The impact of steeping, germination and hydrothermal processing of wheat (*Triticum aestivum* L.) grains on phytate hydrolysis and the distribution, speciation and bio-accessibility of iron and zinc elements

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ABSTRACT

Chelation of iron and zinc in wheat as phytates lowers their bio-accessibility. Steeping and germination (15 °C, 120 h) lowered phytate content from 0.96% to only 0.81% of initial dry matter. A multifactorial experiment in which (steeped/germinated) wheat was subjected to different time (2-24 h), temperature (20-80 °C) and pH (2.0-8.0) conditions showed that hydrothermal processing of germinated (15 °C, 120 h) wheat at 50 °C and pH 3.8 for 24 h reduced phytate content by 95%. X-ray absorption near-edge structure imaging showed that it indeed abolished chelation of iron to phytate. It also proved that iron was oxidized during steeping, germination and hydrothermal processing. It was further shown that zinc and iron bio-accessibility were respectively 3 and 5% in wheat and 27 and 37% in hydrothermally processed wheat. Thus, hydrothermal processing of (germinated) wheat paves the way for increasing elemental bio-accessibility in whole grain-based products.

1. INTRODUCTION

Whole grain food products warrant an important role in a healthy diet as they reduce the risk of developing diet-related disorders such as obesity (Anderson, Smith, & Gustafson, 1994), type II diabetes (de Munter, Hu, Spiegelman, Franz, & van Dam, 2007) and cardiovascular disease (Liu, Stampfer, Hu, Giovannucci, Rimm, Manson, et al., 1999). Their health benefits are mainly ascribed to high concentrations of dietary fiber and other bio-actives such as B vitamins, mineral elements [*e.g.* potassium (K), iron (Fe) and zinc (Zn)] and polyphenols which are particularly present in the peripheral grain tissues (Brouns, Hemery, Price, & Anson, 2012). From the outside to the inside, a wheat grain consists of pericarp, seed coat, nucellar epidermis, aleurone and starchy endosperm (Barron, Surget, & Rouau, 2007). Its embryo is located on the dorsal side of the grain. Most wheat for human consumption is roller milled to separate the energy dense but nutrient poor flour (*i.e.* refined starchy endosperm) from bran tissues (including pericarp, seed coat, nucellar epidermis and aleurone) and embryo (Delcour & Hoseney, 2010). While wheat bran has an excellent nutritional profile, its inclusion in food recipes reduces the perceived organoleptic quality of the resultant products, and hence its main commercial use is as animal feed (Seyer & Gelinas, 2009). Further, the bio-active compounds concentrated in the aleurone have low bio-accessibility as they are entrapped in cells with rigid walls which withstand both conventional milling and digestion by the human enzyme arsenal (Brouns, Hemery, Price, & Anson, 2012). Moreover, about 85% of the phosphorus (P) in a wheat kernel is stored as phytic acid (myo-inositol 1,2,3,4,5,6 hexa*kis*phosphate) which is usually chelated by divalent cations such as those of Fe, Zn, calcium (Ca), manganese (Mn), magnesium (Mg) and copper (Cu) and by trivalent ions such as that of Fe. The resulting phytates are present as granules embedded in protein-rich globoid structures in the aleurone (Schlemmer, Frolich, Prieto, & Grases, 2009). Unfortunately, phytate is only poorly digested or absorbed by humans due to a lack of intestinal phytase enzymes (Sandberg & Andersson, 1988). As a result, the elemental bio-accessibility amounts to only 5 to 10% (Bouis, Hotz, McClafferty, Meenakshi, & Pfeiffer, 2011). Under certain dietary

63 circumstances, this can lead to Fe and Zn deficiencies which mainly affect women and children
64 in developing and even industrialized countries (Miller, 2013; Wessells & Brown, 2012). Iron
65 deficiency affects about two billion people worldwide and mainly results in anemia which itself
66 causes disorders such as poor mental performance, fatigue, neurological damage and cognitive
67 dysfunction (Miller, 2013). Furthermore, 1.3 billion people worldwide risk inadequate Zn intake
68 (Wessells & Brown, 2012) and have an increased risk of developing disorders such as impaired
69 growth and immune functions and diarrhea (Gibson, 2012). Supplementation and/or food
70 fortification are currently used to prevent element deficiencies in humans (Johnson, Smith, &
71 Edmonds, 1998). However, such approaches are not easily available to those living in
72 underdeveloped and developing countries. Moreover, breeding strategies aiming at increasing the
73 mineral elemental content in staple foods (Bouis, Hotz, McClafferty, Meenakshi, & Pfeiffer,
74 2011) are long term efforts which require substantial resources. New approaches are therefore
75 needed to expand the reach of food-based interventions.

76 Wheat germination initiates seedling growth and proceeds once adequate temperature and
77 moisture content conditions are met. Hydrolytic enzymes such as α-amylase, xylanase and
78 phytase are activated and *de novo* synthesized in the aleurone cells to fuel the germination
79 (Miransari & Smith, 2014). The increase in phytase activity during germination makes phosphate,
80 mineral elements and myo-inositol available for plant growth and development (Platel, Eipeson,
81 & Srinivasan, 2010) and, when used in food systems, for human uptake. Moreover, hydrothermal
82 processing of cereal grains at a relative low pH (2.0 to 5.0) and at 37 to 55 °C allows phytase
83 action and, hence, phytate hydrolysis (Fredlund, Asp, Larsson, Marklinder, & Sandberg, 1997).
84 Thus, technologies such as wheat grain steeping, controlled germination and hydrothermal
85 processing result in greater accessibility of mineral elements in the human gastro-intestinal tract.

86 Against this background, this study aimed to hydrolyze phytate structures in wheat grains and,
87 hence, to increase the bio-accessibility of mineral elements by (i) steeping and germination, and
88 by (ii) hydrothermal processing of steeped and germinated wheat grains. We here present, for the

first time, the impact of germination and hydrothermal processing of wheat on the spatial distribution and chemical speciation of Fe and Zn ions. These results were obtained using high-definition μ-X-ray fluorescence microscopy (μXFM) and X-ray absorption near-edge structure (XANES) spectroscopic imaging (De Brier, Gomand, Donner, Paterson, Smolders, Delcour, et al., 2016). The resulting knowledge can be used to develop whole grain-based products with improved mineral bio-accessibility.

2. MATERIALS AND METHODS

2.1 Materials

All chemicals and reagents were of at least analytical grade and purchased from Sigma-Aldrich (Bornem, Belgium) unless otherwise specified. Winter wheat (Cellule, harvest 2015) was kindly supplied by Limagrain (Avelgem, Belgium). A reference wheat (NIST1567) flour sample with certified element composition was obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

2.2 Steeping and germination of wheat grains

Wheat grains were steeped and germinated in triplicate in a pilot-scale micro malting system (Joe White Malting Systems, Perth, Australia) at 15 °C. The steeping was in a clear excess of water. It consisted of three successive wet stages (7, 7 and 3 h at 15 °C) alternated by two intermediate 6 h air rest stages at 15 °C. The imbibed grains were then germinated for 120 h. Samples were withdrawn after steeping and different times of germination (6, 12, 24, 48, 72, 96 and 120 h), flash-frozen using liquid nitrogen (N_2), freeze-dried and milled (IKA-mill, Staufen, Germany) into whole meal (< 200 µm) prior to analysis.

2.3 Hydrothermal processing of (steeped and germinated) wheat

Incubation (in duplicate) of wheat, steeped wheat and wheat germinated for 48 h or 120 h (20.0 g) was in 200 mL 100 mM HCl (pH 2.0) containing 100 mM NaCl or in 100 mM sodium acetate (pH 3.8, 4.0 and 5.0), sodium maleate (pH 6.0) or trishydroxymethyl-aminomethane (Tris)/HCl (pH 8.0) buffers at 20, 40, 45, 50, 55, 60, 65, 70 or 80 °C for 2 to 24 h. After incubation, the grains were rinsed with water to remove residual incubation media from the surface, flash-frozen with liquid N_2, freeze-dried and milled into whole meal prior to analysis.

2.4 Experimental design

To investigate the impact of different hydrothermal treatments on phytate breakdown and to identify the conditions that maximize the phytate breakdown, we used a four-factor I-optimal response surface experimental design. I-optimal experimental designs ensure precise predictions and are, therefore, ideal for the purpose of process optimization (Goos, Jones, & Syafitri, 2016). Our experimental design belongs to the family of response surface designs because we considered the factors' main effects, interaction effects and quadratic effects. The experimental factors were incubation time and temperature, the pH of the incubation medium and the different sample types (wheat, steeped wheat and wheat germinated for 48 or 120 h). The incubation time ranged from 2 to 24 h, the incubation temperature from 20 to 80 °C and the pH from 2.0 to 8.0. In the experiment, 20 different factor level combinations were tested. The resulting phytate responses were then used to fit a response surface model using ordinary least squares regression to identify significant ($p < 0.05$) effects. We started by fitting the full response surface model and used backward model selection to arrive at our final model. The fit of the final model was evaluated using the coefficient of determination (R^2) and the actual-by-predicted plot comparing the observed and predicted response values.

We also performed a follow-up experiment to fine-tune our model and to improve insight into the process. In the follow-up experiment, the temperature was varied from 40 to 70 °C when incubating sound wheat and wheat germinated for 120 h at pH 3.8 for 8 and 24 h. We used a 6 × 2 × 2 full factorial design. The analysis of the data from the follow-up experiment was performed in the same way as for the data from the initial experiment.

2.5 Phytase activity

The phytase activity assay was developed based on Heinonen & Lahti (2007). Whole meal (0.50 g) was suspended in 5.0 ml 25 mM sodium acetate buffer (pH 5.0), extracted [30 min, 150 rotations per min (rpm), 7 °C], centrifuged (10 min, 9,800 *g*, 7 °C) and filtered (Whatman filter, GE Healthcare Life Sciences, Buckinghamshire, UK). An aliquot (100 µl) of the resultant extract was added to 1.0 ml of 100 mM phytic acid (prepared in 25 mM sodium acetate buffer and

adjusted to pH 5.0 with 1.0 M HCl) and pre-incubated at 37 °C. After 3 h of incubation at this temperature, the enzymatic reaction was stopped by adding 4.0 ml color reagent solution [containing 50% *v/v* acetone, 25% *v/v* ammonium molybdate solution (5% *w/v*) and 25% *v/v* sulfuric acid (2.5 M)]. The extinction was measured at 400 nm (Ultraspec 2000 UV/VIS spectrophotometer, GE Healthcare, Uppsala, Sweden) after 10 min. The inorganic orthophosphate released as a result of phytase action is a measure for phytase activity. To correct for the orthophosphate initially present in the whole meal extracts, control samples [where an aliquot (100 µl) of such extract was added to 1.0 ml sodium acetate buffer (pH 5.0) instead of to the phytic acid substrate solution] were also included in the phytase assay. Phytase activity is defined as the amount of enzyme that releases one millimole of phosphate per minute of incubation per gram dry matter at 37 °C.

2.6 Phytic acid content

The phytic acid content in whole meal was determined after acid extraction of myo-inositol phosphates in triplicate with 0.66 M HCl (16 h, 150 rpm, room temperature) as in the Megazyme (Bray, Ireland) K-Phyt assay. Free P was directly measured after extraction by adding a color reagent solution containing ammonium molybdate, ascorbic acid and sulfuric acid, while total P was determined after subsequent breakdown of myo-inositol phosphates with an excess of phytase and alkaline phosphatase. The concentration of free P in the extracts was subtracted from the total P concentration to obtain the content of bound P. The latter was then divided by 0.282 to calculate the amount of phytate under the assumption that the measured P released from enzymatic hydrolysis exclusively originates from phytic acid. The phytate concentration will be defined as weight% of the initial dm (% of initial dm).

2.7 Elemental content

The elemental content in the (processed) wheat samples and digested fractions thereof (see 2.10) was determined using inductively coupled plasma mass spectrometry (ICP-MS). Samples (0.05

g, < 200 µm) were weighed into glass tubes and digested with 1.0 ml ultrapure 25% *v/v* HNO_3 and subsequently with 2.0 mL *aqua regia* (75% *v/v* ultrapure HCl and 25% *v/v* ultrapure HNO_3), followed by dilution with Milli-Q water (18.2 MΩ; Milli-Q Plus, Merck Millipore, Darmstadt, Germany) to 10.0 mL. The elements in the different samples and the NIST1567 wheat reference were analyzed by ICP-MS with an Agilent 7700x (Santa Clara, CA, USA). The reported elements were recovered within 10% of the certified values. The total concentrations of the elements in wheat grains, in mg/kg dm (± standard deviation, n ≥3), were 4,319 ± 164 K; 3,463 ± 132 P; 1,268 ± 34 Mg; 465 ± 7 Ca; 34.8 ± 0.9 Fe; 33.7 ± 1.2 Mn; 21.6 ± 0.3 Zn and 3.3 ± 0.1 Cu.

2.8 Mineral distribution

Scanning X-ray fluorescence (XRF) maps were collected using the hard X-ray Microprobe beamline P06 at the PETRA III synchrotron facility (DESY, Hamburg, Germany) to visualize the distribution of mineral elements in different wheat tissues (pericarp, aleurone and starchy endosperm). This experiment utilized the beamline's cryogenically cooled double crystal Si (111) monochromator for X-ray energy selection, and a pair of Kirkpatrick–Baez mirrors, which for this experiment, were focused to a spot size of 0.97 µm (horizontal) x 0.90 µm (vertical) at a flux of 2 x 10^10 ph/s on the sample. For XRF mapping, an incident beam energy of 10.5 keV was used and the full XRF spectrum for each image pixel collected using the Maia 384C detector system. The samples were analyzed on-the-fly with an encoded motor step (pixel) size of 1.0 µm and a transit time per pixel of 0.25 ms. This very low residence time of the beam at each pixel substantially reduced the risk of beam damage and, hence, of inducing chemical changes in redox sensitive elements such as Fe. Transverse cross sections of the central part of the freeze-dried wheat grains (85 µm) were obtained as in De Brier, Gomand, Donner, Paterson, Smolders, Delcour, et al. (2016). Sections were directly adhered on to Kapton polyimide tape without the need for embedding. For each treatment, two cross sections from two independently sectioned wheat grains were analyzed. We here show representative results of high quality cross sections. Semi-quantitative concentrations for different elements were calculated with GeoPIXE v7.4i

software (Ryan, Clayton, Cousens, Sie, Griffin, & Suter, 1990) in which corrections were made for self-absorption, absorption in air and the response of the detector using standard metal foils.

2.9 Iron and zinc speciation

X-ray absorption near-edge structure (XANES) imaging was used to investigate the chemical speciation of Fe and Zn in the aleurone cells of cross sections from the different wheat samples. In essence, the photon energy was stepped across the absorption edge of Fe and Zn, exciting core electrons in the atoms, resulting in characteristic fluorescence signals. Areas of interest for XANES imaging were determined from the XRF maps obtained previously (Figure S1 supplementary data). XANES imaging "stacks" (about 100 x 500 μm), comprising many XRF maps, were obtained by scanning the entire 2D area of interest at multiple, discrete energies across the relevant absorption edge with a pixel size of 3.0 μm and a dwell time of 0.48 ms. The K-absorption edges of Fe (7,112 eV) and Zn (9,659 eV) were scanned from 7,076 to 7,244 eV and from 9,582 to 9,832 eV respectively, using either 74 or 134 energy steps. From these stacks of XRF maps at discreet energies, XANES spectra can be extracted for each pixel. The obtained data were processed and analyzed using GeoPIXE v7.4i software and Athena v 0.9.24 software. XANES spectra of Fe and Zn reference foils were collected to calibrate the inflection point energy of the spectra. Phytate Fe^{2+}, phytate Fe^{3+} and phytate Zn^{2+} standards were prepared by mixing equal volumes of 30 mM $FeSO_4$, $FeCl_3$ and $ZnSO_4$ with 33 mM of phytic acid. In essence, phytic acid was dissolved in Milli-Q water after which the pH was adjusted to 2.0 with concentrated HCl to protonate the phosphate groups. Next, the Fe and Zn solutions were added to the phytic acid solution and the pH was adjusted to 6.0 with 0.1 M KOH to ensure complete chelation of the minerals. The phytate Fe^{2+} standard was prepared in a N_2-filled anaerobic chamber using deoxygenated Milli-Q water. Finally, the standard solutions were absorbed onto narrow strips of 11 μm thick filter paper (Whatman filter, GE Healthcare Life Sciences, Buckinghamshire, UK) which were wrapped in Kapton polyimide tape to protect them from oxygen and scanned across the relevant absorption edge as described earlier.

2.10 Iron and zinc bio-accessibility

An *in vitro* digestion procedure was carried out as in Minekus, Alminger, Alvito, Ballance, Bohn, Bourlieu, et al. (2014) with slight modifications to adapt it for evaluating the mineral bio-accessibility, *i.e.* the amount of minerals that was released from the food matrix and available for absorption. Mineral absorption itself was not measured in the present study. An aliquot (3.0 g dm) of ground (processed) whole meal (< 200 μm, see 2.2) was suspended in triplicate in 50.0 ml deionized water (pH 7.2) and incubated for 5 min at 37 °C under magnetic stirring (250 rpm). After adjusting the pH to 2.0 with 150 mM HCl and adding 2.0 ml pepsin solution (Sigma P-7012, 2.0 mg/ml), samples were incubated for 120 min at 37 °C under magnetic stirring (250 rpm). Next, 13.4 ml pancreatin solution (Sigma P-3292, 5.0 mg/ml in 150 mM sodium bicarbonate) was added and the pH was adjusted to 6.8 with 1.0 M NaOH after which the mixture was incubated for 3 h at 37 °C under magnetic stirring (250 rpm). Finally, the suspensions were centrifuged (15 min, 9,800 *g*, room temperature) and the obtained supernatants (digested fractions) and residues (undigested fractions) were flash-frozen with liquid N_2 and freeze-dried. The elemental content of the freeze-dried fractions were determined as outlined above and the elemental bio-accessibility was calculated as follows:

$$Mineral\ bio-accessibility\ (\%) = \frac{mass\ of\ element\ in\ digested\ fraction\ (g\ dm)}{total\ mass\ of\ element\ in\ wheat\ (g\ dm)} * 100$$

For each element, the difference between the concentration in intact wheat grains and in the weighted average concentration of all corresponding fractions obtained after the *in vitro* digestion assay, did not vary by more than 12%.

2.11 Statistical analysis

For constructing designs for the initial and follow-up experiment and for estimating the response surface models for phytate breakdown, the JMP Pro 12 software (SAS Institute, Cary, NC) was used**.** A one-way ANOVA ($P < 0.05$) with the Tukey multiple comparison procedure was

245 performed to identify significant differences between mean values of the other responses under
246 study.

247 ## 3. RESULTS AND DISCUSSION

248 ### 3.1 Impact of processing on phytase activity and phytate concentration in wheat grains

249 The endogenous phytase activity (Figure 1) in wheat was relatively high (1.6 mmole
250 phosphate/min/g dm). Eeckhout & De Paepe (1994) and Azeke, Egielewa, Eigbogbo, & Ihimire
251 (2011) already reported that sound wheat contains significant phytase levels. The phytase activity
252 increased linearly from 1.9 mmole phosphate/min/g dm in steeped grains to 5.8 mmole
253 phosphate/min/g dm in wheat germinated for 48 h and further increased to 8.3 mmole
254 phosphate/min/g dm when germinating for 120 h. Our results are in line with those of Bartnik &
255 Szafranska (1987) and Azeke, Egielewa, Eigbogbo, & Ihimire (2011) who observed a 3 to 5 fold
256 increase in activity levels when germinating wheat for 4 to 5 days at 20 to 28 °C. To date, the
257 mechanism leading to the increase in phytase activity during germination is not well understood.
258 It is well-known that gibberellic acid increases phytase synthesis (Gabard & Jones, 1986).
259 However, other research efforts have attributed the increase in phytase activity to activation of
260 pre-formed enzyme (Ou, Cheng, Xing, Lin, Nout, & Liang, 2011). Wheat seeds probably contain
261 a limited amount of pre-formed phytases which are activated within the first hours after
262 imbibition, while further increase in phytase activity during germination can be attributed to *de*
263 *novo* synthesis (Bewley, Bradford, Hilhorst, & Nonogaki, 2013).

264 The phytate concentration of the wheat grains made up 0.96% of the dm. This is within the range
265 of 0.6 to 1.0% of dm reported earlier by Febles, Arias, Hardisson, Rodriguez-Alvarez, & Sierra
266 (2002) for 100 different wheat samples. The increase in phytase activity during germination
267 corresponded with a decrease in phytate content from 0.96% of dm to 0.81% of the initial dm in
268 wheat grains germinated for 120 h (Figure 1). It is of note that the phytate content significantly
269 decreased with 7% after 36 h of germination at 15 °C. Bartnik & Szafranska (1987) and Azeke,

Egielewa, Eigbogbo, & Ihimire (2011) found that the wheat phytate content decreases by 9 to 15% after 2 days and by 24 to 34% after 4 to 5 days of germination at temperatures ranging from 20 to 30 °C, indicating that higher germination temperatures lead to a higher degree of phytate breakdown. Furthermore, prolonged germination times of at least 3 to 5 days are generally needed to decrease phytate concentrations in cereals with more than 30% (Azeke, Egielewa, Eigbogbo, & Ihimire, 2011). Although there was a substantial increase in phytase activity, phytate breakdown during germination was rather limited. Typical germination conditions here used (pH 7.2, 15 °C) are indeed not optimal for endogenous wheat phytase action (pH 4.0 to 6.0; 50 to 60 °C) (Peers, 1953).

3.2 Impact of hydrothermal processing of (germinated) wheat grains on phytate contents

In this section, we report the results of our study on whether the relatively high endogenous phytase activity in germinated wheat could further breakdown phytate and, hence, improve elemental bio-accessibility when it was incubated under different conditions. Endogenous wheat phytase is most active at 55 °C and a pH of 4.5 to 5.0 (Peers, 1953). Incubating flour from (germinated) wheat under these conditions reduced the phytate content by 70 to 90% (Guo, Bian, Zhu, Guo, Peng, & Zhou, 2015). As, to the best of our knowledge, no systematic study of the impact of different process parameters on the hydrolysis of phytate during hydrothermal processing of intact (germinated) wheat grains has been executed yet, we constructed a 20-run I-optimal experimental design to assess the impact of hydrothermal processing of different wheat sample types on the hydrolysis of phytate. The phytate content decreased from 0.96% of dm in unprocessed wheat to 0.44, 0.44, 0.38 and 0.36 % of the initial dm in hydrothermally processed (60 °C, pH 4.0, 8 h) wheat, steeped wheat and wheat germinated for 48 or 120 h, respectively. Since regular wheat already had relatively high phytase activity (see 3.1), no large differences in phytate hydrolysis were observed between regular and germinated wheat after hydrothermal processing.

A response surface model was fit to explain the variation in phytate content as a function of incubation time, incubation temperature, pH and wheat sample type. To optimize the model fit, we used a power transformation for the incubation temperature (*i.e.*, the fourth power of incubation temperature). Our final model had a R^2 value of 79%. It turned out that the incubation temperature had a substantial nonlinear effect on phytate contents ($P < 0.001$) and that the sample type had a significant main effect ($P < 0.001$). The largest phytate breakdown was observed between 55 °C and 75 °C. There was also a significant interaction effect between incubation time and temperature ($P = 0.012$) and between the incubation time and the pH of the incubation medium ($P = 0.004$). Incubation time had a rather strong negative impact on phytate contents when the incubation temperature was as high as 70 °C, but virtually no effect when it was as low as 20 °C. The pH of the incubation medium hardly mattered if the incubation time was about 12 h, but it had a slightly negative nonlinear effect on phytate contents if the incubation time was 2 h and a slightly positive nonlinear effect on phytate contents if the incubation time was 24 h. Regardless of the incubation time, varying the pH level had a rather small effect as long as it was in the range from 3.5 to 6.5 (with a maximal predicted phytate breakdown at a pH of 3.8). Therefore, pH was the least influential factor ($P = 0.013$), even though it was clearly statistically significant. There was no significant interaction effect involving the sample type, so that the interpretation of the sample type's effect was quite simple: the phytate contents in wheat germinated for 120 h were 0.03% smaller than those in wheat germinated for 48 h, the phytate contents in wheat germinated for 48 h were 0.03% smaller than those in steeped wheat and steeped wheat resulted in phytate contents that were 0.03% smaller than those in regular wheat. While the model provided interesting insights regarding the impact of pH of the medium, incubation time and wheat sample type on phytate contents, it was not entirely satisfactory because of its rather low R^2 value and because it was unable to successfully describe the effect of temperature. This is clearly visible in the actual-by-predicted plot (Supplementary data, Figure S2a), where a specific cluster of points appeared well below the 45° line. These points indicate that some observations

with a small observed response could not be predicted well by the response surface model. More specifically, the model predicted a minimal phytate content of 0.30% of the initial dm when wheat grains germinated for 120 h were incubated at a pH of 3.8 for 24 h at 70 °C, while we measured a phytate content of 0.39% of the initial dm when subjecting wheat grains germinated for 120 h to these conditions.

To obtain more insight into this issue, a full factorial follow-up experiment involving six temperature levels in the range from 40 °C to 70 °C was executed (see 2.4). In this experiment, regular wheat or wheat germinated for 120 h was incubated at a pH of 3.8 for 8 or 24 h, building on the conclusions from the initial response surface experiment. Using the data from the follow-up experiment, we fitted a new response surface model with an R^2 of 93% (Supplementary data, Figure S2b). The model involved a large quadratic effect for the incubation temperature ($P < 0.001$), with a minimal predicted phytate content at a treatment temperature of 50 °C. The incubation time had a significant negative main effect on phytate contents ($P < 0.001$), with a minimal predicted phytate content for 24 h of processing. In addition, the interaction between the wheat sample type and the incubation time was significant ($P = 0.010$). For an incubation time as little as 8 h, the difference in phytate content between untreated and germinated samples was about 0.16% while, for an incubation time of 24 h, the difference was about 0.05%. The impact of incubation time on phytate contents was more negative for untreated samples than for samples germinated for 120 h.

In regular wheat, a phytate content less than 0.2% of the initial dm was predicted after hydrothermal processing at temperatures ranging from 40 to 60 °C for 15 to 24 h and a pH of 3.8 (Figure 2A). In wheat germinated for 120 h, a phytate content less than 0.1% of the initial dm was predicted after hydrothermal processing at 42 to 56 °C for at least 15 h and a pH of 3.8 (Figure 2B). That a lower phytate content was predicted in wheat germinated for 120 h than in regular wheat after hydrothermal processing can be explained by the higher phytase activity in the former (see 3.1). The statistical model even predicted that hydrothermal processing of wheat germinated

for 120 h at 50 °C and pH 3.8 for 24 h almost completely degrades the phytate structures (Supplementary data, Figure S3). The prediction model was experimentally validated using several confirmatory tests in which wheat grains germinated for 120 h at 15 °C were hydrothermally treated at this optimal process condition. The comparison between the measured phytate content (0.04 ± 0.01% initial dm) with that predicted by the model (0.01 ± 0.04% initial dm) confirmed the validity of the here developed model to predict the impact of the hydrothermal process conditions on phytate contents. The nearly complete degradation is due to the fact that hydrothermal processing at 50 °C allows optimal endogenous phytase action and may favor the interaction between phytase and its substrate owing to microstructural changes in the phytate globoids (Bergman, Autio, & Sandberg, 2000). The pH only had a modest effect on phytase activity when the grains were incubated at 50 °C indicating that temperature is the predominant factor. Nevertheless, incubation times of at least 18 h were needed to breakdown phytate almost completely.

3.3 Impact of germination and hydrothermal treatment on the mineral distribution in wheat

The impact of wheat processing on the mineral distribution and spatial associations of divalent elements with their main ligand, phytic acid, was studied by determining the distribution of P, Fe and Zn in pericarp, aleurone and starchy endosperm. Moreover, the impact of wheat processing on the distribution of K, the most abundant mineral element in wheat, was studied as well (Figure 3). The profiles in Figure 3 represent the elemental concentration (mg/kg) within the line profile defined by white rectangles and in the direction of the white arrows in the elemental images. Potassium was mainly present in the region corresponding to the aleurone where it probably formed weak soluble complexes with *e.g.* oxalate (199 mg/100 g dm) (Israr, Frazier, & Gordon, 2013). As a result of steeping and germination and certainly hydrothermal processing of germinated wheat, most K was leached into the steeping or incubation media, respectively. Moreover, during germination and mainly during hydrothermal processing K ions probably dissociated from their ligands, became highly mobile (Marschner, 2012) and diffused into the

endosperm (Figure 3). Phosphorus was also present in the aleurone, where it is mainly stored as phytate. During steeping and germination, it was not translocated. However, hydrothermal processing caused loss of P due to leaching of orthophosphates released by enzymatic action. Iron and Zn were mainly present in the region corresponding to the aleurone in wheat where they (partly) were co-located with P suggesting that they were chelated to phytic acid (De Brier, et al., 2016). Nevertheless, variations in Zn speciation exist between the different wheat tissues. Indeed, Zn ions are mainly bound to sulfur containing peptides (*e.g.* metallothionein) in the protein storage vacuoles of the embryo tissues (Persson, Hansen, Laursen, Schjoerring, & Husted, 2009), while it can also be associated with nicotianamine in the endosperm (Eagling, Neal, McGrath, Fairweather-Tait, Shewry, & Zhao, 2014). Germination and hydrothermal processing did not affect the localization of Fe indicating that it was mainly transported to the developing embryo through the aleurone cells. Although Zn was mainly present in the aleurone in the germinated and/or hydrothermally processed wheat grains as well, it was also present in small concentrations in the pericarp after germination. One can assume that Zn partly migrated from the aleurone to the pericarp once it was released from the phytate structures. Although Fe and Zn were still confined to the aleurone and, hence, co-located with P, they were probably no longer chelated to phytate since the phytate structures were almost completely hydrolyzed during hydrothermal processing (see 3.2). To confirm this hypothesis, we studied the impact of germination and hydrothermal processing of wheat on the mineral bio-accessibility (see 3.5).

3.4 Impact of processing on iron and zinc speciation in wheat grains

XANES imaging was here used to determine the oxidation state of Fe and the association of Fe and Zn with phytate in aleurone cells of regular wheat, of wheat germinated for 48 or 120 h, of wheat germinated for 48 h or 120 h and hydrothermally processed (at 60 °C and pH 4.0 for 4 h or at 50 °C and pH 3.8 for 8 h). We also included phytate Fe^{2+}, phytate Fe^{3+} and phytate Zn^{2+} standards in this study.

The XANES spectra can provide information about both valence and species of target ions. In our study, both Fe and Zn were analyzed separately. The "inflection point" of the absorption edge of the XANES spectrum (maximum of the spectrum's first derivative) is indicative of the valence state of the target ion, where a higher energy inflection point indicates a more oxidized ion and a lower one indicates reduction. Other spectral features, including the principal peak (white line) and overall shape of the spectra can be used in comparison and identification of species. When inspecting the Fe K-edge XANES collected from our samples, it was observed that different Fe species were present (Figure 4a). The energies of the inflection point of the absorption edge as well as that of the principal peak of the ferric and ferrous standards were higher for ferric (7,127.9 and 7,131.8 eV, respectively) than for ferrous (7,124.1 and 7,130.8 eV, respectively) compounds (De Brier, et al., 2016). Further, the phytate standards exhibited a typical shoulder feature at around 7,137 eV (De Brier, et al., 2016). The XANES spectra of Fe in the aleurone cells of wheat grains had an inflection point and a principal peak at 7,125.7 and 7,130.9 eV, respectively (Supplementary data, Table S1) which is in line with De Brier, Gomand, Donner, Paterson, Smolders, Delcour, et al. (2016) and suggests that ferrous iron compounds occur in regular aleurone cells. However, the inflection point and the principal peak of Fe in the germinated wheat grains were at 7,128.1 and 7,130.9 eV and in hydrothermally processed germinated wheat grains at 7,128.1 and 7,131.9 eV, respectively. The principal peaks of Fe at higher energies in germinated and hydrothermally processed wheat showed that Fe oxidation had occurred during germination. Fe oxidation negatively influences Fe absorption by the human body, since ferrous ions can readily be absorbed by the intestinal enterocytes, while ferric ions have to be reduced prior to uptake (Waldvogel-Abramowski, Waeber, Gassner, Buser, Frey, Favrat, et al., 2014). The typical Fe-phytate shoulder feature was also present in the Fe XANES spectra of wheat (Figure 4a) and wheat germinated for 48 h, providing direct evidence that Fe-phytate chelates occurred in the aleurone cells. However, the spectra extracted from the aleurone cells in wheat germinated for 120 h and especially in hydrothermally processed (germinated) wheat clearly exhibited other

features at the Fe K-edge, indicating that other Fe species occurred in these samples and that its speciation (partly) changed upon processing. We conclude that Fe was not only chelated to phytate in these samples supporting the outcome of the developed model on phytate breakdown (see 3.2). We here for the first time thus provided direct evidence that Fe in wheat grains is oxidized during steeping, germination and hydrothermal processing and, at the same time, that the chemical environment of Fe drastically changed.

Moreover, we studied the chemical speciation of Zn in aleurone cells of the above-mentioned samples (Figure 4b). The energy position of the inflection point and the principal peak of the Zn XANES spectra were similar for all samples studied and about 9,658 and 9,662 eV, respectively. In line with the spectrum of the Zn-phytate standard, the spectra of aleurone cells of wheat and wheat germinated for 48 h clearly exhibited a shoulder feature at an energy position of about 9,665 eV, indicating that Zn was indeed mainly chelated to phytate structures in aleurone cells and that germination impacted its speciation to only a limited extent. This shoulder feature was less pronounced in the spectra of the hydrothermally processed wheat grains, indicating that other Zn species occurred in their aleurone cells. It is reasonable to expect that the changes in Fe and Zn structures upon wheat processing can increase their bio-accessibility.

3.5 Impact of processing on iron and zinc bio-accessibility in wheat

We showed that during germination and hydrothermal processing of wheat grains, phytate was extensively broken down and that Fe ions were released from their chelates. Here, we investigated with an *in vitro* test whether this decrease in phytate content improved elemental bio-accessibility. First, we evaluated the changes in wheat's elemental content upon germination and hydrothermal processing. Germinating wheat for 5 days at 15 °C significantly impacted neither the Fe nor the Zn contents (data not shown). During hydrothermal processing (50 °C, 8 h) of regular wheat, only 5 and 4% of the total elemental Fe and Zn, respectively, were leached out into the incubation media. However, this process condition used for wheat germinated for 120 h resulted in a 30 and

20% loss of the elemental Fe and Zn, respectively. Since only minor losses of elemental Fe and Zn were observed in hydrothermal processed regular wheat grains, these samples were here used for assessing elemental bio-accessibility.

The Fe bio-accessibility in wheat amounted to 4.6% (Table 1), in line with the findings of Platel, Eipeson, & Srinivasan (2010) and Luo, Xie, Jin, Wang, & He (2014). They reported it to be 6.4 and 4.6%, respectively. The Fe bio-accessibility increased to 14.1% in steeped and germinated wheat which is similar to the 17% bio-accessible Fe in germinated wheat reported by Platel, Eipeson, & Srinivasan (2010). The Zn bio-accessibility in wheat was only 2.5 and increased to 14.6% during germination for 5 days at 15 °C. Platel, Eipeson, & Srinivasan (2010) reported that the Zn bio-accessibility in wheat increased from 11 to 37% during germination. Further, the study of Afify, El-Beltagi, El-Salam, & Omran (2011) also showed that Zn bio-accessibility in sorghum increased from 8 to 18% upon germination. The Fe and Zn bio-accessibility increased even up to 36.6 and 27.4%, respectively, in wheat grains which were hydrothermally processed at 50 °C and pH 3.8 for 8 h (Table 1). This agrees well with Sandberg & Svanberg (1991) who found that hydrothermally processing of wheat bran at pH 4.5 to 5.0 at 55 °C for only 1 h reduced the phytate content by 98% and increased Fe bio-accessibility from 3 to 53%. Finally, we found a good linear relationship between phytate content and Fe bio-accessibility (Supplementary data, Figure S4) suggesting that phytate hydrolysis is absolutely needed to render Fe more accessible under the present conditions.

4 CONCLUSIONS

This study aimed to investigate the hydrolysis of phytate structures in wheat grains and, hence, to increase the bio-accessibility of mineral elements. Germination of wheat for 5 days at 15 °C only led to 15% and thus a limited phytate breakdown. At the same time, the Fe ions were (partially) oxidized and the chemical environment of Fe (and Zn) changed only slightly, which was in line with the rather limited phytate breakdown during germination. As a consequence, the Fe and Zn bio-accessibility increased from 5 and 3% in wheat to 14 and 15% in germinated wheat, respectively. Under the experimental conditions used here, wheat germination had a limited beneficial impact on elemental bio-accessibility. Hydrothermal processing of germinated wheat using conditions allowing optimal phytase action (50 to 60 °C, pH 4.0, 8 to 24 h) led to an almost complete phytate breakdown. The chemical speciation of Fe ions in wheat grains drastically changed upon hydrothermal processing indicating that they were no longer bound to phytate structures. The extensive phytate breakdown in these samples increased the Fe and Zn bio-accessibility further to 37 and 27%, respectively. Thus, hydrothermal processing of (germinated) wheat grains makes the Fe and Zn ions substantially more available for uptake in the human gastro-intestinal tract and these innovative process strategies can pave the way for increasing the content of bio-accessible mineral elements in whole grain-based products.

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Conflict of interest

The authors declare that they do not have any conflict of interest.

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FIGURE CAPTIONS

Figure 1

Phytase activity (—●—) [mmole phosphate/min/g dry matter (dm)] and phytate content (- -●- -) (% of initial dm) as a function of time (h) in wheat grains steeped at 15 °C for 29 h and germinated at 15 °C for 120 h.

Figure 2

Contour plots representing the influence of hydrothermal processing [incubation time (h) and incubation temperature (°C) at pH 3.8] on the predicted phytate content [% of initial dry matter (dm)] in wheat (A) and wheat germinated for 120 h (B).

Figure 3

Tri-color image of iron (Fe; red; maximum concentration of 273 mg/kg), phosphorus (P; green; maximum concentration of 13,900 mg/kg) and potassium (K; blue; maximum concentration of 2,700 mg/kg) distribution in cross sections of a typical wheat grain (A), a wheat grain germinated for 48 h (B) and a wheat grain germinated for 48 h and hydrothermally processed at 60 °C and pH 4.0 for 4 h (C). Elemental concentration profiles of K, P, Fe and zinc (Zn) from a selected area (white box in figure) are shown. The distance (μm) on the X-axes of the profiles correspond to the selected area (350 μm wide) starting at the outer side of the cross section. The letters in the profiles indicate the pericarp (P), aleurone (Al) and endosperm (E) for each selected area.

Figure 4

(A) From top to bottom: iron X-ray absorption near-edge structure spectroscopy spectra of standard reference phytate Fe^{2+} (1) and phytate Fe^{3+} components (2), of aleurone cells selected in cross sections of wheat (3), wheat germinated for 48 h (4) or 120 h (5), wheat germinated

for 48 h and hydrothermally processed at 60 °C and pH 4.0 for 4 h (6) and wheat hydrothermally processed at 50 °C and pH 3.8 for 8 h (7).

(B) From top to bottom: zinc X-ray absorption near-edge structure spectroscopy spectra of standard reference phytate Zn^{2+} (1) and of aleurone cells selected in cross sections of wheat (2), wheat germinated for 48 h (3), wheat germinated for 48 h and hydrothermally processed at 60 °C and pH 4.0 for 4 h (4), wheat hydrothermally processed at 50 °C and pH 3.8 for 8 h (5) or wheat germinated for 120 h and then hydrothermally processed at 50 °C and pH 3.8 for 8 h (6).

FIGURE CAPTIONS SUPPLEMENTARY DATA

Figure S1

X-ray fluorescence image for Fe (350 mg/kg) in a wheat grain germinated for 48 h in which the indicated area (A) was selected for extraction of the X-ray absorption near-edge structure (XANES) spectroscopy. Detail image of a XANES stack for Fe (0.12 weight%) in the aleurone cells of the wheat grain germinated for 48 h (A) taken at the highest energy scanned (7,129.8 eV), above the Fe absorption edge providing a uniform fluorescence signal. This selected area in the wheat grain is a typical region scanned for each XANES stack.

Figure S2

Actual by predicted plot of first model with a specific cluster of points well below the 45° line ($R^2 = 0.79$) (A) and model from follow-up experiment ($R^2 = 0.93$) (B) used to predict the impact of incubation temperature, pH of incubation medium, incubation time and wheat sample type on the phytate content [% of initial dry matter (dm)].

Figure S3

Prediction profiler of the follow-up experiment showing the predicted phytate content [% of initial dry matter (dm)] for the optimal process conditions (wheat germinated for 120 h, 50 °C, pH 3.8, 24 h). The profiler also highlights the impact of incubation temperature, incubation time and type wheat sample on the predicted phytate content.

Figure S4

Linear correlation between iron bio-accessibility (%) and phytate content [% of initial dry matter (dm)] in wheat grains.