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Optimisation of in vitro measurement of available iron from different fortificants in citric fruit juices

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Abstract

The percentage of dialyzable ferrous and total iron were studied in a citric juice (pineapple and passion fruit) fortified with ferrous sulphate, micronised dispersible ferric pyrophosphate and ferrous bis-glycinate in similar concentrations (49.2 mg Fe/l). The in vitro method of Kapsokefalou and Miller (1991) [Kapsokefalou, M., & Miller, D. D. (1991). Effects of meat and selected food components on the valence of nonheme iron during in vitro digestion. *Journal of Food Science, 56*, 352–355.] was optimised for this matrix using 0.15 N PIPES buffer (pH 8.5) to adjust pH during pancreatic digestion. We also studied different pH values of Hepes buffer used in the measurement of iron concentrations with Ferrozine (chromogen solution). The maximum absorbances were obtained with a Hepes buffer pH value of 8.5. Ferrous sulphate was used as a reference salt due to its high bioavailability, although novel compounds, such as ferrous bis-glycinate and micronised dispersible ferric pyrophosphate, showed a high relative iron availability in this juice. Taking into account that percentage of dialysable ferrous iron is considered to be the more available fraction of total iron, the iron fortificant ferrous bis-glycinate proved to be more adequate for fortifing citric juices, giving a 10.7% of dialyzable ferrous iron. Moreover, the percentage of dialyzable total iron from ferrous bis-glycinate (31.0%) was statistically higher than those from ferrous sulphate and micronised ferric pyrophosphate (28.4% and 28.2%, respectively).

Keywords: Iron fortification; Available iron; Citric fruit juice; In vitro digestion

1. Introduction

Considering that iron deficiency is an important nutritional problem that affects ≈20% of the world's population (Walter, Pino, Pizarro, & Lozoff, 1998), and that food fortification according to dietary habits of the affected population represents the most cost-effective, long-term approach to reducing prevalence of iron deficiency (Hurrell, 1997), it is imperative for involved industries or laboratories, to use an accurate, sensitive, practical, rapid and cheap methodology for the control of the iron bioavailability in fortified products.

Bioavailability is an important factor in the nutrition field because of its variations with different foods, food components and gastrointestinal conditions. This concept represents the integration of the various processes whereby an ingested nutrient becomes available: digestion, absorption, transport, utilisation and, elimination (Favier, 1993).

Several approaches have been used to estimate iron bioavailability, including in vitro digestion to measure iron solubility or dialysability and animal studies (Crews, Burrell, & McWeeny, 1983; Forbes et al., 1989; Hazell & Johnson, 1987; Hurrell, Lynch, Trinidad, Dassenko, & Cook, 1988; Miller, Schricker, Rasmussen, & Van Campen, 1981; Sandström & Cederblad, 1987; Wolters et al., 1993; Zemel, 1984). The in vitro digestion cannot reflect the complexity of natural system but

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information from these experiments regarding the effects of enzymes and pH may be applicable to the in vivo situation: they permit a reasonable estimation of trace element availability (Bermejo et al., 2002).

Particularly, the in vitro method developed in 1981 by Miller et al. (1981) has been shown to provide availability measurements that correlate well with in vivo studies. It is one of the most extensively used, is considered useful to predict many inhibitors/enhancing dietary factors and has been applied to examine the influence of processing on mineral availability from foods (Wolters et al., 1993).

Several modifications of this method have been proposed to measure availability of intrinsic or added iron (Hazell & Johnson, 1987; Larsson, Minekus, & Hayenaar, 1997; Luten et al., 1996; Vaquero et al., 1992; Wolters et al., 1993) and all of them used $NaHCO_3$ for pH adjustment during pancreatic digestion/dialysis. Instead of $NaHCO_3$, Kapsokefalou and Miller (1991) proposed the use of 0.15 N PIPES buffer (pH 6.1–6.3) to adjust pH during pancreatic digestion. Differences in the pH regulation procedure, including type and concentration of base or buffer added to the pepsin digest may render dissimilar final dialysate pH values, according to the composition of the food matrix. (Wolfgor, Drago, Rodriguez, Pellegrino, & Valencia, 2002).

On the other hand, in the case of iron, the effect that its solubility in water, oxidation state and extent of complex formation, has on its bioavailability has been evaluated by some authors (Lee & Clydesdale, 1978), and it is generally accepted that only soluble non-heme iron can be absorbed; thus, only a fraction of the soluble iron is available (Wienk, Marx, & Beynen, 1999). Iron exists mainly in the iron (III) form (Martínez, Ros, Periago, & López, 1999), and it is well known that iron (II) is more available than iron (III), because the latter has a low solubility in the gut. However, iron (III) can be reduced to the more soluble iron (II) in the gut by the action of gastric hydrochloric acid and reducing agents, such as ascorbic acid (Quinteros, Farré, & Laganda, 2001). Therefore, it would be interesting to estimate the different oxidation states of the iron added to fortified products to complete the study and knowledge of its availability.

In this work, we have considered that a citric fruit juice could be a very suitable vehicle for iron fortification from a nutritional point of view, for two reasons: (i) it is a beverage that lacks some inhibitors of iron absorption, such phytates or oxalates and (ii) its intake and absorption are faster than those of solid foods. On the other hand, in acid beverages, such as fruit juices, problems with stability of iron fortificants and discoloration are unlikely to occur since the percentage of added iron that remains in the ferrous state is higher at low pH.

Several soluble and insoluble iron compounds have been used to fortify foods. Although soluble iron compounds have high bioavailability, they often cause unacceptable colour and flavour. Ferrous sulphate is a water-soluble compound that has the highest relative bioavailability (RBV) among conventional iron compounds (≈100). It is commonly added to foods but it has been reported to cause a metallic taste in fruit drinks (Hurrell, 2002). Ferric pyrophosphate is a water-insoluble iron compound often use to fortify infant cereals and chocolate drink powders as it causes no adverse colour and flavour changes to food vehicles. However, it is only of low absorption in man. Recently, novel ferric pyrophosphate compounds have been developed, based on small particle size ferric pyrophosphate and encapsulation with a mixture of emulsifiers, so that they remain in suspension in liquid products. Fidler et al. (2004) showed that this novel compound has a similar iron absorption to that of ferrous sulphate from a fortified infant cereal as well as from a yoghurt drink. Ferrous bis-glycinate has the advantage of being soluble in water and does not change the organoleptic properties of the food vehicle. This compound is being increasingly considered in programmes for iron fortification of foods and beverages because it prevents iron from binding to inhibitors in food (Miglioranza et al., 2003; Olivares & Pizarro, 2001).

The objective of this research was to optimize the in vitro method developed by Kapsokefalou and Miller (1991) to determine iron availability in a citric fruit juice fortified with different iron compounds, and in addition to estimate the proportion of iron (II) and total iron present in these beverages.

2. Materials and methods

2.1. Preparation of fruit juice

The fruit juice was obtained from pineapple and passion fruit concentrates (60° Brix), reconstituted to reach 6.4° Brix and a pH of 3.8–3.9. During the manufacturing process, each iron fortificant: ferrous sulphate (Merck, Spain), ferrous bis-glycinate (Ferrochel®, Albion Laboratories, Clearfield, Utah) and micronised dispersible ferric pyrophosphate (SunActive-Fe®, Taiyo Kagaku, Japan) was separately added to reconstituted citric fruit juice and the mixture homogenised in shaking tanks to yield a total iron concentration of 49.2 mg Fe/l (33% DRI). Juices were pasteurised at 90 °C for 30 s and equally divided into 200 ml glass bottles, closed, cooled and stored at room temperature. Total iron and ferrous iron concentrations were analysed in juices after the manufacturing process.

All samples were prepared at the pilot plant of Hero España, S.A. (Murcia, Alcantarilla, Spain).

2.2. In vitro estimation of dialysable iron

To determine the relative iron bioavailability of the different juices, we followed the method developed by Miller et al. (1981) and modified by Kapsokefalou and Miller (1991). To optimise the conditions of the dialysis assay in our juices, we also slightly modified the buffer pH, as described later. Dialysable total iron and dialysable ferrous iron, were used as indicators of non-haem ion bioavailability.

2.3. Reagents

Distilled, deionised water was used throughout the experiment. All glassware was washed with detergent, rinsed with water, soaked overnight in 10% HNO_3 rinsed again and dried. All chemicals were of analytical grade. $FeCl_3$ (Sigma Chemical Co., St. Louis, MO) was used for standard solution.

2.4. Pepsin digestion mixture

Pepsin digestion mixtureAbout 4.0 g porcine pepsin (Sigma P-7000) was suspended in 0.01 N HCl and diluted to 100 ml with 0.1 N HCl.

2.5. Pancreatin–bile extract mixture

About 0.5 g porcine pancreatin (Sigma P-1750) and 3.0 g bile extract (Sigma B-8631) were dissolved in 0.01 N $NaHCO_3$ and diluted to 250 ml with 0.1 N $NaHCO_3$.

2.6. PIPES buffer

PIPES [piperazine-*NN*′-bis(2- ethane-sulfonic acid)] disodium salt (Sigma P-3768) was dissolved in water to reach 0.15 N and adjusted with concentrated HCl to the desired pH.

2.7. Hepes buffer

Hepes [*N*-2-hydroethyl-piperazine-*N*′-2-ethanesulfonic acid] sodium salt (Sigma H-7006) was dissolved in water to reach 0.3 N and adjusted with concentrated HCl to the desired pH.

2.8. Protein precipitant solution (reducing)

Hundred gram of trichloroacetic acid and 50 g of hydroxylamine monohydrochloride were dissolved in water, 100 ml concentrated HCl was added and the volume was diluted to 1 l with water.

2.9. Protein precipitant solution (non-reducing)

This was the same as the protein precipitant solution (reducing) except that hydroxylamine monohydrochloride was omitted.

2.10. Ferrozine chromogen solution

Ferrozine [3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine] disodium salt (Sigma P-9762) was dissolved in water to reach a concentration of 5 mg/ml.

2.11. Dialysis tubing

Spectra/Por®I dialysis tubing (Spectrum, CA, USA) with a molecular weight cut-off of 6000–8000 was cut into 20-cm lengths and soaked in water for at least 1 h prior to use.

2.12. In vitro digestion

Ten ml aliquots of each citric fruit juices fortified with different iron compounds were transferred to 100 ml polystyrene bottles and the pH was adjusted to 2.0 with 0.05 N HCl and mixed with 1 ml pepsin suspension. The mixture was incubated at 37 °C in a shaking water bath for 2 h. At the end of the pepsin incubation, a dialysis bag containing 20 ml PIPES buffer was placed in each bottle. The samples were incubated 30 min. Five ml of the pancreatin/bile mixture was added to each bottle and the incubation continued for another 2 h. At the end of the pancreatin/bile incubation the dialysis bags were removed and rinsed by dipping in water. The pH of each dialysate and retentate was measured at the end of the in vitro incubation.

2.13. Iron determination

Iron concentrations in dialysates (Fe(II) and total) and retentates (Fe(II) only) were measured using a modification of the method proposed by Reddy, Chidambaran, Fonseca, and Bates (1986), as modified by Kapsokefalou and Miller (1991). For total iron measurement, reducing protein precipitant solution (1 ml) was added to 2 ml aliquots of each dialysate. For Fe(II) measurement, non-reducing protein precipitant solution (1 ml) was added to 2 ml aliquots of each dialysate and retentate. Samples were held overnight at room temperature. Subsequently, they were centrifuged at 2575*g* for 10 min. Aliquots of the supernatants (1 ml in duplicate) were transferred to separate tubes. Ferrozine solution (0.25 ml) and Hepes buffer (2 ml) were added to each tube. Absorbance (at 562 nm) was measured immediately after chromogen solution addition for the Fe(II) determination, or 1 h after chromogen addition for the total iron determination. Iron standards were prepared

by diluting an iron solution ($FeCl_3$, 1 mg Fe/ml) with 0.05 N HCl to achieve the following concentrations of iron: 0, 1, 4, 6, 10, 15, and 20 μg/ml. The slope of the regression line and r^2 equalled 99.543 and 0.9999, respectively.

2.14. Calculations

Dialysable ferrous iron (D-Fe(II)), dialysable total iron (D-(Fe(II)+Fe(III))), and total ferrous iron (D-Fe(II) + non-D-Fe(II)), were expressed as percentages of the total iron contained in all samples. It was assumed that dialysable iron had equilibrated across the dialysis membrane by the time the dialysis bags were removed at the end of the digestion.

$$\text{(a) D-Fe(II)\%} = \frac{^{1}[\text{Fe(II)}]_{\text{D}}\ (\mu\text{g/ml}) \times \text{dialysate volume (ml)}}{^{2}\text{Fe}\ (\mu\text{g/ml}) \times 10\ \text{ml}} \times 100$$

[1]Ferrous iron concentration in the dialysate.[2]Iron concentration in beverage.

$$\text{(b) D-[Fe(II) + Fe(III)]\%} = \frac{^{3}[\text{Fe(II)} + \text{Fe(III)}]_{\text{D}}\ (\mu\text{g/ml}) \times \text{dialysate volume (ml)}}{^{2}\text{Fe}\ (\mu\text{g/ml}) \times 10\ \text{ml}} \times 100$$

[3]Total iron concentration in the dialysate.

$$\text{(c) D-Fe(II) + non-D-Fe(II)\%} = \frac{^{1}[\text{Fe(II)}]_{\text{D}}\ (\mu\text{g/ml}) \times \text{dialysate volume (ml)} + ^{4}[\text{Fe(II)}]_{\text{R}}\ (\mu\text{g/ml}) \times ^{5}\text{retentate volume (ml)}}{^{2}\text{Fe}\ (\mu\text{g/ml}) \times 10\ \text{ml}}$$

[4]Ferrous iron concentration in the retentate.
[5]Total volume minus volume of dialysate.

2.15. PIPES buffer pH adjustment for pancreatic digestion

To maintain a physiologically relevant pH (pH 6.5–7.5), in vitro biological systems must be stabilised by the incorporation of a buffer that undergoes reversible protonation. Many buffers are not suitable for biological applications because the pH values of the solutions depend on the concentration of the ionic components and the temperature of the solution. PIPES buffer [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] disodium salt, is a biological buffer capable of possessing both positive and negative charges, that make it suitable for biological applications because its buffering capacity is independent of temperature and concentration of the solution. With the purpose of reaching during pancreatic digestion a pH in the dialysate and in the retentate similar to intestinal pH (6.5–7.5), the selection of appropriate pH for the PIPES buffer is essential.

We have selected several values of PIPES buffer pH (6.3, 6.9, 7.5, 8.0 and 8.5), with the purpose of assessing it during pancreatic digestion and to observe, at the end of in vitro digestion wheather pH values of dialysate, and also retentate, were close to physiological pH.

2.16. Hepes buffer pH adjustment for iron determination

Hepes buffer (*N*-2-hydroxiethyl-piperazine-*N*′-2-ethanesulfonic acid) sodium salt presents the same characteristics as PIPES buffer. In this case, Hepes buffer was used to maintain an appropriate pH for colorimetric reaction between the chromogen solution (Ferrozine) and ferrous iron present in the dialysate and retentate. The formation and stability of the Ferrozine–iron complex has pH requirements, different values are found in the literature (Ceriotti & Ceriotti, 1980; Persijn, Van der Slick, & Riethorst, 1971; Ruutu, 1975; Stookey, 1970).

We have selected a wide range of pH values (from 4.2 to 8.5) for Hepes buffer in order to find which is the most appropriate in the formation of Ferrozine–iron complex, in citric fruit juices.

2.17. Statistical analysis

Results were expressed as means ± SD of five determinations. The data analyses were carried out using the one-way ANOVA test at a significance level of

$P < 0.05$. To explain the relationship between different Hepes buffer pH values, ferrous iron in dialysate and retentate, and dialyzable total iron concentration, a Pearson's correlation analysis was carried out. Statistical analyses were performed using a SPSS programme version 10.0 for Windows (SPSS Inc. Chicago, IC).

3. Results and discussion

3.1. General

Each of the fortified juices was analysed for iron species just before the in vitro digestion process. Total iron concentrations were 57.4 ± 2.76, 50.1 ± 3.00, and 50.1 ± 1.87 mg Fe/l for ferrous sulphate, micronized ferric pyrophosphate and ferrous bis-glycinate, respectively, whereas ferrous iron concentration in each sample were 35.6 ± 1.22, 15.5 ± 1.03 and 27.9 ± 1.52 mg/l, respectively. As we could observe, ferrous iron fortificants had a higher proportion of ferrous valence than ferric pyrophosphate, even though ≈40% and 44% of the ferrous form from ferrous sulphate and ferrous bis-glycinate respectively, had been oxidised to the ferric state at this early stage. That means that, even at the acid pH of our juices (3.8–3.9), the stabilities of ferrous forms added as fortificants are not guaranteed. Juices were analysed immediately after the manufacturing process, and we have to consider that, during the storage period, the percentages of ferric form would probably increase gradually. Since the ferrous form is the more available for our organism and, as Nojeim and Clydesdale (1981) have reported, the more ferrous ion present in the food at the time of consumption, the more likely is it to remain in that form through the digestive tract.

On the other hand, 30% of the micronized ferric pyrophosphate added to the juice was reduced to the ferrous form. Lee and Clydesdale (1980) reported that ferric compounds such as ferric orthophosphate and ferric EDTA, were solubilised to ferrous forms to a greater or lesser extent in an acid-type fruit beverage. Hodson (1970) similarly found that iron from ferric orthophosphate was converted to the ferrous form after 6 months storage of a liquid dietary product. Hurrell (1984) also reported that the reduction potential of a food system affects the chemical state of the iron present and conversion of ferric to ferrous iron is increased by the addition of reducing agents such as ascorbic acid and by lowering the pH. All these conclusions have been obtained for non-protected iron compounds, but in the case of micronized ferric pyrophosphate, the coat that involves the iron should have protected it from changes in the valence. We presume that, due to the heat treatment of juices, rupture of micronised particles could occur and, consequently, loss of ferric iron and

Table 1
Values of PIPES buffer pH required to reach final pH 6.5–7.5, final pH of dialysates and retentates for each citric fruit juices fortified with different iron fortificants

Iron fortificant	PIPES pH									
	pH 6.3		pH 6.9		pH 7.5		pH 8.0		pH 8.5	
	Final D* pH	Final R* pH	Final D* pH	Final R* pH	Final D* pH	Final R* pH	Final D* pH	Final R* pH	Final D* pH	Final R* pH
Ferrous sulphate	5.10 ± 0.02^{a}	5.19 ± 0.01^{b}	6.20 ± 0.00^{a}	6.15 ± 0.02^{a}	6.56 ± 0.02^{a}	6.54 ± 0.02^{a}	6.70 ± 0.02^{a}	6.67 ± 0.02^{a}	6.83 ± 0.01^{b}	6.70 ± 0.01^{b}
MDFP	5.03 ± 0.23^{b}	5.13 ± 0.01^{c}	6.10 ± 0.01^{c}	6.08 ± 0.00^{b}	6.54 ± 0.01^{a}	6.43 ± 0.01^{b}	6.64 ± 0.01^{b}	6.59 ± 0.01^{c}	6.83 ± 0.01^{b}	6.60 ± 0.00^{c}
Ferrous bis-glycinate	5.12 ± 0.17^{a}	5.23 ± 0.02^{a}	6.15 ± 0.01^{b}	6.13 ± 0.01^{a}	6.55 ± 0.00^{a}	6.55 ± 0.00^{a}	6.68 ± 0.01^{a}	6.64 ± 0.01^{b}	6.95 ± 0.01^{a}	6.73 ± 0.01^{a}

Values are means ± SD ($n = 5$).
The non-coincidence of letter in the same column indicates statistically significant differences ($P < 0.05$).
D*, dialysate; R*, retentate.
MDFP, micronized dispersible ferric pyrophosphate.

partial conversion to ferrous form at the low pH of the samples.

3.2. PIPES buffer pH adjustment for pancreatic digestion

We have studied several PIPES buffer, pH values: 6.3, 6.9, 7.5, 8.0 and 8.5, in order to observe which one is required to reach a final digest/dialysate system pH of 6.5–7.5 after pancreatic digestion in our citric fruit juice samples.

In Table 1, dialysate and retentate pH values for each PIPES buffer pH are shown, both initially and at the end of pancreatic digestion. When pH regulation was carried out with PIPES buffer at pH 6.3 and 6.9, pH values in dialysate and retentate, for each citric fruit juice fortified with the three iron compounds, were always below physiological pH. On the other hand, with PIPES buffer at pH 7.5, 8.0 and 8.5, both dialysate and retentate pH values rose until they reached a pH of 6.5–7.5 for any iron fortificant employed. Therefore, any one could be ideal, although we have selected the highest pH (8.5) because, as explained later, the percentage of dialysable iron obtained was higher in this case (see Fig. 1). The selection of the correct pH or molarity of a buffer, such as PIPES, to reach a physiological final digest/dialysate pH in each food matrix would provide conditions for more reliable evaluation of available iron (Wolfgor et al., 2002). It is important to point out that the food matrix employed in this study had a low protein concentration (0.2 g/100 g), allowing a good pH equilibrium between both compartments (final digest/final dialysate). We have considered this factor because Wolfgor et al. (2002) concluded that in dairy matrices, with higher protein concentration, high molecular weight products of protein digestion may remain in digests providing acid conditions and preventing pH equilibrium between both compartments.

3.3. Hepes buffer pH adjustment for iron determination

Iron concentrations in dialysate and retentate were measured using a spectrophotometric method with Ferrozine where the use of Hepes buffer is required for maintaining a correct pH in the formation of Ferrozine-iron (II) complex. Ferrozine (monosodium salt hydrate of 3-(2-piridyl)-5,6-diphenil-1,2,4-triaze-*p*-*p*′-disulfonic acid) is a compound which reacts with divalent iron to form a stable magenta complex (Stookey, 1970). The maximum absorbance is recorded at 562 nm between pH 4 and 9. As the range of pH is so wide, we selected different values of Hepes buffer pH to investigate changes in the dialysate and retentate iron concentration both in its soluble as total form, and to find, which value of Hepes buffer pH gives maximum absorbance of soluble and total iron for each fortificant iron source.

The dialysable ferrous iron, dialysable total iron and non-dialysable ferrous iron concentrations (mg/l) in the citric fruit juice fortified with each iron compound at different values of Hepes buffer pH (4.2, 5.5, 7.5, 8.0 and 8.5) are shown in Table 2. Studies on the use of Hepes buffer to maintain an appropiate pH for colorimetric reaction between the chromogen solution (Ferrozine) and ferrous iron are scarce. Therefore, we thought that, to optimise this method in citric juices, the relationship between the different Hepes buffer pH values and dialysable ferrous iron, non-dialysable ferrous iron and dialysable total iron concentrations should be evaluated.

From Table 2, it is clear that dialysable ferrous iron concentration and retentate ferrous iron, using the iron fortificant ferrous sulphate, increased with the Hepes buffer pH, showing a positive correlation between them ($r^2 = 0.605$, $P < 0.01$; $r^2 = 0.846$, $P < 0.01$, respectively). Also, the highest dialysable ferrous iron (2.12 ± 0.06 mg/l), dialysable total iron (8.16 ± 0.18 mg/l) and non-

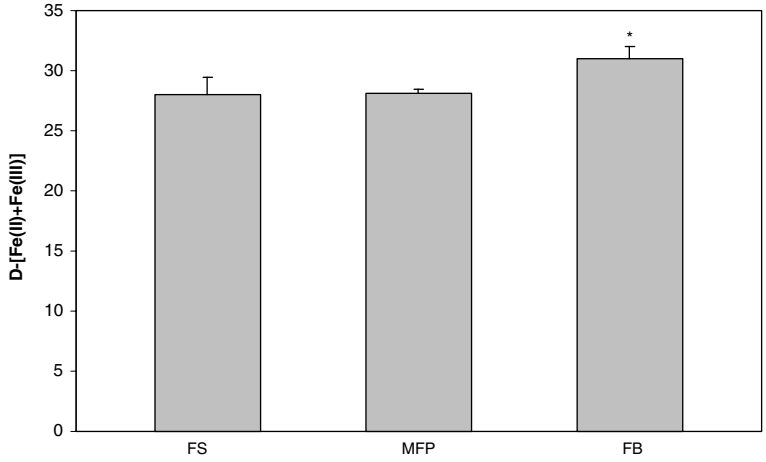

Fig. 1. The sum of ferrous and ferric iron, % D-(Fe(II) + D-Fe(III)), dialysed during in vitro digestion. Dialysable-(Fe(II) + Fe(III)) is expressed as a percentage of the total iron in samples. Each value is the mean ± SE of the three analyses performed in triplicate. *Abbreviations:* FS, ferrous sulphate; MFP, micronised ferric pyrophosphate; FB, ferrous bis-glycinate. *Significant differences for each variable ($P < 0.05$).

Table 2
Iron (II), total iron in dialysates and iron (II) in the retentates in the citrus fruit juice fortified with different iron fortificants using different Hepes buffer pH values; expressed as mg/l

Hepes pH	Ferrous sulphate			Micronized dispersible ferric pyrophosphate			Ferrous bis-glycinate		
	$Fe(II)_{D*}$	Fe $total_{D*}$	$Fe(II)_{R*}$	$Fe(II)_{D*}$	Fe $total_{D*}$	$Fe(II)_{R*}$	$Fe(II)_{D*}$	Fe $total_{D*}$	$Fe(II)_{R*}$
4.2	1.66 ± 0.04^{b}	7.83 ± 0.09^{b}	3.42 ± 0.01^{c}	1.27 ± 0.06^{c}	7.94 ± 0.34^{ab}	3.40 ± 0.11^{c}	3.37 ± 0.37^{a}	9.01 ± 0.23^{a}	7.07 ± 0.25^{a}
5.5	1.04 ± 0.07^{c}	6.69 ± 0.08^{e}	3.14 ± 0.17^{c}	0.98 ± 0.05^{d}	6.58 ± 0.34^{d}	3.56 ± 0.31^{c}	3.96 ± 0.10^{a}	9.40 ± 0.13^{a}	6.72 ± 0.66^{a}
7.5	2.04 ± 0.03^{a}	7.65 ± 0.12^{bc}	4.46 ± 0.05^{b}	2.01 ± 0.10^{a}	7.33 ± 0.34^{bc}	4.52 ± 0.06^{b}	3.59 ± 0.28^{a}	9.19 ± 0.23^{a}	6.73 ± 0.68^{a}
8.0	1.77 ± 0.09^{b}	7.38 ± 0.13^{d}	4.12 ± 0.25^{b}	1.73 ± 0.05^{b}	7.31 ± 0.09^{c}	4.48 ± 0.06^{b}	3.65 ± 0.35^{a}	8.55 ± 0.55^{ab}	6.60 ± 0.54^{a}
8.5	2.12 ± 0.06^{a}	8.16 ± 0.18^{a}	5.26 ± 0.38^{a}	2.09 ± 0.18^{a}	8.00 ± 0.12^{a}	5.99 ± 0.04^{a}	3.66 ± 0.20^{a}	8.20 ± 0.25^{b}	6.66 ± 0.49^{a}

Values are means ± SD ($n = 5$).
The non-coincidence of letter in the same column indicates statistically significant differences ($P < 0.05$).
D*, dialysate; R*, retentate.

dialysable ferrous iron (5.26 ± 0.38 mg/l) concentrations corresponded to Hepes buffer, pH of 8.5, showing significant differences ($P < 0.05$) from the rest of the values.

The same was observed for micronised dispersible ferric pyrophosphate. In this case, in spite of having a different structure and composition, the dialysable ferrous iron, retentate ferrous iron and dialysable total iron concentrations were similar to that of ferrous sulphate, which, also showed a positive correlation between dialysable ferrous iron and retentate ferrous iron concentrations with Hepes buffer pH ($r^2 = 0.772$, $P < 0.01$; $r^2 = 0.926$, $P < 0.01$, respectively). Encapsulated and micronised dispersible ferric pyrophosphate is an emulsified form of ferric pyrophosphate. Like chelation, the coating of iron with emulsifiers may also protect iron from forming non-absorbable complexes and is thus also considered to be a potentially useful approach for improving the bioavailability of iron. The highest values for these variables were reported at Hepes buffer pH values of 8.5 and 7.5, showing significant differences ($P < 0.05$) from other analyses performed at different buffer pH.

Curiously, ferrous bis-glycinate showed a particular behaviour, since it did not show any correlation between different Hepes buffer pH values and ferrous iron concentration in dialysate and retentate. In contrast, there was a negative correlation ($r^2 = -0.650$, $P < 0.01$) between dialysable total iron concentration and different Hepes buffer pH values. The optimum Hepes buffer pH for this iron fortificant could be 5.5, where the results for both dialysable ferrous iron concentration and dialysable total iron concentration were the highest (3.96 ± 0.10 and 9.40 ± 0.13 mg/l, respectively). However, although any Hepes buffer pH value would be useful, to maintain the same conditions of work for the three iron compounds, we selected the Hepes buffer pH of 8.5.

On the other hand, it is important to point out that most ferrous iron remained outside dialysis membrane in all cases, the ferrous iron concentration in the retentate being higher than that in the dialysate (Table 2). Such a situation may be due to the formation of complexes between ferrous iron and high molecular weight compounds of juices during in vitro digestion, which would inhibit its transport across the membrane. This fact should be proved by in vivo experiments, as it would suppose a decrease in the potential availability of the iron compounds used as fortificants. If we calculate total ferrous iron concentration as ferrous iron concentration in the dialysate plus ferrous iron concentration in the retentate (see Table 2), we deduce that samples with ferrous bis-glycinate contain approximately 2-fold the concentrations obtained for ferrous iron and micronised ferric pyrophosphate fortified juices. At the same time, ferrous bis-glycinate samples also showed significantly higher ferrous iron concentration in the dialysates than did the other juices. Therefore, there was a higher it in vitro iron bioavailability if we consider such form as the more absorbable. At the same time, ferrous sulphate and ferric pyrophosphate fortified juices showed similar total ferrous iron concentrations after the digestion process, which confirms that ferrous sulphate is not a stable fortificant being oxidised in a great proportion in this matrix, and that micronised ferric pyrophosphate is removed from its coat and some part transformed to ferrous form.

3.4. Iron availability

After selecting the optimum pH value for Hepes and PIPES buffers (pH 8.5), we calculated the percentages of dialysable total iron (Fig. 1), dialysable ferrous iron and total ferrous iron (Fig. 2) of each iron fortificant compound. The results, presented in Figs. 1 and 2, are expressed as percentages of the total iron in samples analysed after the manufacturing process.

The percentage of dialysable total iron was significantly higher ($P < 0.05$) for ferrous bis-glycinate ($30.9 \pm 1.02\%$), followed by micronised ferric pyrophosphate ($28.2 \pm 0.28\%$) and ferrous sulphate ($27.9 \pm 1.46\%$). These values are slightly superior to those of dialyzable iron in an orange juice (25.0%) reported by Miller et al. (1981), following the original

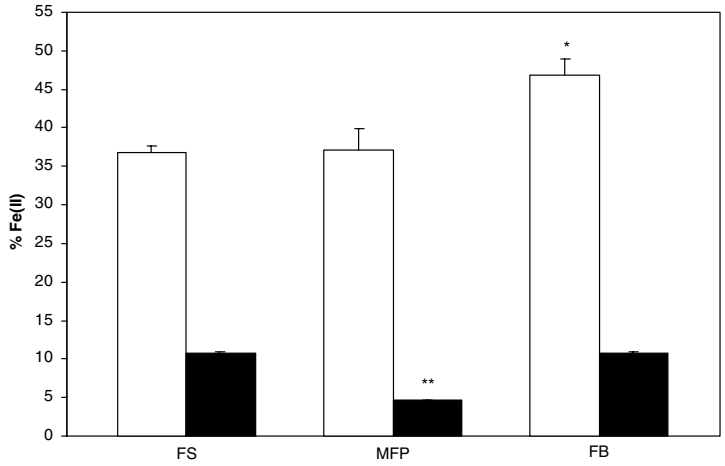

Fig. 2. The percentage of total ferrous iron, % D-Fe(II) + non-D-Fe(II), and dialysable ferrous iron, % D-Fe(II), formed during in vitro digestion. Fe(II) is expressed as a percentage of the total in samples. Each value is the mean ± SE of the three analyses performed in triplicate. □ % D-Fe(II) + non-D-Fe(II); ■, % D-Fe(II). *Abbreviations:* FS, ferrous sulphate; MFP, micronised ferric pyrophosphate; FB, ferrous bis-glycinate. *Significant differences for % D-Fe(II) + non-D-Fe(II) ($P < 0.05$); **Significant differences for % D-Fe(II) ($P < 0.05$).

method. The enhancing effect of fruit juices on iron absorption has been previously demonstrated in several in vivo studies and it is clearly correlated with the ascorbic acid content of the juices. Only from pineapple juice was the absorption of iron relatively high, despite its low ascorbic concentration (Ekmekcioglu, 2000). The high availability of iron fortificants used in our study confirm that these juice (pineaple and passion fruit) are good vehicles for iron fortificants, even without the addition of vitamin C during the manufacturing process.

Fig. 2 shows the percentages of dialysable ferrous and the percentages of total ferrous iron (ferrous iron in dialysate plus retentate) of each iron fortificant added to the citric juice. Ferrous bis-glycinate presented the highest total ferrous iron (46.8 ± 2.09) with statistical significance ($p < 0.05$), while data for micronised ferric pyrophosphate and ferrous sulphate were similar (37.1 ± 2.86% and 36.9 ± 0.84%, respectively). Comparing these results with the ferrous iron values obtained in juices at the moment of the manufacturing process, we can observe that the ferrous valence of iron bis-glycinate increased with respect to ferrous sulphate during the digestion. However, it is important to point out that only a small fraction of ferrous iron was dialyzable; in other words, the ferrous iron in juices could be bind to ligands, forming high molecular weight iron complexes that inhibit its transport across the membrane. Ferric iron constituted the main fraction of total dialyzable iron in our experiment. The available ferrous iron was significantly lower in micronised ferric pyrophosphate juice (4.75 ± 0.15%), while no differences were found between ferrous sulphate and ferrous bis-glycinate (10.8 ± 0.12% and 10.7 ± 0.16%, respectively). This means that, if we consider ferrous sulphate as the reference salt, due to its high bioavailability, ferrous bis-glycinate presents the same proportion of available ferrous form and a better total iron dialysability in this juice. Micronised ferric pyrophosphate also showed promising results because total iron availability was similar to the reference compound, although a lower percentage of dialysable ferrous iron was found. A reason for the high iron availability of micronised dispersible encapsulated ferric pyrophosphate, in spite of the low and medium bioavailability reported for non-encapsulated ferric pyrophosphate, could be its micronised particle size. Particle size of encapsulated ferric pyrophosphate has previously been shown to influence iron absorption (Fidler et al., 2004). The particle size distribution of this compound is within the range of 0.1–2.6 μm and the average particle size is 0.3 μm (Sakaguchi, Rao, Nakata, Nanbu, & Juneja, 2003). This particle size should facilitate its passage through the dialysis membrane and should raise its iron concentration in the dialysate. Morevover, more research to improve the quality of coatings and their resistance to high temperatures is ongoing (Zimmerman, 2004) since a potential barrier to use of encapsulated forms of iron in staple food fortification is the relatively low melting point of the capsules (45–65 °C), which may cause unwanted sensory changes during food preparation (Hurrell, Furniss, & Burri, 1989).

4. Conclusion

The optimisation of the in vitro method to measure iron availability in fortified citric juices includes the selection of a pH value of 8.5 for PIPES and Hepes buffers in order to allow the development of a uniform final pH of the digest/dialysate system similar to physiological conditions, and also to improve the formation and

stability of the ferrozine – iron complex needed for iron assays.

Data in our study lead us to conclude that the iron fortificant ferrous bis-glycinate seems to be the most stable compound when added to pineapple and passion fruit juices, confirming a high ferrous iron availability equivalent to the reference salt, ferrous sulphate. At the same time this iron chelate showed the highest total iron availability in the citric juice. On the other hand, other different aspects must be taken into account when a source of iron has to be selected, such as flavour, colour, stability and oxidation; therefore, more investigations should be performed to study possible variations of sensory and chemical parameters during the storage of the juice which could also cause modifications of iron availability. In vivo studies are also required to validate the use of this citric juice as a vehicle for novel iron compounds.

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