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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)

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An adequate calcium intake during the first years of life is needed for normal growth and development and to prevent rickets. The bioavailability of calcium from infant foods (milk-based formulas and fruit juices containing milk and cereals, FMC), the dietary sources of calcium in these stages of life, has been estimated on the basis of simulated gastrointestinal digestion and calcium solubility and dialyzability values and on the efficiency of transport and uptake by Caco-2 cells. The ranking of samples according to calcium bioavailability depends on the use of solubility or dialyzability as criterion. On the basis of the former, the highest value corresponded to adapted formulas and the lowest to fruit juices. However, when using percentage dialysis, the highest value corresponded to fruit juices and the lowest to follow-up formulas. The highest percentages of transport efficiency and uptake by Caco-2 cells corresponded to calcium from the analyzed fruit juices, followed by toddler, follow-up, and adapted formulas.

KEYWORDS: Calcium bioavailability; in vitro digestion; Caco-2 cells; infant foods

INTRODUCTION

Dietary calcium intake is of interest for human beings in general, but particularly for infants and young children in the first years of life, when growth is accelerated. Insufficient calcium intake in this period is responsible for diseases such as rickets. It is now well recognized that an adequate dietary calcium intake is needed for adequate development and contributes to the prevention of rickets (*1*).

Dietary reference intakes (DRI) for calcium have been established in the range of 210−270 mg per day for infants (0−12 months) and 500−800 mg per day for young children (1−3 years) (*2*). In infants, dietary calcium is provided by human milk and/or infant formulas. According to European legislation (Directive 91/321/CEE), adapted formulas, which can be the only food source for infants up to 4−6 months of age, should afford a minimum calcium content of 50 mg/100 kcal. Children from 1 to 3 years of age have a more varied diet, but milk remains the main dietary source of calcium—usually in the form of follow-up or toddler formulas or even cow's milk or derivates. In recent years, fruit juices containing milk and cereals (FMC) have appeared on the market and are beginning to find widespread acceptance.

Considering that the aforementioned foods are often the main (if not the only) dietary sources of calcium for infants and young children, it is of interest to determine the calcium content of these products (adapted, follow-up, and toddler formulas) and also the proportion of this calcium that can be absorbed and used for physiological purposes (i.e., its bioavailability).

The first step defining bioavailability is nutrient solubility in the intestinal tract (bioaccessibility) (*3*). Calcium must be in a soluble form, usually ionized (Ca^{2+}) or bound to a soluble organic molecule, before it can cross the intestinal wall. Calcium bioaccessibility and therefore absorption and bioavailability depend on its solubility within the intestinal lumen, which in turn depends on the composition of the food of origin. Some baby foods, such as fruit juices, contain oxalates, phytates, and tannins that can negatively affect calcium bioavailability. On the other hand, lactose, proteins, and phosphopeptides—components or compounds that can be released from milk during gastrointestinal digestion—maintain calcium in soluble form until it reaches the distal intestine, where it can be absorbed by nonsaturable routes, thus facilitating the intestinal absorption of calcium (*1*, *4*−*6*). The evaluation of calcium bioavailability in humans would be ideal, but human studies are time-consuming, costly to perform, and impractical for large-scale applications. In addition, the common use of radioisotopes in these studies implies a possible hazard that should be taken into account, especially in the case of infants, who are particularly vulnerable (*7*).

In vitro methods for assessing bioavailability constitute good alternatives to in vivo procedures and are generally based on the simulation of gastrointestinal digestion followed by the

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Table 1. Energy Value and Protein, Fat, Carbohydrate, and Calcium Contents of the Analyzed Samples Referred to a 100 mL Ready-to-Eat Sample[a]

	A[s,l]* [b]	F, FB* [b]	T	TB* [b]	FMC[1]	FMC[2]	FMC[3]
energy (kcal)	67.99	65	65	61.49	60	57	56
protein (g)	1.45	1.95	2.6	1.85	0.6	0.4	0.5
casein (g)	0.73	0.96	1.69	1.19	0.48	0.32	0.4
fat (g)	3.77	3.25	2.5	2.6	0.1	0.1	0.1
carbohydrates (g)	7.05	7.0	8.0	7.68	14.3	13.7	13.5
lactose (g)	7.05	3.49	8.0	2.46	1.3	1.2	1.2
maltodextrins (g)	0	3.49	0	5.23	0	0	0
Ca (mg)	50.96	78	100.0	110.5	55	54	50

[a] A, adapted formula [supplemented with (s) ferrous sulfate or (l) ferrous lactate]; F, follow-up formula; FB, follow-up formula with added bifidobacterium; FMC, juice + cereals + milk (1, pineapple and banana; 2, peach and apple; 3, grape, orange, and banana); T, toddler formula; TB, toddler formula with added bifidobacterium. [b] *, powdered samples were reconstituted according to the instructions of the manufacturer (13% w/v).

determination of how much calcium is soluble (*8*, *9*) or dialyzes through a membrane of a certain pore size (*8*−*16*). Solubility or dialyzability in turn can be used to establish trends in the bioavailability or relative bioavailability values of calcium. In fact, such methods estimate only the fraction of the element available for absorption (bioaccessibility), which constitutes the first step in the in vivo process of mineral absorption (*7*).

The aforementioned in vitro methods have been improved by the incorporation of a human colon carcinoma cell line (Caco-2) presenting many of the functional and morphological properties of mature human enterocytes (*17*−*19*). This system is therefore able to rate different food items in terms of bioavailability. It has been applied to calcium in standard solutions and models (*4*, *20*−*22*) to evaluate the effect of dietary factors upon bioavailability. Studies on the uptake of calcium from baby foods, with the purpose of evaluating calcium bioavailability, are scarce (*7*, *23*, *24*), and none of them include a transport step.

The aim of the present study was to estimate the bioavailability of calcium from different milk-based formulas and fruit juices containing milk and cereals (FMC), on the basis of simulated gastrointestinal digestion and calcium solubility and dialyzability values, and to estimate calcium transport and uptake by Caco-2 cells.

MATERIALS AND METHODS

Samples. A total of nine different samples have been studied: two milk-based adapted infant formulas with the same composition except for the iron salt used for enrichment, ferrous sulfate (A^s) or ferrous lactate (A^l); two follow-up formulas, one with added *Bifidobacterium bifidum* and *Bifidobacterium longum* (FB) and the other without such addition (F); two toddler formulas, likewise one containing *B. bifidum* and *B. longum* (TB) and the other without added bacteria (T); three fruit juices containing different fruit juice proportions (51−55%), skimmed milk (6%), and cereals (1%) (FMC), with the addition of calcium and ascorbic acid, intended for infants and young children (FMC[1], pineapple and banana; FMC[2], peach and apple; and FMC[3], grape, orange, and banana).

Samples were kept in their commercial packaging at 25 °C until analysis. The compositions of the aforementioned samples are shown in **Table 1**.

Materials and Reagents. Enzymes and bile salts were purchased from Sigma Chemical Co. (St. Louis, MO): pepsin (porcine, catalog no. P-7000), pancreatin (porcine, catalog no. P-1750), and bile extract (porcine, catalog no. B-8631). The working enzyme solutions were prepared immediately before use.

Calcium standard solutions were prepared immediately before use by dilution with distilled deionized water (DDW) of a standard solution of 1000 mg/L ($CaCl_2$ in 6.5% HCl, Titrisol, Merck, Barcelona, Spain). Lanthanum solution (5 g/100 mL) was prepared with La_2O_3 (Merck).

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

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 calcium determination, glass and polyethylene material were washed with detergent, soaked in concentrated nitric acid (sp gr = 1.41), and rinsed three times with DDW before use.

In Vitro Digestion. The in vitro procedure described by Jovaní et al. (*24*) was applied to the samples. Reduced amounts of enzymes were used, because the gastrointestinal tract in the early stages of life is not yet fully developed (*12*, *13*).

Demineralization. Pepsin, pancreatin, and bile extract were treated with resin (Chelex-100, Sigma Chemical Co.) according to the procedure described by Jovaní et al. (*24*), to remove the calcium from the enzyme preparations.

Briefly, before use, 0.125 g of pepsin was dissolved in 6 mL of 0.1 N HCl; 2 g of chelating resin was added, and the global mixture was shaken in a vessel for 30 min using a magnetic bar (Variomag Mono, Komet, Germany). The mixture was poured into a 2.0-cm-diameter filtration column to filter out the resin from the pepsin solution. An additional 6 mL of 0.1 N HCl was added to the column, and the filtrate was collected into the pepsin solution.

For intestinal digestion, 0.05 g of pancreatin and 0.310 g of bile extract were dissolved in 12.5 mL of 0.1 M $NaHCO_3$. Then 3.12 g of chelating resin was added, and the resulting mixture was shaken for 30 min using a magnetic bar. The mixture was then poured into a 2.0-cm-diameter filtration column to filter out the resin. An additional 5 mL of 0.1 M $NaHCO_3$ was added to the column, and the filtrate was collected into the pancreatin−bile salts solution.

Solubility. To minimize the possible effects due to the different amounts of calcium present in the samples, the weight contemplated in the study differs for each sample and depends on the calcium content in each case. Accordingly, 10 g of adapted formula, 7 g of follow-up formula, 40 g of toddler formula, 5 g of toddler formula with bifidus, and 80 g of FMC were weighed to obtain calcium amounts of close to 40 mg.

Cell culture grade water (Aqua B. Braun, Braun Medical, S.A., Barcelona, Spain) was added to the sample, and the pH was adjusted to 2.0 with 6 N HCl (pH-meter Crison GLP 21, Barcelona, Spain). The pH was checked after 15 min and, if necessary, readjusted to 2.0, and then an amount of freshly prepared demineralized pepsin solution sufficient to yield 0.02 g of pepsin/g of sample was added. The sample was made up to 100 g with cell culture grade water and incubated in a shaking water bath at 37 °C/120 strokes/min for 2 h (SS40-2 Gran Instruments, Cambridge, U.K.). The gastric digest was maintained in ice for 10 min to stop the pepsin digestion.

To facilitate the intestinal digestion stage, the pH of the gastric digests was raised to pH 5.0 by dropwise addition of 1 M $NaHCO_3$. 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/g of 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. Then the pH was adjusted to 7.2 by dropwise addition of 0.5 M NaOH.

Aliquots of 20 g of sample were transferred to polypropylene centrifuge tubes (50 mL, Costar) and centrifuged at 3500*g* for 1 h at 4 °C (Jouan GT422, Saint Nazaire, France), and the supernatants were used to determine the mineral content (solubilized fraction).

Dialysis. Dialysis comprised a gastric step, common to that of the solubility method, followed by an intestinal step in which dialysis is included. The dialysis bag (cutoff molecular weight of 10−12000 Da; Visking 3-20/322, Medicell, London, U.K.) 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/g of aliquot was added, and incubation was again continued for up to 2 h.

The dialysate was taken to measure its calcium content.

Caco-2 Method. *Cell Culture.* Caco-2 cells were obtained from the European Collection of Cell Culture*s* (ECACC 86010202, Salisbury, U.K.) and were used between passages 70 and 80. The cells were maintained in 75 cm^2 flasks (IWAKI brand) in minimum essential medium (MEM; Gibco BRL Life Technologies) with 10% v/v fetal 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) under a 5% CO_2/95% air atmosphere at constant humidity. Culture medium was changed every 2 days. The cells at 70% confluence were harvested by using trypsin−EDTA solution (2.5 g/L trypsin, 0.2 g/L EDTA). After the cells were detached from the flasks, they were suspended in culture medium.

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, whereas water was added to adjust the osmolarity to 310 ± 10 mOsm/kg (freezing point osmometer, Osmomat 030, Berlin, Germany).

The soluble fraction was selected to carry out retention and transport assays with Caco-2 cells, because it is more similar to the in vivo digest. Moreover, the calcium content of the dialysate was lower than in the case of the soluble fractions, because only the soluble forms of calcium with a molecular weight below the cutoff value of the dialysis membrane are able to dialyze—resulting in low additions to the cells and important variability in the uptake assays.

Calcium 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.) 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.

At 19−21 days after initial seeding, spent culture medium was aspirated from the apical and basolateral chambers, and 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 (130 mM NaCl, 10 mM KCl, 1 mM $MgSO_4$, 5 mM glucose, 50 mM HEPES) was added to the basal chamber, and 1.5 mL of 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%.

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% sodium dodecyl sulfate (SDS).

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

Calcium Determination. Total, soluble, or dialysate calcium contents and calcium of monolayer, transport buffer (transport blank) and the basal chamber contents were measured by atomic absorption spectrophotometry (AAS, Perkin-Elmer, model 2380, Boston, MA). All samples, with the exception of the dialysate, were previously subjected to dry digestion at 450 °C. An amount of lanthanum chloride sufficient to yield a lanthanum final content of 0.2% was added to suppress phosphate interferences (*14*).

Table 2. Calcium: Total, Soluble, and Dialysate Contents[a]

sample	total content (mg/g)	soluble fraction (mg/g)	% solubility	dialysate fraction (mg/g)	% dialysis
A[s]	3.1 ± 0.4	3.1 ± 0.2	97.7 ± 7.2a	1.0 ± 0.07	30.8 ± 2.1ab
A[l]	3.3 ± 0.5	3.3 ± 0.4	99.3 ± 11.4a	0.9 ± 0.09	28.7 ± 2.9ac
F	6.0 ± 0.1	5.6 ± 0.07	92.4 ± 1.3ab	1.4 ± 0.1	24.1 ± 2.3c
FB	6.0 ± 0.2	5.6 ± 0.08	92.8 ± 1.4ab	1.6 ± 0.1	26.0 ± 2.5ac
T*[b]	6.7 ± 0.2	5.4 ± 0.3	80.7 ± 4.6c	1.9 ± 0.2	28.9 ± 3.8ac
TB	9.7 ± 0.3	8.2 ± 0.3	84.1 ± 3.5bc	3.5 ± 0.4	36.3 ± 4.5bd
FMC[1]	0.5 ± 0.01	0.3 ± 0.009	67.6 ± 1.9d	0.2 ± 0.009	39.0 ± 2.0d
FMC[2]	0.5 ± 0.02	0.3 ± 0.005	67.9 ± 1.2d	0.2 ± 0.01	39.4 ± 2.6d
FMC[3]	0.5 ± 0.02	0.3 ± 0.02	68.5 ± 6.1d	0.2 ± 0.005	39.1 ± 1.2d

[a] A, adapted formula [supplemented with (s) ferrous sulfate or (l) ferrous lactate)]; F, follow-up formula; FB, follow-up formula with added bifidobacterium; FMC, juice + cereals + milk (1, pineapple and banana; 2, peach and apple; 3, grape, orange, and banana); T, toddler formula; TB, toddler formula with added bifidobacterium. Mean values ± standard deviation ($n = 10$). Noncoincidence of letters (a–d) denotes statistically significant differences ($p < 0.05$) between values in the same column. [b] *, liquid sample (contents are referred to dry product).

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

Dialysis percentages were calculated as follows: dialysis % = 100 × D/C, where D = calcium content of the dialysate (mg of Ca/g of sample) and C = total calcium content of the sample (mg of Ca/g of sample).

Differences between the calcium content of the monolayer incubated with soluble mineral fraction and the content of monolayer not exposed (retention blank) yield an estimation of the cellular retention (micrograms) of calcium. Transport was evaluated by the difference between the calcium amount in basal chamber and transport buffer (transport blank).

Retention percentages were calculated as follows: retention % = 100 × R/C, where R = calcium retention (μg of Ca/well) and C = total calcium content of the sample (μg of Ca added).

Transport percentages were calculated as follows: transport % = 100 × T/C, where T = difference between calcium amount in basal chamber and transport buffer (blank) (μg of Ca/well) and C = total calcium content of the sample (μg of Ca added).

Due to the differences among samples in terms of the solubility of calcium after in vitro digestion, calcium transport and uptake (retention + transport) efficiencies were expressed as follows: % transport efficiency = (% solubility × % transport)/100; % uptake efficiency = (% solubility × % uptake)/100.

Statistical Analysis. Ten aliquots were used in the solubility and dialysis assays and five in the Caco-2 cell assays. Samples with unequal variances were logarithmically transformed and analyzed by one-way analysis of variance (ANOVA). Selected pairs of means were compared by Tukey's test. Values of $p < 0.05$ were considered to be significant.

A simple regression analysis was also applied to the results with the aim of estimating the possible relationship between the calcium content of the samples and calcium solubility, calcium dialysis, or Caco-2 cell transport and uptake efficiency and also the possible relationship between soluble or dialyzable calcium and Caco-2 cell transport and uptake efficiency.

RESULTS AND DISCUSSION

Total, soluble, and dialysate calcium contents are shown in **Table 2**.

The results obtained in the calcium retention and transport assays are summarized in **Table 3**. The uptake fraction is estimated as the sum of Ca retention and Ca transport.

The analyzed samples had calcium contents in agreement with the EU directive (Directive 91/321/EEC) and also with the contents reported on the label by the manufacturer.

Table 3. Calcium: Retention Transport and Uptake by Caco-2 from Infant Foods[a]

sample	calcium added (μg)	retention (μg)	% retention	transport (μg)	% transport	% transport efficiency	uptake[b] (μg)	% uptake	% uptake efficiency
A[s]	445.7	0.4 ± 0.1	0.1 ± 0.01a	7.8± 0.8	1.8 ± 0.2a	1.7 ± 0.2a	8.2 ± 0.7	1.9 ± 0.2 a	1.8 ± 0.2a
A[l]	485.7	1.2 ± 0.1	0.2 ± 0.01b	7.8 ± 0.9	1.6 ± 0.2a	1.6 ± 0.2a	9.0 ± 0.9	1.9 ± 0.2 a	1.8 ± 0.2a
F	613.9	6.1 ± 2.7	1.0 ± 0.4c	10.9 ± 2.4	1.8 ± 0.4a	1.6 ± 0.4a	17.0 ± 1.7	2.8 ± 0.3 b	2.6 ± 0.3b
FB	450.8	3.2 ± 0.4	0.7 ± 0.1c	8.0 ± 1.7	1.8 ± 0.4a	1.6 ± 0.3a	11.2 ± 2.1	2.5 ± 0.5 b	2.3 ± 0.4ab
T*[c]	403.3	4.9 ± 1.1	1.2 ± 0.1c	70.1 ± 10.7	17.4 ± 1.4b	14.0 ± 1.1b	75.0 ± 10.6	18.6 ± 1.4 c	15.0 ± 1.1c
TB	767.3	5.8 ± 0.5	0.8 ± 0.1c	28.8 ± 2.1	3.7 ± 0.5c	3.1 ± 0.4c	34.6 ± 2.2	4.5 ± 0.6 d	3.8 ± 0.5d
FMC[1]	97.5	1.8 ± 0.3	1.8 ± 0.3d	16.5 ± 1.6	16.9 ± 1.7d	11.5 ± 1.1d	18.3 ± 1.9	18.7 ± 2.0 c	12.7 ± 1.3c
FMC[2]	97.0	1.8 ± 0.4	1.9 ± 0.4d	18.5 ± 0.7	19.1 ± 0.7d	13.0 ± 0.5d	20.3 ± 0.8	21.0 ± 0.8 c	14.2 ± 0.5c
FMC[3]	97.7	1.8 ± 0.4	1.8 ± 0.4d	20.2 ± 0.5	20.7 ± 0.5d	14.2 ± 0.4d	22.0 ± 0.8	22.5 ± 0.8 c	15.4 ± 0.6c

[a] A, adapted formula [supplemented with (s) ferrous sulfate or (l) ferrous lactate]; F, follow-up formula; FB, follow-up formula with bifidobacterium; T, toddler formula; TB, toddler formula with bifidobacterium; FMC, juice + cereals + milk (1, pineapple and banana; 2, peach and apple; 3, grape, orange and banana). Mean values ± standard deviation ($n = 5$). Noncoincidence of letters (a–e) denotes statistically significant differences ($p < 0.05$) between values in the same column. [b] Uptake = retention + transport. [c] *, liquid sample (contents are referred to dry product).

As could be expected, soluble fractions had higher calcium contents (milligrams per gram and percent) than dialysates (milligram per gram and percent) (see **Table 2**). This means that under the applied in vitro assay conditions, only calcium soluble compounds of smaller size than the pore size of the dialysis membrane are dialyzed (cutoff molecular weight 10–12000 Da).

The results obtained show that the ranking of samples according to calcium bioavailability depends on the criterion used. When the solubility percentage was used, the highest value corresponded to adapted formulas and the lowest to FMC. In contrast, on using the dialysis percentage, the highest value corresponded to fruit juices and the lowest to the follow-up formulas (see **Table 2**). The analyzed fruit juices clearly differed from the rest of the samples in terms of total calcium contents and calcium solubility and dialyzability.

The two adapted milk-based formulas analyzed had the same composition, differing only in iron salt (lactate or sulfate used for enrichment). No differences were observed between them in terms of calcium dialysis and solubility percentages (**Table 2**) or in terms of calcium transport and uptake by Caco-2 cells (**Table 3**).

Differences between the values of calcium solubility and dialyzability obtained in this study and those reported by other authors (**Table 4**) can be attributed not only to differences in the composition of the samples but also to the methodology applied—particularly with regard to the amount and activity degree of the enzymes used, the pH values and incubation times during the gastric and intestinal steps, and even centrifugation speed in the case of solubility. The enzyme demineralization applied in this study could also have contributed to the observed differences.

In relation to the matrix composition, components of milk-based formulas or fruit juices such as lactose, casein, organic acids, total calcium content, fat, and fiber can affect calcium bioavailability (*5*, *6*). Lactose is well-known to enhance calcium absorption, because it favors diffusion or passive transport and can influence the active transport of calcium (*25*). The role of lactose may be more important when calcium intake is high, especially in infants and the elderly, in whom solubility is a limiting factor and passive absorption is the predominant route (*1*).

It has been reported that in milk-based infant formulas the composition of the protein fraction affects calcium dialysability—the effect being particularly marked when casein is the main protein fraction (*14*, *25*). When casein is digested, the phosphopeptides released exert a positive effect on calcium absorption, through the inhibition of phosphate precipitation in the intestinal lumen, and thereby maintain calcium in a soluble form available for absorption (*4*, *5*). Phosphopeptides also act at cell membrane level, increasing calcium uptake—although the underlying mechanism is not fully clear (*21*, *22*, *26*).

Table 4. Calcium: Solubility and Dialyzability Percentages in Infant (IF) [Adapted (A) or Follow-up (F)] and Toddler (T) Formulas

infant formula	calcium	ref
Solubility		
IF milk based	19.5 ± 1.3	*8*
IF powdered milk based	60.2	*9*
IF liquid milk based	66.5	
Dialysis		
IF milk based	15.0 ± 1.0	*10*
A milk based	21.9 ± 1.4	*11*
F milk based	31.0 ± 2.5	
T milk based	27.7 ± 0.6	
IF milk based	8.1 ± 0.9	*8*
A milk based	5.0–9.1	*14*
F milk based	14.3–17.7	
IF soy based	7.4–20.9	*15*
IF milk based		*12*
gastric pH 2	20.0 ± 1.1	
gastric pH 4	15.6 ± 1.2	
A casein based	21.2 ± 0.6	*13*
A whey based	13.3 ± 1.2	
A soy based	13.0 ± 1.2	
IF powdered milk based	14.8 ± 2.5	*9*
IF liquid milk based	15.6 ± 0.8	
IF milk based	26.03–34.06	*16*

Toddler formulas were those having the highest casein and calcium contents (**Table 1**) and also yielded the highest percentages of transport efficiency and uptake efficiency among milk-based formulas (**Table 3**)—probably due to their greater casein content. These results are in agreement with the observations of increased calcium absorption by Caco-2 cells when casein phosphopeptides are present (*21*, *22*, *26*).

The presence of *Bifidobacterium* did not affect calcium solubility, although a small increase in calcium dialyzability in toddler formulas has been observed, whereas in these formulas a decrease in transport efficiency and uptake efficiency was also recorded (**Table 3**). This could be explained by the different physical states of the formula, T being liquid and TB being in solid/powdered form. The effect of infant formula manufacturing processes on mineral bioavailability has been described. In this

context, liquid formulas contain emulsifiers of a bipolar nature that can attract divalent cations such as calcium, thereby reducing calcium solubility (*9*). This could explain the lower dialysis percentage of calcium from T (liquid) versus TB (solid).

In addition, the contents of early Maillard reaction products are greater in liquid than in solid/powder milk-based formulas. These products may possibly bind calcium, with absorption of the resulting complex (*27*). This factor would explain the more efficient calcium absorption from liquid infant formulas versus powdered formulas in suckling rats (*9*). Such observations are in agreement with the greater efficiency of Caco-2 cell transport and uptake of calcium from the liquid formula versus the solid preparation (**Table 3**).

In fruit juices containing milk and cereals (FMC), an observation of note is the low calcium content and solubility compared with milk-based formulas (**Table 2**). The low calcium solubility could be attributed to the formation of insoluble compounds with fiber from cereals (*5*, *6*, *28*) and to their lower casein phosphopeptide content in relation to milk-based formulas. On the other hand, juices contain citric and malic acids of low molecular weight that form soluble complexes with calcium (*29*, *30*). This would explain the higher calcium dialysis percentages obtained in fruit juices, when compared to milk-based formulas, and also the superior calcium transport efficiency and uptake efficiency by Caco-2 cells (**Table 3**). Increased calcium retention or absorption from fruit juices fortified with calcium citrate or calcium malate versus from milk (*31*) or water (*30*) has been reported in rats. In addition, the presence of carbohydrates (fructose) increases the effect of the mentioned organic acids (*31*). Although the underlying mechanism remains unclear, the enhancing effect of fructose could involve the formation of a calcium–fructose chelate in a similar way as reported for iron (*32*). The analyzed fruit juices containing skimmed milk and cereals (FMC) contained also fructose—unlike the milk-based formulas.

Solubility percentages of calcium would not be a good indicator of calcium bioavailability, considering that the lowest calcium solubility percentage corresponded to toddler formulas and fruit juices, which yielded the highest calcium percentages of transport and uptake efficiency—probably because in these products calcium is in the form of soluble complexes of low molecular weight (particularly in fruit juices). This would explain the higher dialysis percentages found in these products.

Despite the low calcium content of the considered fruit juices when compared with the analyzed milk-based formulas, these juices can be considered a dietary source of calcium when calcium bioavailability—estimated from the percentages of dialysis and uptake and transport—is taken into account.

The results obtained in the regression analysis showed that calcium content affects solubility and dialyzability, because significant correlations were found between calcium content and solubility percentage ($p = 0.0008$), % solubility = 1/(0.01 + 1.88/calcium content) ($r = 0.9047$), and also between calcium content and dialysis percentage ($p = 0.0063$), % dialysis = 28.12 + 5226.11/calcium content ($r = 0.8241$). A high calcium content implies not only a high solubility percentage but also a low dialysis percentage. On the other hand, fruit juices containing milk and cereals (FMC), which had the lowest calcium contents among the analyzed samples, yielded the highest transport efficiency ($p = 0.0006$), % transport efficiency = 1.99 + 5140.62/calcium content ($r = 0.9142$), and uptake efficiency ($p = 0.0005$), % uptake efficiency = 2.55 + 5433.83/calcium content ($r = 0.9157$)—in agreement with the observation by Ekmekcioglu (*18*) that higher calcium contents are associated with lower absorption performance.

The observed lack of correlation between calcium solubility or calcium dialyzability and calcium transport and uptake by Caco-2 cells indicates that although calcium would be in a soluble form adequate for uptake, other factors are involved in absorption—thus corroborating the interest of incorporating Caco-2 cell cultures to in vitro systems to more closely resemble the conditions found in in vivo assays.

Solubility and total cell uptake (cell retention + transport) do not always show parallel trends. Therefore, these parameters cannot be used indistinctly as indicators of the bioavailability of minerals from foods. In this context, assays in Caco-2 cells offer an estimation of the absorption process and can be considered a better indicator of bioavailability than solubility.

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