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Speciation of bioaccessible (heme, ferrous and ferric) iron from school menus

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Abstract A spectrophotometric method using bathophenantroline as reagent has been optimized for iron speciation [ionic Fe(II) and Fe(III)] in the mineral soluble (bioaccessible) fraction obtained from the in vitro digestion of food dishes. The effect of the precipitant and reducing reagents, and the amount of sodium nitrite added was studied. Heme-Fe was estimated by subtraction of ionic Fe from the total bioaccessible Fe (determined by atomic absorption spectrometry). The method was applied to 13 dishes included in school menus. Soluble Fe was mainly in ionic form (49–100%). With the exception of spinach and potato omelets, a significant linear correlation (r=0.92) was obtained between Fe(II) and bioaccessible Fe. The Fe(II)/Fe(III) ratio increased with increasing meat protein content in the dish. In the analyzed dishes, heme-Fe content depended on meat content and also on the processing procedure applied.

Keywords Iron speciation · Bioaccessibility · Meals · Dishes

Introduction

Speciation of iron (Fe) in foods is of nutritional interest due to the importance of Fe chemical species in determining bioavailability. It is well-known that the absorption of heme-Fe is more efficient (15%) than that of non-heme Fe (<5%) [1].

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Non-heme Fe can be found in foods as Fe(II) or Fe(III), the availability of the former being greater than that of Fe(III), because the latter is less soluble in the intestinal lumen than Fe(II) [2]. Non-heme Fe is reduced within the lumen by dietary or endogenous factors such as ascorbic acid and glutathione, or by ferrireductase at the brush border surface, prior to membrane translocation of Fe(II) [3].

The absorption of non-heme Fe can be affected by compounds released from foods during digestion, that act as enhancers (meat proteins, ascorbic acid) or as inhibitors (phytic acid, fiber, polyphenols) of absorption [4, 5].

In vivo and in vitro methods can be used to estimate iron bioavailability. In vitro models include simulated human gastrointestinal digestion and the measurement of mineral elements which, under these conditions, pass into the soluble fraction or are dialyzed through a dialysis membrane of a certain pore size. In fact, these methods measure mineral bioaccessibility i.e., the fraction of total mineral in the food that is available for uptake by the brush border cell membranes. This represents the first step of bioavailability, which furthermore also comprises metabolization and use for normal body functions. In the case of Fe, such in vitro assays are useful for predicting its availability, due to the good correlations obtained between the results of in vitro and in vivo assays [6], and also for establishing food rankings according to Fe availability.

Different chelating compounds have been used for estimating non-heme Fe from foods, including: ferrozine, bathophenantroline or α–α dipyridyl (with prior elimination of interferences by precipitation) followed by spectrophotometric assay. These methods have been used for the speciation of soluble Fe from vegetal foods [7, 8], and for measuring non-heme Fe in meat products [1, 9–11], and also in dishes or meals [12, 13]. Spectrophotometric methods have also been applied to the speciation of Fe(II) and Fe(III) in beans [14] and in both in vivo [15] and in vitro [16–19] assays of Fe availability, to measure non-heme Fe contents.

Several studies have shown that in meat and seafood, cooking, freezing, freeze-thaw cycling, and storage decrease and increase, heme and non-heme Fe contents,

respectively [20, 21]. On the other hand, the changes experimented by foods during gastrointestinal digestion could affect Fe species in raw and cooked food, upon reaching the small intestine lumen [3].

The present study describes a useful method for Fe speciation [heme and non-heme Fe (ferrous and ferric)] and its application to the bioaccessible Fe fraction of dishes from school menus.

Materials and methods

Samples

Thirteen dishes commonly found on Spanish school menus were analyzed, differing in formulation and preparation methods, and having the following main ingredients: cereals (Cuban style rice, rice with lean meat, spaghetti with sausage and macaroni with tuna), legumes (lentils with sausage and legume stew), meat (chicken in breadcrumbs with vegetable stew, chicken with sauce, potato stew), fish (fried hake and pre-cooked hake filet) and eggs (potato and spinach omelet). The dishes and ingredients are reported in Table 1.

Sampling was carried out in the kitchen of an important catering company in Córdoba (Spain), that cooks and distributes them to different schools. Dishes were homogenized with an electric mill to obtain small particles; these were then, transferred to polypropylene flasks, frozen and stored at −20 °C until analysis.

In vitro digestion: Solubility method

A modification of the method developed by Sahuquillo [22] was used. Distilled-deionized water (DDW) water (Millipore-Milli Q; Millipore Ibérica S:A:, Barcelona, Spain) (60 ml) was added to 30 g of meal, and the pH was adjusted to 2.0 with 6N HCl. After 15 min the pH value was checked, and if necessary readjusted to pH 2. In order to develop the pepsin-HCl digestion, 0.5 g of pepsin solution (1.6 g of pepsin, P-7000, from porcine stomach, (Sigma Chemical Co. St. Louis, MO, USA) in 10 ml of HCl 0.1 M per 100 g of sample was added. The mixture was then incubated for 2 h at 37 °C in a shaking water bath.

Prior to the intestinal digestion step, the pH of the gastric digests was raised to pH 5 by drop-wise addition of 1 M $NaHCO_3$. Then 18.8 ml of the pancreatin-bile salt mixture (0.4 g of pancreatin, P-170, from porcine pancreas and 2.5 g of bile salt B-8631 porcine, Sigma Chemical Co. St. Louis, MO, USA) in 100 ml of 0.1 M $NaHCO_3$.was added and incubation was continued for an additional 2 h. To stop the intestinal digestion the sample was kept for 10 min.

Table 1 Description of the dishes of the school menu

Type	Dish	Formulation/preparation
With cereal base	Cuban style rice	Rice[a], chicken broth, fried tomato[a], sausage[a], sunflower oil, garlic and salt
	Rice with lean meat	Rice[a], lean meat[a], mushrooms, peppers, green peas, tomato, onion[a], sunflower oil, garlic and salt
	Spaghetti with sausage	Spaghetti[a], sausage, tomato, sunflower oil and salt
	Macaroni with tuna	Macaroni[a], tuna, tomato, sunflower oil and salt
With leguminous base	Lentils with sausage (chorizo)	Lentils[a], sunflower oil, mashed tomato, salt, coloring, laurel, chicken broth, carrot, sausage[a], onion, pepper, garlic, potatoes[a]
	Stew	Chickpeas[a], green beans, carrot, pork bone, chicken, potato[a], veal ragout[a], salt pork, chicken broth and salt
With tuber base	Potato stew	Potatoes[a], veal ragout[a], white wine, mashed tomato, carrot, chicken broth, onion, pepper, sunflower oil, laurel, coloring and salt
With meat base	Chicken in breadcrumbs with vegetable stew	Chicken in breadcrumbs[a], vegetables and sunflower oil
	Chicken in sauce	Chicken breast[a], chicken broth, flour, onion, almonds, sunflower oil, potatoes, coloring matter and salt
Fish	Fried hake	Hake filet and sunflower oil
	Precooked hake filet	Hake in breadcrumbs and sunflower oil
	Spanish/potato omelet	Potatoes, egg, sunflower oil and salt
	Spinach omelet	Spinach, eggs, sunflower oil and salt

[a]Main ingredients

in an ice bath. The pH was adjusted to 7.2 by drop-wise addition of 0.5 M NaOH. Aliquots of 20 g of the digested sample were transferred to polypropylene centrifuge tubes (50 ml, Costar Corning Europe) and centrifuged at 3500×*g* for 1 h at 4 °C. Finally the supernatant (soluble fraction) was collected.

Total bioaccessible iron determination

Aliquots of 2.5 g of soluble fraction were dried at 100 °C on a hot plate (Magnetic stirrer hotplate SM6, Bibby Stuart Scientific, UK) and ashed at 450 °C in a muffle furnace (Heraeus M1100/3, Hanau, Germany) during 12 h, adding concentrated HNO_3 to obtain white ashes. These were dissolved in concentrated HCl and DDW up to a volume of 10 ml. Total soluble Fe was measured by flame atomic absorption spectrophotometry (Perkin-Elmer, Model 2380, Norwalk, CT, USA) under the following instrumental conditions: (wavelength: 248.3 nm; slit width: 0.2 nm; lamp current: 30 mA; acetylene flow: 2.7 l min^{-1}; air flow: 17.5 l min^{-1}; nebulizer: impact ball).

Non-heme Fe (ionic) determination

Ionic Fe (Fe (II) + Fe (III)), to 2.5 g of soluble fraction, 0.2 ml of 0.39% sodium nitrite, DDW in sufficient amount to complete the weight to 5 g, 2.5 g of a mixture containing trichloracetic acid-HCl (TCA-HCl) and hydroxylamine hydrochloride, in percentages of 10 and 12.5% (w/v), respectively were added. The whole was incubated at 100 °C for 10 min and then centrifuged (3300×*g*/15 min/ 10 °C). Two ml of supernatant aliquot were transferred to a 1-cm spectrophotometric cell and 1 ml of bathophenantroline solution (12.5 mg of bathophenantroline (4, 7-diphenyl-1, 10-phenanthroline disulfonic acid (Sigma B-1375) and 8.203 g of sodium acetate in 50 ml of DDW) was added. The preparation left to stand for 10 min, and absorbance at 535 nm was then measured against a reagent blank using an UV-visible spectrophotometer (Perkin-Elmer, Lambda-2, Norwalk, CT, USA).

- *Fe(II):* The procedure described for ionic Fe was applied, though using a solution containing TCA-HCl 10% (w/v).
- *Fe(III):* This was calculated from the difference between the ionic Fe and Fe(II) contents.

Heme-Fe content in the mineral soluble fraction of the digest was estimated from the difference between total bioaccessible Fe and ionic Fe.

Statistical analysis

A *t*-test was applied for method optimization and a simple regression model was used to evaluate the possible relation between different Fe species. A probability level of 95% was used throughout the study. Statistical evaluation of the data was carried out with the Statgraphics Plus 4.0 statistical package for Microsoft Windows.

Results and discussion

Optimization of the method

Effect of the protein precipitating solution (TCA-HCl)

The possible co-precipitation of Fe(III) and proteins when TCA is added was reported by Carter [9], who proposed using ascorbic acid to ensure complete reduction of Fe(III) to Fe(II) prior to adding TCA. Ascorbic acid addition was not possible in this study, because a differentiation between Fe(II) and Fe(III) was sought. Thus, an assay was carried out to determine wether the addition of TCA-HCl precipitated heme-Fe while ionic-Fe remained soluble

Effect of the joint or sequential addition of TCA and hydroxylamine To investigate the possible effect upon Fe(II) and Fe(III) assay of the simultaneous or sequential addition of TCA-HCl and hydroxylamine chloride, both reagents were added simultaneously, or first TCA-HCl and after 5 min hydroxylamine hydrochloride, to different Fe(II) and Fe(III) standards, with or without matrix added. Matrix in this case was the soluble mineral fraction of the digest of (a) chicken in breadcrumb with vegetable stew and (b) potato omelet. The results are shown on Table 2.

The application of a means comparison test (*t*-test) showed neither simultaneous nor sequential addition of the reagents (TCA-HCl and hydroxylamine-HCl) to aqueous Fe standard to affect of Fe(II) and Fe(III) assay, though in standards containing added matrix the effect depended on the matrix type, no statistically significant differences ($p>0.05$) being found in chicken in breadcrumb with vegetable stew, while in the case of potato omelet a statistically significant ($p<0.05$) decrease was found in Fe(II) and Fe(III) when the reagents were sequentially added. The oxidation state did not seem to affect Fe losses (see Table 2).

Effect of TCA on heme-Fe It has been reported that TCA treatment together with the incubation conditions applied could cause the release of Fe bound to the heme group, this phenomenon being responsible for an overestimation of non-heme Fe the expense of heme-Fe. This can be minimized by adding $NaNO_2$ to stabilize the Fe bound to the porphyrin ring of the heme group [10, 11].

To evaluate possible overestimation in the determination of non-heme Fe, two matrixes were selected: the soluble mineral fractions from spaghetti with sausage and from fried hake. To both of them we added 10 µl of a 5 mg ml^{-1} hematin solution ([7, 12diethenyl-3, 8, 13, 17-tetramethyl-21H, 23H-porphine-2, 18dipropanoate(4-)-N^{21}, N^{22}, N^{23}, N^{24}]- hydroxyferrate(2-)dihydrogen) (Sigma), containing 440 µg heme Fe ml^{-1}, equivalent to 0.88 µg heme Fe ml^{-1} in the assay. Hematin was added because in the gastrointestinal digestion process the heme group is released from the globin protein fraction and therefore only Fe bound to the porphyrin ring reaches the intestinal lumen [23].

In previous assay, the amount of $NaNO_2$ to be added was selected, it correspond to 0.078 g in the assay, that is 0.2 ml

Table 2. Effect of simultaneous or sequential (5 min delay) addition of hydroxylamine and TCA-HCl in iron determination

Fe added (1 μg ml^{-1})	Simultaneous addition	Sequential addition (5 min delay)
	Fe μg ml^{-1}	Fe μg ml^{-1}
Fe^{+3} aqueous	0.984 ± 0.017	0.956 ± 0.022
Fe^{+2} aqueous	1.04 ± 0.05	0.996 ± 0.033
Fe^{+3} aqueous + matrix[a]	2.11 ± 0.05	1.90 ± 0.03∗
Fe^{+3} aqueous+ matrix[b]	1.63 ± 0.05	1.59 ± 0.04
Fe^{+2} aqueous + matrix[a]	2.10 ± 0.01	1.93 ± 0.03∗
Fe^{+2} aqueous + matrix[b]	1.68 ± 0.05	1.69 ± 0.15

Values are expressed as mean ± standard deviation (n=3)

∗Significant differences $p<0.05$ within a row

[a]Soluble mineral fraction from potato omelet

[b]Soluble mineral fraction from chiken in breadcrumbs with vegetable stew

of a 0.39% w/v $NaNO_2$ solution. Contribution of heme-Fe to the non-heme-Fe determination and the effect of $NaNO_2$ addition, are reported in Table 3.

The addition of 0.88 μg ml^{-1} Fe from hematin (ratio Fe added/ intrinsic or matrix Fe = 1.18) yielded a 10.2% overestimation of the Fe content of spaghetti with sausage. In the fried hake matrix (ratio Fe added to intrinsic or matrix Fe = 2.34) overestimation was reached 27.70%, suggesting a linear relationship between added heme-Fe and the overestimation of non-heme Fe. In both cases $NaNO_2$ addition reduced overestimation to percentage values close to 5%. It has to be noted that the amount of hematin added (0.88 μg ml^{-1}) corresponded to 50% of Fe as heme-Fe. In the analyzed dishes, containing a large variety of vegetable ingredients, the heme-Fe percentage did not reach 50%.

Matrix interference assays To evaluate possible matrix interferences in the determination of ionic Fe the addition's method was applied to different soluble mineral fractions of the analyzed dishes. A *t*-test was applied to compare the slopes of the regression equations corresponding to the added matrix with those aqueous standards; differences between them indicated matrix interferences. The results obtained are reported in Table 4 and show absence of matrix interferences.

Application of the proposed method to the dishes

Iron (total, ionic, Fe(II), Fe(III) and heme-Fe contents) in the mineral soluble fraction of the analyzed dishes (bioaccessible fraction) are reported in Table 5.

Soluble ionic Fe (Fe(II) + Fe(III)) percentages in relation to bioaccessible Fe ranged from 49–100%, suggesting that most of the bioaccessible Fe was ionic Fe. The latter, with the exception of potato and spinach omelets, was mainly Fe(II), and a statistically significant correlation ($p<0.05$), [Fe(II)] = 0.4713 + 0.7806 × [ionic Fe]; r=0.925, was found between soluble ionic Fe and Fe(II) contents.

The ratio between Fe(II) and Fe(III) contents increased with meat protein content in the analyzed dish. This is known as the "meat factor" and seems to be related to the ability of sulfhydryl groups of amino acids such as cysteine to reduce Fe(III) to Fe(II) [24], Fe(II) being more bioavailable than Fe(III) [2]. In intestinal rats contents a strong correlation (r=0.980; $p<$0,001) between Fe absorption and soluble Fe soluble has been reported [15]. The Fe(II) percentage with respect to total soluble Fe was found to be higher when meat proteins were present than when proteins came from eggs and milk. The results obtained in the present study agree with this observation.

In potato and spinach omelets Fe(III) was the most abundant Fe species, respectively representing 85–63%, of global soluble ionic Fe. Non-ionic Fe (2.25±0.10 μg g^{-1}) in potato omelet, probably corresponds to Fe bound to peptides originating from egg white proteins, such as ovotransferrin or from yolk (phosvitin), the latter having been shown to bind more than 50% of yolk Fe(III) [25]. In the case of spinach omelet, the situation is different due to the high oxalate and phytate contents of spinachs [26, 27], that negatively affect Fe solubility. Spinach omelet presented a total Fe content of 17.9 μg g^{-1}, and 62.3% of it was solubilized, while in potato omelet (with a total Fe content of 8.92 μg g^{-1}) 84% was solubilized.

The highest heme-Fe contents corresponded to dishes containing meat as ingredient (Cuban style rice, rice with lean meat, chicken in breadcrumbs with vegetable stew and chicken in sauce) though one of Cuban style rice ingredient

Table 3. Contribution of heme-Fe to non-heme Fe determination and effect of sodium nitrite addition

	Fe μg ml^{-1}	Contribution of heme-Fe (%)
Spaghetti with sausage	0.679±0.039	
+ Hematin[a]	0.797±0.074	10.2±2.8
+ Hematin[a] + $NaNO_2$ (0.2 ml)	0.727±0.033	5.45±1.8
Fried hake	0.342±0.058	
+ Hematin[a]	0.585±0.079	27.70±8,93
+ Hematin[a] + $NaNO_2$ (0.2 ml)	0.389±0.010	
		5.34±1,08

Values are expressed as mean ± standard deviation (n=3)

[a]0.88 μg ml^{-1} of Fe from Hematin

Table 4 Matrix interferences evaluated by the addition method

r = correlation coefficient
CI = Confidence interval for slope (95% probability level)

Set	Regression equation	r	CI
Aqueous standard	$y = 0.2057x–0.0008$	0.9997	0.2001—0.2149
+ Potato omelet	$y = 0.1878x–0.2572$	0.9983	0.1682—0.2074
+ Chicken in breadcrumbs	$y = 0.2224x–0.1273$	0.9992	0.2062–0.2386
+ Spaghetti with sausage	$y = 0.2172x–0.1474$	0.9997	0.2073–0.2272

Table 5. Iron bioaccesible: total, ionic, Fe(II), Fe(III) and heme Fe contents of the analyzed dishes

Sample	Bioaccesible Fe ($\mu g\ g^{-1}$)	ionic Fe ($\mu g\ g^{-1}$)	Fe(II) ($\mu g\ g^{-1}$)	Fe(III) ($\mu g\ g^{-1}$)	Heme Fe ($\mu g\ g^{-1}$)
Lentils with sausage	3.65±0.25	2.81±0.19	2.92±0.28	n.d	0.84±0.19
Stew	7.60±0.96	6.85±0.21	6.57±0.29	0.28± 0.02	0.75±0.18
Cuban style rice	11.03±0.52	8.55±0.69	6.44±0.85	2.11±0.86	2.48±0.68
Rice with lean meat	5.45±0.83	3.92±0.18	2.41±0.08	1.51±0.08	1.53±0.18
Chicken in breadcrumbs with vegetable stew	5.65±0.47	3.71±0.04	2.83±0.13	0.88±0.12	1.94±0.03
Chicken in sauce	7.39±0.84	7.07±0.99	5.80±0.18	1.27±0.18	n.d
Potato omelet	7.48±0.87	5.23±0.10	0.77±0.13	4.46±0.13	2.25±0.10
Spinach omelet	11.15±0.87	11.08±1.27	3.87±0.14	7.21±0.15	n.d
Macaroni with tuna	7.64±0.58	6.75±0.47	5.90±0.43	0.85±0.21	0.85±0.07
Spaghetti with sausage	4.75±0.63	4.86±0.39	4.44±0.14	0.42±0.14	n.d
Precooked hake filet	6.38±1.79	3.61±0.14	3.48±0.26	0.13±0.04	2.77±0.14
Fried hake	3.46±0.01	3.55±0.10	3.43±0.17	n.d	n.d
Potato stew	5.78±0.37	5.60±0.51	5.71±0.19	n.d	n.d

Values are expressed as mean ± standard deviation ($n = 3$)
n.d.: not detectable

was egg, and part of Fe determined as heme-Fe thus could correspond to Fe bound to egg peptides, as mentioned above mentioned in relation to omelets.

Differences in heme-Fe content in chicken-based dishes could be explained by differences in meat content between the two analyzed dishes. Thus, chicken was the main ingredient in chicken in breadcrumbs with vegetable stew, while in chicken in sauce the meat content was much lower, chicken broth possibly being the main contributor to the higher soluble Fe content. Moreover, chicken in sauce required a long cooking procedure (about 2 h) that could have contributed to decrease the heme-Fe content, with a resulting increase in ionic Fe, as has been reported elsewhere [28, 29]. The non-detection of heme-Fe in potato stew containing a small portion of veal, could also be explained by the cooking process involve.

Differences in heme-Fe contents between fried and fillet (precooked) hake could likewise be attributed to differences in their preparation. While fried hake consisteds of a whole hake fillet, directly fried in oil, precooked hake filet was obtained through an industrial process. With the exception of seafood, fish has a low heme-Fe content [30]. The frying process could suffice to destroy the low heme-Fe present. The high non-ionic Fe instead of heme-Fe content found in precooked hake filet could correspond to Fe bound to additives used in formulation of the product.

Considering that the school menu was composed of two dishes, and that the average weight of a serving was respectively 150 g and 200 g/dish for children aged 4–8 and 9–13 years, the estimated average bioaccessible iron supplies per meal were about 2 mg (4–8 year old children) and 2.7 mg (9–13 year old boys and girls), respectively. The iron Recommended Dietary Allowances (RDA) for these population groups are in the range from 8 (younger children) to 10 mg/day (boys and girls). These RDA assume that dietetic iron has a bioavailability of 18% [31]. According to this, the bioavailable amount of iron would be 1.8 mg for children and 1.44 mg for boys and girls. If bioaccessible iron is assimilated to bioavailable iron, then the school meal would suffice to cover the daily iron requirements in this population group, and even in the case that bioaccessibility were lower than bioavailability, the contribution of the school menu to cover the iron requirements is substantial.

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