A Comparative Study of Iron and Zinc Concentration, Localization, Speciation and Bioavailability in Two Wheat Cultivars

Thesis submitted in accordance with the requirements of the University of East Anglia for the degree of Doctor in Philosophy

Submitted by

Tristan Eagling

September 2013

All work is the authors' own unless stated, Caco-2 analysis was performed with the assistance of Anna waver at UEA, and results obtained form part of her thesis.

Abstract

Iron (Fe) and Zinc (Zn) deficiency remains a prevalent nutritional disorder worldwide, disproportionally affecting people of low and middle income countries due to the reliance on non-meat sources in the diet. Cereals, a relatively poor Fe and Zn source, account for over 50% of the energy intake in developing countries, and are a potentially important target for biofortification strategies aimed at improving dietary Fe and Zn content and utilisation.

The effectiveness of a crop in combating Fe and Zn deficiency is largely dependent on Fe and Zn speciation, as different forms vary in their bioavailability. Size exclusion chromatography, coupled with inductively coupled plasma-mass spectrometry is a valuable approach to identify and quantify different forms of Fe and Zn in the grain. Adaptations of the method to quantify and identify nutritionally relevant forms of Fe and Zn (ferritin, Fe-phytate, mono-ferric phytate and Fe-nicotianamine, Zn-nicotainamine) are described, together with the assessment of bioavailability of Fe in wheat using an *in vitro* Caco-2 cell model system.

Speciation of Fe and Zn varied between milling fractions with low molecular weight (LMW) complexes likely to be Fe-deoxymugenic acid/nicotianamine and Zn-nictainamine being the predominant extractable Fe and Zn species in the purest white flour fraction.

During in vitro digestion the speciation of Fe differed between white and wholemeal bread. Overall, the bioavailability of Fe assessed using a Caco-2 cell model system appeared to be much higher in white bread than in wholemeal bread. Addition of ferrous sulphate was a more effective fortificant in white bread compared to wholemeal bread. The quantity of total or soluble Fe did not appear to be related to predicted bioavailability from the Caco-2 cell model, indicating that the effects of Fe speciation and the presence of absorption inhibitors are of greater importance than total or soluble iron when determining Fe bioavailability.

Acknowledgements

This research project would not have been possible without the support of many people. The author wishes to express his gratitude to his supervisor, Professor Zhao, Professor Shewry and Professor Fairweather-Tait, Dr Andrew Neal and Professor Mcgrath who were abundantly helpful and offered invaluable assistance, support and guidance. Particular Thanks for their endless patience in correcting my less than perfect grammar and deciphering the code which is 'Tristan speak'. The author would also like to thank Sarah Durham and everyone in the soil science and analytical department who voluntarily or involuntarily have cleaned up after me over the last 4 years, your patience is appreciated.

Special thanks also to all my graduate friends, especially; Charlotte Lomax, Joe Helps, Joanna Scales, Maddy Berger, Nelly Kinns and Lauren Cowley you all had a part in shielding my supervisors from the true level of my grammatical incompetence. The author would also like to make a special thanks to Laura Seamons who was a ray of sunshine, I hope one day that we can collaborate on an important text entitled 'Would you rather ……?'.

The author would also like to convey thanks to HarvestPlus, and BBSRC for providing the financial funding throughout my project and the COST action for inviting me to their meetings which were an invaluable source of inspiration. The author also wishes to express his love and gratitude to his beloved families; for their understanding & endless love, through the duration of his studies.

Contents

List of tables and figures.

Figures

Tables

Appendices

Appendix 1. Bran yields of 6 cultivars from Brabender mill

Appendix 2. Total minerals in 2012 10 milling fractions. R2 is highlighted in black to show abnormally high mineral concentration.

Appendix 3. Effect of SDS on extraction

Appendix 4. Normalised expression of taFer1 and tafer2 in endosperm and whole grain of wheat (Cadenza)

Appendix 5. SEC chromatograms showing ^{66}Zn speciation in soluble fractions of offal flour in Rialto

Appendix 6 Comparison of low phytic acid wheat and Rialto whole grain

Appendix 7: Chromatogram of mono-ferric phytate after in vitro digestion

Appendix 8. Comparisons of mono-ferric phytate to $FeCl_3$

Appendix 9. Bioavailability of Fe in presence of NA and ascorbic acid

List of Abbreviations and Acronyms

AA	Ascorbic acid
AACC	American Association of *Cereal* Chemists
DMA	2'deoxymugineic acid
ESI	Electrospray ionization
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
ICP-MS	Inductively coupled plasma mass spectrometry (ICP-MS)
IP	inositol phosphate
LPA	Low phytic acid
NA	Nicotianamine
NAS	Nicotianamine synthase

1. Introduction

The Green Revolution saw yields of certain cereals increase as much as 10-fold over the last half of the twentieth century and is often cited as one of the great achievements of plant science. Although its effect on providing food for an ever increasing population cannot be denied, it did not come without a cost. The introduction of high yielding varieties of crops such as rice, maize and wheat heralded a new age of agriculture, one which high micronutrient and protein crops such as legumes were largely ignored in favour of the new more profitable modern cereal varieties: this was most evident in the developing world (*1, 2*).

This has had an effect on human micronutrient deficiency for two main reasons. First, by reducing the diversity of crops, the population in areas most at risk of micronutrient deficiency or "Hidden Hunger" began to get more of their calorie intake from cereals low in micronutrients (*2, 3*). Secondly, the bias towards high yielding varieties seems to have had a negative effect on the total amount of some micronutrients especially zinc (Zn) in the grain. This has been particularly evident in modern wheat varieties (*3-8*). Recently, organisations such as HarvestPlus have attempted to tackle the problem of Hidden Hunger by focusing on increasing the amount of Zn and iron (Fe) in edible tissues of cereals and legumes (*9-11*). One particular focus has been in wheat, as in some developing countries wheat products account for over 50% of the total calorie intake (*3*).

The effectiveness of increasing total Fe and Zn has been questioned as the relevance of this to the Fe and Zn that is bioavailable is variable. One recent HarvestPlus-funded project involved the breeding of high iron beans which showed an increase of Fe of 80% (*11*), however bioavailability studies involving stable isotopes on a target population showed no improvement in the amount of Fe absorbed compared to low Fe beans (*12*). This is probably the most conclusive study to date on the limitation of total Fe as an indicator of bioavailable Fe, but numerous studies in cereals have shown similar results previously (*13-15*). For Zn, the effectiveness of bio-fortification is more difficult to judge as to date, there are no universally established biomarkers for Zn nutrition (*16, 17*). However, it is likely that total Zn is not an accurate indicator of ultimate bioavailability as it, like Fe, is also heavily affected by anti-nutrients which will be discussed in detail further in this chapter.

One possible explanation for the disparity observed between total Fe and bioavailable Fe could be the forms in which Fe exists *in planta*. Not all forms of Fe and Zn have the same bioavailability (*18-*

20). Therefore the form of sequestered Fe and Zn in the plant is of vital importance. Work on speciation of Fe and Zn in cereal grain is still in its infancy and the little work done so far has concentrated on rice (*21, 22*), with one study on the barley embryo (*23*). To date there is no work on either wheat or maize. Although it is likely that micronutrient speciation in wheat will be similar to rice, there are some distinct differences which may affect mineral speciation within the grain, namely that rice does not have a crease and therefore transport route of minerals to the grain is likely to be different. Wheat also remobilises up to 77% of its Fe from leaves while in rice this is thought to be as low as 4% (*24)*. As well as the speciation of these two minerals, one must also consider the interaction with anti-nutrients in the grain which have been shown to reduce bioavailability. Phytic acid has been known to be a major inhibitor of Fe bioavailability since the 1950's (*25*) but phytic acid also forms insoluble complexes with Zn as well (*26-28*). If we are to tackle Fe and Zn deficiency in cereal diets, it is essential that we understand the path that Fe and Zn take between being absorbed from the soil to the point of digestion.

1.1 Mineral transport from root to grain.

Fe and Zn are both essential for plant growth and reproduction and are therefore present in all tissues at all stages of plant development. Both Fe and Zn at high concentrations can be damaging to the cell (*29-31*). In the case of Fe this is due to the Fenton reaction which is catalysed by Fe(II) and produces the hydroxyl radical OH· which puts the cell under oxidative stress (*30*). To prevent this, plants must ensure minimal metals are in their free state and are instead either bound to other chemicals such as organic acids to form non-reactive compounds or stored away from the cytoplasm in the cell vacuole (*31, 32*). To maintain this, a complex transport and metal homeostasis system has evolved. The uptake, sequestration and translocation of these two important minerals is summarised below with particular relevance to wheat.

1.1.1 Uptake from soil

All graminaceous plants including cereals such as rice, wheat and maize, acquire Fe and Zn from the soil by a mechanism known as strategy II (*33*). One advantage of this strategy is it allows the plant to survive at higher soil pH compared to the less resilient plant groups' dependant on strategy I (*31, 34*).

The effect of pH on mineral uptake of plants is well documented; alkaline soils have a much lower level of bioavailability of cationic metals than acidic soils due to precipitation of carbonates and stronger sorption on soil solids at high pH and the increased solubility and competition with

protons for sorption at lower pH (*31, 35*). Strategy I plants are able to absorb insoluble Fe(III) first by solubilising Fe(III) by the release of protons and organic acid which reduces soil pH, then by the activation of ferric-chelate reductase which reduces Fe(III) to its more soluble and available form Fe(II).

Graminaceous plants instead overcome the effects of high pH by releasing phytosiderophores (PS) when they are deficient in Fe or Zn. These PS contain mugineic acids which are non-protogenic amino acids synthesised from methionine (*36*) These chelate Fe and Zn into more soluble forms for uptake by YS1 (yellow stripe 1) transporters (*31, 37*). In wheat, the principal mugineic acid is (DMA) (*38*); in some other cereals other types of mugineic acids may be secreted (*36*). DMA forms a complex with Fe (III) and is transported into the plant by the corresponding Yellow stripe transporters (YS1) (*31, 37*).

Rice was previously thought to be a strategy II plant, but has recently been shown to use a combination of both strategies , as it synthesises and secrets PS as a strategy II plant but also can absorb Fe(II) by OsIRT1 which is expressed in the root tissue during Fe deficiency (*39, 40*).The IRT group of transporters which transports Fe(II) was previously thought to only be found in strategy one plants (*34*), this additional absorption mechanism is thought to allow rice to be better adapted to submerged conditions (*39*).

Zn availability is also reduced in alkaline soils, especially under dry conditions (*31, 41*). Plants take up Zn in a similar way to Fe, as the free ionic form Zn^{2+} or as a Zn-PS complex (*31*). Zn^{2+} uptake is achieved by a zinc-regulated transporter, of the iron-regulated transporter-like protein (ZIP) family (*42*), while in strategy II plants Zn bound to mugineic acids uptake is mediated by YS1(*43*).

1.1.2 Xylem Transport

Zinc and Fe are transported from the root to the shoot via the xylem (*31, 44*). The xylem sap transports minerals through the xylem system from sites of high water potential to sites of low water potential. Long-distance Fe transport in the xylem sap has long been thought to be facilitated by citrate, as plants deficient in citrate show signs of Fe deficiency in vegetative tissues and addition of citrate allowed iron to move through the plant (*45*). Other organic acids such as nicotianamine (NA) may also have a role in long-distance iron transport (*44, 46*). The pH of the xylem (~5.5) means that Fe will be chelated more readily by citrate than NA (*47*). How the iron is transported from the root to the xylem in wheat however is unknown (*31*); although in rice it has

been proposed that OsFRDL1 may transport ferric citrate into the xylem via the pericycle cells in Fe-deficient conditions (*48*)

The transfer of Zn from the root tissue in *Arabidopsis thaliana* to the xylem is meditated by HMA2 (heavy metal ATPase) and HMA4 (*31*), again the transporters in wheat are unknown. Once in the xylem, it too is chelated but the exact chelator is unknown although possible candidates include NA, (*49*), phytosiderophores (*50*), 2'-deoxymugineic acid (DMA) (*51*), malate and metallothionein (LMW cysteine-rich proteins) (*52*). However, it is thought that only a small proportion of minerals transported to the developing grains is supplied via the xylem vessels due to the lack of transpiration and therefore low water potential (*10, 53*).

1.1.3 Phloem transport

Minerals are remobilized from leaves upon senescence and transported via the phloem to the developing grain. The phloem pathway is primarily concerned with movement of carbohydrates, and also transports minerals from source tissues, such as leaves, to sink tissues, such as grain, along an osmotic pressure gradient, and is said to be undirected (*10*). For both Fe and Zn, the YSL (yellow stripe-like transporters) protein transport system has been shown to have a role in grain loading from the phloem as well as root uptake (*31, 49*). In the case of Fe, it is thought that it is transported by YSL proteins as a Fe(II)-NA complex (*30, 54*) (*31*). NA chelates both Fe(III) and Fe(II) *in planta* although Fe(II) is the more stable form (*55*). The proposed role of NA as the primary Fe chelator in the phloem was previously based on numerous reports on the effect of nicotianamine synthase (NAS) genes on Fe translocation in rice (*56, 57*), and on computer modelling of the binding capacity of various metal chelators at phloem pH (*58*).

Recent work in rice using a combination of size exclusion chromatography coupled with inductively coupled plasma mass spectrometry (SEC-ICP-MS) and electrospray ionization (ESI) have found Fe bound as Fe(III)-DMA to be the dominant complex in the phloem sap (*59, 60*). The corresponding Fe(III)-DMA *transporters (*OsYSL18 ,OsYSL15) have also been identified in the rice phloem (61, 62) providing strong circumstantial evidence of DMA's role as a translocator of Fe. OsYSL18 has also been identified in the vascular bundle of the developing wheat grain (*61*) and in the embryo of the mature grain (*62*). This is the only evidence of DMA transport within the grain and no such DMA specific transporters have been found in either wheat or barley (*52*). Another candidate of Fe transporter in the phloem is the OPT (oligopeptide transport) proteins which have

been shown in *Arabidopsis thaliana* to affect seed Fe concentration; this may mean that Fe-peptide complexes may also be transported through the phloem (*31*).

Less is known about the translocation of Zn, although recent work has shown that NA is likely to be the major chelator for Zn in the phloem of rice (*59*), However it is unclear whether Zn^{2+} is transported to the phloem by OsZIP4 or whether it is transported as a Zn-NA complex as in Arabidopsis (*30, 59*). Like Fe, DMA may also have a role in Zn translocation as the expression of *OsDMAS1* is increased in zinc deficient shoots in rice (*51*).

1.1.4 Transport into the grain via the crease

The crease functions as a route for nutrient supply to the grain and is responsible for grain filling (*63*). It consists of a complete phloem strand and a semi-complete xylem strand (*46*). Minerals may enter the crease from both the xylem - from the roots and stem, or the phloem from senescing tissues (*46*). However, minerals from the soil and root system must first be transferred from the xylem to the phloem to enter the grain, which is again thought to be mediated by a YSL transporter (*31*).

Within the crease is the pigment strand where minerals move through the symplast via plasmodesmata (*46, 52, 64*). The cell walls of the pigment strand are impermeable to nutrients from the apoplastic route meaning that nutrient entry to the grain is tightly regulated (*52*). At the end of the pigment strand is the nucellar projection or transfer cells **(Fig. 1)** (*64*). It is here that minerals are transferred into the apoplast or endosperm cavity. Transfer cells are characterised by numerous invaginations which create a much larger surface area (*46*). Minerals are then taken up from the endosperm cavity by influx cells, which are modified aleurone cells, and are then distributed in the symplast through the aleurone, endosperm and embryo (*46, 52*).

In the case of Zn and Mn it has been proposed that there is a direct continuation of the phloem transport system from the crease to the embryo (*63*). This is supported by recent synchrotron imaging which appears to show high concentrations of Zn and Mn in the intermediate tissue indicating a likely route for directed transport (Fig.1). How, and in what form, minerals are transported from the transfer cells to the influx cells and on to the rest of the grain is currently unknown (*49, 65*), although laser capture micro dissection of barley grain into transfer cells of the maternal vascular bundle and other grain tissues followed by microarray analysis found

expression of YSL transporters with specificity for Fe(II)-NA and Zn-NA indicating a possible role of NA as a metal chaperone (*46, 52*).

In rice, OsYSL18 has been identified in the vascular bundle of the developing grain (*61*) and in the embryo of the mature grain (*62*); this is the only evidence of DMA transport within the grain and no such DMA specific transporters have been found in wheat or barley (*52*).

Interestingly, transport of Zn into the grain seems less regulated than for Fe (*66*), and high Zn supply to the plant appears to increase grain Zn (*67, 68*). However, transport of Zn to the endosperm is limited and appears almost independent of external supply as the majority of the additional Zn in high Zn wheat plant was accumulated in the crease (*68*). Contrastingly, however, wheat treated with a foliar Zn as $ZnSO_4$ combined with a high N application rate of 240 kg ha^{-1} showed that Zn was not only freely transported to the grain but also to the endosperm as endosperm zinc concentrations were ~2-fold higher as non-treated grain (*67*). One possible reason for this is that the limiting factor is not transport through the crease, but that the endosperm protein acts as a sink for grain Zn and increases in sulphur and nitrogen (N) enhance this sink (*67, 68*), another possible explanation is that increased N affects production of mugineic acids and NA which facilitate uptake and translocation of Zn (*67*).

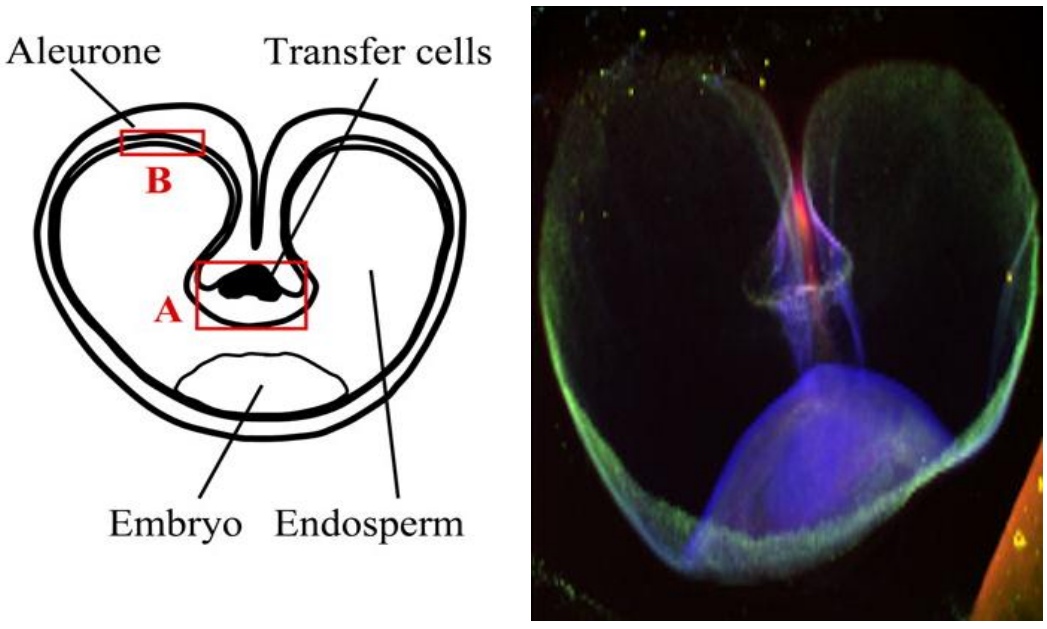


Figure. 1. Mineral localisation in wheat. The left hand side shows a schematic representation of a cross-section of a wheat grain. The right hand side shows synchrotron X-ray fluorescence image of a cross-section of a wheat grain, and reveals zinc (blue), iron (green) and manganese (red) localizations. Image by Andrew Neal.

1.2 Fe and Zn storage in grain

The cereal grain is divided into four tissue types: the embryo, the aleurone, the endosperm and the outer layers (testa and pericarp) (**Fig. 2.**). Analyses of Fe and Zn show that both minerals appear to be localised predominantly in the aleurone and the embryo (in particular the scutellum) (**Fig. 2.**) with very low concentrations in the starchy endosperm (*23, 67, 69, 70*) **(Fig. 1, 2.)**, this pattern is similar in other minerals including phosphorus (P), magnesium and calcium (*70*).

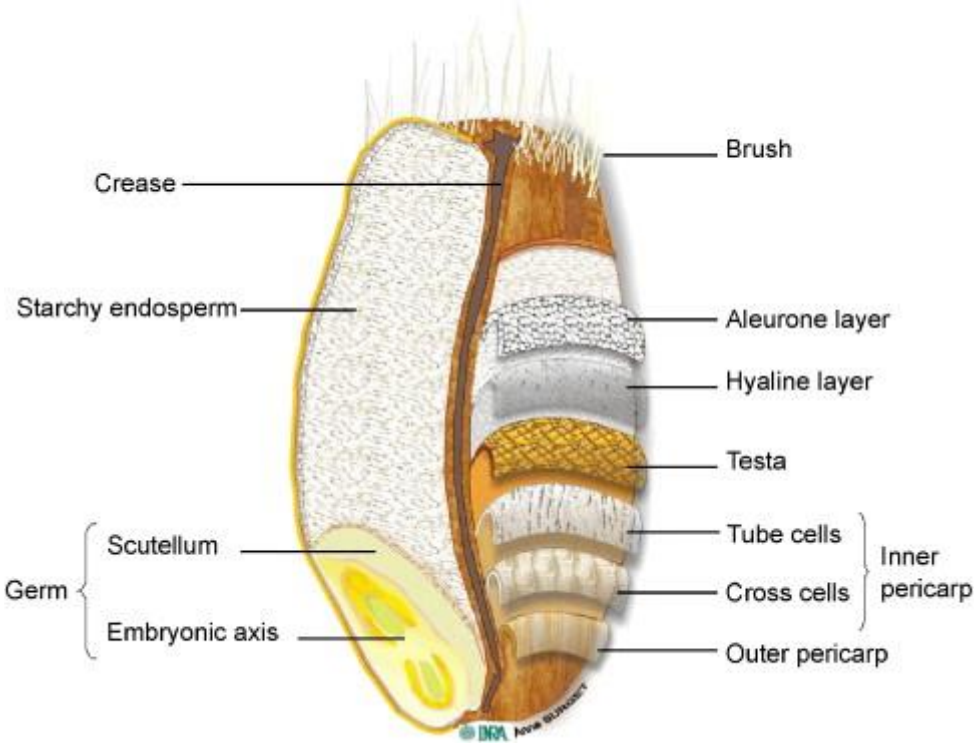


Figure 2. Wheat grain, showing component tissues. Reprinted from (*71*)

1.2.1 Phytate

Phytate is the name given to any salt of phytic acid ($C_6H_{18}O_{24}P_6$), also known as *myo*-inositol hexakisphosphate (IP_6). Phytic acid is the main storage form of P in cereal grains and is often referred to as organic P (*72*). The role of phytate in plants is thought to be as the major storage form of minerals particularly P for germination (*73, 74*).

Phytic acid has a high density of negatively charged phosphate groups which allow it to form very stable insoluble complexes with Fe, Zn and various other minerals (*75, 76*). For the plant, this provides an advantage by reducing any potential toxicity effect of minerals in the grain until the minerals are needed for germination, at which point intrinsic phytase releases the minerals **(Fig. 3)** (*77*) as well as allowing the minerals to be highly localised in phytate granules. However, for

humans phytate is a major problem as these stable insoluble salts are poorly digested in the intestine (*14, 75, 78*). Inhibition of mineral absorption only appears to occur with IP_6 and IP_5. Inositol phosphates with lower numbers of phosphate groups do not inhibit Fe absorption (*79-81*).

In wheat, phytate is heavily localised in phytate globoids in the protein storage vacuoles (PSV) in the aleurone layer of the grain where it has been shown to be localised with Fe, strongly suggesting *in planta* binding (*69, 82*). Imaging by nanoscale secondary ion mass spectrometry (NanoSIMS) has also shown clear localisation of Fe with P and C in the aleurone (*69*). There is also evidence of PSV containing phytate and Fe in the embryo (*46, 77*). Further evidence of the Fe bound to phytate *in planta* is the liberation of Fe by phytase in rice (*83*), barely (*23*) and wheat (*84*). Fe bound to phytate represents approximately 70% of total grain iron which is almost exclusively located in the aleurone (*3*).

For Zn, the story is less clear, and although it seems likely Zn is stored in the PSV whether it too is bound to phytate is still unknown. Although a clear correlation between phytate and Zn has been shown within wheat grain (r=0.37) (*85*), this does not provide evidence of *in planta* binding. A recent speciation study using a combination of SEC-ICP-MS and phytase treatment in the barley embryo found strong evidence that Zn is not bound to phytate at all, but instead appears to be associated with protein (*23*). Treatment of rice bran however did show an effect of phytase at liberating Zn but this was not as clear as Fe (*83*).

1.2.1.1 Phytic acid synthesis

The synthesis of phytic acid or inositol hexakisphosphate (IP_6) is summarised below (**Fig. 3**). The first step of IP_6 synthesis is the conversion of Glucose-6-phosphate to $Ins(3)P_1IP1$ mediated by $Ins(3)P_1$ *myo*-inositol monophosphate synthase (MIPS). The remaining steps involve sequential ATP-dependent phosphorylation of the IPs mediated by Ins phosphate kinases, however this pathway is not yet completely understood (*74, 77*).

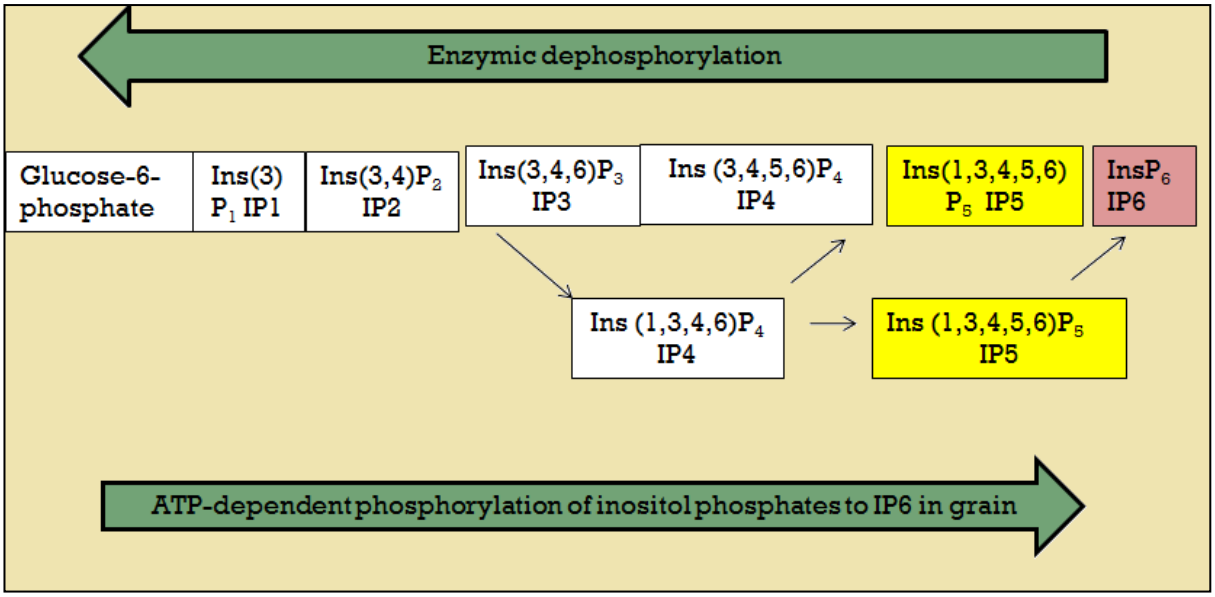


Figure 3. Stepwise synthesis of phytic acid (IP_6)

Due to the ATP-dependant nature of phytic acid synthesis, it is assumed that synthesis occurs in the cytoplasm (*74*)

1.2.3 Nicotainamine and 2-deoxymugenic acid

As previously discussed, the role of DMA as a phytosiderophore and NA as a metal transporter are well documented. However, they have only recently been thought to have a role in mineral storage. Fe bound to NA was first demonstrated in rice in 2009 by a study using SEC-ICP-MS (*22*). Further investigation with ESI-MS also showed the presence of Fe-DMA as well as Zn-NA and Zn-DMA (*21*).

In barley, YSL transporters with specificity for Fe (II)-NA (*46*) and Zn-NA (*52*) have been identified in the developing grain, although to date there is no evidence of any DMA specific YSL transporter there is evidence of genes involved in DMA synthesis in barely within the endosperm (*52*). In wild type brown rice, DMA occurs at ~5-fold the concentration of NA (*86*), the amount of both DMA and NA are also increased by up to 3-fold in transgenic lines with more Fe and Zn translocated to the grain (*86*) indicating a role of DMA in either transport within the grain or storage.

DMA and NA are both zwitterionic (having both a positive and negative charge). When mixed in solution the most abundant complexes formed are DMA-Fe(III) and NA-Fe(II), however both DMA-Fe(II) and NA-Fe(III) are present but at a lower abundance (*87*) . It is likely therefore that the oxidation state of non-phytate bound Fe in the grain affects which of these it will be chelated to (*88*). Both DMA and NA are thought to be present ubiquitously throughout the grain.

1.2.4 Ferritin

Ferritin is a name given to a large protein complex though to be able to complex 4500 Fe atoms per Ferritin molecule (*89*). What form Fe is in the ferritin core is an area of debate. Previously it was thought ferritin oxidised the highly toxic Fe(II) to Fe(III) and stored it in the core (*89*) however it is now thought to exist as a Fe(III)-O-Fe(III) complex and Fe(II) depending on location in the core (*90*). As well as containing Fe, ferritin is also thought to be able to store Zn under high levels of Zn^{2+} (*91*).

In wheat ferritin is ubiquitous; but how much Fe is stored in this state in the grain is not known due in part to difficulties in accurately quantifying ferritin and bound iron (*92*). Ferritin was previously been thought to function as the main iron pool in cereals (*93*), however, data from previous studies (*92, 94*) indicate that only about 1-2% of the total iron in wheat is bound as ferritin.

In *Arabidopsis thaliana* and pea there is strong evidence that ferritin acts as a primary storage form of Fe for germination (*34*) however it has also been argued that its role is in protecting plant tissue from oxidative damage due to free hydroxyl radicals caused by excessive iron (*44, 95-97*). The most likely explanation on this difference in function is that exact nature of each ferritin molecule is likely to depend on its isoform as in plants numerous structures have been identified (*34*).

1.2.5 Other forms of Fe and Zn

Trace amounts of iron are also found in mitochondrial Fe-S metalloproteins such as frataxin (*98, 99*). Less is known about the forms of Zn in plants; citrate, malate and metallothionein have all been identified as possible chelators (*49, 52*), and these low molecular weight (LMW) compounds may also act as storage forms (*51, 52*). In bran of millet, it is also suggested that Zn may be bound to fibre and tannins (*100*). Zn also is an important component of many proteins in plants (*101*).

In cereals there are high levels of metallothioneins (MTs), which are LMW cysteine rich metal-binding proteins, and have been shown to be present in the embryo and aleurone during development 9-28 days post anthesis (*102*). The two main MTs *in planta* are MT_3 and MT_4 which seem to have different metal binding properties. MT_3 is thought to have a temporary role in binding both Cd and Zn for transport, while it has been proposed MT_4 has a role in Zn storage (*102*). The very high activity of MT_2 in barley endosperm treated with Zn may also indicate that Zn

is sequestered by this particular MT protein in the endosperm (*52*). However, how much is Zn bound to MT at grain maturity, or whether this is the case in other species, has not been confirmed. However, metallothioneins provide a possible candidate for the protein Zn sink proposed in previous studies (*67, 68*).

1.3 The importance of transport of minerals to the endosperm

Although transport of minerals to the grain is important, arguably of for human nutrition of more importance is the transport to the endosperm (*3, 70*). Most processed wheat products remove the outer layers and the embryo and are therefore made up almost entirely of the starchy endosperm. Mineral density in the endosperm of cereals is considerably lower than the outer layers (*70, 103*): in wheat, depending on the milling process, this removal of the outer layers can cause a loss of between 40-60% of the total Fe and 40-75% of the total Zn (*70, 103, 104*). However milling is likely to have some beneficial effects on bio-availability as the milling results in a similar reduction in the total phytic acid in the wheat flour as well (*103, 104*).

1.4 Bioavailability of Fe and Zn in wheat

Unfortunately, despite their wide use as a calorie source, cereals remain a very poor source of Fe and Zn with levels of bioavailability in wheat estimated to be 5% for Fe and 25% for Zn (*3*). Caco-2 (described in **4.1**) experiments looking at wheat products prepared using different baking techniques found bioavailability to be between 11-30% for Fe and only 3-4% for Zn (*14*). It should be noted though that flame atomic absorption spectrophotometry was used to measure total minerals absorbed by the cells in these experiments and one flaw of this method is that it will often over-report mineral absorption as a certain amount will stick to the cells without being absorbed (*105*). Also the effectiveness for Caco-2 cells being an accurate measure of Zn absorption has not yet been proven and therefore should not be used as a quantitative indictor (*105*).

1.5 Current strategies for improving Fe and Zn nutrition

Because of the low availability and low total Fe and Zn in cereals, various strategies have been proposed for increasing the bioavailability of mineral nutrients; for the most part this will concentrate on Fe as it is the primary area explored in chapter 5.

The three main strategies for tackling micronutrient deficiency have been identified by WHO (*106*) as:

1. **Supplementation**

2. **Diet diversification**
3. **Fortification**

Supplementation refers to vitamins and minerals concentrated in a synthetic form in capsules, tablets or injections and administered as part of a government policy or individual nutritional interventions. In the case of iron, supplementation has had limited success (*107*). A major problem with iron supplementation is the need for very regular doses, ideally once a day. This can become very expensive; the World Bank estimates such strategies for Fe at 0.5 to 3.17 USD per unit per person (*106*), and any such strategy tends to have low adherence (*108*). To date, the major supplementation strategies for iron has concentrated on pregnant women (*107*).

Diet diversification refers to the education of population groups to cultivate and eat foods rich in micronutrients. As a long-term strategy it is often seen as ideal, however it is not without limitations (*109*). One problem is that countries most affected by mineral deficiency are those least likely to have the resources to cultivate high micronutrient sources which are often more expensive to produce (*109*). This is particularly true for Fe where the ideal foods would be leafy green vegetables and meat which require more resources than other crops commonly grown in these areas.

1.6 Fortification

Fortification has historically referred to the addition of synthetic micronutrients to a food source to improve the nutritional status of a population (*109*). In the west, fortification strategies have been employed since the 1940's and in the case of iron, are mandatory in many countries including the UK and Sweden. In addition to this, many food companies such as those who make infant feed or cereals voluntarily fortify their products to make them more desirable to the consumer (*110*).

In the developing world, fortification of staple foods has been seen as one of the most effective methods to tackle iron deficiency, as staple foods form a large percentage of the populations' calorie intake. Wheat flour is currently the most Fe-fortified food product, with 78 different countries currently implementing or planning fortification programs (*111*). Fortification of wheat flour and other staples with Zn and other minerals has also been shown to be effective (*112*). Iron fortification strategies are expensive and require a well-developed infrastructure to be implemented. As well as benefiting the target group, normally children and menstruating and

pregnant women, they must also be designed so as not to negatively affect members of the population not at risk of Fe deficiency (*110, 113*).

1.6.1 Fortification in the UK

The UK has a mandatory fortification policy for wheat flour which has been in place since 1953. Fe is added exclusively to white flour products to restore the level to that of wholemeal products (*114*). Under current regulations, this means all flour under 16.5 mg kg^{-1} is brought up to at least 16.5 mg kg^{-1} by a process known as recovery (*115*). This would mean most wholemeal flour products would be exempt, however, industry often voluntarily fortify wheat products with Fe (*110*).

1.7 Bio-fortification

Bio-fortification is the process of fortifying staple crops with micronutrients through conventional breeding, fertilization or biotechnology (*3, 9*). This approach has received widespread attention as it circumvents many of the problems of other previously mentioned methods. Adherence is not a problem as populations are able to continue with established dietary habits. Another advantage, in the case of high Fe and Zn cultivars, is once crops are developed there will be no additional cost issues (*3*). Bio-fortified crops are also easily accessible in rural areas as they simply replace established crops; these rural areas are often where Fe deficiency is most widespread (*9*).

1.8 Bioavailability

Bioavailability in nutritional science is defined as 'the amount of an ingested nutrient that is absorbed and available for physiological function' (*18, 105*). The ideal way to measure this is by human studies, however these are expensive, time consuming and very difficult to compare across laboratories (*15*). For Fe, the primary outcome measure for human studies is haemoglobin concentration (*116, 117*), however this is not always ideal in developing countries where other factors such as other nutritional disorders of inflammatory disease can affect haemoglobin levels (*118*). For Zn, it is recommended to measure Zn serum/plasma levels (*16*).

Due to the expensive and difficult nature of human studies, various alternative models have been developed. Animal models provide an attractive alternative, and have been used extensively in Fe

bioavailability studies of edible plants using dogs (*119*), pigs (*120*), mice (*121*) and chickens (*122*). One disadvantage of animal studies is that they are again very expensive and not all labs will have the appropriate licences. Animal studies also rely on an assumption that the animal model digests the food stuff in a similar manner to humans, which is rarely the case. In the case of Fe nutrition, there are many factors which may have an effect including gastric pH, microflora and whether or not the organism in question synthesises ascorbic acid.

1.8.1 Caco-2 Cell Model

The Caco-2 cell model allows for the evaluation of single meal bioavailability studies in a physiological environment which is impossible in human studies (*15*), although it is not always accurate (*123*) and has been shown to compare unfavourably with human studies in studies with beans containing low iron levels (*105*). However, Caco-2 remains the recommended method for measuring Fe availability (*105*). The current preferred method is the use of a human ferritin-specific ELISA assay. The Caco-2 cells are harvested after exposure to a sample which has undergone *in vitro* digestion. The cells are destructively sampled and the total protein and total ferritin are calculated to provide a ng mg^{-1} of protein as ferritn (*15, 124*). Human cells sequester cellular Fe as ferritin, therefore total ferritin expression is an accurate indicator of how much Fe has been absorbed.

The final amount of minerals in the cells can also be measured by ICP-Atomic Emission Spectroscopy (*14*) or ICP-MS, however this method is not preferred as it cannot differentiate between intrinsic minerals and minerals absorbed by the cells, as well as minerals which are adhered on the surface of the cells but not absorbed (*105, 123*).

1.8.2 Absorption of Fe and Zn in the human gut

Dietary Fe can be defined by two main categories haem and non-haem Fe (*125*). The absorption of haem Fe is relatively simple and will only be covered briefly here as is not important to cereals. Haem is freed from myoglobin and haemoglobin by pancreatic enzymes. The degradation products of this stay bound to the haem-Fe and are maintained in a depolymerized state as metalloporphyrin. In this state the Fe cannot interact with possible anti-nutrients such as phytate so is readily absorbed (*126*). Uptake of metalloporphyrin is not competitive with non-haem Fe and occurs at different transport sites (*126*).

Non-haem Fe, however, which makes up the entirety of Fe from plant sources, has a far more complex story. The majority of dietary non-haem Fe is thought to enter the gastrointestinal tract as Fe(III). However, Fe(III) is insoluble above pH 3 and therefore non-bioavailable (*125, 126*). For absorption, it must first be converted to Fe(II). This occurs by the reduction of Fe(III) to Fe(II) by numerous dietary components including ascorbic acid and amino acids such as cysteine and histidine (*125*). This reduction is thought to take place during the initial gastric stages of digestion at acidic pH when the Fe(III) is soluble. Once in the Fe(II) form Fe is taken up by DMT1 (Divalent metal transporter 1) (*125, 127*).

Fe bound to ferritin is also readily absorbed (*19*). This is thought to be because Fe is encapsulated by ferritin - a large protein which protects the Fe from possible oxidation and/or interaction with anti-nutrients in the human digestive system so that it is readily available in the duodenum at the point of human digestion (*128*). However, it has been proposed that the ferritin shell breaks during the gastric stage of digestion and the Fe enters the common digestion pool (*129*).

1.6.3 Factors effecting absorption of Fe

1.6.3.1 Pro-nutrients or enhancers

The absorption of non-haem Fe is therefore very dependent on the presence of pro-nutrients. As already mentioned, ascorbic acid and cysteine are capable of reducing Fe(III) to Fe(II) (*124, 130*) and it is thought that this is how they promote Fe absorption. However, ascorbic acid has also been shown to form an Fe-chelate which is soluble up to pH 11 (*75*). The complete effect of ascorbic acid on Fe absorption is not completely understood - although it clearly has a role in reducing Fe(III), it has also been shown to enhance absorption of Fe(II) (*121*). Some organic acids such as citric acid have also been shown to enhance Fe absorption, but at levels far higher than are likely to be found in the human diet (*130, 131*).

Another pro-nutrient that has been identified which is likely to be of upmost importance in Fe absorption in cereals is nicotianamine (NA). Caco-2 studies have shown that NA is in fact a more potent enhancer of Fe than ascorbic acid, and is twice as effective at enhancing absorption of both Fe(II) and Fe(III) (*121*). The fact that it is able to enhance absorption of Fe(II) so much indicates its role is unlikely to be in reducing Fe. Therefore, it is likely to enhance absorption by another mechanism (*121*). It has been speculated that chemicals containing more hydroxyl groups are able to enhance Fe absorption by forming soluble Fe-chelate complexes (*131*), and also that

organic acids containing more hydroxyl groups were better promoters (*131*). Nicotianamine has three hydroxyl groups (**Fig. 4**) and therefore would be an ideal Fe chelator as should DMA with four **(Fig. 4)**.

Figure 4. Chemical structure of nicotainmine(left) and 2'-Deoxymugineic Acid (right)

1.6.3.2 Anti nutrients or inhibitors

Anti-nutrients are chemicals which reduce the bioavailability of Fe and Zn. The most widely studied is phytate which has been shown to form insoluble complexes with both Fe and Zn (*14, 27, 132-135*). In addition to being insoluble, at intestinal pH 6-7, phytic acid is highly negatively charged and therefore is thought to not be able to cross the lipid bilayer of the plasma membrane in the gut (*135*). When bound to multivalent cations such as Fe and Zn, phytate salts precipitate and are therefore not available for digestion (*135*).

One exception to this appears to be the compound mono-ferric phytate, which is soluble at physiological pH (*119, 136, 137*). Mono-ferric phytate is composed of one phytate molecule bound ionically to Fe(III) rather than ferric phytate which contains 3-4 Fe molecules per phytic acid (*136*) . Due to its solubility it is thought to have some degree of bioavailability. This has been shown in both dog (*119*) and Caco-2 models (*137*). However, it its unknown what the exact level of bioavailability is or even if mono-ferric phytate is able to survive the digestion process and if the Fe is not just added to the digestion pool.

Many polyphenols have been shown to bind Fe (*120, 138*). This was first suggested with the observation that tea has an inhibitory effect on Fe absorption (*139*), but little is known about the interaction with polyphenols and other minerals. Caco-2 studies have shown a negative effect of some polyphenols such as procyanidins on Zn absorption (*140*).

In cereals, the effect of polyphenols was most pronounced in a Caco-2 study on rice which showed that the polyphenol content of 15 different rice varieties, of variable colour (black through to white), was the primary factor in affecting Fe absorption, more so than phytate or

total Fe (*141*). This was also shown in beans where Fe absorption in red varieties (high in polyphenols) was compared to white beans (low in polyphenols) by using Caco-2, chicken (*142*) and pig models (*120*). However more recently, in human studies it has been shown that bean polyphenol content is not an important factor and it is in fact phytic acid that is the only nutritional relevant inhibitor (*143, 144*). The effect of polyphenols therefore remains unresolved and more studies in humans would need to confirm this effect and explain the disparity between human studies and Caco-2 and animal studies. Tannic acid is a polyphenol found in relatively high concentrations in the bran of wheat (*145*) and has been shown to be a more potent inhibitor of Fe than phytate in a Caco-2 system (*137, 146*).

1.7. Biotechnological strategies to improve iron and zinc concentration and bioavailability

It is clear by exploring the complexities involved in Fe and Zn *in planta* transport, and sequestration, that any bio-fortification strategy will need to take into account far more than just total mineral content and an understanding of both translocation mechanisms and of potential enhancers/ inhibitors is essential. One tool to understand this is the use of transgenic crops which can highlight the potential of a certain gene or, where acceptable, replace conventional crops. Below is a summary of some of the current research in biotech strategies aiming at biofortifying cereals.

1.7.1 Ferritin increase

Although representing only a very small percentage of iron in grain, ferritin complexes have been shown to be very bioavailable to humans due to the protein shell protecting the Fe contained inside from anti-nutrients such as phytate (*96*). Increasing the ferritin content of seeds should theoretically increase the amount of bio-available iron in wheat (*19*).

Legumes store as much as 90% of their iron as ferritin (*96*) and therefore various studies have focused on expressing soybean ferritin in cereals (*147-150*). Initial work using the Maize Ubiquitin-1 promoter caused an increase of both ferritin and iron in vegetative tissue but no increase in Fe within the seed of either rice or wheat (*148*). In rice, ferritin expressed using the rice seed-storage protein glutelin promoter, *GluB-1* has been shown to increase total Fe content in seeds up to three-fold (*150, 151*). Similar success has been achieved in maize using seed specific vector pTO126, increasing Fe content in seeds by up to 70% (*148*). It is thought that increased ferritin in the endosperm, and specifically the amyloplasts, aids Fe accumulation by removing free Fe from

the cytoplasm, in turn causing the cell to respond as if it was Fe-deficient and influx more iron (*149*). There is evidence that this Fe is obtained exclusively through the phloem system, as soil iron levels have no effect on Fe levels in seeds of transgenic varieties; conversely, leaves of transgenic lines have less iron and show signs of chlorosis after grain filling; indicating the additional iron had been mobilized from the vegetative tissue (*149*).

Although these studies show promise, no data has been yet generated on the location within the seeds of wheat for ferritin Fe, although it has been reported that some Fe in rice endosperm is in clusters which may be Fe-ferritin (*152*). Recently, synchrotron analysis of wheat expressing endosperm-specific ferritin has shown that the iron is in fact concentrated in the crease and not in the endosperm (*153*). Bioavailability of seed expressing ferritin has been tested, although only in combination with a different treatment such as phytase and cysteine peptides, so results are not conclusive (*147, 150*).

1.7.2 Accelerated senescence

During anthesis, proteins and minerals are remobilised from vegetative tissues to the grain. One strategy to improve the nutrient content of grain is to accelerate plant senescence early, thus beginning grain filling earlier. Land races lines have been shown to do this by a NAC transcription factor (*NAM-B1*) which is non-functional in modern lines (*154*) (*155*). How viable accelerating senescence is as a breeding strategy is yet to be tested. Currently, only RNAi has been used to reduce *NAM* expression and whilst accelerating senescence may increase mineral concentration, it may have a negative effect on grain yield due to reduced carbohydrate loading into the grain a consequence of early senescence (*156*).

1.7.3 Phytate reduction

As previously described, phytate has been identified as the major anti-nutrient in wheat: its reduction is one method which is being explored in stable crops (*157-161*). Viable low phytate mutants (*lpa*) have been isolated in maize, rice, barley and wheat (*157-161*). Low phytic acid mutants are found in two forms: *lpa1* where IP_6 is reduced and free P increased, and *lpa2* in which IP_6 is lower but there is an increase in less phosphorylated inositol phosphates (IP_1-IP_5) (*157*) **(Fig. 5.).** In the case of maize, this is due to disruption in the embryo causing incomplete IP_6 synthesis (*162*). Levels of phytic acid in both *lpa1* and *lpa2* lines have been shown to be notably lower than wild-type and as little as 5% of wild type in some *lpa1* maize (*163*). However, reduction in phytate

has been shown to have negative effects on plants and only a few *lpa* mutants are suitable for breeding (*163*) as phytate is found ubiquitously throughout the plant and is thought to be involved in germination, emergence and stress tolerance as well as its ability to chelate potentially toxic metals (*164*). However it has also been argued that current commercial grain crops have much higher phytate levels due to high P levels used in fertilizer (*165*).

Current phytate reduction strategies concentrate on tissue-specific reduction of phytate to maintain yield and viability (*164*). Recently an ATP-binding cassette (ABC) transporter has been identified in maize which when silenced blocks transport of phytate from its site of synthesis, creating a bottle-neck in phytate accumulation, producing an *lpa1* phenotype (*164*). Using the *Ole* and *Glb* promoters, this gene suppression can be targeted to the embryo with no noticeable effect and produce an 85% reduction in total phytate in the grain (*166*). Greater understanding will allow for a more targeted approach in producing LPA wheat grain without detrimental complications (*164*).

Another approach which has been explored is the introduction of an extrinsic phytase (*147, 167, 168*) or over-expression of intrinsic phytase to reduce phytic acid complexes after synthesis (*164*). The increase of phytase has been shown to produce viable crops and reduce phytic acid by hydrolysing it back to its lower states of phosphorylation **(Fig. 5.)**. Modern wheat lines have been shown to have lower phytase activity than some landraces (*85*). The effectiveness of crops with high phytase activity may be further increased depending on the preparation method of the grain according to optimum pH and temperature (*169-171*). Intrinsic wheat phytase has been shown to have optimum activity at ~50 ºC and ~pH 5.5 (*169-171*) with fungal phytase from *Aspergillus. niger* having optimum temperature of 55 ºC and two pH optima of ~2 and ~6 (*172*). Therefore any cereal crops which use high phytase as a phytate reduction strategy should try and match the phytase to a specific grain end use.

Although low phytate wheat may seem an attractive alternative, there are considerations which have to be made. Phytic acid has been shown to have numerous health benefits in the diet (*173, 174*) and it has even been argued that phytic acid should be included as an essential nutrient or even a vitamin (*175*). The decrease in phytate needed to have an adequate effect on mineral bioavailability is thought to be very high therefore this would have implications on other

nutritional aspects of any crop (*147*).

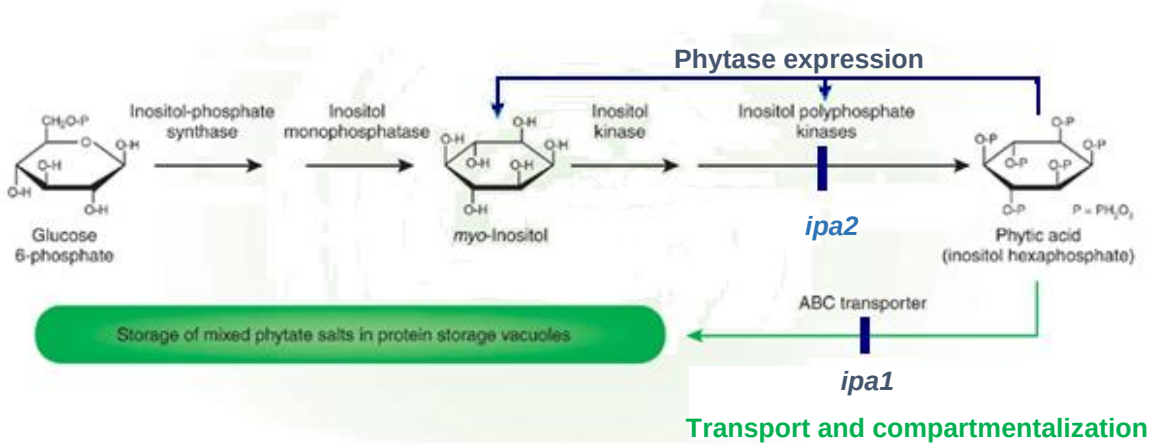


Figure 5. **Low phytate cereals** :Edited from Raboy, V.,(*164*)

The benefits of low phytate stable crops on mineral bioavailability has been tested in various animal (*176, 177*) and human studies (*178, 179*) with positive results; a rise in mineral availability of ~50% for humans has been observed (*180*). The Caco-2 model has also been used to assess maize phytase lines with moderate success with iron absorption being slightly higher in *lpa* mutants compared to wild type varieties (*147*).

1.7.4 Nicotianamine

In the phloem, NA is the primary chelator of many metals including Fe and Zn. Therefore over-expression of the nicotianamine synthase gene (*NAS*) is one possibility to increase grain metal nutrient content. The role of NA and DMA in grain loading of Fe and Zn has been demonstrated in various plant studies (*46, 51, 54*) and over expression of *HvNAS1* from barley was found to double both Fe and Zn within seeds of tobacco plants (*54*). In rice, increases in NA expression throughout the plant have shown a significant increase in grain mineral content in both milled and non-milled fractions, for Zn and Fe there is a >2-fold increase in total amount in milled fraction (*21, 22*). Analysis also showed the increased Fe was of a LMW non-phytate form. The grains of these over-expression lines have been tested on mice and results show that the increase in Fe and Zn is of bioavailable form (*21, 22*).

In another similar study, *NAS* was over-expressed in the endosperm of rice. This also had a positive effect on both Fe and Zn, with increases of between 20-46% and 33-55% respectively in unpolished rice grain (*121*). In polished grain however there was only an increase in Zn and this was only about 15% (*121*). Although the level of Fe in the polished grain was not significantly

higher, the amount of NA in the grain was 9-fold higher than wild-type. Caco-2 analyses showed that Fe in both cooked and uncooked NAS over-expressing samples was increased by 2-2.4-fold compared to wild-type (*121*) indicating that levels of NA may have more of an effect than total Fe.

How viable this strategy would be for wheat however is yet to be proven. Rice and wheat transport Fe differently and NAS expression has been shown to be different under Fe-deficient conditions between barley (a closer relative of wheat) and rice (*181*). In wheat, grain iron is transported from the leaf therefore the amount of available iron in the rhizosphere and leaf tissue will also be a limiting factor.

1.7.5 Transporters

Another strategy to increase the mineral content of grains is the manipulation of metal transporters through biotechnological interventions. Possible candidates are *VIT1* which has been shown to have a role in *Arabidopsis* seed iron concentration (*35*), and HMA4 which may target Zn to the seed, again in *Arabidopsis* (*49*). However neither of these has been studied in cereals. In rice, ubiquitous over-expression of *OsIRT1,* a gene responsible for transport of Fe(II) has been shown to improve grain Fe and Zn (*182*), however the plant was not agronomically viable as height and grain were altered. Increasing expression of YSL transporters has long been thought to be a viable strategy for increasing phloem Fe translocation to the grain (*70*). In rice, successful over-expression of *OsYSL2* under a phloem-specific promoter was able to increase Fe by 4.4-fold in the polished grain (*183*).

1.7.6 Multi gene approach

Due to the complexity of mineral homeostasis, multi-gene manipulation may be the best method for improving bio-available Fe and Zn (*33*). The first attempt at this was in rice where *Phaseolus* ferritin, rice metallothionenin and the *Aspergillus* phytase (*phyA*) were co- expressed resulting in rice grain with increased Fe, and decreased phytic acid content (*150*). A second example is transgenic maize over-expressing both soybean ferritin and *phyA*, which increased grain Fe by 20-70% (*147*). This approach is likely also to have a positive effect on the bioavailability of the Fe (*147*) although this was not tested.

Transgenic rice plants over-expressing the *AtNAS1* gene and *Phaseolus* ferritin under the control of the endosperm-specific globulin promoter provided 6-fold enhancement of Fe accumulation in

grain (*152*). This approach of combining ferritin and nicotianamine has since been extended in rice with similar results. Masuda et al (*86*) expressed soybean *ferritin in the endosperm of rice* combined with enhanced Fe transportation by the over-expression of *HvNAS1,* they also over-expressed *OsYSL2* under the control of the *OsSUT1* and *OsGlb1* promoters theoretically allowing for more Fe-NA to be transported into the grain (*86*). Both of these plants are likely to have vastly improved Fe bioavailability but await human testing.

1.8 Aims of thesis

The aims of this thesis will focus on three main areas **1**) To increase understanding of Fe and Zn localization within the white grain **2**) To gain insight into the speciation of Fe and Zn within the wheat grain and finally **3**) to assess the relevance of both localisation and speciation to bioavailability in a dietary relevant food source.

Chapter 2. Materials and methods

2.1 Materials

2.1.1 Healthgrain samples

Analyses were initially performed on samples of six wheat cultivars (Table. 1.), selected based on previous analyses which showed that three contained high (Rialto, San Pastore, Tiger) and three low (Crousty, Valoris , Riband) concentrations of Fe and Zn (*4*). The samples were grown at three European sites (Martonvásár, Hungary; Saxham, UK; Choryn, Poland) in 2007, as described by Shewry et al. (*184*). Climate and soil conditions of all three sites are reported by Shewry et al. (*184*). A total of 18 grain samples (six cultivars grown at three sites) were obtained.

Table. 1. Origin of Healthgrain wheat varieties

Wheat line	Origin	Growth habit	Year of registration
Crousty	France	Winter	1994
Rialto	UK	Winter	1993
Riband	UK	Winter	1987
San Pastore	Italy	Winter	1940
Tiger	Germany	Winter	2001
Valoris	France	Winter	1998

2.1.2 Felid grown samples

For the 2010 harvest, Rialto (high Fe and Zn) ,Riband (low Fe and Zn) and Sparks (intermediate Fe and Zn) with and without foliar application of Fe and Zn were grown in a field trial at Rothamsted Research in 2010, in three replicated plots (1.8 x 10 m) in a completely randomised block design (**Fig. 6**). Standard farm practices and fertilisation (176 kg N/ha split into two applications) were followed for untreated samples. Treated samples were sprayed with 1kg/ha (220 lts of water per hectare) of Fe-EDTA and Zn-EDTA on two separate occasions five days apart approximately seven months from planting at flowering stage.

For the 2012 harvest Rialto and Riband were grown without foliar Fe and Zn treatment. In four replicate plots (1.8 x 9 m) in a completely randomised block design. Standard farm practices and fertilisation (176 kg N/ha split into two applications) were followed.

Figure 6. **Field grown Rialto, Riband and Sparks 2010 harvest**

2.1.3 Low phytic acid wheat lines

Initial analyses were performed on two lines (A95615-Js-12 333 Mu-4-6/IDO563 and A02568WSA-12-10 GH0607 91-21) obtained from the USDA harvested in 2009. Later some related lines were also obtained for USDA (IDO488, Centennial, Kanto 107, Strelinskaja Mest) these related lines were determined from consulting previously published work on the lines (*72, 157*) . All lines were then grown in the glasshouse at Rothamsted. Glasshouse conditions were 20 Cº day, 15 Cº night with 16 hr period of daylight. Winter wheat (Wheat A02568, Kanto 107, Strelinskaja Mest) were sown first into vermiculite. They were then vernalized for 8 weeks at 4 Cº day and night. The light level is low and is set to run for a period of 8 hr to simulate low light levels during the winter. Spring wheat was sown two weeks before to provide seedlings at similar growth stage

when potted on. Wheat was grown into 13 cm pots. There were four pots of each line and one plant per pot, and treated with the fungicide "Talius" (active ingredient – Proquinazid) to prevent mildew. The plants were watered by hand once a day and were staked and tied as appropriate. Plants were de-leafed to decrease the risk of pests and disease. Biological control amblyseius was applied to help control thrips presence. Wheat was threshed using a handheld threshing block.

2.1.4 High ferritin wheat line

The transgenic high ferritin line (fer0509) and control was obtained from Henrik *Brinch-Pedersen* (Aurhus University, Denmark) and harvested in 2010.

2.1.5 Nicotainamine over expressing rice

Rice was obtained as bran and polished rice flour fractions from Huajia campus, Zhejiang University, China ,where the rice was polished with a rice milling machine JNMJ3 (Taizhou Grain Instrument, Zhejiang, China) for 1 min, 3 times, to obtain polished grain. Samples were harvested from a paddy field on the farm of the Huajia campus, Zhejiang University, with a planting distance of 18×18 cm. The soil contained 10.92 g kg^{-1} total Fe and 87.8 mg/kg total Zn as determined by inductively coupled argon-plasma mass spectrometry (ICP-MS, Agilent 7500ce, CA, USA). Unpolished Wild type grain concentration 13.2 mg/kg Fe and 23.1 mg/kg Zn. EN1 and EN2 unpolished grain concentration was 15.8 and 18.4 mg/kg Fe and 31.74 to 36.99 mg/Zn respectively. Polished Wild type grain concentration 6.1 mg/kg Fe and 22.1 mg/kg Zn. EN1 and EN2 polished grain concentration was 6.0 and 6.2 mg/kg Fe and 28.74 and 29.6 mg/Zn respectively (*121*).

2.2 Chemicals and reagents

All the chemicals used were of analytical grade or high purity. Acids (HNO_3, HCL, acetic acid) were from Fischer chemicals, UK. Elemental Standards were made from stock solutions containing 1000 mg/kg and were from Fisher chemicals, UK. All elemental standards were prepared daily. All water was de-ionized pure water from a Milli-Q Water Purification System (PUR1TE, ONDEO, UK) was used. Gases for ICP-MS (He_2 and H_2 and He_20_2) were from (BOC, Surrey, UK). TRIS-base and EDTA were from Sigma-Aldrich, Austria. Quartz sand (SiO_2) from Fluka (Sigma-Aldrich, Austria). Chelax 100® resin was from Bio-Rad, (USA). Sodium phytate, tannic acid, citrate, ascorbic acid, NaCl, KCl,

and *Sodium dodecyl sulphate* (SDS) were all form Sigma Aldrich, Austria. All glassware was acid washed in 10% HNO_3.

2.2.1 Buffers

All buffers for mobile phase and extraction were degased and made up each day, pH was monitored by a pH meter (Jenway 3540). Buffer containing EDTA for rinsing column was made up every month.

.

2.2.2 Preparation of Fe-DMA and Fe-NA

Both DMA and NA form 1:1 molar complexes with Fe. Fe (II)-NA was prepared from ferric chloride (Sigma Aldrich, Austria) and Fe (III)-DMA was prepared from ferrous sulphate (Sigma Aldrich, Austria). DMA/NA were dissolved and Fe was dissolved separately, appropriate solutions were then mixed immediately to avoid oxidation. Both NA and DMA were purchased from Toronto Research Chemicals Inc., except initial speciation analysis of NA which used NA was a gift from Professor Jian Feng Ma (Okayama University, Japan).

2.2.3 Preparation of mono-ferric phytate

A solution of 100 ml of 0.5 M acetic acid with 10 sodium phytate and l g $FeCl_3$-$6H_2O$ was stirred for 2 hrs until all the sodium phytate was completely dissolved. The resultant solution was allowed to stand briefly, 100ml of 95% ethanol was added and the solution left overnight. The precipitate was then collected by centrifugation (5000 g 10 mins) (Medowrose scientific LTD ,Oxford , UK), washed three times with acetone, allowed to air dry, and stored in a desiccator. The final product was a pinkish crystalline solid and ICP-AES analyses of the product gave a molar ration of 1 Fe to 6 P which is consistent with the expected structure (*136*).

2.2.4 Enzymes

The enzymes protease XIV (bacterial; from *Streptomyces griseus*, pepsin (from porcine stomach mucosa) and phytase (from wheat), cellulase (*Aspergillus niger*) and xylanse (*Thermomyces lanuginosus*) were from Sigma Aldrich, Austria. All enzymes were demineralised according to Chelax® 100 resin instruction (*185*).

2.3 Methods

2.3.1 Moisture content

The moisture content was determined by an electric moisture meter (Extech Moisture Meters UK). Briefly, a small subsample was ground and compacted and electrical resistance was measured.

2.3.2 Milling methods

2.3.2.1 Brabender milling

Milling and separation of flour and bran were performed on a Brabender Quadrumat Junior Mill (Duisburg, Germany) (**Fig. 7**). The grains moisture content was first adjusted to 15% by the addition of deionised water. Bran yields for all samples ranged between 25-30% (**Appendix 1**) and recovery was >95%. However initial results showed that mineral content of the white flour was higher than expected for pure starchy endosperm and there was visual evidence of bran, it therefore concluded the pure starchy endosperm contained some bran contamination, therefore samples were further purified by sieving stage Vortex gene-2 with 150µm sieve.

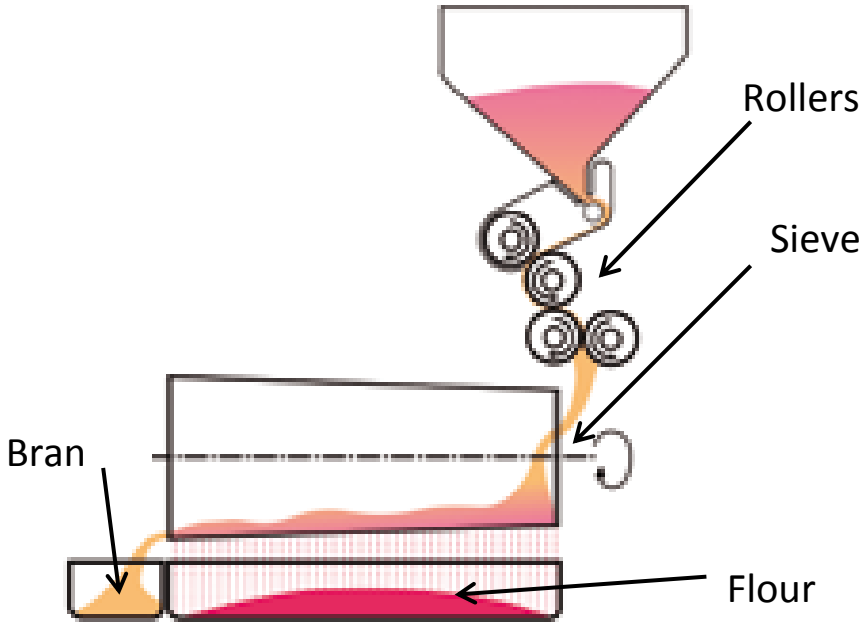


Figure 7. **Schematic of milling process in Brabender mill edited from the Brabender website** *(186)*.

2.3.2.2 Buhler mill

For field grown samples, 2.5 kgs of grain sample was brought to 16% moisture, left to condition for 16 hr, and milled in a Buhler mill (MLU-202) according to Approved Method 26-21.02 (*187*). Tempered wheat was fed at a rate of ≈175 g/min and milled to obtain 74-78% extraction of straight-grade flour. In the milling operation, three break flour streams (first break [B1]; second break [B2]; third break [B3]) and three reduction flour streams (first reduction [R1]; second reduction[R2]; third reduction [R3]), as well as two offal fractions (offal flour and course offal) and to bran fraction (bran flour and course bran) were obtained. All milling was performed at Campden BRI, Gloucestershire, UK.

2.3.4 Harberg falling number

Harberg Falling Number was performed by the sample processing facility at Rothamsted. A 7 g sample of wheat flour is weighed and combined with 25 ml of distilled water in a glass falling number tube with a stirrer and shaken to form a slurry. As the slurry is heated in a boiling water bath at 100 Cº and stirred constantly, the starch gelatinizes and forms a thick paste. The time it takes the stirrer to drop through the paste is recorded as the falling number value.

2.3.5 Ash content

Ash content was determined using AACC Approved Method 08-12(*188*). Briefly 1g of samples was disincarnated in a silica dish and total ash content weighed.

2.3.6 Determining total micro-mineral concentration and soluble macro minerals

Initial test where performed on the six cultivators grown in Hungary. Mineral concentration were analysed using both microwave digest and open-tube digest to determine which method was to be the most accurate

2.3.6.1 Microwave digest.

All digests were performed using MARS Xpress Microwave system in Teflon tubes. 0.4 g±0.05 of sample prepared as described was added to 8 mls of digest solution of (HNO_3 37.5%/ 18MΩ H_20 37.5% /H_2O_2 25%). Samples were then microwaved using the following program.

	Power Max (W)	Power %	Ramp (min)	Temperature (C)	Hold (min)
Stage 1	400	100	8	55	3
Stage 2	800	100	14	115	20
Stage 3	800	100	5	140	15

Samples were then diluted to 25ml volume with 18MΩ H_20 for ICP-OES

2.3.6.2 Open tube digest

All digest were performed on digestion block (Eurotherm,UK). Samples were prepared as described and pre-digested in $HNO_3/HClO_4$ acid mixture for 4 hrs. The following heating regime was then carried out overnight.

Programme	Rise rate $^0C/hr$	Temp ^0C	Dwell hrs
1	60	60	3
2	120	100	1
3	120	120	1
4	50	190	2.5
5	STEP	50	END

After evaporation 20% Aristar HNO_3 was added, before concentrations made up to 20 ml with 18MΩ H_20 for ICP-OES.

Both methods were validated by the use of standards Bcr-189 (whole grain), NIST 8438 (wheat white flour) and NIST 1567a (wheat white flour). Replicated analyse (n = 3) of the standards gave mean values in good agreement with the certified values of all standards. Further analysis used only the open tube method which showed the highest agreement with standards in relation to Fe and Zn, the elements of interest, and BCR-189 was run with all samples to make sure results were comparable. The mean results for Bcr-189 after open tube digest were 67.1±1.7 mg/kg for Fe and 59.1±1.6 mg/kg for Zn compared to certified values of 68.3±1.9 and 56.5±1.7 mg/kg, respectively. Wet digestion procedures in open vessels are at risk of sample contamination and loss of analytes by volatilization compared to the closed tube microwave method (*189*) Due to the nature of our analytes, Fe and Zn, the risk of volatilization was deemed considered to be minimal.

2.3.7 Total minerals of dry mass

Minerals concentrations were determined using inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer optima 7500 DV, Waltham, USA). 500 mg (oven dried at 80 ºC for 4 hrs) of each sample was digested in ultra-pure HNO_3 and $HClO_4$ (87:13 % v/v) in triplicate using digestion blocks (200 ºC, 12 hrs) (Eurotherm MBB151, Durrington, UK). Working conditions for ICP-OES were: RF Power 1.5 kW, auxiliary gas (Ar) flow rate 0.2 l/min, carrier gas (Ar) flow 0.85 l/min. Blank and certified reference materials (CRM, BCR-189, wheat wholemeal flour and NIST1568a, rice flour) were included in each batch of analyses and the values for the CRM were within <5% of the certified values for Fe and Zn. Signal drift was corrected by measuring a standard solution every 10 - 20 samples. Possible Soil contamination was checked by analysing Ti and Al concentration of samples, all results were well below 10ppm indicating no soil contamination.

2.3.8 Macro minerals of soluble fraction

Due to the higher concentration of macro-minerals of interest (P, Ca, S) soluble macro minerals were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer optima 7500 DV, Waltham, USA) as described above except solution were diluted with acid to make 10 ml of final concentration of 5% HNO_3.

2.3.9 Micro minerals in solution

Micro minerals in soluble fractions were determined by inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500ce, Agilent Technologies, Palo Alto, CA, USA) with the following conditions: RF power 1.5 kW, carrier gas 0.85 l/min, nebuliser pump speed 0.2 l/min, H_2 gas 6 ml/min. All samples were measured with three technical replicates and made up to 10 ml with final concentration of 5% HNO_3. Standards of 10 µg/l Fe, Zn and Cu and a blank were used to monitor signal stability, in addition an internal standard containing ^{89}Y which was added to all samples and monitored throughout.

2.3.10 Determination of Nitrogen percentage

Total grain N was determined by the analytical Unit of the Soil Science Department, Rothamsted Research, according to the Dumas digestion method, using a LECO CNS 2000 combustion analyser (Leco, Stockport, UK). Flour fractions were oven dried (overnight 80 Cº) and 0.5 g of samples was analysed, all samples were analysed in duplicate and results presented as means.

2.3.11 Determination of phytate

The phytate content of the samples were determined with a Megaenzyme assay kit (K-PHYT 05/07; Megazyme Int., Wicklow, Ireland). Briefly, either 1 g or 0.5 g of samples with extracted with 10 ml of hydrochloric acid followed by enzymatic dephosphorylation step with phytase and alkaline phosphatase and precipitation using a colour reagent prepared from ascorbic acid in sulphuric and ammonium molybdate. The Absorbance (655 nm) of free phospurous and total phosphorous were then compared to phosphorus standards using a Varioskan spectrometer (Thermo, Finland). All runs included standards and controls and all samples were analysed in triplicate.

2.3.12 Data analysis

ANOVA, Correlation and regression analysis was performed using GenStat 13th edition (VSN, Hemel Hempstead, UK).

Chapter 3. Total Fe and Zn concentrations in different milling fractions of wheat grain

3.1 Introduction

The majority of wheat products consumed in the diet are produced after milling of the grain to give white flour. This consists almost entirely of the starchy endosperm, while the bran, which contains the aleurone layer, embryo and outer layers of the grain (pericarp and testa), is largely discarded (*67*). However, the concentrations of Fe and Zn in the endosperm are much lower than those in the bran (*66, 103*). The amount of white flour which is recovered during processing is referred to as the extraction rate. It is therefore important that biofortification strategies also consider the localisation of minerals within the grain if the end products are to have increased nutritional benefits.

Variation in the concentrations of total Fe and Zn in whole grain of wheat genotypes has been widely reported. For example, a recent study of 130 cultivars grown on the same site showed that the concentrations of Fe and Zn ranged from 28 to 51 mg/kg and from 13 to 35 mg/kg, respectively (*4*). Other studies have shown even greater variation; analyses of lines grown in France showed variation of 19-58 and 14-35 mg/kg for Fe and Zn, respectively (*190*), while in Asian wheat varieties the concentration of Fe ranged from 23 to 49 mg/kg (*191*) and of Zn from 25-56 mg/kg (*192*). Variation from 4-16 mg/kg of Fe and from 5-16 mg/kg of Zn has also been reported for white flour of Chinese wheat cultivars (*103*). Although this range of genotypic variation is encouraging from a plant breeding perspective, it is not clear whether the variation is consistent between different fractions of the grain, and in particular whether the variation reported for whole grain is reflected in white flour from the same genotypes.

Correlations between minerals in grain are also of interest; this is particularly so for the relationship between Fe and Zn as breeding for one mineral could theoretically simultaneously increase the concentration of the other (*8, 193*). Protein is also correlated with Fe and Zn

concentrations and high grain protein from a nutritional standpoint is a desirable trait (*193*). Correlations with other elements are also of interest, including with P, or more importantly phytic acid.

For this chapter the total concentrations of primarily Fe and Zn were determined in a range of cultivars grown on a number of sites. Grain was milled to give white flour and bran (Chapter **2** section **3.2.1**). Two cultivars (Rialto, Riband) were also grown in a field trial so as to have biological replicates and to have enough material for laboratory milling to determine variation in the concentrations of minerals, protein and phytic acid across 10 milling fractions. A field experiment was also conducted to determine the effects of foliar application of Fe and Zn on the mineral concentrations of wheat grain of three cultivars (Rialto, Riband, Spark).

Hypothesis: Cultivars of grain with higher Fe and Zn concentrations within the whole grain will also have higher Fe and Zn concentrations within the white flour fraction.

3.2 Materials and methods

The samples provided from Healthgrain projects were grown as described (**chapter 2**). The three cultivars used to determine the effects of foliar treatment were field grain harvested in 2010 (chapter **2**, **2.1**). Further analysis on 10 milling fractions milled by the Buhler mill (chapter **2, 3.2.1**) were from field grown grain of Rialto and Riband harvested in 2010 and 2012 (chapter **2, 2.2**.) These two cultivars were chosen as they represented high and low Fe and Zn lines. The effects of foliar treatments were also determined on field grown cultivars Rialto, Riband and Spark harvested in 2010. All results are expressed as dry mass (*dm*).

3.2.1 Data analysis

For the multiple environment trials of 6 wheat lines, each site (three in total) was taken as a replicate as each individual plot was not replicated (**Chapter 2).** For Rothamsted field grown lines (Rialto, Riband, Spark) (**Chapter 2**) analysis of variance (ANOVA) was used where appropriate. Correlation coefficients were calculated to determine relationships between minerals across genotype and location, and different milling fractions.

3.3 Results

3.3.1 Samples from the Healthgrain project

Figure 7 shows the concentrations of Fe and Zn in flour and bran of samples of six wheat cultivars grown on three European sites (in Hungary, Poland, UK). Because cultivar differences were consistent across the three sites, only the mean data are presented. The cultivars Rialto, San Pastore and Tiger contained higher concentrations of Fe in the whole grain than Crousty, Valoris, and Riband. This cultivar difference was generally similar for the concentrations of Fe in both bran and white flour. Notably, the mean concentrations of Fe were 43% higher in the wholemeal samples and 43% higher in the white flour of the high Fe cultivars when compared to the low Fe cultivars. Similarly, the mean concentrations of Zn were 16% higher in the wholemeal samples and 58% higher in the white flour of the high Fe cultivars when compared to the low Fe cultivars. The cultivar-specific differences in Fe and Zn concentrations were consistent across the three sites. There were also significant correlations between the mineral concentrations in wholemeal and concentrations in the white flour, for both Fe (r=0.540, n=18, p<0.05) and Zn (r=0.489, n=18, p<0.05) among the different cultivars across the 3 sites.

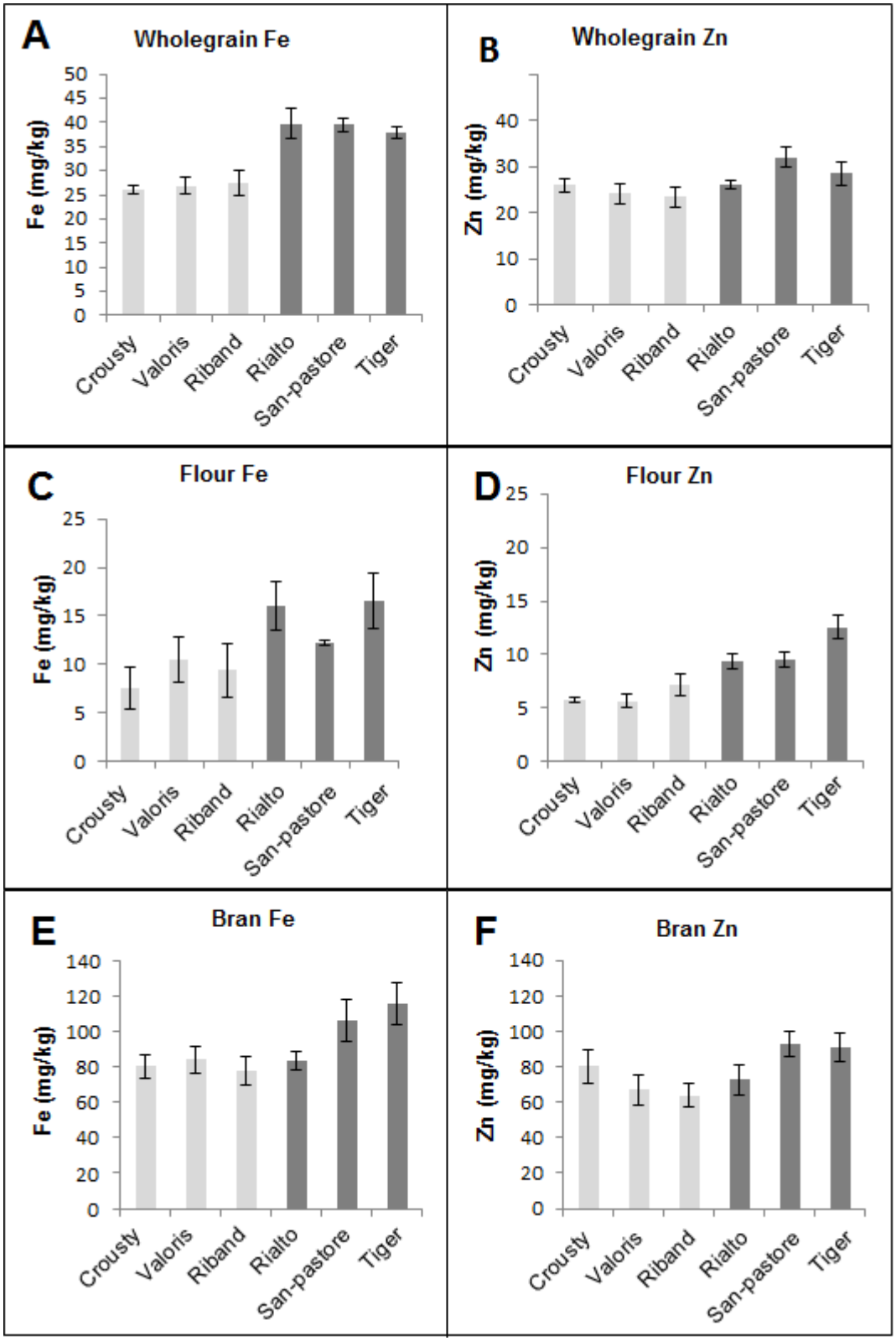


Figure 8. The concentrations of Fe and Zn in wholemeal (A, B), white flour (C, D) and bran fractions (E, F) of six wheat cultivars. Data are means of materials grown in field trials at three sites in 2007 (Hungary, Poland, UK) (mg/kg dm ± SE).

Table 2 The concentrations of P and S in wholemeal, bran and white flour fractions of six wheat cultivars. Data are means of materials grown in field trials at three sites in 2007 (Hungary, Poland, UK) (mg/kg dm ± SE)

Cultivar	P (mg/kg) wholemeal Means ± SE	S (mg/kg) Wholemeal Means ± SE	P (mg/kg) Bran Means ± SE	S (mg/kg) Bran means ± SE	P (mg/kg) White Flour means ± SE	S (mg/kg) White Flour means ± SE
Crousty	3384±139	1584±20	10270±1099	2128±132	826±8	1163±30
Valoris	3350±162	1493±70	8962±287	1877±22	819±17	1042±20
Riband	3658±175	1614±77	9128±1003	1882±64	928±65	1174±140
Rialto	3551±124	1640±29	8888±503	1862±15	1174±85	1199±100
San-P	3880±165	1620±55	10829±794	1997±27	1068±103	1282±82
Tiger	3525±111	1521±40	9864±393	1876±28	1161±17	1335±137

Figure 8 Shows the concentrations of Fe, Zn and P in the white flour fractions in more detail and the effects of environment on grain concentration. The Fe concentration varies the most between the sites by >100% between some cultivars (**Fig. 8a**). The Zn concentration varied less with the highest variation being 50% between sites (**Fig. 8b**). The P concentration was relatively stable across all sites with only low variation, although the high Fe/Zn cultivars (Rialto, San Pastore , Tiger) did appear to have more P than the low Fe/Zn cultivars (Crousty, Valoris, Riband). No statistics were performed as there was only one biological replicate for each site.

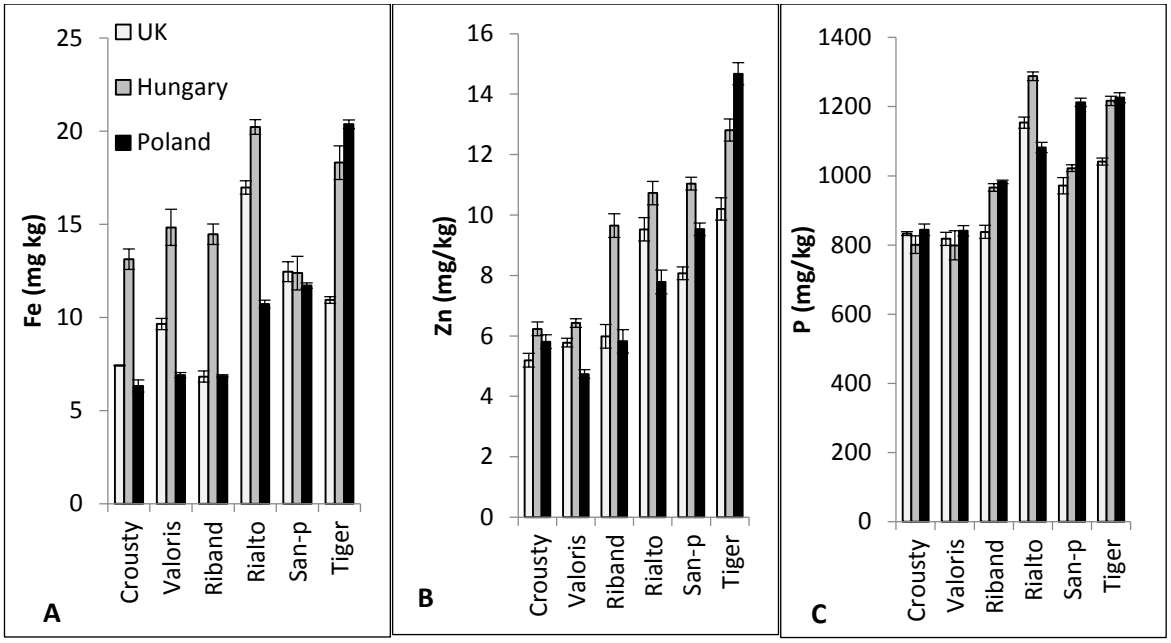


Figure 9. The concentrations of A) Fe B) Zn and C) P in white flour fraction of the six cultivars (Crousty, Valoris, Riband , Rialto, San Pastore [San-p], Tiger) fractions across 3 different sites, error bars represent SE of means of three technical replicates. Rialto, San Pastore and Tiger are lines identified as being high Fe/Zn.

Figure 9 shows the correlation between Fe and Zn in Healthgrain samples. In whole grain samples there was a positive correlation between Fe and Zn (r=0.419, n=18, p<0.05). This correlation was higher in bran (r=0.724 p>0.001), and highest in white flour (r=0.811, n=18, p>0.001). **Figure 9** shows the correlations between total S and Zn. Zn and S were correlated in the white flour fraction, however there was no significant correlation between S and Zn in the bran fraction. **Figure 9** shows the correlations between total P and Fe. Fe and P were correlated in the white flour fraction, however there was no significant correlation between P and Fe in the bran fraction. Similar results were obtained for Fe and S and Zn and P (data not shown).

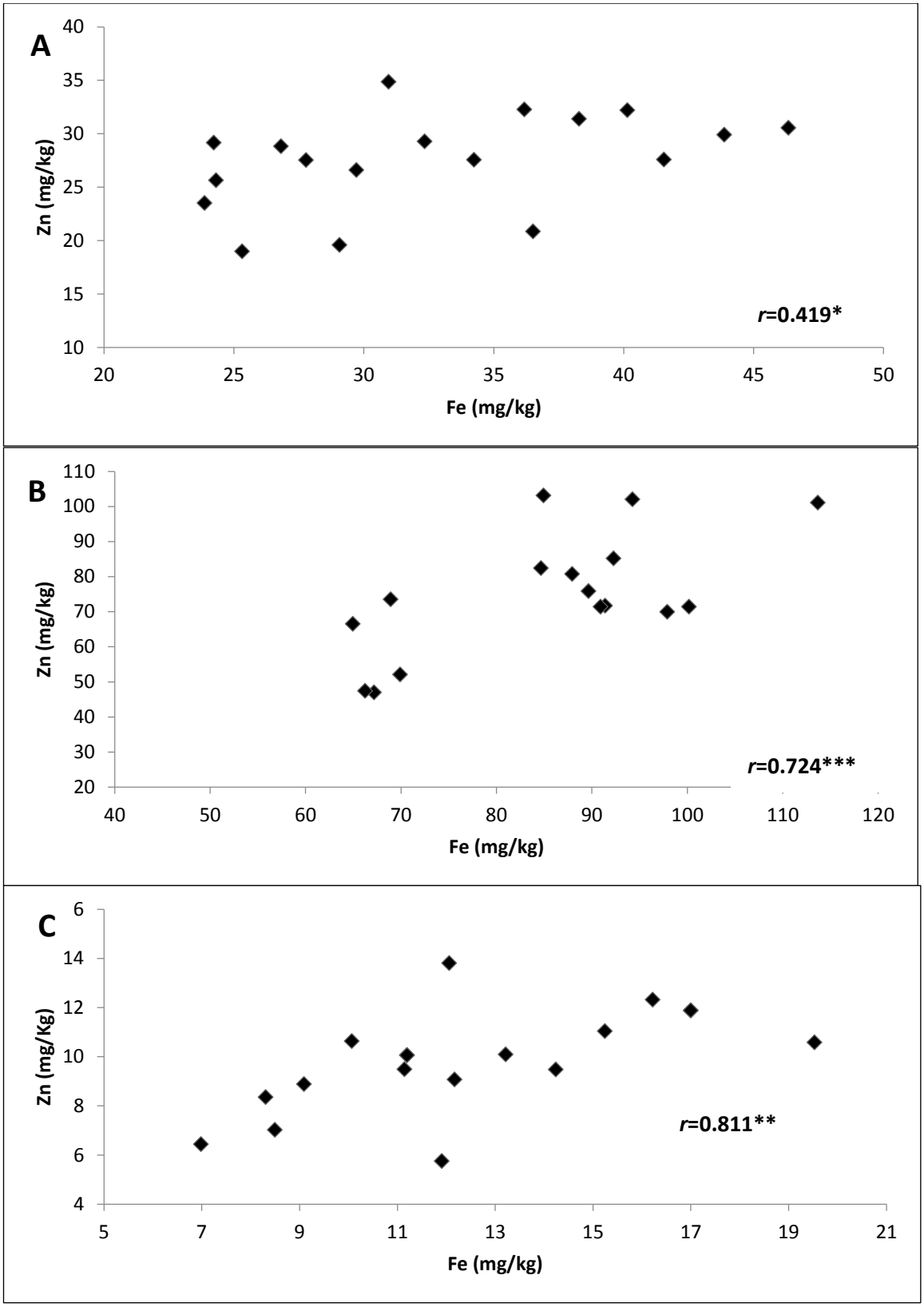


Figure 10. **Correlation coefficients (*r*) between Fe and Zn concentrations in wholemeal (A), bran (B) and white flour fractions (C) of six wheat cultivars**. Data are means of samples grown in field trials at three sites in 2007 (Hungary, Poland, UK) (mg/kg dm). Level of significance: **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (n = 18).

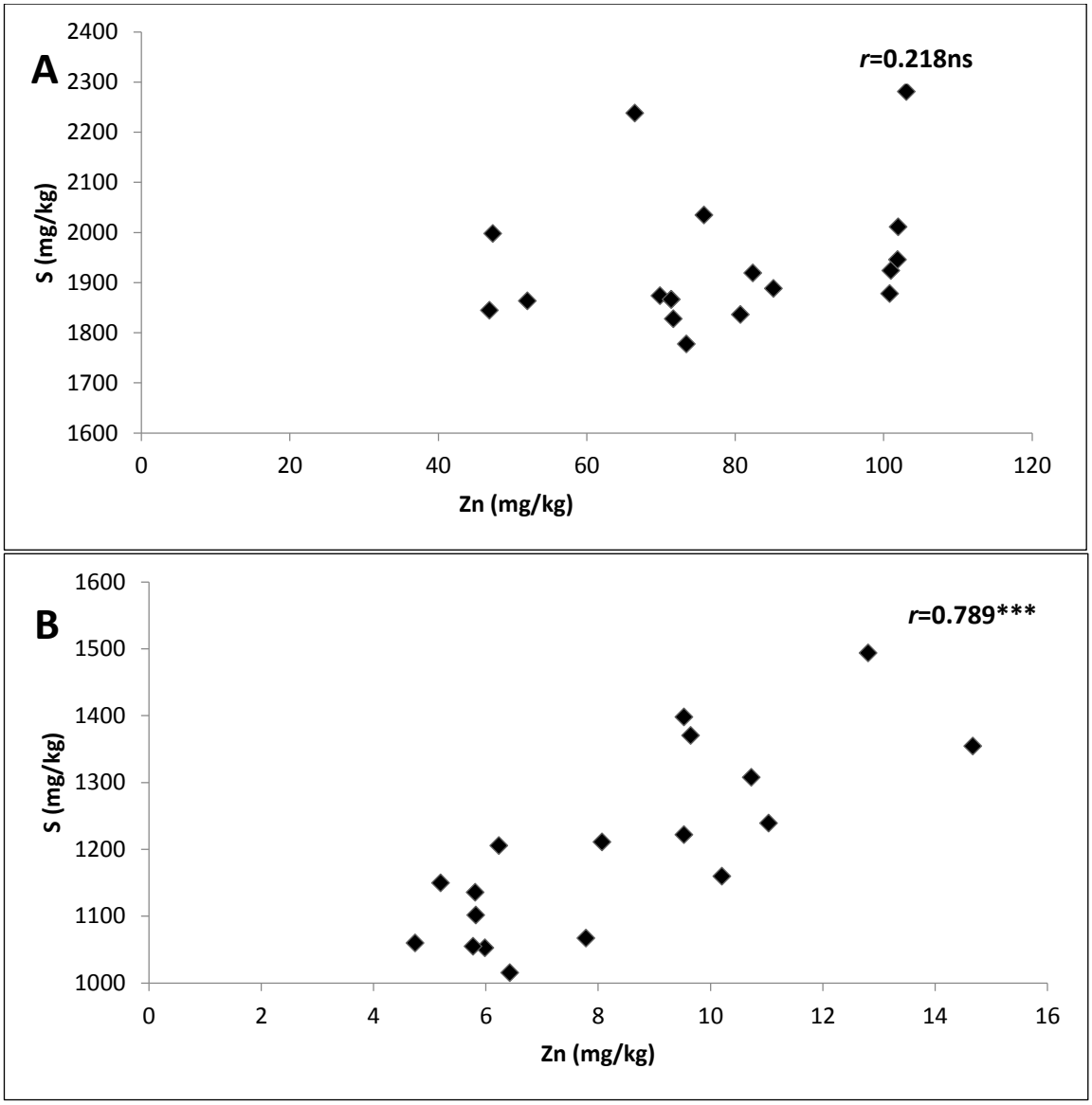


Figure 11. Correlation coefficients (*r*) between Zn and S concentrations in bran (A), and white flour fractions (B) of six wheat cultivars. Data are means of samples grown in field trials at three sites in 2007 (Hungary, Poland, UK) (mg/kg dm). Level of significance: **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (n = 18).

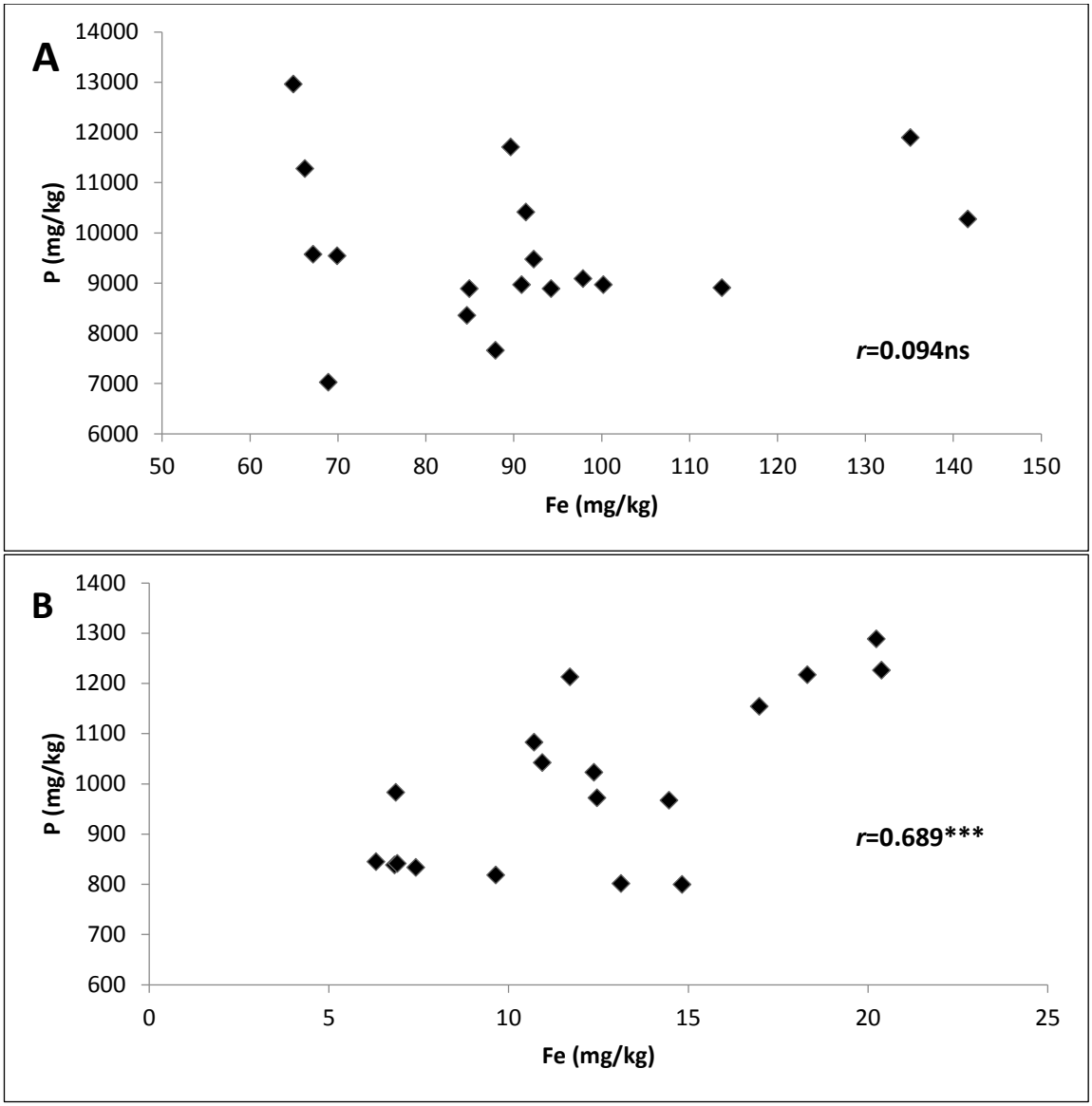


Figure 12. Correlation coefficients (*r*) between Fe and P concentration in A)bran and B)white flour fractions of six wheat cultivars. Data are means of samples grown in field trials at three sites in 2007 (Hungary, Poland, UK) (mg/kg dm). Level of significance: **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (n = 18).

Figure 13 shows the molar ratio of P:Fe. These figures show that in wholegrain flour the cultivars high in Fe/Zn (Rialto, San-pastore, Tiger) had lower P:Fe ratios compared to cultivars low in Fe/ZN (Crousty, Valoris , Riband), with mean ratios of 79:1 compared to 93:1, however in the white flour this relationship was reversed, with high Fe/Zn cultivars having a P:Fe ratio of 80:1, compared to low Fe/Zn cultivars with a P:Fe ratio of 86:1.

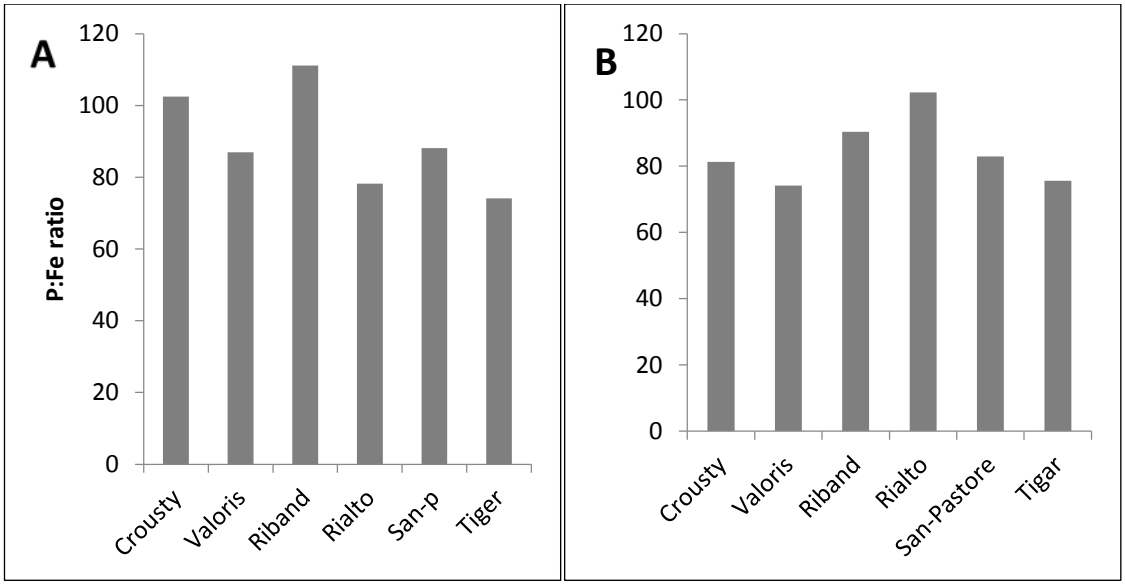


Figure 13. The P:Fe ratio of Healthgrain samples. Data are means wholemeal (**A**) and white flours (**B**) samples grown in field trials at three sites in 2007 (Hungary, Poland, UK) .

3.3.2 Field trial at Rothamsted in 2010 and 2012

Figure 14 shows the effect of foliar treatment of Fe-EDTA and Zn-EDTA on Fe and Zn concentration in wholegrain of Rialto, Riband and Sparks. ANOVA showed that the foliar Fe application had no significant effect on Fe concentration in wholegrain, however; here was a slight effect (mean increase of 1.1 mg/kg) of Zn treatment (p=0.05)

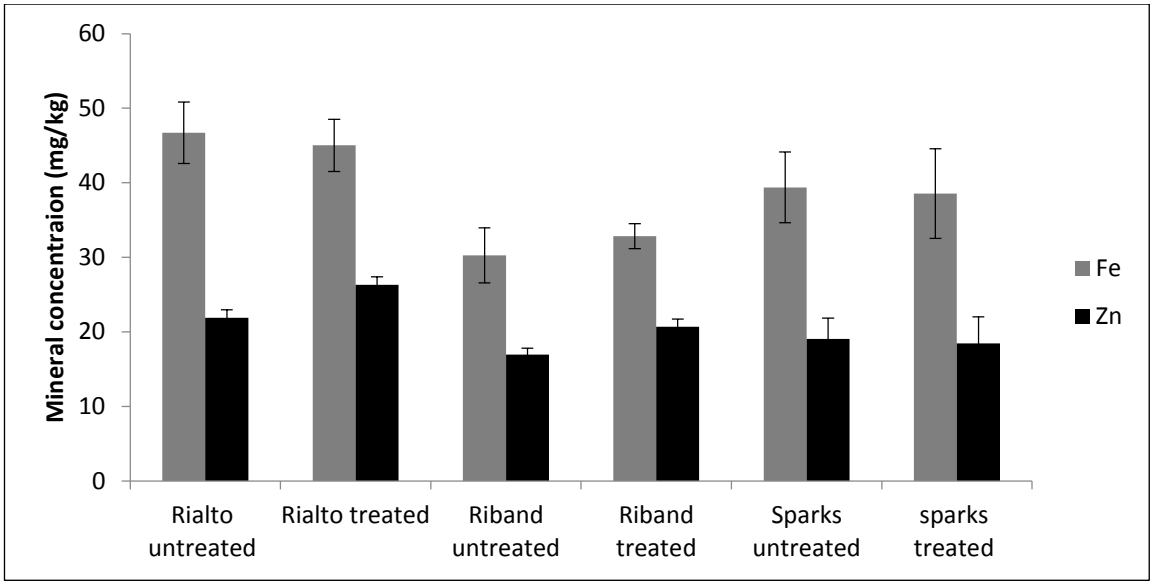


Figure 14. **The concentrations of Fe and Zn in whole meal of the cultivars Rialto, Riband and Spark grown at Rothamsted in 2010 with and without combined foliar treatment of Fe-EDTA and Zn-EDTA. Data are means ± SE of three biological replicates.**

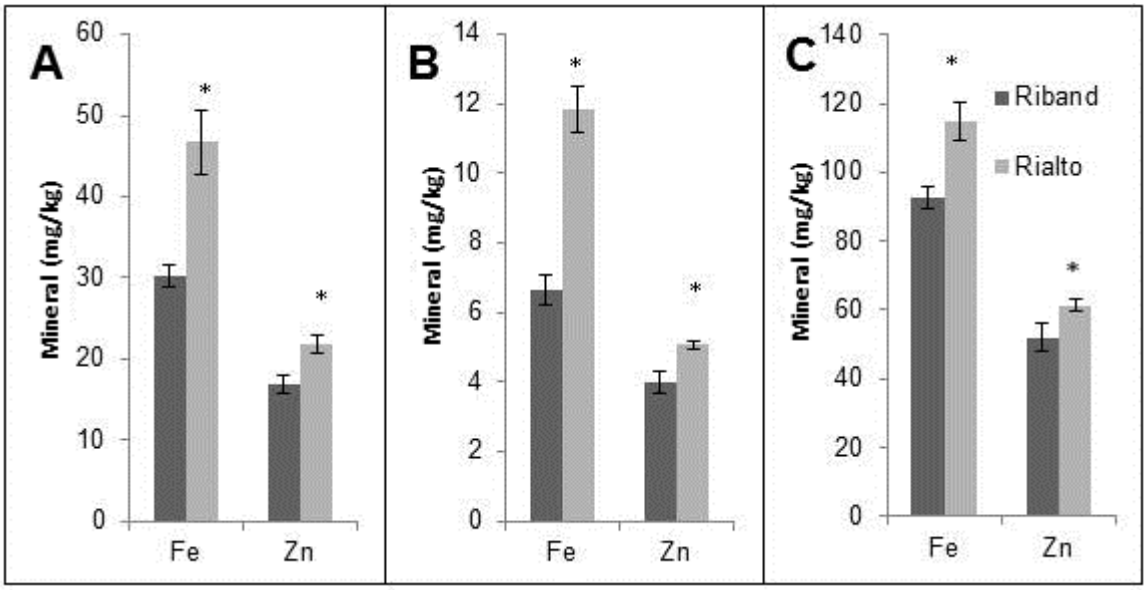


Figure 15. The concentrations of Fe and Zn in (A) whole meal, (B) white flour and (C) bran fractions of the cultivars Rialto and Riband grown at Rothamsted in 2010. Data are means ± SE of three biological replicates, * represent significant differences between cultivars at $p< 0.05$ according to ANOVA.

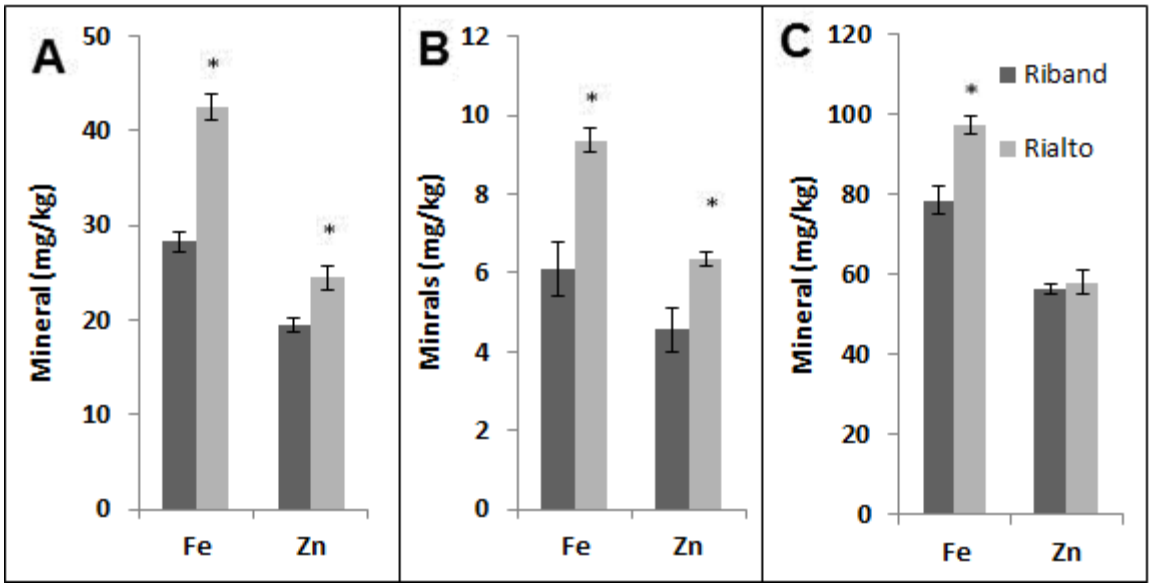


Figure 16 The concentrations of Fe and Zn in (A) whole meal, (B) white flour and (C) bran fractions of the cultivars Rialto and Riband grown at Rothamsted in 2012. Data are means ± SE of four biological replicates, * represent significant differences between cultivars at $p< 0.05$ according to ANOVA.

Figure 15 shows the concentrations of Fe and Zn in grain of Rialto and Riband grown at Rothamsted in 2010. Rialto had significantly ($p<0.05$) higher concentrations of Fe and Zn than Riband, in wholemeal (by 54% and 29%, respectively), bran (by 24% and 18%) and white flour (by 80% and 26%). **Figure 16** shows the same samples grown in a replicate trial in 2012. Rialto again had higher Fe and Zn in wholemeal (by 50% and 26%, respectively), and white flour (by 53% and 40%). Rialto had higher Fe in bran (by 24%), but there was no significant difference between cultivars for Zn in bran. It is notable that the difference in the concentration of Fe was greatest in the white flour fraction in both years. These differences were broadly similar to the data from the multi-site trials (**Fig. 8**).

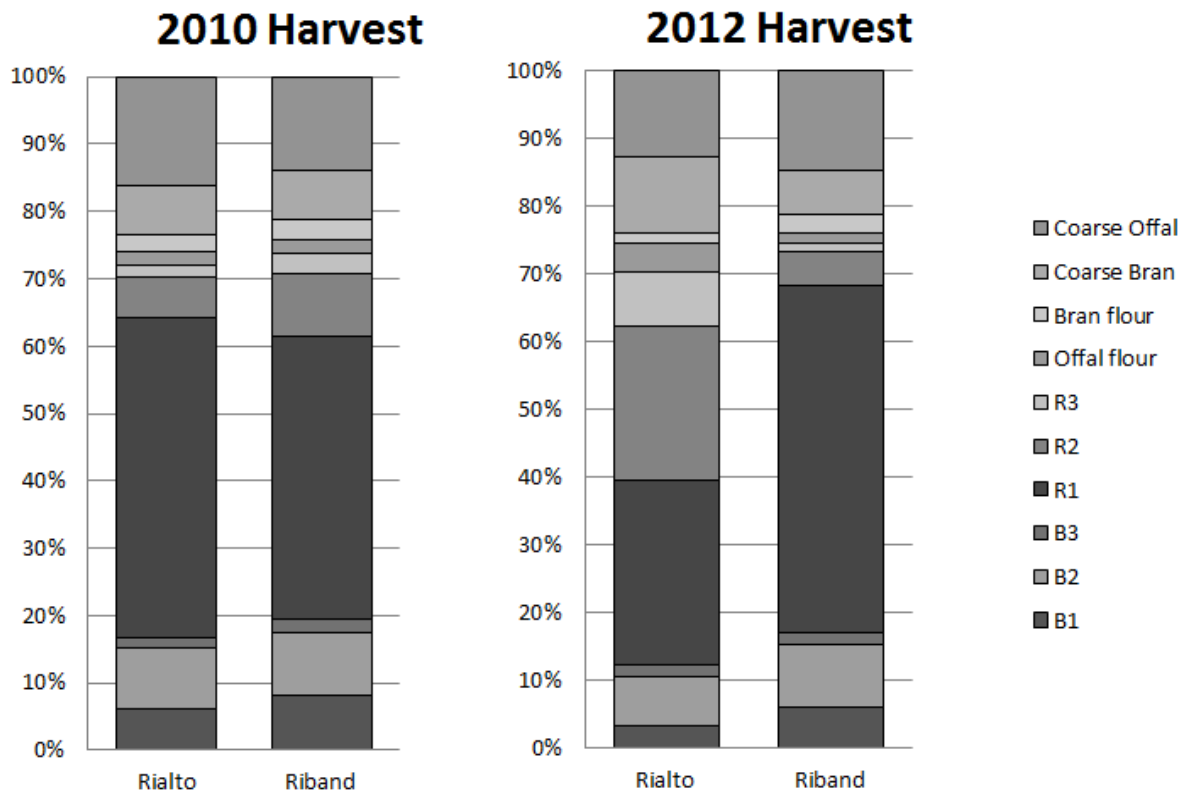


Figure 17. Percentage yields of Buhler milling fractions from Rialto and Riband grown at Rothamsted in 2010 and 2012.

Table 3. Ash content of the 10 milling fractions from the 2010 harvest.

Fraction	B1	B2	B3	R1	R2	R3	Offal flour	Bran Flour	Coarse offal	Coarse Bran
Ash(%)[1]										
Rialto	0.2	0.4	0.7	0.4	0.7	1.3	1.3	1.5	6.1	4.6
Riband	0.3	0.5	1.8	0.4	0.7	1.1	1.3	1.7	6.3	4.9

`

The yeilds of Buhler mill fractions are provided in **Figure 17**. Milling fraction yields for Rialto in 2012 were atypical and therefore most analysis were performed on the fractions from 2010. For the 2012 harvest the R2 fraction of Rialto was abnormally high corresponding to 23% of the total grain, whereas expected yeild of this fraction is 13% (*103*). Considering the total Fe, Zn and P (**Appendix 2**) in this fraction it is clear there is some aleurone contamintion in this fraction as Fe, Zn and P are all much higher than expected. The cause of this is not kown but no further analyses were performed on their fractions due to this anomaly, which would have skewed any results. For the 2010 harvest the break fractions B1 - B3 and the reduction fractions R1 - R3 correspond to

white flour, with R1 accounting for 47.6% and 42.1% of the total grain in Rialto and Riband, respectively (**Fig. 17**). Riband, a soft wheat, had higher yield of the break fractions (B1-3). This has previously been reported as an effect of grain hardness (*194*).

Ash content, an indicator of bran contamination(*195*), decreases closer to the center of the grain, with the first break (B1) and the first reduction (R1) having the lowest ash content in both cultivars. Coarse offal had the highest ash content which was 20-30 times that of the first break fraction (B1) (**Table 3**). The R1 fraction had the lowest ash content (**Table 3**) and is therefore considered to represent the purest starchy endosperm fraction derived from the central part of the grain (*194*).

The total concentration of Fe (**Fig. 18a**) varied greatly between the six flour fractions (R1, R2, R3, B1, B2 and B3), from 9 to 32 mg/kg in Rialto and 5 to 35 mg/kg in Riband, respectively. The differences were even greater between the flour and shorts (offal flour, bran flour, coarse offal and coarse bran) fractions, with a 26 fold difference in Fe concentration between the R1 and coarse offal fractions from Riband.The total concentrations of Zn (**Fig. 18b**) also varied greatly between the six flour fractions (R1, R2, R3, B1, B2 and B3), 2 to 20 mg Zn/kg in Rialto and 2 to 16 mg Zn/kg in Riband. The differences between R1 and coarse offal for Zn concentration was 44 fold.

There was a less dramatic difference in P concentration between the flour fractions and shorts fractions; however, there was still a clear difference between the flour and shorts in both cultivars , with the coarse bran fractions containing ~12 fold greater P then the B1 and R1 (**Fig. 19a**). The concentrations of sulphur were more consistent across all fractions with the greatest varation being only ~2 fold higher in course offal compared to B1 in Riband (**Fig. 19b**).

There was a strong postive correlation between the concentrations of Fe and Zn in the ten milling fractions (r=0.929, n=20, p<0.001) (**Fig. 20**), the strongest correlation being observed between Fe and P (r=0.999, n=20, p<0.001) (**Fig.20c**). Intrestingly this was higher than the corrlation between Fe and phytic acid (r=0.956, n=20, p<0.001) (**Fig 20a**).

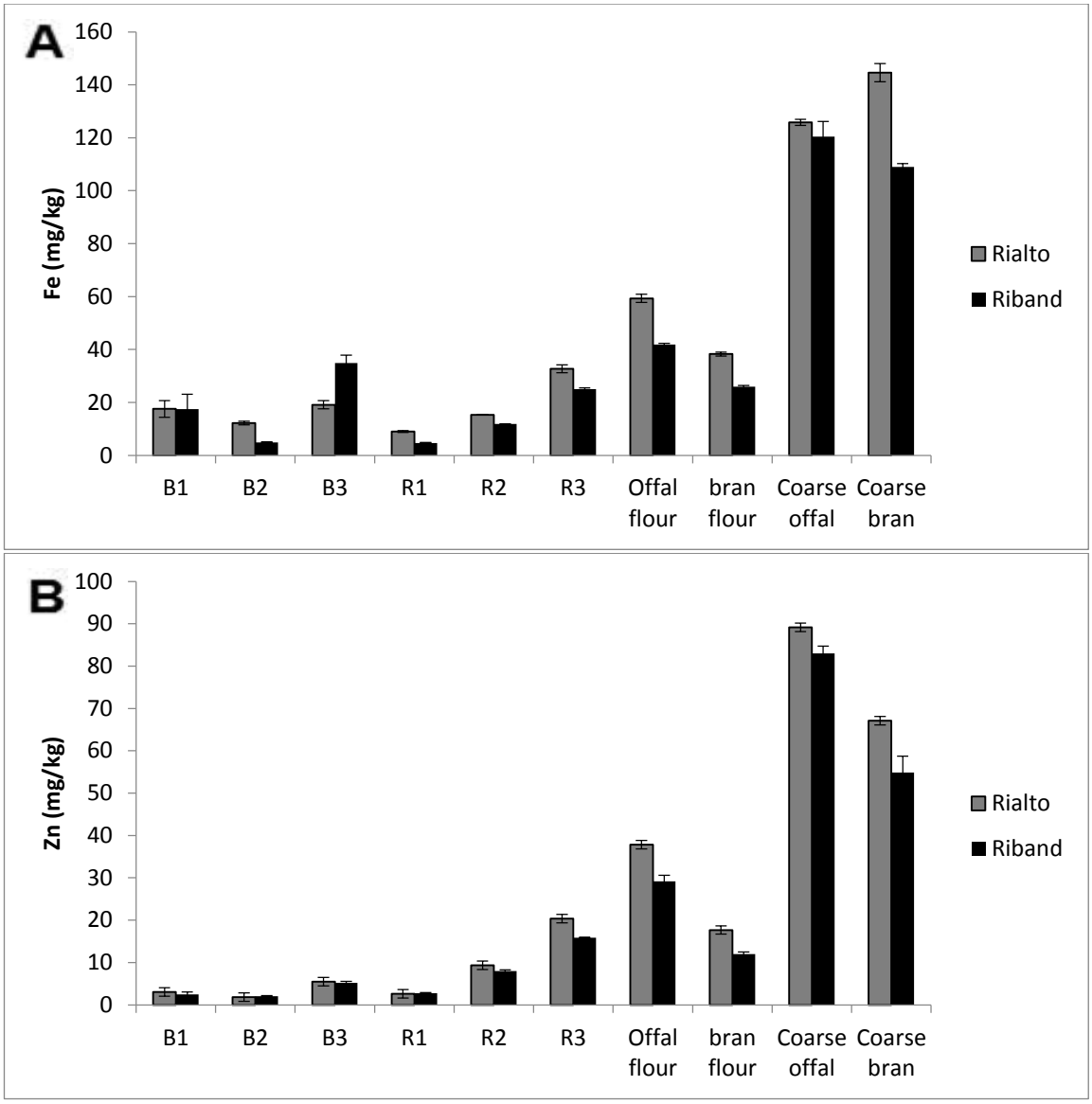


Figure 18. The concentrations of Fe (A) and Zn (B) in 10 fractions obtained by Buhler milling of the cultivars Rialto and Riband, grown at Rothamsted in 2010. Data are means ± SE of three technical replicates.

Phytic acid showed a similar distrubution pattern to Fe and Zn (**Fig. 21**), varation in the white flour fractions was however higher, with differences between B3 and R3 being >300 fold in Rialto. Rialto had higher phytic acid concentrations than Riband in all fractions except offal flour and B3. This was particularly evident for the white flour fractions where concentrations were over 2 fold higher in the reduction fractions (R1-R3) and the first 2 break fractions (B2, B3). Both Fe and Zn showed strong postive correlations with phytic acid across the milling fractions (**Fig. 22**).

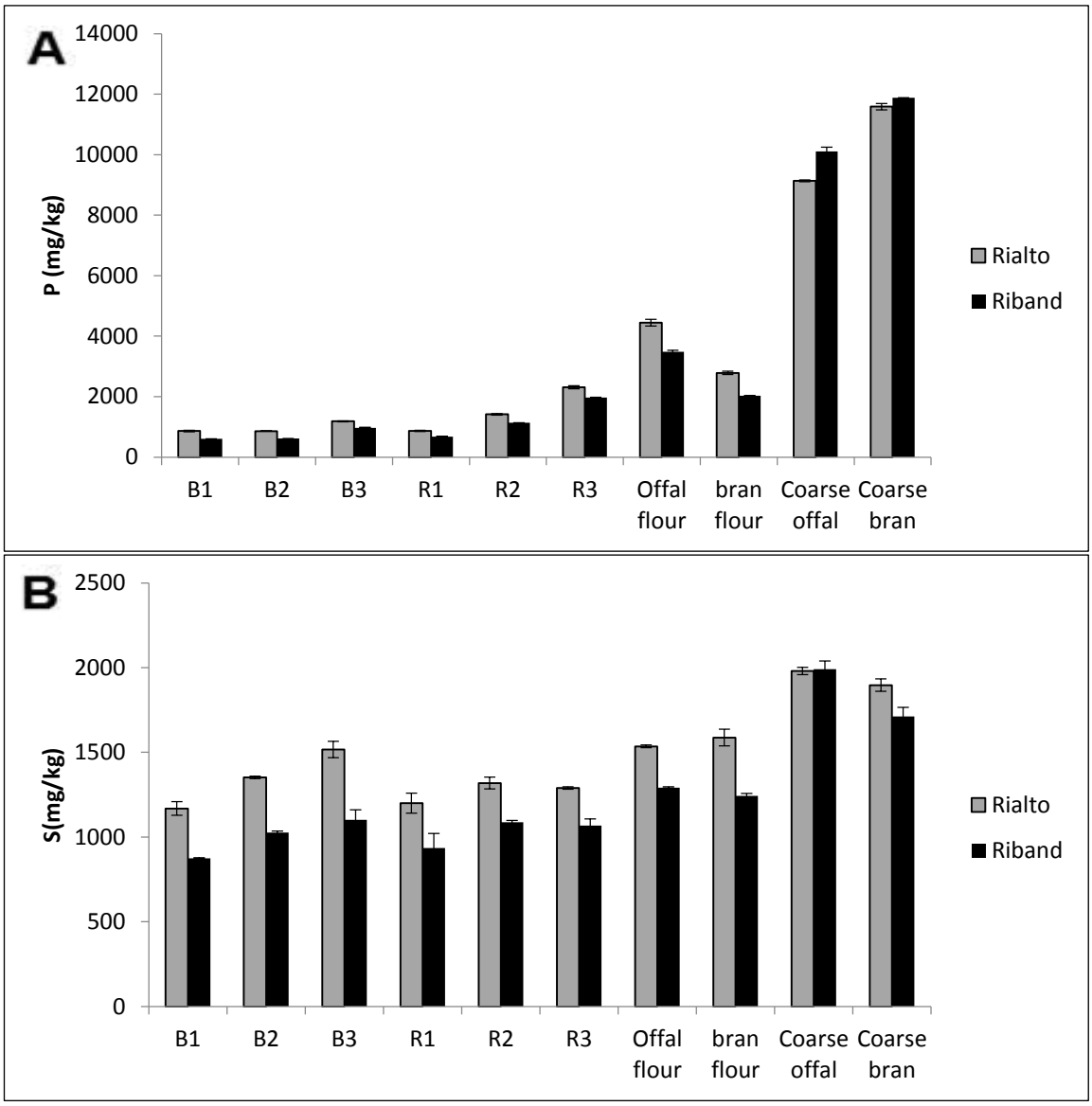


Figure 19. The concentrations of P (A) and S (B) in 10 fractions obtained by Buhler milling of the cultivars Rialto, and Riband grown at Rothamsted in 2010. Data are means ± SE of three technical replicates.

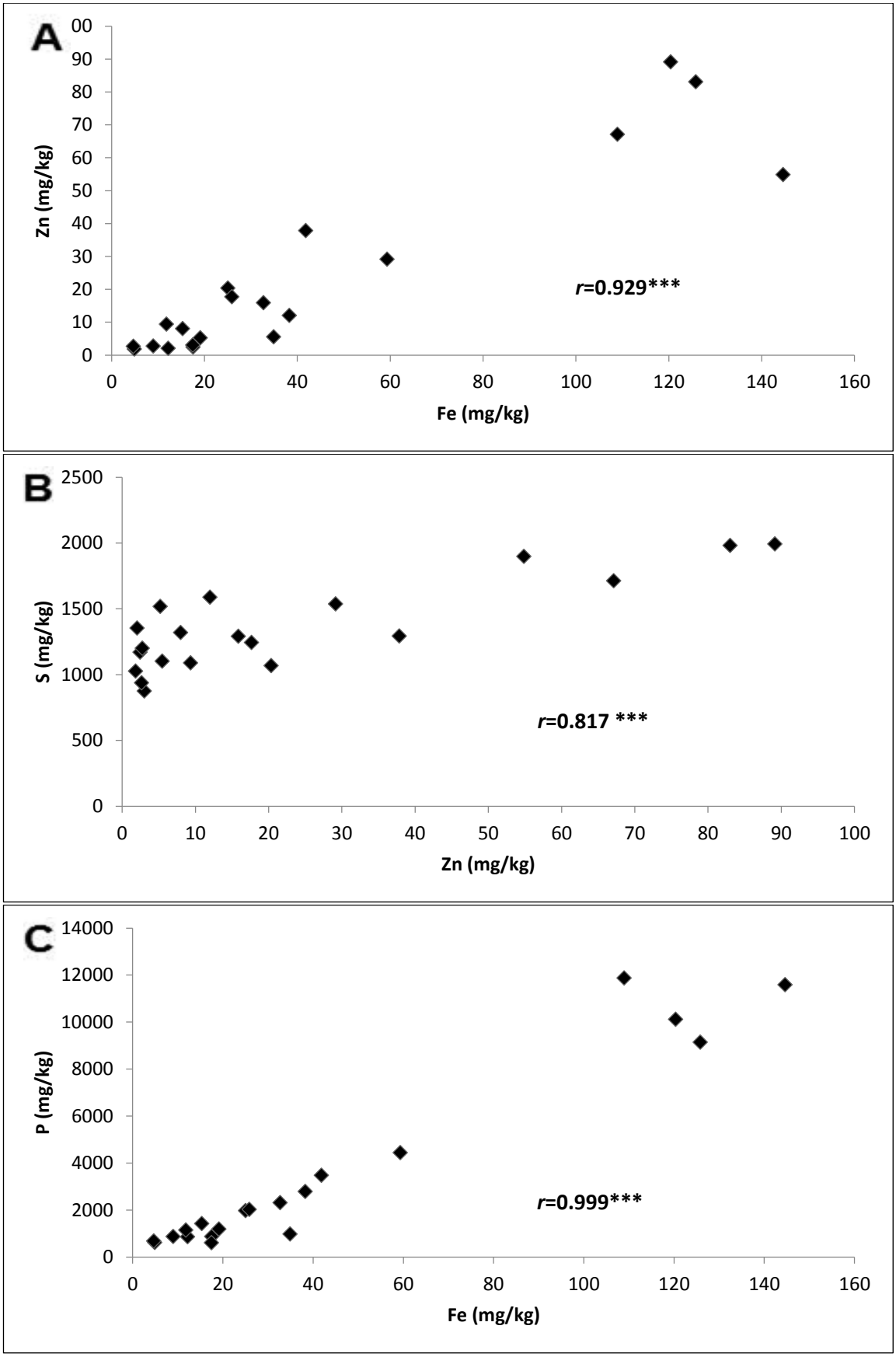


Figure 20. The correlation coefficients (*r*) between Fe and Zn (A), Zn and S (B) and Fe and P (C) in the 10 milling fractions of Rialto and Riband grown at Rothamsted in 2010 (n = 20). Level of significance: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

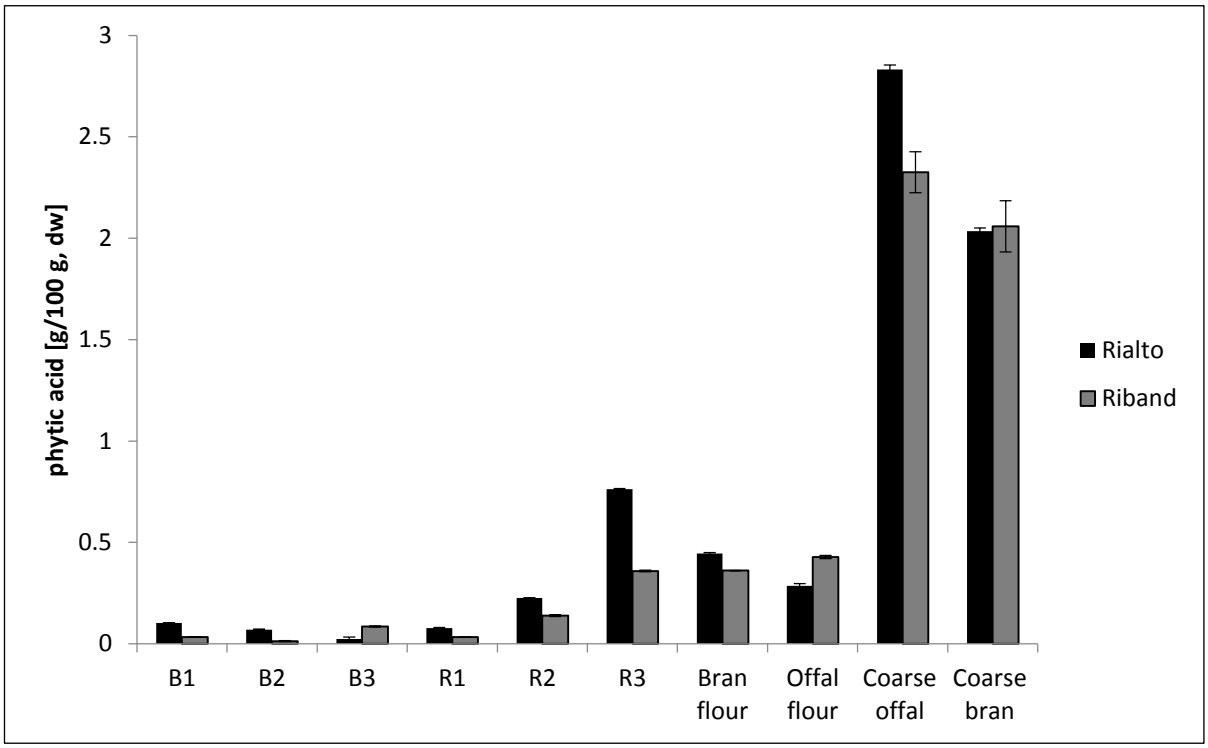


Figure 21. The concentrations of phytic acid in 10 fractions obtained by Buhler milling of the cultivars Rialto, and Riband grown at Rothamsted in 2010. Data are means ± SE of three technical replicates.

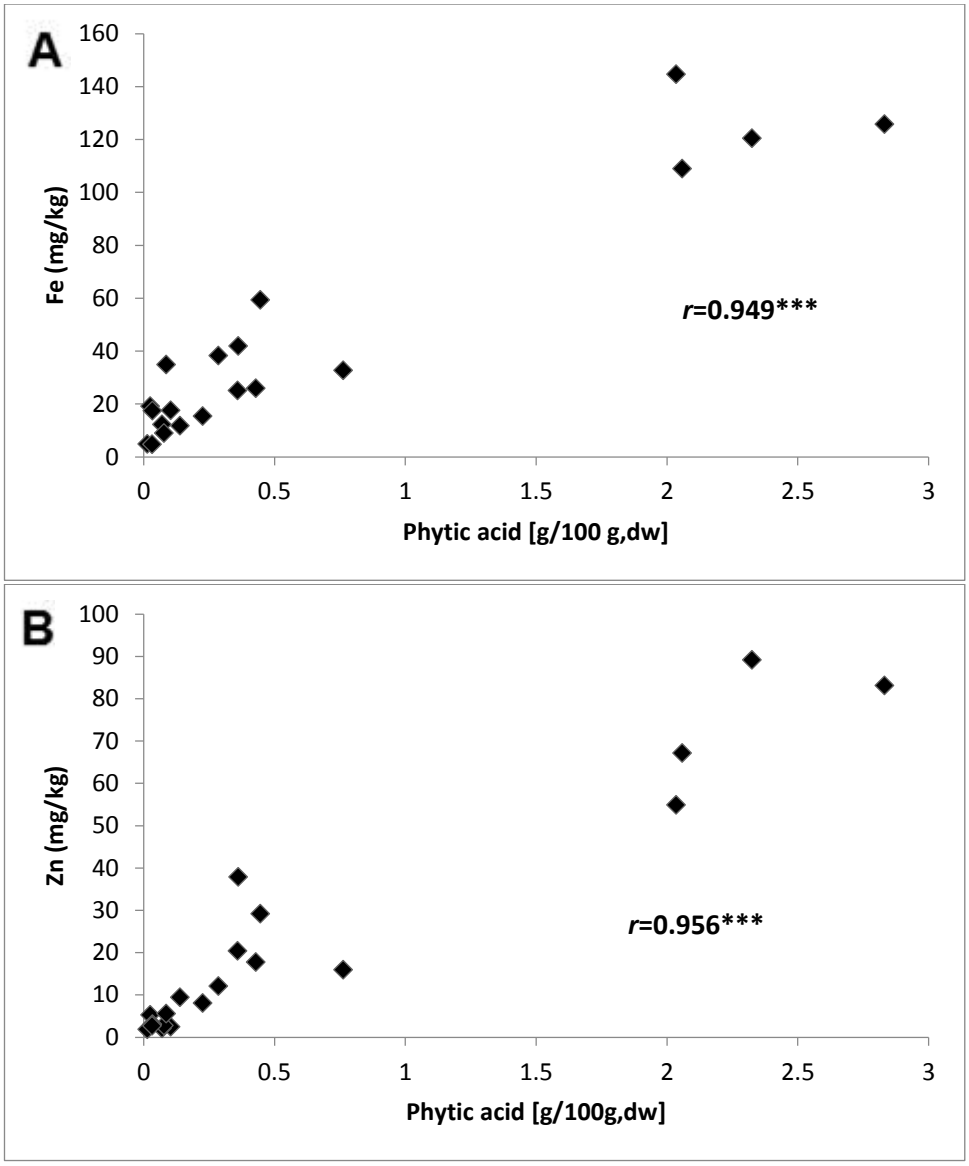


Figure 22. The correlation coefficients (*r*) between Fe and phytic acid (A) and Zn and phytic acid (B) of the 10 milling fractions of Rialto and Riband grown at Rothamsted 2010 (n = 20), Level of significance: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Figure 23 shows the nitrogen content of the 10 milling fractions, which is an indicator of protein content. N content, although higher in bran fractions, did not vary greatly between the bran and flour. Similar to minerals there appears to a radial protein concentration gradient in the flour fractions with B1 and R1 central endosperm fractions having the lowest protein levels. Rialto had higher protein content than Riband in all fractions, except coarse bran. **Figure 24** shows that the concentrations of both Fe and Zn were highly correlated with %N with almost identical *r* values.

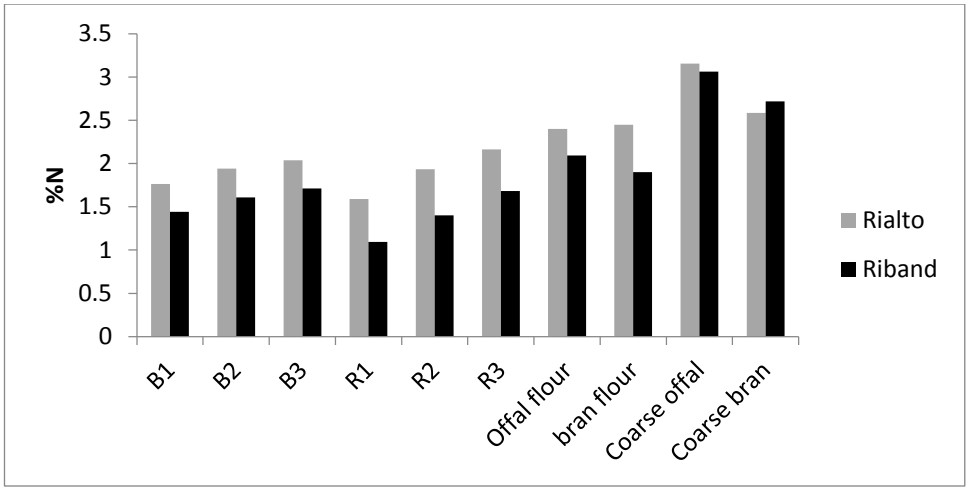


Figure 23. Protein content as %N of 10 fractions obtained by Buhler milling of the two cultivars Rialto and Riband grown at Rothamsted 2010, results are means of two technical replicates.

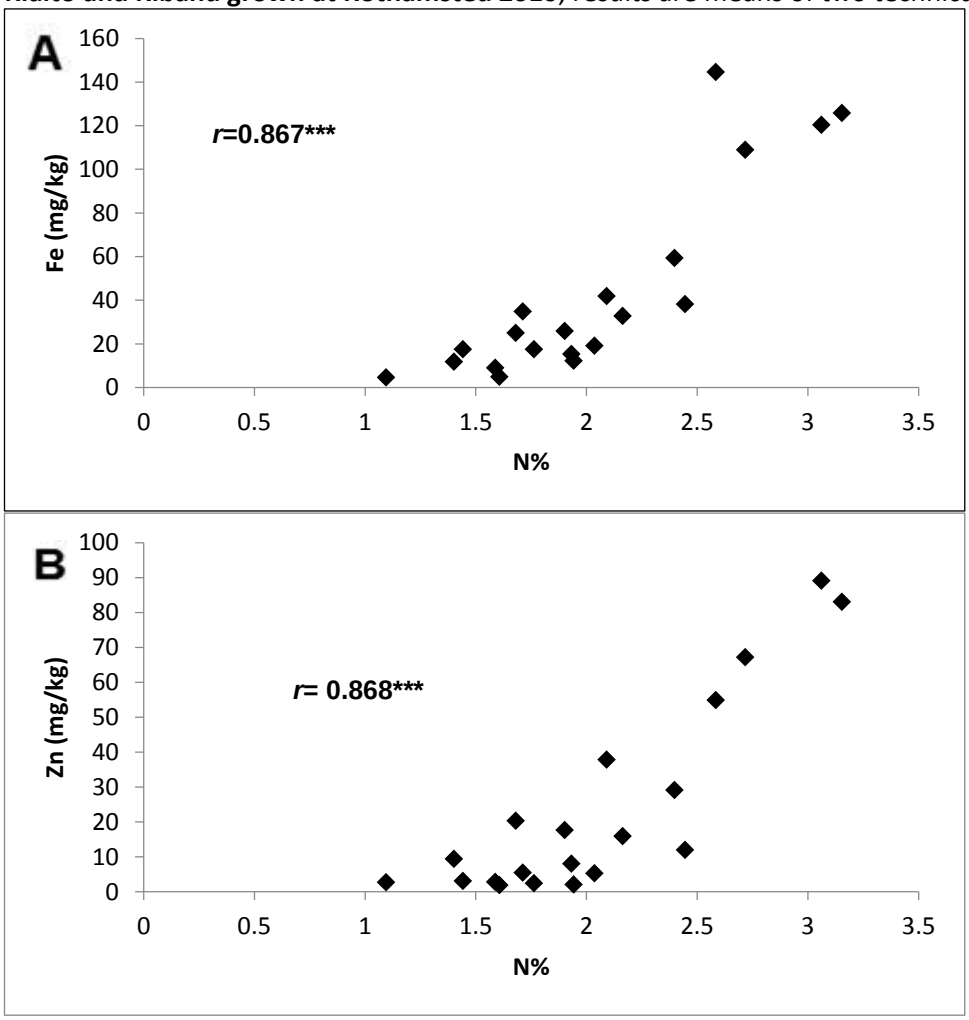


Figure. 24. Correlation coefficients (*r*) between Fe and %N (A) and Zn and %N (B) of the 10 milling fractions of Rialto and Riband grown at Rothamsted in 2010 (n = 20), Level of significance: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

3.4. Discussion

3.4.1 Total Fe and Zn

The initial aim of this study was to determine if there was a relationship between the total concentrations of Fe and Zn in whole grain and in white flour of wheat. There is evidence that the loading of minerals, such as Fe and Zn, into the starchy endosperm is highly regulated and occurs independently of loading into the outer layers that constitute the bran (*46, 68*). Consequently, the concentrations of minerals in the white flour may not reflect those in the whole grain, which is dominated by the minerals stored in the outer layers (especially the aleurone) and the embryo. Analyses were therefore initially carried out on white flour fractions obtained with a Brabender Quadrumat Junior laboratory mill, followed by sieving to reduce contaminating bran, with a more detailed study of six flour and four bran/offal fractions obtained using a Bühler mill, which are more similar to fractions from commercial milling.

The results for total Fe and Zn were largely in agreement with previous studies of wholegrain and various fractions (*5, 103, 196*). In the case of wholegrain, it has been argued that any breeding strategy for Fe and Zn should be targeted to a specific environment due to the high level of genotype x environment interaction (*5, 196*). Our results show that this is also the case for white flour, which showed variation of >100% between experimental sites.

All three cultivars identified from the Healthgrain project which were high in Fe and Zn in wholegrain were also high in the white flour. This was demonstrated by a positive correlation between whole grain Fe and Zn and white flour Fe and Zn (r=0.589 $p<0.01$) and (r=0.485 $p<0.05$) respectively.

The results of the field trial with Rialto and Riband at Rothamsted were in agreement with the multi site analysis with white flour of Rialto being significantly higher in Fe and Zn than that of Riband in both years. Analysis of the 10 milling fractions also showed that Rialto had higher concentrations of both Fe and Zn than Riband in most fractions. The increases in total Zn in the flour fractions from R1 (central endosperm) to R2, R3 derived from the outer endosperm (**Fig. 18b)** are consistent with the localization of Zn in wheat grain determined using laser ablation-ICP-MS (*67*), which showed that the concentration of Zn decreased with increasing distance from the aleurone layer. The comparison of white flour fractions suggests a similar pattern for the spatial distribution of Fe (**Fig. 18a**). The distributions patterns observed for Fe and Zn in milling fractions observed are in agreement with previous results on Chinese wheats (*103*).

3.4.2 Milling

One factor which needs to be considered when comparing milling fractions is how each cultivar responds to shear forces during the milling process, as milling of hard (*e.g.* Rialto) and soft (*e.g*. Riband) wheats may result in different amounts of aleurone tissue being present in the white flour fractions, especially the late break and reduction fractions (i.e. B3, R3). In particular, white flour from hard wheat cultivars contains more aleurone material, especially in the break fractions (B1-3)(*194*). This is because the aleurone cells are more damaged in hard wheats and their contents contaiminate into the flour fractions (*194*).

This is probably why considerably higher levels of phytic acid are present in the reduction and break fractions of Rialto (hard wheat,**Fig. 21**), which were >2 fold higher than in Riband in all white flour fractions except for B3 (which only accounts for 1% of total grain), and also for the higher concentrations of other minerals as well (**Fig 18,19**).

3.4.3 Correlation between Zn and Fe

Correlations between Fe and Zn in wheat have been widely reported (*8, 193, 197, 198*) with significant ($p<0.05$) correlation coefficients (*r*) of 0.659 (*199*) and 0.711 (*198*) in modern wheat. An extensive study of 42 winter wheat varieties grown in central Asia also showed a positive correlation with an r^2 value of 0.63 ($P<0.001$)(*193*). A similar correlation has also been reported in wild emmer wheat with an a r^2 value of 0.214 for 518 ascensions ($P<0.01$)(*200*) .

Our data also showed a positive correlation between Fe and Zn in wholegrain. Interestingly this relationship was more pronounced in the bran fraction than the flour fraction (**Fig. 10a).** This is possibly due to differential loading of the endosperm with Zn and Fe which has been reported in wheat grain (*63, 68*) .

3.4.4 Correlations between Zn and S

Increasing S supply has been shown to have a positive effect on grain Zn (*201*). It has previously been argued that breeding for increased Zn can be achieved by increasing the concentration of S containing amino acids methionine which may act as a sink within the grain (*193*).

Analysis of the 6 Heathgrain project cultivars showed a strong correlation between S and Zn in the white flour**,** but this correlation was not evident in the bran **(Fig. 11)**. The correlation we observed

in flour was stronger than the previously observed correlation in whole grain (*r*=0.53) (*202*). It has been reported that Zn entering the endosperm is under tight control by the crease (*63, 68*). It is possible therefore that this influx of Zn into the endosperm may be influenced by S sink caused by proteins containing thiol groups. Research on barley has found that Zn appeared to be speciated with sulphur in the embryo and therefore is probably bound to thiol containing proteins (*23*).

In grain milling fractions S and Zn are again highly correlated, and Rialto, known to be a high Zn line, has more Zn and more S in all milling fractions than Riband, indicating that this relationship is likely to occur in all tissues.

3.4.5 Protein

Differences in protein levels (determined by %N) between milling factions showed a similar trend to that in minerals and phytic acid but the differences between the flour and bran fractions were much less pronounced and were more in line with differences observed in S content. This is to be expected as proteins are the major form of S in plant cells and S can be used as a crude estimate of protein content.

Correlations between protein and minerals including Fe and Zn have been widely reported (*199, 203*) and shown in both endosperm and bran (*67, 204, 205*). One possible reason for this association is the *Gpc-B1* allele, a quantitative trait locus associated with increased grain Fe/Zn and protein content in wild wheat cultivars (*154, 206*). This effect has been demonstrated by using grain with recombinant chromosome substitution lines (RSLs) with the *Gpc-B1* allele of *T. dicoccoides,* which showed an increase in Fe, Zn and protein in grain of 18%, 12% and 29% respectively, compared to grain containing the allele from modern wheat (*Triticum durum*)(*206*). Our data shows a correlation between Fe/Zn and protein (**Fig. 17b),** indicating that the association of Fe/Zn and protein exists in all fractions.

3.4.6 Phytic acid

Phytic acid is a strong anti-nutrient and a crude way therefore to estimate bioavailability of Fe is to measure the ratio of Fe and P (*207*) as phytic acid makes up over 80% of the total P in cereals (*158*). Our results are encouraging as the grains high in Fe/ZN had a lower P:Fe ratio (**Fig. 11)** than those low in Fe/Zn despite the high Fe/Zn cultivars containing more total P (**Table. 2**). More detailed analysis of both P and Fe in Rialto and Riband shows the two to be highly correlated (**Figs.**

12c, 20a). However it is not advisable to determine Fe:P ratio as an accurate indicator of Fe:phytic acid ratio, especially when examining white flour. In the samples Fe:P ratios in white flour were only slightly higher in Rialto, however in the Buhler milling fractions (R1, R2, R3, B1, B2, B3) it is clear that Fe:phytic acid ratios are considerably higher. The phytic acid to Fe ratios of these two cultivars and the effect of this on bio-availability of Fe are discussed in more detail in **Chapter 5**.

Phytic acid is thought to be highly localised in the aleurone layer and embryo as phytate granules in wheat (*208*), with no strong evidence for phytate granules in the endosperm (*69*). Our data are in agreement with previous studies which show higher concentrations in the bran milling fractions and lower concentrations in the flour fractions (*72, 103*). It is possible that all of the phytate found in our white flour milling fractions may be from contamination from the germ and aleurone which would explain the lower concentration in fractions derived from the centre of grain and would account for the previously discussed the large cultivar differences between the hard and soft wheat. The strong correlations between phytic acid and Fe and Zn may also be indicative of contamination of flour fractions with aleurone layer and germ being responsible for a large portion of the minerals present in the white flour fractions, as previously discussed.

It should be noted that the strong correlation between phytic acid and Zn does not necessarily indicate *in planta* speciation as phytate granules are present in protein storage vacuoles (PSV) (*69, 208*) and therefore any part of the grain containing traces of phytate granules will also contain some protein, another likely chelator of Zn. Interestingly the only flour fraction (B3) which was higher in phytic acid in Riband compared to Rialto was also higher in Fe but not in Zn (**Figs. 14,21**).

3.4.7 Foliar treatment

Our foliar combined treatment was shown to have a minor significant effect on Zn concentration. Foliar treatment of Zn as ($ZnSO_4$) has been previously shown to increase grain Zn concentration by up to 3 fold (*209-212*) and also increases Fe concentration in the grain (*212, 213*). One reason for this could be the timing of our application. It has previously been argued that the application should be in the later stages of wheat development (*214*). However, it has also been suggested that treatment at the booting stage will be most effective (*210*). Our application was during the flowering stage which may have been too late to have an adequate affect. The effect of our Zn treatment was also lesser than has previously been reported (*209-212*).

Our combined application of Fe-EDTA and Zn-EDTA had no effect on Fe concentration in grain whereas foliar application of Fe has been shown to be effective in previous studies (*212*), although not as effective as Zn (*212*). In one field trial, $FeSO_4$ was applied at tilling and heading stages and Fe concentration in grain was increased 21 mg/kg (*212*). However in rice the application of $FeSO_4$ had no effect on Fe in the grain but $ZnSO_4$ increased both Fe and Zn (*215*).

It is possible, therefore, that the sulphur in the previous foliar treatments affected transport to the grain. One possible reason is that sulphur may increase the content of methionine, which in turn may lead to increased production of DMA and NA, which are involved in the uptake and translation of Zn and Fe (*201*).

Taking this into account, it is likely that the reason our foliar treatment was not as effective as previous studies was the chelator used, in this case EDTA. Previous studies in peaches (*Prunus persica (L.) batsch)* have shown EDTA to be an ineffective source of foliar Fe compared to $FeS0_4$ (*216*). One study in wheat further confirms this as it showed that although the Fe-EDTA was taken up by the leaves the plants were able to translocate this Fe to the grain as the plant lacks the relevant transport mechanism (*217*). Our results therefore suggest that EDTA is not an adequate chelating agent for either Fe of Zn.

3.5 Summary

Our data show that breeding of wheat for increased whole grain Fe and Zn is likely to be an effective strategy for improving Fe and Zn concentrations of white flour fractions. However the concentration of P may also be increased in the white flour which may impact on bio-availability. Strong correlations between Fe and Zn indicate that simultaneously targeted breeding for these two traits is likely to be an effective approach.

There was an uneven distribution of minerals in the grain fractions with the bran fractions containing more of all the elements investigated than the white flour, and the milling fractions derived from central endosperm the least. This was least obvious for S and %N, an indicator of protein content, where variation between milling fractions was the lowest.

3.6. Implications of results

The extraction rate is likely to be the most important factor in determining the mineral concentration in the white flour. This is especially relevant when one considers the variation in

extraction rate of wheat products which range from 100% in wholemeal in some rural areas of developing counties to as low as 30% in highly processed products in the west (*218*). The link between phytic acid and Fe and Zn across milling fractions is also likely to affect the bio-availably of these milling fractions and is explored in more detail in chapter 5. The link between Zn and S in the white flour requires further study if the mechanisms of Zn storage in the endosperm are to be understood.

4.1. Introduction

The importance of metal and metalloid (elements which have both properties of metals and non-metals) speciation has become an area of interest for analytical chemists over the last 30 years (*219*). This interest was prompted by the discovery that in both the environment and biological systems metals very rarely exist in a free form and are instead complexed by a plethora of different organic compounds, from organic acids, to large marco molecules, such as the protein ferritin (*219, 220*). This finding promoted research into a new field of speciation. Speciation can be defined as **1**) the distribution of an element between its chemical forms or **2**) the analytical efforts deployed to determine the chemical formulation of an element (*219*).

The importance of speciation studies cannot be understated as the species of a metal has implications not only for its physical state but also for its function, potential toxicity, mobility, and bioavailability within a biological system (*219, 220*). Nevertheless, historically, the analytical chemistry of metals has concentrated on total metals due to the ease of analysis. The analysis of metals tends to revolve around the complete destruction of any ligands prior to analysis (*219*). Current metal speciation studies tend to still rely on using Inductively Coupled Plasma (ICP), either as Inductively Coupled Plasma Mass Spectrometry (ICP-MS) or Inductively Coupled Plasma Atomic Mission Spectroscopy AES-ICP. These methods allow for metal determination, but separation of complexes is first achieved by use of a less destructive system, often involving chromatography (*220, 221*). The first study which used this hybrid system was in 1966 where Kolb et al (1966) coupled a gas chromatograph and a flame atomic absorption spectrometer to determine alkyllead compounds in gasoline (*222*). One advantage of the combination of ICP-MS with chromatography techniques is that it allows the isolation of complexes which contain only the element of interest, in comparison to traditional UV detection methods, used in conjunction with HPLC, which detect all complexes. This is practical and relevant for food matrixes which contain many complexes, of wide molecular sizes (*223*).

The combined use of chromatography and ICP-MS has allowed various species of metals and metalloids to be characterised and quantified in plants, with notable studies investigating selenium (*224, 225*) arsenic (*226*) mercury (*227*) cadmium (*228*) and zinc (*229*).Yet, despite its nutritional importance, Fe has received relatively little attention.

The speciation of both Fe and Zn in wheat grain is important considering the potential bioavailability of these minerals in a widespread food source. However, it is only recently that the forms of these two elements within the grain have begun to be understood. One barrier to this research is that both Fe and Zn in grain are largely insoluble; this is thought to be due largely to the binding of the metals with phytic acid to form insoluble phytate complexes (*134, 230*). One method which has been used to gain insight into the speciation of insoluble metals in grain employs the use of enzymes to liberate insoluble metals from potential chelators, indicating their possible binding. Studies have been performed in barley (*23*) millet (*231*) and rice (*83*) using different combinations of phytase, cellulase, xylanase and protease.

The Identification of soluble Fe and Zn has been achieved by size exclusion chromatography linked to ICP-MS (SEC-ICP-MS) in barley (*23*) and in rice (*21, 22*), with confirmation of speciation also by Electro Spray Ionisation (ESI) (*22*). However, to date virtually nothing is known about Fe or Zn speciation in the wheat endosperm, in part due to their low abundances (*46*).

In this chapter we will use enzymes to determine the forms of insoluble Fe and Zn in different fractions of wheat grain and also further develop the SEC-ICP-MS method to determine Fe and Zn binding in soluble fractions of wheat. We will also assess the effectiveness of the SEC-ICP-MS method by analysing transgenic crops with potentially different Fe and Zn speciation.

Hypothesis. That Fe and Zn speciation differs between milling fractions of wheat grain.

4.2 Methods

4.2.1 Enzyme assay

Enzyme assays were performed using demineralised enzymes as described in chapter 2. 250mg of the milling fractions were extracted in 10 ml of 50 mM Tris-HCl with the addition of dissolved demineralised enzyme were appropriate. Samples were incubated for 18 hrs at 55ºC at pH 5.5, with shaking at 120 rpm. Plastic sterilin vials were used for extraction to minimise any potential metal contamination. The temperature and pH was chosen as they were within the region of activity for all enzymes (protese XIV (pH 5.0-9.0, <80 ºC), wheat phytase (pH 4.0-5.5, 55 ºC), cellulase (pH 5.0-7.0 50-60 ºC), xylanase (pH 4.5-6, 30-60 ºC). By using the same conditions for all enzymes we were also able to discount any effects of temperature or pH on the samples. The milling fractions R1, offal flour and coarse bran (**Chapter 2**) were chosen for investigation as they were representative of different tissue fractions within the grain.

Initially, the changes in speciation of soluble Fe and Zn with enzyme treatment were investigated, however, after demineralisation it was shown that the enzymes had strong chelation capacity to the intrinsic metals in the samples; this effect had also been reported in previous studies (*231*). Therefore it was deemed inappropriate to analyse samples in this way as any complexes, and therefore results, were likely to be heavily influenced by the presence of the enzymes rather than by the action of the enzymes alone.

4.2.1.1 Choice of enzymes

Enzyme type and concentration (**Table 3**) were selected based on previous similar experiments in rice, barley and millet (*23, 83, 231*). The phytase had 0.01-0.04 units of activity /mg, as advised on the packaging, which was lower than the phytase, also from Sigma, which Perrson et al used(*23*) (personal correspondence). To rectify this lower enzyme activity we used more phytase by mass than stated in their paper, however, we could not adjust the units of activity the level in their study without raising the background of Fe to above 10% of the total Fe in some of our samples. The amount of phytase as units of activity per gram of sample in the final experiment equated to ~50% that of Perrson et al (*23*) but 4 times that of Lestienne et al (*231*). Cellulase was added to degrade dietary fibre and weaken its binding capacity to minerals; previously cellulase has been shown to increase soluble Zn and Fe in rice bran (*83*). Xylanse was added to the cellulase as wheat contains large amounts of arabinoxylan, another type of dietary fibre (*231, 232*). In this experiment both cellulase and xylanase were used at a concentration in excess of that used in previous studies (*83, 231*). It has also been reported that protein may form insoluble complexes with Fe and Zn as well, therefore a mix of bacterial proteases (protease XIV) was also used in this experiment, at a concentration in excess of, or approximating, that used in previous studies (*23, 83*). Initially pepsin was tested instead of protease XIV but was shown to have insufficient activity at pH 5.5.

Table 3: Enzymes used in assays and their associated units of activity per gram of sample.

Enzyme	Units of activity per gram of sample
Phytase (from wheat)	5.16-20.64
Protease (protease XIV from *Streptomyces griseus)*	40
Cellulase (*Trichoderma reesei)*	440
Xylanase (*Thermomyces lanuginosus)*	280

4.2.2 Data analysis

All samples were analysed in duplicate which was insufficient to allow statistical analysis of each cultivar using ANOVA. Therefore, all ANOVA results represent pooled data from both cultivars (n=4) and only show the effects of enzyme and not cultivar differences.

4.2.3 Development of extraction method

The primary goal of the speciation analyses was to explore metal speciation in wheat endosperm, however, there is very little soluble Fe or Zn within these fractions, therefore sensitivity and extraction quality had to be maximised if any metal species were to be detected. Therefore, various methods were tested to improve extraction. Sonication is an established method for cell disruption and theoretically could make Fe and Zn more soluble by breaking down cell walls which may prevent their release. Sonication of wholegrain samples was performed for 3 minutes at maximium amplitude (300 ultra soniK, NEY, UK) and total Fe and Fe speciation were compared using ICP-MS and SEC-ICP-MS in 3 samples, however no effects were observed (data not shown).

Sodium dodecyl sulphate (SDS) has previously been used to improve selenium extraction in plants (*233*) therefore, this was tested on wholemeal wheat flour. The extraction of Fe was improved 3 fold however there were unacceptable changes in Fe speciation when analysed by SEC-ICP-MS, indicating the presence of species were unlikely to represent species *in planta* (**Appendix 3**).

Persson *et al* (*23*) used SEC-ICP-MS to analyse barley embryo using quartz sand and a pestle and mortar to homogenise samples and improve extraction. Initially we used the same method, however, when we attempted to measure total soluble Fe in rice endosperm it was found that the soluble Fe was higher than total Fe within the rice endosperm, therefore indicating condemnation during the extraction. One possible cause of this was that metal chelators within

our samples were able to chelate Fe from either the pestle and mortar or quartz sand. To rectify this an agate pestle and mortar was used, as agate contains only trace amounts of Fe compared to porcelain, however there was no reduction in the elevated soluble Fe content. Therefore, it was determined that quartz sand was the likely source of the additional Fe. It has been reported that quartz sand contains Fe as Fe_2O_3 which makes up 0.02% of the total weight (*234*) although this Fe should be insoluble and therefore theoretically should not affect our samples. However, our samples contained chelators such as nicotainamine (NA) which would be able to solubilise this Fe. To test this theory we used a solution of EDTA (EDTA 10mM /50mM Tris HCl pH 7.5) which has a similar chelation capacity to NA, thought to be the strongest metal chelator in cereals (*235*) in place of the sample, to see if any Fe was incorporated into the solution. The solutions were then analysed for total Fe by ICP-MS as described in **Chapter 2**.

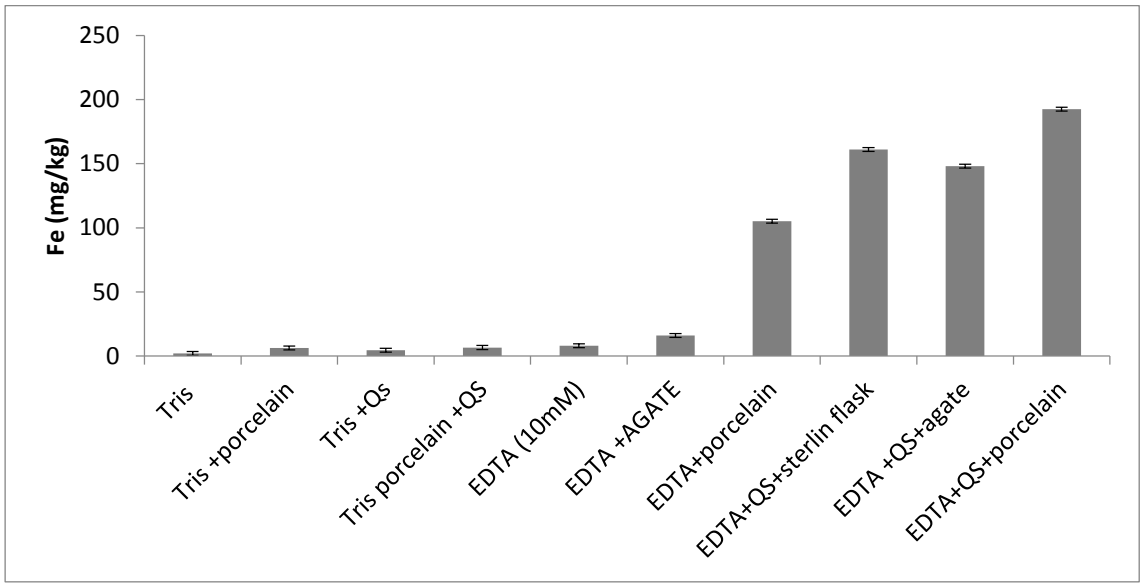


Figure 25. **Effect of sample extraction method** on EDTA 10mM /50mM Tris HCl pH 7.5 (EDTA) and 50mM Tris HCl pH 7.5 (Tris). Porcelain and agate denote porcelain and agate pestle and mortar and QS is quartz sand. SE± represents means of two technical replicates.

The results (**Fig. 25**) showed conclusively that EDTA was able to chelate Fe from agate, porcelain and quartz sand and therefore all of these materials were excluded from further experiments. Attempts were made to acid wash the quartz sand but the amount of EDTA-extractable Fe was only reduced by ~60% which was still insufficient.

4.2.4 Extraction method

Due to the problems with the extraction methods described above we decided to place the samples in plastic sterile vials and incubate at 36º for 18 hours with shaking at 120 rpm which was similar to the conditions used in the control samples for enzyme analysis, This increased incubation time and temperature increased the amount of extractable Fe and Zn without major alterations to speciation. For all white flour fractions (wheat white flour, rice white flour, R1, R2, R3, B1, B2, B3) 140mg of sample was extracted in 7 ml 50 mM Tris HCl pH 7.5. For whole meal and bran fractions (rice bran, offal flour, bran flour, coarse bran, coarse offal), 70mg of sample was extracted in 7 ml 50 mM Tris HCl pH 7.5.

Analysis of low phytic acid lines was only performed on whole grain, which is higher in Fe, therefore it was decided to reduce the extraction time to 1 hr and to reduce the temperature to room temperature. These changes were to try to reduce any potential phytase activity which may have occurred, to compensate for this, increased amounts of sample (500 mg) were extracted in 10 ml Tris HCl pH 7.5.

4.2.5 Size exclusion

All analyses were performed on an HPLC (Agilent 1100 Series, Agilent Technologies, Waltham, USA) coupled to ICP-MS (Agilent 7500ce, Agilent Technologies, Waltham, USA) using the conditions in **Chapter 2**. A Superdex 75 10/300 GL size exclusion column (Glass, 10 × 300 mm, 13 µm cross-linked agarose/dextran, Amersham Biosciences, USA) was used, with an optimum separation range between 0.7 and 70 kDa. The mobile phase was Tris-HCl buffer (50 mM, pH 7.5), pumped through the column isocratically at 0.47 ml/min, at a controlled temperature of 18 ºC. The sample injection volume was 100 µl. Initially a pump speed of 1.0 ml/min was used but it was decided to decrease this to improve separation, however, some initial analyses were performed under these conditions and where these results are presented this is indicated in the figure legend. Molecular size calibration was achieved for both pump speeds using a UV detector (Agilent Technologies, Palo Alto, CA, USA) at 214 nm, using the following standards: apoprotein (6.6 kDa), cytochrome C (12.4 kDa), vitamin B12 (1.35 kDa), Tri-glycine (0.19 kDa) and glycine (0.075 kDa) (all from Sigma-Aldrich). A log-linear regression curve was derived from the plot of molecular size versus retention time. 100 µl 20 mM EDTA/50 mM Tris-HCl buffer (pH 7.5) was injected between flour samples to remove residual metals adsorbed to the column. This wash was

repeated twice after bran fractions due to the higher levels of Fe and Zn in the samples. A more thorough wash with 100 µl of pepsin (1 mg/ml), phytase (2 mg/ml), NaCl (0.5 M) and acetic acid (10%) (all from Sigma-Aldrich), at a flow rate of 0.2 ml/min at 36 ºC, was performed between batches of 30 samples. Finally, 10 mM EDTA/50 mM Tris-HCl buffer (pH 7.5) was run through the column for 40 min each day to remove any residual metals retained by the column, followed by 2 hrs of 50 mM Tris-HCl buffer (pH 7.5) to recalibrate the column.

4.2.6 Ion-pair chromatography.

Ion-pair chromatography had been suggested as a method to confirm speciation results of SEC-ICP-MS. We attempted to combine ion-pair chromatography with ICP-MS to determine at Fe and Zn separation in peaks isolated by SEC-ICP-MS. However we were unable to isolate peaks with adequate concentrations of Fe or Zn for analysis. Analysis of samples also showed unsatisfactory separation for Fe species in all the buffers tested.

4.2.7 Elemental analysis

Extracts separated by SEC were then analysed by ICP-MS. Initially, the conditions for the ICP-MS were similar to those used by Persson et al (*23*) who incorporated O_2 into the reaction gas to allow for the measurement of $^{47}PO+$ and $^{48}SO+$ ^{72}FeO. Therefore, some of the results in this chapter are reported in this mode, where it was deemed appropriate to look at $^{47}PO+$. However we found that the sensitivity for ^{72}FeO and ^{57}Fe, the only two forms of Fe analysable in this gas mode, was insufficient for endosperm fractions. Therefore, most of analyses in this chapter and the next were performed in H_2/He mode with the following conditions: RF power of 1.5 kW, carrier gas supplied at 0.85 L/min, nebulizer pump speed of 0.2 l/min, collision/reaction gas used was H_2 at 3 ml/min and He at 2 ml/min. One advantage of this method is that it allows for the analysis of ^{56}Fe which is the most abundant isotope (91.8 %). H_2 and He were used as the collision/reaction gases as they prevented any polyatomic interference in the determination of this isotope, which is not preventable in He/O2 mode (*23*).

4.2.8 Identification of peaks

SEC-ICP-MS allows for the size of peaks containing elements of interest to be identified. However this is not adequate on its own to confirm identification.

4.2.8.1 High molecular weight peaks (HMW)

Both the rice and wheat samples contained peaks of unknown complexes containing both Fe and Zn, one of them eluted at our void volume (70 kDa+), with another Fe peak which eluted close to the first (~20 kDa) in wheat. The two peaks in wheat appeared to be linked as samples would either contain neither of them or both at similar ratios. No previous information was available on the identification of these peaks. One possibility is that the peaks contained ferritin which theoretically would elute either as a whole molecule (480 kDa) (*236*), which would elute in the void volume, or as subunits (26-28 kDa) (*237*), which are similar in size to the 2nd high molecular weight peak found in wheat.

We were unable to obtain standards of ferritin due to the expense and the difficulty in obtaining ferritin with Fe incorporated in it. It has been reported that legumes contain a much higher percentage of their Fe bound to ferritin (18-42%) (*238*) compared to wheat. Previous SEC-ICP-MS work on soybean found that the majority of the soluble Fe eluted at 70 kDa+ on the same column and accounted for 33% of total Fe (*239*). We therefore analysed a runner bean (*Phaseolus coccineus*) sample extracted under identical conditions to our other samples and found that the majority of soluble Fe eluted at this same HMW form (**Fig. 26**), and that this accounted for 40% of the total Fe. From this it was determined any ferritin in our samples was likely to be present in this peak. However, it is possible that this peak could contain other complexs and is unlikely to be pure ferritin due to the low abundance of Fe-ferritin in wheat (1-2%) (*92, 94*). High molecular peaks were also identified for Zn but we have no indication of possible speciation of these complexes although it is likily they are bound to large proteins due to their size. Therefore these peaks are labeled as unknown HMW peak throughout this study.

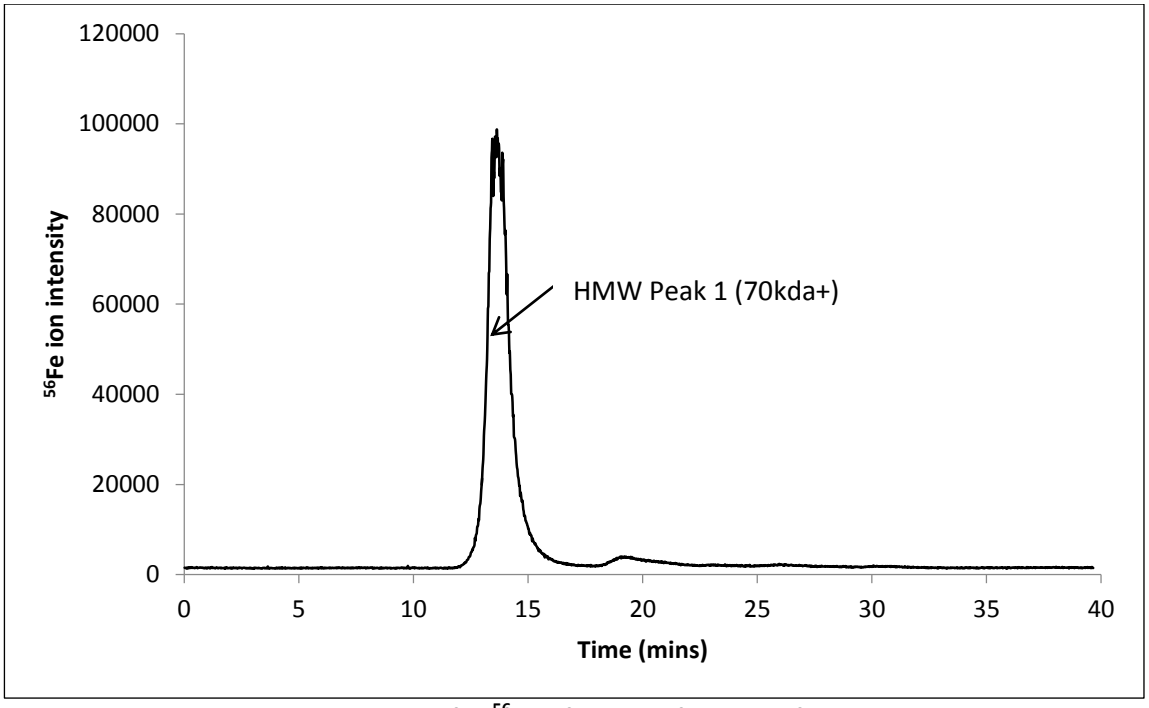


Figure 26. SEC-ICP-MS chromatogram for ^{56}Fe of soluble fraction of *Phaseolus coccineus*. Sample was obtained by extraction for 18 hrs at 36º, in 50mM Tris HCl pH 7.5. The using initial pump speed of 1.0 ml/min total time 40mins.

4.2.8.2 Medium molecular weight peak (MMW)

A MMW peaks were also identified containing Fe. In rice this corresponded to a size of 12.4 kDa and in wheat 5.0 kDa. Based on previous studies and data presented we concluded that both these peaks represented Fe bound as soluble phytate. Persson *et al* (*23*) identified this MMW peak by demonstrating a co-elution of Fe with P in barley embryos. Additional confirmation was achieved by re injecting isolate samples and using ion pairing chromatography linked to He/O_2 mode ICP-MS. They concluded that the peak was an oligomer of Fe bound as different IP's mainly as IP6 with smaller amounts of IP4 and IP5.

In rice a peak was identified corresponding to a complex of 12.4 kDa, which is similar in size to the peak identified by Persson et al (12.5 kDa) (*23*). We also demonstrated using a similar extraction and He/O_2 gas mode ICP-MS that it co-eluted with two P peaks. This indicates that the peak is likely to comprise Fe primarily bound to IP6 and IP5, or to two different oligomers of Fe-IP6. Comparison with mono-ferric phytate a soluble form of Fe phytate prepared as described in **Chapter 2,** also showed almost identical traces for both P and Fe (**Fig. 26**).

In wheat, however, the medium molecular weight Fe peak eluted at a later retention time and corresponded to a molecule of 5 kDa. However, simultaneous analysis of Fe and PO in He/O_2 mode showed that this peak also co-eluted with P. The difference in size is probably due to an effect of the food matrix on the oligomer. Wheat contains different phytases with much greater activity than rice or barely (*240, 241*) and it is likely that during the extraction the ratio of Fe-IP6 and Fe-IP5 was changed by wheat phytase activity to a greater degree in wheat flour than in rice flour or barley embryo. As these peaks represent oligomers rather than the complexes themselves it is likely that even small changes in the ratio of IP5 or lower IPs and IP6 will likely have a large effect on the retention time of said oligomers and account for the changes in size. Interestingly, wheat samples which underwent simulated human digestion conditions had a phytate peak at 12.4 kDa (**Chapter 5**), probably due to deactivation of the phytase enzyme at gastric pH (2.0).

To test this theory, the same synthetic mono-ferric phytate was added to a wholemeal wheat sample to determine if the food matrix changed its retention time. It was found that when extracted in the presence of wheat the majority of the mono ferric phytate eluted as this MMW peak of 5 kDa (**Fig. 27**). Based on this observation and previous evidence of the occurrence of soluble phytate in cereals it was concluded that the MMW peak in wheat was Fe bound as soluble phytate (at an unknown ration IP6 and its lower phosphaltion states). No Zn was identified bound as a MMW peak co-eluting with P.

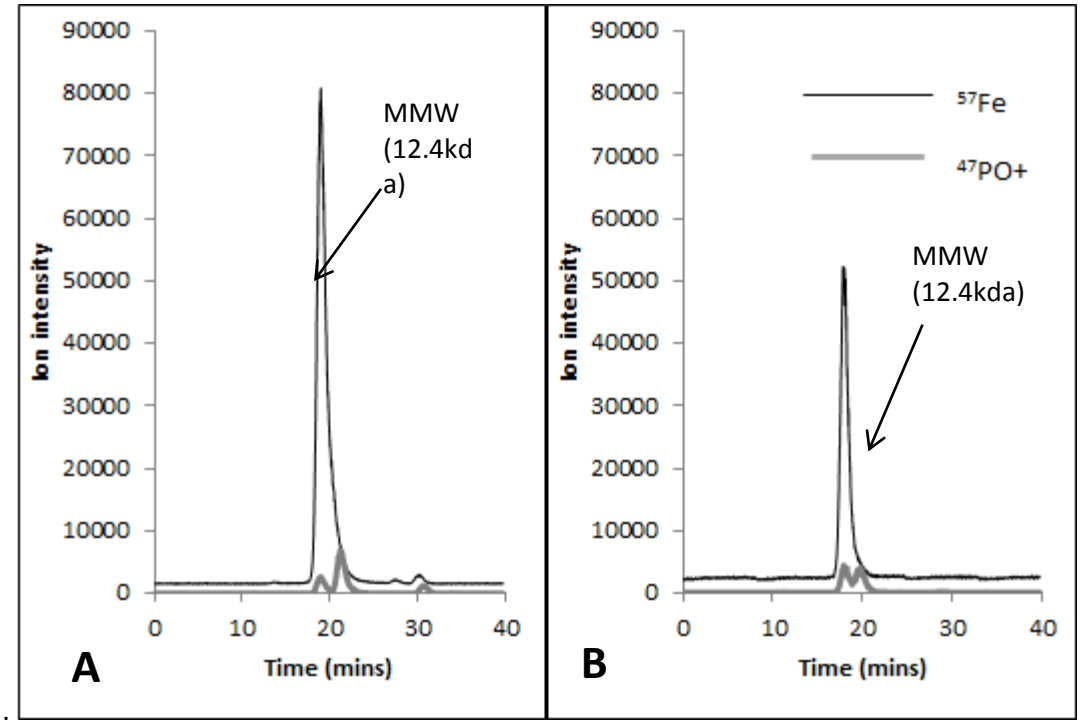


Figure 27. Comparison of rice bran extraction and mono-ferric phytate. SEC-ICP-MS Chromatogram For ^{57}Fe of soluble fraction of **A)** rice bran **B)** mono-ferric phytate. Samples obtained by extraction for 18 hrs at 36ºC in 50mM Tris HCl pH 7.5.Using initial pump speed of 1.0 ml/min for a total time of 40mins.

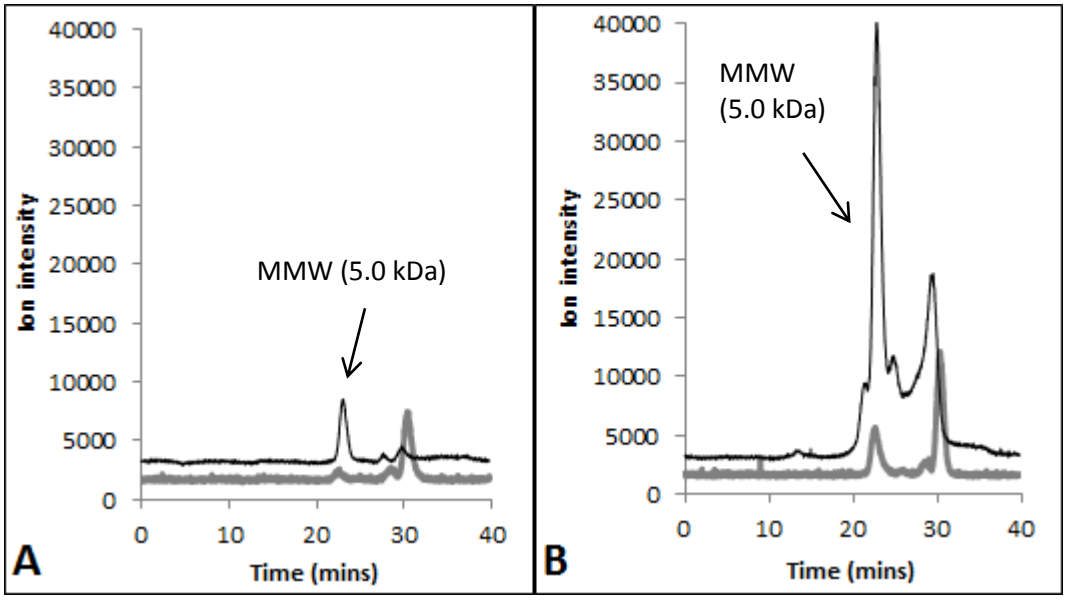


Figure 28. Comparison of wheat wholemeal extraction with and without mono-ferric phyate. SEC-ICP-MS Chromatogram For ^{57}Fe of soluble fraction of **A)** wheat wholemeal flour **B)** wheat wholemeal with mono-ferric phytate (0.1/mg/ml) obtained by extraction for 18 hrs at 36 ºC in 50mM Tris HCl pH7.5. Using initial pump speed of 1.0 ml/min total time 40mins.

4.2.8.3 Low molecular wheat peak (LMW)

Extracts of both rice and wheat contained a LMW peak for both Fe and Zn. In rice these low molecular wheat peaks have been well characterised by both SEC-ICP-MS and ESI-ICP and are thought to be Fe and Zn bound to the nicotainamine (NA) and 2'-deoxymugineic acid (DMA) (*21, 22*).

The LMW peak for Fe and Zn in wheat eluted at an identical time to that present in rice (**Fig. 29, 30**), with peaks eluting at a time corresponding to 1.5 kDa which is similar to that reported by Lee *et al* of 1.3 kDa (*21, 22*) . Confirmation of our LMW peaks was provided by comparisons to standards (prepared as described in **Chapter. 2**) of both Fe and Zn bound to both NA and DMA.

The Fe standards were in the oxidation states they are thought to exist at *in planta* (i.e. Fe(II)-NA and Fe(III)-DMA) (*87*) and the LMW peak was also compared to Fe-citrate, another potential LMW chelator of Fe *in planta*. This LMW peak eluted at the same time as a standard of Fe-DMA; however the retention time was also very similar to that of Fe-NA (**Fig. 29**). Therefore, due to this overlap, it was considered that the LMW peak may contain both Fe-DMA and Fe-NA. Similarly, previous studies (*12, 18*) with polished rice have shown that the LMW peak contains both Fe-DMA and Fe-NA. Based on this evidence we indentified our LMW Fe peak in wheat and rice as an oligmer of Fe-NA/DMA and it is referred to as such in the following text. Fe-citrate eluted at a much later time corresponding to a size of <0.1 kDa, therefore it was assumed that Fe-citrate was not present at suffcient concentration for detection in our samples.

For Zn SEC-ICP-MS also identified a LMW complex of Zn (1.9 ± 0.2 kDa) in the extracts of white flour of both rice and wheat (**Fig. 30**), which was of a similar size to a peak identified in rice using SEC-ICP-MS (*18*). Based on this report and on comparison with Zn-NA and Zn-DMA standards, which eluted at 1.9 kDa and 2.5 kDa respectively (**Fig. 30**), we tentatively identified the LMW peak as Zn-NA, and it is referred to as such in the following text.

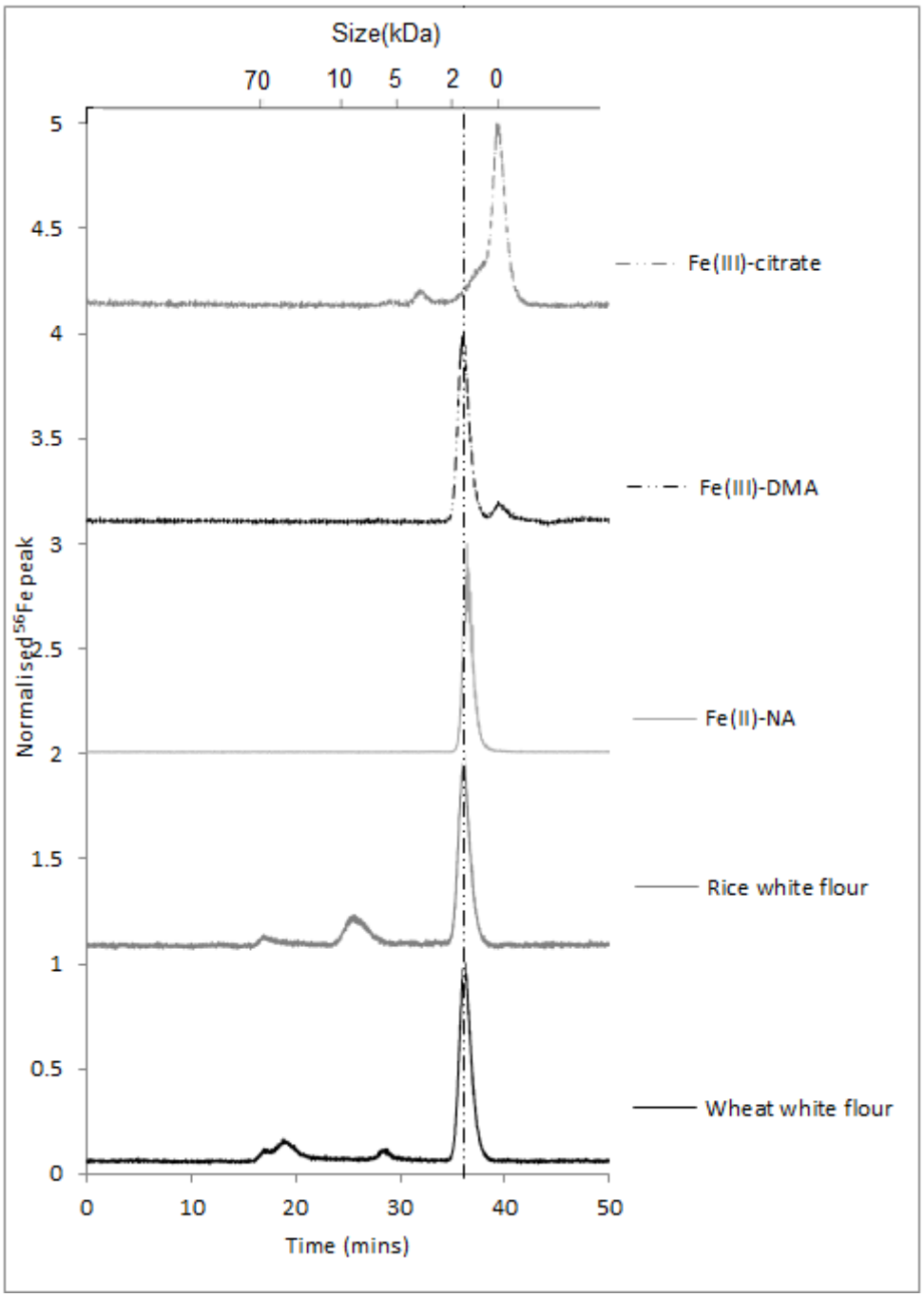
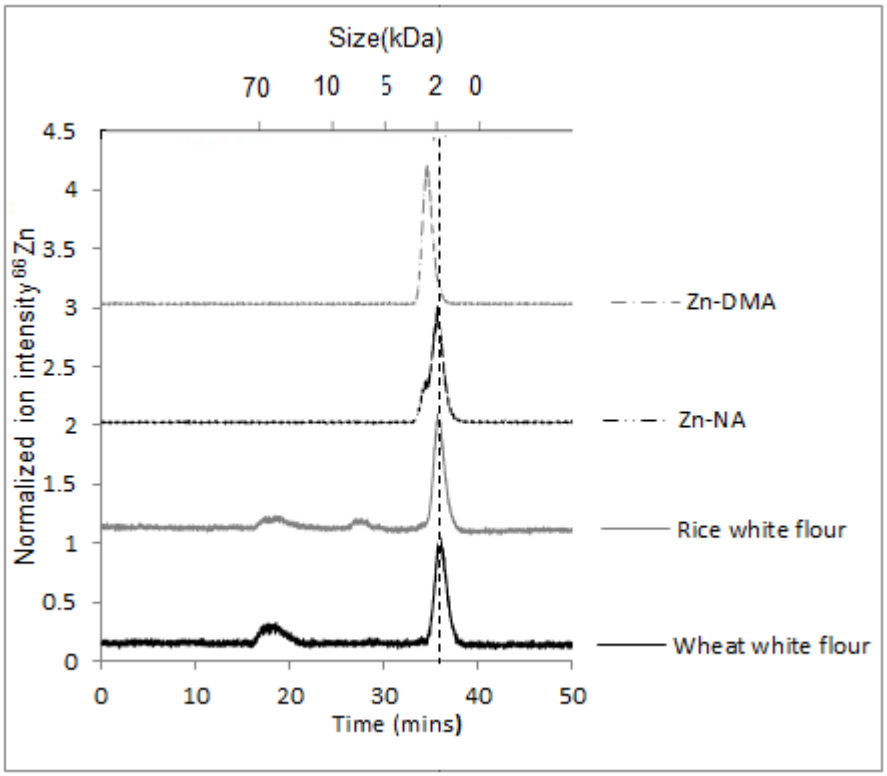


Figure 29. Comparisons of LMW Fe peaks by SEC-ICP-MS. All samples were extracted in 50 mM Tris HCl at pH 7.5 for 1 hr. Rice flour was polished rice and wheat white flour was the R1 milling fraction of Rialto.



.

Figure 30. Comparisons of LMW Zn peaks by SEC-ICP-MS. All samples were extracted in 50 mM Tris HCl at pH 7.5 for 1 hr. Rice flour was polished rice and wheat white flour was from the R1 milling fraction of Rialto.

4.3 Results

4.3.1 Enzyme assays

.

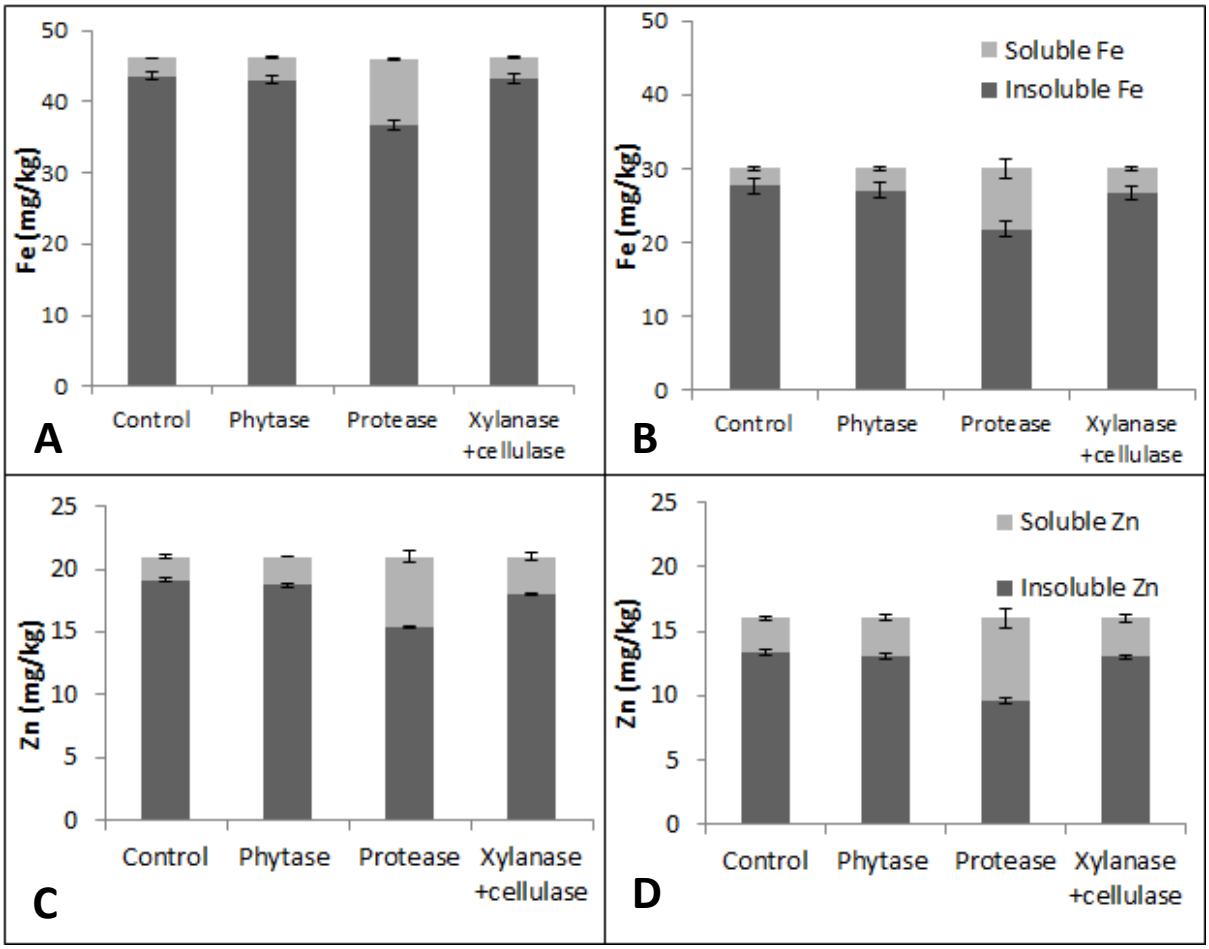


Figure 31. Effect of different enzymes on soluble Fe and Zn in extracts of wholemeal flour, of **Rialto** (**A, C**) **and Riband** (**B, D**). All samples were extracted for 18 hours at 55ºC with shaking (120rpm). Enzyme combinations are as follows: Control, 50mM Tris HCl pH 5.5; Phytase, wheat phytase (5.16 units/g of sample) dissolved in 50mM Tris HCl pH 5.5; Protease, protease XIV from *Streptomyces griseus* (40 units/g of sample) dissolved in 50mMTris HCl pH 5.5; Xylanase+ cellulose, Xylanase from *Thermomyces lanuginosus* (440 units/g of sample) and cellulase from *Trichoderma reesei* (280 units/g of sample) dissolved in 50mM Tris HCl pH 5.5. SE± represents means of two replicates.

Figure 31 shows the effect of enzymes on wholemeal flour of both Rialto and Riband. Very little Fe was soluble under our extraction conditions in either cultivar (5% for Rialto and 7.7% for Riband). Only the protease and cellulase+xylanase (Cl+Xy) treatments had positive effects on soluble Fe (p=0.05) in the order of: protease> Cl+Xy. Higher amounts of Zn were soluble under our extraction conditions (8.3% for Rialto and 16% for Riband).Both protease and Cl+Xy had effects (p=0.05), again in the order of protease> Cl+Xy.

Figure 32 shows the effect of the enzymes on soluble Fe in different milling fractions. In R1 the fraction most represented of endosperm, soluble Fe in our control extraction was 22% and 30% in Rialto and Riband, respectively. Only protease treatment had a significant effect (p= 0.05). In offal flour soluble Fe was 19% and 18% in Rialto and Riband, respectively. Only protease treatment had a positive effect on soluble Fe while, phytase and Cl+Xy had negative effects. In coarse bran soluble Fe in control extractions was 20% in both samples. Both Cl+Xy and protease had positive effects on Fe solubility in order of Cl+Xy>protease. **Figure 33** shows the effects of the enzymes on soluble Zn in different milling fractions. For R1, soluble Zn in the control extraction was 60% and 58%, respectively in Rialto and Riband. Only protease had a positive effect (p=0.05). In Riband all the Zn was made solubilised by treatment with protease. In offal flour, soluble Zn in the control extraction was 9% and 17% in Rialto and Riband, respectively. Only protease had a positive effect (p=0.05). In bran there was no apparent effect on Zn solubility by any enzyme In Rialto. However there was an increase in soluble Zn with treatment with both protease and Cl+Xy in Riband. ANOVA showed no significant effect of any treatment. **Figure 34** Shows the effect of a combination of enzymes. The combination of phytase and cellulase was shown to increase soluble Fe in coarse bran by >50% compared with the control or single enzyme treatments. However there was no observed effect for any treatment on R1.

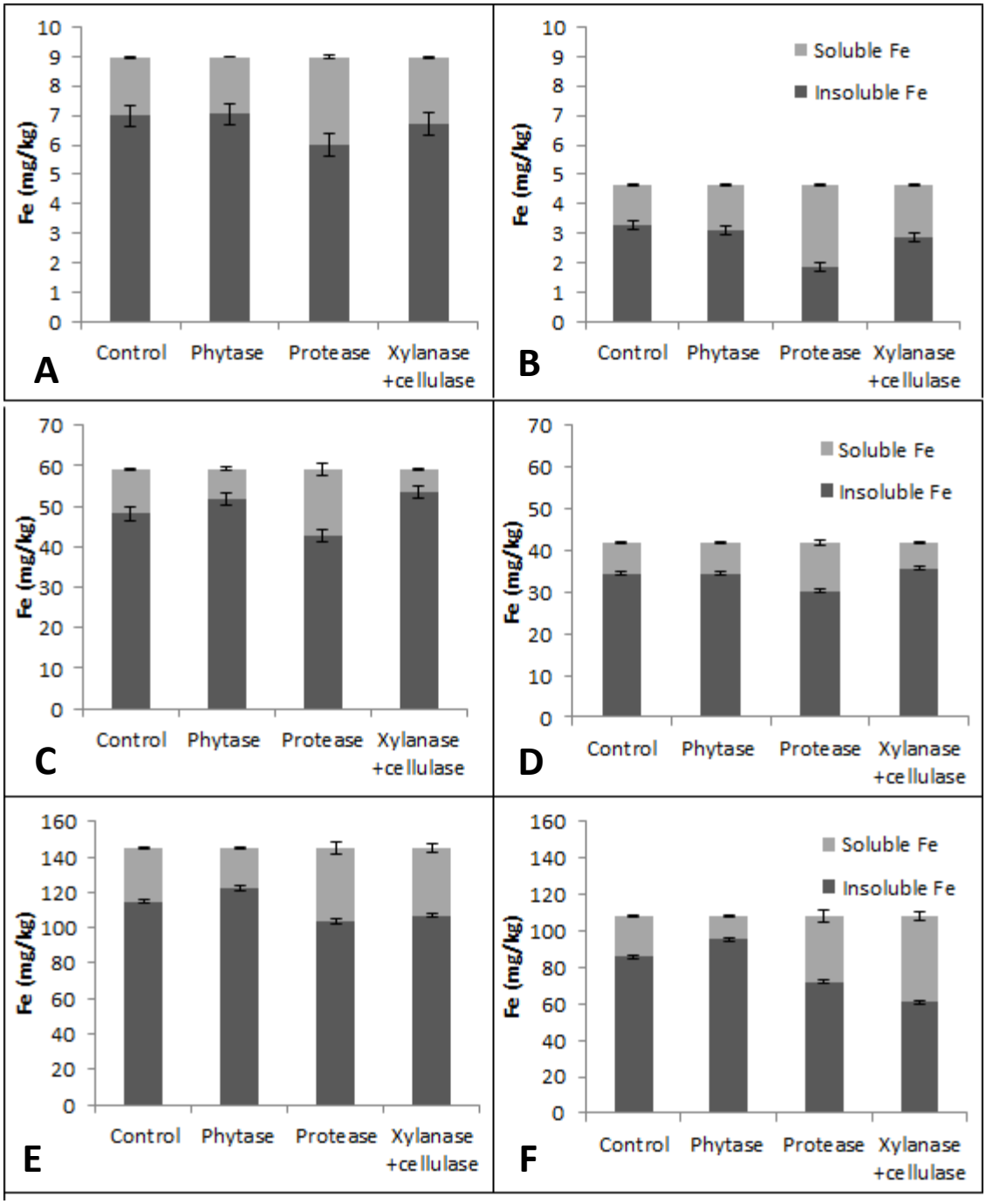


Figure 32. Effect of enzymes on soluble Fe in milling fractions of wheat on the R1 (A,B) , offal flour (C,D) and coarse bran (E,F) of Rialto (A,C,E) and Riband (B,D,F). All samples were extracted for 18 hours at 55 ºC with shaking (120rpm) in Control 50 mM Tris HCl pH 5.5 Phytase: wheat phytase (5.16 units/g of sample) dissolved in 50 mM Tris HCl pH 5.5 Protease: protease XIV from *Streptomyces griseus* (40 units/g of sample) dissolved in 50 mM Trisl HCl pH 5.5 Xylnase+cellulase Xylanase from *Thermomyces lanuginosus* (440 units/g of sample) and cellulase from *Trichoderma reesei* (280 units/g of sample) dissolved in 50mM Tris HCl pH 5.5. SE± represents means of two replicates.

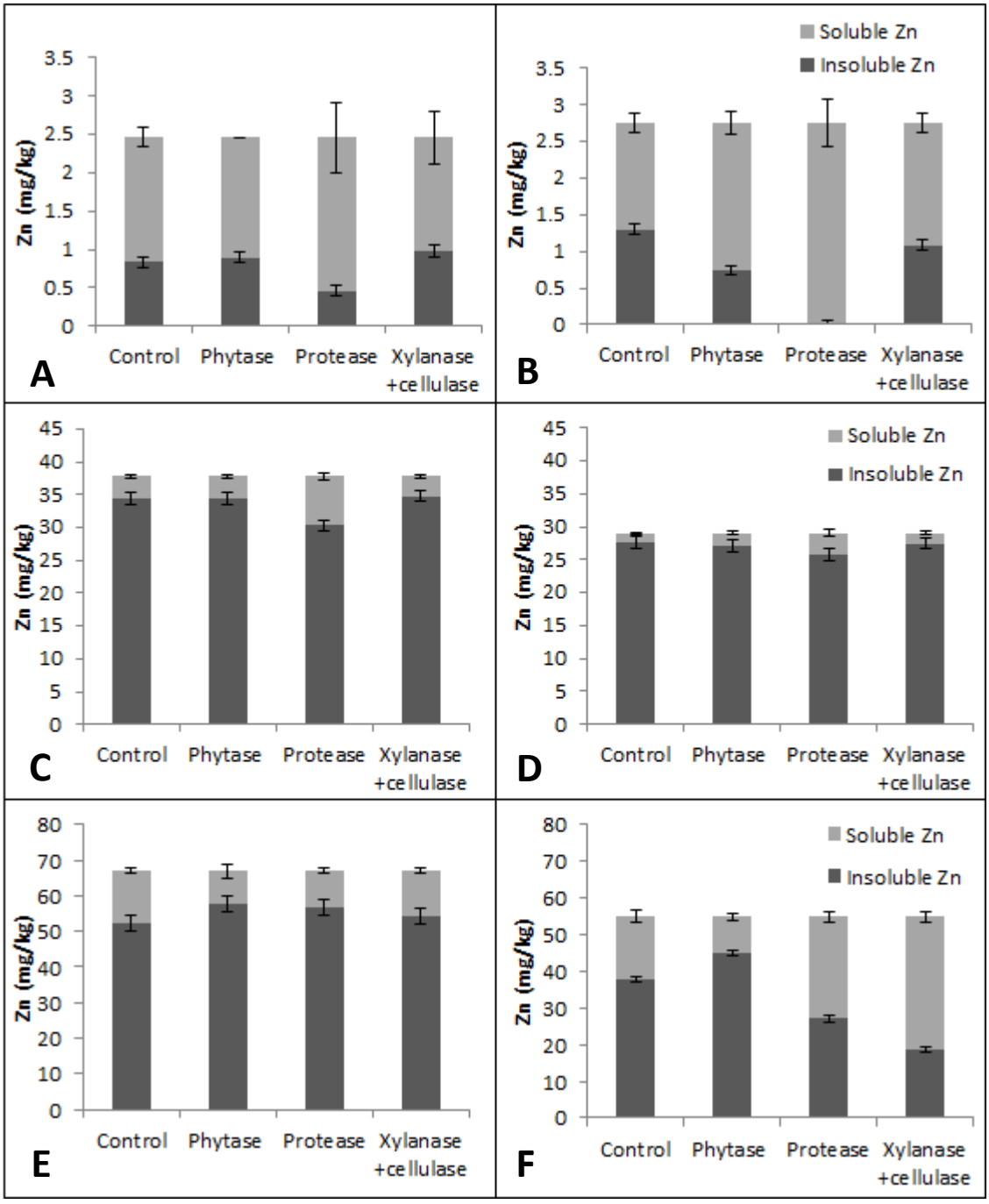


Figure 33. Effect of enzymes on soluble Zn in milling fractions of wheat on the R1 (A, B) , offal flour(C, D) and coarse bran (E, F) of Rialto (A, C, E) and Riband (B, D, F). All samples were extracted for 18 hours at 55 ºC with shaking (120 rpm). Bars represent different enzyme treatments: 50 mM Tris HCl, pH 5.5 with no enzyme addition (Control), Wheat phytase (5.16 units/g of sample) dissolved in 50 mM Tris HCl, pH 5.5 (Phytase), Protease XIV from *Streptomyces griseus* (40 units/g of sample) dissolved in 50 mM Tris HCl pH 5.5 (Phytase), Xylanase from *Thermomyces lanuginosus* (440 units/g of sample) and cellulase from *Trichoderma reesei* (280 units/g of sample) dissolved in 50 mM Tris HCl pH 5.5 (Xylanase+cellulase). SE± represents means of two replicates.

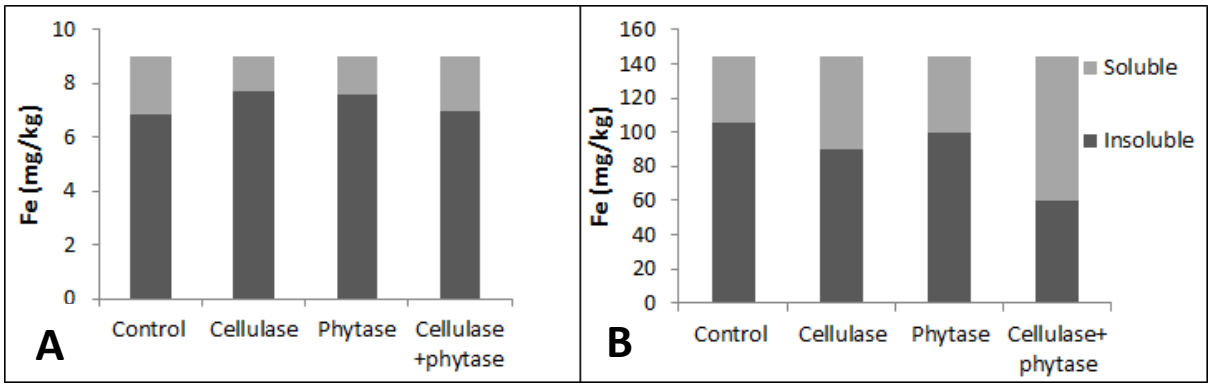


Figure 34. Effect of combined enzymes on soluble Fe on R1 (A) and coarse bran (B), from Rialto. All samples were extracted for 18 hours at 55ºC with shaking (120 rpm).Control, 50 mMTris HCl pH 5.5; Phytase, wheat phytase (5.16 units/g of sample) dissolved in 50 mM Tris HCl pH 5.5; cellulase, cellulase from *Trichoderma reesei* (280 units/g of sample) dissolved in 50 mM Tris HCl pH 5.5; Cellulase +Phytase cellulase from *Trichoderma reesei* (280 units/g of sample) and wheat phytase (5.16 units/g of sample) dissolved in Tris HCl pH 5.5.

4.3.2 Speciation results

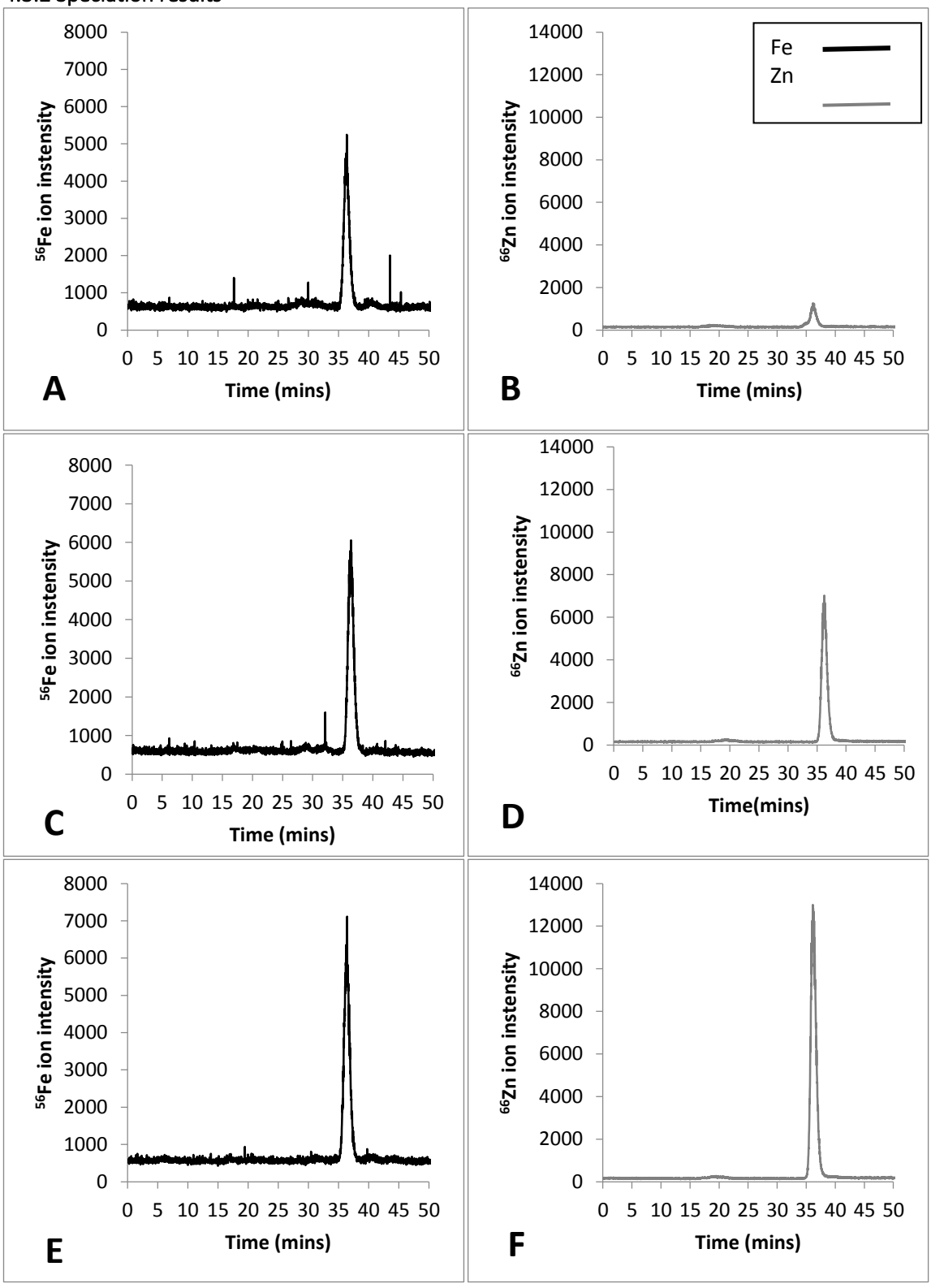


Figure 35. SEC-ICP-MS chromatogram of the soluble extracts of polished rice. ^{56}Fe (left) ^{66}Zn(right) of soluble fraction of polished wild type (Wt) (**A,B**) and two transgenic *OsNAS1* lines EN1, (**C,D**) EN2, (**E,F**) obtained by extraction for 18 hrs at 36ºC in 50mM Tris HCl 7.5.

Figure 35 shows the speciation of Fe and Zn in polished grain of two transgenic NAS overexpressing lines (EN1, EN2) and wild type (Wt). **Figure 36** shows total amounts of soluble

minerals in polished grain of two transgenic NAS overexpressing lines (EN1, EN2) and the wild type (Wt) line. The NAS plants did not have higher concentrations of soluble Fe or Cu but did have more soluble Zn and Mn.

All of the soluble Fe appeared in the LMW form identified as Fe-DMA/NA, with very little difference in Fe intensity indicating no difference in Fe concentration, this was confirmed by ICP-MS (**Fig. 36**). All the soluble Zn was in the LMW form thought to be Zn-NA, and the level of ion intensity was greatly increased in the transgenic lines indicating more Zn was bound in this form. This was confirmed by ICP-MS (**Fig. 36**).

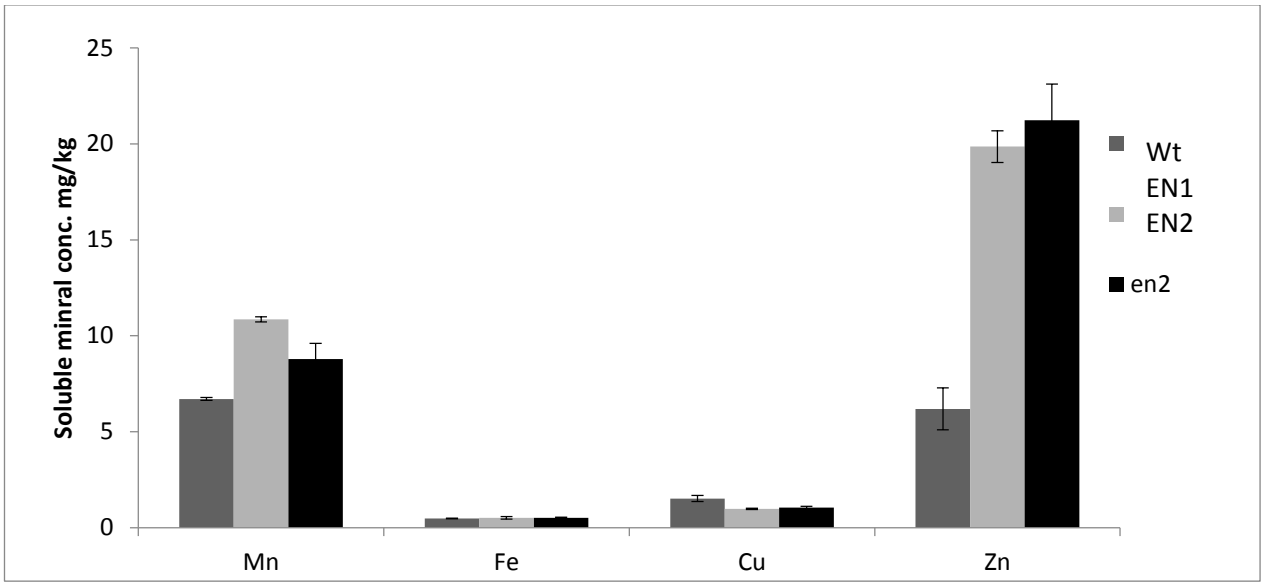


Figure 36. Mineral concentration of polished rice extracts. Determined by soaking at (37º, 18hrs, 120 rpm) in Tris-HCl 50 mM pH 7.5 followed by ICP-MS. Error bars represent SE (n=2).Total Fe and Zn are shown in **2.1.5**

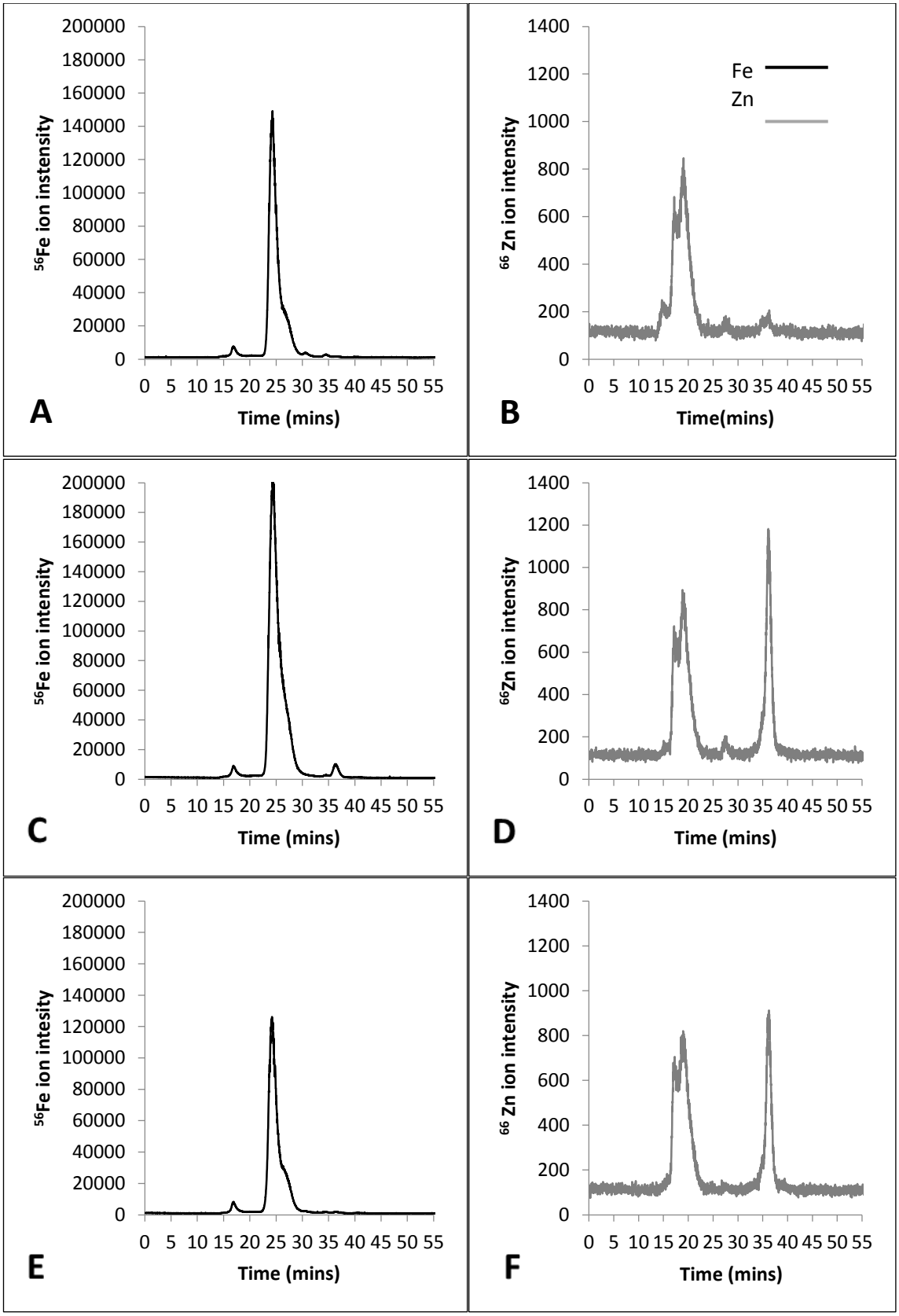


Figure 37. SEC-ICP-MS chromatogram of soluble fractions from rice bran. ^{56}Fe (left) ^{66}Zn(right) of wild type (Wt)(**A,B**) and two transgenic *OsNAS1* lines EN1, (**C,D**) EN2, (**E,F**) obtained by extraction for 18 hrs at 36ºC in 50mM Tris HCl 7.5.

Figure 37 shows Speciation of Fe and Zn in bran of two transgenic NAS overexpressing lines (EN1, EN2) and wild type (Wt) rice. Must of the Fe is in the MMW form identified as Fe-soluble phytate and accounts for over 95% of the soluble Fe (**Fig. 38b**). There does appear to be slightly more Fe bound to Fe-DMA/NA in EN1 but this is not apparent in EN2 (**Fig. 37**). There is also a HMW peak representing ~4% of the total soluble Fe but this did not differ between the genotypes. In rice bran approximately ~33 of the Fe is soluble and this does not vary between genotypes. For Zn there are clear differences between the transgenic and Wt lines, with Zn-NA accounting for 49% and 35% of soluble Zn in EN1 and EN2, respectively, and only 8% in Wt (**Fig. 37, 38**). This also had an effect on increasing soluble and total Zn (**Fig. 38**).

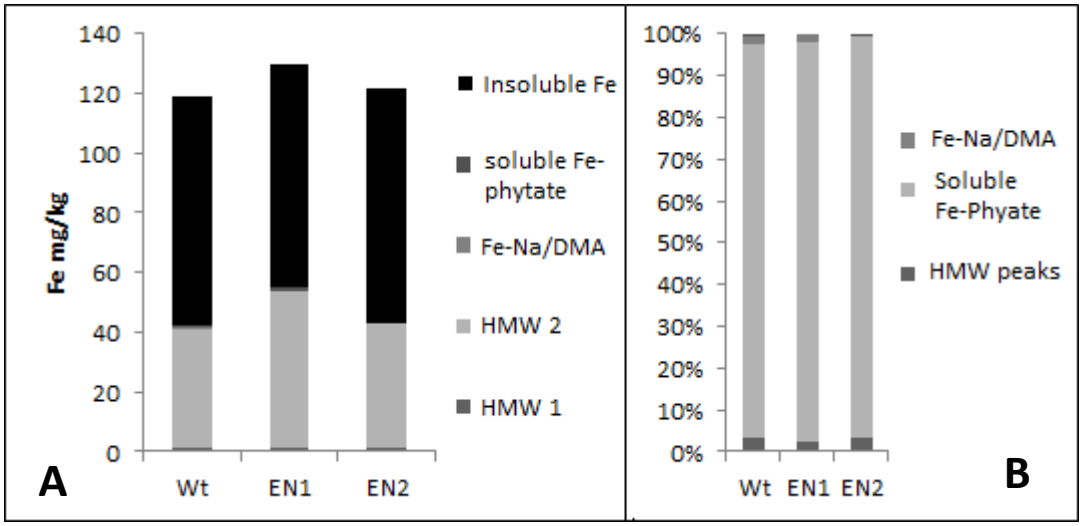


Figure 38. Iron speciation in rice bran samples determined by a combination of SEC-ICP-MS, ICP-MS and ICP-AES. A) Insoluble fraction is calculated by subtracting the soluble fraction determined by ICP-MS from the total dry weight mass determined by ICP-AES. The amounts of each fraction within the soluble fraction are determined by SEC-ICP-MS, by calculating area of each peak. **B)** Shows percentages of the soluble forms of Fe within the bran.

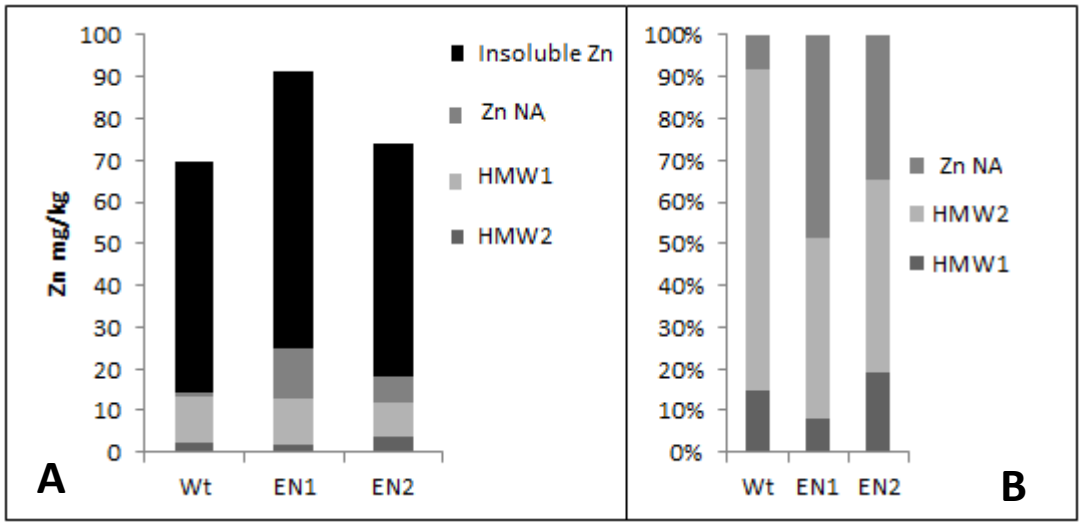


Figure 39. Zinc speciation in rice bran samples determined by a combination of SEC-ICP-MS, ICP-MS and ICP-AES. A) The insoluble fraction is calculated by subtracting soluble fraction demined by ICP-MS from total dry weight mass determined by ICP-AES, amounts of each fraction within

soluble fraction are determined by SEC-ICP-MS, by calculating area of each peak. **B**) Shows percentage of the soluble forms of Zn from within the bran.

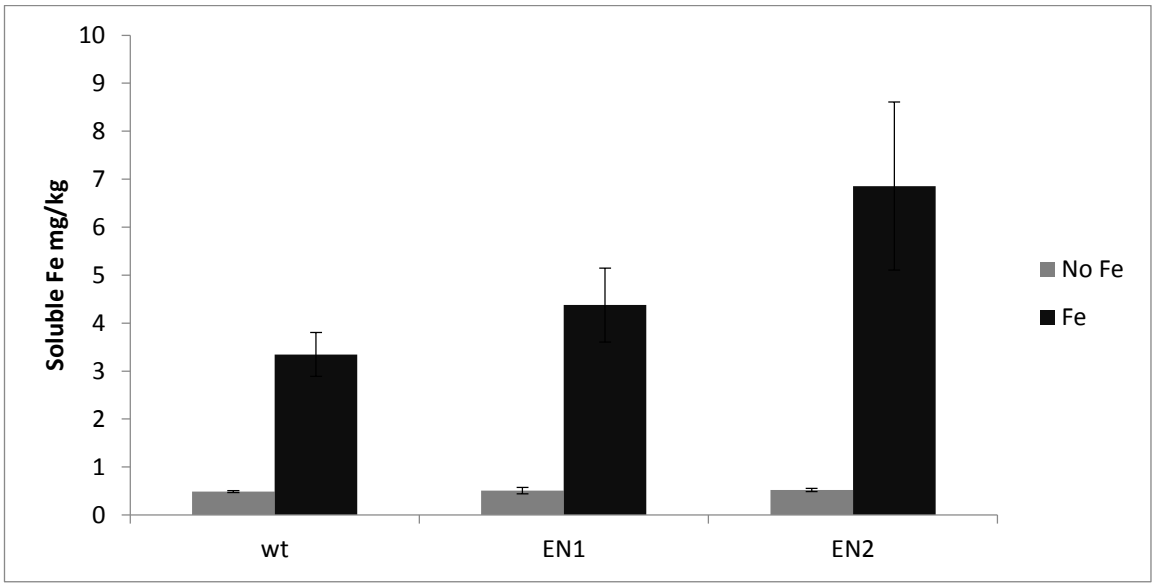


Figure 40. Increase in Fe concentration of polished OsNAS1 over expressing rice when incubated with quartz sand. Fe is determined by ICP-MS, 140 mg of the polished rice samples were either incubated in 7 ml of 50 mM Tris HCl pH 7.5 for 18 hours at 37ºC(No Fe) or under identical conditions with Fe as Fe^2O_3 in 500mg of quartz sand (Fe). Error bars represent SE of two technical replicates.

Figure 40 shows that all lines had significantly more Fe when incubated in the presence of Fe. The increase in Fe was more evident in the two transgenic lines which appeared to recruit more Fe than the Wt line with the amount of soluble Fe in EN1 and EN2 increasing ~8 fold and ~13 fold respectively compare to Wt which only had a ~6 fold increase (**Fig.40**). SEC-ICP-MS of the same samples showed that the additional soluble Fe was mainly in the LMW Fe-NA/DMA form in the transgenic lines with the remainder being bound to soluble phytate (12.4 kDa) which was not present in rice extracted in an Fe free environment (**Fig. 36**)

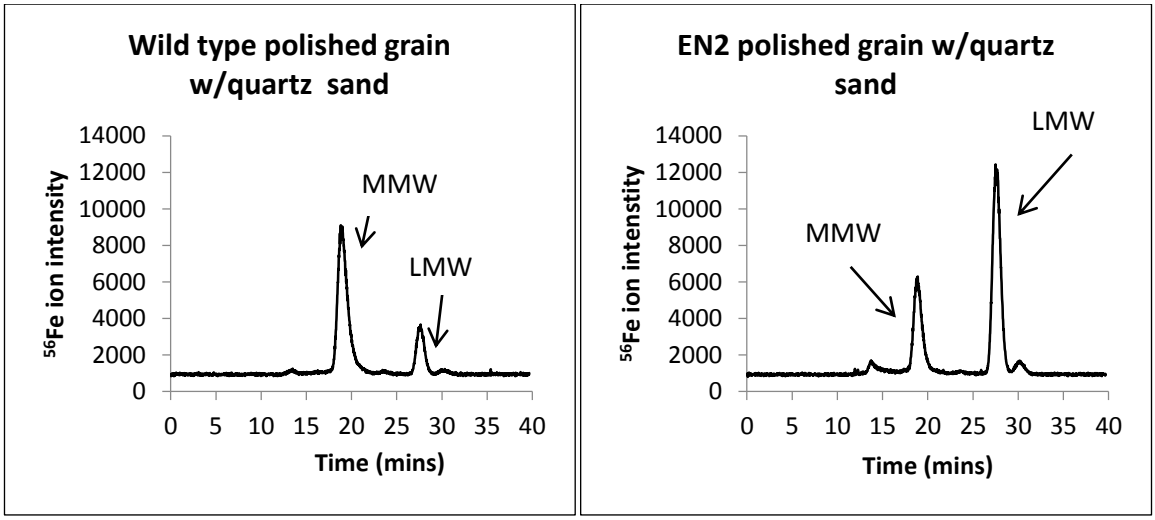


Figure 41. Chromatograms of SEC analyse of ^{56}Fe speciation in soluble fraction of polished rice extracted in the presence of Fe as quartz sand. Pump speed 1.0ml/min, total time 40mins.

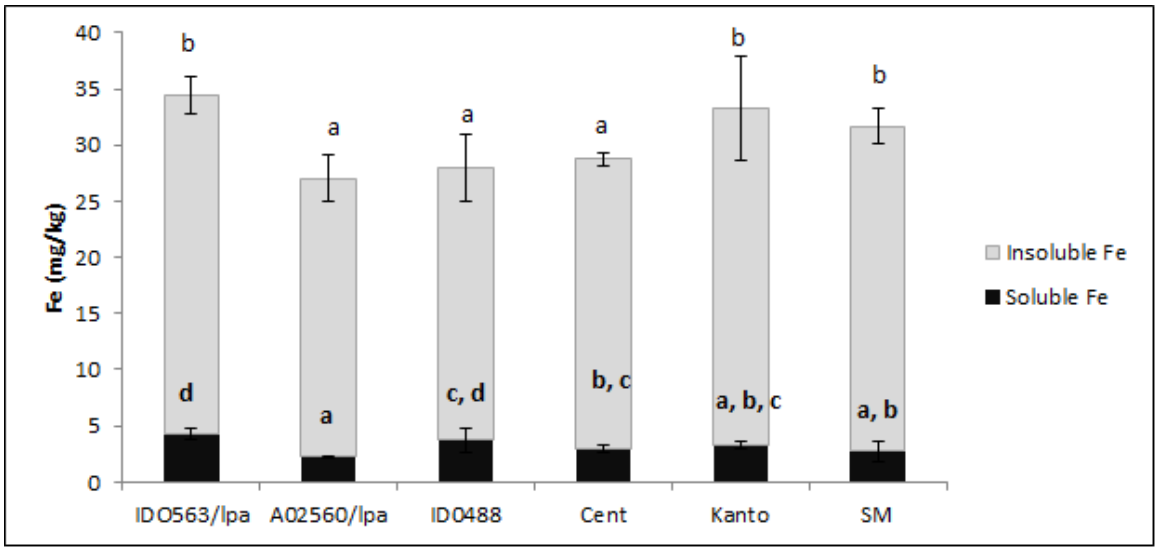


Figure 42. The proportions of soluble and insoluble Fe in wholemeal of low phytic acid lines (*lpa*) and related lines grown in glasshouse. Total Fe is determined by AES-ICP and soluble Fe by ICP-MS after extraction in 50mM Tris-HCl pH 7.5. Bars without a common letter (a−c) are significantly different, p< 0.05. Letters in bold (**a-c**) represent differences in soluble P.

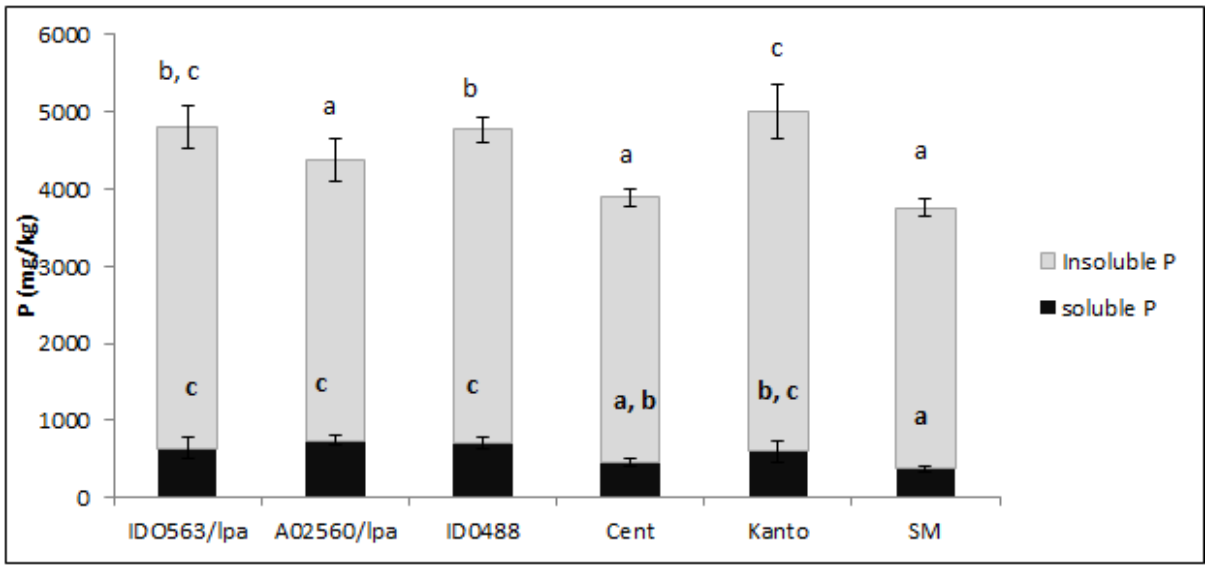


Figure 43: **Shows proportions of soluble and insoluble P in wholemeal of low phytic acid lines (lpa) and related lines grown in glasshouse**. Total Fe is determined by AES-ICP and soluble Fe by AES-ICP after extraction in 50mM Tris-HCl pH 7.5. Bars without a common letter (a−c) are significantly different, p< 0.05. Letters in bold (**a-c**) represent differences in soluble P.

Figures 42 and **43** show total and soluble Fe and P in wholemeal wheat flour of low phytic acid (LPA) lines and potential parents. Total Fe did not appear to be higher in LPA lines than parent lines, soluble Fe was between 10-15% in all lines. Total levels of Fe were not significantly higher than parent lines. Soluble P was higher in both low phytic acid lines compared to related lines except IDO488. Total P was not higher in *lpa* lines compared to related lines. **Figure 40** shows a comparison between speciation of soluble Fe in wholemeal of the TaFER1 overexpressing line Fe0509 and Bobwhite (the control line). There was no apparent difference in any peak between lines.

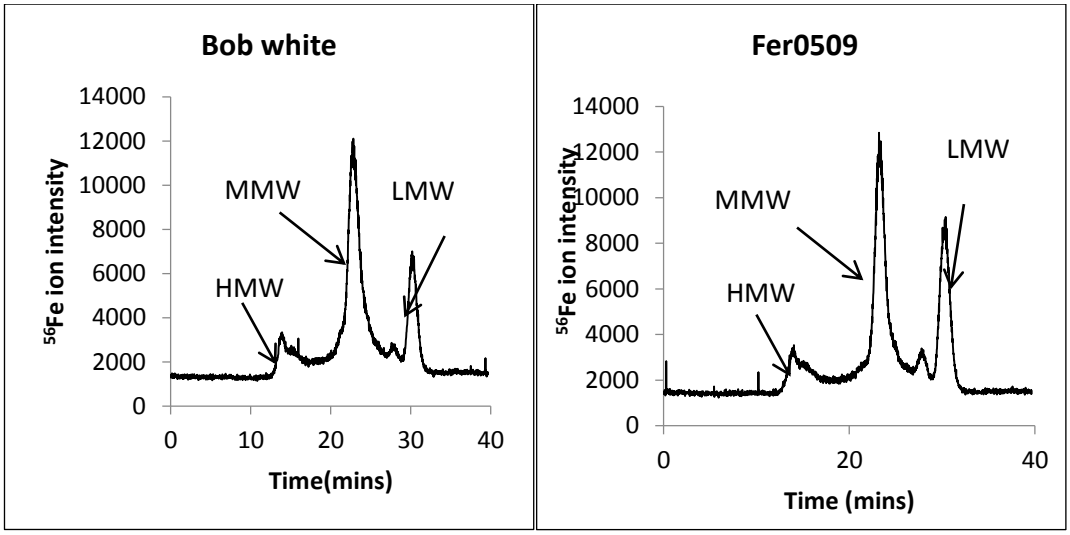


Figure 44. SEC chromatograms showing ^{56}Fe speciation in soluble fractions from wholemeal of the control line (Bobwhite) and *TaFER1* over expressing wheat (fer0509); Pump speed 1.0ml/min total time 40mins.

Figures 45 and **46** show SEC-ICP-MS analyses of the Fe and Zn species in the Tris-HCl extractable fraction of white flour from samples of Riband and Rialto grown at Rothamsted in 2010. The extracts of both cultivars contained two unidentified high molecular weight (HMW) forms of Fe (at 20 kDa ± 0.4 and >70 kDa, respectively). A medium molecular weight (MMW, 5.0 ± 0.1 kDa) peak containing Fe was identified in the extracts of Rialto, but not of Riband. As previously discussed this peak has been identified as soluble Fe-phytate (possibly Fe-IP_6 and/or Fe-IP_5)

The extracts of Riband and Rialto contained similar amounts of a low molecular weight (LMW, 1.5 ± 0.1 kDa) peak of Fe which was identified as Fe-NA/DMA (**Fig. 44**). SEC-ICP-MS also identified a LMW Zn complex (1.9 ± 0.2 kDa) in the extracts of white flour (**Fig. 45**) which had been previously identified as Zn-NA.

The concentrations of soluble Fe differed significantly between the two cultivars (p<0.05), with Rialto having 23% more soluble Fe than Riband, but the total concentrations of soluble Zn (sum of both LMW Zn and Zn associated with HMW compounds) were not significantly different (**Fig. 37.**). Most of the soluble Fe and Zn were bound in LMW complexes and the total amounts of LMW Fe and LMW Zn did not differ significantly between the cultivars (**Fig. 44, 45**).

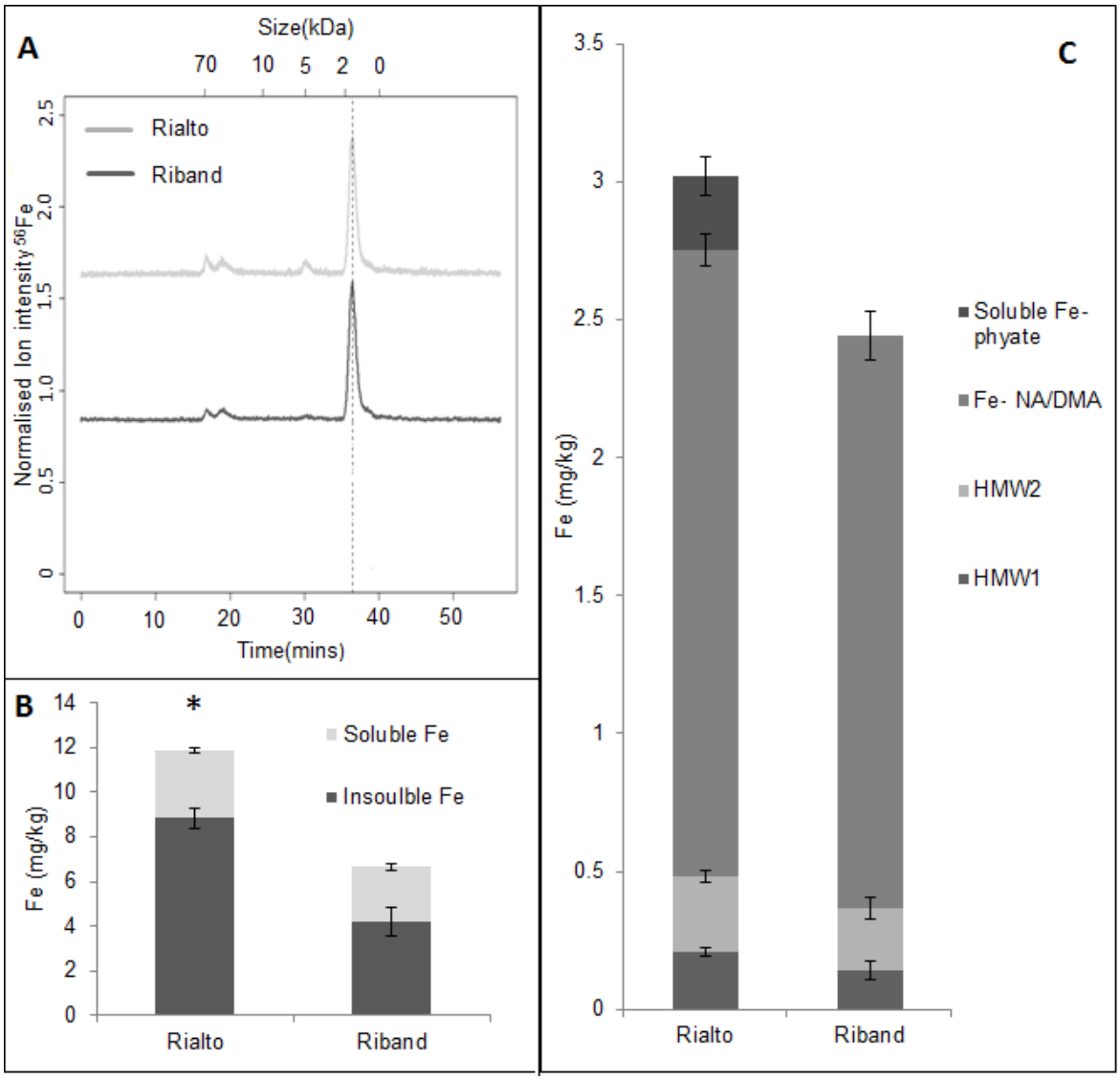


Figure 45. **Determination of Fe speciation in white flour fractions of Rialto and Riband**.
(**A**) SEC-ICP-MS chromatograms (averages of 3 biological replicates). All samples were extracted in 50 mM Tris-HCl pH 7.5. (**B**) Soluble and insoluble Fe determined by a combination of ICP-MS and ICP-AES. (C) Quantification of molecular species of Fe from relative peak areas on SEC-ICP-MS data and ICP-MS of soluble fractions. Data (**B, C**) represent means ± SE of three biological replicates, *represents significant difference (p<0.05).

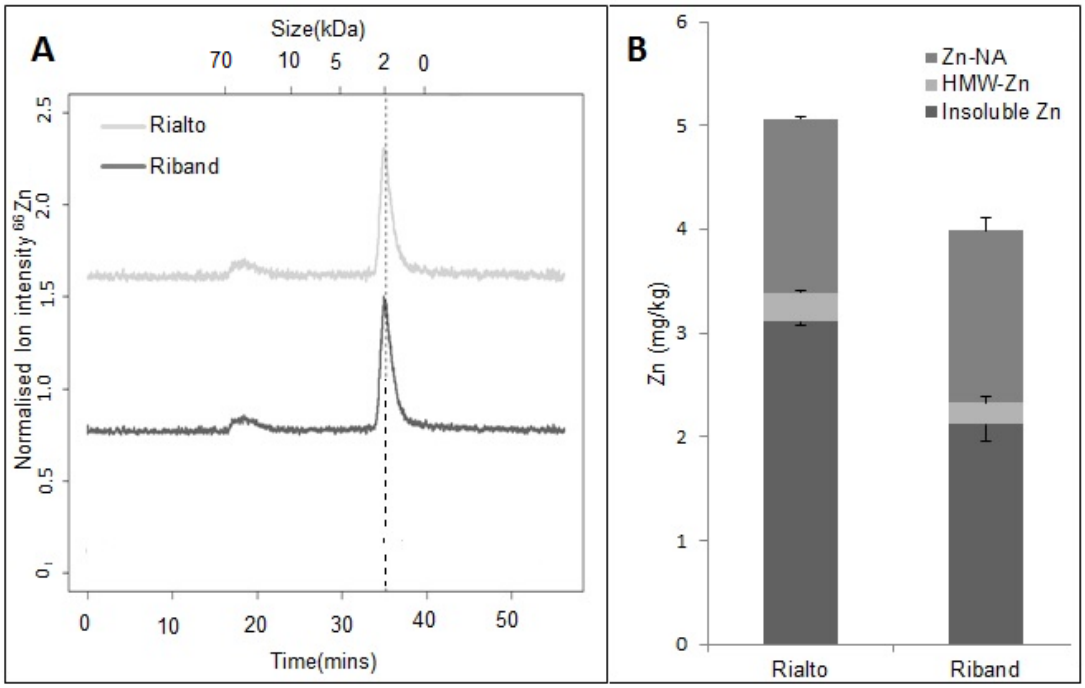


Figure 46. Determination of Zn speciation in white flour fractions of Rialto and Riband. (**A**) SEC-ICP-MS chromatograms (averages of three biological replicates). All samples were extracted in 50 mM Tris-HCl pH 7.5; (**B**) insoluble Zn determined by ICP-AES. (**C**) Quantification of molecular species of Zn from relative peak areas on SEC-ICP-MS and ICP-MS of soluble fractions. Data (B) represents means ± SD of three biological replicates (p<0.05).

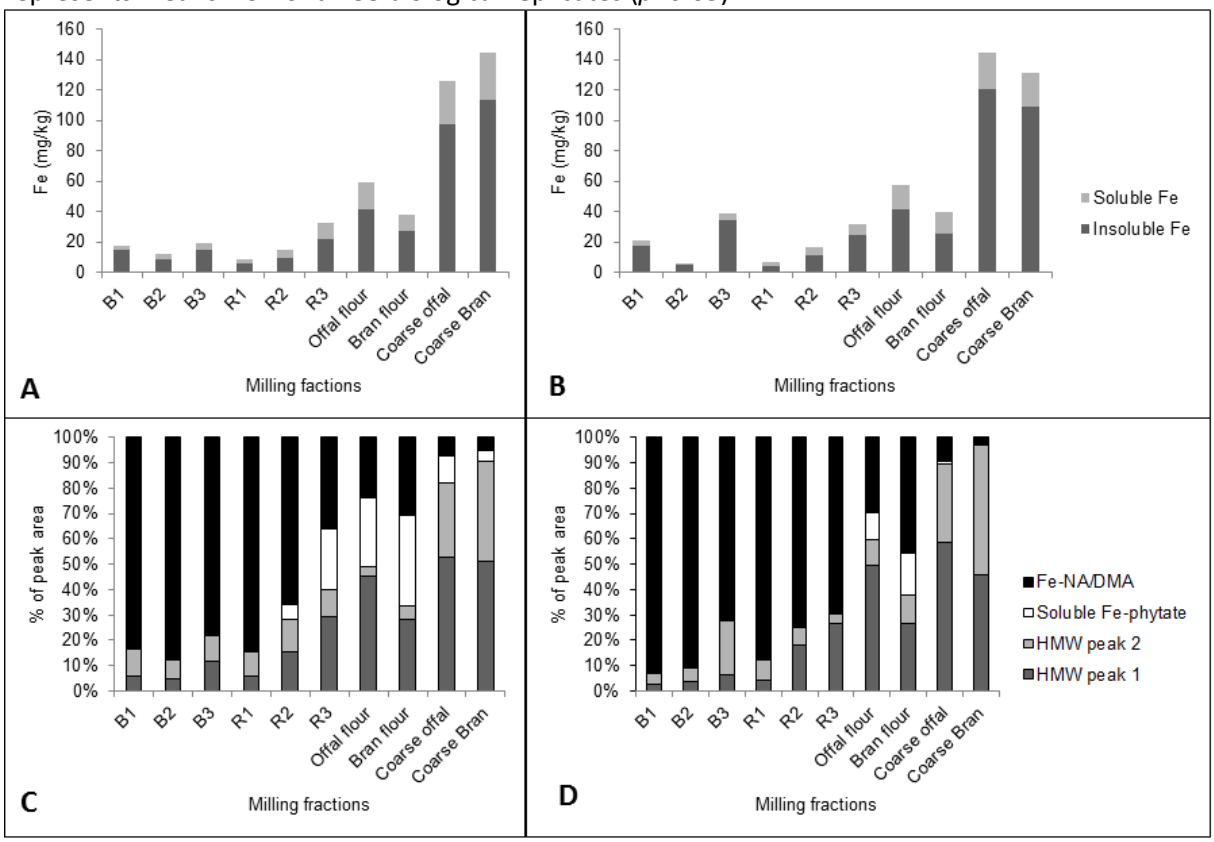


Figure 47. Quantification of the forms of Fe in milling fractions of Rialto and Riband. The SD of the method was shown to be less than 0.5% by analysis of five replicate samples of bran flour from Rialto. C and D show the concentrations of total soluble and insoluble Fe in the same milling fractions as shown in (**A**) and (**B**). Results are means of two technical replicates.

Table 4. Quantification of forms of Fe in grain Rialto and Riband, based on analyses of milling fractions and whole grain

	Fe species in whole grain[1]						
	Fe-HMW1	Fe-HMW2	Soluble Fe-phytate	Fe-NA/DMA	Insoluble Fe	Total calculated	Total Fe determined by ICP-AES[2]
Rialto (mg/kg)	4.1	2.9	0.7	2.3	33	43.1	46.7
%	*9.6*	*6.7*	*1.6*	*5.3*	*76.7*		
Riband (mg/kg)	3.8	3.3	0.12	2.5	24.2	33.8	30.3
%	*11.2*	*9.6*	*0.38*	*7.3*	*71.4*		
Technical error (%)[3]	0.5	0.5	0.2	0.4			

[1]The total concentrations and percentages of each form of Fe were calculated based on the peak relative area on SEC-ICP-MS and total soluble Fe determined by ICP-MS for each milling fraction and the proportions of the milling fractions (**Fig 17**).
[2]Total Fe determined by ICP-OES of whole grain.
[3]Technical error was determined by analyzing five replicate samples of Rialto bran.

Figure 47 shows the speciation of Fe in soluble extracts of 10 fractions from Bühler milling of both Riband and Rialto. The break fractions B1 - B3 and the reduction fractions R1 - R3 correspond to white flour, with R1 accounting for 47.6% and 42.1% of the total mass in Rialto and Riband, respectively (**Table 4**). The R1 fraction has the lowest ash content (**Chapter 3**, **Table 3**) and is therefore considered to represent the purest starchy endosperm fraction derived from the central part of the grain (*26*). Ash content, an indicator of bran contamination, decreases closer to the center of the grain (**Chapter 3, Table 3**). A similar effect is observed with phytic acid content (**Chapter 3, Fig 19**).

The main difference observed between the milling fractions from the two cultivars was the amount of soluble Fe-phytate (**Figures 47c, d**). These results agree with the data in **Figure 45**, showing that soluble Fe-phytate was present in the white flour extracts of Rialto but not of Riband. In addition, more soluble Fe-phytate was present in the bran fractions, especially the offal fractions, of Rialto compared to Riband.

The calculated amounts of soluble Fe species in whole grains of Rialto and Riband are given in **Table 4**, based on the yields of the milling fractions and the SEC-ICP-MS analyses. This shows that the concentration of soluble Fe-phytate was over five-fold higher in Rialto than in Riband, although this form still represents only a small percentage of the total grain Fe (1.6%). The contents of LMW Fe (likely to be Fe-NA and/or Fe-DMA) were similar in the two cultivars but it represented a higher percentage of the total Fe in Riband. A higher proportion of the total Fe was insoluble in Rialto (by 36%) than in Riband.

4.4 Discussion

Our initial hypothesis was proved correct in that variation in Fe and Zn speciation exists between tissue factions in wheat in both insoluble and soluble fractions. Distinct variations between polished rice and bran faction in soluble Fe and Zn speciation was also observed.

4.4.1 Enzyme results

It is clear that the majority of Fe and Zn in wheat is insoluble (**Fig .31**). We show for the first time that the forms of this insoluble Fe and Zn are likely to vary between tissue fractions in wheat. In R1 which is the purest endosperm fraction only protease had an effect on solubility. This effect was most pronounced in Riband, with 100% of the Zn and 53% of the Fe being solubilised compared to 81% of the Zn and 22% of Fe in Rialto (**Fig 32, 33**). These differences between cultivars soluble Zn and Fe after treatment of protease are likely to be explained by the presence of Fe and Zn from the aleurone layer in the R1 fraction of Rialto which was not affected by protease activity. As previously discussed in **Chapter 3** Rialto, a hard wheat, contains more aleurone and bran in its white flour fraction.

Offal flour contains both white flour and some aleurone and germ (*242*) and behaved similarly to R1 for both Fe and Zn, with protease having the greatest effect, However, this was not as pronounced as for R1 (**Fig. 32, 33**), this again is likely to be due to a large proportion of the Fe in tissues originating from the aleurone layer and outer tissues of the grain. The major differences observed were between R1 and coarse bran. Coarse bran is made up of the outer and intermediate pericarp layers, with some aleurone layer and a very small amount of endosperm (*242*).

Phytase was shown to have no significant effect on the release of either Fe or Zn (**Fig. 31-38.**), which contradicts previous studies on barley embryos (*23*), However, it should be noted their

enzyme extractions were only performed on embryo tissue. It would be expected that phytase would have no effect on R1 as pure endosperm should have no phytate. In the milling process the embryo is most likely spread across all fractions but concentrated in the offal flour and bran flour (*195*), however wheat embryo only accounts for 3-3.2% of the total grain (*243*). It would be expected that phytase would have no effect on R1 as pure endosperm should have no phytate The lack of an effect of phytase on other tissues does not necessarily indicate a lack of phytate binding; phytate is stored in insoluble phytate granules within the protein storage vacuoles (PSV). It is therefore possible that phytate granules may be protected *in planta* from extrinsic phytate by hydrolysis resistant protein complexes (*83*) or that they are inaccessible for the enzyme due to fibre (*100*).

In millet, exogenous phytase was only able to liberate Fe in conjunction with xylanase and it was concluded that Fe is bound as phytate-fibre complexes which are resistant to hydrolysis by phytase alone (*100*). A separate experiment was performed to investigate this effect in coarse bran and R1 milling fractions of Rialto (**Fig. 34**). Phytase was shown to release more soluble Fe compared with cellulase alone. It is therefore likely that Fe is bound to insoluble phytate in the outer factions but is protected of the grain by dietary fibre. This is in agreement with Wang *et al* who showed phytase improves both Fe and Zn solubility most effectively in rice bran when combined with cellulase (*83*).

From our data we are able to conclude that insoluble Zn is likely to mainly be bound to protein within the endosperm and a proportion of Fe is also bound to protein. The link between protein and Zn in the endosperm of wheat has been identified previously (*67*) and has been shown in rice using a similar method (*244*) However, there has been no information for Fe speciation until now. The lack of effects of both the phytase and combined phytase and cellulase treatments indicates that neither Fe nor Zn are bound to phytate in the endosperm.

A reduction in soluble Fe with the addition of phytase was also observed in millet (*231*). One reason for this maybe that exogenous phytases, in this case crude wheat phytase, may form insoluble complexes with Fe (*231*). This is likely to be greater in our study as we demineralised our enzymes prior to extractions, thereby increasing the chelation capacity of the proteins or other complexes in the enzyme preparations.

4.4.2 Speciation in transgenic OsNAS rice

We analysed an elite rice line transformed with rice the nicotianamine synthase gene (*OsNAS1*) fused to a rice glutelin promoter. Previous studies had shown that the transgenic lines (EN1, EN2) had increased expression of *OsNAS1* in the endosperm and increased concentrations of NA in polished (8 fold) and unpolished (7 fold) rice grain (*121*). The effect on total Fe and Zn concentration had been reported previously, with increases of Zn in unpolished rice (33.4% to 55.4%), and polished (22.7% to 31.9) grain, but no increase in Fe (*121*). Our data showed no differences in either soluble Fe (**Fig. 37)** or Fe speciation between the transgenic and Wt polished (**Fig. 35**) rice but did show effects on both soluble Zn and Zn speciation (**Fig. 36, 39)**

This does not agree with a another study using SEC-ICP-MS on NAS overexpressing rice which found the Fe-Na peak was significantly increased in transgenic lines (7 fold) and total Fe was increased in the polished rice (2.6 fold). The reason for the differences in Fe concentration in polished grain of Lee *et al* (*22*) compare to Zheung *et al* (*121*) maybe due to the overexpression of a different form of NAS , in this case *OsNAS3,* which is normally only expressed in the roots (*22*). It is therefore likely that the expression of this gene in the shoots and grain enhanced iron transport which would not normally exists within these plant tissues. This also may have contributed to the reported speciation change of Fe. However, it should be noted that the study by Lee et (*22*) used quartz sand in their extraction which, as we have shown, is likely to have contributed to the differences in Fe speciation between their wild type and transgenic lines (discussed below). In addition, in the study by Lee *et al* the *OsNAS3* gene was expressed ubiquitously throughout the plant whereas in Zheung *et al* the expression was endosperm specific, therefore overexpression of NA throughout the plant may have had an effect on increasing uptake and transport to the grain.

The effect on Zn speciation in the polished rice lines overexpressing *OsNAS1* was shown in our SEC-ICP-MS data and was shown to increase Zn as Zn-NAS by 4 fold (**Fig. 35, 37**). Another study by Lee *et al* it was showed that Zn concentration in polished rice increased (2.9 fold) when *OsNAS2* was overexpressed (*22*). This increase is considerably greater than that seen by Zheng et al (22.7% to 31.9%) (*121*). Lee *et al* also showed a greater increase in LMW peaks (16.4 fold) in transgenic lines which also showed the presence of DMA as well as NA in transgenic lines (*245*). Our data on the *OsNAS1* lines strongly suggests that the additional extractable Zn is Zn-NA (**Fig. 35**). These differences are again likely to be due to the expression of different genes. The difference in

targeting of gene expression is also likely to account for the differences in Zn sequestration within the two rice constructs.

Overexpressing *OsNAS1* in the rice endosperm has previously been shown to increase Fe bioavailability 2 fold in polished rice (*121*). However, our SEC-ICP-MS data show no difference in *in planta* Fe speciation. Therefore, this effect is likely to have been caused by an 8 fold increase in unbound NA within the plant, which possible re-associates with Fe during the digestion. This effect of NA during digestion is explored further in **Chapter 5**.

Despite the endosperm specific expression of *OsNAS1* we also observed an effect on Zn speciation within the bran fraction with an increase in Zn-NA (**Fig. 35).** The resons for this is not understood but it is unlikely to be due to endosperm contamination. One possible explanation is that Zn-NA, a highly soluble small compound, was transported throughout the grain from the endosperm as the concentration within the endosperm increased.

4.4.3 Effect of increased NA in presence of Fe

Our initial data and the study of Lee et al (*22*), used the method described by Persson et al (*23*) advocating the use of quartz sand (~0.03% Fe_2O_3) and porcelain (0.52% Fe_2O_3) pestle and mortars in the extraction stage. This showed much greater differences between the amounts of Fe-NA in Wt and transgenic lines (**Fig. 41**) and higher total Fe (**Fig. 40**). This may have some relevance for human nutrition, as cooking and preparation of rice will nearly almost always happen in the presence of Fe due to its abundance in most cookware (*246*). The effect due to cooking using stainless steel or iron cookware on Fe concentration in rice is well reported (*246, 247*).

We showed that NAS rice was able to recruit Fe from quartz sand (used as a proxy for Fe_2O_3 containing cookware) to a greater degree than Wt rice (**Fig. 40**). Although there was no difference in Fe speciation between the lines when extracted without Fe, in the presence of Fe (originating from quartz sand) the increased unbound NA in the transgenic lines affected the speciation profile with an increase in Fe bound to the LMW Fe-NA. The implications of this for human nutrition and the end use potential of NAS rice should not be understated as it clearly shows that if cooked in the presence of Fe, NAS rice will not only contain considerable more Fe than its Wt counterpart but that this Fe will also be in a highly bioavailable form (*121*).

4.4.4 Low phytate wheat lines

It has been argued that low phytic acid lines should have higher soluble P and higher soluble Fe which would be bioavailable to humans. However our results showed that this was not the case for Fe (**Fig. 18.**). IDO563 (a *lpa* spring wheat) did not have higher soluble Fe than one of its related lines IDO488 and AO2568 (*lpa* winter wheat) did not have significantly higher Fe to any lines and significantly lower soluble Fe than Kanto and Centenarial. Soluble P was significantly higher in both lpa mutants compared to controls, apart from IDO488 (**Fig. 43)**.

SEC-ICP-MS showed no effect on Fe speciation compared to its controls (data not shown), however the AO2568 mutant did appear to have a higher percentage of soluble P as inorganic P compared to all controls, which is indicative of the *lpa* phenotype (*157*) (data not shown).

Why the effect of the low phytic mutants did not have more noticeable impacts on Fe speciation is unknown and needs further investigation. But from this data we can conclude that these particular *lpa* mutants would not have improved bioavailability, and this was demonstrated with a dialyzability assay discussed in **Chapter 5**.

4.4.5 Ferritin overexpressing lines

Theoretically, an increase in ferritin would increase the amount of Fe bound to ferritin within the grain. It would therefore affect the speciation profile of the grain, creating a larger HMW peak, which is likely to be ferritin Fe. However, we saw no change in Fe speciation between transgenic and control lines, indicating that the increase in *TaFer1* gene expression in the endosperm did not affect Fe speciation. One possible reason for this is that although the ferritin was increased in the endosperm, additional Fe was not able to be transported to the endosperm (*153*) in sufficiently increased amounts to alter speciation and therefore the ferritin remained without bound Fe in the endosperm.

4.4.6 Speciation in wheat white flour

The data from SEC-ICP-MS show the presence of HMW, MMW and LMW peaks of Fe and HMW and LMW peaks of Zn in white wheat flour (**Fig. 45-47**). Based on matching of retention time (**Fig. 29, 30**) and on previous studies of rice (*18*), the LMW Fe in wheat is likely to be a mixture of Fe-NA

and Fe-DMA. Interestingly, it would appear from our comparisons with standards that Zn-NA is the only form of soluble Zn in the endosperm in both rice and wheat. This is in agreement with other studies which have shown that Zn binds more favorably with NA (*55*) and Zn-NA was the only Zn-chelate in the phloem sap of rice (*59*). However, it was found that Zn was bound to both NA and DMA in transgenic rice overexpressing the *OsNAS2* gene (*21, 245*).

The most notable difference between the two cultivars of wheat was the presence of Fe bound to soluble phytate, which appeared as MMW complexes, in white flour of Rialto but not of Riband. The effect of this on the nutritional value of white flour of Rialto is not known but soluble Fe bound to phytate has been shown to be partly available for absorption (*119*) and the bio-availbilty of this complex is explored further in **Chapter 5**. One explanation for the difference between the two cultivars in soluble Fe-phytate (**Fig. 45, 46**.) could be premature sprouting of Rialto, which is known to be prone to pre-maturity amylase production. This is supported by the determination of the Hagberg Falling Number (HFN), which was lower for Rialto than for Riband (112.3 ± 10.0 compared to 217.0 ± 23.3, $p<0.05$). Although HFN measures the activity of amylase on starch, the synthesis of phytase also occurs during germination (*248*). Increased phytase activity and other enzymes produced during germination could therefore be responsible for the higher soluble phytate in Rialto, as it is possible they would cause the insoluble phytate granules to degrade and release soluble forms of phytate. Another reason could be contamination of the white flour fraction with aleurone tissues which is greatest in hard wheat such as Rialto. This was discussed in more detail in **Chapter 2**. We found no evidence of soluble forms of phytate-Zn in white flour (**Figure. 46**) or other milling fractions **(Appendix 5)**, which is in agreement with previous analysis of barley embryos (*21*).

Analysis of the 10 milling fractions showed that like soluble Fe and Zn were present in different forms in white flour fractions and outer fractions. The endosperm is an unusual tissue in that it is made up almost entirely of starch and has comparatively low amounts of protein when compared to other tissues (*249*). It was no surprise, therefore, that the fractions which contained the highest portion of endosperm (R1, B1) contain little or no Fe bound as either the HMW peak or soluble phytate peak. Instead all of the Fe and Zn was bound as the highly soluble Fe-DMA/NA or Zn-NA form.

The milling fractions corresponding to the outer layers of the grain (offal and bran flour, coarse offal and coarse bran) contain most of the aleurone layer, the embryo and pericarp/testa tissues

(*194*). However, it is not possible to derive precise information on individual tissues from these analyses as each fraction is likely to contain varying proportions of aleurone, embryo and outer layers (*194*). Soluble phytate-Fe was highest in the offal and bran flour fractions (**Fig. 47**). It has previously been shown that a proportion of the Fe remains as soluble mono-ferric phytate, however this varies depending on the phytic acid: Fe ratio (*137*). This is in agreement with our data which show that the concentrations of soluble Fe-phytate appear to be related to total phytate except in the coarse bran and coarse offal, where, although total phytate levels were high, soluble phytate remained low, possibly due to phytic acid : Fe ratios favoring insoluble complexes (*137*) (**Fig 47, Chapter 3, Fig 21**).

In addition to soluble phytate and DMA/NA, Fe is also bound to at least two HMW compounds (**Fig. 45, Table 4**). The identification of these peaks is challenging. HMW1 is excluded from the size exclusion column, suggesting that it is in excess of 70 kDa. HMW2 also elutes very close to HMW1. These high molecular weight peaks seem to be much more abundant in outer layers of the wheat grain. As previously discussed the iron storage protein ferritin may contribute to these peaks due to their large size (*92*). Our own analysis of data on the expression from the Affymetrix GeneChip Wheat Genome Array (*250*) also showed that expression of *TaFER1* and *TaFER2,* the genes responsible for ferritin synthesis, is very low in the endosperm, compared to that of whole grain (**Appendix 4**). However, ferritin is present in low abundance in cereals (1-3% of total Fe) (*94, 251*) and it is therefore likely that the HMW peaks are a combination of Fe and Zn containing macromolecules, most likely proteins. Therefore, their abundance in the outer fractions would be consistent with protein levels being low in the endosperm and higher in the germ and aleurone (*249*).

Polyphenols may also account for part of the HMW peak as they have been shown to bind both Fe (*138, 252, 253*) and Zn (*252, 253*) and tend to have higher abundances in the outer layers of grain. However polyphenols have been analysed by SEC and have been reported to be of a much smaller size ~1kda (*254*), although the size of potential metal containing oligomers is unknown.

Speciation of Zn in bran fractions of Zn is not presented due to difficulties in quantifying the separate peaks. Like Fe the HMW peak was higher in the bran fractions, however there was also another LMW peak in bran fractions which eluted before and overlapped the Zn-NA peak making exact quantification impossible. (**Appendix 5**)

4.4.7 Differences in wheat and rice

The speciation of Fe and in wheat and rice white flour appeared to be very similar (**Fig 29, 45, 46.**), with LMW forms accounting for the majority of the soluble Fe and Zn. However the bran fractions of wheat and of rice appeared to showed obvious differences, soluble Fe being much higher in rice largely due to the higher abundance of soluble Fe-phytate (**Fig. 35**). The unknown HMW peak also formed a greater percentage of soluble Fe in wheat when compared to Wt rice.

4.4.8 Limitation of methods

Enzyme assays can only provide an indication of metal binding in insoluble fractions. As previously discussed metals may be bound to a particular chelator but the appropriate enzyme may not be able to physically reach the complex, due to another macromolecule forming a physical barrier.

SEC-ICP-MS is an effective method for separating potential metal containing complexes but there are limitations in its ability to distinguish metal species of similar size. The range of our column also only allowed for accurate size determination between 70 kDa-and 1 kDa.

Extracting metal species from plants in their *in planta* form is difficult as small chemical or physical changes may have impacts on metal speciation. A major criticism of *in vitro* analysis of metal speciation is that decompartmentalisation of metal chelators occurs during extraction and may result in unavoidable artefacts; *in situ* analysis such as NANO-SIMS (*69*) or Laser ablation (LA)-ICP-MS (*67*) reduces this problem but provides limited information on speciation. Although SEC-ICP-MS offers a relatively fast and accurate way to measure metal species in cereals the results should only be used as an indication of *in planta* speciation rather than as an absolute determination.

4.5 Summary

Insoluble Fe and Zn appear to be bound by phytate, protein and dietary fibre in wheat. However, the degree of binding varies depending on the milling fraction, which is indicative of differential metal binding throughout different tissue fractions of the grain.

Soluble Fe and Zn metal complexes were identified in both wheat and rice. In the white flour (endosperm) of rice and wheat, Fe was bound to DMA and NA, whereas Zn was bound to NA. Fe speciation varied histologically throughout the grain, with soluble phytate and a HMW peak being present in considerably higher amounts in the outer grain fractions.

4.6. Implications of results

The results from this study have increased our understanding of the distribution and forms of Fe and Zn in wheat and rice grain. They also show that DMA and NA are likely to play a role in Fe and ZN storage within the grain of wheat, as has been shown in rice. The results also demonstrate the usefulness of SEC-ICP-MS for the speciation of these minerals within cereals which, combined with detailed bioavailability studies, will allow for more targeted breeding strategies for increased mineral content in wheat grain.

SEC-ICP-MS is useful for assessing transgenic crops which have been bio-fortified as it provides a way to determine the speciation and hence potential bioavailability of the target metal. Our results showed that the transgenic Tafer1 and the *lpa* mutants were unlikely to have improved bioavailability compared to wild type plants. However OsNAS1 rice had clear changes in Zn speciation and potential for improved Zn availability. Although the transformation did not affect Fe speciation, the elevated levels of NA are likely to improve Fe availability once in the digestion pool. However, it is necessary to understand the behavior of the compounds in the GI track to conclude whether the differences may affect bioavailability. This is explored in **chapter 5**.

5.1 Introduction

As discussed in **Chapter 1** bioavailability is the most important factor with regard to Fe nutrition and, although data describing total Fe levels, the location of Fe and speciation of Fe in wheat are useful, the effects on bioavailability can never be more than a crude estimate unless changes introduced by the digestion process are understood. Due to the widespread prevalence of Fe deficiency in humans, Fe is one of the most widely studied minerals with regards to bioavailability but little information is available concerning changes in non-haem Fe speciation during digestion. One previous study did examine Fe speciation changes in meat using a rat digestion model however as previously stated rats are not a suitable model for Fe metabolism in humans (*255*).

The initial aim of this project was to determine the differences between Fe bioavailability in wholegrain of different wheat cultivars. However preliminary experiments with Caco-2 cells (**Appendix 6**) showed that the sensitivity of the model was not sufficient to detect any differences between the samples. In fact, we were not able to detect any differences between the control and wholemeal samples. No previous studies had reported any results on unmodified whole meal flour indicating that the low level of Fe bioavailability was not suitable for the Caco-2 cell system.

However, concurrent speciation work (**Chapter 4**) identified marked differences in Fe speciation between wholemeal and white flour that could theoretically lead to white flour having a higher level of bioavailability than whole meal flour. There is some evidence that this may be the case from human studies (*256-258*). Our own preliminary experiments (**Fig. 48**) also showed that Fe in white flour was significantly more dialyzable than Fe in wholemeal, 2.2% compared to 5.4%, respectively ($P<0.05$). The aim of the experiments in this chapter was to explore the differences between the Fe bioavailability after simulated digestion of whole meal and white flour using the two contrasting cultivars discussed in chapter 2.

It was decided to study unleavened bread rather than raw or leavened samples for two reasons: 1) Many countries where Fe deficiency is most prevalent eat more unleavened than leavened wheat products. For example, it is estimated that 80% of wheat flour in Pakistan is ultimately made into unleavened bread such as chapattis; 2) The addition of yeast to make leavened bread

may complicate any analysis of speciation and make differences between the wheat cultivars harder to determine.

We first determined the Fe bioavailability of our bread samples using the Caco-2 system to test whether if our initial hypothesis was correct. We then measured the amount and speciation of soluble Fe at two stages of digestion. Phytic acid was also measured in all samples. Finally the same samples were fortified with inorganic Fe and again assessed for their bioavailability, soluble Fe and speciation.

To date there is no accurate *in vitro* method of measuring Zn bioavailability (*105*) and therefore Fe availability was the focus of this study.

Hypothesis : That white flour may be a more available source of Fe than whole grain due to the removal of anti-nutrients from the bran layer and more favourable Fe speciation in the endosperm.

5.2 Materials and methods

5.2.1 Preparation of unleavened bread

Bread samples were made from wheat cultivars Rialto and Riband grown in the field at Rothamsted in 2010 (see Chapter 2). As the samples were to be analysed using the Caco-2 cell system, pooled samples of all three biological replicates were used, as it was not practical to analyse all three replicates individually due to the number of experiments that this would require. Whole meal bread was made from the milled whole grain (**see 3.2**) and none of it was discarded (100% extraction rate). Although this is unlike most bread that is commercially available in the UK it is representative of the breads eaten in poor rural parts of many developing countries where iron deficiency is most prevalent. White flour chapattis were made from the white flour fraction (Riband 38±2.1%, Rialto 41±3.4% extraction) described in **3.2**. The white flour fractions were obtained at a much lower extraction rate than would be found commercially. This was done in part to achieve a greater contrast with wholemeal, as preliminary Caco-2 experiments (**Appendix 6**) had found the sensitivity for determining differences between similar wheat samples to be insufficient. Additionally to our knowledge no studies have ever been conducted on such a low extraction rate. The process by which the unleavened breads were made is summarised below.

First 20 g of flour was placed in a silica crucible. Glass was not used as there is a small amount of Fe within glass which could react with intrinsic chelators during the baking process. Then 5.76 ml of deionised water, and 0.4 g of salt was added to the flour, which was then kneaded into a flattened dough ball. The breads were then left to prove at 30º for 30 mins, before being baked at 200º for 15 mins. The breads were then broken up by hand into smaller pieces, approximately 0.5 cm^3, before undergoing flash freezing in liquid nitrogen and then they were freeze dried for 72 hours. Samples were then homogenised and ground to a fine flour in an agate ball mill (Retsch PM400, Düsseldorf, Germany).

Fortification was achieved by adding $FeSO_4$ (30 mg Fe/kg) in solution to the flour with the deionised water before cooking. The process, although not a complete replication of the process of making chapattis, mimicked all the important steps which may affect bioavailability.

5.2.2 *In vitro* digestion

Initially 1 g of sample was used, however due to the very low sensitivity of the Caco-2 system (**Appendix 6**) the samples size was increased to 3 g per digest, which would theoretically triple the Fe presented in the post-digestion solution to the cells. The samples were added to 10 ml 120 mmol/L NaCl and 5 mmol/L KCl at pH 2 and ascorbic acid was added (see below, 5.3.3). The pH was then further adjusted to pH 2 using HCl. For Fe fortified samples the process was identical except that 1 g of sample was used as there was sufficient Fe.

For the gastric digestion 0.5 ml of a pre-prepared porcine pepsin solution was added to each sample. This solution contained 0.2 g pepsin in 5 mL of 0.1 mol/L HCl, which was added to 2.5 g of Chelex-100 (Bio-Rad Laboratories, Hercules, CA) and mixed for 30 mins. The slurry was then poured into a 1.6 cm FlexColumn (Fisher Scientific, UK) and left to settle for 20 mins before the eluent was filtered (Whatman Ashless filter paper 40 125nm) and collected. The column was further eluted with 0.1 mol/L HCl. Samples were then placed on rolling bars (Stuart, UK) for 1 hr at 37^oC. The filter paper step was added after concerns that some Chelex was contaminating the sample rendering some of the soluble Fe insoluble.

For the full simulated digestion experiment, samples were treated as per the gastric digestion procedure but with an additional intestinal digestion stage. First 0.05 g porcine pancreatin and 0.3 g bile extract were dissolved in 25 mL of 0.1 M/l $NaHCO_3$, and Chelex-100 (12.5 g) was added. The mix was then prepared as described above except that the column was further eluted with 10 ml

of $NaHCO_3$ and 2.5 ml added to each sample after which the pH of each sample was adjusted to 5.5-6.0 with 1.0 M $NaHCO_3$. The sample pH was then adjusted to 6.9-7 and the volume made up to 15 ml using 140 mM NaCl, 5 mM KCl pH6.9 solution, before being placed on roller bars for 2 hr at 37ºC.

Samples were centrifuged (21,000 *g*, 10 mins) and the solid phase discarded. The supernatant was then flash frozen in liquid nitrogen and stored ready for the Caco-2 cell analysis.

5.2.3 Addition of ascorbic acid

It is a conventional practice to add ascorbic acid (AA) during Caco-2 experiments. The amount of ascorbic acid is set at a molar ratio (normal 10-20:1 AA:Fe) determined by the amount of Fe in the samples (*141, 259-261*). However, because of the large variations in total Fe in our samples (6.5-77mg/g) an identical amount of AA was added to each sample. The quantity of ascorbic acid added to each digest was 66 µl (200 mM solution) per 1 g of sample, an amount equivalent to one medium glass of orange juice (330ml) with a 45 g serving of bread. Although this meant that some samples would have a higher AA:Fe ratio than the others, the amount of AA chosen was sufficiently high to ensure that it achieved maximal effect as observed in previous studies (*124*). Also the study was designed to compare samples under identical dietary conditions, therefore it was not appropriate to adjust the ascorbic acid levels by over 10 fold between our highest and lowest Fe samples.

5.2.4 Dialyzability analysis

All samples were digested as described in the *in vitro* digestion and placed on 6 well plates as described in 5.3.7, except that no cells were present and the bottom chamber contained 140 mM NaCl, 5 mM KCL pH 6.9 rather than medium. After 2 hours the membranes were removed and the Fe concentration of the solution measured using a colorimetric method. This method was found to be unsuitable because the samples had a slight absorbance at 562 nm and this absorbance varied between each sample. Therefore ICP-MS was used instead (**2.3.9**), which is suitable for solutions with low concentrations of Fe as previously stated.

5.2.5 Amylase digest

Samples (1 g) were shaken (60 rpm) for 5 min at 37º in 5 ml of pH 7 buffer (120 mmol/L NaCl and 5 mmol/L KCl.) with 1 mg of salivary amylase (Sigma) at a concentration approximately 5 times that of human saliva (*262*). A higher concentration was used as it was a proof-of-principle experiment looking for potential effect rather than physiologically relevant results.

5.2.6 Caco-2 Cell Culture Procedures

Unless otherwise stated, all reagents were from Sigma-Aldrich. Caco-2 cells (HTB-37) were from the American Type Culture Collection (Manassas, VA, USA) at passage 20 and stored in liquid nitrogen until seeding. Cells to be seeded were then transferred in Dulbecco's modified Eagle's medium (DMEM, Invitrogen UK; supplemented with 10% fetal bovine serum, 25 mM HEPES solution, 4 mM L-glutamine, and 5 mL 5000 u/mL penicillin/streptomycillin solution) and were maintained at 37 °C in an incubator with humidified atmosphere consisting of 5% carbon dioxide and 95% air.Caco-2 cells were seeded onto collagen-coated 6-well plates (Greiner, UK) at a density of 4.75×10^4 and suspended in 2 mL of supplemented DMEM, which was replaced every 2 days. Cells between passages 32 and 38 were used for experiments at 13 days post seeding, and 24 h prior to experimentation, they were switched to serum-free medium (DMEM supplemented as above with the exception of fetal bovine serum). This caused the cells to be starved of Fe to maximise Fe uptake from samples and increase the sensitivity of the assay.

5.2.7 Caco-2 ferritin assay

A sterilized insert ring, fitted with a dialysis membrane (15 kDa MWCO), was inserted into each 6 well plate, thus creating a two-chamber system. 1.5-mL aliquot of the intestinal digest (defrosted and warmed to 37º) was pipetted into the upper chamber. The plate was covered and incubated on a rocking shaker at 6 oscillations/min for 2 hrs.

When the intestinal digestion was terminated, the insert ring and digest were removed. The solution in the bottom chamber was allowed to remain on the cell monolayer and an additional 1 mL of MEM was added to each well. The cell culture plate was then returned to the incubator for an additional 22 h, after which the cells were harvested for analysis.

Cells were then sonicated (3 X 5 seconds) and stored at -20, before being defrosted at room temperature and kept on ice, and a spectroferritin ELISA assay (Ramco, USA) was carried out following the manufacturer's instructions. Total protein was quantified using a BCA protein assay

(Pierce, USA) according to the manufacturer's instructions and a value for ferritin content (ng/mg total protein) was obtained.

5.2.8 Phytic acid

Analysis of phytic acid was performed using a commercially available kit (K-PHYT 12/12 Megazyme , Ireland), as per the manufacturer's instructions. Perhaps a brief explanation of the principle involved for the assay.

5.2.9 ICP-MS

Total Fe was determined by ICP-MS as described in more details in Chapter 2. All samples were diluted to a final concentration of 5% HNO_3 and fortified samples were diluted 5 fold to the same final concentration, such that the final concentration of Fe in the samples was within a standard (curve 0-200 ug/l).

5.2.10 Speciation of Fe

Speciation results were obtained as described in Chapter 3 except that rather than a Tris HCl extraction, samples were run through an *in vitro* digestion. Samples were then mixed with an equal amount of Tris 20 mM HCl pH 7 prior to injection. This was done to improve the clarity of the peak as undiluted peaks have very wide peaks, which is often the case when the sample solvent (120 mM/l NaCl and 5 mmol/L KCl pH 7) differs from the mobile phase (Tris-HCl 20 mM pH 7).

5.3 Results

5.3.1 Preliminary experiment

Caco-2 assays are expensive and time consuming. An alternative predicative method for Fe availability is to measure dialyzability which, although not providing an accurate measure of bioavailability, can provide an indication of the potential bioaccessibility and therefore

bioavailability of a sample (*14, 105*). Therefore a dialyzability assay was performed on a variety of samples. The experiment was designed to assess four factors to help design further experiments:

1. whether the addition of a pre-digestion amylase stage would improve Fe bioaccessibility;
2. whether Fe in white flour was more dialyzable than wholemeal bread;
3. whether low phytic acid wheat would be a more bioaccessible source of Fe;
4. what effect, if any ,was obtained by the addition of an iron fortificant to wholemeal and white bread samples.

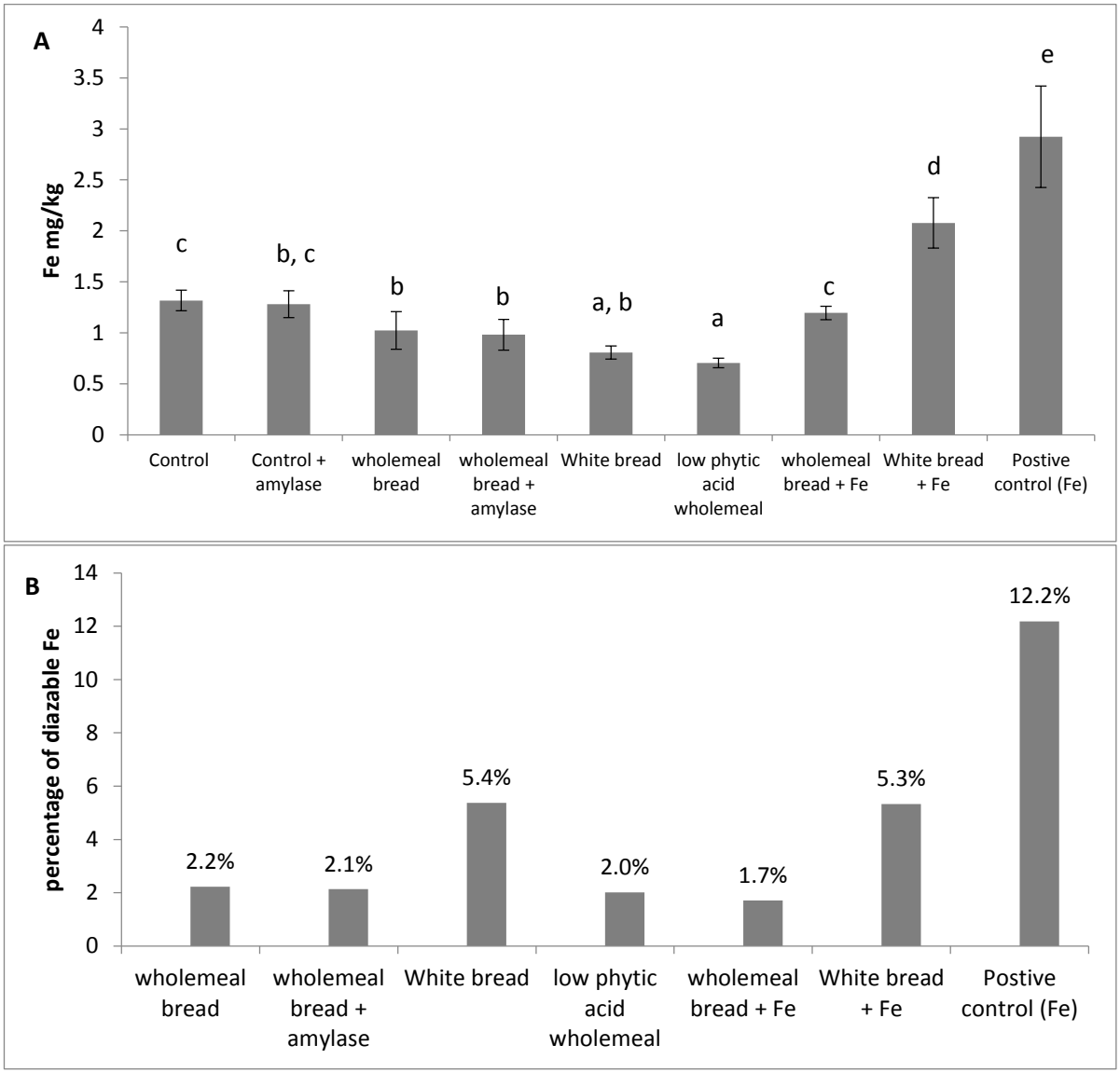


Figure 48. A) Dialyzability Fe after *in vitro* digestion .Data is total dialyzable Fe of 1 g of white or wholemeal bread made from cv. Rialto or cv. IDO5633 (low phytic acid) (see **2.1.3**), either with no treatment or treated with ferric chloride, 24 µg of Fe/g of sample (+Fe) or amylase. Data

represent the mean ± SE (n= 3). Bars without a common letter (a−e) are significantly different, p< 0.05.**B)** Percentage of total Fe dialyzable.

The preliminary results (**Fig. 48**) indicated that the addition of amylase had no effect on Fe dialyzability and therefore this step was omitted from future investigations. The dialyzablity of Fe from breads made from Rialto showed that there was a significant difference between whole meal breads and white breads. Fe dialyzablity was very low and represented only 2.2% of total Fe for wholemeal bread, as opposed to 5.4% (**Fig. 48b**) for breads made from white flour. Therefore it was decided to further explore the differences in bioavailability of wholemeal and white flour.

White bread samples with additional Fe ($FeCl_3$ 24 µg of Fe/g) were found to have significantly more dialyzable Fe than wholemeal bread. Fortified samples, however, were lower in dialyzable Fe than the positive control which contained the same amount of Fe as fortified samples but with no bread. Therefore it was also decided to explore this further.

Low phytic acid (LPA) wholemeal bread was also found to have significantly less dialyzable Fe than Rialto wholemeal, which is opposite to what we had predicted. Because of these results and earlier Caco-2 experiments on LPA breads (**Appendix 6**) it was decided not to look at low phytic acid breads further.

Both controls (control, control +amylase) had higher dialyzable Fe than expected however and were higher than some samples, which showed that the use of the colorimetric method (**5.2.4**) was probably not suitable for our samples and therefore all subsequent Fe quantification was done using ICP-MS.

5.5.2 Fe bioavailability in bread assayed using Caco-2 cell model

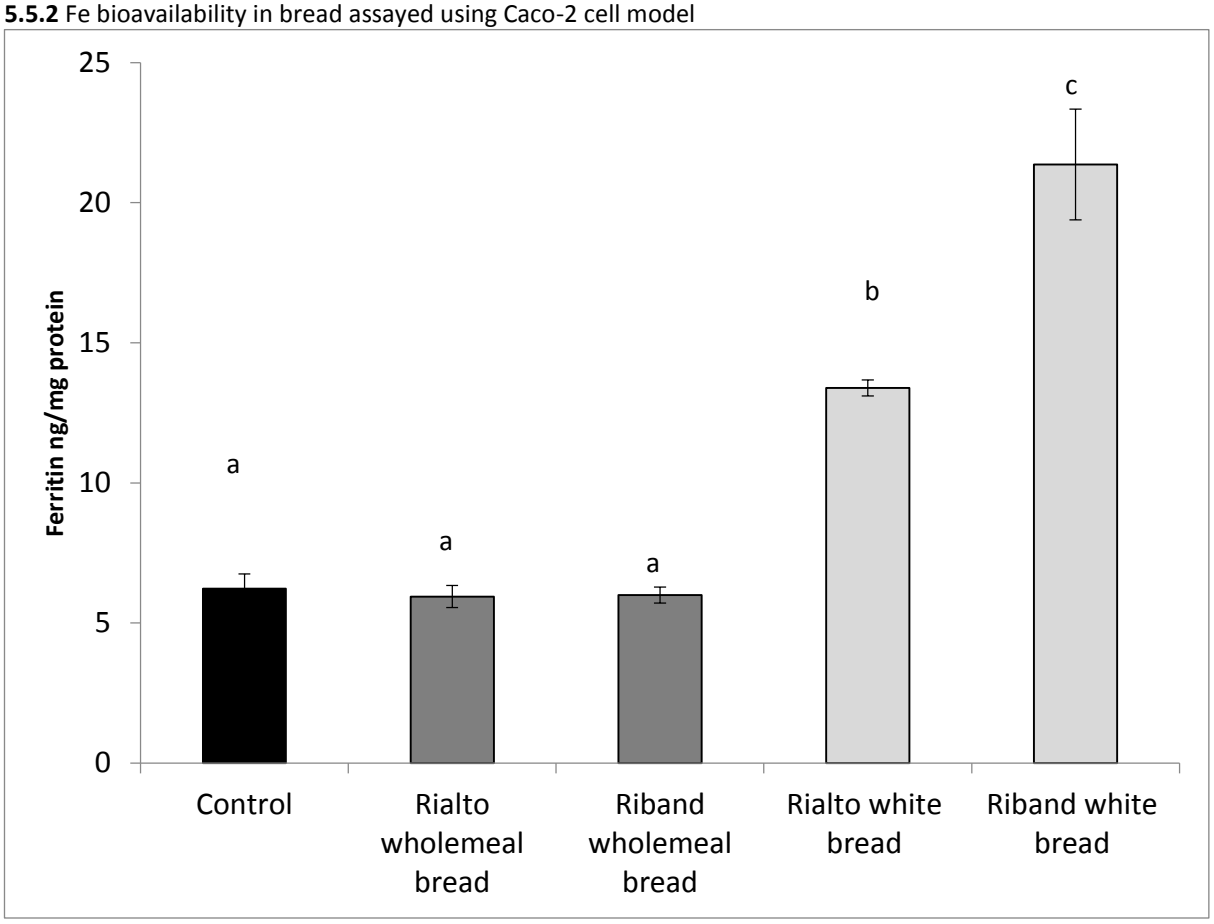


Figure 49. Caco-2 results for bread samples shows ferritin response of Caco-2 cells to 3 g of bread sample with ascorbic acid (132 µM). Results are from two independent experiments, Values (± *se*; *n* = 12) with no letters in common (a-c) are significantly different (LSD 5%)

Results from the Caco-2 analysis showed that neither wholemeal sample was able to initiate a response above that of the control (Fig. 2). Both samples of white flour however had a ferritin response significantly different from the control, with Riband significantly higher than Rialto ($P<0.01$).

5.3.2 Phytic acid concentrations

Figure 50. Amount of phytic acid in different breads. Data represent the mean ± SD (n= 3). Bars without a common letter (a−d) are significantly different, p< 0.05.

Figure 50 shows Results for phytic acid showed that Rialto wholemeal contained more phytic acid than Riband, this was most pronounced in the white bread, where Rialto had three times the total phytic acid.

5.3.3 Soluble Fe and macro minerals

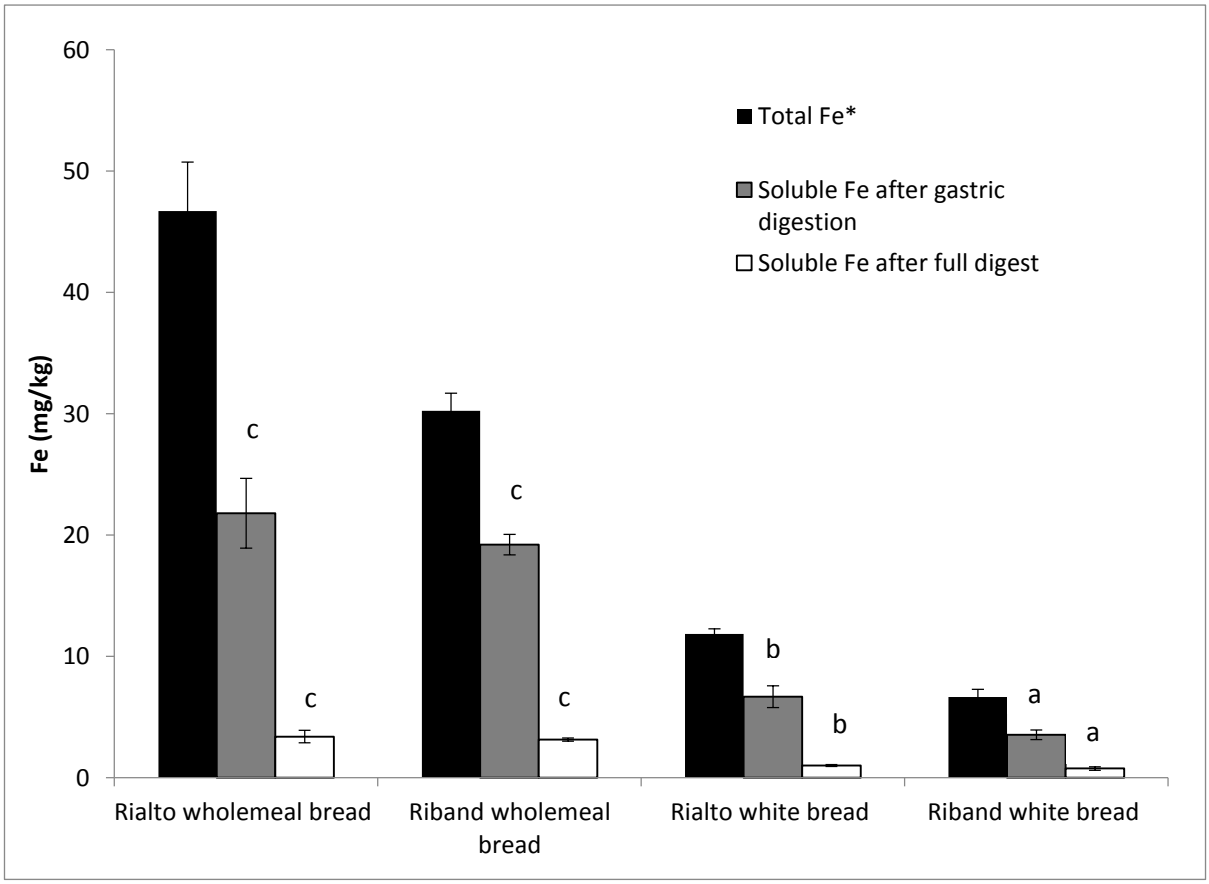


Figure 51. Amounts of soluble Fe at different stages of digestion. Total Fe is determined by ICP-AES ± represents mean of 3 biological replicates. Soluble Fe was determined by ICP-MS after an in vitro digestion ± represents mean of 3 technical replicates. Bars from the same stage of digestion without a common letter (a−c) are significantly different, $p < 0.05$.

During the gastric digestion, more than 50% of the total Fe became soluble in all samples (**Fig. 51**). This was reduced considerably with soluble Fe accounting for ~10% of the total Fe after full digestion in all samples. After gastric digestion, both wholemeal samples had more total soluble Fe than white bread samples ($P<0.05$) and there was no significant difference between the soluble Fe in the wholemeal samples, Rialto white bread was significantly higher in soluble Fe than Riband ($P<0.01$). After full digestion both wholemeal samples had more soluble Fe than white bread samples ($P<0.01$) but there was no significant difference between the two varieties in soluble Fe in wholemeal samples. In contrast, Rialto white bread was significantly higher in soluble Fe than Riband ($P<0.05$).

Table 5. Soluble minerals in full digestion, results were determined by ICP-AES analysis of digested samples. ± represents mean of 2 technical replicates. Values without common letter (a−d) are significantly different, p< 0.05 for that mineral.

mg/kg dw	Ca	Mg	P	S
Rialto wholemeal	47±5 *a*	574±10 *a*	2117±1 *a*	399±23 *a*
Riband whole meal	38±2 b	438±31 *b*	1811±41 *b*	481±35 *b*
Rialto white flour	47±13 *a*	113±4 *c*	314±16 *c*	386±13 *c*
Riband white flour	57±20 *a*	46±17 *d*	122±28 *d*	278±14 *d*

Table 5 shows the results for minerals where P and Mg were significantly different between all samples. Soluble phosphorus was much higher in wholemeal samples than white bread samples and Rialto had more soluble P and Mg than Riband in both breads. Soluble calcium was approximately ~ 15% of total Ca in all samples, and sulphur was ~50% of totals.

5.3.4 Iron speciation

Results for Fe speciation are shown as chromatograms, which are fully explained in **chapter 4**. Briefly, each peak represents a complex containing the target element, with the assumed molecular size of each complex decreasing from left to right. The higher the peak the greater the amount of complex in the soluble fraction.

The three major Fe peaks are summarised below (Table 2), following which the results of Fe speciation at different stages of a simulated digestion are illustrated. P speciation is also shown to, with the LMW peak representing inorganic P. The other peaks in the P chromatograms are assumed to be soluble forms of phytic acid (IP6) and its lower phosphorylation states (IP5-IP1). The organic P shown in these chromatograms will not exclusively be bound to Fe however as phytic acid also binds to magnesium, calcium, copper, Zinc and other minerals. However we were not able to look at these elements simultaneously as this would have reduced Fe sensitivity. It is also likely that some of the smaller organic P peaks are lower IPs not bound to any minerals.

Table 6. Identification of peaks in digested samples

Peak	Size (kDa)	Time	Assumed identification	Evidence for identification
High molecular weight (HMW)	75+	16-18	Likely to be Fe-bound to proteins, probably contains ferritin although unlikely to be exclusively ferritin	See (**chapter 4**)
Medium molecular weight (MMW)	12.4	25	Fe bound to soluble phytate (possibly bound as an oligomer containing both mono ferric phytate and Fe-IP-5)	Similar size to peak identified in Persson et al (*23*). Co-elutes with P peak indicating complexion. Elutes at the same time as mono-ferric phytate standard(**Appendix 7**)
Low molecular weight (LMW)	1.5	36	Fe bound as nicotainamine (Fe-NA) or 2'-Deoxymugineic Acid (Fe-DMA)	Elutes at similar time to standard of Fe-NA, is of similar size to a LMW identified in rice as FE-DMA/NA (*263*) . Elutes at the same time as a LMW complex in rice (**Fig. 29**) which was identified as a ligand of Fe-NA/DMA (*21*).(**Chapter4**)

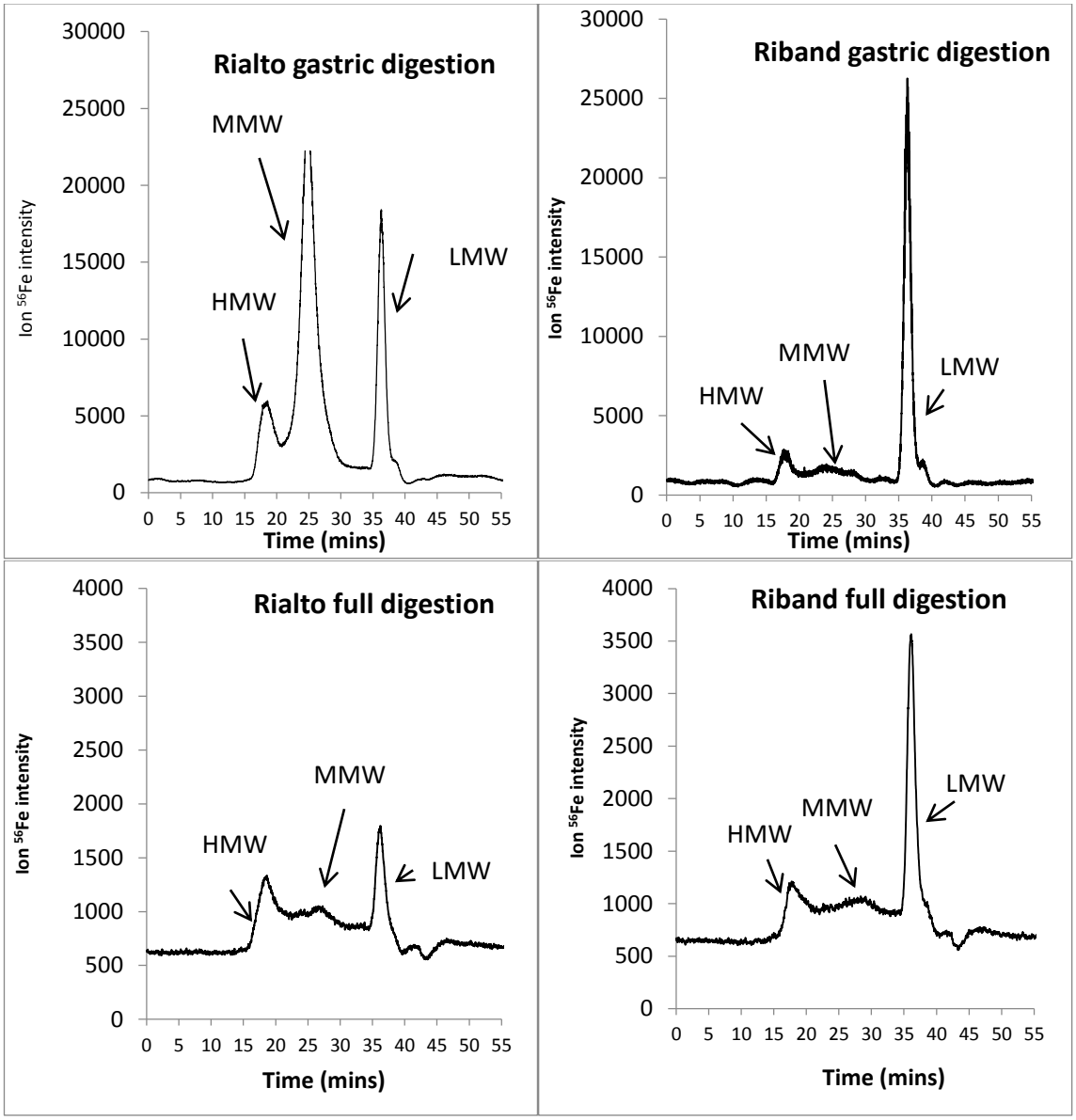


Figure 52. Iron speciation of white bread during digestion. Chromatograms for ^{56}Fe speciation of soluble fraction of 3g of white bread samples after gastric digestion or simulated full digestion, Results are means of 2 replicates.

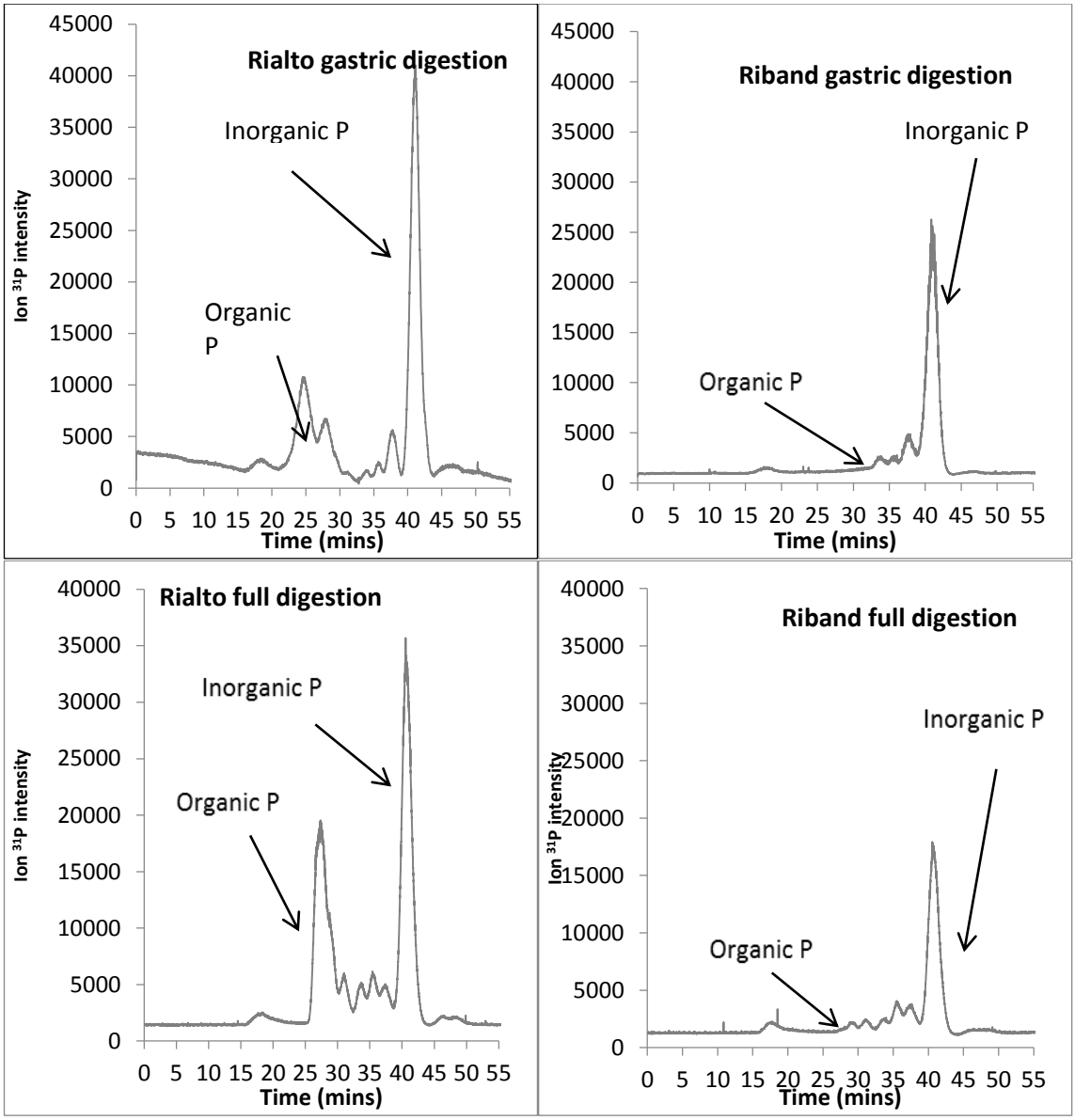


Figure 53. Phosphorous speciation in white bread during digestion. Chromatograms for ^{31}P speciation in the soluble fraction of 3 g of white bread samples after gastric digestion or simulated full digestion. Results are means of 2 replicates.

Speciation analysis showed that Rialto white flour contained soluble Fe-phytate, and the corresponding organic phosphorous during the gastric stages of digestion, while Riband did not (**Figs. 52, 53**). After full digestion it is less clear whether soluble Fe-phytate is still present at a detectable level in either Riband or Rialto. Iron bound to the LMW peak is present at all stages of digestion but makes up a larger portion of the total soluble Fe in Riband at both stages of digestion (**Figs. 52, 53**).

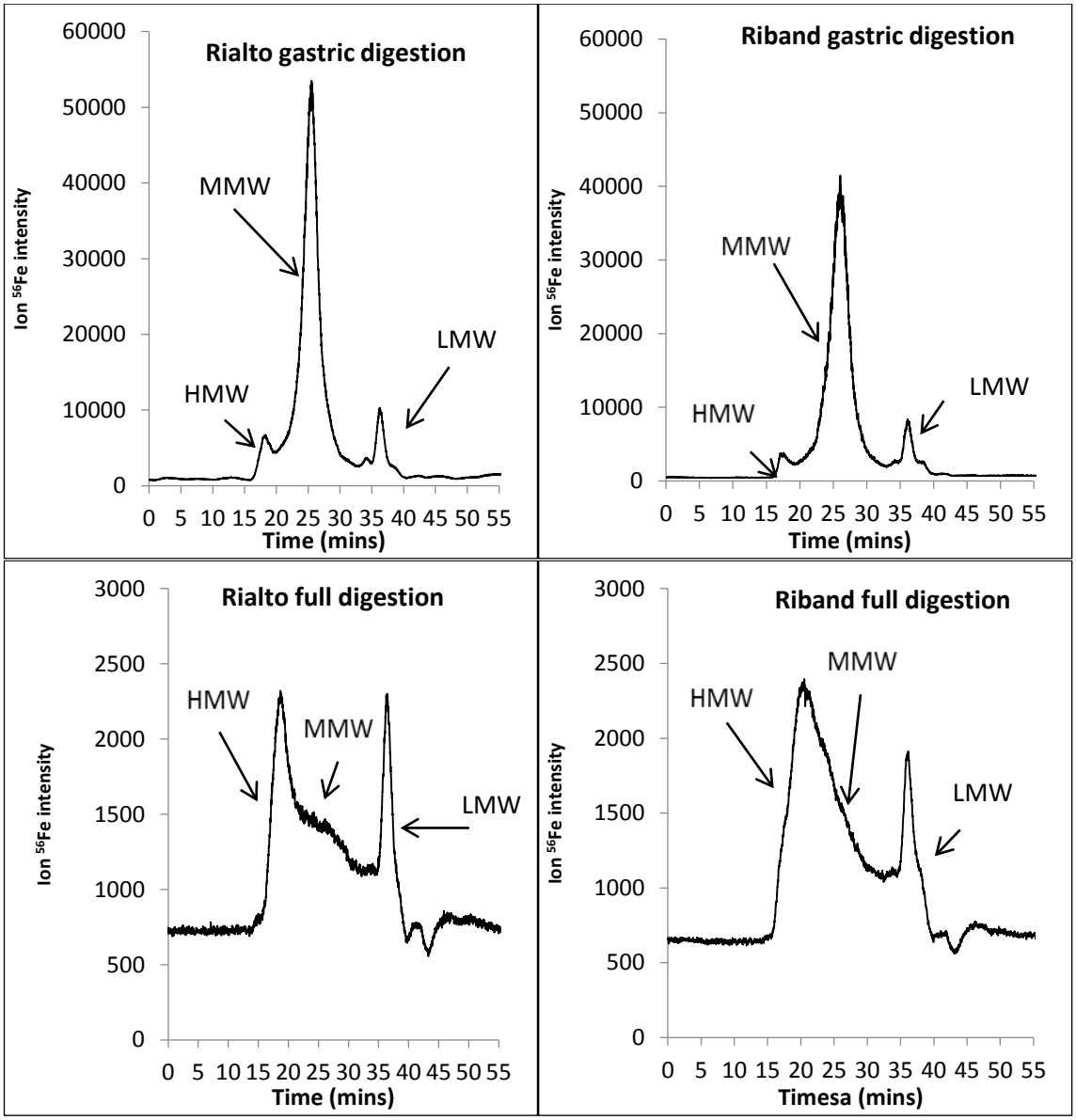


Figure 54. **Iron speciation of wholemeal bread during digestion**. Chromatograms for ^{56}Fe speciation of soluble fraction of 3 g of bread wholemeal samples after gastric digestion or full digestion, results are means of 2 replicates.

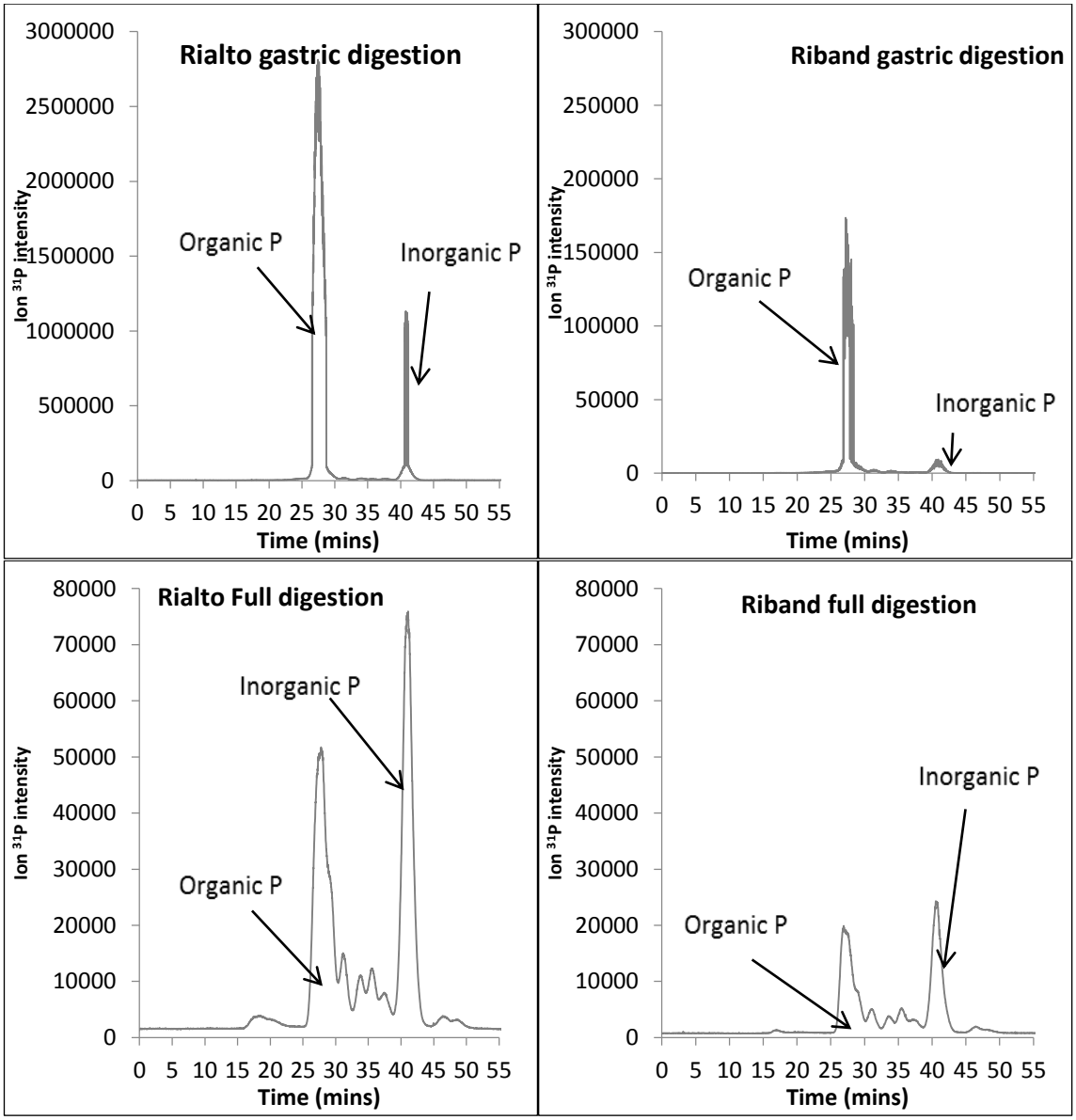


Figure 55. **Phosphorous speciation of wholemeal bread during digestion** .Chromatograms for ^{31}P speciation of soluble fraction of 3 g of wholemeal bread samples after gastric digestion or simulated full digestion, results are means of 2 replicates.

Speciation of Fe in wholemeal flour samples showed that during gastric digestion nearly all the Fe was bound to soluble phytate (MMW) (**Figs. 54, 55**), whereas after full digestion the Fe MMW peak is almost undetectable from the HMW which makes up the majority of soluble Fe (**Fig. 55**). Similar to white flour the intensity of all peaks is reduced at the later stage of digestion (**Figs. 54, 55**). Following the stomach digestion most P is in the organic form, whereas after the full digestion P is mainly in the inorganic form in both cultivars. There are no notable differences between cultivars for Fe or P speciation.

5.3.5 Iron bioavailability and speciation in fortified samples assayed using Caco-2

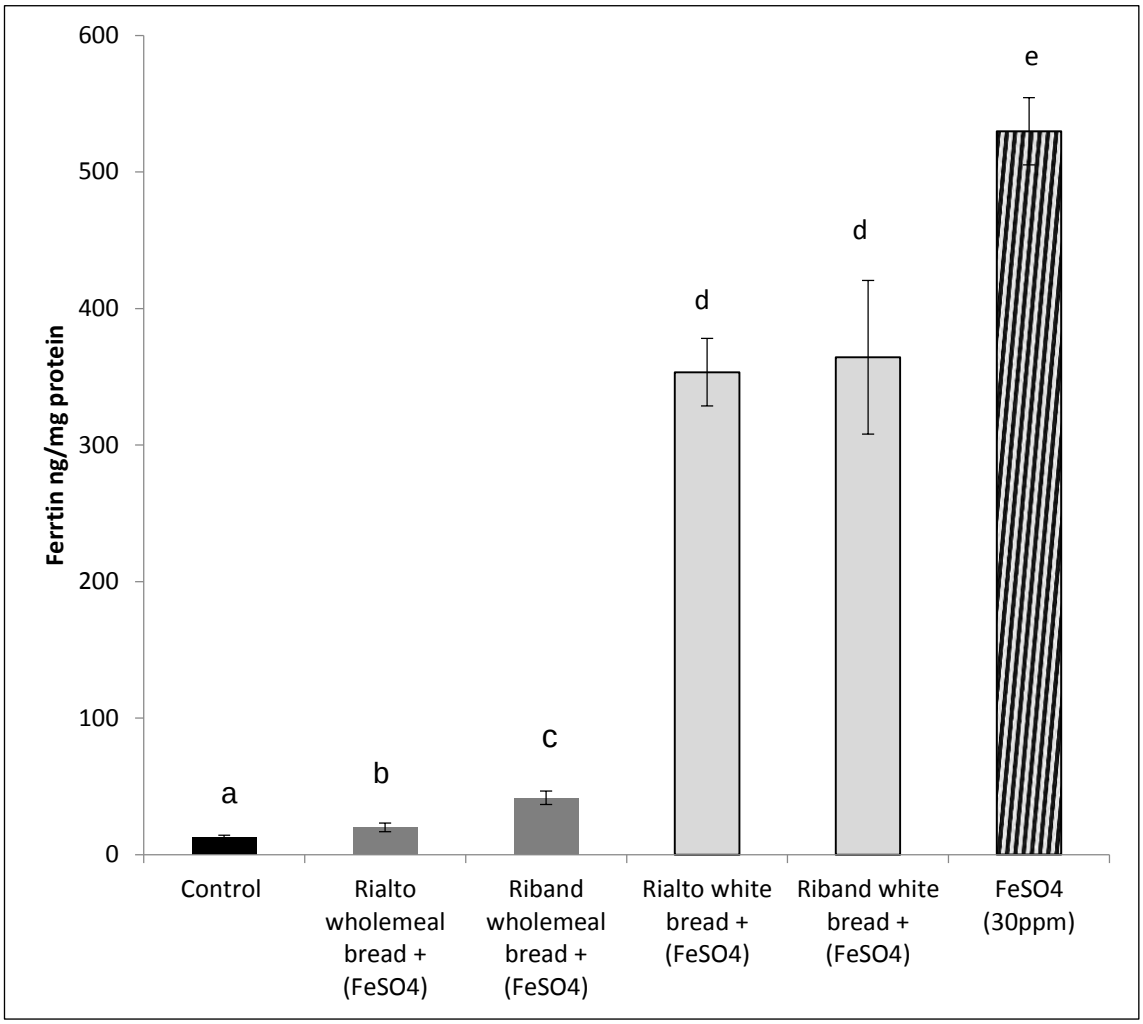


Figure 56. Bioavailability results for fortified bread samples. Shows ferritin response of Caco-2 cells to 1 g of bread sample with ascorbic acid (132 µM). $FeSO_4$ was added to all samples at 30µg/g with the far right bar representing $FeSO_4$ (30 µg) in solution without bread sample. Results are means from two independent experiments. Values (± *se*; *n* = 12) with no letters in common (a-e) are significantly different (LSD 5%).

Results for Caco-2 analysis showed that both Rialto and Riband wholemeal samples were able to initiate a response in Caco-2 cells significantly greater than the control (**Fig. 56**, P<0.01). Both white flour samples produced a ferritin response greater than the control and considerably higher than wholemeal, with Riband being significantly higher than Rialto (P<0.01) (**Fig. 56**). However all wheat samples produced a lower response than $FeSO_4$ on its own.

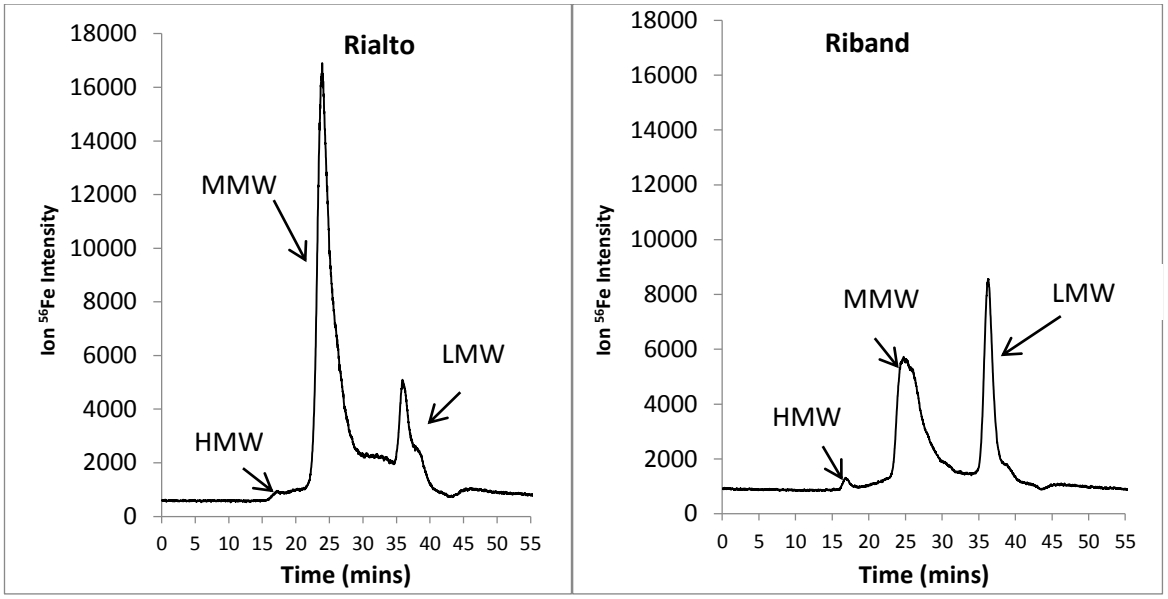


Figure 57. Iron speciation of fortified white bread after full simulated digestion. Chromatograms showing ^{56}Fe speciation in the soluble fraction resulting from 1 g of wholemeal bread samples after gastric digestion or full digestion. Results are means of 2 replicates.

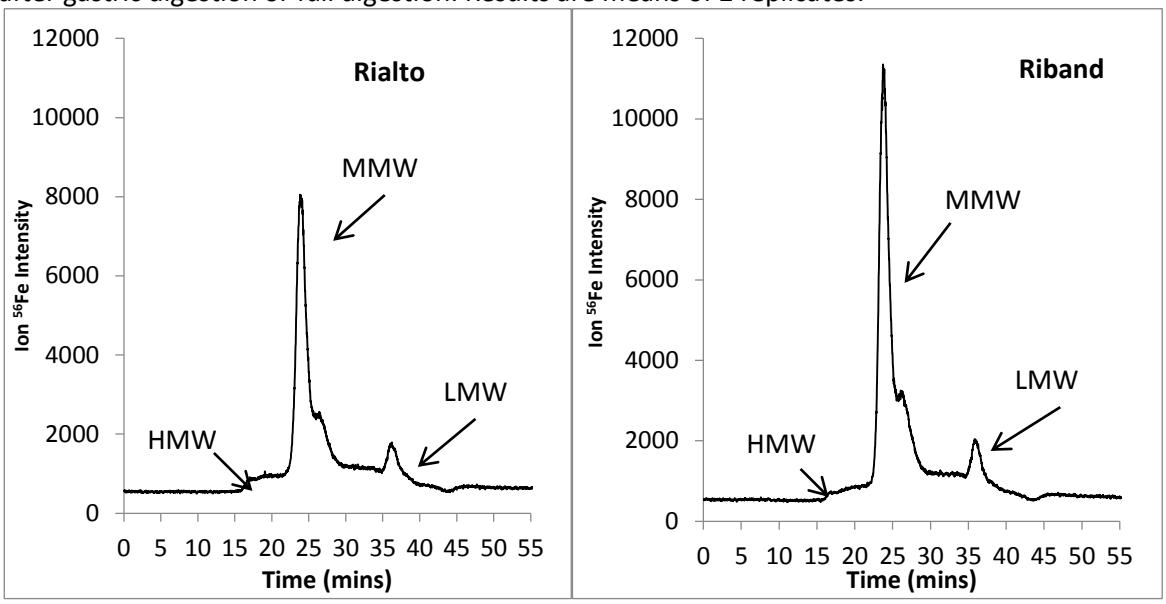


Figure 58. **Iron speciation of wholemeal bread after full simulated digestion** .Chromatograms for ^{56}Fe speciation of soluble fraction of 1 g of wholemeal bread samples after gastric digestion or full digestion, results are means of 2 replicates.

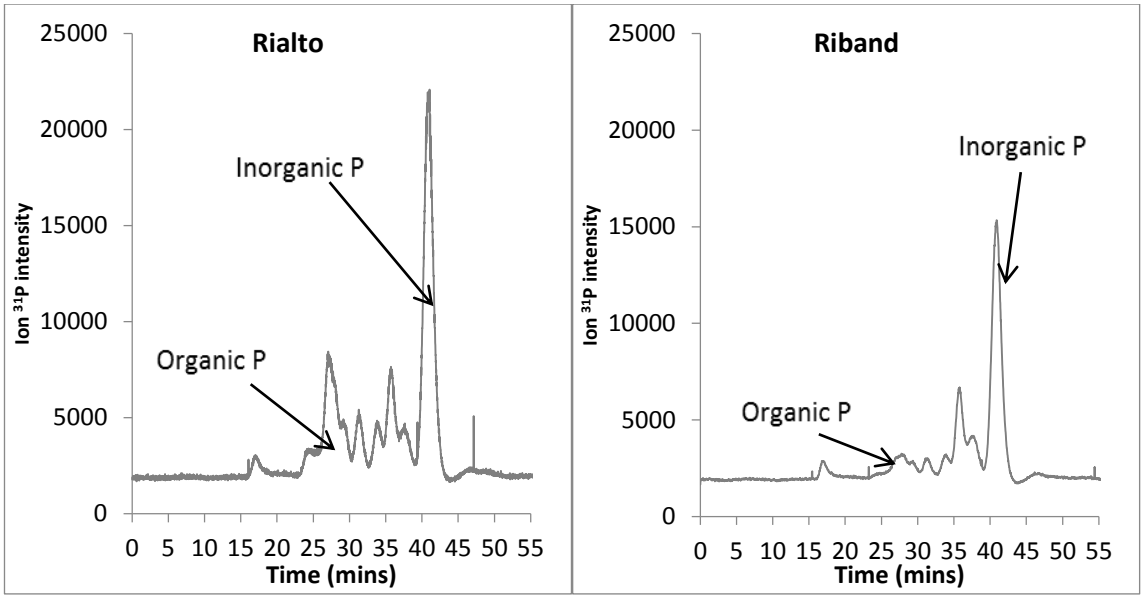


Figure 59. **Phosphorus speciation of white bread after full simulated digestion**. Shows chromatograms for ^{31}P speciation of soluble fraction of 1 g of wholemeal bread samples after gastric digest or full digestion, results are means of 2 replicates.

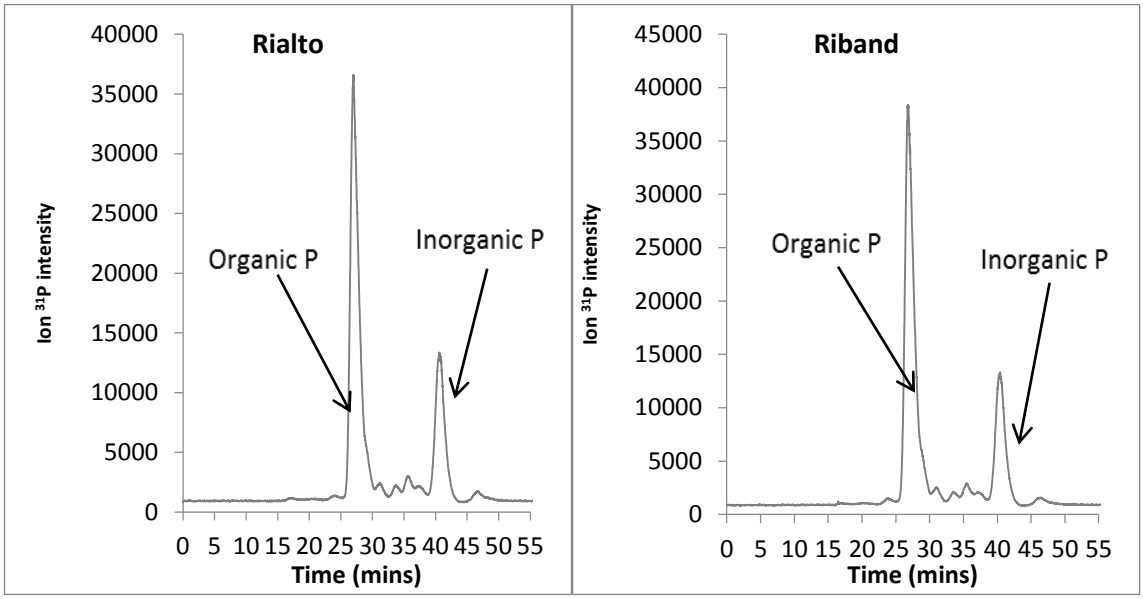


Figure 60. Phosphorus speciation of wholemeal bread after full simulated digestion. Chromatograms for ^{31}P speciation of soluble fraction of 1 g of wholemeal bread samples after gastric digest or full digestion. Data are means of 2 replicates.

Speciation analysis of fortified white bread showed that a much larger percentage of soluble Fe was bound to the low molecular weight form than in wholemeal, where nearly all the additional soluble Fe was bound to soluble phytate (**Figs. 57, 58, 59, 60**). White bread made from Riband had less Fe bound to soluble phytate compared to that from Rialto. Interestingly, organic P appeared to remain higher in fortified breads than in unfortified samples in wholemeal samples although this effect was not noticeable in white bread samples.

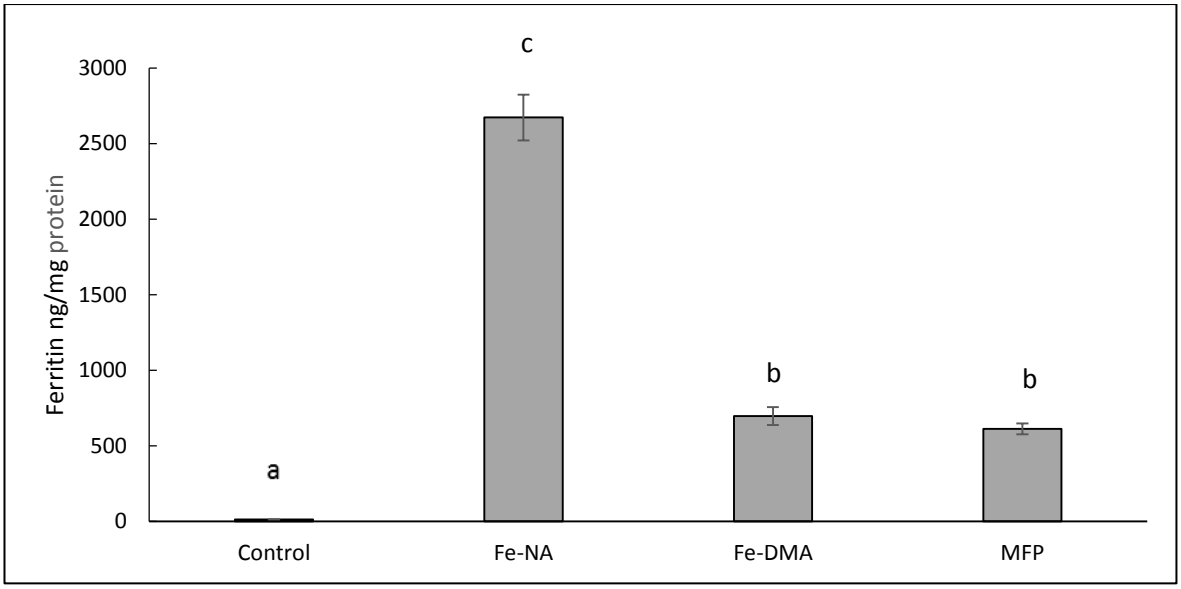


Figure 61. Bioavailability of synthetic Fe compounds. Shows ferritin response of Caco-2 cells to 8 ug of Fe. All compounds where made at a 1:1 molar ratio of nicotainamine (NA), 2'-deoxymugineic acid (DMA) and mono ferric phytate (MFP) .Values (± *se*; *n* = 12) with no letters in common (a-c) are significantly different (LSD 5%).

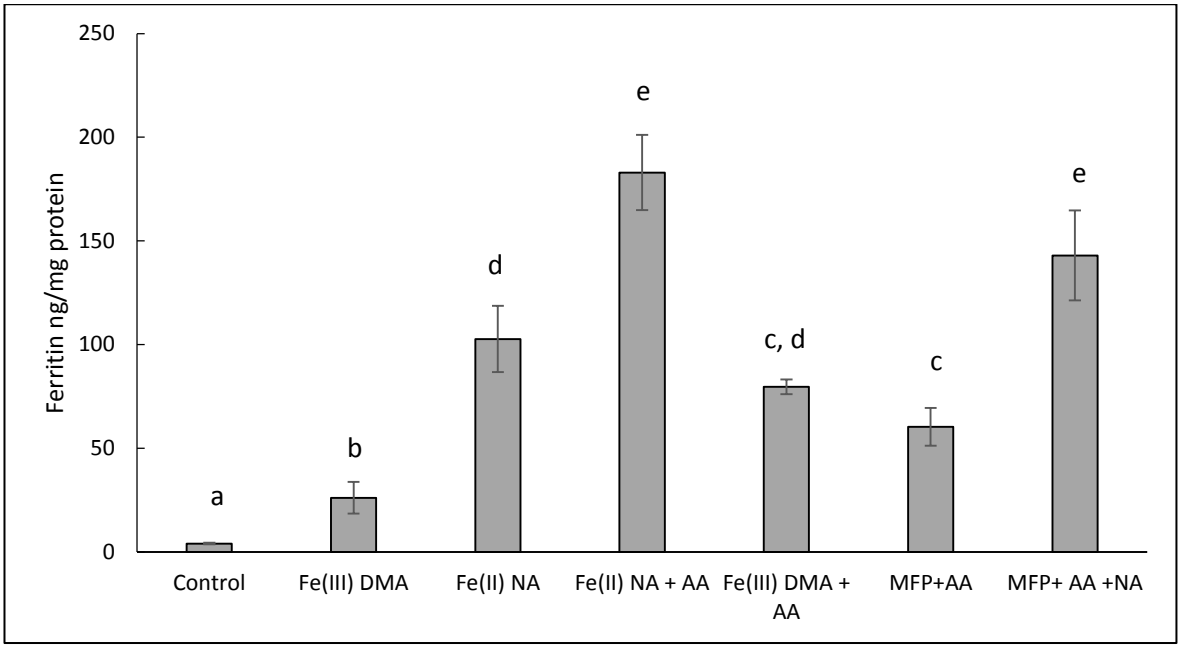


Figure 62. Bioavailability of synthetic Fe compounds with and without ascorbic acid. Shows ferritin response of Caco-2 cells to 1.6ug of Fe with or without ascorbic acid (AA) (132 µM).All compounds where made at a 1:1 molar ratio of nicotainamine (NA), 2'-deoxymugineic acid (DMA) and mono ferric phytate (MFP) .Values (± *se*; *n* = 6) with no letters in common (a-e) are significantly different (LSD 5%).

Figure 61 shows that in the absence of ascorbic acid Fe-NA was significantly more bioavailability then both Fe-DMA and mono ferric phytate. There was no significant difference between Fe-DMA and mono-ferric phytate. All forms of Fe were more bioavailable than control. Figure 62 shows

the effect of ascorbic acid on the same synthetic compounds, Fe with NA and AA was again higher than both DMA and mono ferric phytate. It should be noted that concentrations of Fe used were lower in second experiment therefore direct comparisons cannot be made between **Figures 61 and 62**.

5.4 Discussion

Results from experiments conducted in a Caco-2 model system (**Fig. 49**) showed that white flour was a better source of available Fe than wholemeal flour, which supported our hypothesis. One recent study by Lei et al (*264*) reported similar results; however this was for uncooked flour of different extraction rates. The results also showed that Fe in Riband white flour, despite having less total Fe and less soluble Fe after simulated digestion (**Fig. 50**), was significantly more bioavailable than that in Rialto white flour. This finding is in agreement with published findings in cereals (*13-15*) that total Fe and soluble Fe are not accurate indicators of Fe availability. The next challenge was therefore to determine why this was the case and why wholemeal bread despite containing ~3-5 fold the Fe content of our low extraction white flour (~40%) contained a great deal of unavailable Fe.

The link between phytic acid and Fe availability is well known (*116, 134, 135*). Phytic acid levels were as expected; higher in wholemeal bread samples. It has been reported before that white flour is much lower in phytic acid than wholemeal flour due to the fact that coarse bran and offal containing the highest amount of phytic acid (*265, 266*). Interestingly, the phytic acid content in the white bread samples was ~3 times higher in Rialto than in Riband. As discussed in chapter 1, phytate is almost exclusively located in the aleurone layer and therefore any phytate in white flour fractions is likely to be indicative of aleuronic contamination. This was also evident from Rialto's increased ash content (chapter 3), another marker for aleuronic contamination. Rialto, a hard wheat (hardness index 79), tends to respond differently to Riband, a soft wheat (hardness index 37), with respect to milling, with more aleurone layer ending up in the white flour fraction (*194*). Therefore it is likely that the differences in white flour observed here are due to this contamination rather than *in planta* cultivar differences in phytate localisation.

However, although from our limited data it is clear there is a link between Fe availability and phytate (**Figs. 49, 50**), it has previously been reported that phytate and Fe availability do not correlate well in larger samples of both wheat and rice and other factors also effect Fe availability.

(*13, 261, 264, 267*). Therefore we decided to try and explore in more detail how phytate was affecting Fe absorption and to what degree it was responsible for differences in Fe availability.

5.4.1. Effect of digestion and phytate on soluble Fe

During the gastric digestion (pH 2) it was observed that ~50% of the Fe was soluble in all samples (**Fig. 51**) and appeared to be mainly bound to soluble phytate (**Figs. 52-55**). However the amount of soluble Fe was greatly reduced during the intestinal digestion (pH 7). This is as expected as previous studies have shown that mineral-phytic acid salts are more soluble at acidic pH (*75, 135*). Schelmmer et al. (2009) found that two thirds of phytate was soluble in the stomach of pigs but this was reduced dramatically as the phytate entered the intestinal stage where the phytate salts precipitate (*135*), however no such studies have been performed in humans.

Speciation analysis allowed us to visualise this interaction more clearly. During the gastric stage of digestion most soluble Fe and P in wholegrain and Rialto white flour elutes at around 12.3 kDa, consistent with a soluble phytate oligomer (*23*). However this peak, and consequently the amount of total soluble Fe, is reduced during full intestinal digestion (**Figs. 51, 52, 54**). Notably Riband white flour, which contained considerable less total phytate (**Fig. 50**), did not have a detectable peak of either Fe or P during gastric digestion (**Fig. 50**) and all of the Fe and the majority of P was in the LMW form or inorganic P form respectively.

Results for soluble Fe in wholemeal bread after full digestion were comparable to similar work done on wheat products by Frontela et al. (*14*). Interestingly though results were more comparable with the uncooked samples than the cooked samples, this is likely due to the fact they used leavened bread (sour dough), in which fermentation is well known to reduce phytic acid (*258, 268, 269*), whereas the unleavened baking process would not have had much phytate reducing effect compared to the uncooked samples.

5.5.2. Phytic acid and Iron ratio effect on bioavailability

Chemical interactions between phytic acid and Fe, and consequently Fe bioavailability appear to be affected by the ratio of phytic acid to Fe (*14, 137, 146, 270*). Gahln et al. (2001) predicted that Fe:phytic acid ratios would have to be below 1:10 for Fe to avoid maximum inhibition of phytate. In our own samples Fe:phytic acid molar ratios were 1:5 and 1:8 for Riband and Rialto white bread samples respectively, and 1:13 and 1:12 for Riband and Rialto wholemeal bread respectively. This agrees with Gahln (2001), as we observe a dramatic increase in bioavailability of

the white bread samples (below 1:10 Fe:phyate ratio) despite the fact that they contained less total Fe, and no difference in Fe availability in our wholemeal samples. In our Fe fortified samples the ratios of Fe: phytate were obviously much higher with white bread samples and wholemeal samples, having ratios of 1:1 and 1:2 for Riband and Rialto and 1:6 and 1:7 respectively.

5.6.3. Effect of NA and DMA on Fe bioavailability

The LMW peak identified in Chapter 4 as an oligomer of nicotainamine (NA) and 2'-deoxymugineic acid (DMA) is also likely to have an effect on Fe bioavailability. NA, a precursor of DMA, has been shown to form strong chelates with Fe. It has been shown to act as a promoter to Fe availability using both synthetic Fe compounds and rice in a Caco-2 model (*121*). The mechanism for this observation was not explored by Zheng *et al.* (2010) although they found evidence that the mechanism is not the reduction of ferric Fe to its ferrous state as is the case with AA. Both NA and DMA are strong metal chelators and form stable complexes with Fe^{2+} and Fe^{3+} although the stability of these complexs is reduced at lower pH (*235*).

Studies in rice have shown that an elevated level of NA has been shown to improve bio-availability in mice (*22*). Lee *et al*. (*22*) also reported an increased LMW peak in their transgenic rice which they identified as Fe-NA.

The data on uncooked flour (**Chapter 4**) show that the low molecular weight peak containing NA/DMA as identified in rice is also present in wheat, and it remains present during digestion. The quantity of Fe bound to this peak also appears to correlate with bioavailability. In Riband white flour, for example, a considerably larger amount of Fe remains bound to Fe-DMA/NA, and consequently Riband is found to be more bioavailable. Both white flour peaks also have more Fe bound to LMW than wholemeal samples which were not bioavailable. The same is true for our Fe fortified samples where both white bread samples had a much higher amount of Fe bound as Fe-DMA/NA compared to the less bioavailable wholemeal samples.

Our data on the synthetic compounds showed NA to be a strong promoter of Fe bioavailability, this effect was further complemented by the addition of ascorbic acid (**Fig. 61**), indicating different mechanism of promoting Fe availability, as the amount of ascorbic acid used theoretically would have had a maximum effect of reducing Fe(III). Fe-DMA although shown to be available was not shown to be as available as Fe-NA (Fig). Further analysis of NA showed that NA

was a much greater enhancer of Fe bioavailability when compare to ascorbic acid and had maximum affect at a molar ration of 1:1 (**Appendix 9**)

5.6.4. Competitive binding of phytic acid and DMA and NA

During the second stage (intestinal) of digestion it is likely that considerable re-association of Fe occurs and the three main chelators, DMA/NA and phytate will compete for Fe. Initially it had been predicted that any Fe bound to NA and DMA would remain stable during digestion, as NA and DMA form very stable complexes with both Fe^{2+} and Fe^{3+}, more so than phytate (*55, 271, 272*). However analysis of Fe speciation during digestion appears to show a reduction in Fe-Na/DMA during digestion (**Figs. 53, 55**), indicating that phytic acid may be able to remove Fe from this complex, rendering it insoluble during the rise in pH in the later stages of digestion. A likely cause of this is that at gastric acidity (pH 2), the chelation capacity for Fe of NA and DMA is very low (*55*). Also this is the stage when most iron is soluble, therefore phytic acid, which is at a much higher concentration than NA or DMA, is probably able to compete and scavenge a large percentage of the Fe. However analysis of the synthetic compound of monoferic phytate with addition of an equal molar ratio of NA in the presence of ascorbic acid at pH 7, showed improved bioavailability compared to mono ferric phytate and ascorbic acid alone, indicating NA is able to compete with soluble phytate if the molar ratios are sufficient. The exact nature of this interaction needs further study if we are to quantify the effects of these chemicals on Fe bioavailability.

5.6.5. Effect of Fe Speciation on bio-availability

As shown in **figure 61** Fe-NA is very bioavailable and Fe-DMA has some degree of bioavailability, therefore one can assume that all the Fe in the LMW peak is bioavailable. Fe bound to phytate, which is insoluble at higher pH, will be completely unavailable. However in previous studies Fe bound as soluble phytate has been shown to have some degree of availability (*119, 136, 137*). Caco-2 analysis of a synthetic mono ferric phytate, made as described in Morris and Ellis et al. (*136*) which eluted at the same time for both Fe and P as the Fe-soluble phytate peak in SEC-ICP-MS (**Figs. 53, 55, 57, 59**) (**Appendix 7**), was shown to have some degree of bioavailability, approximately 1/3 of $FeCl_3$ (**Appendix 8**) and considerably less than Fe-NA (**Fig. 60**). Therefore the bioavailability of wheat is likely to be determined by the amount of both the MMW and the LMW peak, with the LMW peak having more weighting with regards to Fe availability.

5.6.6. Calcium

Calcium can act as inhibitor of Fe absorption. In all samples soluble Ca (**Table 5**) was similar and therefore unlikely to have been a factor in explaining differences in bioavailability.

5.6.7. Fortification

Fortification with Ferrous sulphate increased the amount of bioavailable Fe in all samples. Ferrous sulphate has been shown to be an effective fortificant of wheat products in many studies (*273, 274*), however concerns have been expressed about its effectiveness to fortify wholemeal bread (*111, 180, 275*) due to its inability to improve Fe status of people consuming it with wholemeal products. Low extraction flour was shown to be a much better vehicle for Fe fortification than high extraction flour. These results are similar to previous data from Caco-2 cells and wheat bread which showed a similar effect with elemental Fe powders (*274*). Although in Yeung *et al*. (*274*) the differences were less pronounced; this is likely to be due to the fact that in this study leavened bread was used and yeast fermentation has been shown to significantly reduce phytate (*268*). Consequently the phytate Fe ratios were not as different between low and high extraction flour as in unleavened samples.

One interesting result was that Riband responded better to fortification than Rialto for wholemeal flour (**Fig. 56**). It is not usual for manufacturers to consider the effect of genotype, as it is thought the level of fortification would negate any intrinsic Fe or matrix effect (*273*). However it was shown that this is not the case and cultivar genotype can actually have a considerable effect on the eventual bioavailability of fortified bread.

The relatively small increase in bioavailable Fe when compared to white bread of $FeSO_4$ on wholemeal bread (**Fig. 56**) is almost certainly due to the chelation of the additional Fe during the gastric stage by phytic acid. This would also explain why NaFeEDTA is a much better fortificant of wholemeal bread compared to $FeSO_4$, as Fe-EDTA complexes are still relatively stable at pH 2 and would protect the Fe from phytic acid.

Interestingly, the addition of Fe as $FeSO_4$ seemed to have an effect on the amount of organic and inorganic P after digestion; the organic P remained much higher. This is likely due to the effect of adding Fe making the phytate complexes more stable during digestion, although further studies are needed to fully understand this effect.

5.7 Limitations of study

Despite Caco-2 cells being the best *in vitro* model for estimating human Fe bioavailability, it can never be a substitute for human studies. One weakness of the Caco-2 cell model which is rarely considered and is almost certainly relevant is the pH of the duodenum at the site of Fe absorption can vary from 5.5 to 7 (*276, 277*) while in Caco-2 experiments the pH is fixed at 7. The data on Fe speciation clearly shows a strong effect of pH, therefore it is likely that in wholemeal bread much more Fe would remain soluble and therefore be available for digestion within duodenum. Lower pH has also been shown to improve availability of haem Fe and $FeCl_3$ (*277*).

Also the Caco-2 digestion is free of any microbial activity which is not the case within the human digestive system, and there is some evidence that human microflora may be able to produce phytase which would affect Fe bioavailability (*278*). Therefore, human studies should ideally be undertaken to confirm *in vitro* findings.

Although there are some clear patterns in the data described above, the limited number of samples means further studies should be performed with more cultivars to confirm our results. Also although it is clear that extraction rate is a contributing factor to Fe bio-availability only two extraction rates were examined. It would therefore be beneficial to look at more extraction rates and to find an optimum extraction rate for Fe bioavailability.

5.8 Implications of results

The initial hypothesis was proven to be correct; white flour was a better source of Fe than whole meal despite containing ~3 times the total iron. White flour was also shown to be a better vehicle for fortification with $FeSO_4$.

From the data it is clear that bioavailability of Fe is affected by a variety of factors and complex chemical interactions. However a simplistic approach to increasing bioavailability of fortified and unfortified samples may be to simply decrease the extraction rate of flour for bread making. Unfortunately this is unlikely to be commercially viable and would likely have other health implications. Currently, many public health agency advocate the consumption of whole grain products for their perceived health benefit. One of the health benefits often listed is the higher amount of minerals, specifically iron. This chapter and other work by Yeung *et al.* (2012) show

that in the case of unleavened bread this is probably not an accurate message and should be re-evaluated.

Recent epidemiological studies have shown a stark differences between the response of fortification of white flour and wholemeal wheat products to non-NaFeEDTA fortification (*111*). This study provides further evidence to this effect and offers some explanation on a chemical level as to why this is the case.

Despite numerous studies exploring fortification in cereal products (*107, 111, 118, 273, 275*), current UK fortification requirements still demand mandatory fortification of white flour and no fortification of wholemeal. This chapter provides some evidence that this too should be re-evaluated as it can be argued that the focus of fortification should be on all types of flour because of the issues with bioavailability and the desire to improve the Fe status of consumers of wholemeal as well as white flour product.

Chapter 6. General Discussion

6.1 Introduction

Prevalence of both Fe and Zn deficiency remain high with estimates of up to 60% of the world population being at risk of one or both deficiencies (*279*). Current recommended daily intake (RDI) in the US, and dietary reference values (DRV) in the UK, are used to determine how much Fe and Zn should be consumed for 98% of the population to avoid deficiency (*280*). These values however do not take into account the difference in bioavailability of Fe or Zn from different food sources and instead are based on an assumed bioavailability, which for Fe is 10% (*281*). This approach also forms the basis for fortification strategies across the world which in the case of Fe in wheat products means that white flour products are often mandatory fortified, but higher Fe containing wholemeal products are exempt (*114, 115*).

This relatively simplistic approach has in the past also been the basis for many biofortification strategies in which the end goal has been to increase the total amount of Fe and Zn within the grain. The initial aims of this thesis were concentrated not only on total Fe and Zn, but on three other factors which also need to be considered. The three factors which we initially aimed to explore are;

- **Location**
- **Speciation**
- **Bioavailability**

It is the authors belief from our results and previous studies that all these factors are each as important if not more important than total Fe and Zn concentration, when considering biofortification strategies. Bioavailability is also particularly relevant when one considers nutrition labelling of cereal based food products which is currently based on the assumed availably discussed above.

6.2 Location

The data on total concentrations of Fe and Zn in different milling fractions (**Chapter 3**) are in agreement with previous studies (*72, 103, 210*) showing that the white flour (made predominantly of starchy endosperm) is relatively low in Fe and Zn.

Although low in Fe and Zn, the endosperm is an ideal target for biofortification due to its widespread consumption (*3, 46, 67*), it is also an ideal tissue for Fe and Zn biofortification due to the food matrix. We demonstrated in **Chapter 4** that any Fe or Zn which is sequestered in the endosperm of wheat is speciated differently in the bran. In the endosperm SEC-ICP-MS and enzyme analysis demonstrated that the majority of Fe and Zn is bound either to insoluble protein or some combination of nicotainmine or 2'-deoxymugineic acid (**Fig 32, 45, 46, 47**). This Fe and Zn is likely to be more bioavailable than that in the outer fractions of wheat, where insoluble phytate-fibre complexes, soluble proteins and soluble phytate complexes form the majority of Fe species. Less was determined about Zn speciation but it is clear that similarly to Fe, there are marked differences in the speciation between these milling fractions (**Fig 32, 33**).

The endosperm and bran fractions also contain different amounts of anti-nutrients particularly phytate which is much higher in the outer fractions of the grain (**Fig 21, 50**). Therefore, in addition to having a more favourable speciation in the endosperm, Fe and Zn from this tissue are likely to be exposed to less anti-nutrients during digestion.

The bioavailability study in **Chapter 5** demonstrated that these differences have a much larger effect on bioavailability than would be predicted. The general consensus for public health professionals has historically been that although Fe in the endosperm is probably more available, the much lower concentration would mean that whole grain products are still a better source of Fe and Zn (*282, 283*). However we showed that Fe in white bread (made predominantly from endosperm) was considerable more bioavailable than in wholegrain, despite it containing ~4 times less total Fe (**Fig. 49, 51**). This is in agreement with a similar Caco-2 study recently performed on raw flour (*264*). Although we were unable to assess Zn, it is probable that a similar result would be obtained as Zn bioavailability is also thought be effected by speciation (*21*) and the presence of phytate (*284*) in a similar way to Fe.

Most cereals which form part of a modern diet undergo some degree of processing before consumption. This processing can involve polishing, milling, or pearling, all of which remove outer layers of the grain (*46, 285*). This practice is becoming more widespread, and processed or white

flour now accounts for the majority of cereal products consumed, both in the developed and developing world (*286*). However, as previously discussed in **Chapter 3,** the loss of the outer layers also accounts for a loss of the majority of the minerals present in the grain. Nevertheless, all flour including even the most highly processed 'patent flour' contains endosperm, therefore, the breeding of cereals with increased Fe and Zn within the endosperm is ideal for modern diets in the emerging world.

It should be noted that improving Fe and Zn concentration in the endosperm is likely to have limitations, unless the presence of adequate chelating mechanisms can also be enhanced (*46, 52, 153*). As the natural chelating capacity of the endosperm is likely to be very low, additional Fe and Zn may cause toxicity, unless they can be sequestered sufficiently (*88, 153*). This has been demonstrated in genetically modified crops with only crops with increased expression of a chelator such as nicotainamine (NA), or ferritin (*21, 33, 86, 147, 245*) showing dramatically improved Fe and Zn content within the endosperm.

6.3 Speciation

We have shown the speciation of Fe and Zn *in planta* to vary between wheat cultivars and milling fractions (**Chapter 4**). We have also shown that during digestion of wheat products Fe speciation is altered (**Chapter 5**); this is the first study to date to look at speciation of soluble Fe in cereals during digestion. Previous studies have used the amount of soluble Fe and Zn remaining after an *in vitro* digest to estimate the level of bioavailability (*14, 26, 100*), however these studies do not take into account the speciation of the Fe and Zn which are soluble after digestion.

The relevance of *in planta* speciation for determining potential bioavailability is dependent on understanding the relationship between *in planta* speciation and post digestion speciation. However, we have shown this relationship be very complex; one must consider the effect of any preparation, the effect of gastric pH and enzyme interactions, as well as the enzymes and pH shift in the intestinal stage of digestion. In addition to this, *in plant* metals are sequestered largely in the cell vacuoles (*208*), but during milling, baking, and digestion, de-compartmentalisation will occur and interaction with other intrinsic chemicals is likely to occur.

It is clear from our results that Fe, and probably also Zn, bound to DMA/NA does seem in part to survive the digestion process, therefore, flour samples with higher percentages of Fe or Zn in

these forms *in planta* are likely to still have some amount in this form, depending on the food matrix after digestion.

6.4 Bioavailability

As previously discussed, bioavailability is the amount of an ingested nutrient that is absorbed and is available for physiological function (*18*). Analysis of the synthetic compounds of three major soluble forms of Fe identified in digested wheat showed marked variation in bioavailability (**Fig. 60**). This highlights the importance of speciation after digestion as a determinant of potential bioavailability. Fe-NA was shown to be far more bioavailable then both mono-ferric phytate and Fe-DMA. The lack of bioavailability of DMA was unexpected due to its previously discussed carboxyl groups (**Chapter 1**), which have been shown to promote bioavailability, it is the authors belief that DMA's preferential binding to Fe (III) is responsible for this difference as Fe in this oxidation state is less bioavailable and the DMA is likely to protect the Fe (III) from reduction to its more available form Fe (II) during digestion.

The amount of soluble Fe and Zn leftover after *in vitro* digestion have often been used in the literature to predict bioavailability (*287*), However this relies on the assumption that all soluble Fe and Zn has the same bioavailability. Our combined study using SEC-ICP-MS and Caco-2 has proven this is not the case for Fe in unleavened bread. Large differences were observed in speciation, and consequently bioavailability of Fe, between white and wholemeal bread, demonstrating the importance of speciation studies post digestion when assessing breads without a proven means of assessing bioavailability.

Although the data on bioavailability is in reference to Fe, similar assumptions can be made about Zn availability; like Fe, NA has been shown to improve Zn availability and phytate has been shown to reduce Zn bioavailability. Therefore, tissue with a higher ratio of NA to phytic acid, such as that used in white flour, are likely to contain more bioavailable Zn than wholemeal products.

6.5.Rialto vs Riband: a case study

Throughout this study we have concentrated on two different cultivars of wheat in the hope of better understanding the genotypic differences in wheat. It is the comparison between the changes in Fe in these two cultivars which have formed the backbone of this study.

From our initial study on total Fe and Zn (**Chapter 3**), Rialto would appear to be the most nutritionally beneficial cultivar, as it contained more Fe and Zn. This was true for both white flour and wholegrain fractions. However, it should also be noted that Rialto had higher amounts of phytic acid and this was especially true in the white flour fraction.

Further speciation analysis in **Chapter 4** of white flour also showed that Rialto would be likely to be a better source of Fe as it contained 23% more soluble Fe than Riband. Speciation of this soluble fraction did however show that some of this additional Fe was bound as soluble phytate in Rialto, indicating that this portion of the soluble Fe may not be as available as the rest. Differences in soluble Zn were not significant and there were no notable differences in Zn speciation, indicating that despite Rialto's higher total Zn this may not have any effect on final bioavailability.

As previously mentioned bioavailability was not assessed for Zn, however our results for Fe highlight the need for further understanding of the journey Fe takes during the digestion system if we are to try and predict bioavailability. Analysis of soluble Fe during an *in vitro* digestion showed that in white flour Rialto had higher concentrations of soluble Fe at both stages of digestion; however there were no significant differences in wholegrain. Therefore, it would again be reasonable to predict that Rialto white flour is a better source of Fe than Riband.

It is not until one considers speciation during digestion in **Chapter 5** that we start to see the effect of Rialtos higher phytic acid concentration, this is especially true in bread made from white flour. During the gastric stage of digestion for Rialto white flour, large amounts of Fe are bound as soluble phytate, this is not the case for Riband. This appears to have an effect on the final speciation after full intestinal digestion, where it was shown that Riband has a much higher percentage of its final Fe bound as the LMW form, DMA/NA.

The consequence of this on bioavailability as measured by Caco-2 is clear and we see that despite the indications from total Fe, soluble Fe and speciation of raw white flour, it is Riband white bread which contains significantly more bioavailable Fe than Rialto. These results highlight the weakness

in using total Fe, or any speciation of raw and undigested samples, to predict bioavailability. The results for Fe highlight the need for further understanding of the journey Fe takes through the digestive system if we are to try and predict bioavailability in genotypes without expensive and complex *in vitro* speciation and/or bioavailability analysis.

6.6 Implications of results on strategies for improving Fe and Zn global status

6.6.1 Importance of nicotainamine

From our data and previous similar studies, it is clear that NA is very important in determining Fe availability in cereals. Previous studies have shown that NA not only enhances transport to the cereal grain of both Fe and Zn (*22, 40, 86, 121, 152*), but also increases bioavailability of both Fe (*22, 121*) and Zn (*245*).

We have further shown that:

- NA may also act to protect Fe during digestion by forming a stable complex, and reduce the negative effects of phytic acid. (**Chapter 5**)
- NA enhances Fe absorption with optimum effect at 1:1, which is the proposed structure for Fe (II)-NA (*288*)(**Chapter 5**).
- NA increases the capacity of a food to recruit Fe from its surroundings (**Chapter 4**).

6.6.2 Conventional Breeding

Our results show that there is variation in the germplasm for total Fe and Zn, within both wholegrain and white flour (**Chapter 3**). This indicates potential to carry out focused breeding for cultivars with higher endosperm Fe and Zn. However, more investigation is required if we are to breed for crops with more bioavailable Fe and Zn. One possible strategy may be to select cultivars with low phytate and high Fe and Zn, however variation in phytic acid among wheat cultivars is small (*103, 265*). Another possibility is breeding crops with increased concentrations of NA. Our results showed that the percentage of Fe as Fe-NA was 5.2% and 7.3% in Rialto and Riband respectively. To date there are no reports of total nicotainamine levels in the germplasm of wheat, however if sufficient variation can be found it would be an ideal target for breeding strategies.

6.6.3 Agronomic fortification

Although our foliar application did not enrich total Fe and Zn, previous studies have shown agronomic fortification to be an effective strategy for increasing total Fe and Zn (*3, 289*), one study has also looked in detail at the final location of both Fe and Zn in grain demonstrating that it is an effective strategy for endosperm enrichment (67). However, to date, there are limited studies on speciation and bioavailability of additional minerals in cereals due to fertilisation; this is an area for further research if the use of agronomic fertilisation is to be validated.

6.6.4 Biotechnological approaches.

Although many biotechnical approaches report increased Fe and Zn in grain, and in some cases in the endosperm, more work should be done to asses potential bioavailability and post digestion speciation, if we are to understand if and how various modifications could improve Fe and Zn availability. The most promising method to date appears to be enhancing NA in rice, which is in agreement with our results demonstrating the importance of NA, however at time of writing there has been no published attempts to overexpress *nicotianamine synthase* (NAS) in wheat or other staple cereal crops.

6.6.5 Fortification

Although biofortification shows promise, in the short-term scientists are unlikely to achieve the levels of improvement which can be achieved by fortification (*110*). Therefore, fortification remains the major strategy employed to tackle Fe deficiency (*111*). Our results show that white and wholemeal flour fortified with ferrous sulphate have very different levels of bioavailability, with fortified white flour being considerably more available, this is in agreement with recent epidemiological studies looking at the effectiveness of various fortification strategies (*111*). We have also shown for the first time why wholemeal flour does not respond as well to fortification with ferrous sulphate, by monitoring speciation changes of fortified flour.

Historically the effect of the food matrix on potential forticants has been ignored; however we have shown that the chemical properties of the target food can drastically affect bioavailability, providing more evidence that current fortification strategies need to be reassessed to take this into account. The effect of the food matrix also implies that crops genetically modified to have lower anti-nutrients such as phytate and more pro nutrients, for instance NA, will be much better vehicles for fortification.

6.6.6 Nutrition Information food labelling

Cereal products (both fortified and unfortified) such as bread and breakfast cereals in the UK will often include information about their mineral content and how they relate to RDI or DRV. However this information is entirely based on total Fe based on an assumption of 10% availability. From our results and previous studies it is clear that different milling fractions of wheat, and probably other cereals have vastly different level of bioavailability and therefore it is the authors' belief that this should be considered if the information provided is to be used to help the consumer

References

1. Evenson, R. E.; Gollin, D., Assessing the impact of the Green Revolution, 1960 to 2000. *Science* **2003,** *300*, 758-762.
2. Fischer, T. R.; Byerlee, D.; Edmeades, G. O., *Can technology deliver on the yield challenge to 2050?* 2011; p 389-462.
3. Cakmak, I.; Pfeiffer, W. H.; McClafferty, B., REVIEW: Biofortification of Durum wheat with zinc and iron. *Cereal Chemistry Journal* **2010,** *87*, 10-20.
4. Zhao, F. J.; Su, Y. H.; Dunham, S. J.; Rakszegi, M.; Bedo, Z.; McGrath, S. P.; Shewry, P. R., Variation in mineral micronutrient concentrations in grain of wheat lines of diverse origin. *Journal of Cereal Science* **2009,** *49*, 290-295.
5. Oury, F. X.; Leenhardt, F.; Rémésy, C.; Chanliaud, E.; Duperrier, B.; Balfourier, F.; Charmet, G., Genetic variability and stability of grain magnesium, zinc and iron concentrations in bread wheat. *European Journal of Agronomy* **2006,** *25*, 177-185.
6. Fan, M.-S.; Zhao, F.-J.; Fairweather-Tait, S. J.; Poulton, P. R.; Dunham, S. J.; McGrath, S. P., Evidence of decreasing mineral density in wheat grain over the last 160 years. *Journal of Trace Elements in Medicine and Biology* **2008,** *22*, 315-324.
7. Garvin, D. F.; Welch, R. M.; Finley, J. W., Historical shifts in the seed mineral micronutrient concentration of US hard red winter wheat germplasm. *Journal of the Science of Food and Agriculture* **2006,** *86*, 2213-2220.
8. Monasterio, I.; Graham, R. D., Breeding for trace minerals in wheat. *Food & Nutrition Bulletin* **2000,** *21*, 392-396.
9. Nestel, P.; Bouis, H. E.; Meenakshi, J. V.; Pfeiffer, W., Biofortification of staple food crops. *J. Nutr.* **2006,** *136*, 1064-1067.
10. Grusak, M. A., Enhancing mineral content in plant food products. *J Am Coll Nutr* **2002,** *21*, 178S-183.

11. Blair, M. W.; Monserrate, F.; Beebe, S. E.; Restrepo, J.; Flores, J. O., Registration of high mineral common bean germplasm lines NUA35 and NUA56 from the Red-Mottled Seed Class. *J. Plant Reg.* **2010,** *4*, 55-59.
12. Petry, N.; Egli, I.; Gahutu, J. B.; Tugirimana, P. L.; Boy, E.; Hurrell, R., Stable iron isotope studies in rwandese women indicate that the common bean has limited potential as a vehicle for iron biofortification. *The Journal of Nutrition* **2012,** *142*, 492-497.
13. Glahn, R. P.; Cheng, Z.; Welch, R. M.; Gregorio, G. B., Comparison of iron bioavailability from 15 Rice genotypes: Studies using an in itro digestion/Caco-2 cell culture model. *Journal of Agricultural and Food Chemistry* **2002,** *50*, 3586-3591.
14. Frontela, C.; Ros, G.; Martinez, C., Phytic acid content and "in vitro" iron, calcium and zinc bioavailability in bakery products: The effect of processing. *Journal of Cereal Science* **2011,** *54*, 173-179.
15. Glahn, R., *The use of Caco-2 cells in defining nutrient bioavailability: application to iron bioavailability of foods*. 2009; p 340-361.
16. Lowe, N. M.; Fekete, K.; Decsi, T., Methods of assessment of zinc status in humans: a systematic review. *The American Journal of Clinical Nutrition* **2009,** *89*, 2040S-2051S.
17. Hooper, L.; Ashton, K.; Harvey, L. J.; Decsi, T.; Fairweather-Tait, S. J., Assessing potential biomarkers of micronutrient status by using a systematic review methodology: methods. *The American Journal of Clinical Nutrition* **2009,** *89*, 1953S-1959S.
18. Fairweather-Tait, S. J., Bioavailability of trace elements. *Food Chemistry* **1992,** *43*, 213-217.
19. Davila-Hicks, P.; Theil, E. C.; Lonnerdal, B., Iron in ferritin or in salts (ferrous sulfate) is equally bioavailable in nonanemic women. *Am J Clin Nutr* **2004,** *80*, 936-940.
20. Lonnerdal, B., The importance and bioavailability of phytoferritin-bound iron in cereals and legume foods. *Int. J. Vitam. Nutr. Res.* **2007,** *77*, 152-157.
21. Lee, S.; Persson, D. P.; Hansen, T. H.; Husted, S.; Schjoerring, J. K.; Kim, Y.-S.; Jeon, U. S.; Kim, Y.-K.; Kakei, Y.; Masuda, H.; Nishizawa, N. K.; An, G., Bio-available zinc in rice seeds is increased by activation tagging of nicotianamine synthase. *Plant Biotechnology Journal* **2011,** *9*, 865-873.
22. Lee, S.; Jeon, U. S.; Lee, S. J.; Kim, Y.-K.; Persson, D. P.; Husted, S. r.; Schioerring, J. K.; Kakei, Y.; Masuda, H.; Nishizawa, N. K.; An, G., Iron fortification of rice seeds through activation of the nicotianamine synthase gene. *Proceedings of the National Academy of Sciences* **2009,** *106*, 22014-22019.
23. Persson, D. P.; Hansen, T. H.; Laursen, K. H.; Schjoerring, J. K.; Husted, S., Simultaneous iron, zinc, sulfur and phosphorus speciation analysis of barley grain tissues using SEC-ICP-MS and IP-ICP-MS. *Metallomics* **2009,** *1*, 418-426.
24. Singh, S. P.; Vogel-Mikus, K.; Pelicon, P.; Vavpetic, P.; Jeromel, L.; Feng, R.; Chibbar, R.; Roy, J. K.; Mantri, S. S.; Kumar, J.; Tuli, R., New insights into iron transport from maternal tissues to endosperm in mature wheat seed using synchrotron radiation. *Quality Assurance and Safety of Crops & Foods* **2012,** *4*, 141-141.
25. Sharpe, L. M.; Peacock, W. C.; Cooke, R.; Harris, R. S., The effect of phytate and other food factors on iron absorption. *Journal of Nutrition* **1950,** *41*, 433-446.
26. Simpson, C. J.; Wise, A., Binding of zinc and calcium to inositol phosphates (phytate) in vitro. *British Journal of Nutrition* **1990,** *64*, 225-232.
27. Lönnerdal, B., Dietary factors influencing zinc absorption. *The Journal of Nutrition* **2000,** *130*, 1378S-1383S.
28. Nahapetian, A.; Bassiri, A., Variations in concentrations and interrelations of phytate, phosphorus, magnesium, calcium, zinc, and iron in wheat varieties during two years. *Journal of Agricultural and Food Chemistry* **1976,** *24*, 947-950.
29. Rout, G. R.; Das, P., Effect of metal toxicity on plant growth and metabolism. *Agronomie* **2003,** *23*, 3-11.

30. Curie, C.; Cassin, G.; Couch, D.; Divol, F.; Higuchi, K.; Le Jean, M.; Misson, J.; Schikora, A.; Czernic, P.; Mari, S., Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Annals of Botany* **2009,** *103*, 1-11.
31. Palmer, C. M.; Guerinot, M. L., Facing the challenges of Cu, Fe and Zn homeostasis in plants. *Nat Chem Biol* **2009,** *5*, 333-340.
32. Briat, J., Iron dynamics in plants. *Advances in Botanical Research* **2007,** *46*, 137 - 180.
33. Paul, S.; Ali, N.; Sarkar, S.; Datta, S.; Datta, K., Loading and bioavailability of iron in cereal grains. *Plant Cell Tiss Organ Cult* **2013,** *113*, 363-373.
34. Darbani, B.; Briat, J.-F.; Holm, P. B.; Husted, S.; Noeparvar, S.; Borg, S., Dissecting plant iron homeostasis under short and long-term iron fluctuations. *Biotechnology Advances* **2013**.
35. Kim, S. A.; Punshon, T.; Lanzirotti, A.; Li, L.; Alonso, J. M.; Ecker, J. R.; Kaplan, J.; Guerinot, M. L., Localization of iron in arabidopsis seed requires the vacuolar membrane transporter VIT1. *Science* **2006,** *314*, 1295-1298.
36. Suzuki, M.; Takahashi, M.; Tsukamoto, T.; Watanabe, S.; Matsuhashi, S.; Yazaki, J.; Kishimoto, N.; Kikuchi, S.; Nakanishi, H.; Mori, S.; Nishizawa, N. K., Biosynthesis and secretion of mugineic acid family phytosiderophores in zinc-deficient barley. *The Plant Journal* **2006,** *48*, 85-97.
37. Zheng, L.; Huang, F.; Narsai, R.; Wu, J.; Giraud, E.; He, F.; Cheng, L.; Wang, F.; Wu, P.; Whelan, J.; Shou, H., Physiological and transcriptome analysis of iron and phosphorus interaction in rice seedlings. *Plant Physiology* **2009,** *151*, 262-274.
38. Ma, J. F.; Taketa, S.; Chang, Y. C.; Takeda, K.; Matsumoto, H., Biosynthesis of phytosiderophores in several Triticeae species with different genomes. *Journal of Experimental Botany* **1999,** *50*, 723-726.
39. Ishimaru, Y.; Suzuki, M.; Tsukamoto, T.; Suzuki, K.; Nakazono, M.; Kobayashi, T.; Wada, Y.; Watanabe, S.; Matsuhashi, S.; Takahashi, M.; Nakanishi, H.; Mori, S.; Nishizawa, N. K., Rice plants take up iron as an Fe3+-phytosiderophore and as Fe2+. *The Plant Journal* **2006,** *45*, 335-346.
40. Cheng, L.; Wang, F.; Shou, H.; Huang, F.; Zheng, L.; He, F.; Li, J.; Zhao, F. J.; Ueno, D.; Ma, J. F.; Wu, P., Mutation in nicotianamine aminotransferase stimulated the Fe(II) acquisition system and led to iron accumulation in rice. *Plant Physiol* **2007,** *145*, 1647-57.
41. Kutman, U.; Kutman, B.; Ceylan, Y.; Ova, E.; Cakmak, I., Contributions of root uptake and remobilization to grain zinc accumulation in wheat depending on post-anthesis zinc availability and nitrogen nutrition. *Plant and Soil* **2012,** *361*, 177-187.
42. Grotz, N.; Guerinot, M., Molecular aspects of Cu, Fe and Zn homeostasis in plants. *Biochim Biophys Acta* **2006,** *1763*, 595 - 608.
43. Schaaf, G.; Ludewig, U.; Erenoglu, B. E.; Mori, S.; Kitahara, T.; von Wirén, N., ZmYS1 Functions as a proton-coupled symporter for phytosiderophore- and nicotianamine-chelated metals. *Journal of Biological Chemistry* **2004,** *279*, 9091-9096.
44. Morrissey, J.; Guerinot, M. L., Iron uptake and transport in plants: The Good, the Bad, and the Ionome. *Chemical Reviews* **2009,** *109*, 4553-4567.
45. Durrett, T. P.; Gassmann, W.; Rogers, E. E., The FRD3-Mediated Efflux of Citrate into the root vasculature is necessary for efficient iron translocation. *Plant Physiol.* **2007,** *144*, 197-205.
46. Borg, S.; Brinch-Pedersen, H.; Tauris, B.; Holm, P., Iron transport, deposition and bioavailability in the wheat and barley grain. *Plant and Soil* **2009,** *325*, 15-24.
47. Rellan-Alvarez, R.; Giner-Martinez-Sierra, J.; Orduna, J.; Orera, I.; Rodriguez-Castrillon, J. A.; Garcia-Alonso, J. I.; Abadia, J.; Alvarez-Fernandez, A., Identification of a Tri-Iron(III), Tri-Citrate Complex in the Xylem Sap of Iron-Deficient Tomato Resupplied with Iron: New Insights into Plant Iron Long-Distance Transport. *Plant and Cell Physiology* **2010,** *51*, 91-102.
48. Yokosho, K.; Yamaji, N.; Ueno, D.; Mitani, N.; Ma, J. F., OsFRDL1 Is a citrate transporter required for efficient translocation of iron in rice. *Plant Physiol.* **2009,** *149*, 297-305.
49. Palmgren, M.; Clemens, S.; Williams, L.; Kraemer, U.; Borg, S.; Schjorring, J.; Sanders, D., Zinc biofortification of cereals: problems and solutions. *Trends in Plant Science* **2008,** *13*, 464 - 473.

50. Palmgren, M. G.; Clemens, S.; Williams, L. E.; Krämer, U.; Borg, S.; Schjørring, J. K.; Sanders, D., Zinc biofortification of cereals: problems and solutions. *Trends in Plant Science* **2008,** *13*, 464-473.
51. Suzuki, M.; Tsukamoto, T.; Inoue, H.; Watanabe, S.; Matsuhashi, S.; Takahashi, M.; Nakanishi, H.; Mori, S.; Nishizawa, N., Deoxymugineic acid increases Zn translocation in Zn-deficient rice plants. *Plant Molecular Biology* **2008,** *66*, 609-617.
52. Tauris, B.; Borg, S.; Gregersen, P. L.; Holm, P. B., A roadmap for zinc trafficking in the developing barley grain based on laser capture microdissection and gene expression profiling. *J. Exp. Bot.* **2009,** *60*, 1333-1347.
53. Grusak, M. A.; DellaPenna, D.; Welch, R. M., Physiologic processes affecting the content and distribution of phytonutrients in plants. *Nutrition Reviews* **1999,** *57*, 27-33.
54. Ghandilyan, A.; Vreugdenhil, D.; Aarts, M. G. M., Progress in the genetic understanding of plant iron and zinc nutrition. *Physiologia Plantarum* **2006,** *126*, 407-417.
55. von Wiren, N.; Klair, S.; Bansal, S.; Briat, J.-F.; Khodr, H.; Shioiri, T.; Leigh, R. A.; Hider, R. C., Nicotianamine chelates both FeIII and FeII. implications for metal transport in plants. *Plant Physiol.* **1999,** *119*, 1107-1114.
56. Inoue, H.; Higuchi, K.; Takahashi, M.; Nakanishi, H.; Mori, S.; Nishizawa, N. K., Three rice nicotianamine synthase genes, OsNAS1, OsNAS2, and OsNAS3 are expressed in cells involved in long-distance transport of iron and differentially regulated by iron. *Plant J* **2003,** *36*, 366-81.
57. Koike, S.; Inoue, H.; Mizuno, D.; Takahashi, M.; Nakanishi, H.; Mori, S.; Nishizawa, N. K., OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. *Plant J* **2004,** *39*, 415-24.
58. Harris, W. R.; Sammons, R. D.; Grabiak, R. C., A speciation model of essential trace metal ions in phloem. *Journal of Inorganic Biochemistry* **2012,** *116*, 140-150.
59. Nishiyama, R.; Kato, M.; Nagata, S.; Yanagisawa, S.; Yoneyama, T., Identification of Zn–Nicotianamine and Fe–2′-Deoxymugineic acid in the phloem sap from rice plants (Oryza sativa L.). *Plant and Cell Physiology* **2012,** *53*, 381-390.
60. Kato, M.; Ishikawa, S.; Inagaki, K.; Chiba, K.; Hayashi, H.; Yanagisawa, S.; Yoneyama, T., Possible chemical forms of cadmium and varietal differences in cadmium concentrations in the phloem sap of rice plants (Oryza sativa L.). *Soil Science & Plant Nutrition* **2010,** *56*, 839-847.
61. Aoyama, T.; Kobayashi, T.; Takahashi, M.; Nagasaka, S.; Usuda, K.; Kakei, Y.; Ishimaru, Y.; Nakanishi, H.; Mori, S.; Nishizawa, N., OsYSL18 is a rice iron(III)–deoxymugineic acid transporter specifically expressed in reproductive organs and phloem of lamina joints. *Plant Molecular Biology* **2009,** *70*, 681-692.
62. Inoue, H.; Kobayashi, T.; Nozoye, T.; Takahashi, M.; Kakei, Y.; Suzuki, K.; Nakazono, M.; Nakanishi, H.; Mori, S.; Nishizawa, N. K., Rice OsYSL15 is an iron-regulated iron(III)-deoxymugineic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings. *J Biol Chem* **2009,** *284*, 3470-9.
63. Pearson, J. N.; Rengel, Z.; Jenner, C. F.; Graham, R. D., Dynamics of zinc and manganese movement in developing wheat grains. *Aust. J. Plant Physiol.* **1998,** *25*, 139-144.
64. Wang, N.; Fisher, D. B., The use of fluorescent tracers to characterize the post-phloem transport pathway in maternal tissues of developing wheat grains. *Plant Physiology* **1994,** *104*, 17-27.
65. Waters, B. M.; Sankaran, R. P., Moving micronutrients from the soil to the seeds: Genes and physiological processes from a biofortification perspective. *Plant Science* **2011,** *180*, 562-574.
66. Zhang, Y.; Shi, R.; Rezaul, K. M.; Zhang, F.; Zou, C., Iron and zinc concentrations in grain and flour of winter wheat as affected by foliar application. *Journal of Agricultural and Food Chemistry* **2010,** *58*, 12268-12274.
67. Cakmak, I.; Kalayci, M.; Kaya, Y.; Torun, A. A.; Aydin, N.; Wang, Y.; Arisoy, Z.; Erdem, H.; Yazici, A.; Gokmen, O.; Ozturk, L.; Horst, W. J., Biofortification and localization of zinc in wheat Grain. *J. Agric. Food Chem.* **2010,** *58*, 9092-9102.

68. Stomph, T. J.; Choi, E. Y.; Stangoulis, J. C. R., Temporal dynamics in wheat grain zinc distribution: is sink limitation the key. *Annals of Botany* **2011,** *107*, 927-37.
69. Moore, K. L.; Zhao, F.-J.; Gritsch, C. S.; Tosi, P.; Hawkesford, M. J.; McGrath, S. P.; Shewry, P. R.; Grovenor, C. R. M., Localisation of iron in wheat grain using high resolution secondary ion mass spectrometry. *Journal of Cereal Science* **2012,** *55*, 183-187.
70. Brinch-Pedersen, H.; Borg, S.; Tauris, B.; Holm, P. B., Molecular genetic approaches to increasing mineral availability and vitamin content of cereals. *Journal of Cereal Science* **2007,** *46*, 308-326.
71. Saulnier, L.; Sado, P.-E.; Branlard, G.; Charmet, G.; Guillon, F., Wheat arabinoxylans: Exploiting variation in amount and composition to develop enhanced varieties. *Journal of Cereal Science* **2007,** *46*, 261-281.
72. Guttieri, M. J.; Peterson, K. M.; Souza, E. J., Mineral distributions in milling fractions of low phytic acid wheat. *Crop Sci* **2006,** *46*, 2692-2698.
73. Mitsuhashi, N.; Ohnishi, M.; Sekiguchi, Y.; Kwon, Y.-U.; Chang, Y.-T.; Chung, S.-K.; Inoue, Y.; Reid, R. J.; Yagisawa, H.; Mimura, T., Phytic acid synthesis and vacuolar accumulation in suspension-cultured cells of catharanthus roseus Induced by high concentration of inorganic phosphate and cations. *Plant Physiology* **2005,** *138*, 1607-1614.
74. Raboy, V., myo-Inositol-1,2,3,4,5,6-hexakisphosphate. *Phytochemistry* **2003,** *64*, 1033-1043.
75. Lopez, H. W.; Leenhardt, F.; Coudray, C.; Remesy, C., Minerals and phytic acid interactions: is it a real problem for human nutrition? *International Journal of Food Science & Technology* **2002,** *37*, 727-739.
76. Coulibaly, A.; Kouakou, B.; Chen, J., Phytic acid in cereal grains: structure, healthy or harmful ways to reduce phytic acid in cereal grains and their effects on nutritional quality. *American Journal of Plant Nutrition and Fertilization Technology* **2011,** *1*, 1-22.
77. Raboy, V., Approaches and challenges to engineering seed phytate and total phosphorus. *Plant Science* **2009,** *177*, 281-296.
78. Hurrell, R. F., Enhanced iron absorption from cereal and legume grains by phytic acid degradation. In *Bioactive Compounds in Foods: Effects of Processing and Storage*, Lee, T. C.; Ho, C. T., Eds. 2002; Vol. 816, pp 117-129.
79. Sandberg, A.-S.; Brune, M.; Carlsson, N.-G.; Hallberg, L.; Skoglund, E.; Rossander-Hulthén, L., Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans. *The American Journal of Clinical Nutrition* **1999,** *70*, 240-246.
80. Frias, J.; Doblado, R.; Antezana, J. R.; Vidal-Valverde, C., Inositol phosphate degradation by the action of phytase enzyme in legume seeds. *Food Chemistry* **2003,** *81*, 233-239.
81. Blaabjerg, K.; Carlson, D.; Hansen-Møller, J.; Tauson, A. H.; Poulsen, H. D., In vitro degradation of phytate and lower inositol phosphates in soaked diets and feedstuffs. *Livestock Science* **2007,** *109*, 240-243.
82. Bohn, L.; Josefsen, L.; Meyer, A. S.; Rasmussen, S. K., Quantitative analysis of phytate globoids isolated from wheat bran and characterization of their sequential dephosphorylation by wheat phytase. *Journal of Agricultural and Food Chemistry* **2007,** *55*, 7547-7552.
83. Wang, Y.; Cheng, Y.; Ou, K.; Lin, L.; Liang, J., In vitro solubility of calcium, iron, and zinc in rice bran treated with phytase, cellulase, and protease. *Journal of Agricultural and Food Chemistry* **2008,** *56*, 11868-11874.
84. Sandberg, A. S.; Svanberg, U., Phytate hydrolysis by phytase in cereals; effects on in vitro estimation of iron availability. *Journal of Food Science* **1991,** *56*, 1330-1333.
85. Liu, Z. H.; Wang, H. Y.; Wang, X. E.; Zhang, G. P.; Chen, P. D.; Liu, D. J., Genotypic and spike positional difference in grain phytase activity, phytate, inorganic phosphorus, iron, and zinc contents in wheat (Triticum aestivum L.). *Journal of Cereal Science* **2006,** *44*, 212-219.

86. Masuda, H.; Ishimaru, Y.; Aung, M. S.; Kobayashi, T.; Kakei, Y.; Takahashi, M.; Higuchi, K.; Nakanishi, H.; Nishizawa, N. K., Iron biofortification in rice by the introduction of multiple genes involved in iron nutrition. *Sci. Rep.* **2012,** *2*.
87. Weber, G.; von Wiren, N.; Hayen, H., Analysis of iron(II)/iron(III) phytosiderophore complexes by nano-electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. *Rapid Commun Mass Spectrom* **2006,** *20*, 973-80.
88. Wang, M.; Gruissem, W.; Bhullar, N. K., Nicotianamine synthase overexpression positively modulates iron homeostasis-related genes in high iron rice. *Front Plant Sci* **2013,** *4*.
89. Harrison, P. M.; Arosio, P., Ferritins: molecular properties, iron storage function and cellular regulation. *Biochimica Et Biophysica Acta-Bioenergetics* **1996,** *1275*, 161-203.
90. Ebrahimi, K. H.; Bill, E.; Hagedoorn, P.-L.; Hagen, W. R., The catalytic center of ferritin regulates iron storage via Fe(II)-Fe(III) displacement. *Nature Chemical Biology* **2012,** *8*, 941-948.
91. Price, D.; Joshi, J. G., Ferritin: a zinc detoxicant and a zinc ion donor. *Proceedings of the National Academy of Sciences* **1982,** *79*, 3116-3119.
92. Lukac, R. J.; Aluru, M. R.; Reddy, M. B., Quantification of ferritin from staple food crops. *Journal of Agricultural and Food Chemistry* **2009,** *57*, 2155-2161.
93. Seckback, J., Ferreting out the secrets of plant ferritin - a review. *Journal of Plant Nutrition* **1982,** *5*, 369-394.
94. Hoppler, M.; Zeder, C.; Walczyk, T., Quantification of ferritin-bound iron in plant samples by isotope tagging and species-specific isotope dilution mass spectrometry. *Analytical Chemistry* **2009,** *81*, 7368-7372.
95. Roschzttardtz, H.; Conejero, G.; Curie, C.; Mari, S., Identification of the endodermal vacuole as the iron storage compartment in the Arabidopsis embryo. *Plant Physiol.* **2009,** *151*, 1329-1338.
96. Briat, J.-F.; Ravet, K.; Arnaud, N.; Duc, C.; Boucherez, J.; Touraine, B.; Cellier, F.; Gaymard, F., New insights into ferritin synthesis and function highlight a link between iron homeostasis and oxidative stress in plants. *Ann Bot* **2009**, mcp128.
97. Arosio, P.; Levi, S., Ferritin, iron homeostasis, and oxidative damage. *Free Radical Biology and Medicine* **2002,** *33*, 457-463.
98. Smith, B. N., Iron in higher plants: storage and metabolic role. *Journal of Plant Nutrition* **1984,** *7*, 759 - 766.
99. Murgia, I.; Tarantino, D.; Soave, C., Mitochondrial iron metabolism in plants: frataxin comes into play. *Plant and Soil* **2009,** *325*, 5-14.
100. Lestienne, I.; Besancon, P.; Caporiccio, B.; Lullien-Pellerin, V.; Treche, S., Iron and Zinc in Vitro Availability in Pearl Millet Flours (Pennisetum glaucum) with varying phytate, tannin, and fiber contents. *Journal of Agricultural and Food Chemistry* **2005,** *53*, 3240-3247.
101. Broadley, M. R.; White, P. J.; Hammond, J. P.; Zelko, I.; Lux, A., Zinc in plants. *The New phytologist* **2007,** *173*, 677-702.
102. Hegelund, J. N.; Schiller, M.; Kichey, T.; Hansen, T. H.; Pedas, P.; Husted, S.; Schjoerring, J. K., Barley Metallothioneins: MT3 and MT4 Are localized in the grain aleurone layer and show differential zinc binding. *Plant Physiology* **2012,** *159*, 1125-1137.
103. Tang, J.; Zou, C.; He, Z.; Shi, R.; Ortiz-Monasterio, I.; Qu, Y.; Zhang, Y., Mineral element distributions in milling fractions of Chinese wheats. *Journal of Cereal Science* **2008,** *48*, 821-828.
104. Liu, Z.; Wang, H.; Wang, X.-E.; Xu, H.; Gao, D.; Zhang, G.; Chen, P.; Liu, D., Effect of wheat pearling on flour phytase activity, phytic acid, iron, and zinc content. *LWT - Food Science and Technology* **2008,** *41*, 521-527.
105. Etcheverry, P.; Grusak, M. A.; Fleige, L. E., Application of in vitro bioaccessibility and bioavailability methods for calcium, carotenoids, folate, iron, magnesium, polyphenols, zinc, and vitamins B(6), B(12), D, and E. *Front Physiol* **2012,** *3*, 6.
106. The World Health Organsation., *Repositioning nutrition as central to development: a strategy for large-scale action*. 2006; p 246

107. Gomez-Galera, S.; Rojas, E.; Sudhakar, D.; Zhu, C. F.; Pelacho, A. M.; Capell, T.; Christou, P., Critical evaluation of strategies for mineral fortification of staple food crops. *Transgenic Research* **2010,** *19*, 165-180.
108. Viteri, F. E., Iron supplementation for the control of iron deficiency in populations at risk. *Nutrition Reviews* **1997,** *55*, 195-209.
109. WHO, Guidelines on food fortification with micronutrients. In FOA organiztion of the UN: 2006.
110. Akhtar, S.; Ashgar, A., Chapter 24 - Mineral fortification of whole wheat flour: an overview. in *Flour and Breads and their Fortification in Health and Disease Prevention*, Victor, P.; Ronald, W.; Vinood PatelA2 - Victor Preedy, R. W.; Vinood, P., Eds. Academic Press: San Diego, 2011; pp 263-271.
111. Hurrell, R.; Ranum, P.; de Pee, S.; Biebinger, R.; Hulthen, L.; Johnson, Q.; Lynch, S., Revised recommendations for iron fortification of wheat flour and an evaluation of the expected impact of current national wheat flour fortification programs. *Food and Nutrition Bulletin* **2010,** *31*, S7-S21.
112. Ahmed, A.; Anjum, F. M.; Ur Rehman, S.; Randhawa, M. A.; Farooq, U., Bioavailability of calcium, iron and zinc fortified whole wheat flour chapatti. *Plant Foods Hum Nutr* **2008,** *63*, 7-13.
113. Lynch, S. R., The impact of iron fortification on nutritional anaemia. *Best Practice & Research Clinical Haematology* **2005,** *18*, 333-346.
114. Hurrell, R.; Bothwell, T.; Cook, J. D.; Dary, O.; Davidsson, L.; Fairweather-Tait, S.; Hallberg, L.; Lynch, S.; Rosado, J.; Walter, T.; Whittaker, P., The usefulness of elemental iron for cereal flour fortification: A SUSTAIN task force report. *Nutrition Reviews* **2002,** *60*, 391-406.
115. The Minister of Agriculture , The bread and flour regulations *Statutory Instruments* **1998,** *no.141*.
116. Hurrell, R.; Egli, I., Iron bioavailability and dietary reference values. *Am J Clin Nutr* **2010,** *91*, 1461S-1467S.
117. Benito, P.; Miller, D., Iron absorption and bioavailability: An updated review. *Nutrition Research* **1998,** *18*, 581-603.
118. Sadighi, J.; Mohammad, K.; Sheikholeslam, R.; Amirkhani, M. A.; Torabi, P.; Salehi, F.; Abdolahi, Z., Anaemia control: Lessons from the flour fortification programme. *Public Health* **2009,** *123*, 794-799.
119. Lipschitz, D. A.; Simpson, K. M.; Cook, J. D.; Morris, E. R., Absprption of monoferric phytate by dogs. *Journal of Nutrition* **1979,** *109*, 1154-1160.
120. Tako, E.; Glahn, R. P.; Laparra, J. M.; Welch, R. M.; Lei, X.; Kelly, J. D.; Rutzke, M. A.; Miller, D. D., Iron and zinc bioavailabilities to pigs from red and white beans (Phaseolus vulgaris L.) are similar. *Journal of Agricultural and Food Chemistry* **2009,** *57*, 3134-3140.
121. Zheng, L. Q.; Cheng, Z. Q.; Ai, C. X.; Jiang, X. H.; Bei, X. S.; Zheng, Y.; Glahn, R. P.; Welch, R. M.; Miller, D. D.; Lei, X. G.; Shou, H. X., Nicotianamine, a novel enhancer of rice Iron bioavailability to humans. *PLoS ONE* **2010,** *5*.
122. Tako, E.; Blair, M.; Glahn, R., Biofortified red mottled beans (Phaseolus vulgaris L.) in a maize and bean diet provide more bioavailable iron than standard red mottled beans: Studies in poultry (Gallus gallus) and an in vitro digestion/Caco-2 model. *Nutrition Journal* **2011,** *10*, 113.
123. Sandberg, A.-S., The Use of Caco-2 Cells to Estimate Fe absorption in humans - a Critical Appraisal. *Int. J. Vitam. Nutr. Res.* **2010,** *80*, 307-313.
124. Glahn, R. P.; Lee, O. A.; Miller, D. D., In vitro digestion/Caco-2 Cell Culture model to determine optimal ascorbic acid to Fe ratio in rice cereal. *Journal of Food Science* **1999,** *64*, 925-928.
125. Sharp, P.; Srai, S. K., Molecular mechanisms involved in intestinal iron absorption. *World J Gastroenterol* **2007,** *13*, 4716-24.
126. Conrad, M. E.; Umbreit, J. N., Iron absorption and transport - An update. *American Journal of Hematology* **2000,** *64*, 287-298.

127. Andrews, N. C., The iron transporter DMT1. *The International Journal of Biochemistry & Cell Biology* **1999,** *31*, 991-994.
128. Theil, E., Iron, ferritin, and nutrition. *Annu Rev Nutr* **2004,** *24*, 327 - 343.
129. Hoppler, M.; SchÃ¶nbÃ¤chler, A.; Meile, L.; Hurrell, R. F.; Walczyk, T., Ferritin-Iron is released during boiling and in vitro gastric digestion. *The Journal of Nutrition* **2008,** *138*, 878-884.
130. Teucher; Teucher, B.; Olivares; Olivares, M.; Cori; Cori, H., Enhancers of iron absorption: ascorbic acid and other organic acids. *Int. J. Vitam. Nutr. Res.* **2004,** *74*, 403-419.
131. Salovaara, S.; Sandberg, A.-S.; Andlid, T., Organic acids influence iron uptake in the human epithelial cell line Caco-2. *Journal of Agricultural and Food Chemistry* **2002,** *50*, 6233-6238.
132. Krebs, N. F., Overview of zinc absorption and excretion in the human gastrointestinal tract. *The Journal of Nutrition* **2000,** *130*, 1374S-1377S.
133. Afify, A. E.-M. M. R.; El-Beltagi, H. S.; Abd El-Salam, S. M.; Omran, A. A., Bioavailability of iron, zinc, phytate and phytase activity during soaking and germination of white sorghum varieties. *PLoS ONE* **2011,** *6*, e25512.
134. Torre, M.; Rodriguez, A. R.; Saura-Calixto, F., Effects of dietary fiber and phytic acid on mineral availability. *Crit Rev Food Sci Nutr* **1991,** *30*, 1-22.
135. Schlemmer, U.; Frølich, W.; Prieto, R. M.; Grases, F., Phytate in foods and significance for humans: Food sources, intake, processing, bioavailability, protective role and analysis. *Molecular Nutrition & Food Research* **2009,** *53*, S330-S375.
136. Morris, E. R.; Ellis, R., Isolation of monoferric phytate from wheat bran and its biologial value as as iron source to rat. *Journal of Nutrition* **1976,** *106*, 753-760.
137. Engle-Stone, R.; Yeung, A.; Welch, R.; Glahn, R., Meat and ascorbic acid can promote Fe availability from Fe-phytate but not from Fe-tannic acid complexes. *Journal of Agricultural and Food Chemistry* **2005,** *53*, 10276-10284.
138. Siegenberg, D.; Baynes, R. D.; Bothwell, T. H.; Macfarlane, B. J.; Lamparelli, R. D.; Car, N. G.; MacPhail, P.; Schmidt, U.; Tal, A.; Mayet, F., Ascorbic acid prevents the dose-dependent inhibitory effects of polyphenols and phytates on nonheme-iron absorption. *The American Journal of Clinical Nutrition* **1991,** *53*, 537-41.
139. Disler, P. B.; Lynch, S. R.; Charlton, R. W.; Torrance, J. D.; Bothwell, T. H.; Walker, R. B.; Mayet, F., Effect of tea on iron-absorption. *Gut* **1975,** *16*, 193-200.
140. Kim, E. Y.; Pai, T. K.; Han, O., Effect of bioactive dietary polyphenols on zinc transport across the intestinal Caco-2 cell monolayers. *J Agric Food Chem* **2011,** *59*, 3606-12.
141. Glahn, R. P.; Cheng, Z.; Welch, R. M.; Gregorio, G. B., Comparison of iron bioavailability from 15 Rice Genotypes: studies using an in vitro digestion/Caco-2 cell culture model. *Journal of Agricultural and Food Chemistry* **2002,** *50*, 3586-3591.
142. Tako, E.; Glahn, R. P., White beans provide more bioavailable iron than red beans: studies in poultry (Gallus gallus) and an in vitro Digestion/Caco-2 Model. *Int. J. Vitam. Nutr. Res.* **2010,** *80*, 416-429.
143. Petry, N.; Egli, I.; Campion, B.; Nielsen, E.; Hurrell, R., Genetic reduction of phytate in common bean (Phaseolus vulgaris L.) seeds increases iron absorption in young women. *The Journal of Nutrition* **2013**.
144. Abizari, A.-R.; Moretti, D.; Schuth, S.; Zimmermann, M. B.; Armar-Klemesu, M.; Brouwer, I. D., Phytic acid-to-Iron molar ratio rather than polyphenol concentration determines iron bioavailability in whole-Cowpea meal among young women. *The Journal of Nutrition* **2012,** *142*, 1950-1955.
145. MacKown, C. T.; Carver, B. F.; Edwards, J. T., Occurrence of condensed tannins in wheat and feasibility for reducing pasture bloat. *Crop Science* **2008,** *48*, 2470-2480.
146. Glahn, R. P.; Wortley, G. M.; South, P. K.; Miller, D. D., Inhibition of iron uptake by phytic acid, tannic acid, and ZnCl2: Studies using an In vitro digestion/Caco-2 Cell model. *Journal of Agricultural and Food Chemistry* **2001,** *50*, 390-395.

147. Drakakaki, G.; Marcel, S.; Glahn, R.; Lund, E.; Pariagh, S.; Fischer, R.; Christou, P.; Stoger, E., Endosperm-specific co-expression of recombinant soybean ferritin and aspergillus phytase in maize results in significant increases in the levels of bioavailable iron. *Plant Molecular Biology* **2005,** *59*, 869-880.
148. Drakakaki, G.; Christou, P.; Stöger, E., Constitutive Expression of Soybean Ferritin cDNA Intransgenic Wheat and rice results in increased iron levels in vegetative tissues but not in Seeds. *Transgenic Research* **2000,** *9*, 445-452.
149. Qu, L.; Yoshihara, T.; Ooyama, A.; Goto, F.; Takaiwa, F., Iron accumulation does not parallel the high expression level of ferritin in transgenic rice seeds. *Planta* **2005,** *222*, 225-233.
150. Lucca, P.; Hurrell, R.; Potrykus, I., Fighting iron deficiency anemia with iron-rich rice. *J Am Coll Nutr* **2002,** *21*, 184S-190.
151. Goto, F.; Yoshihara, T.; Shigemoto, N.; Toki, S.; Takaiwa, F., Iron fortification of rice seed by the soybean ferritin gene. *Nat Biotech* **1999,** *17*, 282-286.
152. Wirth, J.; Poletti, S.; Aeschlimann, B.; Yakandawala, N.; Drosse, B.; Osorio, S.; Tohge, T.; Fernie, A. R.; Gunther, D.; Gruissem, W.; Sautter, C., Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. *Plant Biotechnol J* **2009,** *7*, 631-44.
153. Neal, A. L.; Geraki, K.; Borg, S.; Quinn, P.; Mosselmans, J. F.; Brinch-Pedersen, H.; Shewry, P. R., Iron and zinc complexation in wild-type and ferritin-expressing wheat grain: implications for mineral transport into developing grain. *J Biol Inorg Chem* **2013,** *18*, 557-70.
154. Uauy, C.; Distelfeld, A.; Fahima, T.; Blechl, A.; Dubcovsky, J., A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. *Science* **2006,** *314*, 1298-1301.
155. Waters, B. M.; Uauy, C.; Dubcovsky, J.; Grusak, M. A., Wheat (Triticum aestivum) NAM proteins regulate the translocation of iron, zinc, and nitrogen compounds from vegetative tissues to grain. *J. Exp. Bot.* **2009,** *60*, 4263-4274.
156. Gregersen, P. L.; Holm, P. B.; Krupinska, K., Leaf senescence and nutrient remobilisation in barley and wheat. *Plant Biology* **2008,** *10*, 37-49.
157. Guttieri, M.; Bowen, D.; Dorsch, J. A.; Raboy, V.; Souza, E., Identification and characterization of a low phytic acid wheat. *Crop Sci* **2004,** *44*, 418-424.
158. Raboy, V., Progress in breeding low phytate crops. *J. Nutr.* **2002,** *132*, 503S-505.
159. Hitz, W. D.; Carlson, T. J.; Kerr, P. S.; Sebastian, S. A., Biochemical and molecular characterization of a mutation that confers a decreased raffinosaccharide and phytic acid phenotype on soybean seeds. *Plant Physiol.* **2002,** *128*, 650-660.
160. Dorsch, J. A.; Cook, A.; Young, K. A.; Anderson, J. M.; Bauman, A. T.; Volkmann, C. J.; Murthy, P. P. N.; Raboy, V., Seed phosphorus and inositol phosphate phenotype of barley low phytic acid genotypes. *Phytochemistry* **2003,** *62*, 691-706.
161. Larson, S. R.; Rutger, J. N.; Young, K. A.; Raboy, V., Isolation and Genetic Mapping of a Non-Lethal Rice (Oryza sativa L.) low phytic acid 1 Mutation. *Crop Sci* **2000,** *40*, 1397-1405.
162. Shi, J.; Wang, H.; Wu, Y.; Hazebroek, J.; Meeley, R. B.; Ertl, D. S., The Maize low-phytic acid mutant lpa2 Is Caused by mutation in an inositol phosphate kinase gene. *Plant Physiol.* **2003,** *131*, 507-515.
163. Raboy, V.; Young, K. A.; Dorsch, J. A.; Cook, A., Genetics and breeding of seed phosphorus and phytic acid. *Journal of Plant Physiology* **2001,** *158*, 489-497.
164. Raboy, V., The ABCs of low-phytate crops. *Nat Biotech* **2007,** *25*, 874-875.
165. White, P. J.; Broadley, M. R., Biofortification of crops with seven mineral elements often lacking in human diets ; iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytologist* **2009,** *182*, 49-84.
166. Shi, J.; Wang, H.; Schellin, K.; Li, B.; Faller, M.; Stoop, J. M.; Meeley, R. B.; Ertl, D. S.; Ranch, J. P.; Glassman, K., Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. *Nat Biotech* **2007,** *25*, 930-937.

167. Chen, R.; Xue, G.; Chen, P.; Yao, B.; Yang, W.; Ma, Q.; Fan, Y.; Zhao, Z.; Tarczynski, M.; Shi, J., Transgenic maize plants expressing a fungal phytase gene. *Transgenic Research* **2008,** *17*, 633-643.
168. Brinch-Pedersen, H.; Olesen, A.; Rasmussen, S. K.; Holm, P. B., Generation of transgenic wheat (Triticum aestivum L.) for constitutive accumulation of an Aspergillus phytase. *Molecular Breeding* **2000,** *6*, 195-206.
169. Leenhardt, F.; Levrat-Verny, M.-A.; Chanliaud, E.; Remesy, C., Moderate decrease of pH by sourdough fermentation is sufficient to reduce phytate content of whole wheat flour through endogenous phytase activity. *Journal of Agricultural and Food Chemistry* **2004,** *53*, 98-102.
170. Liang, J.; Han, B.-Z.; Nout, M. J. R.; Hamer, R. J., Effects of soaking, germination and fermentation on phytic acid, total and in vitro soluble zinc in brown rice. *Food Chemistry* **2008,** *110*, 821-828.
171. Larsson, M.; Sandberg, A. S., Malting of oats in a pilot-plant process. Effects of heat treatment, storage and soaking conditions on phytate reduction. *Journal of Cereal Science* **1995,** *21*, 87-95.
172. Sandberg, A.-S.; Hulthen, L. R.; Turk, M., Dietary Aspergillus niger phytase increasesIron absorption in humans. *J. Nutr.* **1996,** *126*, 476-480.
173. Thompson, L. U., Potential health benefits and problems associated with antinutrients in foods. *Food Research International* **1993,** *26*, 131-149.
174. Zhou, J. R.; Erdman, J. W., Phytic acid in health and disease. *Critical Reviews in Food Science and Nutrition* **1995,** *35*, 495 - 508.
175. Shamsuddin, A. M., Anti-cancer function of phytic acid. *International Journal of Food Science & Technology* **2002,** *37*, 769-782.
176. Poulsen, H. D.; Johansen, K. S.; Hatzack, F.; Boisen, S.; Rasmussen, S. r. K., Nutritional Value of Low-phytate Barley Evaluated in Rats. *Acta Agriculturae Scandinavica, Section A - Animal Science* **2001,** *51*, 53 - 58.
177. Veum, T. L.; Ledoux, D. R.; Shannon, M. C.; Raboy, V., Effect of graded levels of iron, zinc, and copper supplementation in diets with low-phytate or normal barley on growth performance, bone characteristics, hematocrit volume, and zinc and copper balance of young swine1. *J. Anim Sci.* **2009,** *87*, 2625-2634.
178. Mendoza, C.; Viteri, F.; Lonnerdal, B.; Young, K.; Raboy, V.; Brown, K., Effect of genetically modified, low-phytic acid maize on absorption of iron from tortillas. *Am J Clin Nutr* **1998,** *68*, 1123-1127.
179. Adams, C. L.; Hambidge, M.; Raboy, V.; Dorsch, J. A.; Sian, L.; Westcott, J. L.; Krebs, N. F., Zinc absorption from a low-phytic acid maize. *Am J Clin Nutr* **2002,** *76*, 556-559.
180. Mendoza, C.; Peerson, J. M.; Brown, K. H.; Lönnerdal, B., Effect of a micronutrient fortificant mixture and 2 amounts of calcium on iron and zinc absorption from a processed food supplement. *The American Journal of Clinical Nutrition* **2004,** *79*, 244-250.
181. Higuchi, K.; Watanabe, S.; Takahashi, M.; Kawasaki, S.; Nakanishi, H.; Nishizawa, N. K.; Mori, S., Nicotianamine synthase gene expression differs in barley and rice under Fe-deficient conditions. *Plant J* **2001,** *25*, 159-67.
182. Lee, S.; An, G., Over-expression of OsIRT1 leads to increased iron and zinc accumulations in rice. *Plant Cell Environ.* **2009,** *32*, 408-416.
183. Ishimaru, Y.; Masuda, H.; Bashir, K.; Inoue, H.; Tsukamoto, T.; Takahashi, M.; Nakanishi, H.; Aoki, N.; Hirose, T.; Ohsugi, R.; Nishizawa, N. K., Rice metal-nicotianamine transporter, OsYSL2, is required for the long-distance transport of iron and manganese. *Plant J* **2010,** *62*, 379-90.
184. Shewry, P. R.; Piironen, V.; Lampi, A.-M.; Edelmann, M.; Kariluoto, S.; Nurmi, T.; Fernandez-Orozco, R.; Ravel, C.; Charmet, G.; Andersson, A. A. M.; Åman, P.; Boros, D.; Gebruers, K.; Dornez, E.; Courtin, C. M.; Delcour, J. A.; Rakszegi, M.; Bedo, Z.; Ward, J. L., The HEALTHGRAIN wheat diversity screen: Effects of genotype and environment on phytochemicals and dietary fiber components†. *Journal of Agricultural and Food Chemistry* **2010,** *58*, 9291-9298.

185. Nobel, A., Chelex® 100 and Chelex 20 Chelating Ion Exchange Resin Instruction Manual. *Bio-Rad Laboratories* **2000**.
186. Brabender Quadrumat® Junior Precision laboratory mill for production-like flour samples. http://www.brabender.com/fileadmin/dateien/gb/download/nahrungsmittel/download/broschueren/alle%20englisch/15043_E_Quadrumat_Jr.pdf (20/7/2013),
187. AACC, Approved Methods of Analysis, 11th Ed. *American Association of Cereal Chemists.* **2010,** *26-21.02*.
188. AACC, Approved methods of the AACC, 10th edtion. *American Association of Cereal Chemists.* **2000,** *08-12*.
189. Nardi, E. P.; Evangelista, F. S.; Tormen, L.; Saint´Pierre, T. D.; Curtius, A. J.; Souza, S. S. d.; Barbosa Jr, F., The use of inductively coupled plasma mass spectrometry (ICP-MS) for the determination of toxic and essential elements in different types of food samples. *Food Chemistry* **2009,** *112*, 727-732.
190. Oury, F. X.; Leenhardt, F.; Remesy, C.; Chanliaud, E.; Duperrier, B.; Balfourier, F.; Charmet, G., Genetic variability and stability of grain magnesium, zinc and iron concentrations in bread wheat. *European Journal of Agronomy* **2006,** *25*, 177-185.
191. Qureshi, I. M. J.; Khattak, T. N.; Akhtar, J., Determination of iron in different types of wheat flours. *International Journal of Agriculture and Biology* **2002,** *4*, 297-299.
192. Morgounov, A.; Gómez-Becerra, H.; Abugalieva, A.; Dzhunusova, M.; Yessimbekova, M.; Muminjanov, H.; Zelenskiy, Y.; Ozturk, L.; Cakmak, I., Iron and zinc grain density in common wheat grown in Central Asia. *Euphytica* **2007,** *155*, 193-203.
193. Morgounov, A.; Gómez-Becerra, H.; Abugalieva, A.; Dzhunusova, M.; Yessimbekova, M.; Muminjanov, H.; Zelenskiy, Y.; Ozturk, L.; Cakmak, I., Iron and zinc grain density in common wheat grown in Central Asia. *Euphytica* **2007,** *155*, 193-203.
194. Greffeuille, V.; Abecassis, J.; Bar L'Helgouach, C.; Lullien-Pellerin, V., Differences in the aleurone layer fate between hard and soft common wheats at grain milling. *Cereal Chemistry* **2005,** *82*, 138-143.
195. Greffeuille, V.; Abecassis, J.; Lapierre, C.; Lullien-Pellerin, V., Bran size distribution at milling and mechanical and biochemical characterization of common wheat grain outer layers: A relationship assessment. *Cereal Chemistry* **2006,** *83*, 641-646.
196. Joshi, A. K.; Crossa, J.; Arun, B.; Chand, R.; Trethowan, R.; Vargas, M.; Ortiz-Monasterio, I., Genotype × environment interaction for zinc and iron concentration of wheat grain in eastern Gangetic plains of India. *Field Crops Research* **2010,** *116*, 268-277.
197. Peterson, C. J.; Johnson, V. A.; Mattern, P. J., Influence of cultivar and environment on mineral and protein concentrations of wheat-flour, bran, and grain. *Cereal Chemistry* **1986,** *63*, 183-186.
198. Graham, R.; Senadhira, D.; Beebe, S.; Iglesias, C.; Monasterio, I., Breeding for micronutrient density in edible portions of staple food crops: conventional approaches. *Field Crops Research* **1999,** *60*, 57-80.
199. Pomeranz, Y.; Dikeman, E., Minerals and protein contents in hard red winter-wheat flours. *Cereal Chemistry* **1983,** *60*, 80-82.
200. Cakmak, I.; Torun, A.; Millet, E.; Feldman, M.; Fahima, T.; Korol, A.; Nevo, E.; Braun, H. J.; Ozkan, H., Triticum dicoccoides: An important genetic resource for increasing zinc and iron concentration in modern cultivated wheat. *Soil Science and Plant Nutrition* **2004,** *50*, 1047-1054.
201. McDonald, G. K.; Mousavvi Nik, M., Increasing the supply of sulphur increases the grain zinc concentration in bread and durum wheat. In 2009.
202. Gomez-Becerra, H. F.; Erdem, H.; Yazici, A.; Tutus, Y.; Torun, B.; Ozturk, L.; Cakmak, I., Grain concentrations of protein and mineral nutrients in a large collection of spelt wheat grown under different environments. *Journal of Cereal Science* **2010,** *52*, 342-349.
203. Iskander, F. Y.; Morad, M. M.; Klein, D. E.; Bauer, T. L., Determination of protein and 11 elements in 6-milling fractions of 2-wheat varieties. *Cereal Chemistry* **1987,** *64*, 285-287.

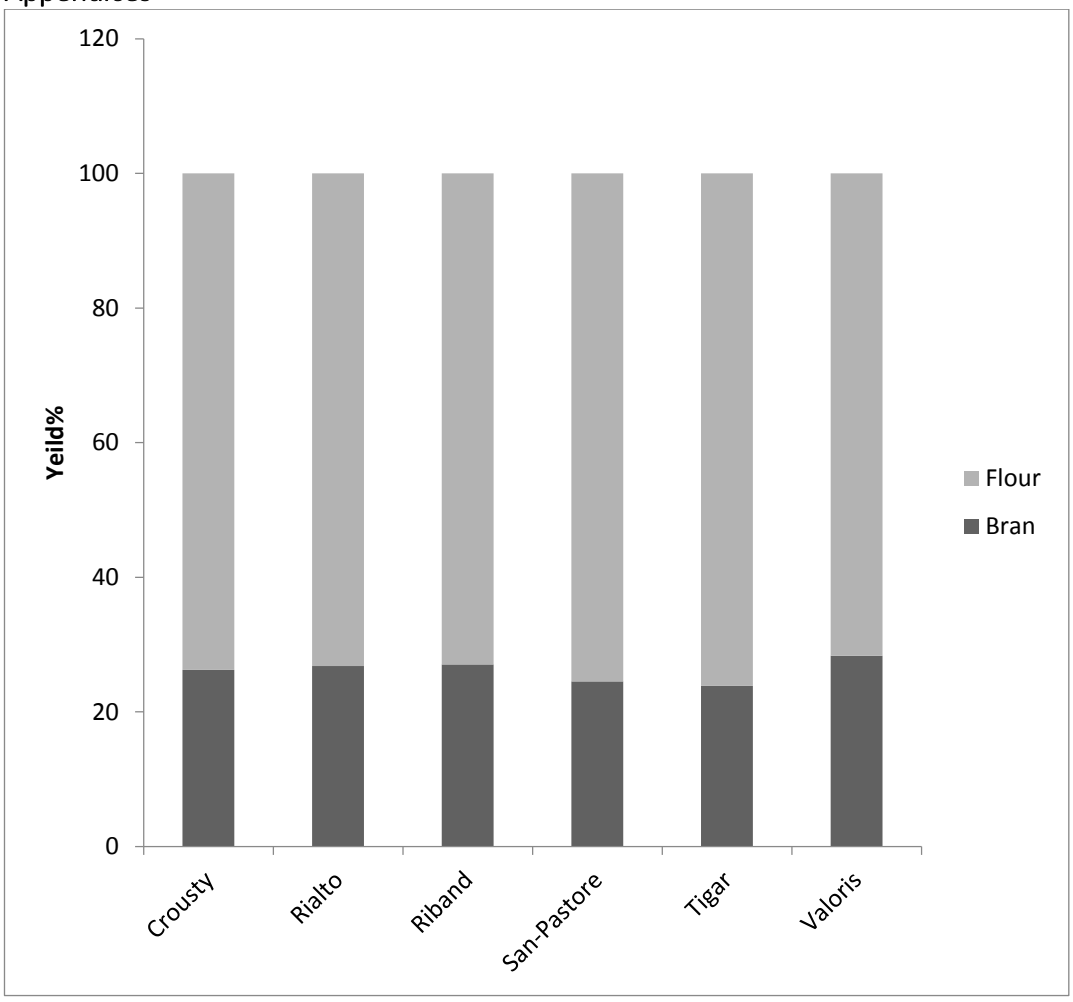
204. Kutman, U. B.; Yildiz, B.; Cakmak, I., Improved nitrogen status enhances zinc and iron concentrations both in the whole grain and the endosperm fraction of wheat. *Journal of Cereal Science* **2010,** *53*, 118-125.
205. Kutman, U. B.; Yildiz, B.; Cakmak, I., Improved nitrogen status enhances zinc and iron concentrations both in the whole grain and the endosperm fraction of wheat. *Journal of Cereal Science* **2011,** *53*, 118-125.
206. Distelfeld, A.; Cakmak, I.; Peleg, Z.; Ozturk, L.; Yazici, A. M.; Budak, H.; Saranga, Y.; Fahima, T., Multiple QTL-effects of wheat Gpc-B1 locus on grain protein and micronutrient concentrations. *Physiologia Plantarum* **2007,** *129*, 635-643.
207. Šimić, D.; Mladenović Drinić, S.; Zdunić, Z.; Jambrović, A.; Ledenčan, T.; Brkić, J.; Brkić, A.; Brkić, I., Quantitative trait loci for biofortification traits in maize grain. *Journal of Heredity* **2012,** *103*, 47-54.
208. Heard, P. J.; Feeney, K. A.; Allen, G. C.; Shewry, P. R., Determination of the elemental composition of mature wheat grain using a modified secondary ion mass spectrometer (SIMS). *Plant J* **2002,** *30*, 237-45.
209. Oliver, D. P.; Wilhelm, N. S.; Tiller, K. G.; McFarlane, J. D.; Cozens, G. D., Effect of soil and foliar applications of zinc on cadmium concentration in wheat grain. *Australian Journal of Experimental Agriculture* **1997,** *37*, 677-681.
210. Ozturk, L.; Yazici, M. A.; Yucel, C.; Torun, A.; Cekic, C.; Bagci, A.; Ozkan, H.; Braun, H.-J.; Sayers, Z.; Cakmak, I., concentration and localization of zinc during seed development and germination in wheat. *Physiologia Plantarum* **2006,** *128*, 144-152.
211. Yilmaz, A.; Ekiz, H.; Torun, B.; Gultekin, I.; Karanlik, S.; Bagci, S. A.; Cakmak, I., Effect of different zinc application methods on grain yield and zinc concentration in wheat cultivars grown on zinc-deficient calcareous soils. *Journal of Plant Nutrition* **1997,** *20*, 461-471.
212. Zeidan, M. S.; Mohamed, M. F.; Hamouda, H. A., Effect of foliar fertilization of Fe, Mn and Zn on wheat yield and quality in low sandy soils fertility. *World Journal of Agricultural Sciences* **2010,** *6*, 696-699.
213. Kutman, U. B.; Yildiz, B.; Ozturk, L.; Cakmak, I., Biofortification of Durum Wheat with zinc through soil and foliar applications of nitrogen. *Cereal Chemistry* **2010,** *87*, 1-9.
214. Rengel, Z.; Batten, G. D.; Crowley, D. E., Agronomic approaches for improving the micronutrient density in edible portions of field crops. *Field Crops Research* **1999,** *60*, 27-40.
215. Fang, Y.; Wang, L.; Xin, Z.; Zhao, L.; An, X.; Hu, Q., Effect of foliar application of zinc, selenium, and iron fertilizers on nutrients concentration and yield of rice grain in China. *Journal of Agricultural and Food Chemistry* **2008,** *56*, 2079-2084.
216. Fernández, V.; Río, V.; Abadía, J.; Abadía, A., Foliar Iron Fertilization of Peach (Prunus persica (L.) Batsch): Effects of iron compounds, surfactants and other adjuvants. *Plant and Soil* **2006,** *289*, 239-252.
217. Nowack, B.; Schwyzer, I.; Schulin, R., Uptake of Zn and Fe by wheat (Triticum aestivum var. Greina) and transfer to the grains in the presence of chelating agents (ethylenediaminedisuccinic acid and ethylenediaminetetraacetic acid). *Journal of Agricultural and Food Chemistry* **2008,** *56*, 4643-4649.
218. Morris, C. F.; Rose, S. P., Wheat. In *Cereal Grain Quality*, Henry, R. J.; Kettlewell, P. S., Eds. Springer Netherlands: 1996; pp 3-54.
219. Donard, O. F. X.; Caruso, J. A., Trace metal and metalloid species determination: evolution and trends. *Spectrochimica Acta Part B: Atomic Spectroscopy* **1998,** *53*, 157-163.
220. Kot, A.; Namieśnik, J., The role of speciation in analytical chemistry. *TrAC Trends in Analytical Chemistry* **2000,** *19*, 69-79.
221. Byrdy, F. A.; Caruso, J. A., Trace-metals speation by HPLC with plasma source-mass spectrometry detection. *Environmental Health Perspectives* **1995,** *103*, 21-23.

222. Kolb, B.; Kemmer, G.; Schlesser, F.; Wiedeking, E., Element specific detection of gas-chromatographically separated metal compounds by atomic absorption spectroscopy. Determination of alkylleads in gasoline. *Z. anal. Chem* **1966,** *221*, 166-175.
223. Dean, J. R.; Munro, S.; Ebdon, L.; Crews, H. M.; Massey, R. C., Studies of metalloprotein species by directly coupled high-performance liquid chromatography inductively coupled plasma mass spectrometry. *Journal of Analytical Atomic Spectrometry* **1987,** *2*, 607-610.
224. Pedersen, G. A.; Larsen, E. H., Speciation of four selenium compounds using high performance liquid chromatography with on-line detection by inductively coupled plasma mass spectrometry or flame atomic absorption spectrometry. *Fresenius J Anal Chem* **1997,** *358*, 591-598.
225. Chan, Q. L.; Afton, S. E.; Caruso, J. A., Selenium speciation profiles in selenite-enriched soybean (Glycine Max) by HPLC-ICPMS and ESI-ITMS. *Metallomics* **2010,** *2*, 147-153.
226. Lombi, E.; Scheckel, K. G.; Pallon, J.; Carey, A. M.; Zhu, Y. G.; Meharg, A. A., Speciation and distribution of arsenic and localization of nutrients in rice grains. *New Phytologist* **2009,** *184*, 193-201.
227. Mestek, O.; Kominkova, J.; Santrucek, J.; Kacer, P.; Malisova, K.; Koplik, R., Analyses of trace metals, peptide ligands of trace metals and mercury speciation in home prepared bread. *Chem. Speciation Bioavail.* **2012,** *24*, 79-88.
228. Vacchina, V.; Polec, K.; Szpunar, J., Speciation of cadmium in plant tissues by size-exclusion chromatography with ICP-MS detection. *Journal of Analytical Atomic Spectrometry* **1999,** *14*, 1557-1566.
229. Ovca, A.; van Elteren, J. T.; Falnoga, I.; Šelih, V. S., Speciation of zinc in pumpkin seeds (Cucurbita pepo) and degradation of its species in the human digestive tract. *Food Chemistry* **2011,** *128*, 839-846.
230. Welch, R. M.; House, W. A.; Ortiz-Monasterio, I.; Cheng, Z., Potential for improving bioavailable zinc in wheat grain (Triticum species) through plant breeding. *Journal of Agricultural and Food Chemistry* **2005,** *53*, 2176-2180.
231. Lestienne, I.; Caporiccio, B.; Besancon, P.; Rochette, I.; Treche, S., Relative contribution of phytates, fibers, and tannins to low iron and zinc in vitro solubility in Pearl Millet (Pennisetum glaucum) Flour and Grain Fractions. *Journal of Agricultural and Food Chemistry* **2005,** *53*, 8342-8348.
232. Saulnier, L.; Guillon, F.; Chateigner-Boutin, A.-L., Cell wall deposition and metabolism in wheat grain. *Journal of Cereal Science* **2012,** *56*, 91-108.
233. Szpunar, J., Trace element speciation analysis of biomaterials by high-performance liquid chromatography with inductively coupled plasma mass spectrometric detection. *TrAC Trends in Analytical Chemistry* **2000,** *19*, 127-137.
234. Kayal, N.; Singh, N., Stepwise complexometric determination of aluminium, titanium and iron concentrations in silica sand and allied materials. *Chemistry Central Journal* **2007,** *1*.
235. Reichman, S. M.; Parker, D. R., Revisiting the metal-binding chemistry of nicotianamine and 2 '-deoxymugineic acid. Implications for iron nutrition in Strategy II plants. *Plant Physiology* **2002,** *129*, 1435-1438.
236. Matzapetakis, M.; Turano, P.; Theil, E.; Bertini, I., 13C–13C NOESY spectra of a 480 kDa protein: solution NMR of ferritin. *J Biomol NMR* **2007,** *38*, 237-242.
237. Masuda, T.; Goto, F.; Yoshihara, T., A novel plant Ferritin subunit from soybean That is related to a mechanism in iron release. *Journal of Biological Chemistry* **2001,** *276*, 19575-19579.
238. Hoppler, M.; Schonbachler, A.; Meile, L.; Hurrell, R. F.; Walczyk, T., Ferritin-iron is released during boiling and in vitro gastric digestion. *J Nutr* **2008,** *138*, 878-84.
239. Koplík, R.; Pavelková, H.; Cincibuchová, J.; Mestek, O.; Kvasnička, F.; Suchánek, M., Fractionation of phosphorus and trace elements species in soybean flour and common white bean seeds by size exclusion chromatography–inductively coupled plasma mass spectrometry. *Journal of Chromatography B* **2002,** *770*, 261-273.

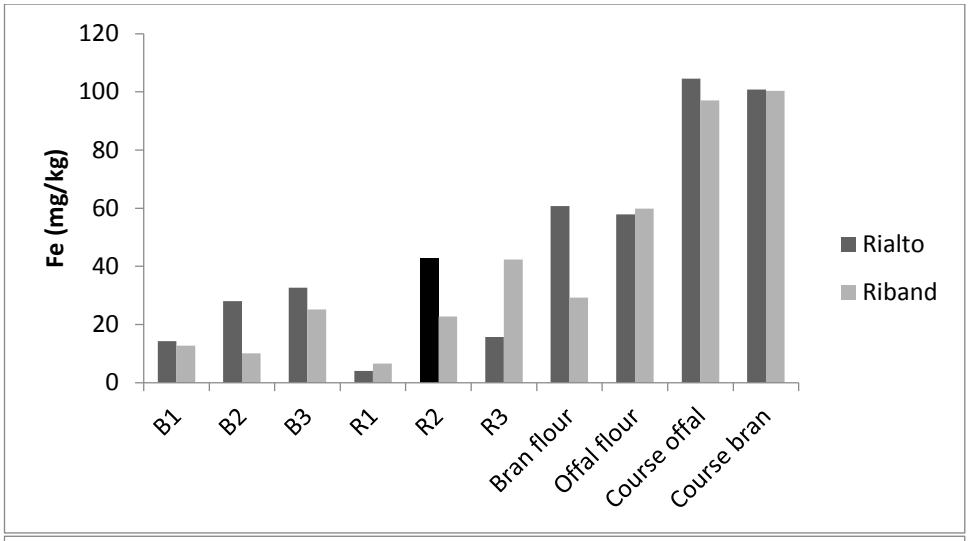
240. Zimmermann, B.; Lantzsch, H. J.; Langbein, U.; Drochner, W., Determination of phytase activity in cereal grains by direct incubation. *Journal of animal physiology and animal nutrition* **2002,** *86*, 347-52.
241. Hayakawa, T.; Toma, Y.; Igaue, I., Purification and characterization of acid phosphatases with or without phytase activity from rice bran(Biological Chemistry). *Agricultural and biological chemistry* **1989,** *53*, 1475-1483.
242. Hemery, Y.; Lullien-Pellerin, V.; Rouau, X.; Abecassis, J.; Samson, M.-F.; Åman, P.; von Reding, W.; Spoerndli, C.; Barron, C., Biochemical markers: Efficient tools for the assessment of wheat grain tissue proportions in milling fractions. *Journal of Cereal Science* **2009,** *49*, 55-64.
243. Barron, C.; Surget, A.; Rouau, X., Relative amounts of tissues in mature wheat (Triticum aestivum L.) grain and their carbohydrate and phenolic acid composition. *Journal of Cereal Science* **2007,** *45*, 88-96.
244. Schjoerring, J. K.; Persson, D. P.; Hansen, T. H.; Laursen, K. H.; Husted, S., Zinc and iron speciation in the cereal grain. In 2009.
245. Lee, S.; Kim, Y.-S.; Jeon, U.; Kim, Y.-K.; Schjoerring, J.; An, G., Activation of Rice nicotianamine synthase 2 (OsNAS2) enhances iron availability for biofortification. *Mol Cells* **2012,** *33*, 269-275.
246. Geerligs, P. D. P.; Brabin, B. J.; Omari, A. A. A., Food prepared in iron cooking pots as an intervention for reducing iron deficiency anaemia in developing countries: a systematic review. *Journal of Human Nutrition and Dietetics* **2003,** *16*, 275-281.
247. Tripp, K.; MacKeith, N.; Woodruff, B. A.; Talley, L.; Mselle, L.; Mirghani, Z.; Abdalla, F.; Bhatia, R.; Seal, A. J., Acceptability and use of iron and iron-alloy cooking pots: implications for anaemia control programmes. *Public Health Nutr.* **2010,** *13*, 123-130.
248. Greiner, R.; Konietzny, U., Phytase for food application. *Food Technology and Biotechnology* **2006,** *44*, 125-140.
249. Shewry, P. R.; Halford, N. G., Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of Experimental Botany* **2002,** *53*, 947-958.
250. Wan, Y.; Poole, R. L.; Huttly, A. K.; Toscano-Underwood, C.; Feeney, K.; Welham, S.; Gooding, M. J.; Mills, C.; Edwards, K. J.; Shewry, P. R.; Mitchell, R. A. C., Transcriptome analysis of grain development in hexaploid wheat. *Bmc Genomics* **2008,** *9*.
251. Reddy, M. B.; Lukac, R.; Maneesha, A., The quantitation of ferritin in the seeds of staple crops: ELISA method development. *The Journal of the Federation of American Societies for Experimental Biology* **2008,** *22*, 694.12-.
252. Bravo, L., Polyphenols: Chemistry, Dietary Sources, metabolism, and nutritional significance. *Nutrition Reviews* **1998,** *56*, 317-333.
253. Karamac, M., Fe(II), Cu(II) and Zn(II) chelating activity of buckwheat and buckwheat groats tannin fractions. *Polish Journal of Food and Nutrition Sciences* **2007,** *57*, 357-362.
254. Yanagida, A.; Shoji, T.; Kanda, T., Characterization of polymerized polyphenols by Size-exclusion HPLC. *Bioscience, Biotechnology, and Biochemistry* **2002,** *66*, 1972-1975.
255. Kapsokefalou, M.; Miller, D. D., Iron speciation in intestinal contents of rats fed meals composed of meat and nonmeat sources of protein and fat. *Food Chemistry* **1995,** *52*, 47-56.
256. Björn-Rasmussen, E., Iron Absorption from Wheat Bread. *Annals of Nutrition and Metabolism* **1974,** *16*, 101-110.
257. Elwood, P. C.; Newton, D.; Eakins, J. D.; Brown, D. A., Absorption of Iron from Bread. *The American Journal of Clinical Nutrition* **1968,** *21*, 1162-1169.
258. Brune, M.; Rossander-Hulten, L.; Hallberg, L.; Gleerup, A.; Sandberg, A. S., Iron absorption from bread in humans: inhibiting effects of cereal fiber, phytate and inositol phosphates with different numbers of phosphate groups. *J Nutr* **1992,** *122*, 442-9.
259. Ariza-Nieto, M.; Blair, M.; Welch, R.; Glahn, R., Screening of iron bioavailability patterns in eight bean (Phaseolus vulgaris L.) genotypes using the Caco-2 cell in vitro model. *J Agr Food Chem* **2007,** *55*, 7950 - 7956.

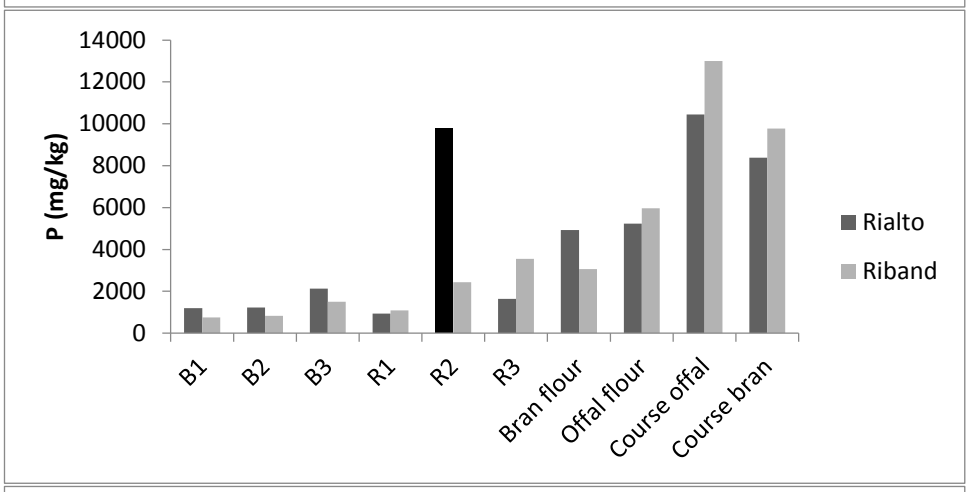
260. Beiseigel, J. M.; Hunt, J. R.; Glahn, R. P.; Welch, R. M.; Menkir, A.; Maziya-Dixon, B. B., Iron bioavailability from maize and beans: a comparison of human measurements with Caco-2 cell and algorithm predictions. *The American Journal of Clinical Nutrition* **2007,** *86*, 388-396.
261. Salunke, R.; Neelam, K.; Rawat, N.; Tiwari, V. K.; Dhaliwal, H. S.; Roy, P., Bioavailability of iron from wheat aegilops derivatives selected for high grain iron and protein contents. *Journal of Agricultural and Food Chemistry* **2011,** *59*, 7465-7473.
262. Lebet, V.; Arrigoni, E.; Amado, R., Digestion procedure using mammalian enzymes to obtain substrates for in vitro fermentation studies. *Lebensmittel-Wissenschaft and Technologie* **1998,** *31*, 509-515.
263. Nishiyama, R.; Kato, M.; Nagata, S.; Yanagisawa, S.; Yoneyama, T., Identification of Zn-nicotianamine and Fe-2'-deoxymugineic Acid in the phloem saps from rice plants (Oryza sativa L.). *Plant and Cell Physiology* **2012**.
264. Lei, J.; Zhang, Y.; Chen, X. G.; Zhang, M. Q.; Bai, L.; Huang, C. Y.; Ivan, O. M., Assessment of iron bioavailability in ten kinds of chinese wheat flours using an in vitro digestion/Caco-2 cell model. *Biomedical and Environmental Sciences* **2012,** *25*, 502-508.
265. Wu, P.; Zhao, T.; Tian, J.-c., Phytic acid contents of wheat flours from different mill streams. *Agricultural Sciences in China* **2010,** *9*, 1684-1688.
266. Anjum, F. M.; Butt, M. S.; Ahmad, N.; Ahmad, I., Phytate and mineral content in different milling fractions of some Pakistani spring wheats. *International Journal of Food Science & Technology* **2002,** *37*, 13-17.
267. Prom-u-thai, C.; Huang, L.; Glahn, R. P.; Welch, R. M.; Fukai, S.; Rerkasem, B., Iron (Fe) bioavailability and the distribution of anti-Fe nutrition biochemicals in the unpolished, polished grain and bran fraction of five rice genotypes. *Journal of the Science of Food and Agriculture* **2006,** *86*, 1209-1215.
268. Hurrell, R. F.; Reddy, M. B.; Burri, J.; Cook, J. D., Phytate degradation determines the effect of industrial processing and home cooking on iron absorption from cereal-based foods. *British Journal of Nutrition* **2002,** *88*, 117-123.
269. Hernandez, M.; Sousa, V.; Villalpando, S.; Moreno, A.; Montalvo, I.; Lopez-Alarcon, M., Cooking and Fe fortification have different effects on Fe bioavailability of bread and tortillas. *Journal of the American College of Nutrition* **2006,** *25*, 20-25.
270. Bullock, J. I.; Duffin, P. A.; Nolan, K. B.; Smith, T. K., Effect oOf phytate on the in-vitro solubilty of Al3+, Ca2+, Hg2+ AND Pb2+ as a function of pH at 37-degrees-C. *Journal of the Science of Food and Agriculture* **1995,** *67*, 507-509.
271. Reichman, S.; Parker, D., Revisiting the metal-binding chemistry of nicotianamine and 2'-deoxymugineic acid. Implications for iron nutrition in strategy II plants. *Plant Physiol* **2002,** *129*, 1435 - 1438.
272. Witter, A. E.; Hutchins, D. A.; Butler, A.; Luther Iii, G. W., Determination of conditional stability constants and kinetic constants for strong model Fe-binding ligands in seawater. *Marine Chemistry* **2000,** *69*, 1-17.
273. Zimmermann, M. B.; Winichagoon, P.; Gowachirapant, S.; Hess, S. Y.; Harrington, M.; Chavasit, V.; Lynch, S. R.; Hurrell, R. F., Comparison of the efficacy of wheat-based snacks fortified with ferrous sulfate, electrolytic iron, or hydrogen-reduced elemental iron: randomized, double-blind, controlled trial in Thai women. *The American Journal of Clinical Nutrition* **2005,** *82*, 1276-1282.
274. Yeung, C. K.; Miller, D. D.; Cheng, Z.; Glahn, R. E., Bioavailability of elemental iron powders in bread assessed with an In vitro digestion/Caco-2 cell culture model. *Journal of Food Science* **2005,** *70*, S199-S203.
275. Kloots, W.; den Kamp, D. O.; Abrahamse, L., In vitro iron availability from iron-fortified whole-grain wheat flour. *Journal of Agricultural and Food Chemistry* **2004,** *52*, 8132-8136.

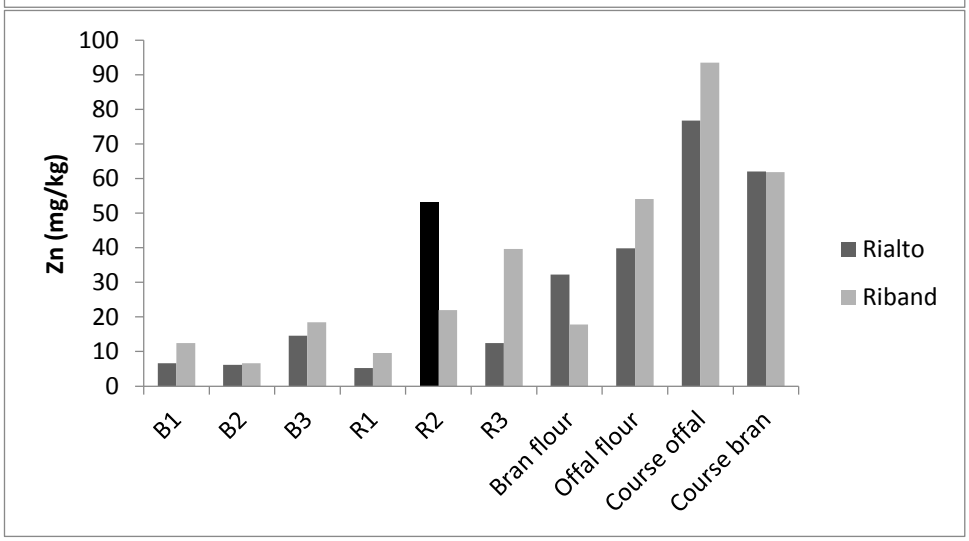
276. Bouhallab, S.; Cinga, V.; Aít-Oukhatar, N.; Bureau, F.; Neuville, D.; Arhan, P.; Maubois, J.-L.; Bouglé, D., Influence of various phosphopeptides of caseins on iron absorption. *Journal of Agricultural and Food Chemistry* **2002,** *50*, 7127-7130.
277. Sørensen, A. D.; Bukhave, K., Iron uptake by Caco-2 cells following in vitro digestion: Effects of heat treatments of pork meat and pH of the digests. *Journal of Trace Elements in Medicine and Biology* **2010,** *24*, 230-235.
278. Sandberg, A.-S.; Andlid, T., Phytogenic and microbial phytases in human nutrition. *International Journal of Food Science & Technology* **2002,** *37*, 823-833.
279. Rawat, N.; Neelam, K.; Tiwari, V. K.; Dhaliwal, H. S., Biofortification of cereals to overcome hidden hunger. *Plant Breeding* **2013**, n/a-n/a.
280. Hauber, U.; Bruce, A.; Neuhauser-Berthold, M., A comparison of dietary reference values for energy of different countries. *Zeitschrift fur Ernahrungswissenschaft* **1997,** *36*, 394-402.
281. Herbert, V., Recommended dietary intakes (RDI) of iron in humans. *The American Journal of Clinical Nutrition* **1987,** *45*, 679-86.
282. Levrat-Verny, M. A.; Coudray, C.; Bellanger, J.; Lopez, H. W.; Demigne, C.; Rayssiguier, Y.; Remesy, C., Wholewheat flour ensures higher mineral absorption and bioavailability than white wheat flour in rats. *British Journal of Nutrition* **1999,** *82*, 17-21.
283. Elwood, P. C.; Benjamin, I. T.; Fry, F. A.; Eakins, J. D.; Brown, D. A.; DE Kock, P. C.; Shah, J. U., Absorption of iron from chapatti made from wheat flour. *The American Journal of Clinical Nutrition* **1970,** *23*, 1267-1271.
284. Gibson, R. S., Zinc: the missing link in combating micronutrient malnutrition in developing countries. *Proceedings of the Nutrition Society* **2006,** *65*, 51-60.
285. Welch, R. M.; Graham, R. D., Breeding for micronutrients in staple food crops from a human nutrition perspective. *Journal of Experimental Botany* **2004,** *55*, 353-364.
286. Slavin, J. L.; Jacobs, D.; Marquart, L., Grain processing and nutrition. *Critical Reviews in Biotechnology* **2001,** *21*, 49-66.
287. Wolters, M. G.; Schreuder, H. A.; van den Heuvel, G.; van Lonkhuijsen, H. J.; Hermus, R. J.; Voragen, A. G., A continuous in vitro method for estimation of the bioavailability of minerals and trace elements in foods: application to breads varying in phytic acid content. *Br J Nutr* **1993,** *69*, 849-61.
288. Weber, G.; von Wiren, N.; Hayen, H., Analysis of iron(II)/iron(III) phytosiderophore complexes by nano-electrospray ionization fourier transform ion cyclotron resonance mass spectrometry. *Rapid Comm Mass Spectrom* **2006,** *20*, 973 - 980.
289. Cakmak, I., Enrichment of cereal grains with zinc: Agronomic or genetic biofortification? *Plant and Soil* **2008,** *302*, 1-17.



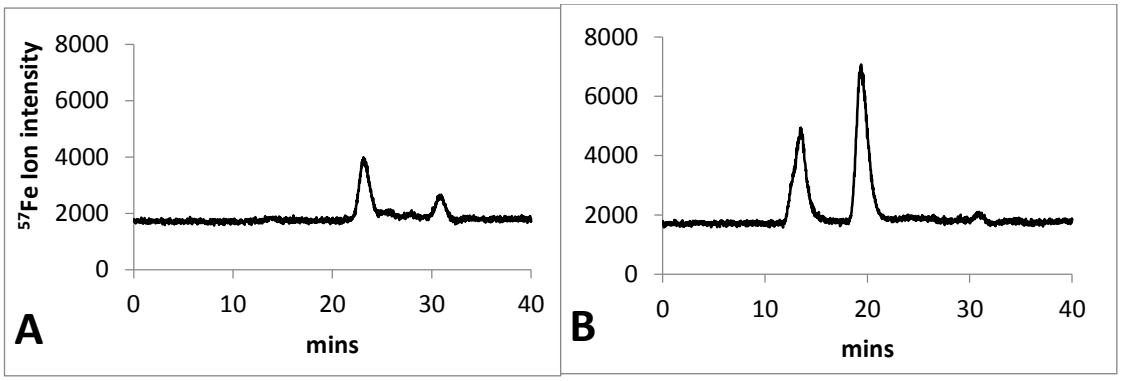
Appendix 1. Bran yields of 6 cultivars from Brabender mill



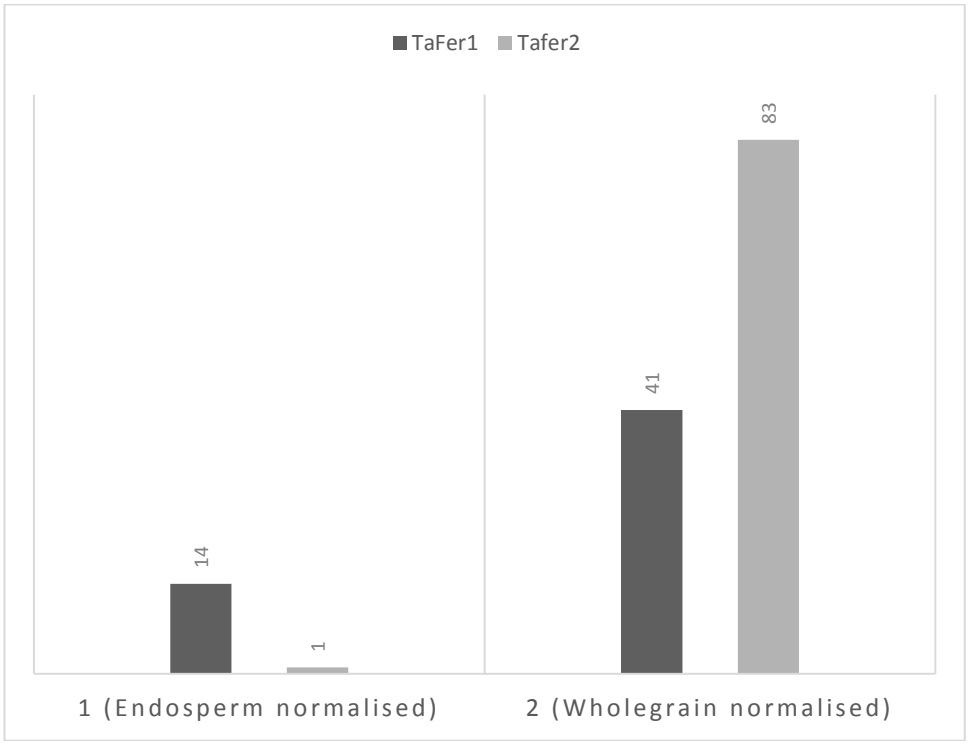




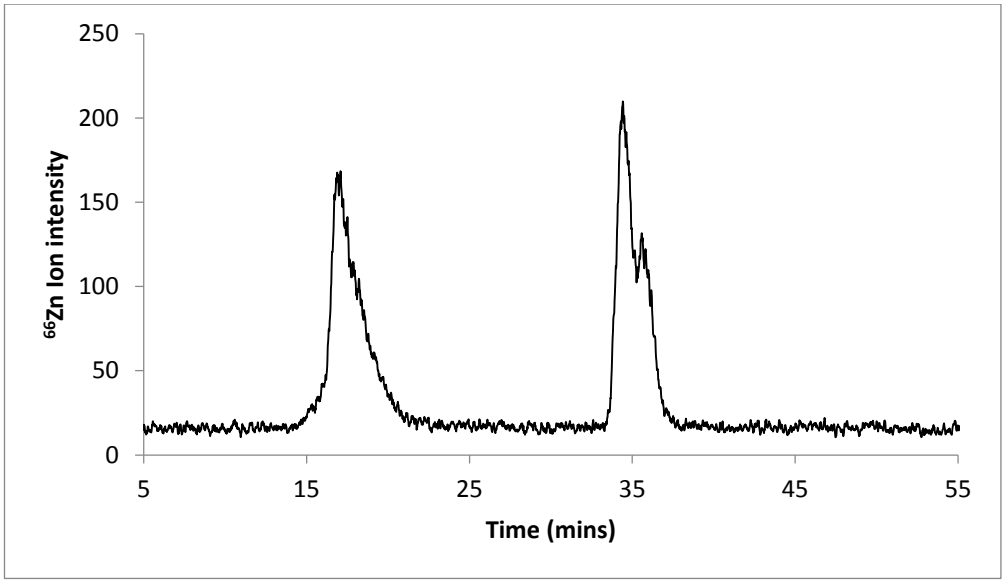
Appendix 2. Total minerals in 2012 10 milling fractions. R2 is highlighted in black to show abnormally high mineral concentration.



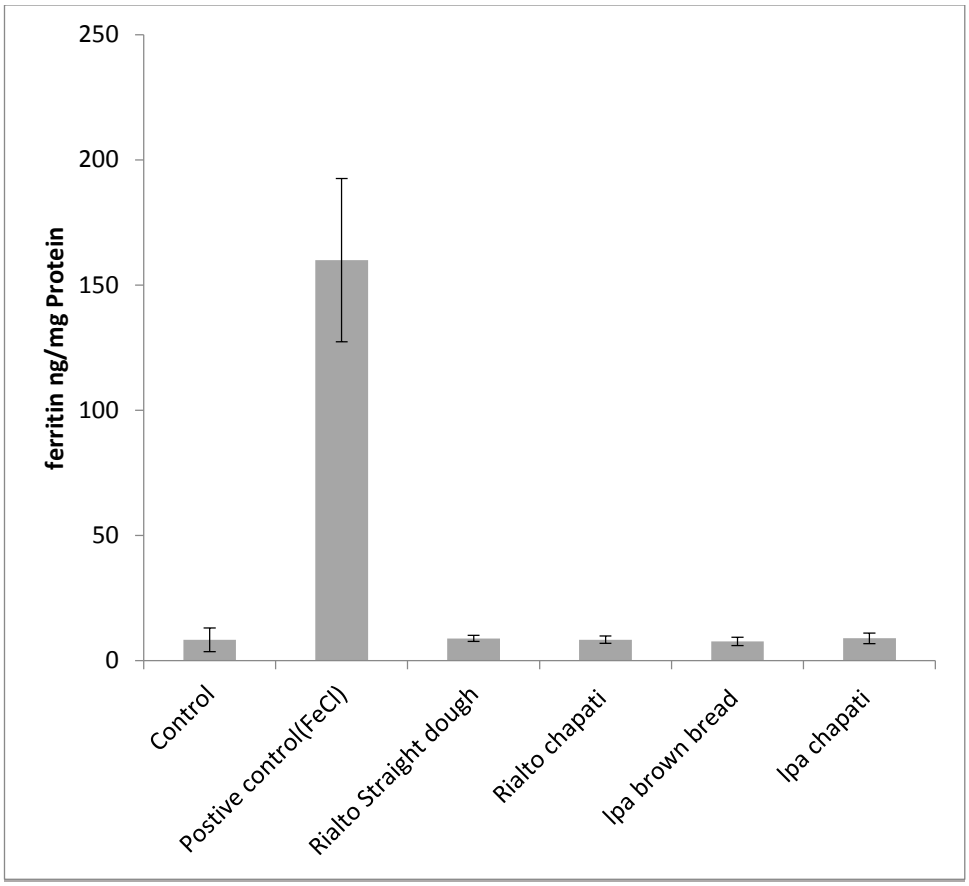
Appendix 3. Effect of SDS on extraction. Chromatogram of Fe57+ signal of San-Pastore whole grain under **a)** Tris-HCl treatment **b)**Tris-HCl with 3% SDS.



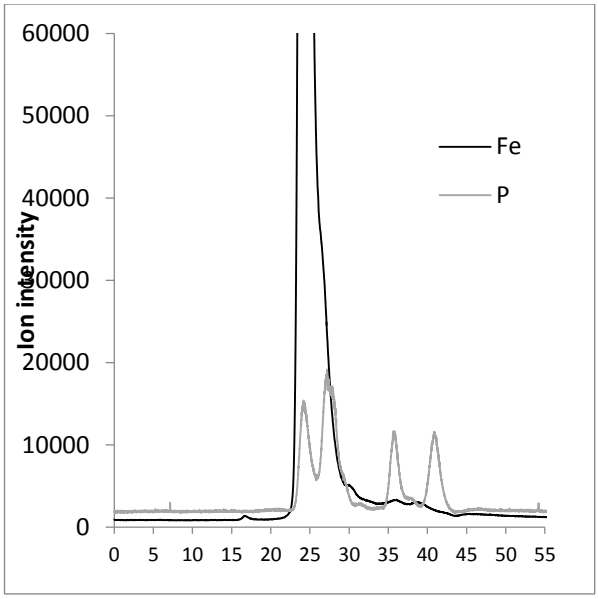
Appendix 4. Normalised expression of taFer1 and tafer2 in endosperm and whole grain of cadenza



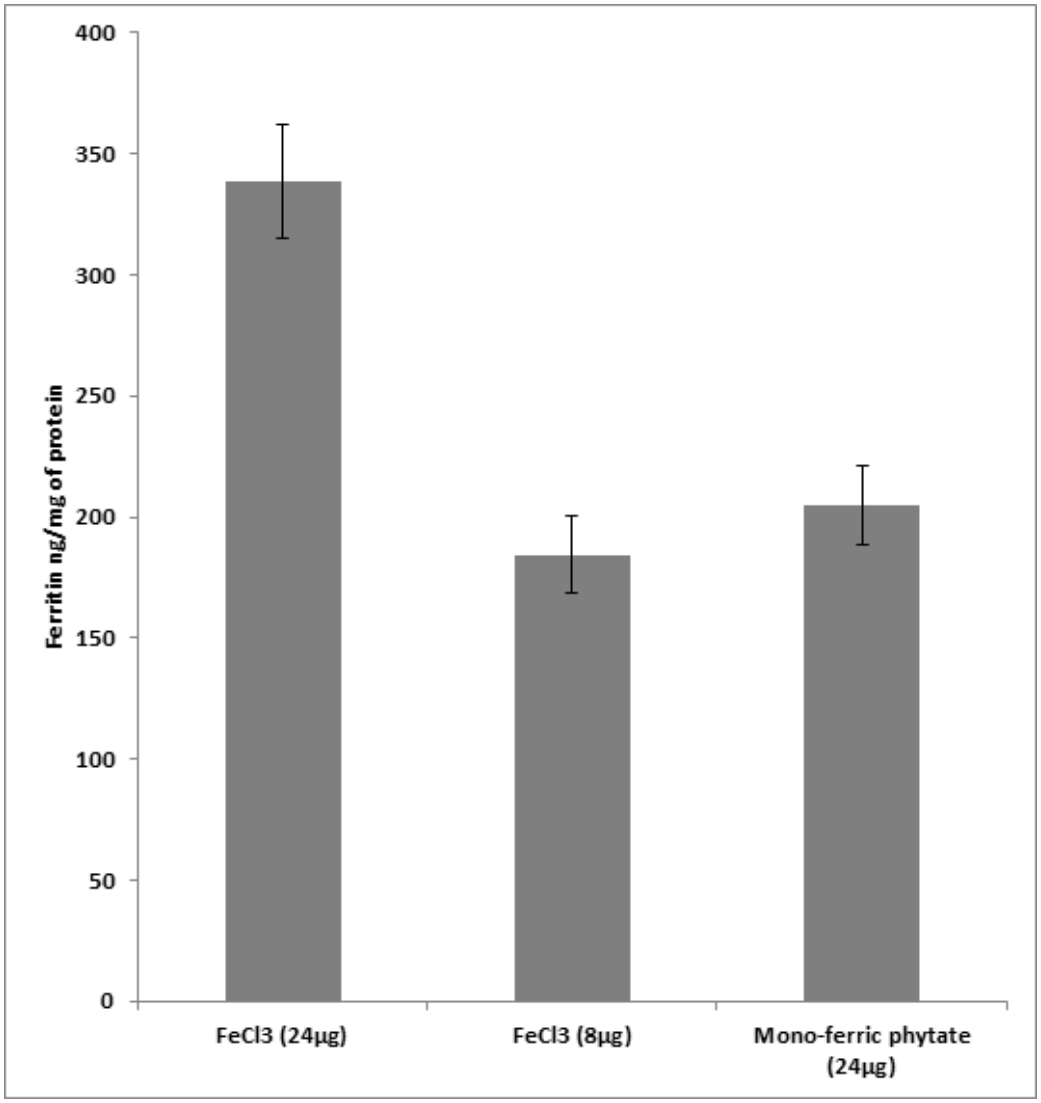
Appendix 5. SEC chromatograms showing ^{66}Zn speciation in soluble fractions of offal flour in Rialto



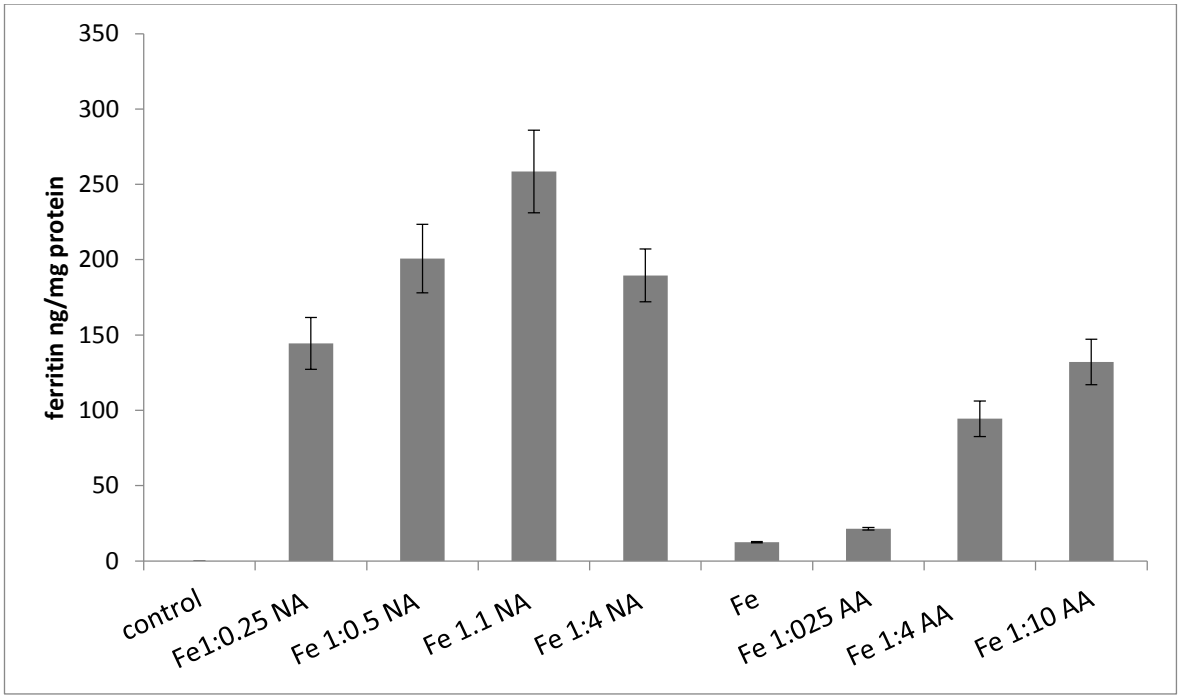
Appendix 6 Comparsion of low phytic acid wheat and rialto whole grain Shows ferritin response of Caco-2 cells to 1 g of bread sample with ascorbic acid (132 µM). Results are from two independent experiments, Values (± *se*; *n* = 12)



Appendix 7: Chromatogram of mono-ferric phytate after in vitro digestion. Shows Fe and P speciation for synthetic mono-ferric phytate after in vitro digestion. The co-elution indicates two oligomers both containing P and Fe. The two peaks are likely to be two isomers of mono-ferric phytate, or one will be Fe-IP5



Appendix 8. Comparisons of mono-ferric phytate to $FeCl_3$. SE ± values are mean (n=6)



Appendix 9. Bioavailability of Fe in presence of NA and ascorbic acid. Shows ferritin response of Caco-2 cells to 3.11 ug of Fe (FeCl) in different molar ratios of ascorbic acid (AA) or nicotainamine (NA) .Values (± *se*; *n* = 6)