Journal of Dairy Research (2002) **69** 473–481. © *Proprietors of Journal of Dairy Research 2002*
DOI: 10.1017/S0022029902005691 *Printed in the United Kingdom*

Influence of enrichment with vitamins and minerals on the bioavailability of iron in cows' milk

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(*Received 25 September 2001 and accepted for publication 28 November 2001*)

Keywords: Iron, milk, dairy products, enrichment, bioavailability.

Iron is an essential element for humans and physiological needs are determined by obligatory loss, growth, menstruation and pregnancy. Although iron metabolism occurs in a 'closed circuit', physiological losses occur and they must be made good. Three factors are important in the study of iron needs: the total amount of iron in the diet; the nature of the iron compound; and other components of the diet (Hallberg *et al*. 1998). Although iron deficiency ensues if insufficient iron is absorbed to meet requirement, the total iron absorbed normally exceeds requirement (Hallberg *et al*. 1997; Jackson, 1997).

That only 5–15 % of the iron in food is actually absorbed is a phenomenon specific to humans (Charlton & Bothwell, 1983). Ferrous forms are absorbed more efficiently than ferric forms (Boccio *et al*. 1997). Certain components of food affect absorption of non-haem iron: fructose, ascorbic acid (Ballot, 1987; Hurrel, 1992), fumarate and some amino acids increase iron absorption (Taylor *et al*. 1986). On the other hand, tannins (in tea, for example) (Tuntawiroon, 1991), polyphenolic compounds (coffee) (Morck *et al*. 1983; Brune *et al*. 1989), phytates (Hallberg *et al*. 1987), phosphates and cellulose plant fibre inhibit absorption of non-haem iron (Rossander *et al*. 1992). The action of HCl in gastric juice is also essential for absorption of non-haem iron (Hurrell, 1992).

Milk contains components necessary for a nutritionally balanced and healthy diet (Favretto & Marletta, 1989; Coni *et al*. 1995). In some population groups, such as children and the elderly, it may constitute the main food (Ziegler & Fomon, 1996). It may be an important dietary source of minerals such as calcium and phosphorus.

In the present study we determined the amount of iron in 40 milk samples including whole, skimmed and low-fat milk, milks enriched with vitamins and minerals, and dairy products. Atomic absorption spectroscopy with electrothermal atomization (ETAAS) was the analytical technique used. We also estimated the influence of enrichment of milk with minerals such as Ca on iron bioavailability using an *in vitro* assay to determine the absorbable fraction of the element.

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materials and methods

Apparatus

We used a Perkin-Elmer 1100B double-beam atomic absorption spectrophotometer equipped with a deuterium background corrector (Perkin-Elmer Corporation, Norwalk, CT 06859, USA). Atomization was performed using a Perkin-Elmer HGA-700 graphite furnace with pyrolytically coated graphite tubes and a L'vov platform; argon of 99·99% purity was the internal gas, and an Fe hollow cathode lamp (Perkin-Elmer) was used. A wavelength of 248·3 nm was selected.

A temperature- and time-controlled digestion block (Selecta; J. P. Selecta, E-08630 Barcelona, Spain) and pyrex tubes were used for sample mineralization. A thermostatic shaking water bath (Selecta), and a Radiometer model 26 pH meter (Radiometer, Copenhagen N.V., Denmark) were used for *in vitro* assays.

Reagents

All solutions were made with double-distilled deionized water (specific resistivity 18 MΩ·cm), obtained by filtering distilled water through a Milli-Q purifier (Millipore, F-91191 Gif-sur-Yvette, France) immediately before use. A standard solution of Fe (Titrisol, $1{\cdot}000 \pm 0{\cdot}002$ g/l) was from Merck (D-64293 Darmstadt 1, Germany). Calibration plots were prepared daily using solutions ranging from 10 to 50 ng/ml. Other reagents were Suprapure nitric acid, Suprapure perchloric acid, and ammonium molybdate and magnesium nitrate used as chemical modifiers. Analytical-reagent grade vanadium pentoxide was used as a catalyst. All reagents were from Merck.

A reference material of certified Fe content (CRM No. 063 R, Trace and major elements in skim milk powder) was supplied by the Community Bureau of Reference (CBR) of the Commission of the European Communities (B-2440 Geel, Belgium).

Reagents used for bioavailability assays were HCl, sodium bicarbonate, sodium hydroxide (in pellets), sodium dodecylsulphate (SDS), ethanol and ethylenediaminetetraacetic acid disodium salt (EDTA Na_2) (Merck); pepsin from porcine stomach mucosa (1:10000 Sigma P-7000; Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), bile salts (approximately 50% sodium cholate and 50% sodium deoxycholate, Sigma B-8756) and pancreatin (EEC 232.468.9, porcine pancreas, Sigma P-1500; Sigma Chemical Co., St Louis, MO); tampon solution (S100; pH $6{\cdot}50 + 0{\cdot}02$ at 20 °C; Radiometer, Demmark) was used to calibrate the pH meter.

For the bioavailability assays, we used Spectra dialysis membranes, dialysis tubing (Visking Size 3-20/32), with a molecular mass cut-off (MMCO) of 12–14 kDa (Medicell International Ltd, London, UK), and dialysis membrane shutters (Medicell International).

To eliminate absorbance due to detergents and samples, all glassware and polyethylene material was washed with double-distilled water after each use, soaked in nitric acid solution (300 ml/l) for 24 h, rinsed several times in double-distilled deionized water, dried in air (Alegría *et al*. 1990; Cornelis, 1993) and kept in a clean place.

Samples

We analysed 40 samples of milk and dairy products of different brands, packed in Tetrabrik® containers. Samples represented the types of milk consumed most frequently in Andalusia (southern Spain), and were classified according to their fat content and enrichment with vitamins and minerals.

Table 1. *Validation of the ETAAS method for determination of iron: recovery of iron from a certified reference material*

(Values are means ± SD, at a 95% confidence level, $n = 10$)

	Fe content (mg/kg DM)			
Reference material	Certified	Obtained	Recovery, %	RSD, %†
No.063 R-BCR skim milk powder	2·32 ± 0·23	2·29 ± 0·10	98·70 ± 0·40	4·37

† Residual standard deviation ($n = 10$).

We analysed 11 whole milk samples, nine skimmed milk samples, eight low-fat milk samples, six low-fat milk samples enriched mainly with vitamins (A, D and E) and minerals (calcium and phosphorus), three skimmed milk samples enriched mainly with vitamins A, D and E, and calcium and phosphorus, one whole milk sample enriched with calcium, and two dairy products. These were not considered milk because all animal fat had been removed, and vegetable fats such as olive oil, sunflower oil enriched in oleic acid, and corn, soy, cotton and coconut oils, palm fat, and certain nutrients (vitamins A, D and E, and Ca and P) had been added. These products are sold in Spain as skimmed milk, and their label contains the information 'skimmed cow's milk with vegetable fat (olive/sunflower oil)'. Three items (each from a different batch) of each type and brand of milk were obtained.

Sample mineralization procedure for determination of total Fe

A 1·0-ml sample was weighed and treated with 5 ml HNO_3 (650 ml/l) and a few micrograms of vanadium pentoxide. The mixture was mineralized in a digestion block at 60 °C for 30 min and at 120 °C for 60 min. Then 1 ml $HClO_4$ (700 ml/l) was added to the samples of whole and low-fat milk, and the mixture kept at 120 °C for 60 min. The solution was transferred to a glass volumetric flask, diluted with double-distilled deionized water to a volume of 25 ml and Fe determined by ETAAS. All determinations were done in triplicate.

Determination of Fe by ETAAS

We optimized analytical conditions for Fe determination by ETAAS using assays of the samples and the reference material of certified Fe content. Results of method validation with the reference material are shown in Table 1. Mean percentage recovery of known amounts of analyte to five different randomly chosen samples, processed as described above, was 98·60 ± 0·75%.

Graphite furnace conditions were optimized on the basis of time-temperature assays. For Fe determination in samples of milk and dairy products, mineralization of the matrix was complete after heating to 1400 °C for 10 s. The atomization temperature yielding maximum signals was 2400 °C. Sample injection volume was 10 μl. This program was slightly modified to determine Fe in the dialysates (bioavailability assays) because of the complexity of the matrix. Mineralization temperature was 1400 °C for 20 s, using 50 μg of $Mg(NO_3)_2$ as matrix modifier (injection volume of 10 μl). We pretreated the graphite tubes with saturated ammonium molybdate and heated them several times in accordance with the temperature program. All determinations were performed in triplicate, and the same procedure was used to run the blanks.

To evaluate the analytical characteristics of the method, the detection limit and the characteristic mass were calculated according to IUPAC rules (Long &

Winefordner, 1983). Precision was measured on ten replicate determinations of each of three different samples (Horwitz *et al.* 1990). To detect possible interferences and matrix effects, the standard addition method was used with five arbitrarily chosen samples; because the blank/addition slope values obtained were close to unity, no further checks were considered necessary (Cuadros *et al.* 1995), and the use of the standard addition method was avoided. Quality parameters of the proposed method are shown in Table 2.

Bioavailability assays

We used the method of Miller *et al.* (1981) with pH, incubation time, sample weight and characteristics of the membranes modified according to the protocol of FLAIR concerted action No. 10 (Shen *et al.* 1996). Before use, the dialysis membranes were conditioned by placing 25 cm membrane (one for each experiment) in a glass beaker containing a solution containing 0·01 M-Na_2-EDTA, 20 g $NaHCO_3$/l, and 1 SDS g/l and boiling for 30 min, rinsing with double-distilled deionized water and storing in ethanol (200 ml/l) at 4 °C. They were then allowed to warm to room temperature for 3–4 h and washed inside and outside with double-distilled deionized water. One end of the membrane was closed with a shutter blind, and the assemblage was left in double-distilled deionized water until use.

Assays were done in three stages. To simulate gastrointestinal digestion, 10·0 g homogenized sample and 80 g double-distilled deionized water were placed in a 250-ml Erlenmeyer flask, the pH was adjusted to 2 with 6 M-HCl; then, 3 ml freshly prepared pepsin solution was added, and the sample volume was brought up to 100 g with double-distilled deionized water. The flask was sealed and incubated in a shaking water bath at 37 °C for 2 h, then placed in an ice-bath for 90 min to stop digestion; during this time titratable acidity was calculated.

Titratable acidity was defined as the number of equivalents of 0·5 M-NaOH required to titrate the combined pepsin-digested pancreatin–bile extract mixture to pH 7·5. This was determined in a 20-g aliquot of the pepsin digest to which 5 ml pancreatin–bile extract mixture had been added, with 0·5 M-NaOH at 20 °C. Samples were left to settle for 30 min and pH was again checked and adjusted to pH 7·5.

To simulate intestinal digestion, a 20-g sample of the pepsin digest was transferred to a 250-ml beaker and incubated in a water bath at 37 °C for 5 min. Then a dialysis membrane tube containing 25 ml double-distilled deionized water and an amount of $NaHCO_3$ equivalent (in moles) to the titratable acidity measured previously, were placed in the 250-ml beaker, and incubated in the water bath at 37 °C for 30 min. Then pH was measured and 5 g pancreatin–bile extract mixture added. Incubation was continued at 37 °C for 2 h. The dialysis tubing was removed, rinsed several times with double-distilled deionized water and dried carefully. Volume and weight of the dialysate were recorded. Fe content of the dialysate was determined by ETAAS. Blank samples were processed in the same way. All determinations were done in triplicate.

RESULTS

Concentrations of Fe were 0·087–0·486 mg/l in the different types of milk and dairy products (Table 3).

Increasing numbers of studies on the nutritional value of diets consumed by different populations conclude that significant differences in nutrient intakes occur,

Table 2. *Quality parameters of the analytical method for determination of iron by ETAAS*

Detection limit† (pg)	Characteristic mass‡ (pg)	Precision§ (RSD, %)	Blank to sample slope ratio‖
6·0	10·0	4·00–5·50	0·996–1·010

† Calculated according to the IUPAC rules (corresponding to three times the standard deviation from blank).
‡ Characteristic mass expressed as pg/0·0044 A-s.
§ RSD, relative standard deviation; measured in 10 determinations on three different samples.
‖ Determined in five samples by the standard additions method.

Table 3. *Iron concentrations in the various milks and dairy products*

	Whole milk ($n = 11$)	Skimmed milk ($n = 9$)	Low-fat milk ($n = 8$)	Enriched† low-fat milk ($n = 6$)	Enriched† skimmed milk ($n = 3$)	Enriched† whole milk ($n = 1$)	Dairy products ($n = 2$)
Average, mg/l	0·201	0·213	0·227	0·153	0·149	0·096	0·184
SD	0·084	0·092	0·147	0·057	0·082	—	0·008
Minimum, mg/l	0·093	0·111	0·093	0·087	0·088	—	0·174
Maximum, mg/l	0·345	0·401	0·486	0·246	0·243	—	0·186
95% confidence level	0·056	0·071	0·123	0·060	0·985	—	0·076

† Enriched with vitamins (A, D and E) and minerals (Ca and P).

Table 4. *Nutritional density of iron (mg/1000 kcal) in the various milks and dairy products*

	Fe nutritional density
Whole milk	2·28–2·48
Low-fat milk	2·65–3·21
Skimmed milk	3·52–4·49
Enriched whole milk	1·43
Enriched low-fat milk	1·76–1·80
Enriched skimmed milk	3·37–3·40
Dairy products	3·18–3·20

depending on the nutritional density (ND) of the diet (Windhan *et al.* 1983*a*, *b*). To evaluate iron content in milk and dairy products, nutritional density was calculated from the following expression (Hansen, 1979): ND = mg of nutrient/1000 kcal. Table 4 summarizes the ND of iron for each type of product analysed according to the Fe concentrations we obtained.

To evaluate differences among the Fe contents of the samples, ANOVA was used after normality of the distribution was confirmed with the Kolmogorov–Smirnov test ($P = 0{\cdot}2308$) and homoscedasticity was checked with the Bartlett test ($P = 0{\cdot}0933$). ANOVA failed to reveal statistically significant differences between the types of milk and dairy products ($P > 0{\cdot}05$).

Assays *in vitro* showed statistically significant differences ($P < 0{\cdot}05$) in the absorbable fraction of Fe in the different types of milk. The absorbable fraction of Fe in whole milk (with an average value of 7·13%) was similar to the percentage in low-fat milk (with an average value of 7·18%). In skimmed milk, the average value obtained was slightly lower at 5·55%. Ca content in these milk samples ranged from 1100 to 1200 mg/l. In milk enriched with Ca (1600 mg/l), the absorbable fraction decreased, being somewhat higher in low-fat milk (5·62%) than in skimmed milk (4·32%) and whole milk (3·79%). The lowest percentage is shown in dairy products

Table 5. *Absorbable fraction of Fe in the various milks and dairy products*

(Values are means ± SD for three replicate assays of each sample)

	Fe content, mg/l	Absorbable fraction, %
Whole milk-1	0·136 ± 0·008	7·05 ± 0·39
Whole milk-2	0·152 ± 0·009	7·21 ± 0·42
Low-fat milk-1	0·112 ± 0·005	7·26 ± 0·50
Low-fat milk-2	0·139 ± 0·006	7·10 ± 0·30
Skimmed milk-1	0·111 ± 0·005	5·65 ± 0·35
Skimmed milk-2	0·145 ± 0·007	5·46 ± 0·20
Enriched whole milk	0·096 ± 0·005	3·79 ± 0·20
Enriched low-fat milk	0·087 ± 0·005	5·62 ± 0·32
Enriched skimmed milk	0·117 ± 0·006	4·32 ± 0·10
Dairy product	0·186 ± 0·007	1·70 ± 0·10

with an average value of 1·70 %. Results are summarized in Table 5. Bioavailability of Fe is expressed as the percentage of Fe dialysed in relation to the total amount of the element in the sample.

DISCUSSION

Although our findings are similar to those of earlier studies, the considerable variability in the data published by different authors is worth noting. Pennington & Young (1990) reported a mean Fe concentration of 0·200 mg/kg in low-fat milk, 0·200 ± 0·300 mg/kg in whole milk and 0·300 ± 0·600 mg/kg in skimmed milk. Favretto & Marletta (1989) found Fe levels between 0·128 and 0·564 mg/kg in whole milk. Wong *et al.* (1978) found Fe concentrations of 0·434 ± 0·104 mg/kg in whole milk, 0·298 ± 0·051 mg/kg in low-fat milk and 0·349 ± 0·044 mg/kg in skimmed milk.

Several authors (Shils *et al.* 1994; Coni *et al.* 1995; García *et al.* 1999) concluded that assessments of the content of trace elements in milk and dairy products should not be based exclusively on calculations from food tables, but should also take into account the loss or gain of trace elements during food processing. Consequently, regular determinations are advisable.

According to the Ministry of Agriculture, Fisheries and Food (2000) the mean daily milk consumption in Spain is 0·325 l/person. From the iron concentrations in the milk samples in the present work, the average daily Fe intake in Southern Spain in the region of Andalusia can be estimated at around 0·06 mg/d. The recommended daily dietary intake of Fe in adults is 10–15 mg (National Research Council, 1989).

Bioavailability of minerals and trace elements has assumed increasing nutritional importance. Bioavailability should be determined by measurements *in vivo* (Southgate *et al.* 1989); however, *in vitro* methods that simulate digestion are an alternative to *in vivo* procedures for calculating the percentage of an element that is transformed into absorbable forms in the digestive tract (Forbes, 1989; Hallberg *et al.* 1997). Although *in vivo* processes cannot be simulated exactly, *in vitro* methods may predict the amount of a given element that can be absorbed from milk (Brown *et al.* 1990). Results of such studies are usually expressed as the soluble fraction of the element under given experimental conditions of pH, enzyme addition, temperature, incubation time, etc. In particular, the *in vitro* procedures based on the method developed by Miller *et al.* (1981) have been shown to provide availability measurements that correlate well with *in vivo* studies (Miller & Berner, 1989). This *in vitro* method is a rapid, simple and cheap alternative to *in vivo* methods for measuring bioavailability of Fe in milk and dairy products.

The observed decrease in the absorbable Fe fraction might be due to the extra Ca in enriched milk and also to other differences of composition (Fox, 1985). Large amounts of Ca in dairy products may lower the solubility of Fe (National Research Council, 1989). When the amount of calcium is increased, the amount of phosphorus increases too (Amiot, 1991). On the other hand, Fe in whole milk is distributed amongst the casein, whey and low molecular weight fraction, and fat fractions. This distribution changes in skimmed milk and leads to differences in absorbable iron (Amiot, 1991). We observed the lowest percentage of absorbable Fe in the dairy products we analysed, which may be due to the fact that their overall composition is different from that of milk.

The mechanism by which calcium affects iron absorption is not clear. Calcium salts may change the physicochemical state of iron salts and render them less available for absorption. Another possible explanation is that competition between calcium and iron for common acceptors of calcium may hinder or prevent the movement of iron from the mucosal cell into the circulation (Van-Dyck *et al.* 1996).

Cabrera *et al.* (1996) measured Fe levels in typical dairy products (condensed and powdered milk, children's milk, yogurt, curd, ice-cream), and determined the absorbable fraction of this element using *in vitro* techniques that simulate human gastric and intestinal digestion. Mean absorbable fraction of Fe in the simulated gastric digestion of dairy products was 15 %, which increased to 20 % after gastric plus intestinal digestion, findings that agree with those reported by National Research Council (1989) and Lombardi-Boccia *et al.* (1994). Different solubilities of Fe in the two phases of digestion indicate the formation during digestion of factors affecting solubility. A critical factor is the formation of complexes at the low pH of gastric digestion. In the intestinal phase of digestion, at pH values of 7·5, intermediate products of protein digestion may undergo other interactions, thereby forming peptide aggregates from which Fe is unavailable; hence the intermediate products of protein digestion may be responsible for Fe binding and solubilization (Lombardi-Boccia *et al.* 1994). Casein may also reduce availability of iron.

Bosscher *et al.* (2001) measured Fe availability from first-age infant formula (with an average value of 41·4 μg/g) and human milk (with an average value of 0·3 μg/g) by an *in vitro* method; Fe availability was 1·02–1·50 % and 8·98–9·10 %, respectively. Hendricks *et al.* (2001) examined the solubility and relative absorption of iron in milk-based liquid infant formulas; mean absorption of Fe was 0·69 % in inorganic formula, 1·86 % in organic formula and 16·59 % in human milk.

Anand & Seshadri (1993) investigated components of foods that influence iron bioavailability, and developed a formula to describe their effects. Bioavailability of iron was enhanced by ascorbic and citric acids, and impaired by calcium phosphate and tannins ($Y = 4{\cdot}29 + 0{\cdot}0137\ A + 0{\cdot}0020\ C - 0{\cdot}0062\ CP - 0{\cdot}0026\ T$; where Y = bioavailability of iron, A = ascorbic acid, C = citric acid, CP = calcium phosphate, T = tannins).

In summary, whenever the overall composition of milk is modified, the bioavailability of some minerals such as Fe is affected. These changes must be assessed and, if possible, quantified. Future research should aim to obtain definitive information on these mineral interactions in a food of great nutritional importance, such as milk. The findings will be useful in designing rational and effective fortification procedures.

We thank Yolanda Garín for translating the original manuscript into English and to K. Shashok for revising the translation.

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