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PAPER

## Development of DRC-ICP-MS methodology for the rapid determination of $^{58}\text{Fe}$ erythrocyte incorporation in human iron absorption studies

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Iron deficiency is the only major nutritional deficiency that still exists in the developed world and iron deficiency anaemia affects nearly one billion people worldwide (Benoist *et al.*, Worldwide prevalence on anaemia 1993–2005, WHO, 2008). However, iron supplements are still not optimally formulated so cheap, side effect-free and well absorbed iron supplements are sought. The development of these requires the determination of iron absorption in animals, volunteers or patients, typically by determining iron isotopic enrichment in blood after administering isotopically labelled iron supplements. Current analytical techniques for isotope ratio work, such as thermal ionisation mass spectrometry (TIMS) and multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS), have a low throughput, due to the requirements of sample pre-treatment and, generally, are not present in clinical or nutritional laboratories. Here we describe a novel, more accessible, dynamic reaction cell inductively coupled plasma mass spectrometry (DRC-ICP-MS) method for the determination of  $^{58}\text{Fe}$  enrichment in samples from nutritional or clinical studies. This is a high throughput method, in which the samples require no pre-treatment other than dilution, that was validated against MC-ICP-MS, and was shown to be fit-for-purpose.

### Introduction

Despite many years of investigation, iron supplements are still not optimally formulated. Generally they rely upon simple ferrous salts which undergo redox cycling in the gut following ingestion and lead to side effects (typically nausea and abdominal pain). Alternatively, ferric iron preparations may be used but these are either poorly absorbed or expensive. Thus, work continues, such as in our laboratory, to identify cheap, side effect-free and well absorbed forms of iron for oral supplementation. Indeed, iron deficiency anaemia affects nearly one billion people worldwide<sup>1</sup> and iron deficiency is the only major nutritional deficiency that still exists in the developed world.<sup>2</sup>

One key assay in nutritional or clinical studies that assess iron supplements or fortificants is an 'absorption and utilisation' test. Absorption means systemic uptake (*i.e.* across the gut not just into the gut) and utilisation, in this context, means the loading of iron into haemoglobin. The gold standard test in humans requires the ingestion of isotopic iron and then the measurement of isotopic enrichment in haemoglobin (washed red cells or whole

blood even) 14 days (d14) after oral ingestion in comparison to baseline (d0), which is immediately pre-ingestion. These days, stable isotopes, especially  $^{58}\text{Fe}$  and  $^{57}\text{Fe}$  are used to avoid any radio-isotopic exposure to the subject. Traditionally, thermal ionisation mass spectrometry (TIMS) has been the reference technique for tracer experiments but it requires lengthy sample pre-treatment procedures, which result in very low throughput and, subsequently, high costs. More recently, multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS)<sup>3,4</sup> has also been used for high precision isotope measurements in blood but, although it allows higher sample throughput than TIMS, it still requires significant sample pre-treatment. Also, like TIMS, it is a specialist instrumentation restricted to the determination of isotopic ratios, and is in very few clinical or nutritional laboratories. A fit-for-purpose assay for the assessment of oral iron absorption should (a) be able to measure whole blood isotopic iron with minimal handling/pre-treatment of the sample, thus allowing a reasonably high throughput without high staff costs, (b) be able to measure isotopic iron enrichment in blood that corresponds to  $\geq 5\%$  intestinal absorption after an oral dose, as much less than this would be considered of little value clinically or nutritionally and (c) use instrumentation that is available in most specialist laboratories, not prohibitively expensive, and has a reasonable sample throughput. Conveniently, the incorporation of dynamic reaction cells (DRC) into traditional quadrupole inductively coupled plasma mass

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spectrometry (ICP-MS) instruments has allowed accurate quantification of isotopes, such as Fe isotopes (and especially  $^{58}\text{Fe}$ ),<sup>5</sup> that were previously inaccessible to ICP-MS due to polyatomic interferences. DRC-ICP-MS, although, not as sensitive, or likely precise and accurate, as MC-ICP-MS for isotope ratio work, is robust, provides a very high throughput and, importantly, is commonly present in clinical and nutritional laboratories carrying out elemental analyses. Previous studies have shown, using basic aqueous fluids which included a simplified 'model' serum, that  $^{58}\text{Fe}$  can be readily assayed in such samples using DRC-ICP-MS technology.<sup>5</sup> We, however, applied this technique to the determination of  $^{58}\text{Fe}$  enrichment in actual whole blood samples of volunteers given  $^{58}\text{Fe}$  labelled iron supplements and, here, we report on the optimised methodology and validation against the reference technique, MC-ICP-MS. We have also made comparative analyses with sector field inductively coupled plasma mass spectrometry (SF-ICP-MS) as, to date, this technique has been the most widely used in nutritional studies of isotopic iron absorption.<sup>6,7</sup>

## Experimental

### Sample collection

Several whole blood samples were collected from healthy volunteers at the MRC-Human Nutrition Research Unit (ethical approval given by the Cambridge Research Ethics Committee, UK). These were pooled into a single sample and used as an in house quality control whole blood.

Whole blood was collected from iron-deficient volunteers participating in an Fe absorption study at the MRC-Human Nutrition Research Unit (ethical approval given by the Suffolk Research Ethics Committee, UK). Baseline whole blood samples (d0 blood) were collected immediately prior to administration of a 60 mg Fe oral supplement labelled with 2 mg  $^{58}\text{Fe}$ , and a second blood sample was collected 14 days after administration (d14 blood). This is a typical protocol that determines iron incorporation into red blood cells and thus gives a direct measure of absorption and bioavailability.

### Specifics of the DRC-ICP-MS methodology

**Reagents.** All standards were prepared by diluting volumetric standards (Fluka, Sigma Aldrich, Dorset, UK). Instrument optimisation was performed using a combined standard (Elan 6100 Setup/Stability/Mass Calibration Solution, Perkin Elmer, Beaconsfield, UK). Nitric acid, ammonia, and butan-1-ol were all of trace analysis grade (Sigma Aldrich, Dorset, UK). Triton X-100 was also from Sigma Aldrich (Dorset, UK). All solutions were prepared in de-ionised water ( $18.2\text{ M}\Omega\text{ cm}^{-1}$ ), produced by a NANOpure Diamond water system (Barnstead, Dubuque, Iowa, USA).

**Sample and standard preparation.** Whole blood diluent was an aqueous solution containing 0.5% Triton X-100, 1% butan-1-ol, 0.5% ammonia, and 0.008% nitric acid. This solution was used for diluting whole blood samples (100-fold) and the in-house quality control whole blood. Blank samples were prepared similarly by diluting UHP water in whole blood diluent. Calcium

spiked samples were prepared by diluting an 85 ppm solution in whole blood diluent.

**Iron isotope analysis.** An Elan DRC<sup>plus</sup> Inductively Coupled Plasma Mass Spectrometer (Perkin Elmer Sciex, Beaconsfield, UK) was used to determine  $^{58}\text{Fe}$ : $^{57}\text{Fe}$  ratios. The sample introduction system consisted of a V-groove nebuliser, a double-pass spray chamber, a demountable quartz torch, and a quartz injector (2 mm internal diameter). Platinum-tipped sample and skimmer cones (Perkin Elmer Sciex, Beaconsfield, UK) were used for all analyses.

Instrument conditions were tuned for optimum signal sensitivity (*via* the measurement of  $^{24}\text{Mg}$ ,  $^{115}\text{In}$  and  $^{238}\text{U}$  isotopes), minimum oxide formation (*via* the measurement of the  $^{140}\text{Ce}$  and  $^{156}\text{Gd}$  isotopes, *i.e.* monitoring the degree of CeO formation at  $m/z = 156$ ) and minimum doubly charged ion formation (*via* the measurement of the  $^{138}\text{Ba}$  and  $^{69}\text{Ga}$  isotope signals, *i.e.* monitoring the degree of  $^{138}\text{Ba}^{2+}$  formation at  $m/z = 69$ ). Further adjustment was then performed to reduce mass bias between  $^{58}\text{Fe}$  and  $^{57}\text{Fe}$  (approximately 5%). Detector voltages were optimised to minimise, but not fully correct, mass bias. Thus, the measurements carried a systematic bias that was consistent within sample pairs (d0 *vs.* d14) which were analysed consecutively, that was cancelled out when calculating the ratio-difference between the two samples. Instrument conditions are described in Table 1.

### Comparative ICP techniques

**SF-ICP-MS.** Samples analysed by SF-ICP-MS for  $^{58}\text{Fe}$ : $^{54}\text{Fe}$  ratios, were first digested in concentrated  $\text{HNO}_3$  at  $110\text{ }^\circ\text{C}$  and then diluted to a nitric acid concentration of  $\sim 0.1\text{ mol L}^{-1}$  vortexing prior to analysis. Sample blanks were taken into account and the isobaric interference of  $^{58}\text{Ni}$  on  $^{58}\text{Fe}$  was corrected mathematically by a concurrently measured  $^{60}\text{Ni}$  isotope signal.<sup>6</sup>

**MC-ICP-MS (Lab 1).** High-precision iron isotope ratios were measured on the Neptune (ThermoFisher Scientific) MC-ICP-MS at the University of Hannover, Germany, as previously described.<sup>8</sup> Briefly, venous blood samples (0.5 mL) were mineralized by microwave digestion and iron was purified by anion exchange. Sample solutions ( $5\text{ }\mu\text{g mL}^{-1}$  Fe in 2%  $\text{HNO}_3$ ) were nebulised and introduced by the ThermoFinnigan stable

**Table 1** Instrument conditions for DRC-ICP-MS analysis of  $^{58}\text{Fe}$ : $^{57}\text{Fe}$

Instrumental Parameter and Unit	Value
Nebuliser Gas Flow ( $\text{L min}^{-1}$ )	0.90–0.93
Sample injection rate ( $\text{mL min}^{-1}$ )	1.0
Plasma Gas Flow Rate ( $\text{L min}^{-1}$ )	15
Auxiliary Gas Flow Rates ( $\text{L min}^{-1}$ )	1.2
RF Power (W)	1100
Lens Voltage (V)	8–12
Sweeps Per Reading	20
Readings Per Replicate	1
Number of Replicates	80
Dwell Time (ms)	50
Integration Time (ms)	1000
DRC gas Argon/5% $\text{H}_2$ ( $\text{mL min}^{-1}$ )	0.3
RPa	0
Rpq	0.45

introduction system (SIS), which consists of a combined cyclonic and Scott-type spray chamber. The isotopes  $^{54}\text{Fe}$ ,  $^{56}\text{Fe}$ ,  $^{57}\text{Fe}$ , and  $^{58}\text{Fe}$  were all measured simultaneously on Faraday collectors. Any residual isobaric interference of  $^{54}\text{Cr}^+$  and  $^{58}\text{Ni}^+$  was corrected for by monitoring  $^{52}\text{Cr}^+$  and  $^{60}\text{Ni}^+$ , respectively. ArO, and ArN interferences were resolved by high-mass resolution techniques.<sup>9</sup>

**MC-ICP-MS (Lab 2).** High-precision iron isotope ratios were measured using the IsoProbe (Isoprime Ltd) Hex-MC-ICP-MS at the Natural History Museum, London.<sup>10</sup> Blood samples were digested in  $\text{HNO}_3/\text{HClO}_4$  mixtures and Fe purification was performed using anion exchange chromatography.<sup>11</sup> Sample solutions ( $2\ \mu\text{g mL}^{-1}$  Fe in 0.1% HCl) were nebulised with a micro-uptake T1-H type nebuliser on an Aridus membrane desolvation system (Cetac Technologies, Omaha, NE, USA). Analyses then were undertaken using a static measurement protocol at  $M/\Delta M = 500$ , with  $^{54}\text{Fe}^+$ ,  $^{56}\text{Fe}^+$ ,  $^{57}\text{Fe}^+$ ,  $^{58}\text{Fe}^+$  and the corresponding interferences being measured on Faraday detectors.  $^{56}\text{Fe}^+$  was measured on a collector using a  $10^{10}\ \Omega$  resistor to avoid saturation of the amplifier, while all other masses were measured using  $10^{11}\ \Omega$  resistors. Isobaric interferences of  $\text{ArO}^+$  and  $\text{ArOH}^+$  were removed by hydrogen supported collision cell technology of the IsoProbe mass spectrometer.<sup>10</sup>

### Calculations

For the purpose of later discussion we have assumed a mildly anaemic (haemoglobin of 11.5g/dL) female subject of 60 kg weight, and  $^{58}\text{Fe}$  absorption ( $^{58}\text{Fe}_{\text{Abs}}$ , %) was calculated from  $^{58}\text{Fe}$  enrichment ( $^{58}\text{Fe}_{\text{Enr}}$ , %) of whole blood (*i.e.* haemoglobin incorporation), according to the following equations:

$$Fe_{\text{Circ}} = 60 \times 69.6 \times 11.5 \times 0.0347$$

$$^{58}\text{Fe}_{\text{Abs}} = \frac{(^{58}\text{Fe}_{\text{Enr}} \times ^{58}\text{Fe}_{\text{Circ}} \times 0.2819 \times 0.01)}{^{58}\text{Fe}_{\text{Dose}}} \times \frac{100}{80}$$

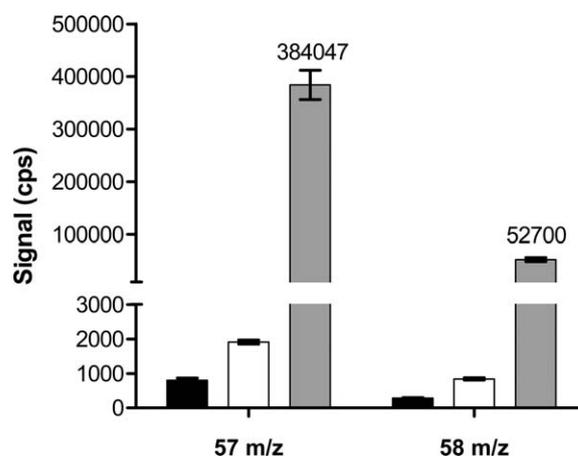
Where  $Fe_{\text{Circ}}$  is total iron circulating in whole-blood (mg) and  $^{58}\text{Fe}_{\text{Dose}}$  is the mass of  $^{58}\text{Fe}$  tracer given (mg). It was assumed that the subject had 69.6 mL blood per kg body weight,<sup>12</sup> 3.47 mg iron per g haemoglobin, and 0.2819% as the natural abundance of  $^{58}\text{Fe}$ .<sup>13</sup> 100/80 is a standard assumption, that of the iron absorbed following ingestion (day 0), 80% is incorporated into haemoglobin by day 14.<sup>14</sup>

### Results and discussion

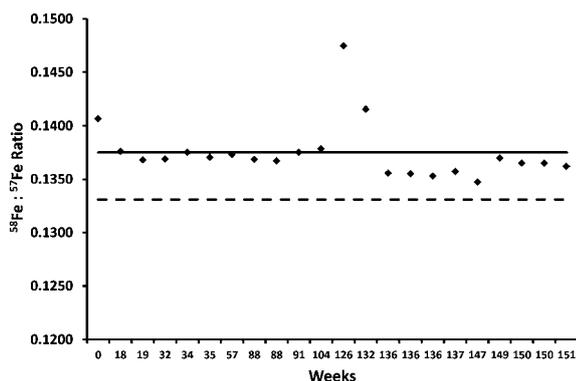
The analytical development described here was driven by need for (i) high throughput and (ii) fit-for-purpose analyses of blood samples from  $^{58}\text{Fe}$  tracer studies. Since sample pre-treatment is typically the bottleneck to analytical throughput, we performed the analysis on whole blood samples that had received no treatment other than dilution. The accurate measurement of isotopes by ICP-MS may be hindered by isobaric interferences, namely overlapping isotopes or polyatomic ion interferences that originate from the plasma gas or components of the sample matrix. In the case of  $^{57}\text{Fe}$  and  $^{58}\text{Fe}$  isotopes in whole blood there is the potential for interference from argon-based and calcium-based polyatomic ions (*e.g.*  $^{40}\text{Ar}^{16}\text{O}^1\text{H}^+$  and  $^{40}\text{Ca}^{16}\text{O}^1\text{H}^+$  for the measurement of  $^{57}\text{Fe}$  and  $^{40}\text{Ar}^{18}\text{O}^+$  and  $^{42}\text{Ca}^{16}\text{O}^+$  for the

measurement of  $^{58}\text{Fe}$ ) and an isobaric interference from  $^{58}\text{Ni}$ .<sup>15</sup> However, under dynamic reaction cell (DRC) conditions ( $\text{Ar}/\text{H}_2$  reaction gas flow =  $0.3\ \text{ml min}^{-1}$ ,  $\text{RPa} = 0$  and  $\text{RPq} = 0.45$ ) the argon based interferences can be attenuated. The isobaric interference by  $^{58}\text{Ni}$  was not corrected in the DRC analyses since this isotope is found at approximately 0.07 ppb in blood<sup>16</sup> whereas  $^{58}\text{Fe}$  is at 1400 ppb (500 ppm total Fe is a fairly typical value) and, thus,  $^{58}\text{Ni}$  represents a negligible 0.005% of the total 58  $m/z$ , that would affect both samples (d0 and d14) similarly. Furthermore, other than dilution, no sample pre-treatment steps were required (*e.g.* NaOH precipitation and ion-exchange separation), which are known to be main sources of Ni contamination.<sup>17</sup> Although whole blood is a rich source of calcium ( $80$  to  $90\ \text{mg l}^{-1}$ ), signal interference from calcium-based polyatomic ions was also negligible under the conditions described here: Fig. 1 shows that the  $m/z = 57$  and 58 signals from a sample diluent blank or a diluent blank spiked with  $85\ \text{mg l}^{-1}$  Ca are similarly negligible when compared to signal from a diluted whole blood sample. Thus, under the DRC conditions described, and on a fit-for-purpose basis, there is no significant argon-based polyatomic ion or calcium-based interference on either  $^{57}\text{Fe}$  or  $^{58}\text{Fe}$ .

In spite of controlling spectroscopic interferences, repeated isotope ratio analysis of an in-house, quality control, whole blood sample showed a consistent ratio bias in relation to isotopic natural abundance (see Fig. 2) presumably due to mass bias. If the bias were consistent then accurate determination of isotopic enrichment in whole blood for two related blood samples (*i.e.* one taken prior to supplementation [sample d0] and one taken fourteen days after [sample d14]) should still be possible. However, repeated analyses of the in-house, quality control, whole blood sample revealed a significant inter-batch variability (RSD 2.0%,  $N = 22$ ), presumably due to mass bias variability over time, which was in contrast to a relatively low intra-batch variability (RSD 0.12%,  $N = 10$ ). Thus, we subsequently analysed d0 and d14 samples in the same analytical batch to minimise this effect. Furthermore, since plasma conditions may vary over a batch, an analytical sequence was implemented such that sample pairs (d0 and d14 from the same subject) were run consecutively (Fig. 3). Therefore, the isotopic ratio



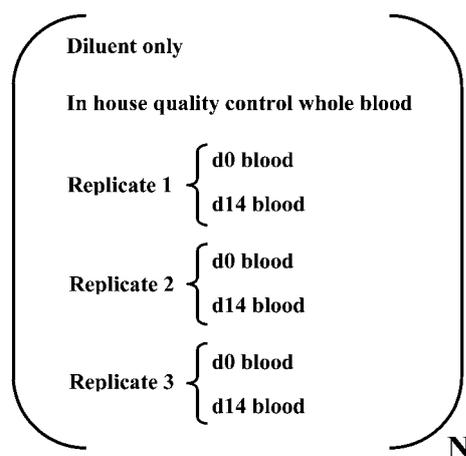
**Fig. 1** Mass counts obtained from sample diluent (black bars), sample diluent spiked with calcium (white bars) and diluted whole blood (grey bars). An 85 ppm Ca solution (subsequently diluted in the whole-blood diluent) was used, as this concentration represents whole blood levels.



**Fig. 2** Repeated analysis of an in-house, quality control, whole blood showed a systematic bias. The bold line represents the mean isotopic ratio and the dashed line represents the natural abundance ratio. Outliers (*e.g.* at weeks 126 and 132) appeared to be related to low instrumental sensitivity for those batches (data not shown).

determined in related d0 and d14 samples was expected to be mostly affected by similar plasma-related biases, which would then be cancelled in the calculation of  $^{58}\text{Fe}$  enrichment.

DRC-ICP-MS was then used for the analysis of whole blood samples from iron-deficient volunteers who were participating in an Fe absorption study and given  $^{58}\text{Fe}$  labelled supplements.<sup>18</sup> Repeated analysis of one sample pair (from one volunteer) over a period of two years in different analytical batches showed that inter-batch variability (3.4%;  $N = 5$  replicates) and intra-batch variability, in which the sample pairs were analysed repeatedly in a single batch (4.4%;  $N = 10$  replicates), were similar. These percentages refer to variability in the measurement of enrichment (*i.e.* the change in the ratio from baseline to day 14). Also, the limit of detection (*i.e.* minimum  $^{58}\text{Fe}$  increment that could be detected), was 0.4% enrichment, whereas the limit of quantification (*i.e.* minimum  $^{58}\text{Fe}$  increment that could be quantified) was 1% enrichment. Thus, this methodology allows the quantification down to 1%  $^{58}\text{Fe}$  enrichment with an associated relative variability of 3 to 4% (*i.e.* the absolute variability is 0.03 to 0.04% of the enrichment).



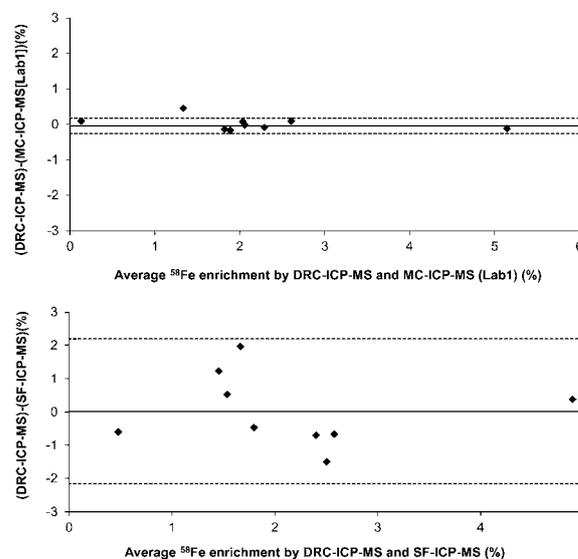
**Fig. 3** Sample analysis sequence implemented for DRC-ICP-MS analysis.  $N$  refers to number of iron deficient volunteers analysed per batch, typically  $N \geq 4$ .

Samples from several volunteers were also analysed by DRC-ICP-MS and compared to MC-ICP-MS (Lab1) and SF-ICP-MS results. Comparison of differences between the analytical methods using a Bland-Altman plot<sup>19</sup> shows that the DRC-ICP-MS results were in agreement with the MC-ICP-MS results (Fig. 4) and, importantly, there was no evidence of increased scatter at greater enrichments (*i.e.* higher  $^{58}\text{Fe}$  concentrations in d14 samples). Additionally, the average difference was low ( $-0.04\%$   $^{58}\text{Fe}$  enrichment) and the 95% confidence range of the bias was narrow ( $-0.26$  to  $0.18\%$   $^{58}\text{Fe}$  enrichment). Comparison to SF-ICP-MS (Fig. 4) also resulted in a low average difference between the two techniques ( $0.02\%$   $^{58}\text{Fe}$  enrichment) but the scattering of the data resulted in a high 95% confidence range for the bias between DRC-ICP-MS and SF-ICP-MS ( $-2.15$  to  $2.2\%$   $^{58}\text{Fe}$  enrichment), and this was about an order of magnitude greater than the range between DRC-ICP-MS and MC-ICP-MS results.

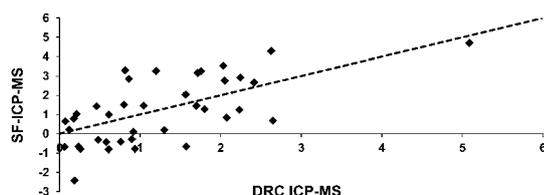
Further analysis of different samples confirmed the poor agreement and low coefficient of correlation between DRC-ICP-MS and SF-ICP-MS methodology ( $r^2 = 0.427$ ; Fig. 5). Moreover, the SF-ICP-MS methodology determined several implausible negative  $^{58}\text{Fe}$  enrichments (*i.e.* an apparent loss of  $^{58}\text{Fe}$  after the volunteers had been given an  $^{58}\text{Fe}$ -labelled iron supplement).

Further agreement was attained by comparison with a second MC-ICP-MS analytical laboratory (Lab 2) and reassured the validity of the original (Lab 1) MC-ICP-MS data (Table 2).

We recognise that for some iron absorption studies, assessing the absorption of two separate supplements or foods may be desirable. In this way, the absorption of iron from the test item, labelled for example with  $^{58}\text{Fe}$ , can be compared to that of a gold standard, such as ferrous sulphate, also labelled with isotopic iron. The test item and gold standard are ingested on separate occasions to prevent possible interactions with absorption. If they are ingested a day apart then clearly separate isotopes



**Fig. 4** Bland-Altman plot showing the differences against the means that were obtained for  $^{58}\text{Fe}$  enrichment as measured by DRC-ICP-MS vs. MC-ICP-MS (Lab 1) or vs. SF-ICP-MS (top and bottom graphs respectively). The solid lines represent the average difference between methods and the dashed lines correspond to the 95% confidence intervals.



**Fig. 5** Comparison between values obtained for  $^{58}\text{Fe}$  enrichment by DRC-ICP-MS and SF-ICP-MS ( $y = 1.083x - 0.1803$ ;  $r^2 = 0.4266$ ). The dashed line represents the unity line ( $x = y$ ). Much of the data in this figure have been previously reported in a conference proceedings.<sup>20</sup>

**Table 2** Summary of all Bland-Altman data for  $^{58}\text{Fe}$  enrichment determined by DRC-ICP-MS, SF-ICP-MS, MC-ICP-MS (Lab 1), MC-ICP-MS (Lab 2)

<b>SF-ICP-MS</b>	AD <sup>a</sup>	0.078%				
	UL <sup>b</sup>	2.6%				
	LL <sup>c</sup>	-2.4%				
	N <sup>d</sup>	38				
<b>MC-ICP-MS (Lab 1)</b>	AD	-0.041%	AD	0.244%		
	UL	0.18%	UL	2.1%		
	LL	-0.26%	LL	-1.7%		
	N	9	N	9		
<b>MC-ICP-MS (Lab 2)</b>	AD	-0.051%	AD	0.191%	AD	0.011%
	UL	0.32%	UL	2.0%	UL	0.33%
	LL	-0.42%	LL	-1.6%	LL	-0.30%
	N	6	N	6	N	6
	<b>DRC-ICP-MS</b>		<b>SF-ICP-MS</b>		<b>MC-ICP-MS (Lab 1)</b>	

<sup>a</sup> Average difference of the bias. <sup>b</sup> Upper limit of the 95% confidence interval. <sup>c</sup> Lower limit of the 95% confidence interval. <sup>d</sup> N = Number of samples compared between laboratories.

(i.e.  $^{58}\text{Fe}$  and  $^{57}\text{Fe}$ ) are required and thus further methodological developments for DRC-ICP-MS of iron isotopes in clinical samples could focus on  $^{57}\text{Fe}$  measurements of whole-blood. If however they are ingested several weeks apart then the same isotope (e.g.  $^{58}\text{Fe}$ ) could be used for both with the new isotope 'baseline' simply being assessed, and subsequently accounted for, for the second, supplement or food.

## Conclusions

The current results show that DRC-ICP-MS can be used to determine accurately the  $^{58}\text{Fe}$  enrichment of blood samples and

can detect a ratio-increase of endogenous  $^{58}\text{Fe}$  as low as 0.4% and with quantification at 1%. These increments represent, respectively, 1% and 3% oral iron absorption from an iron supplement containing 2 mg  $^{58}\text{Fe}$  as a label, in a typical volunteer (60 Kg female with 11.5 g/dL haemoglobin). Since a minimum 5% oral iron absorption is expected for any worthwhile supplemental or fortification strategy, DRC-ICP-MS is clearly adequate for the routine determination of iron absorption in human supplementation or fortification studies.

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