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# Elemental mapping of biofortified wheat grains using micro X-ray fluorescence☆

I. Ramos [a], I.M. Pataco [b], M.P. Mourinho [b], F. Lidon [b], F. Reboredo [b], M.F. Pessoa [b], M.L. Carvalho [a], J.P. Santos [a], M. Guerra [a,*]

[a] Laboratório de Instrumentação, Engenharia Biomédica e Física da Radiação (LIBPhys-UNL), Departamento de Física, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

[b] GeoBioTec, Departamento de Ciências da Terra, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

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ABSTRACT

Micro X-ray fluorescence has been used to obtain elemental maps of biofortified wheat grains. Two varieties of wheat were used in the study, *Triticum aestivum* L. and *Triticum durum* desf. Two treatments, with different nutrient concentration, were applied to the plants during the whole plant growth cycle. From the obtained elemental maps it was possible to extract information regarding the plant's physiological processes under the biofortification procedures. Both macro and micronutrients were mapped, providing useful insight into the posterior food processing mechanisms of this biofortified staple food. We have also shown that these kind of studies can now be performed with laboratory benchtop apparatus, rather than using synchrotron radiation, increasing the overall attractiveness of micro X-ray fluorescence in the study of highly heterogeneous biological samples.



## 1. Introduction

In the last years, the application of micro X-Ray Fluorescence (μ-XRF) imaging to the investigation of elemental distribution within biological tissues and particularly in foods, has been steadily increasing (cf. [7,8,13]) and references therein). Several competing techniques such as Inductive Coupled Plasma Mass Spectrometry (ICP-MS), Scanning Electron Microscope coupled to Energy Dispersive Spectrometry (SEM-EDS), nano Secondary Ion Mass Spectrometry (nanoSIMS) as well as Synchrotron Radiation μ-XRF (SR-XRF) can provide better lateral resolution and lower detection limits than benchtop μ-XRF apparatus, although the sample preparation, beamtime allocation and especially the long times for acquisition and analysis of the spectra, result in the loss of versatility when compared to benchtop spectrometers. A recent discussion of imaging methods, such as the ones enumerated above, for use in hydrated biological samples has been performed by Zhao et al. [16]) highlighting the usefulness of XRF as an elemental distribution analysis tool.

Cereals grains are a very good example of highly heterogeneous systems at the μm or sub-μm range, and thus provide an analytical challenge for XRF imaging [7]. Nevertheless, cereal grain elemental distribution has been investigated with several techniques, especially SR-XRF, for example in barley [8], in foliar biofortified wheat [1] and rice [2]. Rice, in particular, has been given a lot of attention over the last few years, with several groups investigating Hg and As concentration and localization, as it is of major importance due to the fact that it is a major food source especially in Asian countries [2,9]. From the obtained elemental mappings and considering the food processing mechanisms, it is possible to quantify the nutrient losses from the cereal grains to the final product, which is very important for food processing companies. This makes elemental, as well as molecular, mappings a very important tool in the optimization of food processing, especially when dealing with biofortified staple crops [1].

Biofortification is becoming an established technique not only for overcoming nutritional deficits of populations [6], but also for studying the metabolic elasticity and homeostatic processes of plants [5], as well as soil–plant interactions and phytoremediation [14]. In this work, we have studied the elemental distribution in biofortified wheat grains of two different varieties, *Triticum durum* desf. and *Triticum aestivum* L., and compared the elemental concentrations in the internal structures of the grains with those of control grains.

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* Corresponding author.
*E-mail address:* mguerra@fct.unl.pt (M. Guerra).

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The main goal of this work is, not only to show that benchtop μ-XRF can be used as a tool for obtaining elemental maps in highly heterogeneous biological samples competing with other more expensive techniques, but also to understand if this kind of analysis can be used to differentiate element distribution in biofortified and control wheat grains.

## 2. Materials and methods

### 2.1. Biofortified wheat grains

In this work, we have used two sets of wheat grains, one of the varieties is a bread wheat (*Triticum aestivum* L.) and the other from the hard type (*Triticum durum* desf.).

#### 2.1.1. Triticum aestivum L

Certified seeds of *Triticum aestivum* cv. Roxo were washed, sterilized and sown in 3 L pots for subsequent growing in a walk-in ARALAB chamber (10.000 EHF, serial 1084), under environmental controlled conditions (80% relative humidity, 500 ppm of $CO_2$, 22/18 °C day/night 12 h photoperiod, 800 μmolQ $m^{-2}$ $s^{-1}$). After germination, the plants were administered with the following nutrient solutions: 3 mL/L and 15 mL/L of a complete nutritive solution in the plants that originated from fourth generation control seeds (F4 0.3/0.3) and (F4 1.5/1.5), respectively. The complete nutritive solutions were prepared by mixing solutions A and B and completed to a final volume of 1000 mL with distilled water. Solution A containing 0.09 g $MnCl_2 \cdot 4H_2O$ (Merck 1173874), 0.12 g $(NH_4)_6 \cdot MO_7O_{24} \cdot 4H_2O$ (Merck 1182), 0.01 g $H_3BO_3$ (Merck 10043353), 0.016 g $ZnSO_4 \cdot 7H_2O$ (Merck 7446200), 0.08 g $CuSO_4 \cdot 5H_2O$ (Merck 7758987), 0.16 g $FeCl_3 \cdot 6H_2O$ (Sigma-Aldrich 44943) and 12.00 g $C_6H_8O_7 \cdot H_2O$ (Merck 902), dissolved with 50 mL of $H_2SO_4$ 95–97% (Merck 7664939); solution B, contained 111 g $NH_4NO_3$ (Merck 6484522), 30 g $Na_2HPO_4 \cdot 2H_2O$ (Panreac 122507), 65 g $K_2SO_4$ (Scharlau PO02871000), 17 g $CaCl_2 \cdot 2H_2O$ (Scharlau CA01941000) and 4.8 g $MgSO_4$ (Merck 7487889), dissolved in 700 mL of water. Before application, freshly nutrient solutions were prepared. The final grains were harvested 141 days after germination.

#### 2.1.2. Triticum durum desf

Certified seeds of the durum wheat, *Triticum durum* cv. Marialva were washed, sterilized and sown in 3 L pots (4 plants per pot), having a substrate with the same characteristics as the other wheat variety. The experimental design considered two nutritional treatments (application of 15 and 90 mL of the nutrient solution described above, dissolved in 5 L of distilled water treatments (c15) and (c90), respectively). The final grains were harvested 126 days after germination.

### 2.2. μ-XRF

The elemental maps of the wheat grains were obtained using the micro-Energy Dispersive X-Ray Fluorescence (μ-EDXRF) system (M4 Tornado™, Bruker, Germany). This spectrometer consists of an air-cooled micro-focus side window Rh-anode X-ray tube, powered by a low-power HV generator. The system features a poly-capillary X-ray optics, which allowed a spot size of 25 μm for Mo $K_\alpha$. The X-ray generator was operated at 50 kV and 100 μA without the use of filters, to enhance the ionization of low-Z elements. For a better quantification of the heavy elements, a set of filters between the X-ray tube and the sample was used, composed of three foils of Al/Ti/Cu with a thickness of 100/50/25 μm, respectively. All the measurements with filters were performed with 600 μA current. The elemental maps were obtained without filters, while the point spectra for quantification were taken with and without the Al/Ti/Cu set of filters. Detection of fluorescence radiation is performed by an energy-dispersive silicon drift detector, XFlash™, with 30 $mm^2$ sensitive area and energy resolution of 142 eV for Mn $K_\alpha$. In order to better interpret the distribution maps of the elements, the wheat grains were cut in half, some longitudinally, along the crease tissue, and some transversely (as can be seen if Figs. 1, 3, 4 and 5), with a stainless steel surgical blade. The two halves of the longitudinally cut grains were then glued onto a mylar foil with the inner sides upward, and placed on the x, y, z translation stage for analysis. For the cross sectional images, one of the grain's halves was cut again in order to obtain a slice of approximately 2 mm thick and glued onto a mylar foil and placed directly on the stage. Measurements were taken under 20 mbar vacuum conditions and performed directly on the two sides of the grains, first in the mapping mode, and then with point analysis on interest sites. These point spectra were acquired during 200 s, while the mappings were performed with a pixel spacing of 15 μm, in order to completely cover the grain, with a measuring time of 6 ms per pixel. Quantification of the point spectra was performed with the WinAXIL™ software package (Camberra, Belgium), using three reference samples (Bush Branches GBW 07603, Poplar Leaves GBW 07604 and Orchard Leaves NBS 1571) in the compare mode [3,11]. The elemental maps were created with the built-in software from the M4 Tornado™, ESPRIT. The obtained weight concentration was not corrected to the total grain biomass, and hence the values for the individual structures within the grain present much higher concentration than the literature values for the whole grain. This is due to the fact that the endosperm, which accounts for more than 80% of the grain biomass, is mainly composed by low atomic weight elements that cannot be measured by this technique. Regarding the mapping software, it is important to refer that, due to the low acquisition time at each pixel, the code does not deconvolve the pixel spectra because of the very low statistics. Instead, it uses Regions Of Interest (ROI) in the spectra, located around the $K_\alpha$ or $L_\alpha$ lines of the selected elements. This results in some inter-elemental dependence on the image hue intensity, as some characteristic lines of a given element can fall inside of the ROI of another element. For example, if one wants to perform an elemental mapping with this software of a sample containing As and Pb, the map would be exactly the same for both elements, as the ROI is the same. The geometrical constraints of the spectrometer do not allow the x-ray beam to hit the sample perpendicularly, instead the sample is irradiated from the side at a 50° angle, making a 100° angle with the detector. This, most of the times results in shadows in the elemental maps, which have to be analyzed bearing this in mind.

## 3. Results and discussion

Elemental maps were obtained for all of the grains, and the qualitative results are completely consistent with each other (except in some few cases, pointed throughout the text). We have thus chosen to present the results for 2 radomly selected grains of each variety, one control grain of the *Triticum aestivum* cv. Roxo type, labeled (F4 0.3/0.3) and the other biofortified (F4 1.5/1.5). For the *Triticum durum* cv. Marialva variety we chose one of the control grains, (c15), and one from the highest concentration treatment (c90). In Fig. 1, we present the maps obtained for these chosen grains. The color scale in the image is only indicative of the relative concentration for each individual element, as it is obtained from scaling the hue according to the minimum and maximum X-ray intensity within the specific ROI. That is the reason why, for elements which are near the detection limit of our spectrometer, the images become too noisy to analyze even with image filters. The superposition of the chosen elements maps was performed by adding the images as layers in the acquisition software.

We have also collected point spectra on the internal structures of the grain, and although the lateral resolution of the spectrometer positioning software does not allow a very good identification of the micrometer sized structures, we were able to extract some quantifiable information from the Scutelum and Radicle structures in the embryo, and from the vascular bundle and bran structures. Mass fraction, in μg/g, of the