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Iron release from spinach: effects of treatment on levels of iron (II) and iron (III) released *in vitro*

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Abstract

The release of iron from spinach, under *in vitro* conditions to mimic stomach conditions was investigated to determine the levels of iron (II) and iron (III) expected to be present in chyme that would enter the small intestine if a diet containing spinach alone were consumed. In the presence of hydrochloric acid only (0.01 M) and no pepsin, the average percentage release was 22% for fresh spinach, 33% for frozen and 45% for vacuum-dried. The maximum release occurred at 1 h in all cases other than fresh spinach which showed a gradual increase over a 4 h experimental period. The effect of pepsin on the rate of release was also investigated, with maximum releases of 26% when pepsin was present at 0.06%, and 41% at 0.19% pepsin. In all cases, only $Fe(aq)^{2+}$ could be stated to be released: there were no significant differences observed between values observed for $Fe(aq)^{2+}$ and total iron $[Fe(aq)^{2+} + Fe(aq)^{3+}]$ analyses [using 1,10-phenanthroline complexation, $\lambda_{max} = 510$ nm ($\epsilon = 10900$ M^{-1} cm^{-1})]. The absolute release, in the presence of hydrochloric acid only, in μmols g^{-1} (dry matter) was consistent throughout eight shop-bought fresh spinach batches, with no correlation of release to total% Fe, leading to the conclusion that free inorganic $Fe(aq)^{2+}$ only is released, not iron contained within non-haem proteins. The higher values for frozen, vacuum-dried and in the presence of 0.19% pepsin may be indicative of some breakdown of cellular structures.

Keywords: Iron; Bioavailability; Non-haem proteins

1. Introduction

The availability of iron in the human diet is of primary importance in terms of preventing the onset of anaemia and maintaining the levels of haemoglobin in the blood. The bioavailability of iron from the diet however depends upon the source: in general approximately 10% of dietary iron is absorbed, in a ''balanced'' diet in the form of haem-iron as found in meat (Kapanadis & Lee, 1995). The absorption of this form of iron is high and so most meat-eaters have few problems with iron-deficiency unless there are underlying biochemical or physiological problems. The remainder of the dietary iron is in the form of inorganic and non-haem iron sources and these are less well-absorbed during digestion (Kapanadis & Lee, 1995). In a vegetarian diet, there is therefore more of a likelihood of a low iron availability for uptake and hence the associated iron-deficiency anaemia. The low absorption of iron from such sources therefore leads to speculation as to whether the iron is actually released from plant sources during the digestive process, or whether the iron is released in a form that is unusable by the body upon bile neutralisation. The fact that iron exists mainly in the iron (III) form (Martinez, Ros, Periago, &, Lopez, 1999) reduces its bioavailability (Johnson, Smith, & Edmonds, 1998) since this form is, in general, less soluble than that of iron (II) at less acidic pHs.

Research has been undertaken which has shown that various organic complexes such as fibre can bind minerals (Leigh & Miller, 1983) and so alter absorption during digestion, however little research has been done on which ionic form minerals are present in when soluble iron is released. The relevance of the form of iron released is of importance due to the changing pH levels during the digestive process. Non-haem iron that is not free iron (II) or iron (III) ions within the foodstuff will normally be present in the form of a metal–protein complex. For example, iron–sulphur proteins including

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ferredoxins are present in vegetable foodstuffs. Such proteins would be expected to release iron, if any is released, during digestion in the stomach where proteases are present. The release of either ionic state of iron in the stomach is of little consequence since the high acidity will allow both to be soluble even if uncomplexed, e.g. by citrate. However, later in digestion after bile neutralisation, problems with the solubility of iron (III) complexes may occur due to the higher pH levels.

The general low absorption of non-haem iron, regardless of physiological iron status, would lend itself to the probability that the release of iron from protein sources is not occurring during the timescale of digestion in acidic conditions. This study has undertaken to look at the breakdown and release of iron from spinach under *in vitro* conditions to determine the ionic form present under the conditions expected in the stomach. Spinach has been chosen since it has a high non-haem iron–sulphur cluster content containing a variety of iron–sulphur cores, (Beinert, Holm, & Münck, 1997) and there is considerable variation in the literature of the release of iron from spinach. Spinach is viewed as a suitable plant material to study due to its high iron content and range of non-haem iron containing proteins. If these iron–sulphur protein molecules are broken down by digestion, the iron may be expected to "drop out" of the protein cluster easily due to the effects of oxygen on similar core clusters, (Beinert et al., 1997; Emptage, Dreyer, Kennedy, & Beinert, 1983) and so high levels of $Fe(aq)^{3+}$ species would be expected. Analysis for $Fe(aq)^{2+}$ and $Fe(aq)^{3+}$ has been performed using complexation by 1,10-phenanthroline (Davies, Kipling, & Sykes, 1973) with any interference effects due to chlorophyll, and complexation of $Fe(aq)^{2+}$ by citrate and oxalate considered. (Gillooly, Bothwell, Torrance, MacPhial, Derman, Bezwoda, Mills, Charlton, & Mayet, 1983).

2. Experimental

2.1. Spinach analysis

Spinach was purchased from J Sainsburys PLC, Middlesbrough, or Safeways, Stockton-on-Tees, in batches over a period of 18 months. The spinach was classified for these experiments as either "fresh" (used as purchased), "frozen" (samples of the spinach are frozen at −20 °C in an Electrolux ER2946/47B fridge-freezer), "homogenised" (samples of spinach are blended with ~20 mls of liquid, samples prefixed by fresh or frozen to indicate state of spinach prior to homogenisation) or vacuum-dried (samples of spinach are placed in a vacuum dessicator for 3 days). Each "sample" of spinach had a wet (fresh) weight of 30.0 g.

From each batch, 30.0 g samples were weighed, then vacuum dried to establish a dry–wet (fresh) weight correlation. The dry material was then digested using micro-Kjeldahl apparatus with concentrated nitric acid (only, no catalytic tablet). The resultant solution was analysed using ICP–MS (inductively coupled plasma-mass spectrometry) and AAS (atomic absorption spectrometry) to determine total iron concentration. The remaining spinach in the batch was weighed into samples and subject to one or more of the treatments. Prior to digestion, samples of spinach leaves were cut into smaller pieces to mimic the effects of mastication: in addition, frozen samples were allowed to defrost for 15 min at 37 °C.

2.2. The in vitro digestion process

2.2.1. Acid digestion

A sample of spinach, (equivalent to 30 g wet weight), was placed in a flat-bottomed volumetric flask and 250 ml of hydrochloric acid (0.010 ± 0.001M) added. (Both at 37 °C prior to mixing.) Samples were then placed in a laterally shaking water bath maintained at 37 °C and shaken continuously throughout the 4 h experiment. At hourly intervals, aliquots of solution were removed and analysed for $Fe(aq)^{2+}$, $Fe(aq)^{3+}$ [via reduction to $Fe(aq)^{2+}$], and chlorophyll. These extracted aliquots were not returned to the solution, but the amount of iron removed was accounted for in later calculations. The intention was to remove the need for centrifugation [to eliminate chlorophyll and other cellular material interference in the spectra obtained (λ range : 300–900 nm)] as part of the analytical procedure to reduce any loss of iron via iron precipitation onto glassware (Pierson & Clark, 1983) Calibration curves for interference effects were therefore produced as part of this study.

2.2.2. Enzyme digestion

The same techniques as for acid-only digestion were utilised with a solution of pepsin enzyme also present. Several concentrations of pepsin solution (porcine stomach pepsin, Sigma p7000) were used: 0.5 and 4%, to yield actual concentrations during digestion of 0.06 and 0.19%, respectively. Activity of the enzyme was studied using bovine haemoglobin with the 4% stock solution stored for no more than 3 days under acidic conditions (0.1 M HCl), at 4 °C. The higher acidity was used to study activity as this is the storage pH required; weaker hydrochloric acid solutions were used throughout the experimental conditions, and pepsin activity confirmed to be constant over the 4 h experimental periods.

2.3. Iron analysis

$Fe(aq)^{2+}$ was analysed by complexation with 1,10-phenanthroline ("phen") to form $[Fe(phen)_3]^{2+}$ using

visible spectrophotometry ($\lambda_{max} = 510$ nm, $\epsilon = 10{,}900$ M^{-1} cm^{-1}) as described previously (Beinert et al., 1997). No interference by $Fe(aq)^{3+}$ occurs at the wavelength used. The analysis also needed to account for any contribution made at this wavelength by chlorophyll or other cellular components present in the solution. Investigation via visible spectroscopy determined that an interference at the analysis wavelength occurred, and it was therefore accounted for during calculations using spectroscopic measurements of the extracted solutions prior to 1,10-phenanthroline complexation (i.e. the calibration curves mentioned previously).

$Fe(aq)^{3+}$ concentrations were determined via reduction to $Fe(aq)^{2+}$ using hydroxylamine hydrochloride followed by 1,10-phenanthroline complexation as described earlier.

2.3.1. Mass balance experiments

Mass balance experiments were performed using ICP–MS to determine absolute levels of iron in solution upon digestion and in the remaining solid matter. The latter was analysed by filtering the solid from the digestion solution, vacuum drying and then digesting in concentrated HNO_3. Five-fold dilution using deionised water occurred before analysis.

2.3.2. Effects of citrate and oxalate on $Fe(aq)^{2+}$ analysis

Standard solutions of $[Fe(H_2O)_6]^{2+}$ were prepared in 0.01 M HCl. Complexation with 1,10-phenanthroline was studied over 9 h in the presence of oxalic and citric acids. The ratios of iron to oxalic or citric acid studied were 3:1, 1:1 and 1:3. For all ratios, no evidence of any alteration in the value at 510 nm was observed indicating that 1,10-phenanthroline is a stronger chelating agent for $Fe(aq)^{2+}$ than citrate or oxalate under the analytical conditions.

3. Results

Twelve batches of spinach were used in this study. Preliminary studies concentrated on the release of iron (II) and (III) ions during digestion in the presence of hydrochloric acid only. Later studies then incorporated the enzyme, pepsin, to allow a determination of the relative roles of acid and enzyme on fresh spinach only. The effects of the enzyme were not as noticeable as expected, with some pre-treatment of spinach, namely vacuum-drying, giving a greater release of iron. The characteristics of each batch of spinach used are shown in Table 1. It is noteable that both % water and % iron vary considerably and so either general inferences can be made by averaging a number of experiments (indicated in parentheses throughout the paper) or direct comparison within each batch must be considered to show specific examples of differences. Both scenarios are utilised throughout this paper. In all experimental runs, the values for $Fe(aq)^{2+}$ and total $Fe(aq)^{2+}$ ($= Fe(aq)^{2+} +$ reduced $Fe(aq)^{3+}$) did not differ within the bounds of experimental error so allowing no conclusion that $Fe(aq)^{3+}$ may have been present in solution.

3.1. Acid-only digestion of spinach

3.1.1. Fresh versus frozen

Batch A allows a direct comparison of the release of iron from fresh and frozen samples under acid-digestion only. Fig. 1 shows the cummulative release of iron in μmols per gram dry matter over the 4 h experiment. A gradual increase could be observed for the fresh sample [maximum = 1.53 μmols g^{-1} (dry matter)], however the maximum release observed for frozen samples [1.82 μmols g^{-1} (dry matter)] occurred only after 1 h. Homogenisation of both fresh and frozen samples from batch A produced surprising results with a maximum release occurring on homogenisation, and a gradual decrease over the 4 h reaction period (Fig. 1). The maximum release for both the fresh and frozen samples were slightly reduced (1.13 and 1.61 μmols g^{-1}(dry matter) respectively) compared with the non-homogenised samples. [The maximum possible release of iron would be 4.83 μmols g^{-1} (dry matter) if 100% release occurred.]

Vacuum-drying (i.e. dessication) of the spinach was also investigated to determine the effects of different treatments other than freezing to allow storage of spinach to prevent spoilage. The nutritional content has importance and so the amount of iron released from vacuum-dried spinach was considered. Six analyses from two differing batches of spinach produced similar

Table 1
Characteristics of the batches of spinach

Batch	% Dry matter	% Total iron (of dry matter)	Sample treatments
A	9.00	0.027	Fresh, frozen, homogenised-fresh, homogenised-frozen
B	9.80	0.020	Vacuum-dried
C	6.50	0.020	Vacuum-dried
D	9.05	0.036	Fresh
E	7.03	0.011	Fresh
F	7.63	0.059	Fresh
G	8.26	0.008	Fresh
H	10.9	0.059	Fresh
I	9.97	0.034	Fresh
J	10.1	[a]	Fresh
K	10.5	[a]	Fresh
L	7.87	0.011	Fresh[b]

[a] Contamination of Keljdahl digestions occurred, rendering analyses unreliable.

[b] This batch only was bought as "prewashed".

levels of release of iron when measured in μmols g^{-1} (dry matter). The average release (6 samples) is shown in Fig. 2 with a maximum release (2.51 μmols g^{-1}(dry matter)) again occurring after 1 h, comparable with the trend observed for frozen samples.

3.2. Acid-enzyme digestion

For every sample of spinach undergoing acid and enzyme digestion, a control digestion of acid only was also performed to ascertain that a true comparable effect was occurring. A small, insignificant effect due to pepsin was observable when pepsin was present at 0.06% (batch D and E) however a more noticeable effect was observed for 0.19% pepsin as shown in Fig. 2 (average of batches F–K). The maximum average release for 0.19% pepsin present compared with acid only digestion of fresh spinach was 2.29 μmols g^{-1} (dry matter) compared with 1.20 μmols g^{-1} (dry matter). These results are averages due to the heterogeneous nature of the spinach (and indeed any foodstuff).

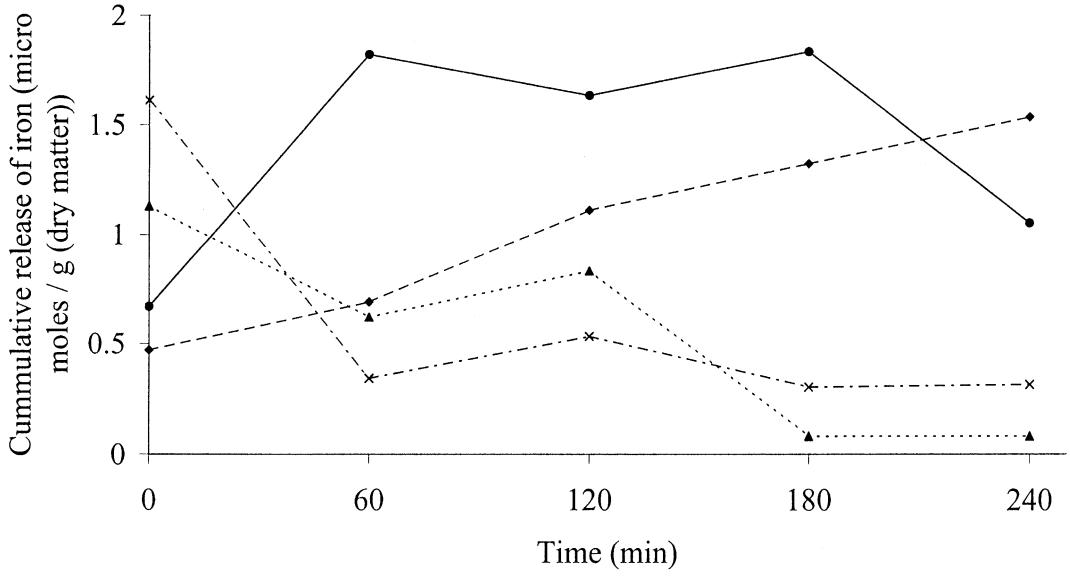

Fig. 1. Release of $Fe(aq)^{2+}$ analysed as $[Fe(phen)_3]^{2+}$ from spinach Batch A normalised to μmols iron per gram dry matter. Initial release from 30 g fresh weight equivalents into 250 ml of 0.01 M HCl at 37 °C with 4 ml extractions removed per hour for analyses. Differences between pre-treatments are shown: ◆, fresh; ●, frozen; ▲, homogenised fresh; **X**, homogenised frozen.

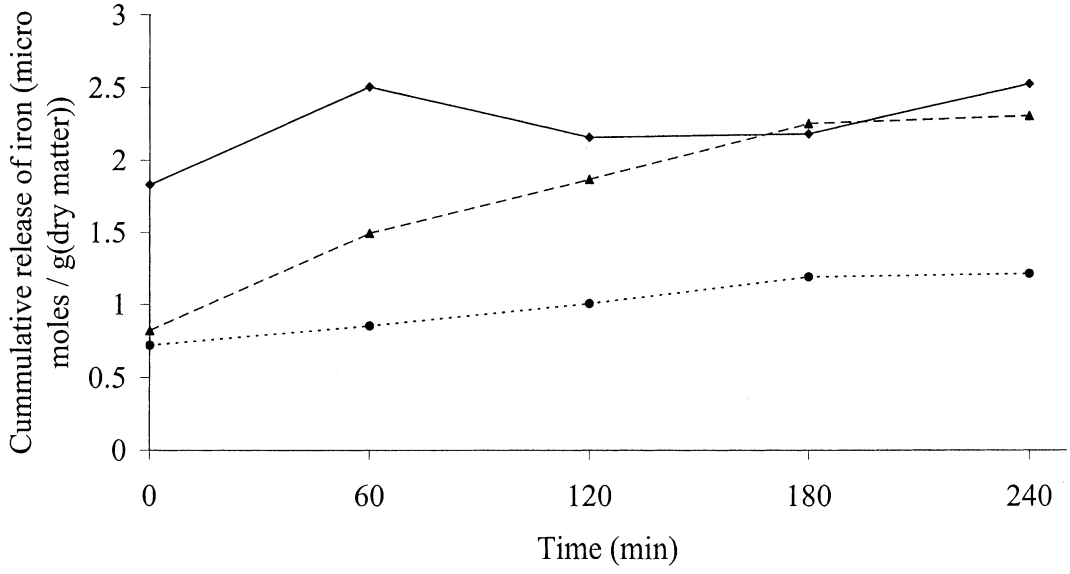

Fig. 2. Release of $Fe(aq)^{2+}$ analysed as $[Fe(phen)_3]^{2+}$ from spinach normalised to μmols iron per gram dry matter. Initial release from 30 g fresh weight equivalents into ca. 262.5 ml of 0.01 M HCl at 37 °C with 4–8 ml extractions removed per hour for analyses. Differences between pre-treatments and digestion conditions are shown: ◆, vacuum-dried (average of 2 batches, 6 digestions); ●, fresh (average of 8 digestions); ▲, fresh with 0.19% pepsin added (average of 8 digestions).

3.2.1. Calibration plots

The digestion of fresh spinach in the presence of acid and enzyme, allowed calibration plots to be produced for general guidelines as to the expected levels of chlorophyll and other plant materials that may be released with time. These plots are shown in Fig. 3, and allow two formulae to be derived, assuming a volume of 250 ml for digestion per gram dry matter spinach in acid only (262.5 ml when enzyme present) to estimate interference absorbance effects (A_i) by chlorophyll and other cellular components on absorbance due to $[Fe(phen)_3]^{2+}$ at 510 nm, 37 °C, (t in min):

$$\text{Acid only} : A_i = 0.000147\ t + 0.0162$$

$$\text{Acid} + 0.19\%\text{pepsin} : A_i = 0.00015\ t + 0.0313$$

Table 2
Percentage iron release during *in vitro* digestion when calculated as an average based upon g dry weight

Treatment	Average % release
Acid-only digestion	
Fresh	22±9
Frozen	33±14
Homogenised fresh	20±1
Homogenised frozen	29±16
Vacuum-dried	45±15
Acid-enzyme digestion	
Fresh (no enzyme)	22±9
Fresh (0.06% pepsin)	26±4
Fresh (0.19% pepsin)	41±9

Actual absorbance ($[Fe(phen)_3]^{2+}$) can then be calculated from measured absorbance ($[Fe(phen)_3]^{2+}$) by subtraction of A_i.

3.3. Percentage releases per treatment and/or digestion

The percentage release of each treatment and/or digestion were averaged since similar levels of iron were released throughout all experiments as μmols per gram dry matter. The heterogeneous nature of all plant materials determined that an average total iron was determined for all batches of spinach since it was acknowledged that variation would occur between samples from each batch. An average value for % total iron of dry matter was determined as 0.031, which would yield a maximum release of 5.55 μmols per gram dry matter. The % release of each treatment and/or digestion are summarised in Table 2 showing that vacuum-drying allows the greatest release of iron.

3.4. Mass balance experiments

These were performed on batches H and I to assess the effects of organic species on the 1,10-phenanthroline complexation analytical technique. ICP–MS analyses for absolute levels of iron and so will also account for any $Fe(aq)^{2+}$ complexed to species that can competitively compete with 1,10-phenanthroline. Results are presented in Table 3. Good correlation was observed for batch H, however, batch I was less comparable. The lack of homogeneity between samples within batch I can also be seen since the original sample for characteristic

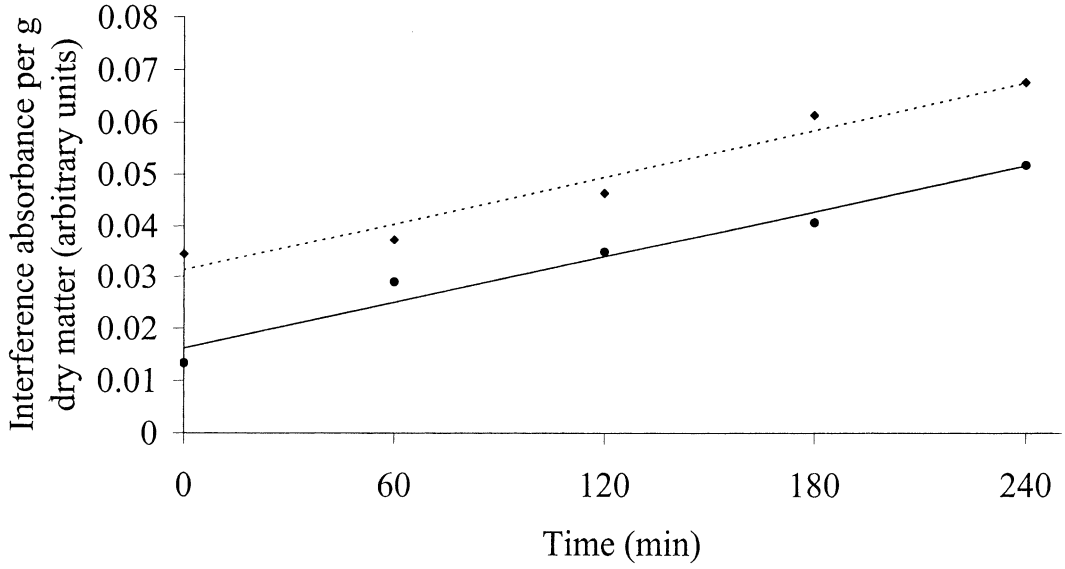

Fig. 3. Calibration plots to show the absorbance values per g dry matter to be used when 30 g fresh spinach is digested in 250 ml hydrochloric acid (●) and pepsin (◆) (additional 12.5 ml volume addition for enzyme plot) over a period of time. Absolute absorbance readings for subtraction from iron analyses can then be calculated by multiplying by gram dry matter in 30 g fresh weight per spinach batch. These plots are only a guideline for shop-bought spinach and error may occur depending on growth conditions.

Table 3
Mass balance experiments for batches H and I

Batch	Digestion conditions	μmols released (cummulative): phen complexation					Final μmols: ICP–MS			Initial μmols: ICP–MS
		0 min	60 min	120 min	180 min	240 min	Solution	Solid	Total	
H	Acid only	3.16	3.43	5.76	4.40	4.13	4.70	31.3	36.0	34.6
H	Acid + enzyme	2.86	4.27	5.15	5.78	5.19	5.74	30.2	35.9	34.6
I	Acid only	4.76	1.14	1.47	3.41	2.09	3.71	20.4	24.1	18.2
I	Acid + enzyme	1.98	3.25	5.22	4.94	5.52	6.22	21.8	28.0	18.2

analysis had a lower level of iron than those two samples digested in acid and acid and enzyme solutions.

4. Discussion

The non-homogeneous nature of plant materials, leads to the discussion of estimation of release of minerals, including iron, from samples upon digestion. Age of plant when picked, hours of daylight during growth and even time of day or year when harvested can all be expected to affect the levels of minerals present and their bioavailability. Analysis of shop-bought foodstuffs therefore does not allow "control" of such parameters: however it does reflect the food consumed by the general population. Twelve batches of spinach were purchased from large supermarkets within the borough of Stockton-on-Tees. The non-homogeneous nature can clearly be indicated by the variation in total iron content determined and no means of correlation to age of the plants was possible due to no requirement by labelling laws. The lack of homogeneity within a batch is highlighted by the mass balance experiments for batch I, however batch H indicates that some homogeneity can also be observed. This demonstrates the need for caution in generalisation of levels of absolute iron in any vegetable foodstuff chosen to be studied analytically.

Interestingly however, comparable absolute amounts of iron released were observed in all batches under fresh, acid-only digestion. These absolute amounts however did not correlate with % total iron in each batch, leading to the assumption that the iron released was not dependent upon the plant itself, i.e. not intracellular contents which may depend upon age of membranes, cellular structures, etc. but rather extracellular iron from the plant. This would account for comparable levels if the presumption is made, that the spinach is grown under "standardised" agricultural conditions (i.e. levels of fertilisers, pesticides, etc.). To check that levels of iron released were not due to extraneous iron that may have been present on the surface of plants, prewashed spinach was also analysed. The total iron and iron released upon acid-only digestion, was comparable however with non-prewashed batches and gave no indication that contamination may have occurred.

The release of iron in all experiments was assumed to be soluble iron (II) only since no significant difference was observed between analyses for $Fe(aq)^{2+}$ and solutions in which the $Fe(aq)^{3+}$ had been reduced to $Fe(aq)^{2+}$. The levels released from fresh spinach in the presence of 0.01 M HCl alone were not noticeably increased upon the addition of pepsin at 0.06%. However, at 0.19% an effect was observed. The iron released without enzyme present is, as stated earlier, believed to be free inorganic iron in extracellular fluids. The low level of release is not thought to be indicative of iron release from non-haem proteins. This idea of two such pools of iron has previously been proposed by Zhang, Hendricks, and Mahoney (1989) The increase in the presence of enzymes may indicate the start of a breakdown of cellular structures releasing intracellular iron. Similar effects are expected upon freezing and vacuum drying due to destruction of cellular walls and membranes. Interestingly the release of iron from frozen samples was lower than for vacuum-dried samples or digestion in the presence of enzymes, indicating perhaps that this processing method (freezing) is less destructive with regard to cellular structures incorporating iron within them. However, the levels recorded in this study for frozen spinach digested in hydrochloric acid alone are higher than previous reports. (Miller, 1987) Miller also demonstrated a low release of iron in spinach that had been subjected to the canning process. This low release would compare with the decrease in levels of iron in solution observed upon homogenisation of the spinach prior to digestion. Homogenisation would appear to allow the release of strong iron chelators that can interfere with iron analyses by 1,10-phenanthroline complexation. These species appear to chelate the iron from solution after 1 h indicative of slow equilibration kinetics occurring. Experiments to assess the possible chelators have indicated that oxalate and citrate cannot interfere with this analytical procedure. At first glance, other possibilities to consider would include cellulose, phytates, fibre, other organic molecules including tannic and gallic acids. Reinhold, Garcia, and Garzon (1981) have noted however that cellulose does not bind significantly to iron (II) at the pH studied.

Oxalic and citric acids have been investigated in this study due to the range of opposing statements on their role in iron absorption during the digestive process. No interference effects were noted on this analytical procedure. This indicates that they cannot complex competitively against 1,10-phenanthroline, not that they are not complexing in the digestive tract. These findings concur with those of Leigh and Miller (1983), who also investigated the effect of fibre. Other organic acids (Gillooly et al., 1983) and cellulose and pectin may appear likely candidates for further investigation, however the range of organic acids is large and will require extensive kinetic investigation, beyond the scope of this paper. Phytic acid, (Hallberg, Rossander, & Skånberg, 1987; Simpson, Morris, & Cook, 1981) and phenolic compounds (Brune, Rossander, & Hallberg, 1989) including tannic acid have been investigated but again it has been for their role in iron absorption rather than interference effects on iron analyses. Complexes forming due to $Fe(aq)^{2+}$ complexing with galloyl ligands would be measured to some extent in these experiments, firstly as a possible interference effect and so excess/lower than average levels of galloyl-compounds in any one batch of spinach may account for slight variations from the calibration levels. However, the absorbance maxima would be expected to be at a higher wavelength than 510 nm (Brune et al., 1989). The relative kinetics of the competition between galloyl and 1,10-phenanthroline are not known at present and thus further study is required. Data obtained in this study did not show a noticeably higher measured interference effect for batch I when compared with batch H however, to explain the more noticeable disparity in the results obtained for batch I by ICP–MS of the final solution and analysis by 1,10-phenanthroline complexation, complexation by some organic species in competition with 1,10-phenanthroline must be postulated at this point in time.

5. Conclusions

The conclusions drawn from this study are that differing trends can be observed in iron released into solution when monitored over a period of time designed to mimic digestion. Interference effects on analytical procedures need to be investigated further before comparisons of iron present in solution can be extrapolated to that which is available for uptake. The lack of measurement of iron in solution does not therefore definitively correlate with a lack of iron released from spinach since disparity between colourimetric and other analytical techniques have been observed in this study in some batches of spinach. However, it does appear on current analytical data that iron is probably not released from non-haem iron protein clusters under simulated in vitro digestion conditions.

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