JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2004,** *52,* 4887−4892 **4887**

Enzymolysis Approach to Compare Cu Availability from Human Milk and Infant Formulas

E. PEÑA,[†] R. DOMÍNGUEZ,[†] A. BERMEJO,[†] J. A. COCHO,[‡] J. M. FRAGA,[§] AND P. BERMEJO*,[†]

Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Chemistry, University of Santiago de Compostela, Avda de las Ciencias, E-15782 Santiago de Compostela, Spain, and Laboratory of Metabolic and Nutritional Disorders, and Department of Pediatrics, Hospital Clínico Universitario, E-15706 Santiago de Compostela, Spain.

The purpose of the present paper is to develop an easy and quick in vitro method to compare copper availability from breast milk and infant formulas. This study focuses on the differences caused by the use of pH 2.0 (adult gastric pH) or pH 5.0 (newborn gastric pH) in the first stage of the enzymolysis. pH affects Cu solubility, a possible estimator of the availability. Selection of a digestor, times of enzymolysis, centrifugation parameters, and Cu determination by ETAAS were discussed as well. Percentage of Cu solubility was larger from breast milk (gastric pH 2.0, 65.3 ± 14.0 vs 40.0 ± 13.9%; gastric pH 5.0, 61.2 ± 16.5 vs 26.6 ± 10.3%), but the soluble content was larger from infant formulas for both pHs (gastric pH 2.0, 245.3 ± 82.1 vs 113.0 ± 103.4 ng mL^{-1}; gastric pH 2.0, 169.3 ± 76.9 vs 75.3 ± 21.9 vs ng mL^{-1}).

KEYWORDS: Solubility; copper; milk; in vitro digestion; pH; ETAAS

INTRODUCTION

This study focuses on Cu, an essential trace element for humans with big importance for growth and children development (*1*). It is a component of a large group of metalloenzymes and proteins with diverse biologic functions: cell respiration, bone formation, gastrointestinal function, nervous system development, etc. (*2*).

A combination of low copper intake, low bioavailability from the diet and high intakes of iron, zinc, or manganese might pose a threat to copper status (*3*−*6*). Cu deficiency is more frequent among preterm infants than among term infants (*7*). Thus, in the first four months of life, plasma Cu level in healthy term infants is mainly dependent on the body stores (*6*, *8*), whereas Cu deficiency symptoms are reported in preterm infants with an immature liver. Regarding toxicity (*9*), for infants that are not breastfed, exposure might occur from infant formulas and drinking water (Cu leaching from domestic piping). There are also possibilities of dermal exposure (e.g., some cosmetics and folk medicine).

Present understanding of Cu bioavailability is limited, and much of our knowledge comes from in vitro studies where adult gastric conditions have been applied. In vivo studies are difficult to perform, because there are obvious limitations (*3*). The use of radioisotopes is not recommended in humans, and moreover, ^{64}Cu and ^{67}Cu have very short half-lives (12.7 h and 2.6 days, respectively) (*10*). Moreover, the natural abundance of the stable isotope ^{65}Cu is high (30.83%) (*11*), which makes it necessary to administrate big amounts to determinate changes in the isotope ratio using mass spectrometry. Another serious obstacle to determine Cu absorption using in vivo methods is that very sensitive equipment is required because of the small amounts of Cu present in breast milk and infant formulas and the small variations observed in the concentrations.

So, the purpose of the present paper is to design a method to evaluate Cu availability after performing an in vitro enzymolysis in similar conditions to those corresponding to the newborn (*12*−*14*) (higher gastric pH than for adults with a limited action of enzymes and biliary salts, short digestion times). The study seeks to explain as well how the gastric pH used in the in vitro simulation is an important parameter to take into account when developing a method to estimate mineral availability from breast milk or infant formulas.

MATERIALS AND METHODS

Chemicals. Digestive enzymes (porcine pepsin, P-7000, EC 3.4.23.1 (1:10 000); porcine pancreatin, P-1750) and sodium bicarbonate ACS reagent, S-6014, were obtained from Sigma Chemicals Co. (Saint Louis, MO). Hydrogen chloride acid (HCl) 37%, copper stock standard solution (1000 g L^{-1}), and Triton X-100 were purchased from Merck (Darmstadt, Germany). Magnesium nitrate was obtained from BDH (Poole, UK) All chemicals were of the highest purity available, and all solutions were prepared using ultrapure water of 18 MΩcm specific resistivity from a Milli-Q purification system, Millipore Corp., (Bedford, MA).

* To whom correspondence should be addressed. Tel.: (34) 600942346. E-mail: pbermejo@usc.es.
[†] University of Santiago de Compostela.
[‡] Laboratory of Metabolic and Nutritional Disorders, Hospital Clínico Universitario.
[§] Department of Pediatrics, University Clinical Hospital.

10.1021/jf030781y CCC: $27.50
Published on Web 07/02/2004

All glassware and polyethylene material were washed prior to and after use with soap and water. Afterward, they were kept in 10% nitric acid for at least 48 h, washed three times with ultrapure water, and stored dry for their use.

Instrumentation. Enzymolysis were carried out in a Boxcult incubator, situated on a Rotabit orbital-rocking platform shaker, J. P. SELECTA S. A. (Barcelona, Spain). A pH-meter Crison 500, Crison Instruments, S. A. (Barcelona, Spain) was used for in vitro digestion as well, and a 2K15 laboratory centrifuge with a rotor model 12141 from SIGMA GmbH (Osterode, Germany) was adequate to separate soluble fractions of digests.

The use of the Boxcult incubator for the enzymolysis procedure was compared with the use of the Stomacher Lab Blender, Model 400, Seward Medical Limited (London, United Kingdom). Seward Stomacher Lab Blender Bags were used for sample treatment.

An L8-Beckmann ultracentrifuge(Palo Alto, CA) with a rotor SW-40 was used to obtain milk whey and to carry out the comparison between soluble fractions obtained by ultracentrifugation and centrifugation.

Finally, a Perkin-Elmer 1100 B atomic absorption spectrophotometer equipped with a deuterium lamp as a background correction system, a HGA-700 graphite furnace atomizer and an AS-70 autosampler (Perkin-Elmer, Darmstad, Germany) was used for carrying out copper measurements.

Procedures. *Samples.* Women from Galicia, North West of Spain, donated breast milk samples through the cooperation of the Hospital Clínico Universitario of Santiago de Compostela. Trained personnel using a motorized pump collected them in polyethylene containers, with care to avoid touching the inner wall of the device or flask. Samples were stored at −20 °C until treatments were performed. Regarding infant formula samples, solutions were prepared by dissolving milk powder using ultrapure water, according to the manufacturer instructions.

An L8-Beckmann ultracentrifuge with an SW-40 rotor was used to obtain milk whey by ultracentrifugation at 31000 rpm (160000*g*) and 4 °C. The sample was taken out with a micropipet after fat separation (*15*).

In Vitro Gastrointestinal Digestion Method. This method is based on the one proposed by Lönnerdal et al. (*12*), with modifications to provide the best working conditions. Samples have suffered a two staged digestion: with pepsin (gastric enzymolysis) and pancreatin (intestinal enzymolysis). Furthermore, the use of pH 2.0 (adult pH) and 5.0 (children pH) in the gastric stage was evaluated, and the obtained results were compared.

A 30-mL aliquot of milk for infant formulas (20 mL for breast milk, due to the shortage of the sample and using the proportional amount of the reagents) were used at least in triplicate to carry out the digestion. Blanks of reagents used in the gastric and intestinal digestion were also performed in triplicate to avoid contamination problems. In the gastric stage of the digestion, samples were introduced into Erlenmeyer flasks, and pH was acidified to 2.0 or 5.0 using HCl 2 and 5 M. 1.0 mL of 0.2 mg pepsin mL^{-1}, equivalent to 3200 kU L^{-1}, was added (dissolved in HCl 0.1 M). Samples were incubated in the Boxcult at 37 °C for 50 min using 100 rpm as stirring speed. Afterward, the enzymolysis was stopped by placing the digests in an ice-water bath to cool. Portions of the gastric digests were centrifuged at 15300 rpm (19890*g*) for 30 min at 4 °C, to obtain gastric soluble fractions.

Similarly, aliquots of gastric digests (15 mL) were neutralized to pH 7.0 with $NaHCO_3$ 1.5 M, and 1.0 mL of 0.15 mg pancreatin mL^{-1} (dissolved in $NaHCO_3$ 0.1 M) was added. Samples were incubated for 30 min and placed in the ice-water bath to cool, and centrifugation was carried out to obtain gastrointestinal soluble fractions.

The following terminology was introduced in this study: 2G, soluble fraction of the gastric digest, gastric pH 2.0; 5G, soluble fraction of the gastric digest, gastric pH 5.0; 2I, soluble fraction of the gastrointestinal digest, gastric pH 2.0; and 5I, soluble fraction of the gastrointestinal digest, gastric pH 5.0.

Cu Determination by ETAAS. Cu contents in milk, milk whey, and soluble fractions were determined using a previously proposed procedure (*15*). Instrumental and graphite furnace conditions are shown in **Table 1**. Magnesium nitrate, 0.01 g L^{-1} was used as a chemical modifier and Triton X-100 to avoid problems in sample injection. Measurements were carried out by the addition procedure with copper concentrations added between 0 and 30 μg L^{-1} and using a different dilution for each sample.

Table 1. Instrumental and Graphite Furnace Conditions for Cu Determination

step	T (deg C)	t_{ramp} (s)	$t_{holding}$ (s)	Ar flow (mL min^{-1})
dry_1	90	10	10	300
dry_2	140	10	10	300
dry_3	200	50	10	300
$pyrolisis_1$	400	15	10	300
$pyrolisis_2$	1500	25	5	300
atomization	2100	0	5	0 (read)
clean	2650	1	5	300

λ: 324.8 nm; integration time: 3.5 s
slit: 0.7 nm; injection volume: 20 μL
hollow cathode lamp current: 15 mA; pyrolitic graphite tubes with L'vov platform

Table 2. Cu Concentration in Soluble Fractions of Digests, Obtained after Using 4 Different Gastric Digestion Times (ng ml^{-1})

	gastric pH 2.0		gastric pH 5.0	
time (min)	gastric digest (2G)	gastroint. digest (2I)	gastric digest (5G)	gastroint. digest (5I)
30	196.2 ± 7.8	156.0 ± 6.3	60.6 ± 9.2	110.6 ± 1.4
50	220.9 ± 8.8	186.2 ± 7.5	53.2 ± 1.4	112.5 ± 14.8
120	233.9 ± 11.7	161.2 ± 5.2	94.1 ± 3.5	140.9 ± 31.1
180	245.5 ± 7.4	188.5 ± 11.2	87.5 ± 6.4	174.9 ± 7.1

RESULTS AND DISCUSSION

Effect of Gastric Digestion Time on Cu Solubility. In availability studies, the digestion time used is variable, according to the purpose of the study. Food transit in the newborn is shorter than in the adult, and this fact has to be taken into account when developing a method to mimic infant digestion. So, to simulate newborn digestion, four different gastric digestion times were studied, always using an intestinal digestion time of 30 min. An infant formula was used in this study, and two gastric pHs, 2.0 and 5.0, were evaluated. The results are shown in **Table 2**. As it can be observed, there is not a clear trend in Cu content in the gastric and gastrointestinal soluble fractions when changing the gastric digestion time. Then, 50 min was selected because it is the time reflected in bibliography as the staying time for both breast milk and milk formulas in the stomach of the newborn (*16*).

Comparison between Ultracentrifugation and Centrifugation to Obtain Soluble Fractions. Centrifugation is the most common approach, due to its simplicity, to obtain an estimation of the availability. To know the best conditions to perform the separation of the soluble fractions, a comparative study between the use of ultracentrifugation and centrifugation for different times was performed. An infant formula and a breast milk sample were digested using both gastric pHs (2.0 and 5.0) and five replicates for each sample. Copper determination was performed after centrifugation (the Sigma 2K15 operating at 19890*g*, 31 000 rpm for 30, 60, or 120 min) or ultracentrifugation (160000*g* for 10 min). All centrifugations were performed at 4 °C.

The results showed that there was only a slight difference between the different time periods (**Table 3**). To compare these results obtained with the ultracentrifugation and the centrifuga-

Table 3. Cu Concentration in Soluble Fractions of Digests Obtained after Using Ultracentrifugation or Centrifugation for Different Times (ng mL^{-1})

	breast milk			
	ultracentr.	cent. 30 min	cent. 60min	cent. 120 min
gastric dig.(2G)	307.7 ± 8.2	308.1 ± 6.7	316.4 ± 2.7	311.2 ± 12.9
gastroint. dig.(2I)	303.6 ± 7.6	297.9 ± 12.4	291.4 ± 11.5	284.3 ± 10.3
gastric dig.(5G)	55.3 ± 1.0	46.7 ± 6.0	41.6 ± 2.6	39.3 ± 0.1
gastroint. dig.(5I)	111.9 ± 1.1	98.0 ± 6.9	87.0 ± 2.2	87.0 ± 1.1
	infant formula			
	ultracentr.	cent. 30 min	cent. 60min	cent. 120 min
gastric dig.(2G)	238.8 ± 4.0	236.5 ± 13.6	229.9 ± 3.6	268.2 ± 8.9
gastroint. dig.(2I)	161.6 ± 9.4	193.5 ± 6.3	172.3 ± 3.6	179.0 ± 9.4
gastric dig.(5G)	49.9 ± 4.7	54.3 ± 5.2	43.7 ± 1.4	44.6 ± 6.0
gastroint. dig.(5I)	111.9 ± 2.3	74.7 ± 4.0	80.4 ± 2.2	75.8 ± 7.1

Table 4. Cu Concentration in Soluble Fractions of Digests after Using Two Different Digestors, Boxcult and Stomacher

	[Cu] (ng mL^{-1})	
	Boxcult	Stomacher
gastric dig. (2G)	494.7 ± 8.8	513.3 ± 13.9
gastroint. dig. (2I)	391.8 ± 20.4	361.7 ± 21.5
gastric dig. (5G)	161.2 ± 18.1	189.5 ± 27.0
gastroint. dig. (5I)	348.6 ± 12.4	305.1 ± 17.2

tion at different times, *t*-paired tests were applied for a significance level of 95% (*17*). Thus, no significant differences were found between centrifugation for 30 min and ultracentrifugation for 10 min (infant formula: $|t| = 0.07 < t_c = 3.18$, $\alpha = 0.05$; human milk: $|t| = 2.33 < t_c = 3.18$, $\alpha = 0.05$). Therefore, centrifugation at 19890*g* (15300 rpm) for 30 min at 4 °C was used throughout all this work, to perform fraction separation after gastric and intestinal digestions.

Comparative Study of the Use of Stomacher and Incubation Camera Boxcult. The in vitro method using the Boxcult incubation camera for the digestion was compared with the one using the Stomacher Lab Blender, Model 400, Seward Medical Limited (London, UK). The Stomacher is an instrument that homogenizes samples inside a plastic bag, with two paddles acting in a similar way to the movements in the stomach, hence the name Stomacher.

Copper was determined in the soluble fractions of an infant formula after in vitro enzymolysis using the Stomacher and the Boxcult incubation camera, and both pHs 2.0 and 5.0 for the gastric digestion. The obtained results are presented in the **Table 4**.

Stomacher is not a thermostatic system, but the mechanical shaking makes the bag temperature reach almost 37 °C. This fact might explain why there is not significant difference between both systems. It can be concluded that the shaking system is not an important parameter in the extraction efficiency. The digestion in the shaking incubator using Erlenmeyer flasks was the preferred method, because it makes the control of the temperature and sample handling easy (pH determinations, transfer to centrifugation tubes), and the contamination risk seems to be less than that with bags, which break sometimes.

Cu Determination in Soluble Fractions. *Calibration and Standard Addition Graphs.* To evaluate the possible matrix effect, standard addition graphs were compared in the same concentration range for the gastric and gastrointestinal soluble fractions of an infant formula and breast milk sample obtained using both gastric pHs 2.0 and 5.0 (**Table 5**). *A* is the absorbance (peak area), and the concentration is expressed in ng mL^{-1}. There is a statistically significant difference between the slope of the calibration graph and the slope of the different addition graphs. Nevertheless, it's possible to use the same addition graph for performing Cu determination in all the digested samples.

Table 5. Calibration Curves

calibration	$A = 0.015$ [Cu] + 0.001 $r = 1.000$	$A = 0.015$ [Cu] + 0.001 $r = 1.000$
	standard additions	
	breast milk	infant formulas
gastric dig. (2G)	$A = 0.010$[Cu] + 0.260 $r = 0.999$	$A = 0.010$[Cu] + 0.177 $r = 1.000$
gastroint. dig. (2I)	$A = 0.010$[Cu] + 0.229 $r = 1.000$	$A = 0.010$[Cu] + 0.087 $r = 1.000$
gastric dig. (5G)	$A = 0.011$[Cu] + 0.052 $r = 1.000$	$A = 0.010$[Cu] + 0.054 $r = 1.000$
gastroint. dig. (5I)	$A = 0.010$[Cu] + 0.1953 $r = 0.999$	$A = 0.010$[Cu] + 0.054 $r = 0.999$

Table 6. Precision of the In Vitro Method ($n = 5$)

	breast milk			infant formula		
	avg	SD	RSD (%)	average	SD	RSD (%)
	[Cu] (ng mL^{-1})					
gastric dig. (2G)	455.8	8.76	1.9	368.4	24.8	6.7
gastroint. dig. (2I)	482.6	25.9	5.4	350.6	15.0	4.3
gastric dig. (5G)	77.4	2.47	3.2	29.5	3.87	13.1
gastroint. dig. (5I)	462.7	14.6	3.1	345.4	16.6	4.8

Precision of the digestion procedure. To know the repeatability of the digestion procedure, five replicates of an infant formula and breast milk were performed at different pH values. The relative standard deviations were calculated in all cases, and results are shown in **Table 6**, where it can be observed that the overall proposed digestion procedure have a good precision.

An infant formula sample was analyzed two different days as an approximation to evaluate the interday precision of the method as well, with RSD (%) ranging from 0.8 to 10.0%.

Sensitivity. The sensitivity was studied through three parameters: limit of detection (LOD), limit of quantification (LOQ), and characteristic mass. LOD and LOQ are defined as 3 and 10 SD/S respectively, where SD is the standard deviation of 11 measurements of a blank, and S is the slope of the standard addition graph. The characteristic mass (m_o) is defined as the mass of analyte that provides an integral absorbance of 0.0044.

By performing the necessary determinations, values listed in **Table 7** were achieved. *A* is the absorbance of the blank. The achieved values are enough to determine copper content in all the samples.

Applications. The in vitro digestion method was applied to two pools of breast milk (samples 1 and 6) and to 8 individual breast milk samples from the same mother (transitional milk, samples 2−5, 7−10). All the women that kindly provided the samples were from Galicia, northwest of Spain. Due to the shortage of volume, five of them were digested using pH 2.0 in the gastric stage (samples 1−5) whereas samples 6−10 were treated using pH 5.0. Regarding the infant formulas, 12 cow's milk based samples were digested simultaneously using both gastric pHs. These formulas were recommended for the first 6 months of lactation and were marketed in Spain.

Table 7. Sensitivity of the Method

Limits of Detection and Quantification					
	A	SD	*S*	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
breast milk	0.006	7.1 10^{-4}	0.009	0.2	0.8
inf. formula	0.006	9.7 10^{-4}	9.7 10^{-3}	0.3	1.0

Characteristic Mass for Copper Determination in Soluble Fractions of Digests (pg)		
	breast milk	infant formula
gastric dig. (2G)	6.2	4.7
gastroint. dig. (2I)	7.8	8.6
gastric dig. (5G)	12.0	6.8
gastroint. dig. (5I)	10.4	9.7

Table 8. Cu Concentration in Milk, Milk Whey, and Gastric and Gastrointestinal Digests of Breast Milk Samples (ng mL^{-1})

			gastric pH 2.0	
sample no.	milk	milk whey	gastric digest (2G)	gastroint. digest (2I)
1	337.8 ± 9.9	119.8 ± 6.7	308.1 ± 6.7	297.9 ± 12.4
2	100.2 ± 0.1	51.5 ± 2.8	77.9 ± 4.8	66.5 ± 6.1
3	113.3 ± 4.3	55.3 ± 1.4	91.8 ± 3.2	67.4 ± 0.8
4	113.3 ± 1.4	61.6 ± 1.9	92.6 ± 3.4	69.7 ± 4.2
5	125.1 ± 2.8	115.4 ± 0.1	86.1 ± 5.7	63.7 ± 3.5
			average	113.0
			SD	103.4

			gastric pH 5.0	
sample no.	milk	milk whey	gastric digest (5G)	gastrointest. digest (5I)
6	120.3 ± 8.4	67.9 ± 2.9	46.7 ± 6.1	98.0 ± 6.9
7	123.3 ± 9.9	61.9 ± 4.9	28.6 ± 1.3	82.3 ± 2.6
8	123.3 ± 1.4	61.0 ± 1.2	34.3 ± 3.7	82.3 ± 4.8
9	139.3 ± 7.1	65.8 ± 1.8	26.2 ± 0.8	74.7 ± 0.8
10	104.6 ± 1.4	90.5 ± 1.4	19.3 ± 1.2	39.2 ± 2.3
			average	75.3
			SD	21.9

Table 9. Cu Concentration in Milk and Milk Wheys of Infant Formula Samples (ng mL^{-1})

sample no.	milk	milk whey
1	267.0 ± 0.1	97.9 ± 3.0
2	765.2 ± 29.7	255.9 ± 4.4
3	749.5 ± 7.4	344.1 ± 5.8
4	646.3 ± 0.1	285.5 ± 4.3
5	684.3 ± 7.6	129.6 ± 2.1
6	339.1 ± 6.4	21.7 ± 2.1
7	823.0 ± 4.9	242.0 ± 10.3
8	554.3 ± 5.4	116.9 ± 8.4
9	762.5 ± 0.1	174.8 ± 7.8
10	534.6 ± 0.1	77.7 ± 6.3
11	786.4 ± 44.9	199.3 ± 17.3
12	655.2 ± 7.4	76.1 ± 3.2

Contents determined in milk, milk whey and milk digests are shown in **Tables 8**−**10**. It can be observed that there is a tendency to a decrease of the concentration of Cu from 2G (gastric digest pH 2.0) to 2I (the corresponding gastrointestinal digest). However, there is a strong tendency of the Cu concentration in the soluble digests to increase from 5G (gastric digest pH 5.0) to 5I (the corresponding gastrointestinal digest). Then, it is clearly shown that it is vital to perform an adequate selection of gastric pH when performing in vitro studies to compare availability of Cu from breast milk and infant formulas.

Table 10. Cu Concentration in the Gastric Digests and Gastrointestinal Digests of Infant Formulas (ng mL^{-1})

	gastric pH 2.0		gastric pH 5.0	
sample no.	gastric digest (2G)	gastrointest. digest (2I)	gastric digest (5G)	gastrointest. digest (5I)
1	236.5 ± 13.6	193.5 ± 6.3	54.3 ± 5.2	74.7 ± 4.1
2	370.2 ± 22.4	240.3 ± 17.8	39.9 ± 2.7	174.6 ± 15.1
3	430.5 ± 9.1	333.5 ± 12.1	135.5 ± 10.1	236.8 ± 20.4
4	368.4 ± 24.8	350.6 ± 15.1	30.8 ± 3.1	345.4 ± 16.6
5	399.4 ± 12.2	195.0 ± 4.5	18.0 ± 1.6	122.0 ± 1.9
6	170.0 ± 2.9	63.0 ± 3.6	15.0 ± 0.9	35.8 ± 1.0
7	498.9 ± 1.0	333.2 ± 15.5	73.5 ± 1.0	203.8 ± 7.0
8	420.9 ± 13.7	288.3 ± 11.4	115.2 ± 2.7	206.7 ± 12.5
9	454.8 ± 9.4	216.1 ± 24.3	60.5 ± 4.9	159.1 ± 6.7
10	353.7 ± 11.9	169.9 ± 2.1	90.1 ± 6.2	118.8 ± 4.8
11	433.6 ± 17.3	329.8 ± 16.7	83.0 ± 2.7	199.5 ± 9.8
12	393.2 ± 12.6	230.7 ± 3.6	115.0 ± 4.7	153.8 ± 4.2
	average	245.3		169.3
	SD	82.1		76.9

Table 11. Solubility in the Gastrointestinal Digests of Breast Milk Samples (%)

sample no.	gastric pH 2.0	sample no.	gastric pH 5.0
1	88.2 ± 3.7	6	81.5 ± 5.8
2	66.4 ± 6.1	7	66.7 ± 2.1
3	59.5 ± 0.7	8	66.7 ± 3.9
4	61.5 ± 3.7	9	53.6 ± 5.7
5	50.9 ± 2.8	10	37.5 ± 2.2
average	65.3		61.2
SD	14.0		16.5

It seems that, using pH 2.0 at the gastric digestion, a bigger liberation of the Cu might happen, mainly from the casein, and this liberation is reflected later in the highest Cu solubility for infant formulas. However, this effect is minor for breast milk samples.

This fact is probably due to different metal distribution among the components of each kind of milk. For samples under study, a 25.9% of the Cu is in the infant formula whey, whereas a 57.2% is in the breast milk whey. Results in bibliography shows that 77% of the copper appears in milk whey from breast milk, whereas 47% of the Cu is bound to low molecular weight compounds for cow milk, and 44% is bound to casein (*10*). For infant formulas, 37% of the copper is in the whey, 54% in the casein and 9% in the fat (*18*).

Availability Estimation. The solubility percentage was used as an indicator of availability

$$S(\%) = [\mathrm{Cu}]_{\text{gastrointestinal digest}}/[\mathrm{Cu}]_{\text{milk}} \times 100$$

In **Tables 11** and **12**, percentages of Cu in the gastrointestinal digests are shown.

It can be observed that only small differences exist between the average percentage of Cu using pH 2.0 or pH 5.0 in the gastric digestion of breast milk. It can be observed too, that for infant formulas, solubility is minor when samples were digested at pH 5.0 in the gastric stage. There is a linear relationship between copper concentration in the gastrointestinal digests using pH 2.0 in the gastric stage and copper concentration in the gastrointestinal digests using pH 5.0 in the gastric stage (**Figure 1**). There has been a decrease in solubility of more than 30%. Sample 4 was not included in this calculation.

Solubility data expressed as percentages were statistically analyzed (*17*) with the following conclusions:

Table 12. Cu Solubility in the Gastrointestinal Digests of Infant Formula Samples (%)

sample no.	gastric pH 2.0	gastric pH 5.0
1	72.3 ± 2.3	28.1 ± 1.5
2	31.4 ± 2.3	22.9 ± 2.0
3	44.5 ± 1.6	31.6 ± 2.7
4	54.3 ± 2.6	53.4 ± 2.6
5	28.5 ± 0.6	17.8 ± 2.8
6	18.6 ± 1.1	10.6 ± 2.9
7	40.5 ± 1.9	24.8 ± 0.9
8	52.0 ± 2.0	37.4 ± 2.3
9	28.3 ± 3.2	20.9 ± 0.9
10	31.8 ± 0.4	22.2 ± 0.9
11	42.0 ± 2.1	25.4 ± 1.2
12	35.3 ± 0.5	23.5 ± 0.6
average	40.0	26.6
SD	13.9	10.3

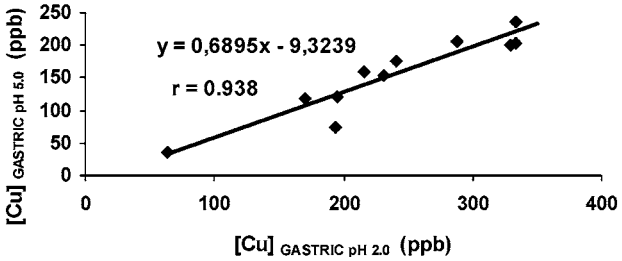

Figure 1. Cu Content in the Gastrointestinal Digests of Infant Formula Samples using both Gastric pHs (Adult pH: 2.0, Newborn pH: 5.0).

There is no percentage of solubility or content difference between gastrointestinal digests of breast milk using the different gastric pHs (65.3 ± 14.0 vs 61.2 ± 16.5%, $|t| = 0.20 < 2.31 = t_c$, $\alpha = 0.05$; 113.0 ± 103.4 vs 75.3 ± 21.9 ng mL^{-1}, $|t| = 0.80 < 2.31 = t_c$, $\alpha = 0.05$).

Percentage of Cu solubility and content is bigger in the gastrointestinal digests from the infant formulas when pH 2.0 (adult gastric pH) was used (40.0 ± 13.9 vs 26.6 ± 10.3%, $|t| = 2.57 > 2.09 = t_c$, $\alpha = 0.05$; 245.3 ± 82.1 vs 169.3 ± 76.9 ng mL^{-1}, $|t| = 2.24 > 2.07 = t_c$, $\alpha = 0.05$).

Cu from breast milk is more soluble than Cu from infant formulas, using pH 2.0 for gastric digestion (65.3 ± 14.0 vs 40.0 ± 13.9%, $|t| = 3.32 > 2.13 = t_c$, $\alpha = 0.05$) or pH 5.0 (61.2 ± 16.5 vs 26.6 ± 10.3%, $|t| = 4.32 > 2.57 = t_c$, $\alpha = 0.05$). However, considering the high amounts of Cu in infant formulas, the soluble amount is bigger than that for breast milk, even using pH 5.0 in the gastric stage (pH 2: 245.3 ± 82.1 vs 113.0 ± 103.4 ng mL^{-1}; $|t| = 2.52 > 2.46 = t_c$, $\alpha = 0.05$; 169.3 ± 76.9 vs 75.3 ± 21.9 ng mL^{-1}, $|t| = 3.73 > 2.14 = t_c$, $\alpha = 0.05$).

Considering previous studies, there is not a large number of publications about Cu bioavailability, and results are difficult to compare. Cabrera et al. (*19*) examined different dairy products (condensed milk, powdered milk, children's milk, whipped cream, yogurt, custard, cream cheese, curd, creme caramel, and ice-cream). This work has several shortcomings: they found that 85% of Cu is in the soluble fraction, but this result is the mean for the 10 different dairy products, no contribution from blanks was observed in any step, and they used pH 3.0 in the gastric digestion. There is another work about the addition of organic salts to a liquid infant formula that produced an increase of the solubility; solubility of Cu gluconate was 11.4%, whereas solubility of Cu sulfate was only 3% (*20*).

Mineral dializability after simulated digestion has been used as well, as an in vitro technique to evaluate availability (*21*). Thus, Barberá et al. digested different kinds of formulas marketed in Spain and determined that the percentage of dialysis ranged from 15.8 to 23.5% (*22*−*23*). They attempted to establish relationships about mineral dializability and infant formula composition (*24*−*27*). The disadvantage of their approach is that all their studies were carried out using pH 2.0 in the gastric stage.

Regarding in vivo studies, very few of them have assessed Cu absorption from milk in children, due to technical and ethical limitations. Comparing stable isotopic extrinsic tag with ^{65}Cu and chemical balance method, Ehrenkranz et al. (*28*) found that Cu absorption was higher from breast milk than from infant formula in very-low-birth-weight infants (net Cu absorption, 61.5 ± 14.0% from preterm human milk (PTHM) vs 16.6 ± 20.6% from preterm formulas; ^{65}Cu absorption, 69.8 ± 14.0% from preterm human milk vs 39.6 ± 21.6% from preterm formulas). Fortified preterm breast milk showed similar values to those of PTHM. Similarly, Olivares et al. (*29*) evaluated the effect of age and copper intake on copper absorption in infants during the first 3 months of life, using ^{65}Cu as a tracer. These two parameters did not affect apparent copper absorption (range from 46 to 95%, average ≈ 80%), within ranges tested.

Therefore, these results agree with ours to some extent, because even with the percentage of solubility from infant formulas being smaller than that from breast milk, the total concentration available for absorption seems to be enough. More research is needed now to propose fortification levels in formulas, adequate processing, and to select bioavailable and safe copper compounds to avoid potential physicochemical reactions that could affect the final product or children health (*6*, *7*, *30*−*32*).

Conclusion. An in vitro method was developed to compare copper availability from products used as baby nourishment. This method does not claim to be able to replace in vivo methods for availability studies, but it could provide useful information, obtained in experimental conditions easy to control. Thus, this study contributes to the better understanding of gastric pH influencing Cu solubility from breast milk samples and infant formulas. Results showed that infant formula samples are more affected by changes in the pH, and there is a linear relationship between copper content in the gastrointestinal digests obtained using pH 2.0 (adult pH) or 5.0 (newborn pH) in the gastric stage. The percentage of Cu solubility is always bigger from breast milk, but infant formulas can provide enough elements for subsequent absorption.

ACKNOWLEDGMENT

We thank the collaboration of Jani Madetoja (Erasmus student from Helsinki University, Finland) in this work.

LITERATURE CITED

(1) Shaw, J. C. Trace element in the fetus and young infant. II. Copper, manganese, selenium, and chromium. *Am. J. Dis. Child*. **1980**, *134*, 1, 74−81.

(2) Moreno Rojas, R.; Cañal Ruiz, C.; Zurera Cosnao, G. Importancia del cobre en la nutrición infantil. *Alimentaria* **2002**, *10*, 107−116.

(3) Lönnerdal, B. Copper nutrition during infancy and childhood. *Am. J. Clin. Nutr*. **1998**, *67*, 1046S−1053S.

(4) O'Dell, B. L. Mineral interactions relevant to nutrient requirements. *J. Nutr*. **1989**, *119*, 1832−1838.

(5) Johnson, M. A.; Smith, M. M.; Edmonds, J. T. Copper, iron, zinc, and manganese in dietary supplements, infant formulas, and ready-to-eat breakfast cereals. *Am. J. Clin. Nutr*. **1998**, *67*, 1035S−1040S.

(6) Lönnerdal, B.; Hernell, O. Iron, zinc, copper and selenium status of breast-fed infants and infants fed trace element fortified milk-based infant formula. *Acta Paed*. **1994**, *83*, 367−373.
(7) Kaup, S. M. Aspects of mineral bioavailability in infant nutrition. *Int. Dairy J*. **1998**, *8*, 435−441.
(8) Jochum, F.; Fuchs, A.; Cser, A.; Menzel, H.; Lombeck, I. Trace mineral status of full-term infants fed human milk, milk-based formula, or partially hydrolyzed whey protein formula. *Analyst* **1995**, *120*, 905−908.
(9) Oskarsson, A.; Palminger-Hallén, I.; Sundberg, J.; Petersson-Grawé, K. Risk assessment in relation to neonatal metal exposure. *Analyst* **1998**, *123*, 19−23.
(10) Lönnerdal, B. Effects of milk and milk components on calcium, magnesium, and trace element absorption during infancy. *Physiol. Rev.* **1997**, *27*, 643−669.
(11) Hurrel, R. F.; Davidsson, L.; Kastenmayer, P. Stable isotopes techniques to measure mineral and trace element absorption in man. In *Bioavailability '93; Nutritional, Chemical, and Food Processing Implications of Nutrient Availability. Proceedings, part 1*; Schlemmer, U., Ed; Bundesforschungsanstalt für Ernährung: Kalsruhe (Germany), 1993; pp 223−232.
(12) Lönnerdal, B.; Yuen, M.; Glazier, C.; Litov, R. E. Magnesium bioavailability from human milk, cow milk, and infant formula in suckling rat pups. *Am. J. Clin. Nutr.* **1993**, *58*, 392−397.
(13) Lönnerdal, B.; Glazier, C. An approach to assessing trace element bioavailability from milk in vitro. *Biol. Trace Elem. Res*. **1989**, *19*, 57−69.
(14) Bermejo, P.; Peña, E. M.; Domínguez, R.; Bermejo, A.; Cocho, J. A.; Fraga, J. M. Iron and zinc in hydrolised fractions of human milk and infant formulas using an in vitro method. *Food Chem.* **2002**, *77*, 361−369.
(15) Bermejo-Barrera, P.; Domínguez-González, R.; Bermejo-Barrera, A. Direct copper determination in whole milk, nonfat milk, and whey milk by electrothermal atomic absorption spectrometry. *Fresenius J. Anal. Chem.* **1997**, *357*, 457−461.
(16) Chevallier, B. *Nutrición Infantil*; Masson, S. A., Ed.: Barcelona, 1997, pp 19−45.
(17) Miller, J. C.; Miller, J. N. *Estadística para Química Analítica*, 2nd ed.; Addison-Wesley Iberoamericana: Buenos Aires, Argentina, 1993.
(18) Bermejo, P.; Peña, E.; Domínguez, R.; Bermejo, A.; Cocho, J. A.; Fraga, J. M. Distribution of Cu, Fe, and Zn among Infant Formula Components. *V Simposio Centroamericano y del Caribe de Química Ambiental y Sanitaria*, La Habana, poster presentation, 2002.
(19) Cabrera, C.; Lorenzo, M. L.; De-Mena, C.; López, M. C. Chromium, copper, iron, manganese, selenium, and zinc levels in dairy products: in vitro study of absorbable fractions. *Int. J. Food Sci. Nutr*. **1996**, *47*, 331−339.
(20) Hendricks, G. M.; Ming, R. G.; Kindstedt, P. S. Solubility and relative absorption of copper, iron and zinc in two milk-based liquid infant formulae. *Int. J. Food Sci. Nutr*. **2001**, *52*, 419−428.
(21) Fairweather-Tait, S. J. Bioavailability of trace elements. *Food Chem*. **1992**, *43*, 213−217.
(22) Barberá, R.; Farré, R.; García, R.; Guillen, A.; Lagarda, M. J. Estimate of the bioavailability of iron, copper, and zinc in infant formulae-studies "in vitro". In *Bioavailability '93; Nutritional, Chemical, and Food Processing Implications of Nutrient Availability. Proceedings, part 2*; Schlemmer, U., Ed; Bundesforschungsanstalt für Ernährung: Kalsruhe (Germany), 1993; pp 243−247.
(23) García, R.; Alegría, A.; Barberá, R.; Farré, R.; Lagarda, M. J. Dialyzability of iron, zinc, and copper of different types of infant formulas marketed in Spain. *Biol. Trace Elem. Res*. **1998**, *65*, 7−17.
(24) Alegría, A.; Barberá, R.; Farré, R.; Lagarda, M. J.; Mandin, A. In vitro bioavailability of copper, zinc, and iron in infant formulas supplemented with sopper salts. In *Metal ions in Biology and Medicine*; Collery, P.; Corbella, J.; Domingo, J. L.; Etienne, J. C.; Llobet, J. M., Eds.; John Libbey Eurotext: Paris, France, 1996, Vol 4, pp 9−11.
(25) Jovaní, M.; le-Masle, M.; Alegría, A.; Barberá, R.; Farré, R.; Lagarda, M. J.; Clemente, G. In vitro interactions between calcium, zinc, copper, and iron in milk- and soy-based infant formulas. *Food Sci. Technol. Int.* **2000**, *6*, 25−31.
(26) Jovaní, M.; Alegría, A.; Barberá, R.; Farré, R.; Lagarda, M. J.; Clemente, G. Effect of proteins, phytates, ascorbic acid, and citric acid on dialisability of calcium, zinc, and copper in soy-based infant formulas. *Nahrung* **2000**, *44*, 114−117.
(27) López, J. C.; Lozano, A.; Alegría, A.; Barberá, R.; Farré, R.; Clemente, G. Mathematic predictive models for calculating copper, iron and zinc dialysability in infant formulas. *Eur. Food Res. Technol*. **2001**, *212*, 608−612.
(28) Ehrenkranz, R. A.; Gettner, P. A.; Nelli, C. M.; Sherwonit, E. A.; Williams, J. E.; Ting, B. T.; Janghorbani, M. Zinc and copper nutritional studies in very low birth weight infants: comparison of stable isotopic extrinsic tag and chemical balance methods. *Pediatr. Res.* **1989**, *26*, 4, 298−307.
(29) Olivares, M.; Lönnerdal, B.; Abrahams, S. A.; Pizarro, F.; Uauy, R. Age and copper intake do not affect copper absorption, measured with the use of ^{65}Cu as a tracer, in young infants. *Am. J. Clin. Nutr*. **2002**, *76*, 641−5.
(30) Lönnerdal, B.; Jayawichrama, L.; Lien, E. L. Effect of reducing the phytate content and of partially hydrolyzing the protein in soy formula on zinc and copper absorption and status in infant Rhesus monkeys and rat pups. *Am. J. Clin. Nutr.* **1999**, *69*, 490−496.
(31) Lönnerdal B.; Kelleher, S. L.; Lien, E. L. Extent of thermal processing of infant formula affects copper status in infant Rhesus monkeys. *Am. J. Clin. Nutr.* **2001**, *73*, 914−9.
(32) Rosado, J. L. Zinc and copper: proposed fortification levels and recommended zinc compounds, *J. Nutr*. **2003**, *133*, 2985S−2989S.

Received for review December 1, 2003. Revised manuscript received May 13, 2004. Accepted May 13, 2004. This work was partially supported by the research project 1997, CEO12 Laboratorios Ordesa (Barcelona, Spain) and R-PETRI PTR-1995/0384-OP, Secretaría de Estado de Educación, Universidades, Investigación y Desarrollo, Ministerio de Educación y Cultura (Spain).

JF030781Y