Redox-active labile iron in fortified flours from the Brazilian market

Ferro lábil redox-ativo em farinhas fortificadas do mercado brasileiro

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ABSTRACT

Objective

To quantify the fraction of redox-active labile iron in iron-fortified flours acquired on the Brazilian market.

Methods

Samples of wheat flour, maize flour and breadcrumbs were extracted with buffers that mimic gastric juice, saliva and intestinal juice. Redox-active labile iron levels were assessed through the reaction of autoxidation of ascorbic acid catalyzed by iron in the presence of a fluorescence probe.

Results

Redox-active labile iron represents 1% to 9% of the total iron in the flour and breadcrumb samples, with the lowest values found under gastric juice conditions and the highest in the more alkaline media. Redox-active labile iron possibly arises from the decomposition of an iron-phytic acid complex. A positive correlation between redox-active labile iron and total iron was found in saline biomimetic fluids.

Conclusion

Redox-active labile iron may be a risk factor for people with impaired antioxidant defenses, such as those who are atransferrinemic or iron overloaded (e.g. thalassemic). Total iron can be used to predict redox-active labile iron absorption at each stage of the gastrointestinal tract after ingestion of iron-fortified flours.

Indexing terms: Brazil; flour; fluorescence; iron; redox activity.

RESUMO

Objetivo

Quantificar a porcentagem de ferro lábil redox ativo em farinhas fortificadas adquiridas no comércio popular.

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Métodos

Amostras de farinha de trigo, fubá e rosca foram extraídas com tampões miméticos de suco gástrico, saliva e suco intestinal. Os níveis de ferro lábil redox ativo foram determinados por meio da reação de auto-oxidação do ácido ascórbico catalisada pelo ferro, em presença de uma sonda fluorimétrica.

Resultados

A fração de ferro lábil redox ativo representa entre 1% e 9% do ferro total nas farinhas estudadas, sendo os menores valores encontrados em condições miméticas do suco gástrico e os maiores nos meios mais alcalinos. Há indícios de que o ferro lábil redox ativo origina-se da decomposição de um complexo entre ferro e ácido fítico. Observa-se uma correlação positiva entre ferro lábil redox ativo e ferro total nas condições de salinidade dos fluidos biomiméticos estudados.

Conclusão

Ferro lábil redox ativo pode ser um fator de risco para pacientes atransferrinêmicos, sistemicamente sobrecarregados com ferro (por exemplo, talassêmicos) ou aqueles com defesas antioxidantes comprometidas por enfermidades. A quantidade de ferro total pode ser preditiva dos níveis de ferro lábil redox ativo absorvidos em cada etapa do trato gastrintestinal após a ingestão de farinhas fortificadas.

Termos de indexação*: Brasil; farinha; fluorescência; ferro; redox atividade.*

INTRODUCTION

Iron deficiency anemia is one of the main nutritional disorders today, affecting approximately 37% of the world's population[1]. Although there are no official statistics in Brazil, regional studies point towards a significant increase in the prevalence of this deficiency in risk groups in recent decades, with 50% - 83% of infants up to two years of age being considered anemic[2]. As in other developing nations, there is enough evidence to associate the iron status of the Brazilian population, irrespective of social class, with the relatively low bioavailable iron content of the major ingredients of the national diet (based mostly on grains and vegetables)[2,3].

Iron fortification of food is a cost-effective approach to providing iron for the population[4]. Fortificants differ markedly in chemical identity, bioavailability, likelihood of causing adverse sensory changes, advantages and usage restrictions. Thus, the food vehicle, food packaging and target population are very important factors to consider when choosing a specific iron compound and absorption enhancers.

After similar efforts throughout the world, Brazil started fortifying maize and wheat flours with 4.2mg of Fe per 100g of flour in 2002[5]. Approved additives include ferrous sulfate, ferrous fumarate, reduced iron, electrolytic iron, sodium iron EDTA and ferrous bisglycinate. The cost of this operation, as demonstrated previously[6], is negligible. Today it costs less than US$0.02 to fortify 100kg of flour with ferrous sulfate[7].

Although this approach has proved successful in decreasing iron deficiency worldwide[4,8], a number of studies have addressed the potential long-term side effects associated with the regular ingestion of highly bioavailable iron forms in food, at least for some segments of the population[8]. A basic assumption is that food fortificants might induce transient but systematic iron overload episodes which may be, at the onset of several clinical conditions, in part due to the propensity of iron to integrate physiological pools of weakly bound forms with potential pro-oxidant activity. In blood, these forms have been collectively termed labile plasma iron (LPI)[9] and defined as a heterogeneous fraction of iron associated with albumin and/or low molecular weight ligands such as citrate, hormones and amino acids. Interestingly, part of LPI has been shown to catalyze the autoxidation of ascorbic acid *in vitro* and in plasma samples at physiological ascorbate levels[9,10]. Ascorbic acid is one of the most promising enhancers of dietary iron absorption[11]. The pro-oxidant deleterious activity of the iron-ascorbate mixture both in food matrices[12,13] and in experimental animal

models[14-18] has been previously reported, although its relevance to daily meals is still elusive.

In this study, a fluorescence-based method was employed to quantify the redox-active, labile iron (RALI) in extracts of iron-enriched flours available in Brazilian supermarkets.

METHODS

The following reagents were used without further purification unless otherwise noted: ferrous ammonium sulfate (FAS), reduced iron (Fe^0), ascorbic acid, nitrilotriacetic acid (Cromoline, Diadema, Brazil); 1,2,3-dihydrorhodamine dihydrochloride (DHR; Biotium, Hayward, USA); deferiprone (L1; Apotex, Toronto, Canada); HEPES (*N*-2-hydroxy-ethylpiperazine-*N*'-2-ethanesulfonic acid), Chelex-100 (Sigma, St. Louis, USA). Iron-free HBS (HEPES 20mM, NaCl 150mM, pH 7.4) was obtained by treatment with 1g. $100mL^{-1}$ Chelex-100 resin.

Samples of fine (2 brands) or coarse (4 brands) maize flours, wheat flour (6 brands), tapioca flour (4 brands) and breadcrumbs (3 brands) were acquired in a supermarket (city of São Paulo, São Paulo state, Brazil) and used without processing.

Flour extraction

Artificial gastric juice (pH 1.2), saliva (pH 6.5) and intestinal juice (pH 6.8) were prepared as described[19]. Samples of *ca*. 1g of flour were treated with 5.0mL of each extractant or 5.0mL Milli-Q water (blanks) in plastic tubes and shaken at 130xg at 37ºC for 30 minutes in a Tecnal TE-421 incubator (Tecnal, Piracicaba, Brazil). Approximately 1.0mL of each suspension was transferred to plastic vials and centrifuged at 11 300 x*g* for 5 minutes. The supernatants were collected and kept in a refrigerator.

Redox-active Fe assay

The detailed protocol and its rationale have been described elsewhere[9]. In brief, quadruplicates of 20μL aliquots of the extracts were transferred to clear-bottom, 96-well plates (TPP, Trasadingen, Switzerland). Two of the replicates were treated with 40μM ascorbate and 50μM DHR in iron-free HBS. The other two received the same treatment plus 100μM deferiprone (L1). Standard Fe samples were prepared by the dilution of stock solutions of nitrilotriacetate:FAS (10:1 molar ratio) in the same solvents used for extraction and treated as the unknown samples. Fluorescence was recorded for 40 minutes at 37°C using a Tecan Genios microplate reader (Tecan, Mannendorf, Switzerland) with a 485/535 nm excitation/emission filter pair. The slopes *(r)* of DHR fluorescence curves against time were calculated from measurements taken between 15 and 40 minutes. Calibration curves were constructed by plotting the difference ($r_{no\ L1}$ - $r_{plus\ L1}$) against Fe concentration. The detection limit for iron is 2μM. Analysis of variance (ANOVA, $p<0.05$) followed by group comparison with Fisher's LSD (Protected t-Test) were performed with GB-STAT® software (version 9.0; Dynamic Microsystems).

Total Fe content

One gram of each sample was accurately weighed and treated with 10mL of concentrated HNO_3 in a beaker covered with a glass plate. The reaction was allowed to proceed for 5h at 100ºC over an electric plate. Two mL of H_2O_2 (30%) were added and the solution was refluxed for 1h at the same temperature. Finally, 2mL of concentrated HCl were added and, after 2 hours, the reflux was stopped. After cooling to room temperature, the acid extract was filtered to a 50mL volumetric flask and the final volume was adjusted. Triplicates were analyzed in a Spectro CIROS-CCD ICP-AES (Spectro Analytical Instruments, Kleve, Germany) spectrometer.

RESULTS AND DISCUSSION

Coarse maize flour (4 brands), tapioca flour (4 brands) and finely crushed breadcrumbs (2 brands) had no detectable amount of RALI. Since

these flours and breadcrumbs are not legally subjected to iron fortification, they where excluded from further studies.

The producers of each brand were personally contacted for information on the exact nature of the iron fortificant applied in the specific batches acquired for this study. All the flours were fortified with reduced iron (325 Tyler Mesh). Currently, this is the only iron fortificant used by Brazilian flour industries, for reasons that include low cost, ease of homogenization, lack of hygroscopic properties and absence of visual effects (e.g., brown spots in wheat flour are often observed due to oxidation of $FeSO_4$).

The total iron content of all the flour and breadcrumb samples was above the minimum required by law (Table 1; mean: 6.0mg/100g^{-1}; required: 4.2mg/100g^{-1}).

Total iron and RALI values for maize flour, wheat flour and breadcrumbs are shown in Table 1. The breadcrumbs brand in which RALI was found to be present had iron-fortified wheat flour in its preparation (according to the producer).

The strongly acidic solutions generated low concentrations of RALI. When observed, they were always significantly lower (p<0.05) than the RALI concentrations generated in intestinal juice. This result could be explained by pH effects on the fluorescence signal in the assay, since it is known that low pH values may decrease the quantum yield of a fluorescence probe[20] under steady conditions, resulting in a bias towards low values when solutions such as gastric juice are used. However, the advantage of this method is that a kinetic curve of the fluorescence is constructed, so that curve slopes rather than end-point measurements are used to assess iron concentrations[9]. Prior to the experiments, the slopes of the fluorescence curves were not affected by the pH of the medium (data not shown).

Therefore, the reason for the low RALI concentrations in gastric juice must be sought elsewhere. Stable iron complexes such as Fe(EDTA) show optimum formation under very acidic conditions. Iron thus effectively competes with H_3O^+ for the ligand-binding sites at low pH, but with increasing alkalinity it tends to be mobilized from the complex due to the formation of stable polymeric (hydr)oxides (in the extreme case[21], solid $Fe(OH)_3$; K_{sp}=2.0x10^{-39}). This is the thermodynamic reason underlying previous observations that the absorption of iron fortificants is prevented in the stomach and favored in the more alkaline environment of the duodenum[22]. Among the natural chelating agents present in flours, phytic acid (PA; myoinositol-6-phosphate) is the most prominent, forming very stable

Table 1. Redox-Active Labile Iron (RALI) and total iron in flours and breadcrumbs from different sources.

	RALI (ppm)[a]								Total Fe (ppm)[a]	
	I		S		G		W			
	M	SD	M	SD	M	SD	M	SD	M	SD
Maize 1	2.44	0.15	1.68	0.12	nd		nd		46.65	0.93
Maize 2	1.61	0.07	0.78	0.29	nd		nd		46.61	1.43
Breadcrumbs	3.59	0.15*	2.69	0.24	2.40	0.57	2.36	0.05	81.71	0.01
Wheat 1	4.49	0.29*	4.08	1.12*	1.69	0.35	4.64	0.23*	52.87	0.04
Wheat 2	3.68	0.28*	2.44	0.37	2.01	0.01	3.36	0.43*	65.32	0.57
Wheat 3	3.42	0.09*	3.31	0.25*	2.16	0.10	2.45	0.06	76.63	0.12
Wheat 4	1.82	0.12	1.62	0.83	nd		0.82	0.14	66.09	0.42
Wheat 5	2.89	0.87	1.48	1.01	nd		3.39	0.46	52.28	0.17
Wheat 6	2.09	0.47	1.91	0.65	nd		2.04	0.24	53.91	1.24

[a] Results are expressed as means and standard deviation - SD (n:2 for RALI and n:3 for Total Fe).
Asterisks(*) indicate significant differences from gastric juice according to analysis of variance (ANOVA, p<0.05) and comparison of the groups by Fisher's LSD (Protected t-Test).
nd: not detectable. I: artificial intestinal juice; S: artificial saliva; G: artificial gastric juice; W: water.

complexes[23] with iron (K_{ML} ~ 10^{25} - 10^{30}). Not surprisingly, PA is the major inhibitor of iron absorption from cereals, where it is present in the considerable amounts of 1% - 6% on a weight basis[24-26]. PA also renders iron non-redox-active even in the presence of ascorbic acid, suggesting its possible role as an antioxidant agent[27]. In order to gain some insight into the effect of pH on the stability of Fe-PA complexes, computer simulations with CHEAQS software[28] were performed adopting the pK_a values for PA derived from ^{31}P-NMR measurements[29] and the equilibrium constant[23] logK_{ML} of 25 for the $Fe(PA)_3$ complex formation. For simplicity, only the first deprotonation of PA was considered in the calculations (Table 2). At acidic pH, only the complex $Fe(PA)_3$ is stable in solution, but approximately 16% of the iron is mobilized to other less stable forms at pH 7. The fact that RALI is observed in some cases at pH=1.2 indicates that more complete databases (e.g. allowing for each of the twelve PA protonation events, different Fe:PA stoichiometries or variable ionic strengths) are required to properly calculate the equilibrium values. Thus, both experimental and simulated results suggest that RALI originates from the dissociation of a Fe^{3+}-PA complex formed in the flour during its extraction.

The protective effect of PA against polymerization of iron (hydr)oxo compounds can be observed when it is removed from the computation. As expected, even at pH 2 most of the metal is precipitated as oxide, and there are virtually no soluble iron species at pH 7.

A positive correlation between RALI and total iron in the flours was observed for the mimetic biological extractants but not for ultra-pure water (Figure 1). This indicates that total iron values can be used to predict the amount of redox-active metal mobilized at each stage of the gastrointestinal tract. Importantly, the results show that it is possible to determine the concentration of RALI formed in the small intestine, where dietary Fe absorption occurs[30]. In agreement with the above discussion, the formation of RALI in gastric juice is observed only at relatively high (>50ppm) total iron contents.

The absence of a linear correlation between RALI and total iron in pure water may be explained by considering the chemical nature of the fortificant (reduced iron). It may be assumed that any factors which favor the Fe^0 to Fe^{3+} conversion are operative during the extraction procedure. Iron corrosion in aqueous medium is known to depend on the oxygen concentration - which was the same for all extractants, since all the extractants were prepared with ultra-pure water - but also to be greatly accelerated by increased ionic strength[21]. Accordingly, we found that only the high salinity extractants mobilized iron in a predictable manner.

From the data in Table 1, it was found that *ca.* 1-9% of the total iron in the flour and breadcrumb samples is labile and redox-active when in the presence of physiological concentrations of ascorbic acid under the experimental conditions. These values are comparable to the 2-6% low molecular weight and redox-active iron found in parenteral iron supplements[10]. The relevance of

Table 2. CHEAQS PRO** simulation (I=0) of pH and phytic acid (PA) concentration effects on iron speciation (2μM).

	pH=2.0			pH=7.0	
	PA=0	PA=10μM	PA=0	PA=10μM	
Soluble species (%)*	$[Fe(H2O)_6]^{3+}$ (19.75)			$[Fe(OH)_2]^+$	(2.14)
	$[Fe(OH)]^{2+}$ (8.68)	$Fe(PA)_3$ (100)	none	$[Fe(OH)_4]^-$	(13.59)
	$[Fe(OH)_2]^+$ (0.27)			$Fe(OH)_3$ *aq*	(0.02)
				$Fe(PA)_3$	(84.25)
Insoluble species (%)*	Fe_2O_3 (71.30)	none	Fe_2O_3 (100)	none	

*Percent of total Fe concentration. *aq*: aqueous; **Verweig W. CHEAQS PRO [computer program][28].

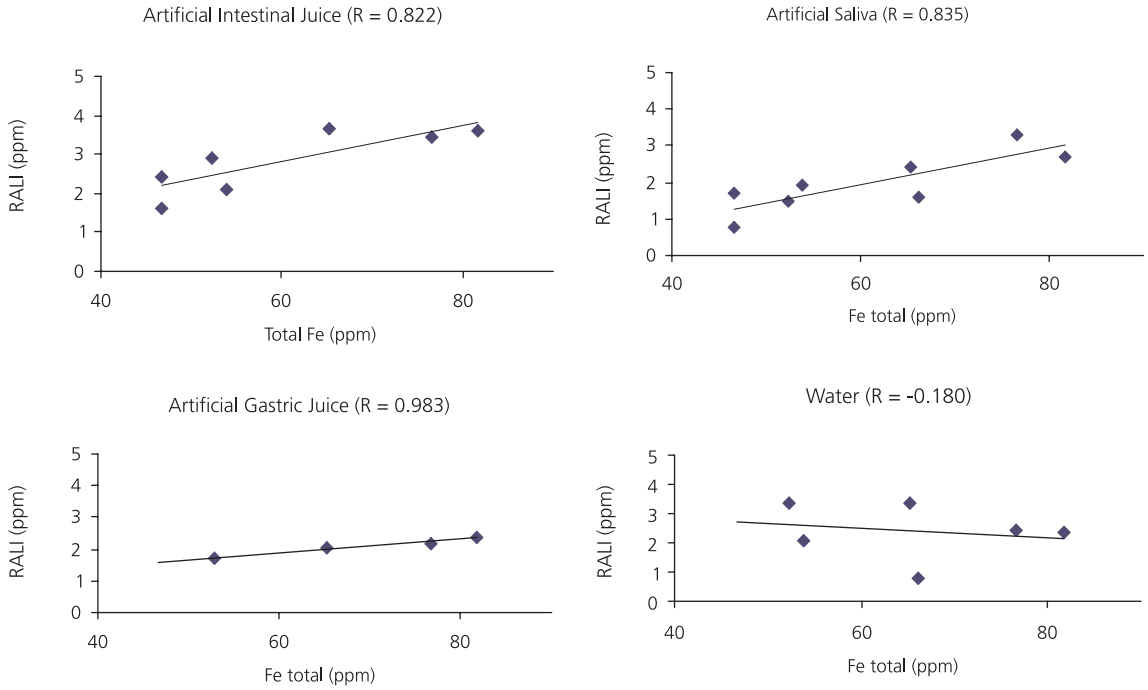

Figure 1. Correlation between total iron and redox-active labile iron (RALI) in fortified flours subjected to different extraction systems (values are taken from Table 1). Numbers in parenthesis show the correlation coefficients.

this information to the clinical evaluation of long--term exposure to dietary RALI in poorly nourished (with impaired antioxidant defenses), iron overloaded or atransferrinemic subjects deserves further investigation.

In conclusion, we found that the combined presence of iron and ascorbate favor the presence of RALI after extraction of iron-fortified flours, due to the redox activity of "free" (and not PA-bound) iron. A saline environment is required to accelerate Fe^0 to Fe^{3+} solubilization. Our results and computer simulations indicate that the decomposition of an iron-PA complex is the probable source of RALI. There is a positive correlation between RALI and total iron in the biomimetic fluids, RALI representing *ca.* 1%-9% of the total iron.

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