Citrate-coated cobalt ferrite nanoparticles for the nano-enabled biofortification of wheat

Yazmín Stefani Perea-Vélez[a], Rogelio Carrillo González[a], Ma. del Carmen A. González-Chávez[a*], Jaco Vangronsveld[b,c], Iván Ortiz Monasterio[d], Daniel Tapia Maruri[e]

Table S4 Followed protocols to evaluate the nutritional quality of wheat grains.

Variable	Method	Procedure	Reference
P concentration in grain	Vanadomolybdo phosphoric acid colorimetric method	Grain samples were ground into powder and acid digested.	[1]
Degree of biofortification		The degree of biofortification was expressed as a percentage, and it was calculated according to the following formula: $\text{Degree of biofortification} = \left(\frac{\text{FeF} * 100}{\text{FeC}}\right) - 100$ Where: FeF: Fe content in grains from plants fertilized; FeC: Fe content in grains from control plant	[2]
Soluble Fe and Zn in grains	Atomic absorption by the flame method	500 mg of ground wheat grain were extracted with 25 mL of Tris HCl buffer (50 mM, pH 7.5) in Falcon tubes by shaking at 120 oscillations per min for 18 h at 37 °C. Afterward, the samples were centrifuged at 13 000 rpm for 10 min. The supernatant was filtered through a 0.2 µm membrane filter and stored until its analysis.	[3]
Phytic acid concentration in grain	Modified colorimetric Wade reagent method	A sample of 0.5 g of powdered grain was mixed with 10 mL of 2.4% HCl solution (v/v) in a Falcon tube and agitated at 220 rpm for 16 h. The acid extract was centrifuged at 3000 rpm at 10 °C for 20 min. The supernatant was transferred to a Falcon tube containing 1 g NaCl and shaken at 350 rpm for 20 min. The mixture was then allowed to settle at 4 °C for 60 min. The mixture was then centrifuged at 3000 rpm at 10 °C for 20 min. One mL of supernatant was diluted 25 times in a centrifuge tube with deionized water. Three mL of this diluted sample was combined with one mL of modified Wade reagent (0.03% $FeCl_3$ $6H_2O$ + 0.3% sulfosalicylic acid), one mL of deionized water. The reaction mixture was vortexed for 30 s and centrifuged. The absorbance of the reaction mixture was measured at 500 nm after 15 min on a spectrophotometer (Varian, Cary 50 Scan UV-Vis). The concentration of phytic acid in the extract was estimated using a calibration curve of sodium phytate from 3 to 48 mg L^{-1}. After the interpolation with a standard curve of phytic acid, the result obtained was multiplied by 0.282 to express the content of phytate phosphorus in the sample because this constant corresponds to the molar ratio of P in the phytic acid molecule.	[4,5]
Phytic acid: Fe and Zn molar ratios		The concentrations of phytic acid, Fe, and Zn were converted into moles by dividing their respective molar mass and atomic weights. The molecular weight of the phytate used was 660.04 g mol-1, and the atomic weights 55.84 g Fe mol^{-1} and 65.38 g Zn mol^{-1} were used	[6,7]
Bioaccessibility test	INFOGEST	A sample of 5 g of whole grain was boiled at 95 ± 5 °C with drinking water with a ratio of 1:12 (w:w grain:water) until wheat grains were soft. Through the extraction procedure, the temperature was kept at 37 ± 2 °C, and constant mixing conditions were obtained by placing the tubes in an orbital incubator at speed of 55 rpm. The mastication process was simulated by placing a sample of soft wheat grain and 5 mL of simulated saliva fluid (SSF; 4mL of electrolyte stock solution 1.25x, 0.025 mL of $CaCl_2(H_2O)_2$ 0.3M solution, and 0.975 mL of distilled water), in a manual mincer. The grains were minced to a paste with a similar consistency to the mustard. The swallowable bolus was then incubated for 2 min at 37± 2 °C. Afterward, the bolus (from the oral phase) was mixed with simulated gastric fluid (SGF; 8 mL of electrolyte stock solution 1.25x, 0.005 mL of $CaCl_2(H_2O)_2$ 0.3M solution, 0.667 mL of Pepsin, 0.48 mL of Lipase), and the pH of the sample was adjusted to pH 3 with HCl 5 M solution. 1.228 mL of distilled water was added to the sample to obtain a final ratio of food to SGF of 1:1 (v/v). The tubes containing the sample were incubated for 2 h. For Fe bioaccessibility in the gastric phase, the samples were centrifuged at 4000 rpm for 5 min, and the supernatant was filtered through a 0.2 µm nylon membrane filter. The filtrates were then placed in boiling water for 5 min, to stop the digestion reaction and freeze for further analysis. On the other hand, the gastric chyme was mixed with simulated intestinal fluids (SIF; 8 mL of electrolyte stock solution 1.25x, 0.04 mL of $CaCl_2(H_2O)_2$ 0.3M solution, 5 mL	[8]

		of pancreatin, 3 mL of bile salts, 3.46 mL of distilled water, 0.5 mL of NaOH 5M solution) to achieve a final ratio of 1:1 (v/v). The mixture was then incubated for two h. Finally, the digestion mixture was centrifuged at 4000 rpm for 5 min. The supernatant was filtrated through a 0.2 µm nylon micro filter. The elemental analysis was done within 24 h by ICP-MS (ICP-OES, Agilent 725-ES).	
Protein concentration in grains	Kjeldahl method	An aliquot of 5 mL of the acid-digested grain sample was mixed with 30 mL of distilled water, and 20 mL of NaOH. The sample was distilled and titrated with 0.01 N H_2SO_4 solution. The protein content was calculated by multiplying the amount of total nitrogen with the conversion factor of 6.25 which assumes the nitrogen content of proteins in foodstuffs is 16%.	[9,10]
Soluble sugars in grain	Enzymatic method	Soluble sugars and free amino acids were analyzed in a 50 mg sample of powdered wheat grains and extracted with 2 mL of ethanol 80% (v/v) in a water bath at 80°C for 40 min. The extract was then cooled and centrifuged at 13,000 rpm for 10 min. The supernatant was kept at 4 °C until its analysis. For the quantification of soluble sugar, an aliquot of 10 µL of the extract was placed in a micro plaque. Then, 150 µL of reaction mix (500 mM HEPES pH 8, 200 mM KCl, 200 mM $MgCl_2$, ATP, NAD, H_2O), and 0.15U (10 µL; enzyme diluted with reaction mix) of hexokinase enzyme was added. The micro plaque was then incubated at 37°C for 40 min. and the absorbance was then measured at 340 nm in a micro plaque lector (BioTek©). Afterward, 0.12 U of phosphoglucoisomerase was added to the samples, and the micro plaque was incubated for 40 min at 37°C. The absorbance was then recorded once again at 340 nm. The previous process was repeated by adding 0.12 U of invertase, and the concentration of glucose, fructose, and sucrose was estimated through the function of a calibration curve from 0.1 to 0.5 µM of each sugar.	[11]
Free amino acids in grain	Method described by Jones et al[12]	100 µL of ethanolic extract was mixed with 50 µL of the ninhydrin color reagent. The mixture was incubated in bathwater at 80°C for 30 min. After cooling, 950 µL of ethanol 50% (v/v) solution was then added, and mixed. An aliquot of the sample was placed in a micro plaque and the absorbance was measured at 570 nm in a micro plaque lector (BioTek©). The amino acid concentration was calculated through the function of a calibration curve of glycine from 10 to 200 µM.	[12]

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