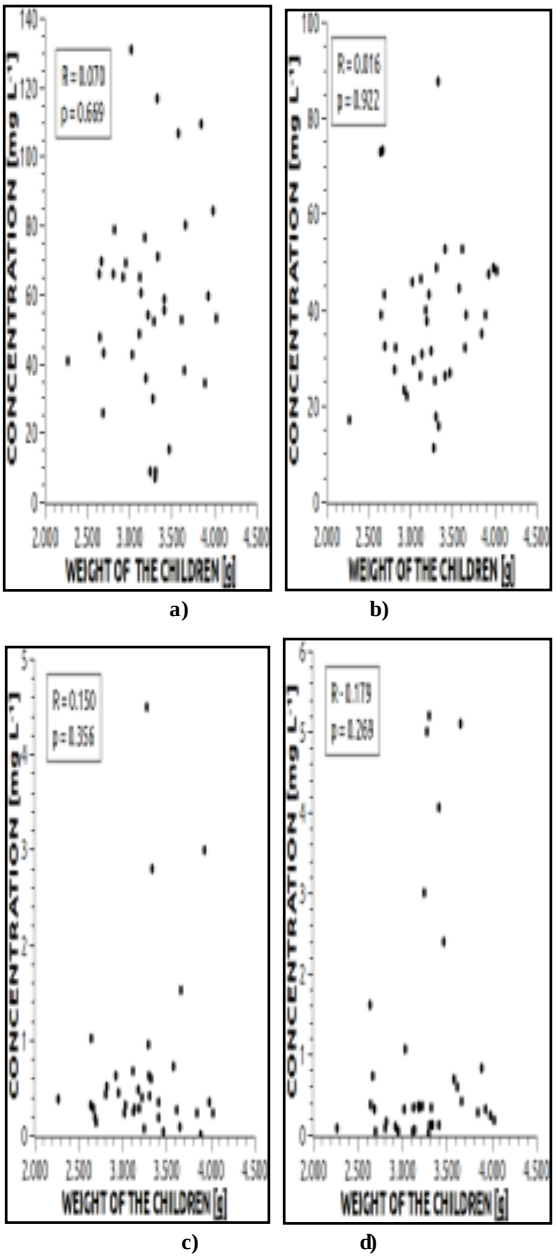


Figure 4. Correlation between weight of the child and in umbilical cord blood for the elements: a) Ca; b) Mg; c) Cr and d) Mn

In statistical terms, the probability of obtaining results outside of the region of conclusion by the regression variance analysis (if $p < 0.05$, the correlation between the experimental and simulated data is significant at 5%). In the Analysis of correlations between concentration of maternal blood and that of the neonate, it may be observed that for all the elements there is a positive association between maternal blood contents and that of the neonate. Therefore, the nutritional state of the child (in regard to the level of these elements present in the blood) depends directly on the nutritional state of the mother. This is also the case for Cr, an element that may be either essential or toxic for the child depending on concentration and oxidation level. Therefore, maternal intoxications from this element would have increase in the blood levels of the neonate as a consequence and possible mutagenic and teratogenic effects during gestation. An evaluation of a possible correlation between the total concentration of the analyte with the body mass of the neonate was also conducted. Figure 4 shows the correlations between neonatal weight and the concentration of the same four elements.

In accordance with Figure 4, there was no significant correlation found between the concentrations of the elements studied in neonate blood and neonate weight. This result is probably due to the fact that the values of Ca, Mg and Mn in neonate blood were above the minimum necessary for growth of the child.

It was observed that the weight of neonates was situated in a range of values near the mean standards for neonates, according to data from the Brazilian Ministry of Health, at 2.5 to 3.0 kg[27]. According to this finding, neonate weight is considered low when it is less than 2.5 kg. In this study, only one neonate exhibited weight less than the standard, which allows the supposition that the levels of element concentrations in UCB did not affect the weight of the neonates.

Correlations between the other neonate parameters and elements in UCB were investigated as well. (Table 10).

Table 10. Correlations coefficients (R) between children's parameters and elements concentration in umbilical cord blood and respective significance levels (p) of the correlations (n = 26)

Children's Parameters	Elements in Umbilical Cord Blood			
	Ca	Mg	Cr	Mn
Height (kg)	R = 0.165 p = 0.422	R = 0.223 p = 0.274	R = 0.004 p = 0.986	R = 0.165 p = 0.422
Cephalic Perimeter (cm)	R = 0.187 p = 0.360	R = 0.330 p = 0.100	R = 0.444 p = 0.023	R = 0.423 p = 0.031
Thoracic Perimeter (cm)	R = 0.178 p = 0.384	R = 0.087 p = 0.672	R = 0.226 p = 0.266	R = 0.334 p = 0.096
Capurro (wk)	R = 0.030 p = 0.883	R = 0.158 p = 0.441	R = 0.274 p = 0.176	R = 0.093 p = 0.651
Apgar 1 min	R = 0.325 p = 0.105	R = 0.113 p = 0.581	R = 0.242 p = 0.233	R = 0.048 p = 0.815
Apgar 5 min	R = 0.236 p = 0.246	R = 0.057 p = 0.781	R = 0.125 p = 0.544	R = 0.389 p = 0.063

Most correlations showed no significance at 5% between neonate parameters and elements in UCB. Only two correlations were significant at 5%: Cr *vs* cephalic perimeter (R = 0.444, p = 0.023) and Mn *vs* cephalic perimeter (R = 0.423, p = 0.031). However, the significance of these correlations should be considered with caution. For Cr, two observations were much higher compared to others. A high concentration could mask the obtained correlation level of significance. If excluded, a non-significant correlation is obtained (R = 0.143, p = 0.505). The same was observed for

Mn, with three noticeably elevated results (R = 0.125, p = 0.570). Unfortunately, in this study, no reasons for this high concentration were found. Considering that no significant correlation between neonate parameters and elements concentration in UCB for all other elements and parameters was found, possible associations between Cr and Mn in umbilical cord and cephalic perimeter should be thoroughly investigated in future studies before any conclusion.

4. Conclusions

1. The preparation and instrumental methods exhibited high analytical efficiency in the determination of Ca, Cr, Mg and Mn presenting recovery percentages from 98.68% and 108.22%. Low variation was evident in repeatability and reproducibility percentages, indicating the significant precision and accuracy of the proposed methodology.

2. The placenta did not block transport of the elements from mother to the fetus since the concentrations of each element did not differ between the two age groups.

3. The analyzed contents of neonate umbilical cord blood were positively related to those detected in the maternal blood. Therefore, the nutritional state of the neonate (in relation to Ca, Mg and Mn), or the presence of contamination (in relation to Cr), depends on the concentrations of these elements in the maternal blood.

4. The levels of the elements in umbilical cord blood did not affect the weight of neonates.

ACKNOWLEDGEMENTS

We gratefully acknowledge the generous support of the Research Support Foundation of the State of São Paulo (Fundação de Amparo à Pesquisa do Estado de São Paulo) in their financial grant for this study (Fapesp processes 1999/09864-9 and 2011/06710-4).

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