



HAL
open science

Mass spectrometry-based analytical developments to link iron speciation to iron bioavailability in maize

Shuanglong Wang, Laurent Ouerdane, Owen Hoekenga, Joanna Szpunar, Ryszard Lobinski

► **To cite this version:**

Shuanglong Wang, Laurent Ouerdane, Owen Hoekenga, Joanna Szpunar, Ryszard Lobinski. Mass spectrometry-based analytical developments to link iron speciation to iron bioavailability in maize. Food Chemistry, 2019, 294, pp.414-422. 10.1016/j.foodchem.2019.05.014 . hal-03133490

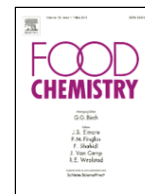
HAL Id: hal-03133490

<https://hal.science/hal-03133490>

Submitted on 6 Feb 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Mass spectrometry-based analytical developments to link iron speciation to iron bioavailability in maize

Shuanglong Wang^a, Laurent Ouerdane^{a,*}, Owen Hoekenga^b, Joanna Szpunar^a, Ryszard Lobinski^a

^a CNRS/Université de Pau et des Pays de l'Adour, Institut des sciences analytiques et de physico-chimie pour l'environnement et les matériaux, UMR5254, 64000 Pau, France

^b Cayuga Genetics Consulting Group LLC, Ithaca, NY 14850, USA

ARTICLE INFO

Keywords:

Speciation
Iron
Maize
Bioavailability
Mass spectrometry
HILIC
Phytate
Mugineic acid

ABSTRACT

A sequential fractionation procedure based on (i) water extraction, (ii) hexane extraction, (iii) saccharification, and (iv) proteolysis was developed to provide the first ever data on the molecular distribution of iron in maize. This was completed by the operational determination of the iron bioavailability using an *in-vitro* simulated model for gastro-intestinal digestion. The coupling of hydrophilic interaction chromatography (HILIC) and size exclusion chromatography (SEC) with the parallel detection by inductively coupled plasma mass spectrometry (ICP-MS) and high resolution electrospray mass spectrometry (HR-ESI-MS) allowed the identification of water-soluble Fe(III)-mugineate, Fe(III)-(citrate)₂, and Fe(III)₂-(phytate)₂. The procedures were applied to study some well characterized maize varieties having shown previously differences in iron bioavailability during cell culture and animal model feeding studies. The combined analytical methods developed in this work could unambiguously discriminate low from high Fe bioavailable seeds in these closely related maize varieties.

1. Introduction

Iron (Fe) is an essential nutrient for all living organisms. Inadequate intake of iron results in iron-deficiency anemia (IDA), especially in children and women who are or may become pregnant, and is a serious problem worldwide (Bodnar, Proulx, Scott, Beavers, & Reddy, 2013; Eagling, Wawer, Shewry, Zhao, & Fairweather-Tait, 2014; La Frano, de Moura, Boy, Lonnerdal, & Burri, 2014). One of the main reasons of IDA is the poor availability of iron in foodstuffs of plant origin in comparison to the forms of iron present in animal-based foods. Therefore, there is a considerable interest in enhancing iron bioavailability from staple foods (Oikeh, Menkir, Maziya-Dixon, Welch, & Glahn, 2003).

Maize is the third most important cereal crop in the world. It supplies not only energy in the form of carbohydrates, fat and proteins but also vitamins and many minerals, including iron. However, only 5–20% of the iron in maize can be assimilated by man (Bachir Daoura & Hash, 2015). Therefore, different breeding and genetic engineering strategies were investigated to increase the bioavailability of iron in maize with the goal of increasing the concentration of iron-binding chelators and proteins, decreasing the concentration of absorption in-

hibitors and increasing the concentration of absorption promoters (Clemens, 2014).

Iron bioavailability is defined as the proportion of iron in a food which is digested, absorbed, and ultimately utilized for normal body functions (Fairweather-Tait, Phillips, Wortley, Harvey, & Glahn, 2007). Several methods have been developed over time to assess the iron bioavailability in foodstuffs. Firstly, the chemical balance method is based on the determination of the difference between the iron taken in with food and iron excreted with feces (Wienk, Marx, & Beynen, 1999). Another widely used method to assess the iron bioavailability/solubility is based on the use of Caco-2 (human colon adenocarcinoma) cells model (Fairweather-Tait et al., 2007; Glahn, Cheng, Welch, & Gregorio, 2002; Glahn, Lee, Yeung, Goldman, & Miller, 1998; Glahn, Wien, van Campen, & Miller, 1996). This approach allows the evaluation as iron source of different types of non-heme iron whereas heme iron is directly absorbed (Au & Reddy, 2000). The rat hemoglobin repletion method is also a standard method used by the Association of Official Analytical Chemists. Finally, isotopic methods can be used; they are based on labelling food with an iron isotope intrinsically (biosynthetically) (Hurrell & Egli, 2010) or during the iron absorption measurement (Cook et al., 1972).

* Corresponding author.

Email address: laurent.ouerdane@univ-pau.fr (L. Ouerdane)

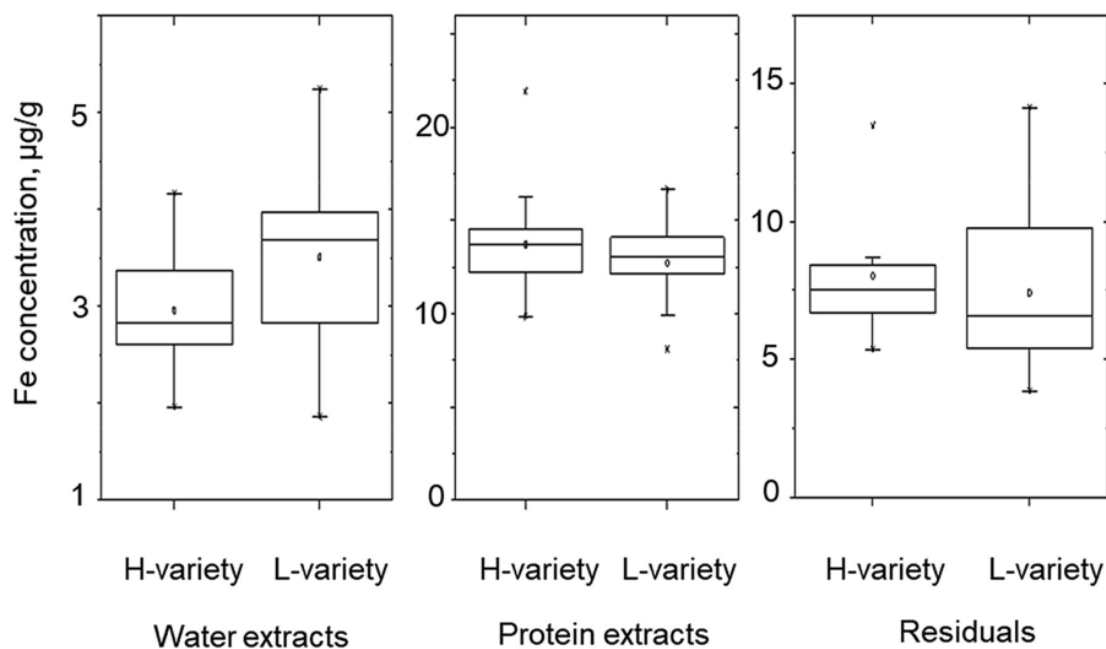


Fig. 1. Statistical evaluation (box-plot, ANOVA method) of iron concentrations during maize fractionation in low iron bioavailable variety (L-variety) and high iron bioavailable variety (H-variety).

The bioavailability of iron strongly depends on its chemical form. Hence, the description of the bioavailability of iron in food on the molecular level and linking this data with the operational approaches is of potential interest. The relevant analytical approaches are based on chemical fractionation or fractionation under simulated gastrointestinal conditions which are followed by the identification of the released species (Bou Khouzam, Szpunar, Holeman, & Lobinski, 2012). Despite numerous efforts, there remain several limitations in methodology, especially on the level of the iron species identification, and the truly molecular characterization is seldom achieved (Weber, Neumann, & Römheld, 2002).

Regarding the iron speciation in foods, most of the emphasis has concerned phytic acid, an abundant chelator of metals and a primary storage form for phosphorous in seeds. Iron-phytate complexes (Persson, Hansen, Laursen, Schjoerring, & Husted, 2009) are assumed to have low bioavailability and thus represent a nutritional liability rather than a nutritional asset. Iron complexes with other ligands, such as mugineic acid (Weber et al., 2002) or citrate (Teucher, Olivares, & Cori, 2004), are expected to have higher bioavailability. However, they are poorly documented in the literature and only a few works referred to the presence of iron mugineic acid complexes exist. For maize, there is no information on the distribution of iron amongst its major chemical constituents (e.g. proteins or polysaccharides) or on the chemical speciation of iron.

It is expected that variabilities in Fe speciation in maize seeds could be related to differences in Fe bioavailability. The goal of this work was to develop an analytical methodology able to provide information on the fractionation and speciation of iron in maize. For this purpose, the protocols of the enzymatic fractionation were re-visited to provide the first data of the operationally defined iron chemical distribution using maize varieties that were developed to have different levels of iron bioavailability (Hoekenga, Lung'aho, Tako, Kochian, & Glahn, 2011; Lung'aho et al., 2011). The combinations of hydrophilic interaction chromatography (HILIC) and size exclusion chromatography (SEC) with the parallel detection by inductively coupled plasma mass spectrometry coupling of spectrometry (ICP-MS) and high resolution electrospray mass spectrometry (HR-ESI-MS) are now well developed approaches to identify iron complexes in biological matrices (Choubassi

et al., 2017). They were applied here to investigate well characterized maize varieties having shown difference in the *in vivo* bioavailability with an attempt to correlate the chemical information with bioavailability data obtained using the Caco-2 cells method (Hoekenga et al., 2011; Lung'aho et al., 2011).

2. Experimental

2.1. Maize samples and sample preparation

Six previously described maize varieties were used for this study. Four of them are two pairs of partially inbred sister lines derived by backcrossing recombinant inbreds to either the B73 inbred parent or the Mo17 inbred parent (Lung'aho et al., 2011) and are referred to as HB, LB, HM and LM. The letter H stands for presumed high Fe bioavailability, L: presumed low Fe bioavailability, B: backcrossed to the B73 parent, M: backcrossed to the Mo17 variety. These four varieties were grown on the experimental farms of Cornell University (2013), Iowa State University (2014, due to the generosity of Prof. Michael Muszynski), and the University of Missouri (2014, due to the generosity of Prof. David Braun). The other two varieties used were experimental hybrids made by crossing the four lines above to create high (HH, HB and HM) and low (LL, LB and LM) types (Hoekenga et al., 2011). The experimental hybrids were used for animal feeding studies to validate the results of the Caco2 bioassays. In the poultry feeding study, differences in iron concentration were by far smaller (12%) than those observed in Caco2 estimated bioavailability (67%) or animal feeding response (40%). This suggests that iron speciation is a key determinant for iron bioavailability and thus analytical chemistry would be highly complementary to bioassays for assessment of nutritional quality.

2.2. Reagents and standards

Analytical reagent grade chemicals: acetonitrile, formic acid, acetic acid, nitric acid, ammonium acetate and ammonia were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). The iron standard solution was from SCP Science (Canada) and citric acid from Acros Organics (Belgium). Ultrapure water (18 MΩ cm) was obtained

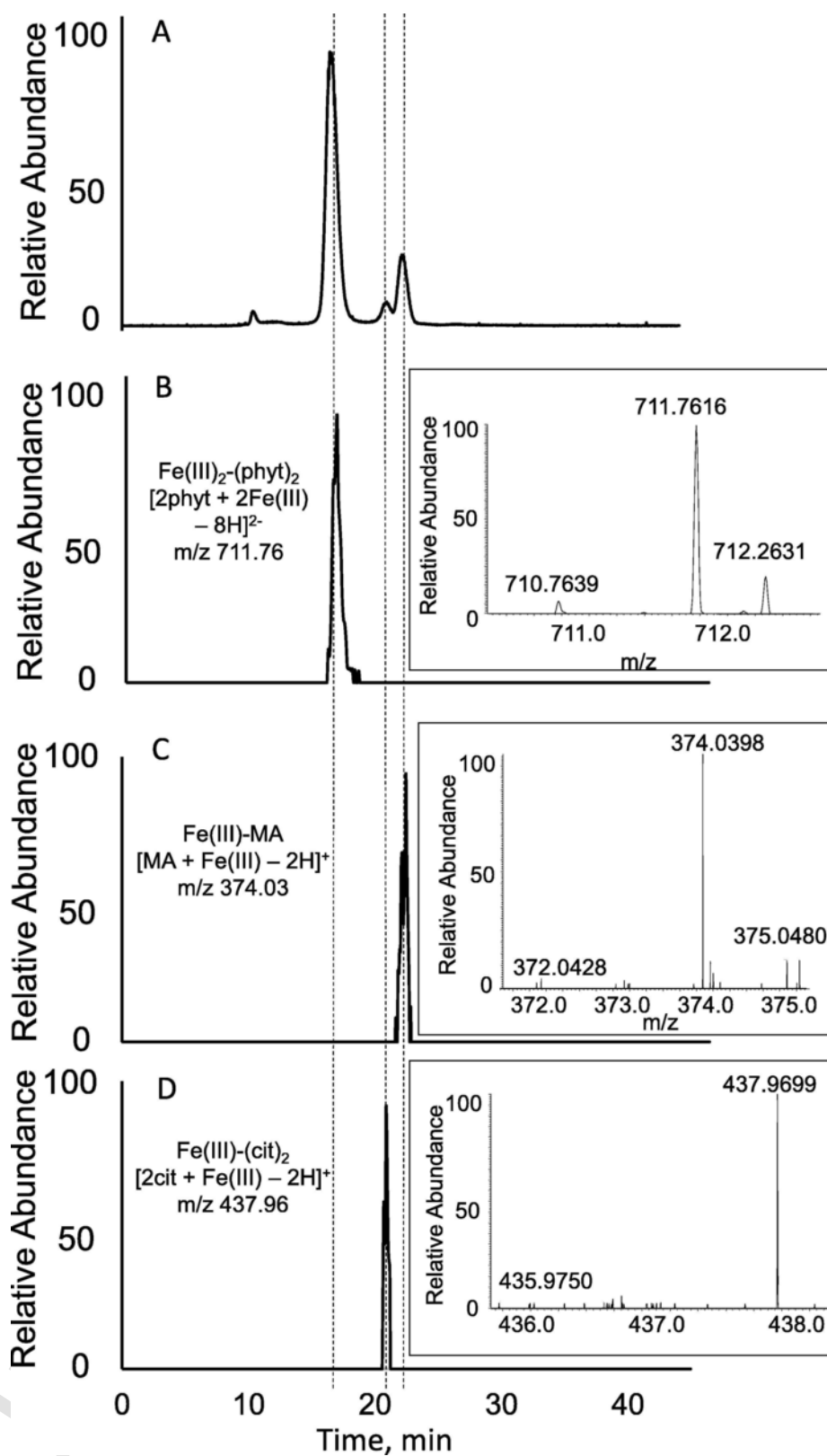


Fig. 2. Identification by SEC-ICP-MS and SEC-ESI-MS of iron species in maize extracts: A) ^{56}Fe SEC-ICP-MS chromatogram and extracted ion chromatograms of B) $\text{Fe}_2\text{-phytate}_2$ (m/z 711.76), C) Fe-mugineate (m/z 374.03) and D) Fe-(citrate) $_2$ (m/z 437.96) were recorded. ESI mass spectra observed at peak apex are shown in the insets.

from a Milli-Q system (Millipore, Bedford, MA). Thermally stable α -amylase (3000 u/mL, 0.1 mL), amyloglucosidase (3260 u/mL, 50 μL), and pepsin (Sigma P-7000; 10,863 u/mg) enzyme solutions for sequen-

tial extraction were diluted with water. The enzymes for simulated gastrointestinal extraction were: (i) 0.2 g pepsin (porcine, 800–2500 u/mg) was dissolved in 5 mL of 0.1 M HCl, mixed with 2.5 g Chelex-100

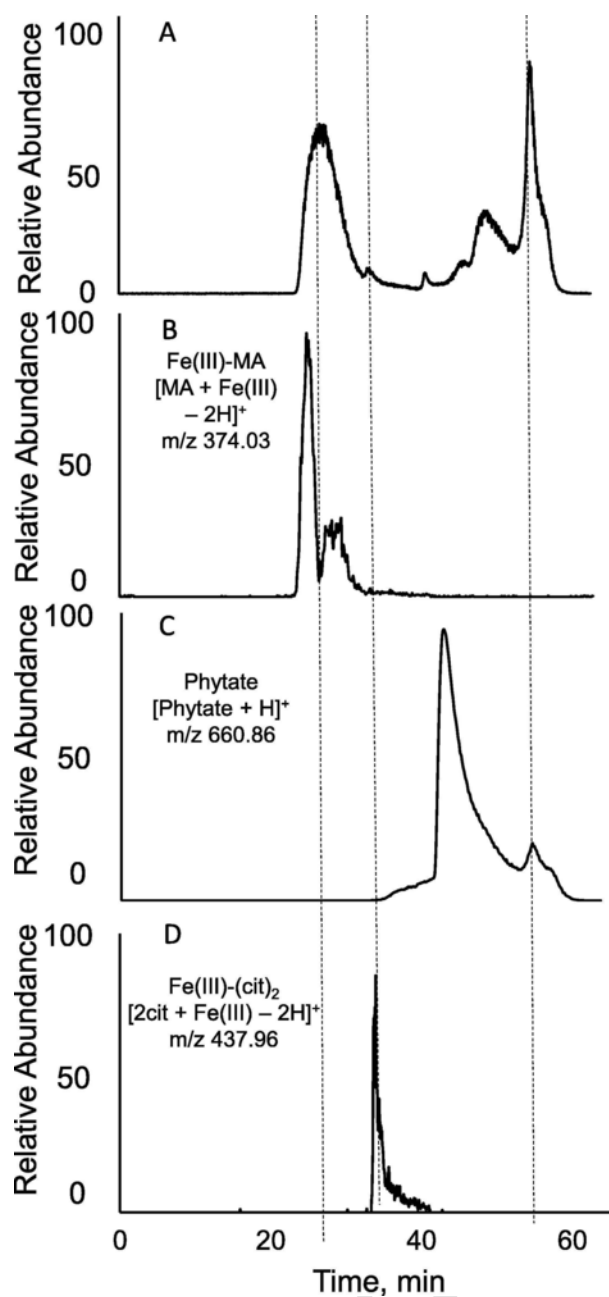


Fig. 3. Identification by HILIC-ICP-MS and HILIC-ESI-MS of iron species in maize extracts. A) ^{56}Fe HILIC-ICP-MS chromatogram and extracted ion chromatograms of B) Fe-mugineate (m/z 374.03), C) phytate (m/z 660.86) and D) Fe-(citrate) $_2$ (m/z 437.96) were recorded.

and shaken for 30 min on a table shaker. The solution was centrifuged to remove Chelex-100 and made up to 10 mL; and (ii) 0.05 g pancreatin 9049-47-6 (4x USP specification) and 0.3 g bile extract (glycine and taurine conjugates of hydoxychoholic acid and other bile salts) were dissolved in 35 mL of 0.1 M NaHCO_3 and shaken with 12.5 g of Chelex-100 for 30 min. Chelex-100 was removed by centrifugation. The enzyme solutions were stored at -20°C (Glahn et al., 1998).

2.3. Instrumentation

A DigiPrep heated-block digester (SCP Science, Courtaboeuf, France) was used to perform the digestion of the samples and the extracts for the total Fe determinations. The iron determination was car-

ried out using an Agilent 7500 model ICP-MS (Agilent 7500, Tokyo, Japan).

For the chromatographic couplings an Agilent Model 7700 s ICP-MS equipped with a collision cell was used. Size-exclusion chromatographic separations were performed using a 1200 series HPLC pump (Agilent) as delivery system. The columns used were: Superdex Peptide HR 10/30 (300 mm \times 10 mm i.d) purchased from GE Healthcare Bio-Science AB, Sweden. Hydrophilic ion interaction chromatography (HILIC) was performed using an Agilent 1100 capillary pump (Agilent) equipped with a 100 mL/min $^{-1}$ splitter module. The column used was a TSK gel amide 80 (250 mm \times 1 mm i.d.) (Tosoh Bioscience, Germany). The HPLC-ICP-MS coupling was carried out by connecting the exit of the column by means of polyetheretherketone (PEEK) tubing to a MicroMist nebulizer (Glass Expansion, Romainmôtier, Switzerland). The ICP-MS conditions were optimized daily for highest intensities and lowest interferences using a standard built-in software procedure. The H_2 collision cell mode was used to exclude interferences that may occur for Fe.

The ESI-MS/MS instrument was hybrid linear ion trap-orbital ion trap analyzer (LTQ Orbitrap) Velos (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source (H-ESI II). It was operated in the positive ion mode with the electrospray voltage set at 3 kV. The source and capillary temperature were 120°C and 280°C , respectively.

2.4. Total metal concentration determination

A 50-mg sample was digested with 2.5 mL 70% HNO_3 and 2.5 mL 30% H_2O_2 (3 h at 80°C). The digest was made up to 50 mL and analyzed with no further dilution for Fe. The ^{54}Fe , ^{56}Fe , and ^{57}Fe isotopes were monitored. An internal standard Rh was added to control the plasma stability. Blanks were analyzed in parallel, the quantification was carried out by the method of standard additions at three concentration levels. The method was validated by the analysis of a wheat flour standard reference material (NIST 8436 Durum Wheat Flour).

2.5. Sequential extraction

Each kernel sample of these plants grown on farms was subsampled from finely ground maize grain, pooled from 100 seeds. The following steps of sequential extraction were first tested individually and then performed sequentially (Table S1) in the following order:

- water extraction: a 250-mg sample was vortexed with 500 mg of water for 2 min and centrifuged for 3 min prior to the collection of the supernatant (Šolínová, Kašička, Sázelová, & Holý, 2009) that was dried at 60°C in a DigiPREP prior to total metal concentration determination of supernatant and residues.
- hexane extraction: a 200-mg sample was weighed in a 10-mL glass screw-top tube and 8 mL of hexane was added. The mixture was vortexed for 1 min at medium speed and then shaken for 1 h at room temperature using a horizontal shaker. Finally, the slurry was set off for 1 h to collect the supernatant that was evaporated to dryness under a stream of nitrogen prior to total metal concentration determination of supernatant and residues. During sequential extraction, the residue was also evaporated to dryness under N_2 prior to a further extraction step (Moreau, Dickey, Johnston, & Hicks, 2009).
- saccharification (starch digestion): a 100-mg sample was mixed with 5 mL of slurry containing 0.1 mL of α -amylase (3000 u/mL). The pH of the slurry was adjusted to 6.5 using 0.1 M sulfuric acid and the slurry was heated at 90°C for 12 min. After cooling down and centrifugation, the supernatant was collected. In order to hydrolyse α -(1,6) and α -1,3 glycosidic linkages from the non-reducing

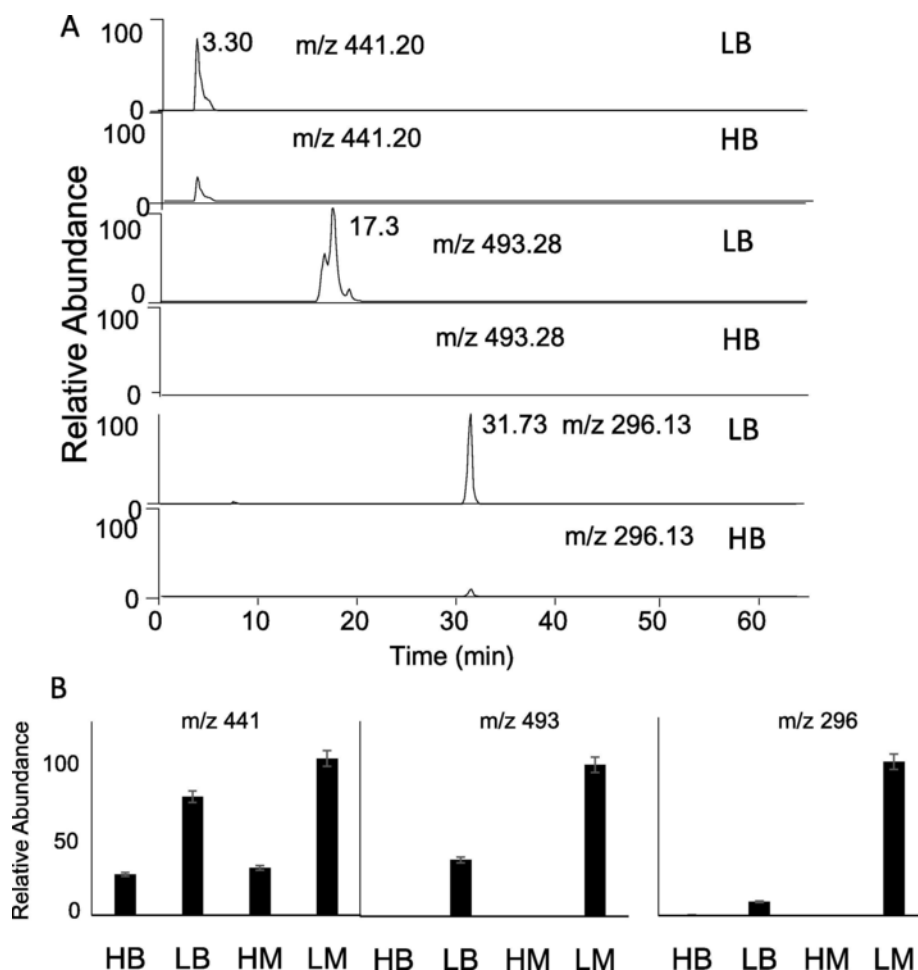


Fig. 4. Plant specificity in terms of polyamine-derived compounds content by HILIC-ESI-MS. A) For each plant variety, LB and HB, extracted ion chromatograms were recorded for the m/z 441.20, m/z 493.28 and m/z 296.13. B) For each m/z , chromatograms were normalized to the most intense signal obtained from HB or LB variety (H and L stand respectively for high and low Fe bioavailability varieties).

ends of amylose and amylopectin (in addition to the α -(1,4) glucan linkages hydrolyzed by α -amylase), β -D-glucose myloglucosidase (3260 u/mL, 50 μ L) was added, pH was adjusted to 4.5 using 0.1 M sulfuric acid. The solution was maintained at 40°C for 6 h and then centrifuged, to remove the supernatant (Al-Rabadi, Gilbert, & Gidley, 2009) that was dried at 60°C in a DigiPREP prior to total metal concentration determination of supernatant and residues.

- d) proteolysis: sample from step C was suspended in 15 mL of water containing 50 μ L of pepsin solution (840 units/L in 0.1 M KH_2PO_4 buffer; pH 2.0) and incubated at 37°C for 3 h in a shaking water bath. The supernatant was recovered by centrifugation (Holden & Mertz, 1984) and was dried at 60°C in a DigiPREP prior to total metal concentration determination of supernatant and residues.

Evaluation of efficiency in solubilizing plant matrix of each extraction step was performed by weighting the dried solid residue resulting after each extraction and by comparing it to the original sample mass. Iron was determined in the supernatants and the residues to close iron mass balance. Blanks were run in parallel by testing each extraction step protocols without adding maize sample. Obtained blank solutions after extraction step were then analyzed as sample supernatants. The procedures were finally used in sequence (step a, b c and then d) to characterize the iron distribution by treating this time as sample for step b, c and d the remaining solid residue resulting from preceding step.

2.6. Simulated gastro-intestinal enzymatic extraction

0.2 g sample was suspended in 10 mL of water containing 0.2 mL of pepsin solution and shaken at 37°C (water bath) for 60 min. The solution was neutralized with 1 M NaOH. Then, 1 mL of pancreatin/bile salt solution was added followed by pH adjustment to 7.0 with NaOH. The solution was made up to 6 mL with 120 mM NaCl/5 mM KCl solution and shaken at 37°C for 2 h. The supernatant was pipetted off after centrifugation (Glahn et al., 1998).

2.7. Size-exclusion chromatography - ICP-MS analyses

20- μ L aliquot of supernatants obtained after extraction (or a standard solution) were eluted from the SEC column using 5 mM ammonium acetate buffer at pH 6.2 as a mobile phase at a flow rate of 0.7 mL min⁻¹.

2.8. HILIC-ICP-MS/ESI-MS/MS analyses

10- μ L aliquot of the extracts were mixed with 20 μ L of acetonitrile and centrifuged (3 min at 13 200 rpm). The supernatants were eluted from the HILIC column using a binary mobile phase gradient: Eluent A was 10 mM ammonium formate buffer at pH 5.5 and Eluent B was acetonitrile. The gradient started with 10% of A and increased linearly to 75% of A within 45 min. The ESI-MS spectra were obtained in the

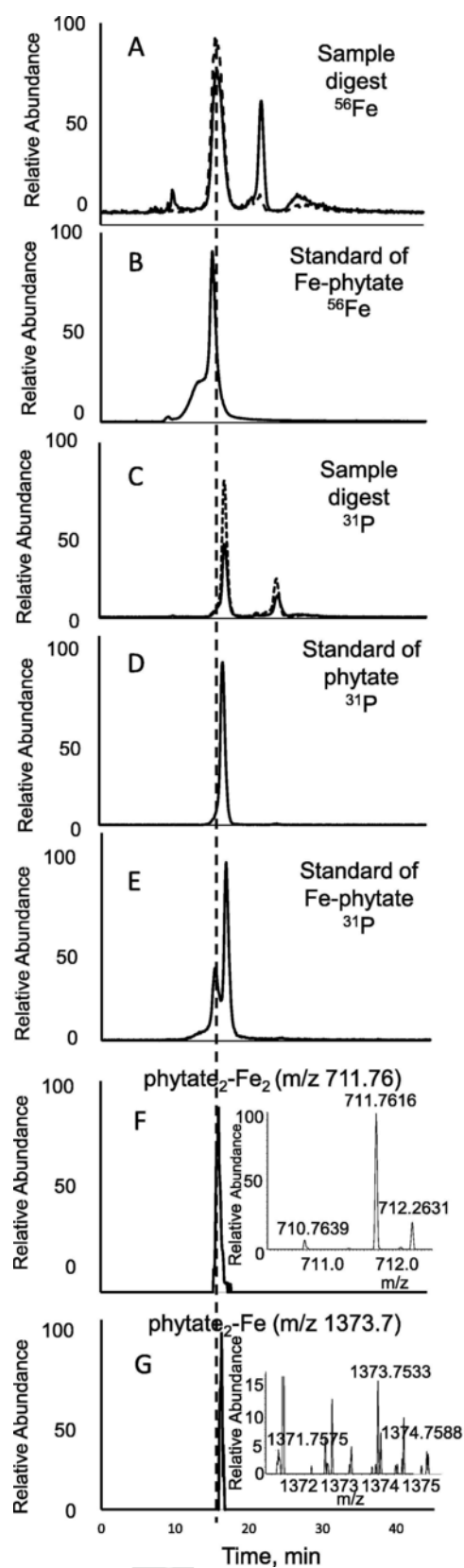


Fig. 5. Preservation of Fe-phytate complex during gastrointestinal digestion of maize. SEC-ICP-MS chromatogram of maize digests following A) ^{56}Fe and C) ^{31}P signals (plain lines for HH variety and dotted line for LL variety) and standards: B) standard of Fe-phytate complex, ^{56}Fe detection, D) standard of phytate, ^{31}P detection, E) standard of Fe-phy-

tate ^{31}P detection. SEC-ESI-MS XIC chromatograms of phytate-Fe complexes F) m/z 711.76 and G) m/z 1373.7 in maize digest.

150–1200 m/z range with the resolution set at 100 000. The ions were fragmented by collision-induced dissociation (CID) at the energy level of 45%. Both positive and negative mode analyses were performed.

3. Results and discussion

The objective of the work is to define a chemical molecular signature of iron (association with different molecular fraction and/or speciation) and to attempt to use it to find differences between the different maize varieties investigated, which have previously been reported to differ in bioavailability and produce larger iron feeding responses *in vitro* and in animals than can be explained by the differences observed in iron content alone.

3.1. Total iron determination

The HB, LB, HM and LM varieties were found not to differ in terms of the iron content. Indeed, the iron contents measured (4 individual seeds of each analyzed in triplicate) were the following: HB $20.6 \pm 0.2 \mu\text{g/g}$, LB $21.8 \pm 0.4 \mu\text{g/g}$, HM $22.8 \pm 0.8 \mu\text{g/g}$, LM $18.0 \pm 1.9 \mu\text{g/g}$, LL $26.0 \pm 0.4 \mu\text{g/g}$ and HH $21.6 \pm 0.4 \mu\text{g/g}$. The iron concentration measured in the NIST8436 Durum Wheat Flour reference material was for the different series of data included in the paper measured on different days: $12.7 \pm 2.2 \mu\text{g/g}$ (certified value: $14.1 \pm 0.5 \mu\text{g/g}$).

3.2. Development of iron fractionation method in maize

The objective was to solubilize maize completely in the mildest possible conditions without mineralization so as to approximate solution chemistries relevant to biological digestion rather than to maximize differences possible in the analytical chemistry laboratory. Maize contains typically (w/w): carbohydrate 66.0–75.9%, protein 5.2–13.7%, fat 2.2–5.7%, and crude fiber: 0.8–2.9%. The rest is moisture 9.5–12.5% and mineral residue 1.2–2.9% (Shobha, Sreeramasetty, Puttarama, & Gowda, 2010). Consequently, a number of procedures targeting the dissolution of the individual components were adapted from literature. They were: extraction of water soluble components with water (Sázelová, Kašička, Ibáñez, & Cifuentes, 2009); fat removal with hexane (Mullen, Boateng, Hicks, Goldberg, & Moreau, 2009); starch degradation with α -amylase and amyloglucosidase (Al-Rabadi et al., 2009), and proteolysis with pepsin (Mertz et al., 1984). The capacity of the procedures used independently or in sequence was investigated using weight loss, as an indicator of the efficiency of the approach. The sample used was commercially available maize kernels purchased in a local shop as animal feed.

No mass loss was observed for water extraction. Extraction with hexane resulted in a $5 \pm 1\%$ mass loss. The saccharification and proteolysis resulted respectively in a loss of $69.5 \pm 0.5\%$ and $11.5 \pm 0.5\%$ of mass. Saccharification and proteolysis were independent from each other, in the sense that the same loss of mass was observed for proteolysis of the residue upon removal of starch. In conclusion, over 85% of the corn mass was removed after sequential defatting, saccharification and proteolysis.

In parallel, the iron mass balance was controlled (Table S1). None of the extraction procedures when applied individually and directly to the sample allowed the complete recovery of the iron. About 15% of iron could be extracted to water and the most efficient extracting agent was pepsin allowing the recovery of 70% of iron. No iron was found to be associated with the polysaccharide fraction and 15% of iron was observed in the residue.

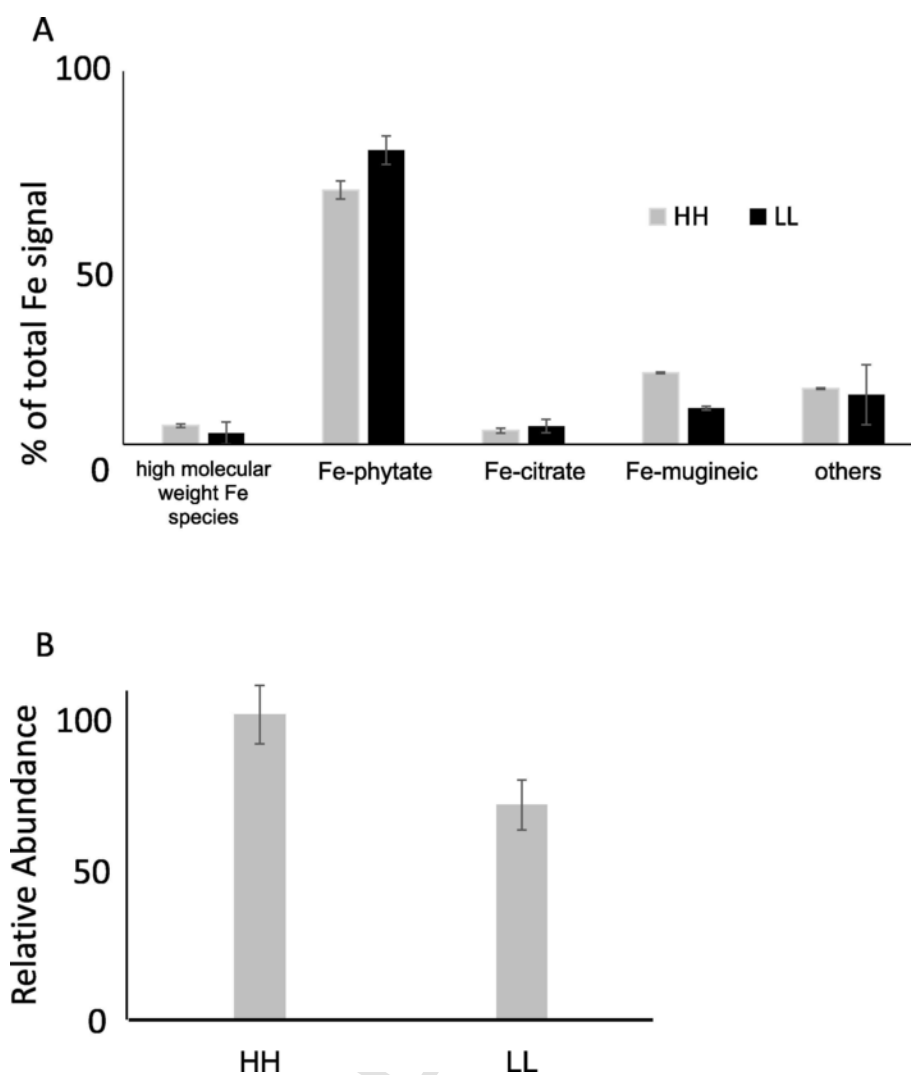


Fig. 6. A) Relative contribution of each Fe-complex in maize gastrointestinal digests by integrating the peak areas of SEC-ICP-MS chromatograms. B) Fe/P ratio within the phytate peak obtained by SEC-ICP-MS (P and Fe signals at 16 min) after gastrointestinal digests (HH and LL stand respectively for high and low Fe bioavailability hybrids).

The sequential application of the extraction procedures leads to the recovery of over 90% of iron from the sample (Table S1).

3.3. Iron fractionation in maize

The sequential procedure has been applied to the samples in order to investigate the difference between them in terms of the iron binding forms. Four varieties of maize samples (10 biological replicates grown in 3 different American states (IA, MO and NY) and 3 analytical replicates of each) were analyzed. The data presented in Table S2 confirms that iron is principally bound to water insoluble proteins (50–70%) or present in the water-soluble extract (15–20%). The fractions of seed iron associated with fat and starch are essentially absent, being within or close to the statistical error.

Fig. 1 shows the boxplot summarizing the statistical evaluation of the results obtained using the ANOVA method. The variations between the samples are statistically very small, as the only significant difference at $p < 0.05$ was that observed in the water fraction where the low available varieties showed higher Fe soluble fraction.

3.4. Speciation of water-soluble iron in maize

The observation of a possible correlation between the maize variety and water-soluble iron lead us to investigate the chemical speciation of iron in this fraction. The two mechanisms investigated for the separation of iron species included size-exclusion and HILIC as shown in the chromatograms of respectively Figs. 2 and 3. They are, to our best knowledge, the first records of the molecular distribution of water-soluble iron in maize seed.

Size exclusion chromatography with ICP-MS detection offers the advantage of fine fractionation of iron species according to the molecular mass using a direct injection and simple mobile phase thus reducing the changes in speciation (Flis et al., 2016). A representative chromatogram is shown in Fig. 2A. It shows one major and three minor peaks. The major Fe peak coelutes with phosphorous, which suggests the presence of phytate. The m/z of Fe-phytate is difficult to observe by ESI-MS as the coeluting free form is causing signal suppression. Note that “Fe-phytate” is frequently referred to as a non-bioavailable form of Fe because of its poor solubility but nevertheless it stays the most concentrated water soluble species of iron. Electrospray mass spectrum indicates the presence of phytate₂-Fe₂ complex at m/z 711.76 (Fig. 2B). To our knowledge, this is the first observation of Fe-phytate complexes

by molecular mass spectrometry. Concerning the small chromatographic peak at about 10 min, it could correspond to water-soluble protein-complex. The remaining two peaks could be identified by electrospray MS as corresponding to iron complexes with mugineic acid (Fig. 2C) and citric acid (Fig. 2D).

HILIC, introduced to speciation analysis by Ouerdane, Mari, Czernic, Lebrun, and Lobinski (2006), allows the fractionation of metal complexes in biological extracts according to their hydrophilicity. The chromatograms in Fig. 3A show several peaks. First broad and intense peak was identified by electrospray MS as corresponding to the iron-mugineic acid complex (m/z 374.03) (Fig. 3B). In ESI-MS experiment, the signal drop of this complex at the apex of the ICP-MS peak around 25 min is due to a concentrated coeluting compound that lead to signal suppression. The broad massif of peaks in the elution range of hydrophilic compounds (40–60 min) corresponds to the complexes with phytate (its free form appearing at m/z 660.86, Fig. 3C). There are likely to be several types of iron-phytate complexes present but ionization of iron-phytate complexes during HILIC-ESI-MS coupling as tested with Fe-phytate standards is too weak. Phytate does not match, in terms of intensity to the ionization of iron in ICP-MS as phytate complex may be more strongly retained (as more hydrophilic) and be released later, which can explain the poor peak definition. HILIC – ESI-MS confirms the presence of Fe-(citrate)₂ (Fig. 3D) despite a lower column recovery of this complex by HILIC as observed elsewhere (Flis et al., 2016).

The morphology of neither HILIC nor SEC chromatograms acquired for all the studied samples did not show statistically important differences between the H and L-type samples (or depending of their geographical origins) for iron species present in water extract. However, some other detected molecules (see Fig. 4A–B) are of potential interest as they are systematically concentrated in the group of LB and LM plants but almost absent in their counterpart, HB and HM plants. However, no significant differences were observed between HH and LL plants.

Interestingly, these three species were putatively identified by MS and MS/MS experiments to be part of the family of alkaloid derived from polyamines: conjugated polyamines, [C₂₄H₂₉N₂O₆]⁺ at m/z 441.20172 (terrestrisamide-like compound) and [C₂₈H₃₇N₄O₄]⁺ at m/z 493.28089 (aphelandrine-like compound) or degraded polyamines, [C₁₁H₂₂NO₈]⁺ at m/z 296.13369 (pyrrolidine-containing compound). Natural polyamines, such as putrescine, spermidine, and spermine, which are present in maize in large amounts, are assumed to play a role in non-heme iron uptake and iron bioavailability from nutrients (Lescoat et al., 2013). Putrescine was shown to activate Caco-2 cell iron uptake and to induce an increase in the ferritin level. Moreover, polyamines are likely to serve as vectors for the intracellular delivery of other iron chelators (Bergeron, McManis, Franklin, Yao, & Weimar, 2003).

The compound at m/z 493.28 was found to be much more concentrated in LM and LB group plants (500 to 1000 times more) than in plant of HB and HM groups. This compound was tentatively identified as a molecule similar to aphelandrine (spermine conjugated to hydroxycinnamic acids and cyclized). The amine conjugation and cyclization of polyamines could reduce their iron binding capacity. The presence of these conjugated polyamines in L plants and the quasi-absence in H plants could be due to a difference in plant stress response or plant maturation between these two types of plants. It could then result in higher level of plant matrices cross linkages and amine conjugation in LB and LM plants, which could participate to the lower iron bioavailability of this plant group. Indeed, free amine molecules like polyamines are known to help Fe assimilation (Lescoat et al., 2013), a capacity they could lose if they are conjugated, cyclized or degraded. Moreover, this type of cross linking reaction, if it would be occurring also with proteins and large organic acids present in seeds, could lead

to a denser plant matrix, especially in globoids and storage vacuoles, which would result in iron trapping and, therefore, in lower iron bioavailability by slowing down its release during digestion. Finally, even if the presence of polyamine conjugated forms would not be directly linked to the observed lower Fe bioavailability, they could be an indicator of a difference in plant metabolism that would lead to change in the way Fe is stored in seeds, which would be explaining the lower Fe bioavailability.

3.5. Iron release in simulated gastro-intestinal conditions

Another attempt to correlate the chemical speciation of iron with the observed difference of iron bioavailability from H and L varieties was made using simulated gastro-intestinal conditions to be as close as possible to real condition. The samples did not show a statistically significant difference in the iron extraction either after the first step (pepsin) or the second (after addition of pancreatin). The ratio of the “total Fe/total P” in the obtained extract was the same for the H and L varieties.

A final attempt to discriminate the H and L varieties on the basis of the chemical speciation of iron was based on the comparison of the iron speciation in the gastrointestinal digest as shown in Fig. 5A–G. The SEC-ICP-MS chromatogram of the LL variety (Fig. 5A) shows a similar morphology to that of the aqueous extract with a major peak corresponding to the presence of phytate. A comparison of this chromatogram with that of the HH variety shows no significant difference in terms of the height of the iron-phytate peak, however peak corresponding to the iron mugineic acid was noticeably more intense (Fig. 5A). A similar observation could not be done with LB/HB or LM/HM varieties that gave similar results. Electrospray mass spectrum at phytate migration time indicates the presence of phytate₂-Fe₂ complex at m/z 711.76 (Fig. 5F) and phytate₂-Fe at m/z 1373.7 (Fig. 5G) in sample digest. According to migration time and peak width by SEC of Fe-phytate complexes, as followed by Fe signal in Fig. 5A for sample digest and in Fig. 5B for Fe-phytate standard, slightly larger complexes than the observed ones are likely to be present even if they were not detected. Note that the level of soluble phytate is evaluated following the phosphorus signal intensity of phytate peak in SEC-ICP-MS (Fig. 5C). The presence of a shouldering at the left of the phytate peak when P signal is recorded confirms the formation of structure larger than free phytate corresponding to phytate complex with Fe and/or salts in sample digest (Fig. 5C), phytate standard (Fig. 5D) and Fe-phytate standard (Fig. 5E).

The Fe mass contribution of each Fe-complex, which eluted by SEC-ICP-MS in maize gastro-intestinal digests was calculated. By integrating the peak area of SEC graph, it was observed that Fe(III)-MA complex was significantly more concentrated in HH plants than in LL plants after gastro-intestinal digestion in Fig. 6A, which was interestingly not the case for water extracts. Mugineic acid is known to enhance Fe uptake in Caco-2 cells experiments (Tristan Eagling et al., 2014), which could in part explain the difference in iron bioavailability. Moreover, the Fe/P signal ratio of the phytate peak (Fig. 6B) showed that the proportion of phytate binding iron in LL plant is lower than in HH plant. It means that phytate/Fe ratio is higher in LL plants, which could result in lower iron bioavailability. This higher proportion of free phytate would result in lower iron bioavailability through competition with other iron chelators that would not have inhibitory effect on iron uptake as phytate has. It is even more interesting that total Fe content and total phytate content in these two types of plants are identical, which demonstrates the interest to do Fe speciation study in gastrointestinal extract as it can show difference in Fe to phytate ratio that are not detectable when only total amount of these species are taken into account. The difference in term of speciation between HH and LL variety is mainly visible after gastrointestinal digest, which could imply a

difference in Fe distribution and organization (in the way it is associated to proteins or to phytate for example) in seed compartments, cells and subcellular structures.

4. Conclusions

The study allowed the first insight into iron speciation in maize kernels. Iron was found to be principally bound to water insoluble proteins (50–70%) or present in the water-soluble extract (15–20%). The fractions associated with fat and starch did not contain iron. The couplings of HILIC and SEC chromatography with ICP-MS and HR-ESI-MS allowed the detection and identification of Fe-citrate, Fe-mugineate and Fe-phytate complexes. These species were detected and compared in genetic varieties of maize known to have different iron bioavailability from Caco-2 cell experiments. The study allowed the demonstration, especially in gastrointestinal extracts, that fine iron speciation as well as the search for other molecular features can permit to give first chemical information to explain the difference in iron bioavailability observed using Caco-2 cells experiments between plants (Hoekenga et al., 2011; Lungaho et al., 2011). Through the determination of precise Fe speciation and fractionation in the plant, the analytical methodology developed here permitted to distinguish accurately and in a reproducible manner near isogenic maize varieties having only different iron bioavailability. Finally, some findings would merit deeper investigations in the future, especially on how they correlate with the different genetic background of the low and high Fe bioavailable crop varieties: 1) the significant higher concentrations in conjugated polyamine concentrations in the extract of some low bioavailable varieties; 2) the significant higher concentrations of iron-mugineate complex in the gastrointestinal extract of some high bioavailable variety and 3) the higher proportion of phytate compared to Fe in the size exclusion chromatographic peak of Fe-phytate complex in the gastrointestinal extract of some low bioavailable varieties.

Declaration of Competing Interest

None.

Acknowledgements

The authors thank Dr. Ivan Baxter (Danforth Plant Sciences Center, St. Louis, MO) for valuable discussions during this study. Shuanglong Wang was supported by a fellowship from the Chinese Scholarship Council.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2019.05.014>.

References

Al-Rabadi, G.J.S., Gilbert, R.G., Gidley, M.J., 2009. Effect of particle size on kinetics of starch digestion in milled barley and sorghum grains by porcine alpha-amylase. *Journal of Cereal Science* 50 (2), 198–204.

Au, A.P., Reddy, M.B., 2000. Caco-2 cells can be used to assess human iron bioavailability from a semipurified meal. *The Journal of Nutrition* 130 (5), 1329–1334.

Bachir Daoura, G., Hash, C.T., 2015. Breeding for high grain Fe and Zn levels in cereals. *International Journal of Innovation and Applied Studies* 12 (2), 342–354.

Bergeron, R.J., McManis, J.S., Franklin, A.M., Yao, H., Weimar, W.R., 2003. Polyamine–Iron Chelator Conjugate. *Journal of Medicinal Chemistry* 46 (25), 5478–5483.

Bodnar, A.L., Proulx, A.K., Scott, M.P., Beavers, A., Reddy, M.B., 2013. Iron bioavailability of maize hemoglobin in a Caco-2 cell culture model. *Journal of Agricultural and Food Chemistry* 61 (30), 7349–7356.

Bou Khouzam, R., Szpunar, J., Holeman, M., Lobinski, R., 2012. Trace element speciation in food: State of the art of analytical techniques and methods. *Pure and Applied Chemistry* 84 (2), 169–179.

Choubassi, G., Aszyk, J., Pisarek, P., Bierla, K., Ouerdane, L., Szpunar, J., Lobinski, R., 2017. Advances in mass spectrometry for iron speciation in plants. *TrAC Trends in Analytical Chemistry*, in press, <https://doi.org/10.1016/j.trac.2017.1011.1006>.

Clemens, S., 2014. Zn and Fe biofortification: The right chemical environment for human bioavailability. *Plant Science* 225, 52–57.

Cook, J.D., Layrisse, M., Martinez-Torres, C., Walker, R., Monsen, E., Finch, C.A., 1972. Food iron absorption measured by an extrinsic tag. *Journal of Clinical Investigation* 51 (4), 805–815.

Eagling, T., Neal, A.L., McGrath, S.P., Fairweather-Tait, S., Shewry, P.R., Zhao, F.-J., 2014. Distribution and speciation of iron and zinc in grain of two wheat genotypes. *Journal of Agricultural and Food Chemistry* 62 (3), 708–716.

Eagling, T., Wawer, A.A., Shewry, P.R., Zhao, F.-J., Fairweather-Tait, S.J., 2014. Iron bioavailability in two commercial cultivars of wheat: Comparison between whole-grain and white flour and the effects of nicotianamine and 2'-deoxymugineic acid on iron uptake into Caco-2 cells. *Journal of Agricultural and Food Chemistry* 62 (42), 10320–10325.

Fairweather-Tait, S., Phillips, I., Wortley, G., Harvey, L., Glahn, R.P., 2007. The use of solubility, dialyzability, and Caco-2 cell methods to predict iron bioavailability. *International Journal for Vitamin and Nutrition Research* 77 (3), 158–165.

Flis, P., Ouerdane, L., Grillet, L., Curie, C., Mari, S., Lobinski, R., 2016. Inventory of metal complexes circulating in plant fluids: A reliable method based on HPLC coupled with dual elemental and high-resolution molecular mass spectrometric detection. *New Phytologist* 211 (3), 1129–1141.

Glahn, R.P., Cheng, Z., Welch, R.M., Gregorio, G.B., 2002. 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* 50 (12), 3586–3591.

Glahn, R.P., Lee, O.A., Yeung, A., Goldman, M.I., Miller, D.D., 1998. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. *The Journal of Nutrition* 128 (9), 1555–1561.

Glahn, R.P., Wien, E.M., van Campen, D.R., Miller, D.D., 1996. Caco-2 cell iron uptake from meat and casein digests parallels in vivo studies: Use of a novel in vitro method for rapid estimation of iron bioavailability. *The Journal of Nutrition* 126 (1), 332–339.

Hoekenga, O.A., Lung'aho, M.G., Tako, E., Kochian, L.V., Glahn, R.P., 2011. Iron biofortification of maize grain. *Plant Genetic Resources* 9 (2), 327–329.

Holden, B.A., Mertz, G.W., 1984. Critical oxygen levels to avoid corneal edema for daily and extended wear contact lenses. *Investigative Ophthalmology and Visual Science* 25 (10), 1161–1167.

Hurrell, R., Egli, I., 2010. Iron bioavailability and dietary reference values. *The American Journal of Clinical Nutrition* 91 (5), 1461S–1467S.

La Frano, M.R., de Moura, F.F., Boy, E., Lonnerdal, B., Burri, B.J., 2014. Bioavailability of iron, zinc, and provitamin A carotenoids in biofortified staple crops. *Nutrition Reviews* 72 (5), 289–307.

Lescoat, G., Gouffier, L., Cannie, I., Lowe, O., Morel, I., Lepage, S., ... Gaboriau, F., 2013. Involvement of polyamines in iron(III) transport in human intestinal Caco-2 cell lines. *Molecular and Cellular Biochemistry* 378 (1), 205–215.

Lungaho, M.G., Mwaniki, A.M., Szalma, S.J., Hart, J.J., Rutzke, M.A., Kochian, L.V., Glahn, R.P., Hoekenga, O.A., 2011. Genetic and physiological analysis of iron biofortification in maize kernels. *PLoSOne* 6.

Mertz, E.T., Hassen, M.M., Cairns-Whittem, C., Kirleis, A.W., Tu, L., Axtell, J.D., 1984. Pepsin digestibility of proteins in sorghum and other major cereals. *Proceedings of the National Academy of Sciences* 81 (1), 1–2.

Moreau, R.A., Dickey, L.C., Johnston, D.B., Hicks, K.B., 2009. A process for the aqueous enzymatic extraction of corn oil from dry milled corn germ and enzymatic wet milled corn germ (E-Germ). *Journal of the American Oil Chemists' Society* 86 (5), 469–474.

Mullen, C.A., Boateng, A.A., Hicks, K.B., Goldberg, N.M., Moreau, R.A., 2009. Analysis and comparison of bio-oil produced by fast pyrolysis from three barley biomass/byproduct streams. *Energy Fuels* 24 (1), 699–706.

Oikeh, S.O., Menkir, A., Maziya-Dixon, B., Welch, R., Glahn, R.P., 2003. Assessment of concentrations of iron and zinc and bioavailable iron in grains of early-maturing tropical maize varieties. *Journal of Agricultural and Food Chemistry* 51 (12), 3688–3694.

Ouerdane, L., Mari, S., Czernic, P., Lebrun, M., Lobinski, R., 2006. Speciation of non-covalent nickel species in plant tissue extracts by electrospray Q-TOFMS/MS after their isolation by 2D size exclusion-hydrophilic interaction LC (SEC-HILIC) monitored by ICP-MS. *Journal of Analytical Atomic Spectrometry* 21 (7), 676–683.

Persson, D.P., Hansen, T.H., Laursen, K.H., Schjoerring, J.K., Husted, S., 2009. Simultaneous iron, zinc, sulfur and phosphorus speciation analysis of barley grain tissues using SEC-ICP-MS and IP-ICP-MS. *Metallomics* 1 (5), 418–426.

Sázelová, P., Kašička, V., Ibáñez, E., Cifuentes, A., 2009. Extraction and separation of water-soluble proteins from *Bacillus thuringiensis*-transgenic and non-transgenic maize species by CZE. *Journal of Separation Science* 32 (21), 3801–3808.

Shobha, D., Sreeramasetty, T., Puttarama, N., Gowda, K.P., 2010. Evaluation of maize genotypes for physical and chemical composition at silky and hard stage. *Karnataka Journal of Agricultural Sciences* 23 (2), 311–314.

Šolínová, V., Kašička, V., Sázelová, P., Holý, A., 2009. Chiral analysis of anti-acquired immunodeficiency syndrome drug, 9-(R)-[2-(phosphonomethoxy)propyl]adenine (tenofovir), and related antiviral acyclic nucleoside phosphonates by CE using β -CD as chiral selector. *Electrophoresis* 30 (12), 2245–2254.

Teucher, Olivares, Cori, 2004. Enhancers of iron absorption: Ascorbic acid and other organic acids. *International Journal for Vitamin and Nutrition Research* 74 (6), 403–419.

Weber, G., Neumann, G., Römheld, V., 2002. Speciation of iron coordinated by phytosiderophores by use of HPLC with pulsed amperometric detection and AAS. *Analytical and Bioanalytical Chemistry* 373 (8), 767–771.

Wienk, K.J.H., Marx, J.J.M., Beynen, A.C., 1999. The concept of iron bioavailability and its assessment. *European Journal of Nutrition* 38 (2), 51–75.

UNCORRECTED PROOF