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**Estimation of the iron bioavailability in green vegetables using an *in vitro* digestion/Caco-2 cell model**

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**Abstract**

It is estimated that over 30% of the global population is anaemic, half of which is due to iron deficiency. The bioavailability of iron from vegetables is low and variable, and influenced by food composition and matrix. We have therefore determined the relative bioavailability of iron in five types of green vegetable, spinach, broccoli, savoy cabbage, curly kale and green pepper, by measuring the ferritin response in a simulated digestion/Caco-2 cell model. Savoy cabbage gave the highest ferritin response and analysis of the digest showed that the iron was present in low molecular weight fractions which contained glucose, fructose, organic acids and amino acids. The addition of fructose 1,6-biphosphate to the Caco-2 cells increased iron uptake 2-fold. These results demonstrate that cabbage was the best source of bioavailable iron out of the vegetables studied and suggest that the formation of complexes with fructose derivatives contribute to increase the iron bioavailability.

## 1. Introduction

In 2010, the World Health Organisation (WHO) estimated that iron deficiency anaemia (IDA) affects one third of the world's population (Lopez, Cacoub, Macdougall, & Peyrin-Biroulet, 2016). There are four main strategies to mitigate IDA in populations: biofortification of crops, fortification of foods, iron supplementation, and nutritional education to encourage dietary modification and diversification. Amongst these, providing guidelines to educate consumers to change dietary habits is considered to be the most sustainable and affordable approach (Zimmermann & Hurrell, 2007).

Iron occurs in diets in two forms, as haem and non-haem iron. Haem iron occurs only in animal foods and is highly bioavailable (~25%), whereas the bioavailability of non-haem iron, which is present in plant-based foods, is lower and more variable (0-20%) due to other factors that can inhibit or enhance its absorption (Hurrell & Egli, 2010; Zimmermann & Hurrell, 2007). Although increased intake of animal foods (haem-iron) could alleviate iron deficiency, cultural preferences and concerns about sustainability are leading to increased consumption of plant-based foods. More importantly, reduced intake of red meat and increased consumption of vegetables have clear health benefits (Dominguez et al., 2018; Trichopoulou et al., 2014; Willett et al., 2019). It is therefore important to identify plant foods which are good sources of bioavailability of iron to replace animal-based foods. Furthermore, it is not only necessary to determine the iron content of plant-based foods, but also how much iron is taken up by the intestinal mucosal cells. This is dependent on the chemical form of iron in the intestinal lumen (i.e. availability for absorption) and the physiological requirements of the individual (Lynch et al., 2018). The bioavailability of dietary iron is defined as the proportion (or percentage) of ingested iron from a meal that is absorbed and used for normal functions in the body (Fairweather-Tait & de Sesmaisons, 2018). Simulated digestion

combined with the determination of uptake by Caco-2 cells is widely-used for predicting iron bioavailability from foods (Diego Quintaes, Barbera, & Cilla, 2017; S. Fairweather-Tait et al., 2005). For example, it was recently demonstrated that the bioavailability of iron from pea seeds varied at different stages of development due to phytate content (Moore et al., 2018). Similarly, Cai et al., (2017) used simulated digestion and uptake by Caco-2 cells to estimate the bioavailability of iron in a number of Chinese vegetables in order to provide recommendations for consumers. The aim of this study was therefore to determine the bioaccessibility and bioavailability of iron in commonly consumed green vegetables in order to underpin dietary recommendations for alleviating IDA.

## 2. Material and Methods

2.1 Reagents

Chemicals, enzymes and hormones were purchased from Sigma-Aldrich, UK unless otherwise stated.

2.2 Vegetables and cooking methods

Five vegetables, spinach (*Spinacia oleracea*), broccoli (*Brassica oleracea* var. *italica*), savoy cabbage (*Brassica oleracea* var. *sabauda*), curly kale (*Brassica oleracea* var. *acephala*) and green pepper (*Capsicum annuum*) were purchased from the local (UK) supermarket in 2014. Additionally, two more savoy cabbage samples were purchased in 2016 from a local supermarket. Vegetables were boiled (except for green pepper that was used raw) at 95-100°C in a 2 L glass beaker previously washed in 10% HCl and rinsed with Milli-Q (18.2 MΩ) $H_2O$ to remove any traces iron from the glass. Different cooking procedures and times were used based on domestic cooking. About 400 g samples of broccoli (chopped in heads) or cabbage (chopped in wedges) were boiled in 1.2 L Milli-Q (18.2 MΩ) $H_2O$ for about 15 min, while 360 g of curly kale and 200 g of baby spinach were boiled for 8 min in about 1.6 L Milli-Q (18.2 MΩ) $H_2O$. After cooking, all the

vegetables were drained for 15 min at room temperature, placed in bags, frozen at -20°C, freeze-dried (Coolsafe 110-4 pro, ScanVac), and finely ground. The ground samples were stored in in the dark in sealed bags at 4°C in a desiccator, with silica gel beads to prevent moisture.

2.3 Determination of iron in the vegetables and simulated digests

The concentrations of iron in the dry ground vegetables (500 mg) and simulated digests (400 µl) samples were determined as previously described (Rodriguez-Ramiro et al., 2017) by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), using a Jobin Yvon Horiba – ULTIMA 2C machine equipped either with a glass concentric nebuliser (1 mL/min sample flow rate) or a concentric PFA micro-flow nebuliser (0-2 mL/min sample flow rate), for dry ground or simulated digests, respectively. The ICP-OES was also equipped with a 50 mL glass cyclonic spray chamber and a radial torch with a 3 mm internal diameter (i.d.) alumina injector according to Rodriguez-Ramiro et al., 2017.

The fractions of bioaccessible and precipitated iron were determined using the following equations (A) and (B) respectively:

(A)          $[(\%) \text{ Bioaccessible Fe}] = [(\text{Fe}_{bioaccessible})/\text{Total Fe}] \times 100;$

(B)          $[(\%) \text{ Precipitated Fe}] = [(\text{Total Fe} - \text{Fe}_{bioaccessible})/\text{Total Fe}] \times 100;$

where $\text{Fe}_{bioaccessible}$ is the fraction of iron which is soluble after simulated gastrointestinal digestion (see below); Total Fe refers to the iron content of the vegetable sample used for simulated digestion (Table 1). This will equate to an iron concentration of 50 µM in the final volume of simulated digestion (15 mL). Amounts are expressed as percentages of the total iron concentrations (50 µM) in the digested samples.

## 2.4 Determination of the molecular weight of iron and phosphate fractions in the simulated digests

Size-exclusion chromatography coupled with inductively coupled plasma mass spectrometry (SEC-ICP-MS) was used to determine the molecular size of compounds which co-eluted with iron and phosphate. Analyses were performed on a HPLC (PerkinElmer LC 200 Series HS, Seer Green, Bucks, UK) comprising an injector and a high-pressure pump equipped with PEEK tubing (0.17 mm i.d.). Minerals were quantified with an ICP-MS (PerkinElmer NexION 300XX, Seer Green, Bucks, UK) equipped with a glass Meinhard nebuliser and Chromera software (PerkinElmer v. 4.1.0). The ICP-MS settings were: gas flow 1L/min; auxiliary gas flow 1.2 L/min; plasma flow 18 L/min; RF power 1600 Watts; cell gas flow He 3.9 mL/min. The column was a Superdex Peptide 10/300 GL (10 x 300 mm, GE Healthcare Bio-Sciences, Sweden). The column was mass calibrated at 280 nm with try-glycine (0.189 kDa), vitamin B12 (1.35 kDa), cytochrome (12.4 kDa), apoferritin (443 kDa) and blue dextran (2000 kDa) for the void volume. The sample (100 μL) was injected into the column at room temperature and the analysis was performed in isocratic mode with ammonium nitrate (200 mM, pH 7.6) as mobile phase and a flow rate of 0.6 mL/min..

## 2.5 Metabolite profiling of fractions from cabbage digests

Sample preparation for $^1$H-NMR was carried out as described previously (Shewry, Corol, Jones, Beale, & Ward, 2017). Freeze dried samples (30 mg) were extracted in triplicate using 80:20 deuterated water : deuterated methanol ($D_2O$:$CD_3OD$) containing 0.01% w/v $d_4$–trimethylsilylpropionate (TSP) (1 mL) as internal standard. $^1$H-NMR spectra were acquired under automation at 300°K using an Avance Spectrometer (BrukerBiospin, Coventry, UK) operating at 600.0528 MHz and equipped with a 5 mm selective inverse probe. Spectra were collected using a water suppression pulse sequence with a 90° pulse and a relaxation delay of 5 s. Each spectrum was acquired using 128 scans of 64,000 data points with a spectral width of 7309.99 Hz. Spectra were automatically Fourier-transformed using an exponential window with a line broadening value of 0.5

Hz. Phasing and baseline correction were carried out within the instrument software. [1]H chemical shifts were referenced to $d_4$-TSP at δ0.00.

^{1}H-NMR spectra were automatically reduced, using Amix (Analysis of MIXtures software, BrukerBiospin), to ASCII files containing integrated regions or 'buckets' of equal width. Spectral intensities were scaled to the $d_4$-TSP region (δ0.05 to −0.05). The ASCII file was imported into Microsoft Excel for the addition of sampling/treatment details. Signal intensities for characteristic spectral regions for major metabolites were extracted via comparison to library spectra of known standards run under identical conditions and quantified relative to the known volume of internal standard.

2.6 *In vitro* simulated gastrointestinal digestion

Simulated gastrointestinal digestion was performed as described by Glahn, Lee, Yeung, Goldman, & Miller (1998) with minor modifications as in Moore et al., (2018). Experiments were conducted using similar iron concentrations (50 µM) in every vegetable digestion. To achieve this, different amounts of the ground dry vegetables (Table 1) were first added to 10 mL of pH 2 buffer saline-solution (140 mmol/L NaCl, 5 mmol/L KCl) followed by the addition of pepsin (0.04 g/mL) and incubated for 90 min on a rolling table at 37°C to simulate gastric conditions. Because iron bioavailability was expected to be low, and to ensure that all of the iron released during digestion remained in solution, ascorbic acid (AA) was added to the gastric digestion (molar ratio of 1:10, Fe:AA) at this step. The pH of the samples was then gradually adjusted to pH 5.5, bile (0.007 g/mL) and pancreatin (0.001 g/mL) added, the pH readjusted to pH 7, and the sample incubated for 60 min on a rolling table at 37°C to simulate intestinal conditions. Samples were then centrifuged at 3000 x *g* for 10 min and the supernatants used for cell culture. A volume of 1.5 mL of the digest was applied to an upper chamber consisting of a Transwell insert fitted with a 15 kDa molecular weight cut-off dialysis membrane (Spectra/Por 7 dialysis tubing, Spectrum laboratories, Europe) suspended over Caco-2 cell monolayers grown in collagen-coated 6 well plates. Vegetable digests

were incubated over the cells for one hour at 37°C in a humidified incubator containing 5% $CO_2$ and 95% air. The inserts were then removed, an additional 1 mL of supplemented Eagle's minimum essential medium (MEM) added, and cells were incubated for a further 23 hours prior to harvesting for ferritin analysis.

All experiments were performed using the following controls: a) a blank digestion without any vegetable digests or added iron to ensure no iron contamination in the *in vitro* digestion/cellular system and b) a reference digestion of 50 µM of ferrous sulphate heptahydrate ($FeSO_4$) dissolved in 0.1 M HCl with 500 µM of AA.

2.7 Cell culture

Caco-2 cells (HTB-37) were obtained from American Type Culture Collection (Manassas, VA, USA) at passage 20 and stored in liquid nitrogen. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 25mM HEPES solution, 10% foetal bovine serum, 1% penicillin (5000 u/mL) and 1% L-glutamine (200 mM) (ThermoFisher Scientific, UK) and 1% MEM non-essential amino acids solution (Sigma-Aldrich, UK). Cells were maintained at 37°C in a humidified incubator containing 5% $CO_2$ and 95% air. Caco-2 cells between passages 30-36 were seeded onto collagen-coated 6- and 12-well plates (Bio-Greiner, UK), for simulated digestion and co-treatment studies respectively, at a density of 5 x $10^4$ cells/cm$^2$ and the media was replaced every 2 days. Cells were used on days 13-15 post-seeding according to the method developed by Glahn et al., (1998). In order to ensure low iron levels in media, at 24 hours prior to the initiation of the *in vitro* digestion experiments, the DMEM medium was replaced by MEM without foetal bovine serum supplemented with 10 mmol/L PIPES buffer [piperazine-N, N' –bis-(2-ethanesulfonic acid)], 26.1 mM $NaHCO_3$, 19.4 mmol/L glucose, 1% antibiotic-antimycotic solution, 11 µmol/L hydrocortisone, 0.87 µmol/L insulin, 0.02 µmol/L sodium selenite ($Na_2SeO_3$), 0.05 µmol/L triiodothyronine and 20 µg/L epidermal growth factor as previously reported (Glahn et al., 1998). Co-treatments of iron (added as Fe solubilised in 1% HCl, high-purity standards, USA) plus sugars

(fructose or fructose 1,6-biphosphate (F16BP) were diluted in MEM before adding to the Caco-2 cells. Working solutions of sugars were freshly prepared to expose the cells to a concentration of 25 µM of iron and 5 mM of sugar (iron:sugar ratio, 1:200).

2.8 Cell harvest and ferritin analysis

Following 24 hours incubation, the cellular medium was aspirated, the cells were rinsed with Milli-Q (18.2 MΩ) H$_2$O and subsequently lysed by scraping in 100 µL (12-well plates) or 200 µL (6-well plates) of CelLytic M (Sigma-Aldrich, UK). Cell pellets were kept on ice for 15 min and stored at -80°C. For analysis, samples were thawed and centrifuged at 14,000 x $g$ for 15 min. Cellular debris was discarded and the supernatant containing the proteins was used for ferritin determination using the Spectro Ferritin ELISA assay (RAMCO, USA). The ferritin concentrations in the samples were determined using a microplate reader at an excitation wavelength of 500 nm according to the manufacturer's protocol. Ferritin concentrations were normalised to total cell protein using the Pierce Protein BCA protein assay (ThermoFisher Scientific, UK).

2.9 Statistical analysis

Data are presented as mean values with associated standard errors of the means (SEM). The data set was tested for homogeneity of variances using the test of Levene. For multiple comparison, one-way ANOVA followed by a Bonferroni test was used when variances were homogenous or by Tamhane test when variances were not homogenous. Statistical significance was set at $p < 0.05$. The statistical analysis was performed using the SPSS package (version 23; SPSS Inc., Chicago, IL, USA).

## 3. Results and Discussion

3.1. Bioaccessibility and bioavailability of iron in green vegetables

The bioaccessibility of iron after simulated digestion (at pH 7) of the different vegetables purchased in 2014 (**Fig.1a)** was calculated based on the total iron content of each sample (**Table 1**). Cabbage

had the highest proportion of bioaccessible iron (16.2%), followed by pepper (12.2%), kale (11.8%) and broccoli (9.7%), with spinach (6.6%) having the lowest proportion. A number of studies have reported variation in the proportion of bioaccessible iron depending on the type of food, the matrix composition and the dietary components released with the iron during the simulated digestion. For example, Suliburska & Krejpcio (2014) reported that the percentage of bioaccessible iron varied from 5.8% - 32.1% in cereal groats, 14.2% - 21.4% in rice, 9.4% - 32.6% in pulses and 2% - 51.1% in nuts. Previous studies in our group have also demonstrated differences in the proportions of bioaccessible iron (from 1.4% to 12%) in breads, relating to effects of dough fermentation on phytate content (Rodriguez-Ramiro et al., 2017). In addition, a study of African green leafy vegetables showed that combinations of vegetables used to constitute Amaranth recipes contribute to differences in bioaccessible iron fractions (from 6.8% to 25%) after simulated digestion (Kruger, Mongwaketse, Faber, van der Hoeven, & Smuts, 2015).

We then estimated the bioavailability of iron in the vegetable digests by determining ferritin accumulation as a measure of iron uptake by Caco-2 cells (**Fig. 1b**). This showed 53 ± 3.7 ng ferritin/mg of protein (mean ± SEM) in cells exposed to a control digest containing 50μM of $FeSO_4$ plus added AA (500 μM) compared with 8.2 ± 3.7 ng ferritin/mg of protein in the blank digests. In order to determine the relative uptake of iron between the vegetable digests, we expressed the ferritin accumulation as a percentage of the accumulation in the control digest (containing 50μM $FeSO_4$ plus added 500 μM AA). The cabbage digest showed a significant increase in ferritin accumulation compared to the blank control which corresponded to 81% of iron uptake in the control cells exposed to $FeSO_4$. By contrast, spinach did not differ significantly from the blank control. Broccoli, pepper and kale gave ferritin concentrations of 58%, 44% and 20% compared to the $FeSO_4$ control, respectively. This allowed the five vegetables to be ranked in order of iron uptake in Caco-2 cells: cabbage > broccoli ≥ pepper > kale > spinach.

Because cabbage showed the highest iron uptake, we then compared iron bioaccessibility and bioavailability in three different samples of savoy cabbage, grown in different years (2014 and 2016) and two regions in the UK. This showed that the percentage of bioaccessible iron was similar in the two samples grown in Lincolnshire in 2014 and 2016 (16.2% and 18.4%, respectively) but higher in the sample grown in Suffolk in 2016 (22.4%) (**Fig. 2a**). However, no significant differences in iron uptake were observed when digests of the three samples were applied to Caco-2 cells (**Fig. 2b**).

Our results are consistent with a study which reported high iron absorption from cabbage and broccoli and low iron absorption from spinach in a group of women who consumed the vegetables labelled with an iron isotope (Gillooly et al., 1983). In addition, Genannt Bonsmann, Walczyk, Renggli & Hurrell (2008) reported that the absorption of iron from a meal comprising a wheat bread roll with spinach was slightly lower (8.4%) than from a similar meal with kale (11%) in a human randomised crossover trial, although the difference was not statistically significant. Similarly, Cai et al. (2017) also reported low iron bioavailability from spinach when compared to other Chinese green leafy vegetables using the *in vitro* digestion/Caco-2 cell model.

Vegetables may vary in their contents of dietary factors that can influence the iron bioavailability. For example, food tables show that the content of ascorbic acid, a well-known enhancer of iron absorption, is higher in cabbage and broccoli than in spinach (Finglas et al., 2015). This, together with the fact that we used larger amounts of cabbage or broccoli than spinach to obtain similar concentrations of iron during digestion, could have contributed to the higher iron bioavailability that we observed. However, other dietary factors may promote iron absorption (such as organic acids (Salovaara, Sandberg, & Andlid, 2002, 2003)) or inhibit it (such as polyphenols, phytate or calcium (Hurrell & Egli, 2010). Thus, the relative amounts of bioavailable iron determined for the different vegetables may have resulted from complex interactions between the iron content and other dietary

factors. Because cabbage contained the highest amount of bioavailable iron it was investigated in more detail.

3.2 Identification of iron and phosphorus in fractions from cabbage after simulated digestion

The cabbage digests were separated on the basis of molecular weight by SEC-ICP-MS to identify fractions enriched in iron. Similar patterns were observed amongst the three samples with most of the iron present as low molecular weight (LMW) components and only traces in in high molecular weight (HMW) fractions (**Fig. 3a**). Two major LMW iron-rich fractions eluted at 28 (peak 1) and 31 min (peak 2) with masses of about 700 and 300 Daltons, respectively. The amounts of phosphate were also determined, showing three phosphate-rich fractions eluting at 27 (peak1), 30 (peak2) and 32 min (peak3) with masses of 760, 350 and 160 Dalton, respectively. A fourth peak was detected at 36 min, but the molecular size could not be determined as it eluted at the column volume (**Fig. 3b**). Comparison of the iron and phosphorus chromatograms showed partial co-elution (**Fig. 3c**).

$^1$H-NMR spectroscopy was used to determine the individual components present in the iron-rich peaks 1 and 2 (eluting at 28 and 31 min) (**Fig. 3d**). This showed that peak 2 contained glucose ($2 \pm 0.61$ mg/g) and fructose ($1.56 \pm 0.39$ mg/g) as well as organic acids (citric acid ($0.63 \pm 0.07$ mg/g) and acetic acid ($0.05 \pm 0.01$ mg/g)) and amino acids (proline ($0.55 \pm 0.05$ mg/g), alanine ($0.15 \pm 0.06$ mg/g), threonine ($0.13 \pm 0.04$ mg/g) and valine ($0.09 \pm 0.02$ mg/g)) (all expressed as mean $\pm$ SEM). By contrast, no components were detected in peak 1.

Organic acids have been reported to promote iron absorption (Gillooly et al., 1983; Salovaara, Sandberg, & Andlid, 2002, 2003), which has been attributed to their ability to chelate iron to generate soluble complexes, and to their pH lowering properties, which increases iron solubilisation from the food. The presence of citric acid in the iron peak (**Fig. 3d**) therefore suggests that complex formation may increase iron availability. It has also been reported that some sugars can form iron

complexes (Spasojevic et al., 2009) and increase iron uptake into intestinal cells (Christides & Sharp, 2013). This is in agreement with earlier studies showing that fructose and other sugars can form highly stable low molecular weight complexes with iron (Charley, Sarkar, Stitt, & Saltman, 1963) that are absorbed across the intestinal mucosa of rodents (Pollack, Kaufman, & Crosby, 1964; Stitt, Charley, Butt, & Saltman, 1962). In addition, a recent study showed that rats fed a modest amount of fructose (similar to the amounts present in sweetened beverages) (3%, fructose, w/v) had increased iron levels in the liver (Song et al., 2013). It has also been suggested that sugars vary in their ability to sequester iron, with phosphorylated forms having higher avidity (Spasojevic et al., 2009). In particular, Bajic et al., (2011) demonstrated using $^{31}$P NMR spectroscopy that fructose 1,6-biphosphate (F16BP) forms stable complexes with $Fe^{2+}$. According to food tables (Finglas et al., 2015), cabbage contains around 0.9 g fructose, 1 g glucose, and 0.4 g sucrose (g/100 g of edible portion). The co-elution of iron and phosphorus observed by SEC-ICP-MS of the cabbage digests suggests that the sugars eluting in the LMW iron peak could be, at least partially, phosphorylated, which may contribute to increased iron bioavailability.

3.3 Effect of fructose and F16BP on iron uptake in Caco-2 cells

In order to determine whether metabolites identified by NMR spectroscopy of the cabbage digests could modulate the iron bioavailability in Caco-2 cells, we performed cellular co-treatments of iron plus fructose or one of its phosphorylated forms, the F16BP **(Figure 4)**. Fructose or F16BP, without the addition of iron, did not affect ferritin formation compared to the blank control. Similarly, co-treatments of fructose with iron did not increase ferritin formation in Caco-2 cells. However, the addition of F16BP to the iron treatment significantly increased ferritin formation by 2-fold compared to the iron control. These results support the hypothesis that phosphorylated sugars chelate iron with higher avidity than non-phosphorylated forms, increasing iron solubility during digestion and facilitating its uptake by the cells. In contrast to Christides & Sharp (2013), we did not observe any effects of fructose on iron uptake by Caco-2 cells. This may due to differences in

fructose concentration in the two studies. The ratio of iron:fructose (1:200) in our study was close to the amount of fructose present in cabbage, whereas Christides & Sharp (2013) used a much higher ratio of iron:fructose (1:2000) (which was used to simulate sweetened beverages). Indeed, when hepatic cells were exposed to iron with increasing concentrations of fructose (iron:fructose from 1:1000 up to 1:15000), an increase in iron uptake was only observed at the highest concentration of the sugar (Christides & Sharp, 2013), which indicates that fructose only chelates iron only above a threshold ratio.

## 4. Conclusion

We have determined the relative bioavailability of iron using an *in vitro* digestion/Caco-2 cell model. This showed that the relative bioavailabilities were in the order: cabbage > broccoli ≥ pepper > kale > spinach. ^{1}H-NMR spectroscopy of a major LMW iron-containing fraction from the cabbage digest showed the presence of sugars, organic acids and amino acids, indicating that the iron could have been present in complexes with these components. We also showed that the uptake of iron by Caco-2 cells was increased by the addition of F16BP, indicating that the higher bioavailability of iron in cabbage digests could result from the formation of complexes with sugars.

## 5 Acknowledgements

**6 Conflict of interest**

The authors declare that they have no conflict of interest

**Figures Caption**

**Figure 1:** Bioaccessible and bioavailable iron in green vegetables (spinach, broccoli, savoy cabbage, curly kale, and green pepper) purchased in 2014. a) Relative amounts of bioaccessible and precipitated iron after simulated gastrointestinal digestion, shown as percentages of total iron in the vegetables b) Iron uptake in Caco-2 cells from the different vegetable digests, measured as ferritin formation expressed relative to the ferrous sulphate control. Data shown are means ± SEM (n = 6-9). Means without a common letter differ ($p < 0.05$).

**Figure 2:** Bioaccessible and bioavailable iron in three samples of savoy cabbage samples grown in different years (2014 and 2016) and two regions in the UK. a) Relative amounts of bioaccessible and precipitated iron after simulated gastrointestinal digestion, shown as percentages of total iron in the samples b) Iron uptake in Caco-2 cells from the different samples, measured as ferritin formation expressed relative to the ferrous sulphate control. Data shown are means ± SEM (n = 6-9). Means without a common letter differ ($p < 0.05$).

**Figure 3:** Iron identification in savoy cabbage. Representative chromatograms of a) iron, b) phosphorus and c) the overlapping of iron and phosphorus in digests separated by SEC-ICP-MS. Chromatograms represent means of three independent digests. d) Metabolite profiles (determined by ^{1}H-NMR) of the iron-containing LMW fractions of cabbages digests. 1, glucose; 2 fructose; 3, citrate; 4, glutamine; 5, glutamate; 6, proline; 7, acetate; 8, alanine; 9, threonine; 10, valine, isoleucine and leucine.

**Figure 4:** Uptake of iron by Caco-2 cells supplied with iron (Fe 25 μM) with or without the addition of fructose and fructose 1,6-biphosphate (F16BP). Data shown are means ± SEM (n = 12). Means without a common letter differ ($p < 0.05$).

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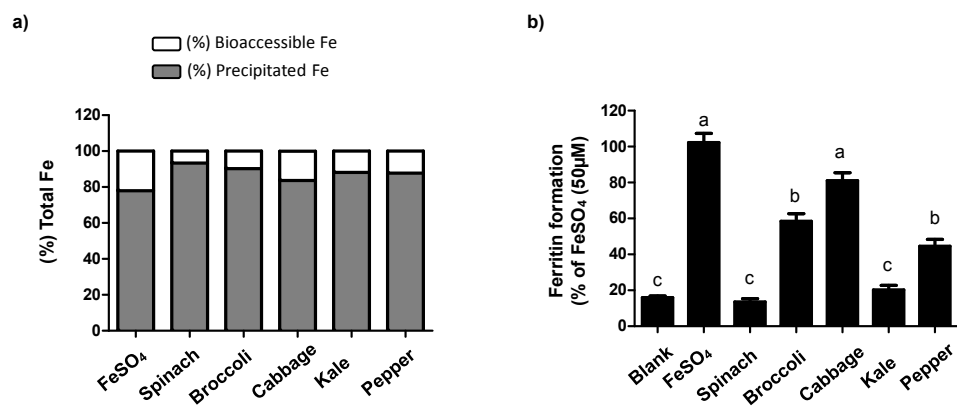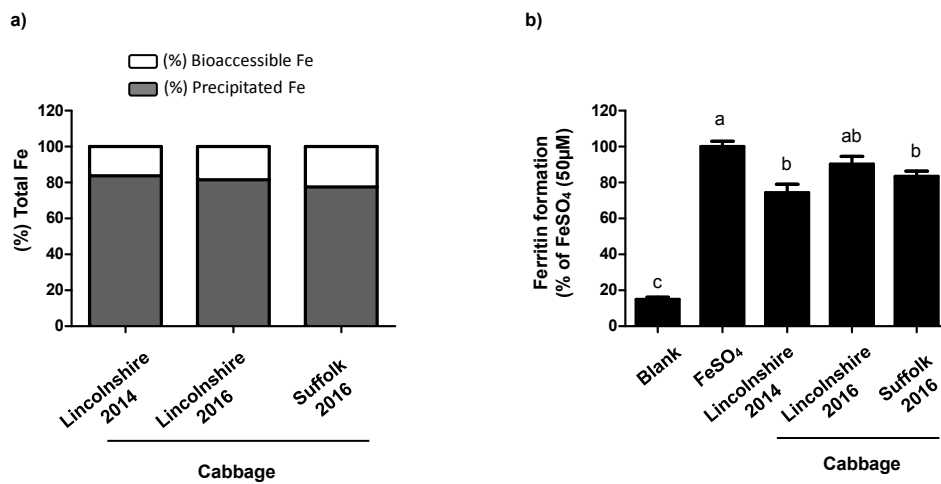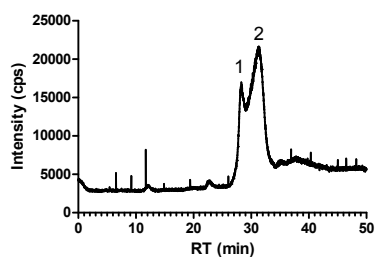
**Figure 1**

a)



b)

**Figure 2**

**Figure 3**
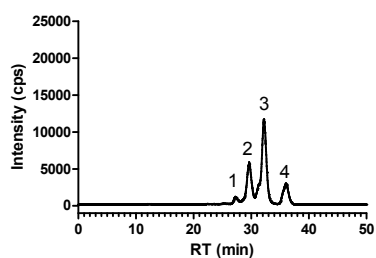
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b)


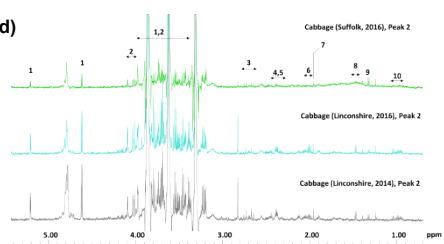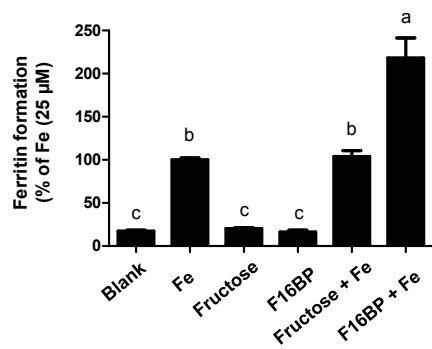
c)



— [Fe] Intensity

— [P] Intensity
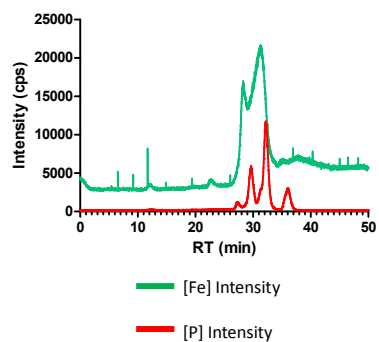
d)

**Figure 4**

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

**Table 1**

Iron content in dry weight (DW) powder of green vegetables and amount of material used for the simulated digestion.

| Vegetable | | Fe (µg/g) DW | Weight of dried vegetable in digestion (g) (50 µM) |
|---|---|---|---|
| Spinach (Boiled) | | 107 ± 1.11 | 0.39 |
| Broccoli (Boiled) | | 47 ± 0.41 | 0.89 |
| Curly Kale (Boiled) | | 55 ± 0.44 | 0.76 |
| Green Pepper (Raw) | | 40 ± 0.23 | 1.04 |
| Savoy Cabbage (Boiled) | Lincolnshire (2014) | 37 ± 0.15 | 1.13 |
| | Lincolnshire (2016) | 34 ± 0.10 | 1.23 |
| | Suffolk (2016) | 41 ± 0.33 | 1.02 |

Spinach, broccoli, curly kale and green pepper were purchased from the local supermarket (Norwich, UK) in 2014. For savoy cabbage, the sample purchased in 2014 was grown in Lincolnshire and the samples purchased in 2016 in Lincolnshire and Suffolk, according to the product label of the supermarket. Data represent the mean ± SEM of 3-4 samples replicates.

*Highlights:*

- The relative bioavailability of Fe in digests of five vegetables was in the order cabbage > broccoli ≥ pepper > kale > spinach

- Low molecular weight Fe was released during simulated digestion of cabbage

- Sugars, organic acids and amino acids were detected in the low molecular weight Fe fractions of cabbage digest

- Fructose 1,6-biphosphate increased the uptake of iron supplied to Caco-2 cells

- The higher bioavailability of iron in cabbage digests could result from complexation with sugars