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ORIGINAL CONTRIBUTION

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Bioavailability of vitamins A and E from whole and vitamin-fortified milks in control subjects

Abstract *Background* Dairy products fortified with vitamins and minerals represent a growing market that is of interest to those sectors of the population with unbalanced diets and increased needs. However, there is little information on the bioavailability of micronutrients in milk products at dietary intake levels. *Aim of the study* To evaluate the bioavailability of vitamins A and E in whole milk and fortified whole and skimmed milk in control subjects. *Methods* A single-dose bioavailability study was performed using three commercially available milks (unfortified whole milk and whole and skimmed milk fortified with vitamins A and E). Nineteen volunteers (10 women and 9 men) ingested 430 ml of each milk on different days. The contents of retinol and α-tocopherol provided in the milks and the retinyl esters and α-tocopherol in triglyceride-rich lipoprotein fractions (TRL) from plasma collected for 6.5 h postprandially were assayed using a quality-controlled HPLC method. The relative absorption of vitamins A and E from milks was calculated on the basis of area under the curve (AUC) versus time curve estimations, adjusted for plasma volume and expressed as percentage of the amount of nutrient provided. *Results* The total amounts of retinol and α-tocopherol provided ranged between 0.48 and 4.15 μmol and 0.41 and 32.49 μmol, respectively. The AUC value of retinyl palmitate in TRL was higher for fortified whole milk than for the other two milks (unfortified whole and fortified skimmed milk). The percent relative absorption of vitamin A did not differ among the three types of milk. The AUC for α-tocopherol was no different after the ingestion of any of these milks. *Conclusion* The mean percentage of retinol absorption was apparently similar for the three types of milk, regardless of the amount of fat ingested with each type of milk and the vitamin A provided.

Key words bioavailability – vitamin A – vitamin E – milk – humans

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Introduction

Milk is an effective delivery vehicle for fat-soluble vitamins, has a long-standing tradition of safety, and is a widely accepted food for enhancing normal growth and development [1]. It is a good source of certain fat-soluble vitamins (i.e., vitamin A), and the fortification of frequently consumed foods has been proven to be an effective and low-cost way to increase the micronutrient supply and reduce the incidence of micronutrient deficiencies [2–6].

A number of factors can influence the final vitamin content in a food, whether related to the treatments and transformations to which the food is subjected in its preparation for consumption or to the storage conditions, and some of these factors may affect the bioavailability of the micronutrients [7]. Moreover, there are several factors that can influence the bio-

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Table 1 Characteristics of the subjects at entrance (9 men, 10 women)

Characteristics	Mean ± SD
Age (years)	22.2 ± 3.0
Body mass index (kg/m^2)	22.3 ± 2.0
Total cholesterol (mmol l^{-1})	4.4 ± 0.8
HDL-c (mmol l^{-1})	1.5 ± 0.4
LDL-c (mmol l^{-1})	2.6 ± 0.8
Triglycerides (mmol l^{-1})	0.7 ± 0.5
Serum retinol (μmol l^{-1})[a]	1.76[a] ± 0.4
Serum retinyl palmitate (nmol l^{-1})	7.1 ± 4.8
Serum α-tocopherol (μmol l^{-1})	24.4 ± 4.1

HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol.
[a]1.9 and 1.4 μmol l^{-1} in men and women, respectively.

availability of vitamins, some of them associated with the individual (nutritional status, age, and lifestyle) [8–10], while others are related to the food (matrix effect, amount of fat, molecular bonds, amount consumed, and chemical forms of the nutrient) [7, 11–13].

Data on the bioavailability of micronutrients (natural or added) from dairy products in humans are very limited and, to our knowledge, only refer to vitamin B12 and folic acid [14, 15], and there is no information related to vitamins A and E. In this context, our aim was to evaluate the bioavailability (relative absorption) of vitamins A and E from commercially available whole and fortified milks in control subjects.

Methods

Study design, subjects, and types of milk

The bioavailability study consisted of a single-dose pharmacokinetic assay involving three types of commercially available milk: whole milk, whole milk fortified with vitamins A and E, and skimmed milk fortified with vitamins A and E. The three types of milk were consumed by all the subjects at 1-week intervals.

Nineteen apparently healthy volunteers (9 men and 10 women) were enrolled in this bioavailability study. Inclusion criteria were: 20–31 years of age, body mass index of 20–25 kg/m^2) and serum retinol and α-tocopherol levels >1.05 and >20 μmol l^{-1}, respectively, indicative of adequate nutritional status and body reserves. Exclusion criteria included consumption of vitamin and mineral supplements, habitual use of drugs or oral contraceptives, dieting, pregnancy, lactation, high-intensity exercise, or chronic or metabolic diseases. The characteristics of the volunteers at the beginning of the study are shown in Table 1.

The volunteers were asked to consume a retinol and provitamin-A-carotenoid-free diet (with the aid of a list of foods that was given to them) over the 24 h preceding each assay in order to reduce possible interferences from previous meals. This length of time was considered to be sufficient to "wash" the intestine by mobilizing any chylomicron particles containing retinol and provitamin A carotenoids from previous meals [16]. After an overnight fast, the volunteers were cannulated and blood samples collected before breakfast (baseline). For the three assays, a common breakfast, consisting of 430 ml of milk plus 10 unfortified biscuits, was provided. The volunteers were asked to consume the breakfast within 10 min, after which blood samples were taken 90 min later and hourly for 5 h.

The three milks were of the same commercial brand (with a high market share) and the fortified milks also contained vitamin D, folic acid, calcium, and phosphorus. The contents of vitamins A and E in the fortified milks were, as specified in the nutrition facts label, 120 μg/100 ml (4.2 μmol l^{-1}) and 1.5 mg/100 ml (34.83 μmol l^{-1}), respectively.

The study procedures were performed in accordance with the Ethical Committee for Clinical Investigation of Hospital Universitario Puerta de Hierro. Subjects were informed about the study and gave their written consent.

Reagents and chromatography

All-*trans*-retinol, retinyl acetate, retinyl palmitate, α-tocopherol, tocopheryl acetate, and ammonium acetate were purchased from Sigma Chemical Co. (Spain). Butylated hydroxytoluene (BHT), potassium hydroxide (KOH), pyrogallic acid, tetrahydrofurane, isopropanol, and water (HPLC grade) were obtained from Carlo Erba (Spain). Ethanol, hexane, acetonitrile, methanol, methylene chloride (HPLC grade), and sodium chloride were from Merck (Spain). Reference materials for the analysis of milks were Infant Formula (SRM 1846) from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) and whole milk powder (CRM 421) from the Laboratory of the Government Chemist (LGC, Teddington, UK).

An ALC/GPC chromatograph (Model 201, Waters Associates, Milford, MA, USA) equipped with a M45 pump, manual injector (Rheodyne) and a data acquisition system (Millenium Station, Waters Assoc., Milford, MA, USA) was used. The chromatographic system consisted of a Spheri-5-ODS column (Brownlee Applied Biosystems, San José, CA, USA) (5 μm, 4.6 mm × 220 mm) with RP-18 guard column (7 μm) with acetonitrile/methylene chloride/methanol (70:20:10, v/v/v) in isocratic elution at a flow rate of 1.3 ml min^{-1} [17]. Ammonium acetate (0.025 M) was

added to the methanol. Analytes were detected with a photodiode array detector (PDA 996, Waters Assoc., Milford, MA, USA) set at 326 nm for retinol and retinyl esters and 295 nm for α-tocopherol and tocopheryl acetate. Retinol, retinyl acetate, retinyl palmitate, α-tocopherol, tocopheryl acetate, and β-carotene were identified by comparing their retention times and online UV-spectra with those of authentic standards, and quantified against standard calibration curves. Other retinyl esters were identified by absorption spectra and quantified, together with retinyl palmitate, using the retinyl palmitate curve.

Analysis of vitamins A and E in the milks

Aliquots of the milks provided to the volunteers were collected and analyzed (in quadruplicate) for vitamins A and E content on the same day of the study. These samples were processed simultaneously by two extraction protocols (with and without previous alkaline hydrolysis of the matrix) to assess both the chemical forms and the total content of the vitamins, as previously described [18]. Quality control was performed using reference materials for milk analyses (from NIST [USA] and LGC [UK]) and the results obtained with the method were in good agreement with the certified values.

Analysis of vitamins A and E in triacylglycerol-rich lipoproteins fractions

Triacylglycerol-rich lipoprotein fractions (TRL) were prepared from plasma (EDTA 7.5%) according to the protocol described by Griffiths et al. [19]. Plasma obtained within 20 min of blood collection was stored at −20°C until analysis. After slow thawing at 4°C, duplicate 0.5 ml samples of plasma from each timepoint were transferred to Eppendorf tubes, overlaid with saline solution (density 1.006 kg l^{-1}) and centrifuged at 12,600*g* for 2 h. The upper layers (TRL fraction) were carefully aspirated, the duplicates were pooled and vitamins A and E were extracted as described elsewhere [20]. Briefly, the TRL fractions obtained from 1 ml of plasma were mixed with 1 ml of ethanol, vortexed and extracted twice with 1.5 ml of methylene chloride/hexane (1:5). The organic phases were pooled, evaporated to dryness and reconstituted with tetrahydrofurane/ethanol (1:1) to be injected onto the HPLC. Using the protocols described, quantification limits were less than 6 nmol l^{-1} for retinol, <1.1 nmol l^{-1} for retinyl palmitate, and <90 nmol l^{-1} for α-tocopherol.

The short-term and long-term precision and accuracy of the analytical method was within accepted values, as verified periodically through our participation in the Fat-Soluble Quality Assurance Programme conducted by the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA).

Statistical analysis

The baseline characteristics of the subjects and the vitamins A and E content in milk and in TRL are expressed as the mean plus or minus standard deviation. The normal distribution of the data was assessed using the Kolmogorov–Smirnov test and the differences between subjects at the beginning of the study by means of Student's *t* test. Except for serum retinol levels, there were no statistically significant differences between men and women at baseline.

The areas under the curves (AUC) of the postprandial responses in TRL fractions versus time were calculated by the trapezoidal method after correction for baseline concentrations. There was no difference between sexes regarding the AUC for the three types of milk (ANOVA) and, thus, the men and women were grouped for the subsequent analyses. Differences in postprandial retinyl esters and α-tocopherol concentrations in TRL fractions after the ingestion of the three types of milk were estimated by paired samples *t* test to assess statistical differences ($p < 0.05$) between groups.

Percentages of relative absorption during the period of study (6.5 h) were calculated on the basis of the AUC values for retinyl esters and α-tocopherol in TRL fractions, correcting for plasma volume (assuming 4% body weight) [21], and expressed against the dose supplied for each type of milk, as determined by HPLC analysis.

Due to the discrepancies between the vitamin content of the milks used as specified in the nutritional label and that determined using a quality-controlled method in our lab, the final percent relative absorption was calculated using the total amount of vitamins as quantified in our laboratory.

Results

Vitamins A and E content in milks used in the bioavailability studies

Vitamins A and E, chemical forms, content, and amounts provided in the study are shown in Table 2. As mentioned in the "Methods" section, the retinol and α-tocopherol contents did not match those reported in the nutritional label; the vitamin E con-

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Table 2 Retinol and α-tocopherol content of the milks used in the study

Milk	Chemical forms of vitamin A	Retinol content (μmol l^{-1}) (mean ± SD)	Retinol supplied (μmol/430 ml) (range)	Chemical forms of vitamin E	α-Tocopherol content (μmol l^{-1}) (mean ± SD)	α-Tocopherol supplied (μmol/430 ml) (range)
Whole (n = 3)[a]	Retinyl palmitate	1.46 ± 0.36	0.48–0.92	α-Tocopherol	1.87 ± 0.63	0.41–1.08
Fortified whole[b] (3.6% fat) (n = 4)	Retinyl palmitate and acetate	7.39 ± 1.35	2.19–4.15	α-Tocopherol tocopheryl acetate	53.03 ± 12.48	16.78–32.49
Fortified skimmed[b] (0.3% fat) (n = 3)	Retinyl acetate	3.33 ± 0.82	1.08–1.99	α-Tocopherol tocopheryl acetate	45.14 ± 12.63	11.01–27.53

[a]Number of cartons analyzed.
[b]Content as specified on the nutritional label: 4.2 μmol retinol l^{-1} and 34.9 μmol α-tocopherol l^{-1}.

centration was higher in both fortified milks, while the vitamin A content was higher in the fortified whole milk and lower in the fortified skimmed milk. The presence of β-carotene in all the milks used was negligible and, for this reason, only free retinol and retinyl esters were used for calculations of the vitamin A absorption.

Postprandial response in the triglyceride-rich lipoprotein fraction (TRL)

Following the consumption of the three types of milk, the concentrations of retinyl palmitate and other long-chain retinyl esters increased in the TRL fractions during the postprandial period. Regardless of the type of milk consumed, the concentration of retinyl palmitate in TRL peaked at ca. 3 h after consumption, and baseline levels were recovered at the end of the period monitored (6.5 h).

The increment in retinyl esters differed depending on the type of milk consumed. As can be seen in Fig. 1, the maximum concentrations reached (after correction for baseline concentrations) after ingestion varied from one type of milk to another. Fortified whole milk provoked an increase of 31.7 nmol retinyl esters l^{-1} (95% CI: 15.8 and 47.9; p = 0.001), greater than that of whole milk, as could be expected, since fortified whole milk provides four times more retinol than unfortified whole milk (Table 2). In contrast, skimmed milk plus vitamins resulted in an increase of 7.8 nmol l^{-1} (95% CI: 3.12 and 12.6; p = 0.003) over that observed with whole milk, despite the fact that fortified skimmed milk provides twice as much retinol as whole milk (Table 2).

The comparison of the response to the ingestion of the two fortified milks in terms of retinyl esters shows that fortified whole milk provoked a greater increase in their concentration (24.06 nmol l^{-1} 95% CI: 9.7 and 38.3; p = 0.002) than fortified skimmed milk, a circumstance that is in accordance with the nearly twofold higher retinol content in fortified whole milk (Table 2).

The AUC corresponding to the response of retinyl esters in TRL for the three assays are shown in Table 3. The most marked response was observed for vitamin-fortified whole milk and the least marked for unfortified whole milk. However, when the AUC response was adjusted for the vitamin A provided to the volunteers, the response in TRL for the three types of milk did not differ significantly (Fig. 2). Similarly, although the absorption ranged widely for all the milks (Table 3), the mean percent absorption was apparently similar for the three types, regardless of the amount of fat ingested with each (i.e., whole

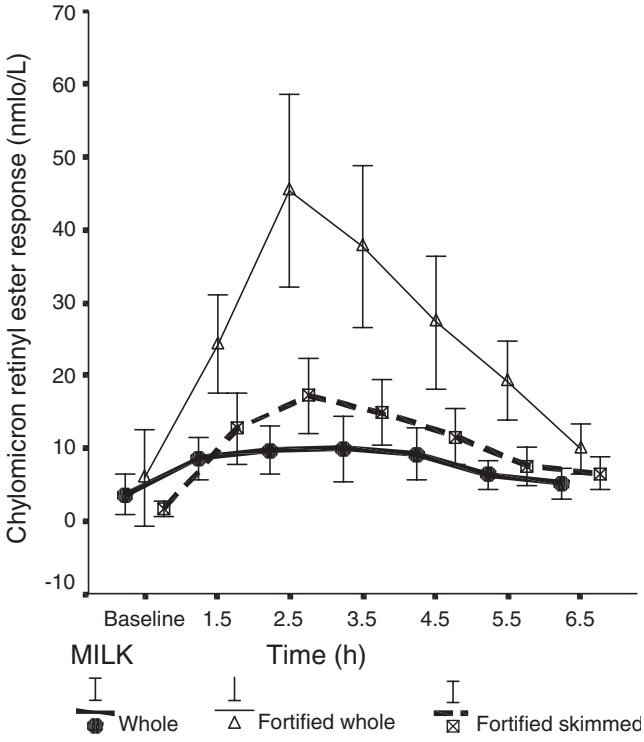

Fig. 1 Retinyl ester response during the postprandial period after the intake of different types of milk. Mean (95% CI)

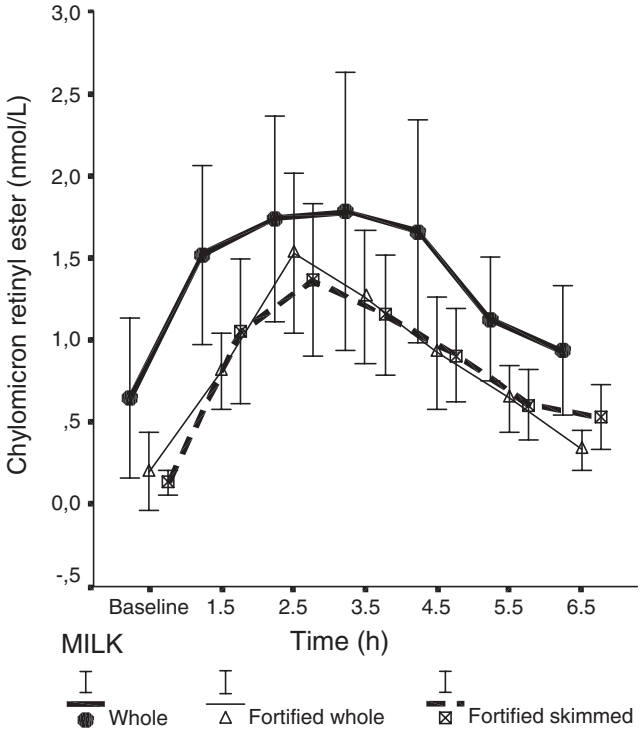

Fig. 2 Dose-adjusted response of retinyl esters in TRL fractions. Mean (95% CI)

versus skimmed) and the amount of vitamin A provided (ca. 0.5–4.0 μmol/430 ml milk).

Between-subject variability in vitamin A response, and thus absorption, was high for the three types of milk (67% for whole milk, 57% for fortified whole milk, and 78% for fortified skimmed milk). Within-subject variability in vitamin A response was 38.5% (95% CI: 28.7 and 48.4) for the three types of milk and significant correlations were observed, especially between the two fortified milks (Spearman $r = 0.753$, $p < 0.001$).

For α-tocopherol, the response in the TRL fractions during the postprandial period did not differ with respect to the baseline at any time-point of the study for any of the milks consumed. Regardless of the dose provided with each milk, the AUC values for α-tocopherol ranged between 1200 and 1400 nmol/l^{-1}/h^{-1}, with a wide within-group variability, and no significant differences were observed in the AUCs corresponding to the three milks.

Discussion

Our study provides information on the absorption of two fat-soluble vitamins (A and E) from whole milk and fortified whole and skimmed milks. At the present time, there is very little information on the bioavailability of vitamins from dairy products, and what information there is focuses on vitamin B12 and folic acid [14, 15]. In our study, the amounts of vitamins A and E ingested were nutritional quantities, not pharmacological doses, and they were provided by a "standard" breakfast, not in concentrates or capsules, a circumstance that differs with respect to previous reports [13, 22, 23].

Stable isotope dilution techniques are considered more accurate and less speculative for the study of nutrient bioavailability, although this approach also has certain disadvantages, such the costs associated with the necessary methodology and the concern as to

Table 3 Response of retinyl esters in TRL fractions during the postprandial period ($n = 19$)

Milk type	AUC (nmol l^{-1} h^{-1}) (mean ± SD)	C_{max} (nmol l^{-1})	Time C_{max} (h)	%Mean absorption (95% CI)
Whole (3.6% fat)	5.4 ± 3.7	10.0 ± 7.0	3.0 ± 1.1	16.4 (11.04–21.7)
Fortified whole (3.6% fat)	22.9 ± 10.6	47.8 ± 24	3.0 ± 0.8	13.6 (9.9–17.4)
Fortified skimmed (0.2% fat)	9.3 ± 5.5	18.3 ± 10.8	2.7 ± 0.9	13.4 (8.4–18.4)

whether the labeled forms undergo absorption and metabolism to the same extent as the endogenous forms. Thus, considering the aim of the present study, i.e., the study of the bioavailability of vitamins A and E from commercially available milks, the use of (intrinsically or extrinsically) labeled vitamins would have been difficult to address and was not among our objectives. An alternative approach is the study of TRL fractions since they represent newly absorbed lipids from recent meals [16, 19]. In this respect, although we did not characterize the TRL fraction and, thus, cannot assure that the entire fraction was isolated, these fractions provided a retinyl ester profile clearly indicative of intestinal origin in the postprandial period (several long-chain fatty acid esters with palmitate representing about two-third of the total), constituting at least qualitative evidence of the nature of the isolated fraction.

It should be pointed out that, in this study, the amounts of the nutrients ingested, as well as the chemical forms of the vitamins present in the different types of milk, were determined on the same day of the assay. This is important since the analysis of the vitamin content in these milks demonstrated a wide variability with respect to that indicated on the label, a circumstance that we had observed in previous analyses using fortified milk [18], coinciding with findings reported by other authors [24, 25].

Interestingly, in this bioavailability assay, the times within which the maximum concentration and subsequent restoration of baseline levels were reached also agreed with those reported elsewhere [23, 26], although in the latter studies, the amounts of nutrients ingested were pharmacological doses and the quantity of fat ingested was also much greater than in our study. Previous studies have reported percentages of absorption of more than 70% [27, 28] or less than 75% [11, 29], depending on the quantity and quality of the fat ingested with the nutrient. In our case, the percentages of relative absorption of vitamin A from the three types of milk were lower, between 2% and 44%. However, it should be noted that the available data on the percent absorption of vitamin A refer to capsules and, in these cases, since the chemical compound is not in the interior of a structure (matrix), the nutrient will be more accessible and, thus, more bioavailable to the organism.

Another factor that can influence the absorption of vitamins is the amount of nutrient ingested [11]. At higher intakes, the absorption capacity is lower owing to limited micellar incorporation, limited capacity for intracellular translocation and limited incorporation into chylomicrons [7]. In our study, the postprandial response of retinyl esters in the TRL fraction was related to the amount provided, except in the case of fortified skimmed milk, in which, as we indicated above, the amount of fat ingested is lower.

The bioavailability of fat-soluble vitamins from the diet is also greatly influenced by fat, since it can provide a hydrophobic environment in which these compounds can become solubilized, and contribute to the stimulation of biliary secretion and, consequently, to the formation of micelles and to the increase in the amount of fat-soluble compounds available for their absorption [7]. However, researchers do not agree with respect to the influence of dietary fat on vitamin absorption. Some studies indicate that the amount of fat ingested in the diet favors the absorption of certain vitamins and related compounds [13], while other authors have observed that vitamin absorption is similar regardless of the consumption of diets with high or low fat content (3–30 g of fat) [22, 30]. In our study, the approximate amount of fat provided (according to the nutritional labels) in the breakfast ranged between 6.2 and 20.8 g, depending on the type of milk ingested (skimmed or whole, respectively). When we compared the increase in the retinyl ester concentration at the different time points after the ingestion of fortified skimmed milk with that observed with unfortified whole milk, the response produced was similar, despite the fact that the amount of nutrient provided was greater with the fortified milk. It could be, in this case, that the low amount of fat influences vitamin A absorption. In addition, despite the fact that the mean percent absorption was apparently similar for the three types of milk, this percentage showed a great within-subject and between-subject variability (60–80%), the latter possibly related to individual differences in fat absorption and/or clearance [11].

On the other hand, some authors affirm that the intake of vitamins A and E in different chemical forms (natural or synthetic) influences the percent absorption, which, in the case of vitamin E, is higher with natural forms than with synthetic preparations [1, 7, 13, 31]. However, the bioavailability of vitamin A when assessed in terms of the different retinyl esters (for example, acetate or palmitate) does not differ [11], a fact that is consistent with our results in the sense that, under the assay conditions (dose, fat, chemical forms), the percentages of relative absorption of vitamin A from the different types of milk was not significantly different.

With respect to vitamin E, the percentages of absorption obtained with the larger quantities provided by capsules ranged between 10% and 95% [1, 30, 32–34], differences that may be due to the variety of experimental and methodological conditions applied in the studies [32]. In our study, the analysis of the TRL fractions did not show a significant increase in α-tocopherol during the postprandial period following the ingestion of any of the milks assayed, a fact that

impeded us from calculating the percent absorption. It could be that the amount of nutrient provided by these three types of milk was insufficient to provoke an evaluable response. In fact, in other studies of the bioavailability of vitamin E in humans [13, 22], an increase in the levels of α-tocopherol in chylomicrons was observed after the ingestion of 300 μmol of vitamin E (in the form of tocopheryl acetate), a much greater amount than that provided in our study with the three types of milk (tocopherol, 0.41–32.19 μmol). However, the response does not appear to depend solely on the amount of nutrient since, using higher amounts, Jeanes et al. [13] did not find variations in the concentrations of labeled α-tocopherol in chylomicrons when the capsule was taken with skimmed milk or with water. Thus, the apparent lack of response in the present study could be attributable, at least in part, to a combination of different factors, including the amount of nutrient ingested, amount of fat, chemical forms of the nutrient, etc.

In summary, the amounts of retinol absorbed from milk increase in relation to the amount provided although the mean percent relative absorption is apparently similar for whole and fortified milks, regardless of the amount of fat ingested with each type of milk (i.e., whole versus skimmed) and the vitamin A provided.

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