JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 4901−4906 **4901**

Fortification of Milk with Calcium: Effect on Calcium Bioavailability and Interactions with Iron and Zinc

SARA PERALES, REYES BARBERÁ, MARÍA JESÚS LAGARDA, AND ROSAURA FARRÉ*

Nutrition and Food Chemistry, Faculty of Pharmacy, University of Valencia, Avda. Vicente Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain

Calcium solubility, dialysability, and transport and uptake (retention + transport) by Caco-2 cells as indicators of calcium bioavailability have been estimated in the in vitro gastrointestinal digests of milk and calcium fortified milk. A significant linear correlation ($p < 0.05$) was obtained between calcium uptake and the amount of soluble calcium added to the cells, and also between percentage calcium uptake and the calcium measured in the analyzed samples. The solubility, dialysis, transport, and uptake values are higher ($p < 0.05$) for calcium fortified milks than for nonfortified milks; that is, calcium fortification increases not only calcium content but also its bioavailability. An inhibitory effect of calcium from fortified milks upon iron absorption was found. The observed effect of calcium from fortified milks upon zinc bioavailability depends on the in vitro method used, zinc solubility and dialysis decrease in calcium fortified milks, and percentage zinc uptake remains unchanged.

KEYWORDS: Calcium; bioavailability; milk; in vitro estimation

INTRODUCTION

Calcium is an essential nutrient required for critical biological functions such as nerve conduction, muscle contraction, mitosis, blood coagulation, and structural support of the skeleton (*1*). Adequate intake of calcium has been demonstrated to reduce the risk of chronic diseases such as osteoporosis, hypertension, colon cancer, breast cancer, kidney stones, polycystic ovary syndrome, ovarian cancer, and a number of other disorders (*1, 2*). The dietary reference intakes (DRIs) for calcium have been established for different ages in the range 800−1300 mg/day (*3*).

Milk and dairy products are the best natural sources of calcium; indeed, 75−89% of all ingested calcium comes from these sources (*4*). Dairy products have high calcium contents of high bioavailability and can be obtained at a low cost in relation to their nutritional value. In addition, these products provide other essential nutrients, and their intake improves the overall nutritional quality of the diet of children and adolescents (*2*). However, the consumption of milk is declining in industrialized countries—leading to inadequate calcium intake. This is one of the reasons why the food industry has developed calcium-fortified foods of use in securing adequate calcium intake (*2, 5*). In clinical studies carried out in humans, the efficacy of dietary calcium fortification has been demonstrated by an improvement in calcium balance when the mineral intake increases (*6, 7*).

However, the fortification of food with nutrients must take into account possible negative interactions with other nutrients such as divalent metals. The inhibitory effect of calcium on iron and zinc absorption is the most debated and well-documented example of this (*8*−*11*).

From a nutritional point of view, it is of interest to know the calcium content of milk and the fraction of this calcium that can be absorbed and utilized, i.e., its bioavailability. Conceptually, bioavailability can be viewed as the integral sum of a four component process consisting of (a) ingestion, (b) bioaccessibility (solubility), (c) absorption, and (d) first-pass effect in the liver. After the ingestion process, bioaccessibility represents the fraction of the external dose of element that is released from food matrixes and is available for absorption from the gastrointestinal tract (*12*). The ideal approach would be to assess mineral bioavailability in humans, but such studies are time-consuming, costly to perform, and impractical for large-scale applications (*13*). In vitro methods constitute a good alternative to in vivo methods for the assessment of calcium bioavailability from milk. In vitro methods are generally based on the simulation of gastrointestinal digestion, followed by the determination of how much calcium is soluble (*5, 14*) or dialyses through a membrane of a certain pore size (*14*−*16*). Solubility or dialysability, in turn, has been useful to establish trends in the bioaccessibility or relative bioavailability values of calcium from milk.

The aforementioned in vitro methods have been improved by the incorporation of a human colon carcinoma cell line (Caco-2) with many of the functional and morphological properties of mature human enterocytes (*17*). This system has been used to estimate calcium bioavailability in different foods and also from a commercial human milk fortifier (*18*). These authors used the model to evaluate the effect of different factors (calcium salts, ascorbic acid, and peptic pH) on the bioavail-

* Correspondence author: phone, +34-96-3544950; fax, +34-96-3544954; e-mail, rosaura.farre@uv.es.

10.1021/jf0601214 CCC: $33.50
Published on Web 05/28/2006

ability of calcium, iron, and zinc (*19*). The present study has the originality of evaluating the effect of calcium fortification of commercial cow milks by using milks fortified or not with calcium from the same manufacturing company, estimating not only calcium uptake but also calcium transport. To our knowledge, the uptake and transport by Caco-2 cells of calcium contained in cow milk and fortified cow milk have not been investigated to date.

The few studies on the bioavailability of calcium from cow milk (*5*−*7*, *14*−*16*), together with the lack of in vitro studies designed to estimate the effect of milk fortification with calcium, have led to the present study, which evaluates the effect on calcium, iron, and zinc bioavailability of fortification of milk with calcium. Different in vitro methods (solubility, dialysability, and uptake and transport by Caco-2 cells) are used to this effect in order to compare the results obtained which each one of them.

MATERIALS AND METHODS

Samples. A total of ten different cow milks from five different manufacturers (A−E) were analyzed. Two milks, one of them with calcium added, were collected from each manufacturer. Samples were kept at 25 °C in their commercial package until analysis.

Materials and Reagents. Enzymes and bile salts were purchased from Sigma Chemical Co. (St. Louis, MO): Pepsin (Porcine: cat no. P-7000), pancreatin (Porcine: cat. no. P-1750), and bile extract (Porcine: cat. no. B-8631). The working solutions of these enzymes were prepared immediately before use.

Calcium, zinc, and iron standard solutions were prepared immediately before use by dilution with distilled deionized water of a standard solution of 1000 mg/L (Titrisol, Merck, Barcelona, Spain).

Transport buffer contained 130 mM NaCl (Merck), 10 mM KCl (Merck), 1 mM $MgSO_4$ (Sigma Chemical Co.), 50 mM HEPES (Gibco, Paisley, Scotland), and 5 mM glucose (Sigma Chemical Co.) at pH = 7. The transport buffer was incubated at 37 °C until starting the assay.

Mineral removal buffer contained 150 mM NaCl (Merck), 10 mM HEPES (Gibco), and 1 mM EDTA (Sigma Chemical Co.) at pH = 7.

All reagents used were of reagent grade, and Millipore-Milli Q distilled−deionized water (Millipore Ibérica S.A., Barcelona, Spain) was used throughout the experiments.

For mineral determination, glass and polyethylene materials were washed with detergent, soaked in concentrated nitric acid (sp gr = 1.41), and rinsed three times with distilled−deionized water before use.

In Vitro Digestion. The in vitro procedure was described by Perales et al. in 2005 (*20*).

Solubility. A gastric followed by an intestinal digestion were applied to 35 g of sample. For gastric digestion the pH was adjusted to 2.0 and an amount of freshly prepared demineralized pepsin solution sufficient to yield 0.02 g of pepsin/sample was added. The sample was incubated in a shaking water bath at 37 °C/120 strokes/min for 2 h. To facilitate the intestinal digestion stage, the pH of the gastric digests was raised to pH 5.0. Then an amount of freshly prepared and previously demineralized pancreatin/bile solution sufficient to provide 0.005 g of pancreatin and 0.03 g of bile salts/sample was added, and incubation was continued for an additional 2 h. To stop intestinal digestion, the sample was kept for 10 min in an ice bath. The pH was adjusted to 7.2, and the digests were centrifuged at 3500 g for 1 h at 4 °C. The supernatants were used to determine the mineral content (solubilized fraction) and were applied in cell culture assays.

Dialysis. Dialysis comprised a gastric step, common to that of the solubility method, followed by an intestinal step where dialysis is included (dialysis bag: cutoff molecular weight 10−12000 Da; Visking 3-20/322, Medicell, London, U.K.). Dialysate was collected to measure its mineral content. The dialysis bag (containing 25 mL of cell culture grade water and an amount of $NaHCO_3$ equivalent to the titrable acidity (previously measured)) was placed in the flasks that contained 20-g aliquots of the pepsin digest. Incubation was continued for 30 min, after which an amount of freshly prepared and previously demineralized pancreatin/bile solution sufficient to yield 0.001 g of pancreatin and 0.006 g of bile salts per gram of aliquot was added, and incubation was again continued up to 2 h. The dialysis bag (dialysate) contents were taken and used to determine the mineral content.

Caco-2 Method. *Cell Culture*. Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC 86010202, Salisbury, U.K.) and were used between passages 75 and 90. Stock cultures were maintained in minimum essential medium (MEM; Gibco BRL Life Technologies, Scotland, U.K.) supplemented with 10% v/v foetal bovine serum (FBS), 1% v/v nonessential amino acids (Gibco), 1% v/v L-glutamine (Gibco), 1% v/v antibiotic solution (penicillin−streptomycin) (Gibco), and 0.1% v/v fungizone (Gibco) at pH 7.2−7.4. The cells were maintained at 37 °C in an incubator (Nuaire, NU-4500, Minnesota) under a 5% CO_2, 95% air atmosphere at constant humidity. Culture medium was changed every 2 days.

Sample Preparation. The gastrointestinal digests from the solubility assay were heated for 4 min at 100 °C to inhibit sample proteases and then cooled by immersion in an ice bath. Aliquots of 20 g of the inactivated digests were transferred to polypropylene centrifuge tubes and centrifuged at 3500 g for 1 h at 4 °C. Glucose (5 mM final concentration) and HEPES (50 mM final concentration) were added to the supernatant fraction to make it similar to the culture medium and facilitate cell viability, while water was added to adjust the osmolarity to 310 ± 10 mOsm/kg (freezing point osmometer, Osmomat 030, Berlin, Germany).

In Caco-2 retention and transport assays, the soluble fraction obtained with the solubility method was used instead of the dialysate, because the soluble fraction is more similar to the digest obtained under in vivo conditions. Besides this, the use of dialysate dramatically reduced the amount of mineral added to cells, because only the soluble forms of mineral with a molecular weight lower than the cutoff value of the dialysis membrane are able to dialyze—resulting in very low mineral content that increased variability in the uptake assays.

Mineral Retention and Transport. To evaluate mineral retention and transport, the cells were seeded onto polyester membrane chamber inserts (24-mm diameter, 0.4-μm pore size; Transwell, Costar Corp., NY) at a density of 35×10^4 cells/filter, with 2.5 mL of medium in the basal chamber and 1.5 mL of suspended cells in the apical chamber. The transwell filters were placed into six well plates dividing an apical or a donor-like compartment from a basal or acceptor compartment.

Nineteen to twenty-one days after initial seeding, spent culture medium was aspirated from the apical and basolateral chambers, and the apical and basolateral cell surfaces of the monolayer were washed three times with phosphate buffered saline at 37 °C. Then, 2.5 mL of transport buffer was added to the basal chamber, and 1.5 mL of heated soluble mineral fraction was added to the apical chamber. Cell cultures were incubated at 37 °C under 5% CO_2 with 95% relative humidity for 2 h. Cell viability after 2 h of exposure to the soluble mineral fraction was assessed by trypan blue exclusion and was typically 80−95%. Moreover, the development of functional tight junctions in the monolayer of Caco-2 cells was monitored by determining transepithelial electrical resistance (TEER) with a Millicell electrical resistance system (Millicell-ERS, Millipore Ibérica S.A., Barcelona, Spain). Any cultures with TEER lower than 250 Ω cm^{-2} were discarded.

After incubation for retention study, the apical compartment was aspirated, the insert was removed, and the monolayer was washed three times with buffer solution at 4 °C to remove nonspecifically bound mineral and residual medium. The cells were lysed by adding 1 mL of 2% SDS (sodium dodecyl sulfate).

The basal chamber solution was pipetted off for the determination of mineral transport across the monolayer.

Calcium, Zinc, and Iron Determination. To estimate calcium bioavailability and the possible effect of calcium enrichment on iron and zinc bioavailability, calcium, iron, and zinc were determined in the sample (total), soluble, or dialyzed fraction and in the cell monolayer, the transport buffer (transport blank), and the basal chamber contents; all measurements were done by flame atomic absorption spectrometry (AAS, Perkin-Elmer, model 2380, Boston, MA). All samples, with the exception of the dialysate, were previously subjected to dry digestion at 450 °C. To dissolve the ashes, 3 mL of concentrated HCl (sp gr = 1.19) was added; the vessel was covered with a watch glass and gently warmed (≈70 °C) for 3.5 h, leaving at the end of

Table 1. Calcium: Total, Soluble, and Dialysate Contents and Transport and Uptake by Caco-2 from Milk and Fortified Milk[a]

	calcium content		in vitro digestion		Caco-2 method			
manufacturer	label (mg/L)	measured (mg/g)	solubility (%)	dialysis (%)	Ca soluble added (μg)	retention (%)	transport (%)	uptake (%)
A	1.2	1.17 ± 0.03	82.91 ± 1.67 a	29.06 ± 0.67 a	506.20	0.79 ± 0.06 a	6.40 ± 0.30 a	7.20 ± 0.29 a
	(1.6)	(1.32 ± 0.03)	(89.39 ± 1.35) b	(30.30 ± 1.28) b	(538.60)	(0.89 ± 0.03) a	(7.12 ± 0.21) b	(8.01 ± 0.16) b
B	1.2	1.17 ± 0.03	82.91 ± 0.90 a	28.21 ± 1.22 a	484.60	0.76 ± 0.04 a	6.06 ± 0.07 a	6.82 ± 0.10 a
	(1.6)	(1.34 ± 0.03)	(88.81 ± 0.76) b	(30.59 ± 0.83) b	(530.90)	(0.72 ± 0.07) a	(7.53 ± 0.11) b	(8.24 ± 0.14) b
C	1.2	1.14 ± 0.01	86.84 ± 0.68 a	29.82 ± 0.98 a	501.04	0.72 ± 0.08 a	6.72 ± 0.05 a	7.44 ± 0.09 a
	(1.6)	(1.34 ± 0.01)	(88.06 ± 0.69) b	(30.59 ± 0.97) b	(545.43)	(0.87 ± 0.06) a	(7.29 ± 0.05) b	(8.16 ± 0.09) b
D	1.2	1.15 ± 0.01	86.09 ± 1.39 a	28.69 ± 0.66 a	514.09	0.71 ± 0.06 a	6.43 ± 0.11 a	7.14 ± 0.11 a
	(1.6)	(1.34 ± 0.01)	(87.31 ± 0.66) b	(29.85 ± 0.40) b	(540.21)	(0.88 ± 0.07) a	(7.12 ± 0.08) b	(8.00 ± 0.12) b
E	1.2	1.15 ± 0.01	85.22 ± 0.78 a	29.57 ± 1.21 a	516.71	0.71 ± 0.08 a	6.45 ± 0.10 a	7.16 ± 0.17 a
	(1.6)	(1.36 ± 0.01)	(86.76 ± 0.25) b	(30.88 ± 0.67) b	(548.04)	(0.77 ± 0.04) a	(7.09 ± 0.11) b	(7.87 ± 0.10) b

[a] Mean values ± standard deviation ($n = 5$). Values in parentheses correspond to calcium enriched milk. Noncoincidence of on line letters following the data (a, b) denotes statistically significant differences ($p < 0.05$) for solubility or dialysis of milk and fortified milk from the same manufacturer.

heating about 1 mL of liquid. The solution was then transferred to a 10 mL volumetric flask, and the volume was completed with water.

An amount of lanthanum chloride sufficient to yield a lanthanum final content in the assay of 0.2% (v/v) was added to suppress phosphate interference for calcium determination. The standard calibration procedure was used in FAAS determination. Quality assurance of the analytical method can be consulted in Ruiz et al. (*21*) and Jovaní et al. (*22*).

Solubility percentages were calculated as follows: solubility % = 100*S*/*C*, where *S* = soluble mineral content (μg/g sample) and *C* = total mineral content of the sample (μg/g sample).

Dialysis percentages were calculated as follows: dialysis % = 100*D*/*C*, where *D* = mineral content of the dialysate (μg/g sample) and *C* = total mineral content of the sample (μg/g sample).

Differences between the mineral content of the cell monolayer incubated with a soluble mineral fraction and the mineral content of the nonexposed cell monolayer (retention blank) yield an estimation of the cellular retention (μg) of calcium, zinc, and iron. Cellular transport was evaluated by the difference between the mineral amount in the basal chamber and the amount in the transport buffer (transport blank).

Retention percentages were calculated as follows: retention % = 100*R*/*C*, where *R* = mineral retention (μg/well) and *C* = mineral soluble added (μg).

Transport percentages were calculated as follows: transport % = 100*T*/*C*, where *T* = cellular transport (μg/well) and *C* = mineral soluble added (μg).

Uptake corresponded/ was defined as the sum of retained and transported (retention + transport) mineral.

Statistical Analysis. To assess the significant differences between calcium, iron, and zinc bioavailability (solubility, dialysis, and transport and uptake by Caco-2 cells) from milk fortified or not with calcium, the paired data t-test was applied.

A simple regression analysis was also applied to the results obtained for calcium with the aim of estimating the possible relationship between (i) solubility or dialysis vs total content and (ii) transport or uptake vs solubility, dialysis, or soluble calcium added to the Caco-2 cells.

A multiple regression analysis was applied to evaluate the relationships between (a) (i) the iron or zinc content and calcium content of milks and (ii) the solubility or dialysis of iron or zinc and (b) (i) the iron or zinc and calcium soluble added to the cells and (ii) the transport or uptake of iron or zinc.

RESULTS AND DISCUSSION

Total, soluble, and dialysate calcium content and calcium retention, transport, and uptake are reported in **Table 1**. The discrepancies between the calcium contents found by analysis and those stated on the label by the manufacturer (see **Table 1**) deserve mention. It was observed that after milk was transferred from its package to another container, an insoluble residue remained in the package. The calcium content of this residue was three times higher in fortified milks (0.175 mg calcium/g milk) than in nonfortified milks (0.060 mg calcium/g milk). Thus, not all added calcium remains soluble during milk storage, and this would explain the differences between the actual calcium contents found and the contents stated by the manufacturer. Differences have also been reported by De la Fuente et al., 2004 (*5*), in skimmed milk enriched with calcium.

Statistically significant differences ($p < 0.05$) in calcium solubility and dialysis percentages between fortified and nonfortified milks were found. In both cases (solubility and dialysis), the highest values corresponded to fortified milks (see **Table 1**). In the same way, calcium fortified milks yielded higher transport and uptake (retention + transport) values in Caco-2 cells versus the values for nonfortified milks (see **Table 1**), while in all cases (fortified and nonfortified milk) the calcium retention percentages were similar. Caco-2 cell uptake percentages cannot be directly compared to in vivo uptake, but results obtained in Caco-2 cell assays corroborate the hypotheses established in vivo (*23*).

The increase in soluble calcium added to Caco-2 cells results in an increase in calcium uptake. A plausible explanation is that milk is fortified with calcium and vitamin D, and the latter increases the uptake of calcium by Caco-2 cells. Vitamin D-mediated up-regulation in CaT1 mRNA levels that may be an important part of the control of apical calcium influx and net apical-to-basolateral transcellular calcium transport induced by vitamin D has been identified in assays with Caco-2 cell lines (*24*).

Among other factors, the fractional absorption of calcium is dose-dependent (*4*, *25*). This dose-dependent effect was observed in the present study, where percentage calcium uptake increased with the amount of soluble calcium added (see **Table 1**). In this context, a significant linear correlation ($p < 0.05$) was obtained between calcium uptake and the amount of soluble calcium added to the cells (Ca % uptake = $-2.953 + 0.0202 \times$ soluble Ca added; $R = 74.719$) and also between percentage calcium uptake and the calcium measured in the analyzed samples (Ca % uptake = $1.877 + 4.577 \times$ Ca measured; $R = 81.060$).

Total, soluble, and dialysate iron contents and iron retention, transport, and uptake are summarized in **Table 2**. Compared to the case of calcium, similar solubility and dialysis percentages were recorded for iron, indicating that most of soluble iron was bound to complexes with a molecular size smaller than the pore size of the dialysis membrane used.

Table 2. Iron: Total, Soluble, and Dialysate Contents and Transport and Uptake by Caco-2 from Milk and Fortified Milk[a]

		in vitro digestion		Caco-2 method			
manufacturer	Fe measured (μg/g)	solubility (%)	dialysis (%)	Fe soluble added (μg)	retention (%)	transport (%)	uptake (%)
A	0.92 ± 0.05	47.26 ± 3.51 a	46.84 ± 3.01 a	1.27	18.46 ± 4.00 a	73.60 ± 3.72 a	92.06 ± 4.39 a
	(0.95 ± 0.01)	(59.05 ± 3.61) a	(30.95 ± 2.35) b	(1.31)	(14.87 ± 0.73) a	(68.83 ± 3.96) b	(83.69 ± 3.33) b
B	0.92 ± 0.07	47.37 ± 9.12 a	40.73 ± 4.34 a	1.15	14.61 ± 0.77 a	69.37 ± 3.83 a	83.98 ± 3.17 a
	(0.95 ± 0.12)	(56.24 ± 7.39) a	(33.89 ± 4.21) b	(1.19)	(13.28 ± 1.53) a	(65.49 ± 9.56) b	(78.77 ± 8.07) b
C	0.88 ± 0.05	54.74 ± 9.42 a	50.44 ± 5.67 a	1.22	15.13 ± 0.79 a	71.38 ± 2.23 a	86.51 ± 2.80 a
	(0.99 ± 0.07)	(52.86 ± 4.30) a	(35.02 ± 4.98) b	(1.24)	(11.70 ± 1.68) a	(61.54 ± 1.02) b	(73.24 ± 1.79) b
D	0.89 ± 0.09	49.21 ± 10.38 a	45.11 ± 5.27 a	1.29	11.86 ± 0.98 a	61.79 ± 0.39 a	73.65 ± 1.14 a
	(0.99 ± 0.07)	(54.19 ± 5.81) a	(29.86 ± 3.42) b	(1.26)	(11.90 ± 0.62) a	(58.44 ± 1.76) b	(70.34 ± 1.21) b
E	0.90 ± 0.07	46.17 ± 8.18 a	50.98 ± 6.15 a	1.29	13.54 ± 0.39 a	64.28 ± 2.05 a	77.82 ± 1.85 a
	(0.99 ± 0.05)	(51.44 ± 6.82) a	(29.94 ± 3.35) b	(1.31)	(12.23 ± 0.62) a	(59.82 ± 0.73) b	(72.06 ± 1.15) b

[a] Mean values ± standard deviation ($n = 5$). Values in parentheses correspond to calcium enriched milk. Noncoincidence of on line letters following the data (a, b) denotes statistically significant differences ($p < 0.05$) for solubility or dialysis of milk and fortified milk from the same manufacturer.

Table 3. Zinc: Total, Soluble, and Dialysate Contents and Transport and Uptake by Caco-2 from Milk and Fortified Milk[a]

		in vitro digestion		Caco-2 method			
manufacturer	Zn measured (μg/g)	solubility (%)	dialysis (%)	Zn soluble added (μg)	retention (%)	transport (%)	uptake (%)
A	3.79 ± 0.28	89.53 ± 0.91 a	14.26 ± 0.81 a	1.70	18.20 ± 1.72 a	19.69 ± 1.22 a	37.90 ± 1.90 a
	(3.86 ± 0.08)	(83.43 ± 2.38) b	(7.71 ± 0.65) b	(1.97)	(18.07 ± 1.28) a	(20.00 ± 1.99) a	(38.07 ± 1.53) a
B	3.53 ± 0.04	90.22 ± 2.08 a	15.62 ± 1.92 a	1.81	18.00 ± 1.35 a	19.93 ± 1.67 a	37.94 ± 2.24 a
	(3.76 ± 063)	(82.45 ± 3.05) b	(7.96 ± 2.08) b	(1.88)	(18.22 ± 1.47) a	(19.64 ± 2.39) a	(37.86 ± 3.06) a
C	3.84 ± 0.04	89.04 ± 1.02 a	14.54 ± 1.41 a	1.78	18.56 ± 1.66 a	18.82 ± 1.54 a	37.37 ± 1.11 a
	(3.80 ± 0.03)	(83.97 ± 0.72) b	(7.65 ± 0.88) b	(1.82)	(17.81 ± 1.45) a	(19.72 ± 1.86) a	(37.53 ± 2.37) a
D	3.86 ± 0.02	88.91 ± 1.00 a	14.24 ± 0.57 a	1.77	18.03 ± 1.67 a	19.34 ± 0.97 a	37.38 ± 0.87 a
	(3.90 ± 0.03)	(85.52 ± 0.53) b	(7.50 ± 0.33) b	(1.79)	(18.57 ± 0.81) a	(19.35 ± 0.70) a	(37.91 ± 1.21) a
E	3.86 ± 0.02	88.01 ± 0.43 a	13.95 ± 0.57 a	1.80	18.96 ± 2.25 a	19.72 ± 1.40 a	38.68 ± 1.78 a
	(3.79 ± 0.01)	(86.23 ± 0.54) b	(8.13 ± 0.69) b	(1.81)	(19.06 ± 0.92) a	(19.57 ± 1.26) a	(38.63 ± 1.58) a

[a] Mean values ± standard deviation ($n = 5$). Values in parentheses correspond to calcium enriched milk. Noncoincidence of on line letters following the data (a, b) denotes statistically significant differences ($p < 0.05$) for solubility or dialysis of milk and fortified milk from the same manufacturer.

No statistically significant differences ($p < 0.05$) were found between the solubility percentages of iron from calcium fortified and nonfortified milk. However, the dialysis percentage of iron was higher ($p < 0.05$) in milk not fortified with calcium than in fortified milk from the same manufacturer (see **Table 2**). This fact shows the possible competitive effect of calcium and iron in the dialysis process, where a negative correlation between total calcium content in milk and iron dialysis percentage (iron dialysis % = 141.149 − 0.081 × total calcium; $R = 88.540$) was found.

The mentioned effect of calcium on iron dialysis has been previously reported by Pérez et al., 2002 (*16*). These authors mentioned a decrease of about the 50% in the iron dialysis percentage in milk fortified with calcium (3.79%) when compared to nonfortified milk (7.13%), which was attributed to calcium fortification.

In Caco-2 cells, statistically significant differences ($p < 0.05$) were found in iron uptake and transport (see **Table 2**), with lower values for calcium fortified milks when compared to nonfortified milk.

Although the majority of experiments carried out in animals have demonstrated an inhibitory effect of calcium from dairy products upon iron absorption, this finding has not been universal (*9, 11,* 26−28). In several in vivo studies, a negative effect of calcium on iron bioavailability has been reported (*8, 9, 11*). A 45% decrease in iron absorption with the simultaneous intake of dietary sources of calcium and iron has been reported (*29, 30*). However, in other publications, the increase in dietary calcium intake—afforded by the increased consumption of dairy products—exerted no negative effect on iron absorption (*31*). The mechanism by which calcium influences iron absorption has not been elucidated (*26*). It has been stated that the inhibition of iron absorption by calcium is the same for non-heme and heme iron. Because heme and non-heme iron are absorbed by different receptors on the mucosal surface, inhibition by calcium must be located within the mucosal cell at some transfer step common to the two kinds of iron (*29*). It has also been stated that an effect of calcium on the luminal surface receptors that mediate iron uptake into enterocytes might provide a more plausible explanation for the inhibitory effect of calcium than competition for an intracellular transport mechanism (*26*). Furthermore, Hallberg and Hulthén (*32*) observed a strong dose−effect relation between the amount of calcium in a meal and the reduction in non-heme iron absorption and suggest one-site competitive binding at a receptor pathway for calcium.

Total, soluble, and dialysate zinc content and zinc retention, transport, and uptake are reported in **Table 3**. In the analyzed samples, the negative effect of calcium on zinc bioavailability was observed, because statistically significant differences ($p < 0.05$) in the solubility and dialysis percentages (see **Table 3**) were found between milk fortified or not fortified with calcium. The decrease was particularly relevant in the case of the dialysis percentages, where 50% reduction was found in calcium fortified milk when compared to nonfortified milk. The negative effect of calcium on the dialysis percentage of iron and zinc could be explained by a competitive effect in the passage across the dialysis membrane (the higher the calcium content, the higher will be the competitive effect). Anions from calcium salts can

form soluble compounds with a higher affinity for iron or zinc than for calcium, compounds that depending on their size could prevent the dialysis of the element component of the complex. On the other hand, milk digests contain phosphopeptides from casein, which present clusters of phosphorylated serine that bind calcium and other cations such as zinc and iron. However, covalent bindings developed between iron and phosphoserine are stronger (approximately 100 times) than ionic binding of zinc or calcium (*33*). These cations bound to phosphopeptides should be too large to diffuse across the dialysis membrane.

The differences found in solubility and dialysis percentages disappeared when retention, transport, and uptake by Caco-2 cells were taken into account (see **Table 3**). It has been reported that excess calcium in the diet can impair zinc availability (*9*, *10*, *27*). However, calcium−zinc interaction seems to be conditioned by the presence of phytic acid in the diet (*9*, *10*). The analyzed samples did not contain phytate—a fact that could explain the lack of a negative effect of calcium on zinc bioavailability.

Results obtained from the applied in vitro methods show an increase in calcium solubility, dialysis, transport, and uptake by Caco-2 cells in calcium fortified milks when compared to nonfortified milks; that is, calcium enrichment not only increases calcium content but also increases its bioaccessibility/bioavailability. The three applied methods are useful for differentiating between calcium fortified and nonfortified milks. In calcium added milks, the theoretical increase in calcium content is 33%, though the mean increase in analyzed calcium is about half this figure (15.9 ± 0.01%) and the mean increase in percentage uptake is 12.7 ± 4.62%.

In summary, the bioavailability of calcium is higher from calcium fortified milk than from nonfortified milk, though calcium fortification exerts a negative effect on the percentages of iron dialysis and uptake. It should be noted that although milk is not a good dietary source of iron, it is an important component in a nutritionally balanced diet. Moreover, considering the observed calcium−iron interaction, the intake of iron supplements at times different from milk ingestion would be wise. In any case, cow's milk is not a suitable vehicle for fortification with iron, unless measures are taken to improve iron bioavailability.

LITERATURE CITED

(1) Miller, G. D.; Jarvis, J. K.; McBean, L. D. Review: The importance of meeting calcium needs with foods. *J. Am. Coll. Nutr.* **2001**, *20*, 168−185.

(2) Nicklas, T. A. Review: Calcium intake trends and health consequences from childhood through adulthood. *J. Am. Coll. Nutr*. **2003**, *22*, 340−356.

(3) *Dietary Reference Intakes for calcium, phosphorous, magnesium, vitamin D and fluoride*; Food and Nutrition Board, Institute of Medicine, National Academy Press: Washington, DC, 2000; pp 250−287.

(4) Cáceres, E.; García, M. L.; Toro, J.; Selgas, M. D. Aspectos fisiológicos y nutritivos del calcio. *Aliment., Equipos Tecnol.* **2003**, 64−72.

(5) De la Fuente, M. A.; Belloque, J.; Juárez, M. Mineral contents and distribution between the soluble and the micellar phases in calcium-enriched UHT milks. *J. Sci. Food Agric.* **2004**, *84*, 1708−1714.

(6) Kohls, K. Calcium bioavailability from calcium fortified food products. *J. Nutr. Sci. Vitaminol*. **1991**, *37*, 319−328.

(7) Chan, G. M.; Hofman, K.; McMurry, M. Effects of dairy products on bone and body composition in puberals girls. *J. Pediatr*. **1995**, *126*, 551−556.

(8) Lönnerdal, B. Effects of milk and milk components on calcium, magnesium and trace element absorption during infancy. *Physiol. Rev.* **1997**, *77*, 643−669.

(9) Jovaní, M.; Barberá, R.; Farré, R. Review: effect of some components of milk- and soy-based infant formulas on mineral bioavailability. *Food Sci. Technol. Int*. **2001**, *7*, 191−198.

(10) Cámara, F.; Amaro, M. A. Nutritional aspect of zinc availability. *Int. J. Food Sci. Nutr*. **2003**, *54*, 143−151.

(11) Amaro, M. A.; Cámara, F. Iron availability: an updated review. *Int. J. Food Sci Nutr*. **2004**, *55*, 597−606.

(12) Causy, D. Case studies of the impact of understanding bioavailability: arsenic. *Ecotoxicol. Environ. Saf.* **2003**, *56*, 164−173.

(13) Jovaní, M.; Barberá, R.; Farré, R.; Martín de Aguilera, E. Calcium, iron and zinc uptake from digests of infant formulas by caco-2 cells. *J. Agric. Food Chem*. **2001**, *49*, 3480−3485.

(14) Roig, M. J.; Alegría, A.; Barberá, R.; Farré, R.; Lagarda, M. J. Calcium bioavailability in human milk, cow milk and infant formulas-comparison between dialysis and solubility methods. *Food Chem.* **1999**, *65*, 353−357.

(15) Roig, M. J.; Alegría, A.; Barberá, R.; Farré, R.; Lagarda, M. J. Calcium dialysability as an estimation of bioavailability in human milk, cow milk and infant formulas. *Food Chem.* **1999**, *64*, 403−409.

(16) Pérez, A.; Lorenzo, L.; Cabrera, C.; López, M. C. Influence of enrichment with vitamins and minerals on the bioavailability of iron in cow's milk. *J. Dairy Res*. **2002**, *69*, 473−781.

(17) Pinto, M.; Robine-Leon, S.; Appay, M. D.; Kedinger, M.; Triadou, N.; Dussaulx, E.; Lacroix, B.; Simon-Assmann, P.; Haffen, K.; Fogh, J.; Zweibaum, A. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line caco-2 in culture. *Biol. Cell*. **1983**, *47*, 323−330.

(18) Etcheverry, P.; Wallingford, J. C.; Miller, D. D.; Glahn, R. P. Calcium, zinc, and iron bioavailabilities from a commercial human milk fortifier: a comparison study. *J. Dairy Sci.* **2004**, *87*, 3629−3637.

(19) Etcheverry, P.; Wallingford, J. C.; Miller, D. D.; Glahn, R. P. The effect of calcium salts, ascorbic acid and peptic pH on calcium, zinc and iron bioavailabilities from fortified human milk using an in vitro digestion/Caco-2 cell model. *Int. J. Vitam. Nutr. Res.* **2005**, *75*, 171−178.

(20) Perales, S.; Barberá, R.; Lagarda, M.; Farré, R. Bioavailability of calcium from milk-based formulas and fruit juices containing milk and cereals estimated by in vitro methods (solubility, dialyzability, and uptake and transport by Caco-2 cells). *J. Agric. Food Chem*. **2005**, *53*, 3721−3726.

(21) Ruiz, C.; Alegría, A.; Barberá, R.; Farré, R.; Lagarda, M. J. Direct determination of calcium, magnesium, sodium, potassium and iron in infant formulas by atomic spectroscopy. Comparison with dry and wet digestions methods. *Nahrung* **1995**, *39*, 497−504.

(22) Jovaní, M.; Alegría, A.; Barberá, R.; Farré, R.; Lagarda, M. J.; Clemente, G. Effect of proteins, phytates, ascorbic acid and citric acid on dialysability of calcium, iron, zinc and copper in soy-based infant formulas. *Nahrung* **2000**, *44*, 114−117.

(23) Delgado-Andrade, C.; Seiquer, I.; Navarro, M. P. Changes in calcium absorption and subsequent tissue distribution induced by Maillard reaction products: in vitro and in vivo assays. *J. Sci. Food Agric*. **2006**, *86*, 271−278.

(24) Fleet, J. C.; Eksir, F.; Hance, K. W.; Wood, R. J. Vitamin D-inducible calcium transport and gene expression in three Caco-2 cell lines. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **2002**, *283*, G618−G625.

(25) Teucher, B.; Dainty, J. R.; Fairweather-Tait, S. J. High bioavailability of calcium in fortified Horlicks. *Eur. J. Clin. Nutr*. **2001**, *55*, 778−781.

(26) Lynch, S. R. The effect of calcium on iron absorption. *Nutr. Res. Rev*. **2000**, *13*, 141−158.

(27) Sandström, B. Micronutrients interactions: effects on absorption and bioavailability. *Br. J. Nutr*. **2001**, *85*, 181−185.

(28) Jackson, L. S.; Lee, K. The effect of dairy products on iron availability. *CRC Crit. Rev. Food Sci. Nutr.* **1992**, *31*, 259−270.

(29) Hallberg, L. Does calcium interfere with iron absorption? *Am. J. Clin. Nutr*. **1998**, *68*, 3−4.
(30) Whiting, S. J. The inhibitory effect of dietary calcium on iron bioavailability: a cause of concern? *Nutr. Rev.* **1995**, *53*, 77−80.
(31) Ames, S. K.; Gorham, B. M.; Abrams, S. A. Effects of high compared with low calcium intake on calcium absorption and incorporation of iron by red blood cells in small children. *Am. J. Clin. Nutr*. **1999**, *70*, 44−48.
(32) Hallberg, L.; Hulthén, L. Prediction of dietary iron absorption: an algorithm for calculating absorption and bioavailability of dietary iron. *Am. J. Clin. Nutr*. **2000**, *71*, 1147−1160.
(33) Peres, J. M.; Bouhallab, S.; Neuville, D.; Maubois, J. L.; Devroede, G.; Arhan, P.; Bougle, D. Mechanisms of absorption of caseinophosphopestide bound iron. *J. Nutr. Biochem*. **1999**, *10*, 215−222.

Received for review January 16, 2006. Revised manuscript received May 9, 2006. Accepted May 10, 2006. This study is part of the project AGL 2000-1483 financially supported by the FEDER/CICYT (Spain). Thanks to the Generalitat Valenciana for the financial support (03/003) given to the "Bionutest". Sara Perales is the holder of a grant by the Generalitat Valenciana.

JF0601214