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High-precision Fe isotopic analysis of whole blood for biomedical purposes without prior isolation of the target element

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Abstract

Recently, it has been documented that Fe isotopic analysis of whole blood and serum by means of multi-collector ICP-mass spectrometry (MC-ICP-MS) is providing promising results in a biomedical context and thus, there is a demand for simple, fast and reliable methodologies, providing high sample throughput. In this work, the possibility of Fe isotopic analysis by MC-ICP-MS directly in acid-digested whole blood and thus, without prior Fe isolation was evaluated. The influence of the main mineral matrix elements and the effect of potentially remaining organic compounds were first systematically evaluated using synthetic solutions. The Fe isotopic composition was biased low in the presence of matrix elements such as Na and K, while it was biased high for glucose concentrations ≥ 1% (w/v). Nevertheless, after dilution of the whole blood sample digest to 0.75-1.5 mg L^{-1} of Fe, followed by adequate correction for instrumental mass discrimination using a combination of internal (with admixed Ni) and external correction, MC-ICP-MS isotope ratio measurements provided accurate and precise results. For actual samples, the Fe isotopic data obtained agree well with those using the reference procedure, based on prior chromatographic isolation of Fe out of acid-digested blood.

Keywords: Fe isotopic analysis, whole blood, MC-ICP-MS, biomedical applications

1. Introduction

Recently, several research groups have demonstrated that isotopic analysis of Fe in whole blood or serum might reveal potentially very useful clinical information. Van Heghe *et al.* have established a clear link between an individual's whole blood Fe isotopic composition and his/her Fe status.[1] Hotz *et al.* have identified the extent of mobilization of storage Fe (liver) and the dietary iron absorption efficiency as governing factors.[2,3] It was also demonstrated that patients suffering from hereditary hemochromatosis (HH)[1,4] or anemia of chronic disease (ACD)[1] show an altered Fe isotopic composition in whole blood compared to healthy subjects. The HH and ACD populations investigated by Van Heghe *et al.* show a difference in $^{56}Fe/^{54}Fe$ isotope ratio in whole blood of +0.5‰ and -0.3‰ compared to the reference population, respectively.[1,5–7] The number of patient samples was too low to assess gender-based differences. For tracing down and quantifying such small differences, a high isotope ratio precision is mandatory, making multi-collector ICP-mass spectrometry (MC-ICP-MS) the preferable technique. However, Fe isotope ratio data with MC-ICP-MS can be compromised by instrumental mass discrimination and spectral interference, mainly from $^{54}Cr^+$, $^{58}Ni^+$ and Ar- and Ca-based molecular ions.[8,9]

To overcome spectral overlap, medium or high mass resolution, potentially in combination with aerosol desolvation, can be used with MC-ICP-MS.[10–15] With the standard sample introduction system, relatively high concentrations of the target analyte, *i.e.* $\geq$ 1 mg L^{-1} are typically preferred to minimize the effect of spectral interference.[16]

Correction for instrumental mass discrimination can be accomplished *via* external correction in a sample-standard bracketing (SSB) approach, or a combination of such external correction with internal correction using Ni or Cu as an admixed internal standard. Both methods have been proven suitable for high-purity Fe solutions.[10,17] The double spike ^{57}Fe-^{58}Fe approach, combined with SSB, was also used, mainly in the context of environmental and geological studies.[18–20] However, it needs to be stressed that both the matrix composition and the target element concentration affect the extent of mass discrimination. Such effects can obscure the small, natural isotope ratio differences that need to be revealed.[21] The origin of mass discrimination is still poorly understood, although several contributions, such as a shift in the ionization equilibrium,

collisional scatter, energy consumption for matrix decomposition, space-charge effects and ambipolar diffusion, have been suggested.[9,22–27]

As a result, the sample preparation procedure preceding isotopic analysis of whole blood Fe is a critical issue. Typically, whole blood is first digested with HNO_3 or a mixture of HNO_3 and H_2O_2 to set the Fe free (predominantly from hemoglobin) in ionic form, followed by the chemical isolation of the target element from the concomitant matrix and residual organic components that potentially affect the extent of mass discrimination. Anion exchange chromatographic methods, based on the high affinity (and thus, partition coefficients) of Fe-chloride complexes towards an anion exchange resin, such as AG MP-1, are the most frequently used for the isolation of Fe from biological samples.[7,10,13,28] Since on-column fractionation effects occur due to the slightly different elution behavior of the different Fe-isotopes on the column, the recovery of Fe needs to be quantitative.[28] Chromatographic isolation of Fe is effective and provides better recoveries and a more effective target element / matrix separation than the more conventional Fe precipitation methodology. However, the chromatographic approach is labor-intensive and time-consuming, thus reducing the sample throughput. Relatively large amounts of highly pure acid (about 20 mL of concentrated HCl per sample) and additional evaporation / re-dissolution steps are required to make the final matrix suited for isotope ratio measurements with MC-ICP-MS.

Stenberg et al.[14] have compared different sample preparation procedures for the isotopic analysis of Fe in Seronorm whole blood reference material by MC-ICP-MS, including microwave-assisted acid digestion with and without Fe isolation *via* anion exchange chromatography and *via* selective Fe precipitation with different reagents. Although no differences in the Fe isotopic composition were found following the approaches mentioned, the authors recommended precipitation of Fe with NH_3 based on practical considerations, e.g., high sample throughput. Based on the high concentration of Fe in whole blood samples (~350 mg L^{-1}, healthy subjects) we decided to perform and evaluate the Fe isotopic analysis of whole blood by MC-ICP-MS without matrix separation, *i.e.* directly after acid digestion and dilution of the sample digest. Synthetic solutions were used to systematically investigate the effect of concomitant matrix components on the Fe isotope ratio results. The MC-ICP-MS instrument settings were optimized using an actual whole blood sample. Eleven whole blood samples were

analyzed directly (digestion & dilution) and after chromatographic Fe isolation, and the results obtained were compared to one another.

2. Experimental

2.1. Reagents and standards

Ultrapure water (resistivity > 18.2 MΩ cm) was obtained from a Milli-Q Element water purification system (Millipore, France). *Pro analysis* purity level nitric acid (14 M) was used after sub-boiling distillation. *Optima* grade hydrochloric acid (12 M) was obtained from Fisher Chemical (UK) and used as such. Ultrapure hydrogen peroxide 9.8 M was acquired from Sigma-Aldrich (Belgium). All sample manipulations were carried out in a class-10 clean laboratory.

The Fe isotopic reference material IRMM-014 from the Institute for Reference Materials and Measurements (IRMM, Belgium) was used for external correction in a sample-standard bracketing approach. A single-element Fe standard solution acquired from Inorganic Ventures (The Netherlands; lot D2-FE03110) was used as in-house isotopic standard solution (further referred to as A&MS-Fe) for preliminary experiments and for validating the isotope ratio measurements. Single-element standard stock solutions (1,000 mg L^{-1}) acquired from Inorganic Ventures were used for the preparation of synthetic solutions, for mass discrimination correction using an internal standard (Ni) and for quantification of the target element. Also glucose (Merck, Germany) was used for the preparation of synthetic solutions.

AG MP-1 strong anion exchange resin (100-200 mesh, chloride form) and polypropylene chromatographic columns (Bio-Rad, Belgium) were used for the Fe isolation procedure.

2.2 Instrumentation and measurements

A Thermo Scientific Neptune MC-ICP-MS instrument (Germany) was used for the Fe isotope ratio measurements. A PFA concentric nebulizer (100 μL min^{-1}) and double spray chamber, with a cyclonic and a Scott-type sub-unit, were used for the introduction of the solutions into the plasma. Measurements were performed in medium resolution,

in static collection mode and using six Faraday collectors connected to 10^{11} Ω amplifiers. Instrument settings and data acquisition parameters are shown in Table 1. Ni was used as an internal standard at the same concentration as the target element Fe. The Fe isotope ratios were calculated off-line after removal of outliers using a 2s-test. Mass bias correction was performed by means of i) external correction in a sample-standard bracketing approach (SSB) and ii) a combination of internal correction (with Ni) by means of the revised Russell's law (using a regression line to establish the correlation between the correction factors for Fe and Ni) and SSB.[29] The isotopic composition is expressed in delta notation (δ^{56}Fe, δ^{57}Fe, ‰), calculated against the isotopic reference material IRMM-014 following eqn.1, where x is 56 or 57.

$$\delta^{x}Fe_{sample} = \left(\frac{{}^{x}Fe/{}^{54}Fe_{sample}}{{}^{x}Fe/{}^{54}Fe_{IRMM-014}} - 1 \right) \times 1000 \qquad (1)$$

Elemental concentrations were determined using an Element XR single-collector sector field ICP-MS instrument (Thermo Scientific). For the introduction of solutions into the ICP, a combination of a 200 µL min^{-1} quartz concentric nebulizer and a cyclonic spray chamber was used. Ga was used as internal standard to correct for matrix effects and instrumentation instability.

A Shimadzu UVmini-1240 UV-Vis spectrophotometer was used to assess the presence of organic matter in the sample.

2.3. Samples and sample preparation

Whole blood samples of 11 individuals, provided by the Ghent University Hospital (Belgium), were analyzed. One of these samples (from a hemochromatosis patient and available in a large quantity) was used to optimize the analytical protocol and to evaluate its analytical performance. Synthetic samples were also analyzed to evaluate matrix effects. These were prepared using the A&MS-Fe standard and major, minor and trace elements (Cu, Zn, Mn, Mg, Ni, Ca, Br, P, S, Na and K), mixtures of these elements and/or glucose as an organic compound.

About 3 mL of blood was digested in a closed Teflon Savillex beaker using a mixture of 7 mL of HNO_3 and 1.5 mL of H_2O_2. The digestion was carried out at 110 ºC overnight (~16 h). The digest thus obtained was evaporated to dryness and re-dissolved in 2 mL of concentrated HNO_3. Then, two aliquots of 1 mL were separated for a second evaporation step. After that, one aliquot was re-dissolved in 0.42 M HNO_3 for direct Fe isotope ratio measurement and the other one was re-dissolved in 5 mL of 8 M HCl + 0.001% H_2O_2 for chromatographic Fe isolation.

Chromatographic isolation of Fe was carried out according to Van Heghe et al.[7] For this purpose, 5 mL of the sample aliquot were loaded onto the column containing 2 mL of previously cleaned AG MP-1 anion exchange resin. The matrix was eluted with 8 mL of 8 M HCl + 0.001% H_2O_2, followed by Cu, eluted with 12 mL of 5 M HCl + 0.001% H_2O_2. Finally, Fe was eluted with 10 mL of 0.6 M HCl. The purified Fe solution was evaporated to dryness at 95 ºC twice to remove residual chloride. The final residue was re-dissolved in 0.42 M HNO_3 for the isotope ratio measurements. Procedural blanks, treated in the same way as the samples, were also included.

Ethical approval was obtained for using these samples by an independent commission connected to the Ghent University Hospital. Patients and individuals forming the reference population signed an informed consent.

3. Results and discussion

3.1. Evaluation of the effect of concomitants using synthetic solutions

Fe isotope ratio measurements *via* MC-ICP-MS are potentially adversely affected by the presence of matrix components, as these might affect the ion transmission efficiencies of the target nuclides.[9,16] Even after chemical isolation, residual matrix may alter the extent of mass bias.[11] To evaluate the effect of blood matrix components, synthetic solutions containing the A&MS-Fe standard (500 μg L^{-1}), individual matrix elements (Cu, Zn, Mn, Mg, Ni, Ca, Br, P, S, Na and K) and combinations of these were used. The average Fe isotopic composition of the pure A&MS-Fe standard obtained during a period of 1 year is: 0.46 ± 0.07 ‰ for δ^{56}Fe and 0.69 ± 0.10 ‰ for δ^{57}Fe (n=25). The

δ^{56}Fe values in the synthetic solutions after correction for mass discrimination using the combined internal & external correction approach are presented in Fig. 1. As can be seen, among the elements studied, Na and K showed the highest influence on the Fe isotope ratio results. The presence of these easily ionizable elements alters the plasma conditions, *i.e.* temperature and electron density, which might explain the effect on the extent of mass discrimination. Shifts of -0.11, -0.17 and -0.13 ‰ were observed in the presence of 1 mg L^{-1} of Na, of K and of a mixture of Na, K, S, P and Br (at 1 mg L^{-1} each), respectively. Levels higher than 2 mg L^{-1} of S (characterized by a high ionization energy and thus, low ionization efficiency) also led to significantly deviating δFe values, but in this case towards higher values.

In contrast, the presence of the other elements studied, whether assessed individually or together, did not generate any deviation of the δ^{56}Fe value outside of the 0.07‰ experimental uncertainty. It had also been previously pointed out that matrix elements should not affect the accuracy of Fe isotope ratio data for an element/Fe ratio < 2.[10,30] However, a deterioration in the precision was reported for increasing concentrations of the matrix elements due to instability of the instrumental mass bias.[30] This effect can be alleviated *via* correction for mass discrimination using the combined approach of internal & external correction for mass discrimination.

A synthetic solution simulating the mineral composition of whole blood after the acid digestion was prepared by addition of the following components to A&MS-Fe standard solution containing 500 µg L^{-1} of Fe: 2 mg L^{-1} of Na, 1.5 mg L^{-1} of K and S, 200 µg L^{-1} of P and 20 µg L^{-1} of Cu, Zn, Br, Mg, Mn, Ca and Ni. For this mixture, the δ^{56}Fe value was 0.12 ± 0.06 ‰ after SSB correction and 0.53 ± 0.04 ‰ after the combined correction approach with ^{62}Ni/^{60}Ni and SSB (n=4). This synthetic solution was also admixed with different amounts of glucose to assess the effect of potentially remaining organic components. The δ^{56}Fe values obtained for the synthetic blood solutions with different amounts of glucose are shown in Fig. 2. An increasing concentration in organic C concentration led to an increasing bias in the δFe values. The influence of organic substance shifted the isotope ratio results in the opposite direction than did Na and K, *i.e.* the δ^{56}Fe value was increased by 0.15‰ after the SSB correction, and by 0.08‰ after the combined internal & SSB correction in the presence of 1% (w/v) of

glucose. The experimental δ^{56}Fe value for the synthetic solution containing all expected matrix components and 1 % (w/v) of glucose was 0.31‰. This value corresponds well with that expected based on the individual extents of bias observed, *i.e.* 0.27‰ (compensation of effects).

3.2. Optimization of experimental conditions using actual whole blood

Fe isotope ratio measurements were performed in a whole blood sample (obtained from a hemochromatosis patient and available in a large quantity) after acid digestion. Instrument settings, such as gas flow rates, sampling depth and RF power, affect the properties of the plasma, especially the temperature, and consequently, the vaporization rate and atomization and ionization efficiencies.[31–33] Thus, these settings were optimized using the acid-digested whole blood (Table 1).

Different sample dilutions were performed. The results obtained after mass discrimination correction using the combination of internal correction & SSB are shown in Table 2. The isotopic composition of this sample, obtained following the reference procedure, *i.e* after sample digestion and chromatographic Fe isolation (n=6), is also included in this table. As can be seen, for a concentration range between 0.75 and 1.5 mg L^{-1} of Fe, accurate and precise results were obtained after correction for instrumental mass discrimination using the combined correction approach. For higher dilution, the results deviated by about -0.35 ‰ and -0.14 ‰ from the reference values when using SSB alone and the combined correction approach, respectively. This deviation can be tentatively explained by vaporization fractionation and analyte losses from the zone of efficient ion sampling from the ICP by diffusion.[16] Thus, 1 mg L^{-1} of Fe was selected as the optimum concentration for isotopic analysis in acid-digested whole blood. As the concentration of the internal standard Ni used for the mass discrimination correction is also of importance, especially when matrix components are present, the effect of the Fe/Ni ratio was evaluated. A 1:1 ratio provided the most precise results and thus, 1 mg L^{-1} of Ni was further used for the mass discrimination correction.

The organic matter present in the measurement solutions, as estimated using the UV absorption at 254 nm, was a factor of 30 higher in the digested sample than in the chromatographically purified Fe fractions. After SSB correction, the delta values

deviated from the 'true' value, but correction with the combined approach provides accurate results up to a level of 1% of organic C.

Solutions of isotopic reference material IRMM-014, used as external standard (external correction for mass discrimination) and as wash solution used in-between two measurements were also matrix-matched *via* addition of matrix elements (simulated blood concentrations) to minimize changes in the ICP conditions. Every sample was measured 10 times. Although a slight improvement was obtained using matrix-matched IRMM-014 when relying on SSB correction only, the results still deviated from the reference value by about 0.5‰. However, with the combined mass discrimination correction, accurate and precise results were obtained using either matrix-matched or non-matrix-matched IRMM-014 standard. For reasons of simplicity, the non-matrix-matched IRMM-014 standard was used in all further work for SSB correction.

3.3 Application to human blood samples

To proof the concept of the study, 10 samples of human whole blood were analyzed with and without Fe analyte isolation. The δ^{56}Fe values obtained for the digested samples and for the purified Fe fractions are illustrated in Fig. 3. The samples were measured in duplicate. All sample results fall on the normal mass-dependent fractionation line, *i.e.* $\delta^{57}Fe = 1.47\ \delta^{56}Fe$. No significant differences at a 95% confidence level were observed between the results obtained using both methods. In all cases, the experimental $|t|$ values were < the critical value.

The precision was evaluated as repeatability (internal and external precision) and reproducibility. The internal precision, expressed as the standard deviation within one measurement (45 subsequent cycles), was 0.04 ‰ for δ^{56}Fe and 0.07 ‰ for δ^{57}Fe. The external precision, expressed as standard deviation for 10 measurements of the whole blood sample measured in one measurement session (one day), was 0.02 ‰ for δ^{56}Fe and 0.03 ‰ for δ^{57}Fe. This is similar to the external precision obtained for the samples after Fe isolation (0.02 ‰ for δ^{56}Fe and 0.04 ‰ for δ^{57}Fe).

The reproducibility obtained, as standard deviation of the sample measured 8 times on different days within 4 months, was 0.03 ‰ for δ^{56}Fe and 0.06 ‰ for δ^{57}Fe. In addition, no decrease in the sensitivity was observed after 3 measurement sessions (measurement days) in a row and the condition of the cones was still adequate. Thus, accurate and precise results were obtained for the Fe isotopic composition in whole blood after acid digestion followed by the adequate dilution of the sample under the conditions described and provided that instrumental mass discrimination was corrected for using a combination of internal correction *via* the revised Russell's law and SSB.

4. Conclusions

It was shown that direct isotopic analysis of whole blood Fe is possible after acid digestion and adequate dilution (to 0.75 and 1.5 mg L^{-1} of Fe) provided that instrumental mass discrimination is corrected for using a combination of internal correction using ^{62}Ni/^{60}Ni and external correction *versus* IRMM-Fe isotopic reference material in a standard-sample bracketing approach. As a result, chromatographic Fe isolation preceding the MC-ICP-MS Fe isotope ratio measurement can be avoided. Similar results were obtained following this approach and following the reference procedure (including chromatographic Fe isolation) for actual whole blood samples. This simple methodology allows to increase the sample throughput and reduce the amount of acids, cost and time required. Further routine use of the method without Fe isolation will have to reveal long-term robustness and the burden on the instrument. At least, the suggested approach can be relevant for specific clinical applications that require a prompt response.

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Figure captions

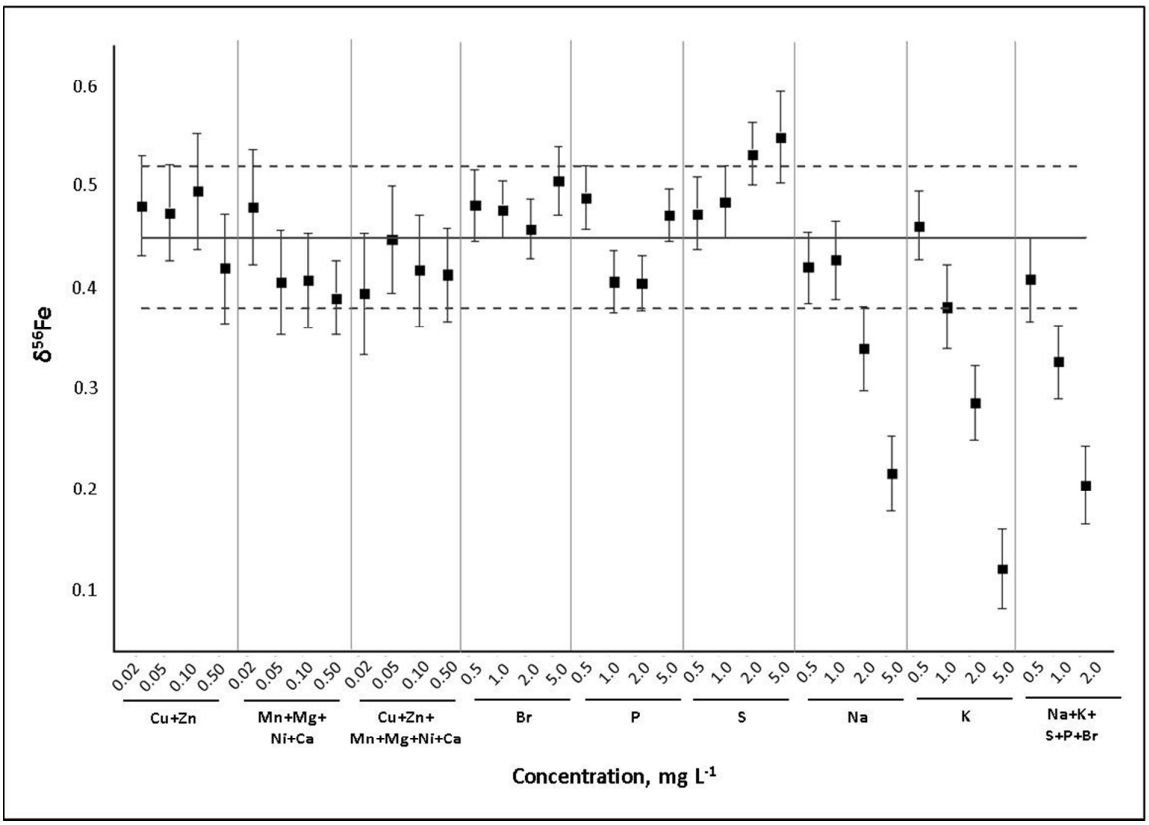


Figure 1. Influence of concomitant elements present in whole blood on the δ^{56}Fe measurement results. The solid line indicates the average value and the dashed lines 2 times the standard deviation for the A&MS-Fe in-house standard. Error bars indicate the standard deviation for 3 measurements.

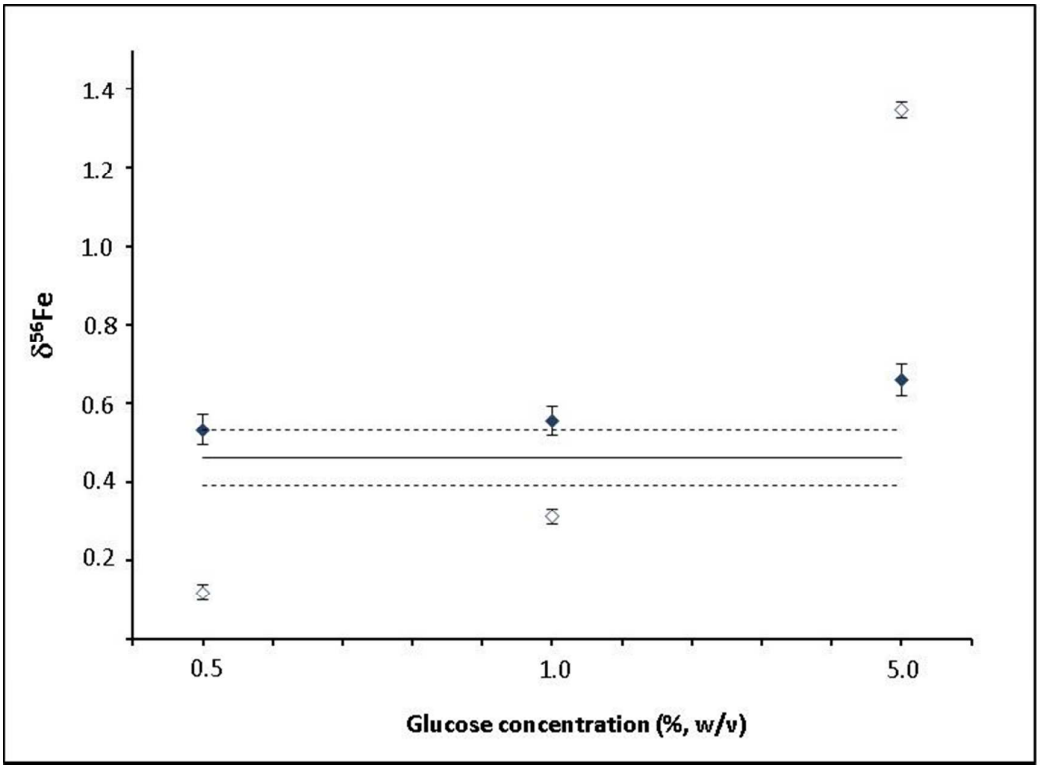


Figure 2. Influence of the glucose content on the δ^{56}Fe measurement results for a synthetic solution also containing the whole blood matrix elements. The solid line indicates the average value and the dashed lines 2 times the standard deviation for the A&MS-Fe standard. Filled symbols indicate the data obtained after SSB correction and open symbols after the combined (internal & SSB) correction. Error bars indicate the standard deviation of 2 measurements.

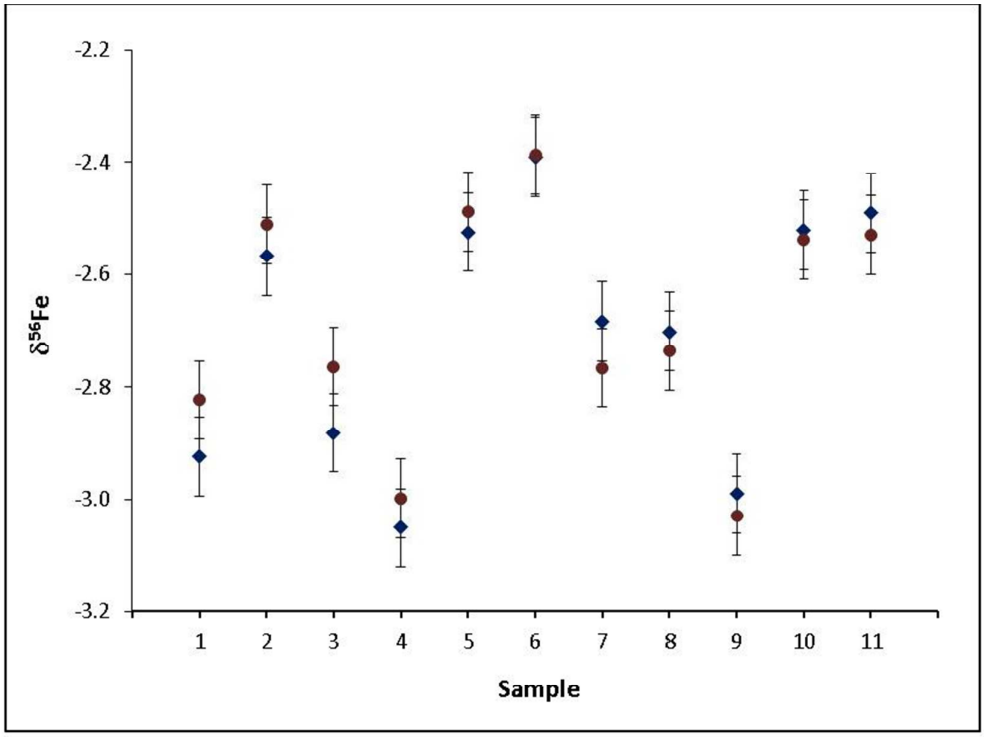


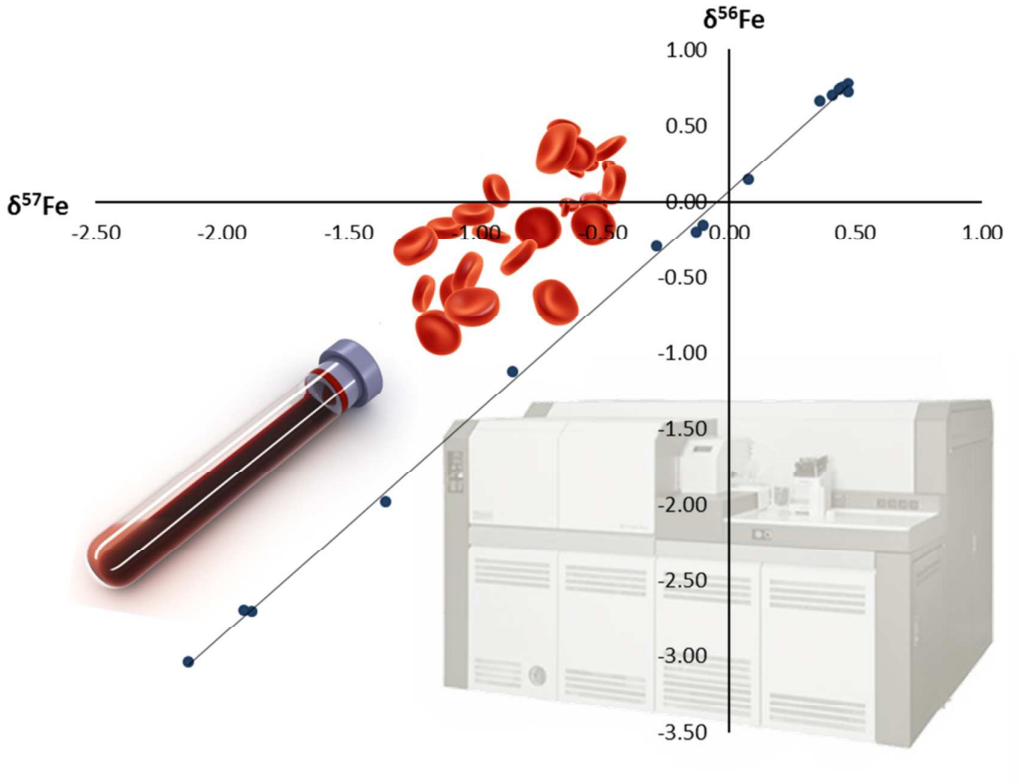
Figure 3. Delta Fe results for actual whole blood samples with and without Fe isolation. Red circles indicate the results obtained after Fe isolation and blue diamonds those without Fe isolation. Error bars represent the external precision as 2 times the standard deviation (0.07‰ for δ^{56}Fe ~~and 0.10‰ for δ^{57}Fe~~).

Table 1. Instrument settings and data acquisition parameters for the Neptune MC-ICP-MS.

RF power (W)	1275
Guard electrode	Connected
Sampling cone and skimmer	Ni standard sampling cone 1.1 mm aperture diameter NI H-type skimmer, 0.8 mm orifice diameter
Lens settings	Optimized for maximum signal intensity
Ar flow-rates (L min^{-1})	
plasma gas	15
auxiliary gas	0.75
nebulizer gas	0.9-1.0
Sample uptake rate (μL min^{-1})	100
Resolution mode	Medium
Acquisition mode	Static; multi-collection
Number of blocks	9
Number of cycles	5
Integration time (s)	4.194
Cup configuration	L4: ^{54}Fe; L2: ^{56}Fe; L1: ^{57}Fe; C: ^{58}(Fe+Ni); H1: ^{60}Ni; H3: ^{62}Ni

Table 2. Isotopic analysis of Fe in whole blood without Fe isolation at different dilution factors.

[Fe], mg L^{-1}	SSB		SSB and exponential law	
	$\delta^{56}Fe$	$\delta^{57}Fe$	$\delta^{56}Fe$	$\delta^{57}Fe$
0.30	-2.83±0.08	-4.12±0.11	-2.62±0.06	-3.78±0.10
0.50	-2.85±0.04	-4.17±0.07	-2.64±0.04	-3.87±0.07
0.75	-2.96±0.14	-4.29±0.18	-2.47±0.03	-3.57±0.06
1.00	-3.28±0.06	-4.86±0.21	-2.45±0.03	-3.59±0.05
1.50	-3.35±0.27	-4.93±0.39	-2.44±0.02	-3.58±0.04
Reference value	*-2.50±0.05*	*-3.71±0.09*	*-2.49±0.05*	*-3.72±0.11*



Accurate and precise isotopic analysis of Fe in whole blood using multi-collector mass spectrometry is possible after acid digestion and dilution, provided that mass discrimination is corrected for using a combination of internal and external correction.