Technical aspects of zinc and iron analysis in biofortification of the staple food crops, wheat and rice

James Stangoulis[A]

[A]Flinders University, School of Biological Science, Bedford Park, South Australia, Email james.stangoulis@flinders.edu.au

Abstract
A major priority for any breeding initiative is to have in place effective tools for assessing the genetic variation of the trait of interest. Ideally, these technologies should be relatively low in cost and also rapid in their analysis to allow for high throughput. In its early years, the HarvestPlus program has relied heavily on ICP-OES for its micronutrient analysis as it has very good sensitivity, can have a throughput of around 500 samples per day and is able to detect Al and Ti which are seen as indicators of soil contamination in plant tissue samples. The down-side to the technology is the high cost of analysis and the degree of specialization needed to run the equipment. Colorimetric techniques such as Dithizone (for Zn) and Perl's Prussian Blue (for Fe) have been developed for high throughput screening and are currently in use within some breeding programs. Newer technologies are also being explored and they include NIRS and both hand-held and bench-top XRF. Results are promising and research in this area is continuing. Research into ways of minimizing harvest and post-harvest soil contamination of plant tissues has also lead to more robust protocols and development of "contaminant-free" equipment for use by all research disciplines working in biofortification.

Key Words
Biofortification, HarvestPlus, iron, zinc, analysis.

Introduction
It is estimated that over three billion people are afflicted with micronutrient malnutrition (Welch and Graham, 2004) and a new biofortification initiative is currently underway through the HarvestPlus challenge program (www.harvestplus.org) to provide better Fe, Zn and pro-Vitamin A carotenoid nutrition to many of those afflicted. Plant breeding programs working in biofortification of staple food crops such as rice and wheat aim to screen germplasm, varieties and elite lines for Fe and Zn-dense traits to identify genotypes that might be used as donor parents. Initial analysis from these research groups has shown that the micronutrient content, particularly Fe, is affected by production and post-harvest handling. Processing of grains plays a critical role in retaining, as well as losing micronutrients, especially during harvesting, threshing, drying, dehulling and milling. Technical strategies are needed to overcome the constraints of screening field harvested seed for analysis of micronutrients. This paper describes recent developments in this crucial area of post-harvest handling of seed, with a particular focus on rice and wheat, as well as more recent developments in rapid analysis techniques.

Materials and methods
Crop harvesting and processing methods for plant breeders are reported in Stangoulis and Sison (2008) while methods for rapid screening techniques are presented in Choi *et al.* (2007).
Micronutrient analyses were performed by ICP-OES through the Waite Analytical Service according to the method reported in Zarcinas *et al.* (1987).

Results and discussion
Crop sampling and processing methods
Crop sampling and processing methods have been developed and published (Stangoulis and Sison 2008) and are available for many staple food crops. These protocols will be updated as new research leads to improvements in sampling and handling methods. For example, in the wheat sampling protocols, there has been no method available to adequately remove soil contamination from field harvested seed, but new research is now available to show that a 5-10 second polishing using a modified commercially available Kett rice polisher, will remove Al and Ti from the surface of the grains, with these elements being indicators of soil contamination (Stangoulis *et al.* Data unpublished). This will now allow wheat breeders to harvest their biofortification plots in the same way as their other breeding plots.

For wheat, the post-harvest treatment of seed to reduce contamination is a little more simpler when compared to rice because there doesn't have to be a polishing step as the target populations for introduction of the biofortified wheat crop, mostly eat ground whole grained products like chapatti. But for rice, the target populations mostly eat milled, polished rice. This created an added complexity to the post harvest sampling method as all commercially available dehulling equipment give some form of contamination; mostly Zn due to the rice seed coming into contact with the black rubber-like compounds in the equipment. In the early days of biofortification research at IRRI, this problem was removed by manual hand-dissection of grains, where paddy rice was broken open by Teflon-coated forceps to extract the brown rice seed. It was imperative that new equipment was found to dehull the grains without contamination and various rubber-type commercially bought products were tested and a polyurethane product with 7 ppm Fe and 3 ppm Zn was found to have the lowest level of Fe and Zn respectively. The rollers in the dehuller were recoated with the new low nutrient compound and tested for contamination and were found to not contaminate samples (Figure 1). Rollers coated with the contaminant-free compound were sent to all NARES in South East Asia who were working on biofortifying rice and this eliminated the need for hand-dehulling. This was also taken one step further with the modification of the commercially bought Kett Mill, where the Zn contaminating black rubber compound in the polishing chamber was replaced with the new contaminant free compound and this greatly reduced Zn contamination. In 2007, over 10 new Kett Mills were converted and sent out to NARES programs to be used in their breeding programs. For rice, the research is still continuing to provide a contaminant-free polishing mill that is able to take very small sample sizes, with a focus on a small Chinese mill that will take under 5 g of sample. For wheat, the ability of the Kett rice polisher to remove soil contamination has lead to a satisfactory completion of protocols for this crop.

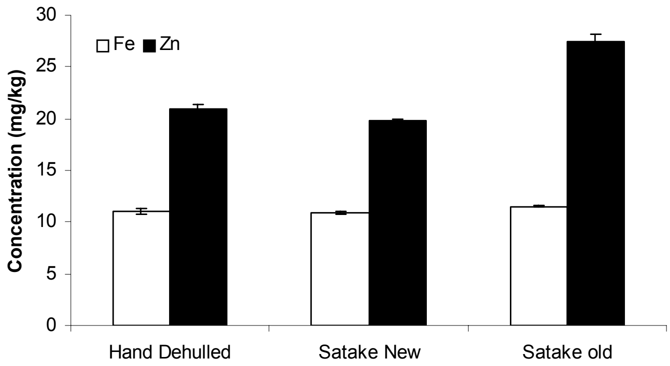

Figure 1. Effect of roller composition on Fe and Zn concentration (mg/kg) of Langi rice. For hand dehulled samples, values represent the mean of 4 replications ± SE, while for the remaining, values represent means of 10 replications ± SE. "Satake New" has modified rollers where the old black rubber was removed and polyurethane was recoated onto the metal rollers.

Micronutrient analysis

Pfeiffer and McClafferty (2007) summarised the various analytical methods and state of the art high throughput screening techniques available to plant breeders. They highlighted the importance of Inductively Coupled-Optical Emission Spectrometry (ICP-OES) over other accurate analytical tools (i.e. AAS) for the simple reason that it is sensitive enough to analyse for Al and Ti which are used as indicators of soil contamination. As the authors rightly point out, detecting contamination while assaying micronutrient concentration is complicated and references are not available to guide the research community but increases in Al and Ti in the analysis are the best indicators that we have at the moment as these elements don't readily enter the plant and their presence in analysis at significant levels is therefore an indication that the result may be inaccurate.

HarvestPlus collaborators have also been using high throughput screening techniques to try and reduce the throughput through the more expensive ICP-OES. High throughput techniques include colorimetric techniques such as modified Perl's Prussian Blue and 2,2 Dipyridal (Choi *et al.* 2007; Ozturk *et al.* 2006). Correlations between Fe determined by ICP and the 2,2 Dipyridal colorimetric method in rice, wheat, maize, sweet potato and cassava ranged between 0.88 and 0.98. The strategy is to eliminate up to 85% of screened material by using these techniques while those considered to have higher levels of Fe and Zn would then be evaluated more extensively by more accurate methods such as ICP-OES and AAS to identify the Fe and Zn-dense genotypes.

For future analysis, the indirect method of NIRS is being evaluated at CIP for its use in screening for Fe and Zn in various crops and the benefit of this technology is that the sample does not have to be digested before analysis and one can screen on whole grains. Also, given that many breeding programs are already using NIRS for traits such as protein, there is no extra work needed in sample preparation. XRF technology is also showing very promising results. The Thermo bench-top XRF has been evaluated and one can see from Figure 2 that the XRF gives comparable results to the ICP-OES for both rice and wheat. The benefit of using this technology is the ease of use which is of paramount importance to NARES in developing countries, the high throughput that one can achieve (analysis time is 3 min per sample) and there is no digestion of sample before analysis. For smaller grains such as rice, it is also possible to screen using whole grains which again reduces the time that one has to put into preparing the sample for analysis. Results so far are showing that XRF technology has potential to allow plant breeders to run their own high throughput analysis.

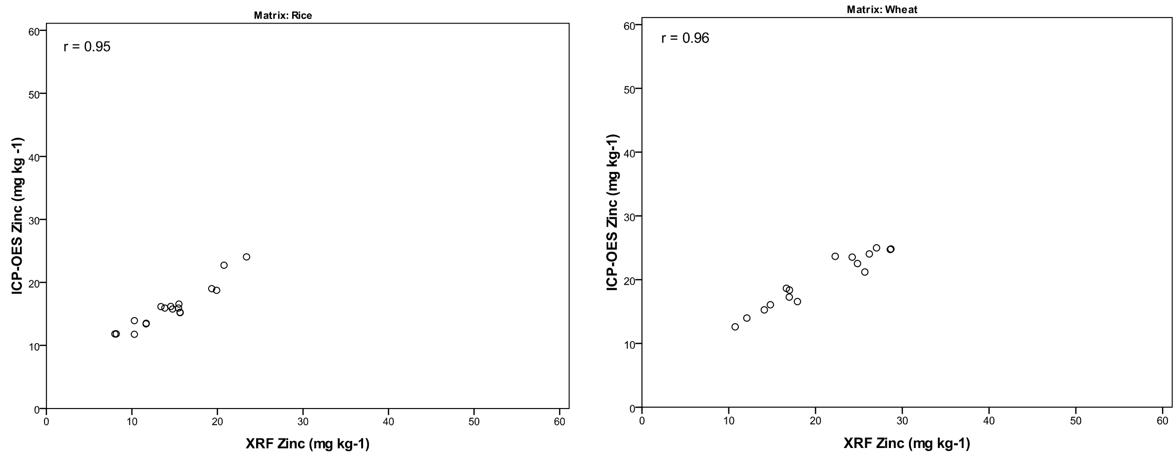

Figure 2. Correlation between XRF analysed Zn and ICP-OES analysed Zn for rice and wheat. XRF analysis time was three minutes.

Conclusion

The advent of biofortification has lead to extensive research programs to try and develop Fe and Zn-dense staple food crops. Plant breeders have had to rethink the way in which they screen for Fe and Zn-dense genotypes and this has required the elimination of soil and machinery contamination. New contaminant-free equipment has been identified and in some case, old types of equipment commonly used in plant breeding have had to be modified. Plant breeders now have protocols to follow to minimise contamination and they also have analytical tools to fasten the screening process. With technologies such as XRF, it is hoped that the complexity of selection in the breeding process will be reduced and further enhance the rapid release of new biofortified crops.

References

Choi EY, Graham RD, Stangoulis JCR (2007) Rapid semi-quantitative screening methods for determination of iron and zinc in grains and staple foods. *Journal of Food Composition and Analysis* **20**, 496-505.

Ozturk L, Yazici MA, Yucel C, Torun A, Cekic C, Bagci A, Ozkan H, Braun HJ, Sayers Z, Cakmak I (2006) Concentration and localization of zinc during seed development and grain germination in wheat. *Physiologia Plantarum* **128**, 144-152.

Pfeiffer WH, McClafferty B (2007). Biofortification: breeding micronutrient-dense crops. Chapter 3. In 'Breeding major food staples for the 21st century'. (Eds MS Kang, PM Priyadarshan) pp. 61-91. (Blackwell Scientific: Oxford).

Stangoulis J, Sison C (2008). 'Crop Sampling Protocols for Micronutrient Analysis'. HarvestPlus Technical Monograph Series 7. ISBN 978-0-9818176-0-6.

Zarcinas BA, Cartwright B, Spouncer LR (1987). Nitric acid digestion and multi-element analysis of plant material by inductively coupled plasma spectrometry. *Communications in Soil Science and Plant Analysis* **18**, 131-146.