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RESEARCH ARTICLE

Development of a new bread type supplemented iron and folic acid– Chemical and technological characterization

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ABSTRACT

Bread is a staple food prepared by baking a dough of flour and water. The virtually infinite combinations of different flours and differing proportions of ingredients has resulted in the wide variety of types, shapes, sizes, and textures available around the world. Considering the worldwide consume of this staple food, this study aimed to develop and assess the chemical and technological characteristics of a new biofortified blend, containing wheat, locust bean flours, iron and folic acid (applied in the form of powder or microcapsules), for the production of bread with nutritional and prophylactic characteristics for human health. Besides bread wheat properties for baking, locust wheat flours was added to the blend in a small amount (0.5%) to increase water absorbance through its polar amino groups of proteins, whereas folic acid and iron inclusion considered the human needs on a daily basis. An 85.89- and 3.93-fold increases for folic acid and iron was carried out through fortification. It was found that, relatively to wheat flour T65, the contents of some minerals (Ca, K, Si), fatty acids (C16:0, C16:1, C18:0, C18:1; C20:1) and sugars (raffinose, sucrose, glucose and fructose) were significantly higher in locust bean flour. Upon blends iron and folic acid fortification, toughness, deformation work / gluten strength and the elasticity index prevailed when powder was used, whereas minimum values were obtained for ash, toughness and gluten strength in the standard blend. Moreover, significant differences were not found for fatty acids. In bread biofortified with folic and iron in the form of powder, all fatty acids (excepting C18:2 and C18:3) prevailed, but lower values were found for sugars and total soluble solids. Moreover, breads height, weight, specific volume remained higher in standard bread, but upon application of benzoic acid or methyl 4-hydroxybenzoate lower shelf life values were found. Although from a hedonic perspective, consumers preferred the standard bread, the biofortified blend revealed a high-quality index suitable for development of a functional staple food incorporating iron and folic acid (in the form of powder or microcapsules). Nevertheless, folic acid as proved to be highly labile during baking, but incorporation of microcapsules slightly limited this degradation. Considering the shelf life of the biofortified bread, pulverization with methyl p-hydroxybenzoate seemed to be the most effective additive.

Keywords: Bread fortification; Bread wheat flour; Folic acid; Iron; Locust bean flour

INTRODUCTION

The proportion of external iron exchange is normally very limited in the human body. In adult males' total losses from exfoliated cells and secretions ranges between 0.5 and 1.0 mg daily. These losses are matched by iron absorption from food, remaining the total iron content of the body fixed within relatively narrow limits. Yet, in female adult's greater iron losses occur during menstrual bleeding (between 4 and 50 mg) and if deficiency develops during pregnancy severe folic acid is decisive in the prevention of pregnancy complications, namely neural tube defects (Scholl, 2000), which may cause anencephaly or spina bifida, and also prevent the occurrence of other types of birth defects (Rao, 2006), including certain heart defects and limb malformations (Oakley, 2002). Yet, folic acid is

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a water-soluble vitamin (B_9) that cannot be stored in the human body, being necessary to ingest it daily (Ball, 2004; Lieberman, 2003). Accordingly, the development of a blend for bread production (which is a staple food worldwide) with addition of iron and folic acid can became a prophylactic functional food for maintaining public health.

Despite protein heterogeneity, albumin, globulin, gliadin and glutenin prevail in bread wheat flour. Prolamines (*i.e.*, gliadin and glutenin) represent about 80% of total protein and are responsible for gluten network formation, being responsible for retention of carbon dioxide generated during fermentation and for the mechanical behavior of dough (Lucas et al., 2019). Besides, protein contents also determine the viscoelastic properties of the masses. Elasticity and strength of the dough are largely determined by glutenin, while gliadins give viscosity. Starch dominate, namely in the form of granules within amyloplasts in the endosperm of bread wheat grains. Two starch types of polysaccharides, amylopectin (a branched polymer) and amylose (a linear molecule) occurs, with relative proportions of 75% and 25%, respectively (Bagulho, 2008). During baking two processes develops in starch (Scheuer et al., 2011): during heating in the presence of sufficient moisture, gelatinization, blowing up the granules (due to water absorption and therefore losing crystallinity); retrogradation, which determines a new crystallization of gelatinized starch chains. Triacylglycerols prevails in wheat flour, being fatty acids a relevant fraction (Becker, 2007). Some of these fatty acids are essential for health, namely palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) acids, but can also be implicated in the development of aroma and taste and also influence texture (Sgarbieri, 1987; El-Dash et al., 1990). Besides, the levels of minerals and vitamins are also very low in wheat grain (about 2 % - Lidon et al., 2019).

The proximate composition of locust bean flour contains lower protein and fat contents than wheat flour, but the opposite occurs with ash due to a higher mineral content (Akubor, 2016). Additionally, locust bean flour imparts dark color in blends flours (Lund and Smith, 1982). Locust bean flour also contains a high amount of total carbohydrates, which explains why is sweet and cherished by people. From a technological point of view locust bean flour also absorbs more water than wheat flour because might contain more hydrophilic groups of proteins and carbohydrates than the lipophilic groups (Akubor, 2016). Indeed, polar amino groups of proteins are the primary sites of protein – water interactions (Kinsella, 1981) and can absorb water up to 200% its weight whereas carbohydrates absorb only 15% of its weight (Zhurasvskaya, 1986). Locust bean flour is also stable from a storage point of view, since moisture content of above 15% can cause mold growth (Enwere, 1998).

In the baking blends, the texture and crumb of the final product is largely determined by bread wheat flour and water. During baking three stages prevail (Scheuer et al., 2011): during mixing/ kneading evolution of gluten viscoelastic properties with incorporation of air, followed by fermentation and cooking (which defines sensorial characteristics due to moisture vaporization, temperature rise, volume increase, changing of dough viscosity into bread crumb elasticity and spongy dough characterization).

Bread has a shelf-life coupled to the development of mold. At the macroscopic level, the identification of the fungus genus is possible namely through colony diameter, presence or absence of edge, zonation, roughness, elevation and exudate, texture and color. At the end of bread shelf life three mold genus prevail (Freitas and Figueiredo, 2000; Almeida et al., 2010): *Eurotium* (forms yellow colonies), *Trichoderma* (with rapid growth and white colonies, but therefore forms green or yellow tufts after one week of growth) and *Cladosporium* (develops black colonies). To preserve bread, preservative food additives such as benzoic acid and methyl p-hydroxybenzoate can be used (Lidon and Silvestre, 2010; Silva and Lidon, 2016). The pH range that provides the best efficiency for benzoic acid is 2.5 to 4.0, whereas methyl p-hydroxybenzoate is efficient in a pH range between 3 and 8, although being more active at low pH (Lidon and Silvestre, 2010; Silva and Lidon, 2016). Preservative food additives can change their efficiency (Lidon and Silvestre, 2010; Silva and Lidon, 2016) in the presence of other microorganisms and growth inhibitors (namely, salt, vinegar, sugar, product composition, water content) and additionally can also affect the sensorial characteristics of bread.

The aim of the present study was to present and discuss, following an integrated chemical and technological approach, a new blend with wheat T65 and locust bean flours enriched in iron and folic acid that can improve nutrition and add human health.

MATERIALS AND METHODS

The moisture content in bread wheat and locust bean flours was determined according to ISO 712: 2009. Five g of sample was weighed, for a previously dried and tared weighing device, together with the lid. Then, the open filter weigher, containing the sample for analysis together with the lid, was placed in the oven for 90 min at 130 ºC. The filter weigher was removed from the oven, closed and placed in the desiccator to cool. Weighed every 15 minutes to the nearest 0.001 g, until a constant weight was obtained. The water content, expressed as a percentage, was calculated according to equation 1.

$$H(\%) = 1 - \left(\frac{batch\ weight\ after\ drying\ (g)}{test\ mass\ (g)} \right) x100 \quad (1)$$

The ash content in bread wheat and locust bean flours was determined according to ISO 2171: 2007. Sample was weighed (5 g) into a crucible. The crucible was placed in the muffle at 900 ºC, until the product ignited, and remained there for 120 minutes, until total combustion. The crucible was removed from the muffle, allowed to cool for 1 min on a plate of heat-resistant material and then in the desiccator. When the crucible reached room temperature, it was weighed. The ash content was calculated according to equation 2.

$$A(\%) = \left(\frac{mass\ of\ the\ residue\ (g)}{sample\ mass\ (g)} \right) x100 \quad (2)$$

Protein quantification was performed on bread wheat and locust bean flours, following ISO 20483: 2013. The Kjeldahl method was adapted, which consists of three distinct stages, digestion, distillation and titration. In the digestion process, the organic nitrogen was transformed into ammonia ($NH4^+$) and the organic compounds were converted into H_2O and CO_2. Digestion was carried out at 400 ºC, with concentrated H_2SO_4 and a catalytic mixture, composed of sodium or potassium sulfate, which increased the boiling point of sulfuric acid, and copper sulfate pentahydrate. This digestion took about four hours. Before distillation, the digested sample was treated with NaOH to form ammonia (which was distilled by steam entrainment). The distillate was titrated with a 0.1N HCl solution, in the presence of a colored indicator. As this analysis does not allow to differentiate protein nitrogen (constituent of proteins) from non-protein nitrogen (NNP), the term crude protein (CP) was attributed to the result of the analysis. The CP content was determined by multiplying the total N content of the sample by the nitrogen conversion factor (N x 5.70).

The enzymatic activity / fall index was determined according to ISO 3093: 2009 (E). Flour was weighed, considering its humidity, and it was placed in a falling number tube. Then, 25 mL of distilled water was added to the flour and stirred continuously for 1 min. The tube with the solution was then placed in the apparatus and the time, in seconds, that the viscosimetric stirrer took to pass through the formed gel was recorded.

The contents of wet and dry gluten were carried out according to ISO 21415-1 and ISO 21415-4, respectively. About 10 g of flour was weighed and placed in a mortar. Distilled water (5 mL) was slowly added to the sample and kneaded continuously with the pestle to form a ball. Subsequently, it was kneaded by hand, taking care to collect all the particles of the sample in the mortar, to avoid losses. The sample was placed in a watch glass and left covered with a damp cloth, resting for 20 minutes. The sample was washed in running water to remove all starch from the flour. The excess water was dried and the wet gluten was weighed. Dry gluten was obtained by weighing after drying. The contents of wet and dry gluten (GS) were calculated using equations 3 and 4.

$$WG(\%) = \left(\frac{dry\ gluten\ mass\ extracted\ after\ washing\ (g)}{sample\ (g)} \right) x100 \quad (3)$$

$$DG(\%) = \left(\frac{mass\ of\ dry\ gluten\ (g)\ x\ sample\ (g)}{(100 - moisture\ of\ flour\ (\%)} \right) x100 \quad (4)$$

Mineral analysis was carried out using an X-ray fluorescence spectrometer (Niton XRF Analyzer Thermo Scientific - Mobile Test Stand). This X-ray fluorescence spectrometry was based on three basic concepts: an X-ray source (to irradiate the sample) and a detection system for measuring radiation from the sample (Brouwer, 2013). Yet, quantification of iron followed Reboredo et al. (2018). Approximately 1 g of sample was weighed into a 50 mL Erlenmeyer. In the hood, 10 ml of nitric acid (65%) was added to the samples and heated to 120 ºC until completely evaporated. Then, 2 mL of nitric acid (65%) and 3 ml of perchloric acid were added, and it was heated again until complete evaporation. The obtained residues were filtered with a 2% hydrochloric acid solution, until a 50 mL volumetric flask was made. To read the iron content in the samples, the Atomic Absorption Spectrophotometer equipment (model AAnalyst 200, manufactured by Perkin Elmer, with the software program AA WinLab) was used. Calibration curves were constructed to obtain equations that correlate the absorbance measured with the concentration in mg L^{-1} of iron present in the sample. The evaluation of the calibration line or linearity was performed using the correlation coefficient, whose value was close to 1.

Total lipids were extracted from the flours according to Zayas and Lin (1989). Approximately 1 g of flour per sample was weighed into a centrifuge tube and 20 mL of hexane. The tubes were placed in a beaker and the samples were shaken for 15 minutes on a shaking plate (Selecta, Agimatic 5), at 700 rpm, at room temperature. Samples were then placed in a centrifuge (Heraeus, Biofuge 28RS) at 4500 G, at 15ºC, for 10 minutes. The supernatant was collected, and 20 mL of hexane were added again, with stirring and centrifugation again under the described conditions. The extracts from the first and second extractions were combined and dried under nitrogen flow

in a water bath (40 ºC). The dry extract was resuspended in 600 µL of ethanol: toluene (1:4). Then, saponification and methylation of total lipids was carried out as reported by Metcalfe et al. (1966), with the addition of heptadecanoic acid (C17:0, internal standard) and 2 mL of methanol - BF3 in each tube. For each sample 2 methylations were carried out. Methylated fatty acids were analyzed using a gas-liquid chromatograph (Varian CP-3380, USA), coupled to a flame ionization detector. For the separation of fatty acids, a 30 m DB-Wax capillary column (J&W Scientific), with 0.25 mm internal diameter and 0.25 µm film thickness, was used. The injector and detector were kept at 200 ºC and 250 ºC, respectively. Hydrogen was used as the carrier gas (1 mL / min), and a flow partition of 1/50. The chromatographic peaks were identified by comparing the retention times of each fatty acid methyl ester with mixtures of standards (Sigma, Supelco and Restek). The results were presented as a percentage of each fatty acid identified in the lipid fraction.

Soluble sugars were cold extracted according to Medlicott and Tompson (1985). Sample of flour (400 mg) was weighed into a centrifuge tube, to which 10 mL of cold water. Tubes were placed in a glass with ice on a shaking plate for 30 minutes and then submitted to ultrasound for 5 minutes. Centrifugation was performed at 15000xg (20 minutes, 4 ºC). The supernatant was collected in a glass vial. The process was repeated under the same conditions, for washing the precipitate. The extracts from the first and second extractions were added and cold filtered. The aqueous extract was subjected to new filtration, the sugars were analyzed by High Performance Liquid Chromatography (Waters, USA), coupled to a refractometric detector (Waters, 2414), equipped with a SugarPak 1 column (Waters 6.5 X 300 mm).

The determination of folic acid in blends and breads was performed based on a microbiological procedure with ELISA reading, according to AOAC 960.46 and AOAC – International (2005). The VitaFast Folic Acid kit was used. Sterile water (2 mL) was added to the standard folic acid flask and homogenized. Then, standard dilutions were prepared in sterile flasks (1.5 - 2 mL). Then sample (1 g) was homogenized, weighed and placed in a sterile 50 mL flask and 30 mL of phosphate water was added. The sample was stirred, and the pH was adjusted to 7 - 7.5 (using 1 N NaOH solution). Phosphate water (10 mL) was added and sample was placed in a water bath, at 90 °C, for 30 minutes. The vial was shaken every 6 minutes. Rapid cooling to below 30 °C was carried out. Then 1.5 mL of sample prepared in a sterile flask was stirred and filtered. One or more dilutions were performed, when necessary. Thereafter, a microtiter plate was prepared and 150 µL of standard folic acid was pipetted into each well. Then, 150 µL of samples were pipetted into the wells. It was homogenized and the plate was covered with adhesive film, so that the wells were well closed. The plate was incubated, at 37 °C, for 48 hours, in the absence of light. In this last stage, the adhesive film was compressed to guarantee the sealing of the wells, and the microplate was placed in an inverse position on a flat surface. The plate was shaken to homogenize the microorganisms, and the plate was again inverted to the normal position. The adhesive sheet was removed diagonally. The bubbles present were removed with the pipette tip and turbidity was measured with the ELISA reader at 610-630 nm.

Colorimetric parameters of flours and blends was determined according to the color system proposed by the Commission Internationale L´Eclairage (CIE). This system uses a three-dimensional space with 3 axes, where: L* is the axis of luminance, which refers to the human perception of luminosity. Numerically, for absolute black, L is equal to zero, and for absolute white, L is equal to 100; a* is the axis from green to red, with -120 being the maximum for green and 120 the maximum for red; b* is the axis from blue to yellow, with -120 being the maximum for blue and 120 the maximum for yellow (Minolta, 1994). The equipment was previously calibrated, with white. Subsequently, the samples were placed in a specific glass for color reading. For each flour and blend, 3 readings were performed.

The alveograph test simulates the behavior of the dough during fermentation and assesses the viscoelastic characteristics of wheat flour. This test was carried out according to ISO 27971 (E): 2008. Wheat flour was weighed (250 g) and placed in the kneader. Then, the blister was put into operation and an amount of NaCl 2.5% (w/v) solution was added, determined by the moisture of the flour. Leave to knead until 8 minutes. Five samples are extracted and placed in the isothermal rest chamber until 28 min. Thereafter, each sample was insufflated until the mass burst. The main parameters evaluated in this test were: toughness (P), extensibility (L), deformation work or gluten strength (W) and the elasticity index (I.e).

The farinnograph test was carried out according to ISO 5530: 2013 and allowed to measure the resistance of the mass through a mechanical action. Flour was weighed with the precision of 0.01 g, based on the moisture content. The flour was placed in the kneader and a volume of water was added to achieve the maximum consistency, corresponding to 500 UF. When it was found that the flour and water formed a dough, the walls of the kneader were scraped with a plastic spatula. After 12 min, the test is finished, and the parameters are read on the graph. From the reading of the farinograph test, the following parameters were obtained: absorption of the dough (Abs), which indicates the amount

of water added to the flour, expressed as a percentage, so that it reaches the standard consistency that corresponds to 500 farinograph units (UF); the development time (DT), which corresponds to the period of time expressed in minutes, from the beginning of the kneading until the moment when the dough reaches the consistency of 500 UF; the stability time (ST), which reveals the time interval during which the mass maintains maximum consistency and is measured by the time that the curve is above 500 UF and the degree of weakening (DW), which indicates the difference in UF between the maximum consistency and the consistency 12 min after the start of the curve.

Breads production were prepared, in an automatic machine, according to the proposal of Portuguese Standard 2100 - 3 (2003), with wheat flour (T65) and 0.5% locust bean flour. The ingredients were used in the proportions referred to in the Standard, with the exception of the salt (that was corrected to 1.0%, according to the recommendation of WHO) and water (whose quantity was tested to find the most suitable hydration. Additionally, the addition of folic acid and powdered iron heptahydrated (blend 1) and microencapsulated (blend 2) was further carried out. The content of iron incorporated was 7 mg / 100 g of flour in the formulations, complying with WHO recommendations and the requirements of legislation in several countries where fortification is mandatory. The content of folic acid incorporated in the formulations was 250 μg of folic acid / 100 g of flour. Baking lasted approximately 4 hours. Weight was determined and expressed in grams. The production of microcapsules of folic acid and iron sulfate heptahydrate were carried out according to Lopera et al. (2009) but drying by lyophilization. For the freezing and drying of the suspensions, a bench freeze dryer (Biobase brand, model BK-FD10P) was used. The freezing stage took place for 2 hours and 48 minutes, at -52.5 ºC. Then, the frozen samples were placed in the drying chamber for 26 hours and 45 minutes, at -40 ºC and at a pressure between 45 - 50 Pa.

To evaluate the volume of the bread, the seed displacement method with rapeseed seeds was used. The volume of seeds needed to fill the empty form, and the volume of seeds to complete the volume of the form containing the baked bread was measured. The volume of the bread was obtained considering the difference in volumes. The specific volume of the bread was determined according to equation 5.

$$SV\,(\%) = \left(\frac{weight\ of\ seeds \times specific\ volume\ of\ rapeseed\ seeds}{weight\ of\ bread} \right) \quad (5)$$

For determination of bread shelf life, the additives benzoic acid 99.5% (E210) and methyl 4-hydroxybenzoate 99.0 - 100.5% (E219) were used (100 mL of each 0.1% additive) as solutes and ethyl alcohol 70% as solvent. Each sample consisted of 3 slices of bread sprayed (crumb and crust) with 15 mL of the additive solution. After spraying, the solvent was allowed to evaporate, and the samples were placed in microperforated plastic bags. All samples were photographed on the day of application of the additives (day zero). The bags were closed and placed in a transparent box at room temperature (≈ 23 ºC). Molds were identified and observed at microscopic level using a binocular magnifying glass (Leica M165 C - Leica Microsystems). The number of days that each sample remained without visible molds was recorded.

The sensorial evaluation test was carried out with 67 untrained evaluators, of both genders, aged between 10 and 70 years. Each evaluator received three samples of bread, with random numbering, together with an evaluation form with a hedonic scale of nine points. This scale corresponded to a score from 1 to 9 for the "very unpleasant" and "very pleasant" extremes, respectively, for the characteristics of color, aroma, flavor and overall appreciation. The results were analyzed by sensory profile, in accordance with ISO 13299: 2016.

The experimental results were statically evaluated by one-way analysis of variance (ANOVA) to determine significant differences between the analyzed samples. Significance was also determined by the F test using the Tukey test ($p \leq 0.05$) for comparative study of means.

RESULTS AND DISCUSSION

Wheat flour T65 is the finest flour mostly used for industrial or homemade bakery (namely, for production of white bread, cakes, pies or pastries). It is extracted from the central part of the endosperm, having a light tone, fine granulometry and high amount of gluten. The moisture content of this wheat flour T45 ranged around 12.5% (Table 1), therefore remaining within the optimal limits for wheat flours (Silva et al. 2010). The ash content was found (Table 1) to be low (0.69%), being a relevant benchmark since, far from correlating with total mineral content, it also influences color flour (Remelgado, 2016). Indeed, the whiter color is closely linked to a higher quality grade of the white flour (Silva, 2003). In this context, X-ray fluorescence spectrometry analysis showed that (Table 1), relatively to the locust bean flour, wheat flour T65 showed a higher content of P and S (about 2.195 and 3.301 $g.kg^{-1}$, respectively). The contents of Ca, K and Si (in $g.kg^{-1}$) was *ca.* 0.456, 3.170 and 1.115 in wheat T65 flour, while in locust bean flour prevailed with values of 11.671, 24.733 and 1.473 $g.kg^{-1}$, respectively. These differences between both

Table 1: Mean values (*n* = 3) of moisture, protein, ash, fall rate, glute, minerals, fatty acids sugars, color, alveographyc and pharynographic parameters in locust bean and/or bread wheat (T65) flours. Letters a and b indicate significant different between both flours (*P*< 0.05). According to Mazliak (1983), Double Bond Index = [(%monoenes + 2 x % dienes + 3 x % trienes) / (% saturated fatty acids)]

Analytical parameters		Wheat flour T65	Locust bean flour
Moisture (%)		12.50±0.01[a]	7.02±0.00[b]
Ash (%)		0.69±0.01[b]	2.55±2.37[a]
Color parameters	L*	91.12±0.59[a]	33.30±0.32[b]
	a*	(-0.26)±0.02[a]	11.16±0.07[b]
	b*	9.39±0.08[a]	19.34±0.15[b]
Gluten (%)	Wet	25.7±0.11	---
	Dry	9.1±0.01	---
Protein (%)		12.12±0.14	5.09±0.17
Falling number (s)		319±2.73	---
Minerals ($g.kg^{-1}$)	Ca	0.456±0.009[b]	11.671±0.137[a]
	K	3.170±0.055[b]	24.733±0.305[a]
	P	2.195±0.035[a]	1.317±0.029[b]
	Si	1.115±0.027[b]	1.473±0.047[a]
	S	3.301±0.026[a]	1.712±0.033[b]
Fatty acids	C16:0 (mol %)	18.22±0.73[b]	24.71±1.59[a]
	C16:1 (mol %)	0.19±0.04[b]	1.70±0.11[a]
	C18:0 (mol %)	1.56±0.17[b]	5.20±0.32[a]
	C18:1 (mol %)	14.74±0.62[b]	46.59±1.28[a]
	C18:2 (mol %)	60.57±0.72[a]	15.50+±0.45[b]
	C18:3 (mol %)	3.43±0.13[a]	2.96±0.15[a]
	C20:1 (mol %)	0.97±0.16[b]	1.73±0.16[a]
	Σ <1% (mol %)	0.33±0.01[b]	1.60±0.38[a]
	Total ($mg.g^{-1}_{DW}$)	16.87±0.26[a]	2.87±0.04[b]
	Double Bond Index	7.39±0.30[a]	2.93±0.19[b]
Sugars ($mg.g^{-1}_{DW}$)	Rafinose	8.7±0.1[b]	31.0±0.6[a]
	Sucrose	23.5±0.8[b]	118.4±1.9[a]
	Glucose	1.4±0.0[b]	111.5±0.3[a]
	Frutose	0.6±0.1[b]	37.1±1.6[a]
Total soluble solids	($mg\ g^{-1}_{DW}$)	34.2±0.9[b]	298.1±4.3[a]
Color parameters	L*	91.12±0.59[a]	33.30±0.32[b]
	a*	(-0.26)±0.02[b]	11.16±0.07[b]
	b*	9.39±0.08[a]	19.34±0.15[a]
Farinogram	Abs (%)	60.85±0,14	---
	DT (min)	3.12±0,63	---
	ST (min)	6.45±0,14	---
	DW (UF)	74.00±5,77	---
Alveogram	P (mm)	99.80±0,23	---
	L (mm)	63.92±0,40	---
	W ($E^{-4}J$)	211.83±3,32	---
	I.e.	1.56±0,01	---

types of flours are related with the diverse genetic makeup, agronomic characteristics and productivity among cultivars and species, being in general somewhat higher then reports from other authors (Peterson et al., 1983; Rennan et al., 2008; Dias et al., 2009a, b; Akubor, 2016), but indicating a greater nutritional value. Furthermore, following the CieLab scale, it was found (Table 1) that the colorimetric values of parameters L*, a* and b* of the wheat flour were 91.12, -0.26 and 9.39, respectively. Although parameters a* remained typical for white wheat flour, the values for L* and b* remained lower and higher, respectively (Oliveira, 2012). Accordingly, the wheat flour T65 showed a high brightness and tending towards white, although with a slight hue to yellow, as relatively to the parameter L they were slightly below 94. Moreover, relatively to the wheat flour T65, the locust bean flour revealed (Table 1) a lower moisture (ca. 7.02%), higher ash contents (about 2.55%, therefore indicating augmented levels of minerals) and a lower value for the colorimetric parameters L*, whereas a* and b* remained significantly higher (33.30, 11,16 and 19.34, respectively). These colorimetric parameters pointed, relatively to the wheat flour T65, a darker luminosity, a

slightly green and yellow tendency. It was found that the contents of total fatty acids of the wheat flour T65 was (Table 1) ca. 16.87 $mg.g^{-1}_{DW}$, whereas locust bean flour showed a significant lower amount (2.87 $mg.g^{-1}_{DW}$). The level of total fatty acids of the wheat flour T65 remained within the usual range (Silva et al., 2010) and further indicated a Double Bond Index of 7.39. Moreover, the locust bean flour revealed a lower Double Bond Index of 2.93, clearly pointing that degree of unsaturation of total fatty acids prevails in the wheat flour T65, which might favor rancidification. In this context, the fatty acid profile of both flours showed significant differences (Table 1). Wheat flour T65 showed, relatively to locust bean flour, only higher amounts of linoleic acid (C18:2), being the amounts of linolenic acid (C18:3) similar. Nevertheless, palmitic acid C16:0, oleic acid C18:1 and linoleic acid C18:2 prevailed relatively to the remaining fatty acids (Table 1). Besides, total soluble solids was found to be, relatively to wheat flour T65, 8.72 fold higher in locust bean flour (Table 1) and raffinose, sucrose, glucose and fructose also prevailed in locust bean flour (3.56, 5.04, 79,64 and 61.83 fold, respectively). Following a nutritional perspective, both flours are good sources of fatty acids and carbohydrates, and therefore can constitute a good caloric matrix for a staple functional food development. Furthermore, the amount of wet and dry gluten contents (Table 1) in wheat flour T65 was *ca.* 25.7 and 9.1, respectively. Considering that gluten cannot be found in locust bean flour, the wet gluten of bread wheat determines the hydratation capacity in the blend mixture (Stanley, 2007). Additionally, the wet gluten is also responsible for retaining the fermentation gas in the dough, giving lightness to the fermented products, and therefore being related to the final quality of the products, providing a better texture, shape and expansion (Scheuer et al., 2011). Like gluten, also the protein content, relates to the quality of the finished product (*i.e.,* texture and appearance). It was found (Table 1) that the contents of protein in wheat T65 and locust bean flours was *ca.* 12.12 %, and 5.09%, respectively. For products with harder texture than cakes, such as bread, high levels of protein are desirable (Stanley, 2007). Indeed, considering the manufacture of French bread as a reference, the range of protein contents for bread must be around 10.5 – 13.0 % (Guarienti, 1996), which points that wheat flour T65 can be consider of excellent quality for bread making, whereas the locust bean flour must be used only as supplementary. The measured fall index of wheat T65 flour, which is directly related to the activity of the α-amylase, was (Table 1) 319 s. As the ideal fall index for bread making must range between 201-350 s (Guarienti, 1996), α-amylase activity causes the best saccharification of starch molecules during the bread making process, resulting in breads with a sticky and moist internal texture. For the wheat flour T65, the farinograph test revealed (Table 1) average values of Abs, DT, ST and DW close to 60.85 %, 3.12 min, 6.45 min and 74.00 UF, respectively (Table 1), which indicated that it is a medium flour since it has an absorption near by 60%, development time between 2.5 - 4.0 min, stability the 3-8 min interval and weakening inside 60-100 UF. Concerning to toughness (P), extensibility (L), overall gluten strength (W) and the elasticity index (I.e) of the wheat flour T65, it was found that wheat flour T65 showed average values of 99.80 mm, 63,92 mm, 211.83 $E^{-4}J$ and 1.56, respectively. Although dependent on the P value, the higher the L value, the greater the bread volume, with the balance reflecting the P/L ratio associated with the value of W. Accordingly, the wheat flour T65 showed a medium-strong overall gluten strength since had a W-value ranging from 201-300 $E^{-4}J$ (Guarienti, 1996).

The levels of the standard blend showed low levels of folic acid and iron (*ca.* 9.33 μg/100g and 1.65 mg/100g, respectively), but after biofortification average values of *ca.* 85.89- and 3.93-fold increases occurred, respectively. In this context, the standard blend having locust bean (0.5%) and bread wheat flours showed (Table 2), relatively to the wheat flour T65, lower average values of toughness (88.9), overall gluten strength (188 $E^{-4}J$) and elasticity index (1.29), but an higher of extensibility (69.2). Moreover, upon blend biofortification the average values of P, W and I.e. became significantly higher relatively to the standard blend (whereas the opposite occurred with L). Yet, P, L and W remained significantly lower with microcapsules biofortification. Accordingly, considering the intrinsic properties of all the blends, it was found that the standard blends and biofortified with microcapsules, relatively to the wheat flour T65, changed to a medium overall gluten strength (Guarienti, 1996). Besides, the level of ashes also became significantly higher in the biofortified blends, but the opposite occurred with moisture (Table 2). Relatively to the fatty acid composition, only the amounts of palmitic acid (C16:0) were significantly lower in the standard blend, whereas relatively to sugars contents biofortification with powder revealed lower levels of raffinose and fructose, still without significant changes in the contents of total soluble solids (Table 2).

Standard bread only showed (Table 3) a slight decrease of folic acid, but biofortified breads with powder or microcapsules displayed much higher and significant decreases (to about 13.32% and 27.80%, respectively). Moreover, iron contents, relatively to blends composition, only decreased significantly in the biofortified bread with powder (Table 3). Accordingly, it must be pointed that the high temperatures of baking bread strongly degrade folic acid, mostly if added to the blends as powder, whereas the lost rates of iron is highly restricted. In this context, biofortified bread, relatively to the standard bread, showed

Table 2: Mean values (*n* = 3) of moisture, ash and alveographyc parameters of blends (standard and biofortified with powder or microcapsules) produced according to the Portuguese Standard 2100 - 3 (2003), with locust bean (0.5%) and bread wheat flours. Letters a and b indicate significant different between both flours (*P*< 0.05)

Analytical parameters	Standard blend	Fortified Blend (folic acid and iron heptahydrated)	
		Powder	Microcapsules
Moisture (%)	13.73±0.01^{a}	13.64±0.01^{b}	13.61±0.01^{c}
Ash (%)	0.75±0.01^{c}	0.77±0.00^{b}	0.78±0.00^{a}
P (mm)	88.9±0.41^{c}	98.7±0.40^{a}	94.2±0.58^{b}
L (mm)	69.2±0.54^{a}	64.2±0.63^{b}	60.7±0.35^{c}
W ($E^{-4}J$)	188±1.53^{c}	233.3±1.45^{a}	196.5±2.60^{b}
I.e.	1.29±0.01^{b}	1.55±0.03^{a}	1.56±0.02^{a}

Table 3: Mean values (*n* = 3) of folic acid, iron, fatty acids, sugars and total soluble solids in blends and breads (standard and biofortified with powder or microcapsules) produced with locust bean (0.5%) and bread wheat flours. Letters a - d indicate significant different between both flours (*P*< 0.05)

Analytical Parameters		Blend			Bread		
		Standard	Fortified (Powder)	Fortified (Microcapsules)	Standard	Fortified (Powder)	Fortified (Microcapsules)
Folic acid (µg/100g)		9.33±0.67^{d}	801.33±0.67^{a}	801.00±1.00^{a}	8.67±0.67^{d}	106.67±1.76^{c}	222.67±1.45^{b}
Iron (mg/100g)		1.65±0.13^{c}	6.54±0.10^{a}	6.43±0.16^{a}	1.82±0.13^{c}	4.12±0.12^{b}	6.44±0.03^{a}
Fatty acids	C16:0 (mol %)	17.9±0.65^{c}	19.42±0.35^{b,c}	18.92±0.07^{b}	20.10±0.83^{a,b}	21.88±0.28^{a}	20.71±0.64$^{a.b}$
	C16:1 (mol %)	0.12±0.00^{c}	0.13±0.00^{c}	0.13±0.00^{c}	0.60±0.10^{b}	0.98±0.05^{a}	0.55±0.03^{b}
	C18:0 (mol %)	1.38±0.07^{c}	1.20±0.03^{c}	1.20±0.01.^{c}	2.33±0.17^{b}	5.33±0.94^{a}	3.72±0.73$^{a.b}$
	C18:1 (mol %)	14.28±1.67^{a,b}	12.21±0.31^{b}	12.57±0.03^{b}	14.2±0.12^{a,b}	17.4±0.87^{a}	17.53±0.51^{a}
	C18:2 (mol %)	62.05±1.02^{a}	62.74±0.15^{a}	62.87±0.03^{a}	58.44±0.58^{b}	49.41±0.74^{c}	53.21±0.79^{b,c}
	C18:3 (mol %)	3.46±0.03^{a}	3.36±0.01^{a}	3.50±0.00^{a}	3.33±0.04^{a}	3.00±0.05^{b}	3.00±0.11^{b}
	C20:1 (mol %)	0.53±0.01^{a}	0.49±0.00^{a}	0.51±0.00^{a}	0.53±0.05^{a}	0.47±0.03^{a}	0.49±0.03^{a}
	Σ <1% (mol %)	0.93±0.02$^{a.b}$	0.97±0.03$^{a.b}$	0.93±0.01$^{a.b}$	1.59±0.09^{a}	1.54±0.35^{a}	0.80±0.04^{b}
Sugars ($mg.g^{-1}_{DW}$)	Rafinose	5.95±0.09^{a}	5.52±0.10^{b}	5.96±0.09^{a}	0.34±0.03^{d}	0.54±0.03^{c}	0.59±0.02^{c}
	Sucrose	16.15±0.73^{a}	14.15±0.32^{a}	16.07±1.05^{a}	52.48±1.33^{b}	36.96±0.97^{c}	55.27±1.22^{b}
	Glucose	1.38±0.08^{a}	1.14±0.07^{a}	1.35±0.05^{a}	n.d.	n.d.	n.d.
	Frutose	1.00±0.08^{c,d}	0.88±0.07^{d}	1.31±0.10^{c}	4.41±0.18^{a}	2.04±0.06^{b}	2.47±0.18^{b}
Total Soluble Solids ($mg\ g^{-1}_{DW}$)		24.49±0.71^{a}	21.69±0.28^{a}	26.69±1.07^{a}	5.72±0.15^{b}	3.95±0.10^{c}	5.83±0.14^{b}

Table 4: Mean values (*n* = 3) of height, weight, specific volume, humidity and shelf life (without food additives, or with application of benzoic acid – E210, and methyl-4-hydroxybenzoate – E219) of breads (standard and biofortified with powder or microcapsules) produced with locust bean (0.5%) and bread wheat flours. Letters a - c indicate significant different between both flours (*P*< 0.05)

Analytical Parameters		Standard Bread (Without additives)	Fortified Bread (folic acid and iron heptahydrated)	
			Powder	Microcapsules
Height (cm)		10.83±0.07^{a}	10.43±0.03^{c}	10.57±0.03^{b}
Weight (g)		605.33±0.88^{a}	603.67±0.67^{b}	601.33±0.33^{b}
Specific volume ($cm^3.g^{-1}$)		3.69±0.14^{a}	3.80±0.01^{a}	3.79±0.01^{a}
Moisture (%)		24.40±0.21^{c}	26.07±0.18^{a}	25.10±0.12^{b}
Shelf life (days)	Without additives	4.67±0.33^{b}	6.33±0.33^{a}	6.00±0.00^{a}
	Benzoic acid (E210)	5.67±0.33^{b}	7.67±0.33^{a}	8.33±0.33^{a}
	Methyl 4-hydroxybenzoate (E219)	6.33±0.33^{b}	8.33±0.33^{a}	8.67±0.33^{a}

(Table 4) significant height and weight decreases (still with a higher height decrease with powder, but without significant deviations of weight for between both biofortified forms). The specific volume did not vary significantly among biofortified and standard breads, whereas the highest humidity was found with biofortification using powder (Table 4). Interesting was to notice that in biofortified bread with powder, the low height corresponded to the highest values for specific volume and humidity, which correlated with the significant highest overall gluten strength of the blend (Table 3). Considering the lability of fatty acids, it was further found that after bread baking only linoleic C18:2 and linolenic (C18:3) acids (in biofortified breads) became significantly degraded due to the high temperatures applied (Table 3). Moreover, the other fatty acids in general displayed higher values mostly due to dehydration during

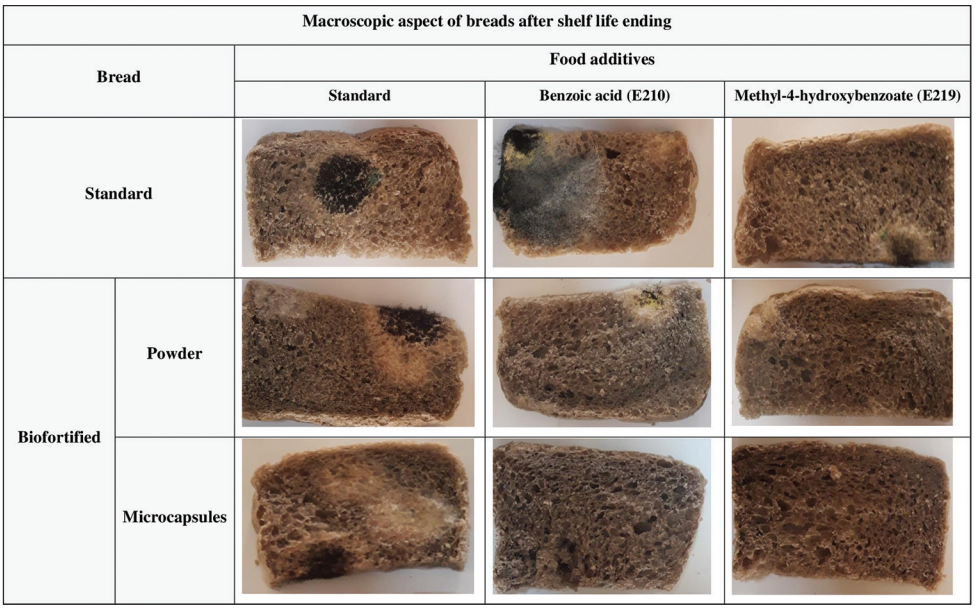

Fig 1. Macroscopic aspects of bread (standard and biofortified with powder or microcapsules) without food additives or treated benzoic acid – E210, and methyl-4-hydroxybenzoate – E219

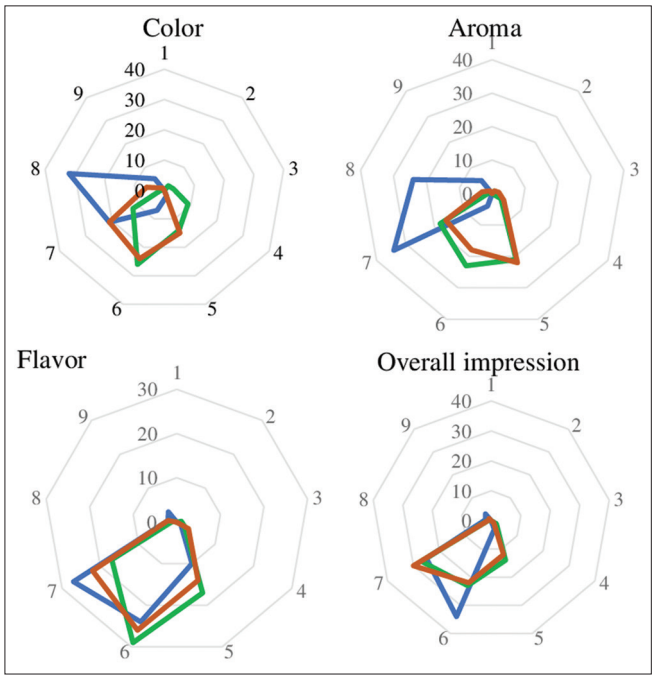

Fig 2. The sensorial evaluation test (n = 3) carried out with 67 untrained evaluators, of both genders, aged between 10 and 70 years. Scale corresponded to a score from 1 to 9 for the "very unpleasant" and "very pleasant" extremes, respectively. Standard bread ; Biofortified bread (powder) ; Biofortified bread (microcapsules)

breads processing. Incorporation of food additives to increase the bread's shelf life showed (Table 4; Fig. 1) that benzoic acid and methyl p-hydroxybenzoate had a significantly higher efficiency, in all bread types. Methyl 4-hydroxybenzoate (E219) increased about 2 days all bread types, showing an apparent higher efficiency than benzoic acid (E210). Interesting was to note that biofortified bread independently of the application of food additives revealed a higher shelf life. Regardless of the incorporation of these food additives, after shelf-life ending, they developed without an apparent sequence, yellow, green and black molds (Fig. 1), corresponding to the genera *Eurotium*, *Trichoderma* and *Cladosporium*, respectively (Freitas and Figueiredo, 2000; Almeida et al., 2010). Independently of food additives application It was found that for color, aroma, flavor and overall impression parameters, the standard sample obtained the highest scores on the hedonic scale, followed by the sample enriched with microencapsulated iron (Fig. 2).

CONCLUSION

Iron deficiency is one of the most common causes of anemia, and blood loss is the most common cause of iron deficiency in adults, namely women after menopause. Additionally, folic acid participates in the synthesis of nucleic acids, in the metabolism of lipids and in the transformation of some amino acids, being important namely for fetal development. Considering that the concept of bread quality is closely related to consumer's destiny, this work developed a biofortified bread with iron and folic acid that can contribute to prevent or correct nutritional deficiencies implicated in the development of anemia and abnormalities with fetal development. The quality parameters vary according to the different types of flour and different industrial uses. In this context, independently of the heterogeneity of locust bean and wheat T65 flours quality, a nutritional blend can be developed incorporating both flour at a ratio of 99.5 – 0.5%. This flour matrix

still as a high quality index (*i.e.,* namely levels of minerals, protein, fatty acids, sugars and gluten) that can further be used for development of a functional staple food incorporating iron and folic acid (in the form of powder or microcapsules). Nevertheless, folic acid as proved to be highly labile during baking, but incorporation of microcapsules slightly limited this degradation. Considering the shelf life of the biofortified bread, pulverization with methyl p-hydroxybenzoate seems to be the most effective additive. Moreover, from a hedonic perspective, consumers preferred the standard bread (*i.e.,* nom biofortified).

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Authors' Contributions

All authors contributed equally to the experimental design, discussion of results and preparation of the manuscript.

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