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Isotopic Analysis of Fe in Human Red Blood Cells by Multiple Collector–ICP-Mass Spectrometry

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Precise $^{56}Fe/^{54}Fe$ and $^{57}Fe/^{54}Fe$ isotopic ratios on human red blood cell (RBC) samples have been measured using multiple collector–ICP-mass spectrometry (MC-ICPMS). The mass spectrometric interferences on Fe isotopes (*e.g.*, $^{56}ArO^+$ and $^{57}ArOH^+$) were successfully minimized by a dry plasma condition achieved by a desolvating nebulizer sample-introduction technique. In order to eliminate possible variations in the measured isotopic ratios due to non-mass spectrometric interferences, Fe was separated from remaining organic compounds and major co-existing elements using an ion chromatographic technique. The resulting precisions of the $^{56}Fe/^{54}Fe$ and $^{57}Fe/^{54}Fe$ ratio measurements were 0.12‰ and 0.20‰, respectively, which were high enough to detect the isotopic variation of Fe in nature. For an inter-laboratory comparison, all of the Fe isotopic ratio data were normalized by the ratios for the IRMM-014 international isotopic standard. A series of 12 RBC samples were collected from one person through monthly-based sampling over a period of one year. These were analyzed to test possible seasonal changes in the $^{56}Fe/^{54}Fe$ and $^{57}Fe/^{54}Fe$ ratios. Moreover, in order to test possible variations in the $^{56}Fe/^{54}Fe$ and $^{57}Fe/^{54}Fe$ ratios among different people, RBC samples were collected from five volunteers (four males and one female). The $^{56}Fe/^{54}Fe$ and $^{57}Fe/^{54}Fe$ ratios for a series of 12 RBC samples collected over a one-year period show 3.06‰ and 4.51‰ lower than the values of IRMM-014, and no significant seasonal change could be found in the ratios. The lack in seasonal changes in the Fe isotopic ratios could be explained by a small contribution of the daily net-intake of Fe (1 – 2 mg/day) onto the total amount of Fe in the human body (2 – 4 g). The $^{56}Fe/^{54}Fe$ and $^{57}Fe/^{54}Fe$ ratios for RBC samples collected from four male samples did not vary measurably, whereas the Fe isotopic ratios for a female RBC were 0.3‰/amu heavier than the mean value of four male samples. This difference in Fe isotopes among the individuals can be the result of a difference in uptake efficiency of the Fe through a dietary process from the digestive tract. The data obtained here demonstrate that the isotopic ratios of trace metals can provide new information about metabolic efficiencies of the metallic elements.

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Introduction

The isotopic composition of elements can be fractionated through various chemical and physical reactions. Light elements, such as H, C, N, O or S, have been widely used as "stable isotope elements" because of their large isotopic variations in nature. This is mainly due to the large mass difference between the isotopes. The isotopic data have contributed significantly to the solutions of many research problems in various fields, including biology, Earth and planetary sciences and environmental science.[1] In order to extend the versatility of the stable isotope technique, many attempts have been made to obtain precise and accurate isotopic data for heavier elements, such as Fe, Cu or Zn. However, sensitive and precise isotopic ratio measurements of these elements have been difficult due to analytical problems arising from low ionization efficiency, which is mainly due to their high ionization potentials. Recently, sensitive and precise isotopic ratio measurements by a multiple collector–ICP-mass spectrometer (MC-ICPMS) has been described.[2,3] A multiple-collector system enables all isotopic signals to be detected simultaneously, thus eliminating the deterioration of analytical precision due to unstable signal intensity. Pioneering studies have revealed that natural stable isotope fractionations of many elements heavier than S (*e.g.*, Fe, Cu, Zn, Ge or Mo) are common in various geochemical samples.[3-10] Beard *et al.*[5] first demonstrated that the Fe isotopic composition of ferrous Fe produced by Fe-reducing bacteria was 0.7‰ per mass unit [‰/amu] lighter than that of ferrihydrite substrate; they concluded that natural isotopic fractionations could be due to biological effects.

Iron is an essential element to the human body for oxygen transport (as hemoglobin), oxygen storage (as myoglobin), and other physiological functions. Labeled Fe isotopes have been widely used to understand the bioavailability of Fe in human nutrition.[11] However, neither the distribution of Fe isotopic ratios nor the isotopic fractionation of Fe through biological processes has been well studied. Recently, variations in Fe

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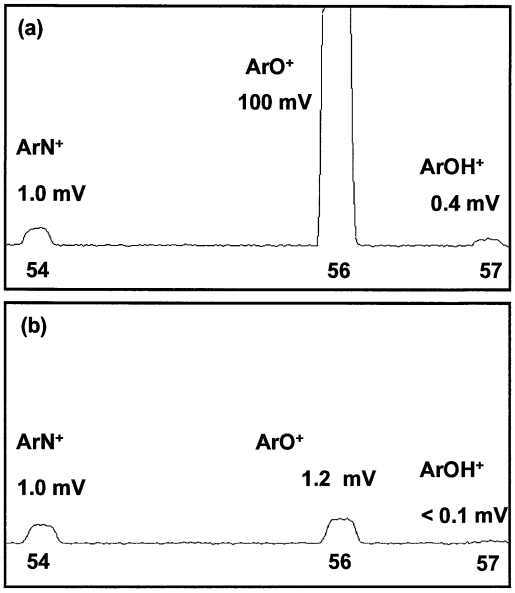

Fig. 1 Mass spectrum of the mass region (53.5 – 57.5 u) obtained from (a) a wet plasma condition achieved by conventional nebulization of deionized water, and (b) dry plasma condition achieved by the desolvating nebulizer system.

isotopes for human blood were reported by Walczyk and Blanckenburg.[9] They demonstrated that human blood samples had low Fe isotopic ratios (–1.0 to –1.6‰/amu) relative to a reference material produced from a natural ore sample (IRMM-014). Zhu *et al*.[10] and Stenberg *et al*.[12] also reported a natural stable isotopic fractionation of Fe in blood. This pioneering work revealed that the Fe isotopic composition of blood is different among individuals, and that an isotopic variation could be attributed to a difference in the uptake, or metabolic efficiencies. In this study, in order to test a possible seasonal change in the Fe isotopic ratios, a series of red blood cell (RBC) samples was collected every month from one person over a period of one year. The Fe isotopes for five RBC samples collected from five volunteers were also analyzed to investigate any potential difference among the individuals.

Experimental

Instrumentation

The MC-ICPMS instrument used in this study was a Nu Plasma 500 (Nu Instruments, Wrexham, UK). The operation conditions, such as the torch position, Ar gas-flow rates and lens settings, were adjusted so as to maximize the signal intensity of ^{56}Fe. A multiple collector array of Faraday cups allows for the simultaneous detection of Fe isotopic signals, thus providing better precision and reliable isotopic ratio data. Faraday collectors were used to measure the following isotopes: ^{53}Cr, ^{54}Fe + ^{54}ArN + ^{54}Cr, ^{56}Fe + ^{56}ArO and ^{57}Fe + ^{57}ArOH. ^{54}Cr was monitored at mass 53 for a possible isobaric interference on ^{54}Fe. However, no correction at mass 54 was required because of low Cr intensity in this study. Faraday cups and electrometers with 10^{11} ohm feedback registers were used to measure each isotope. No correction for the collection efficiency was made in this study. Details of the instrument and the operating parameters are summarized in Table 1.

Table 1 Details of the instrument and the operation parameters

1) MC-ICPMS instrument	
Nu instruments	Nu plasma
2) ICP ion source	
ICP	27.12 MHz
Power	1.35 kW Forward, < 5 W Ref.
Argon gas flow rates	
Cooling	13 l/min
Auxiliary	0.7 l/min
Nebulizer	0.9 l/min
3) Mass spectrometer	
Ion energy	4000 V
Extraction	2400 V
Analysis mode	Static
Ion detection	Analogue by Faraday
Typical transmission	3 V/μg g^{-1}
Integration time	10 s
Scan settled time	5 s
Number of cycles	60 cycles/run
Total analysis time	600 s/run
4) Desolvating nebulizer system	
Sweep gas	3 l/min
Spray chamber temp.	70˚C
Desolvator temp.	160˚C
5) Blank subtraction	
Blank mode	On-peak baseline measurement
Integration time	10 s
Number of cycles	60 cycles/run
Total analysis time	600 s/run
6) Normalizaion	
Mass bias correcion	Sample-standard bracketing method

Desolvating nebulizer system

The introduction of a solvent (H_2O) into the plasma ion source results in the production of argide ions, such as ArN^+, ArO^+ or $ArOH^+$. In order to improve the precision and accuracy of the Fe isotopic ratio measurements, these interfering signals must be reduced. It is widely recognized that because these polyatomic signals can be suppressed by minimizing the water load into the ICP, the dry plasma condition achieved by laser ablation sample introduction,[13] or desolvating nebulizer techniques,[2] has been generally used for the isotopic analysis of Fe. We firstly adopted a laser ablation technique for the isotopic analysis of Fe for geochemical samples. Although *in-situ* Fe isotopic data can be derived from solid samples, the precision of the ^{57}Fe/^{56}Fe ratio measurement was 0.2% (2SD), which was not high enough to detect a small variation in the Fe isotopic ratios in nature. In this study, a desolvating nebulizer system (Aridus, Cetac, Omaha, USA) was used to minimize the water load into the plasma. The solvent H_2O could be effectively removed by a PFTE membrane filter at 160˚C, and the resulting signal intensities of ArO^+ and $ArOH^+$ signals were 1.2 mV and < 0.1 mV, respectively, which were 1/80 and < 1/4 of the levels of the conventional solution nebulization technique (Fig. 1). The operation parameters of the Aridus are also summarized in Table 1. In the case of the Aridus desolvating nebulizer, a small amount of N_2 is normally added to the carrier gas. However, N_2 gas was not added in this study because its addition resulted in the production of an ArN^+ signal that interferes with the $^{54}Fe^+$ isotope. Since there was no significant difference in the signal intensity of ArN^+ (~1 mV) between conventional solution nebulization and the Aridus desolvating nebulizer, indicating that the source of nitrogen atoms could be

originated from the engulfed air at the ICP torch.[2,13] Although the polyatomic ions were not completely removed, the contribution of these mass spectrometric interferences could be minimized by an "on-peak" baseline-subtraction technique. The signal intensities of ArN^+, ArO^+ and $ArOH^+$ were monitored before and after measurement of the Fe signals, and the blank subtracted signal intensity was used to calculate the Fe isotopic ratios. The resulting contribution of these argide ions were < 0.2‰ for ^{56}Fe and ^{57}Fe, and < 0.5‰ for ^{54}Fe.

Samples and sample decomposition

About 10 ml of heparinized blood samples was collected according to the procedures of the ethical code at Juntendo University. RBC samples were prepared by removing blood plasma and white blood cells. One milliliter of RBC sample was mixed with 4 ml of conc. HNO_3, 0.5 ml of conc. H_2O_2 and 0.5 ml of conc. $HClO_4$. These were then heated up to 120 – 160˚C for 20 min by a microwave oven (MLS-1200 MEGA, Milestone, Bergamo, Italy).[14] The dissolved sample solution was then subjected to the chemical separation procedure described below. Most of the organic components were decomposed at this stage.

Chemical separation

In the MC-ICPMS measurement, in order to obtain precise and accurate isotopic data, a correction of the mass-discrimination effect is strongly recommended. The correction factors for the mass-discrimination effect observed in MC-ICPMS are typically larger than those observed in conventional thermal ionization mass spectrometry (TIMS).[15] It is widely believed that the space-charge effect in the plasma or vacuum interface region could be the main source of the mass discrimination effect.[16] The space-charge effect results in a preferential transmission of the heavier ions. A typical mass-discrimination effect for Fe observed in MC-ICPMS is 3.5%/amu, but this is strongly dependent upon the levels of the co-existing components. In order to minimize possible erroneous measurements due to matrix effects, the analyte Fe must be chemically separated from major elements and residual organic components. In this study, anion-exchange chromatography using AG-MP1 (Bio-Rad Lab., Hercules, USA) was adopted.[3,17] The decomposed sample solutions were heated to dryness on a hot plate. The resulting cake was redissolved in 1 ml of 6 M HCl, and then loaded onto an AG-MP1 anion exchange resin column (1.6 ml). After removing major elements (*e.g.*, Ca, Mg, or Na) and residual organic matrices by 30 ml of 6 M HCl, Fe was eluted and collected by 10 ml of 2 M HCl. The recovery of Fe through this procedure was > 96%. No significant isotopic fractionation through the present chemical separation procedures could be found (< 0.1‰/amu).

Chemical blank

All of the acid used in this study was of analytical grade or higher. Nitric acid, hydrogen peroxide and perchloric acid were TAMA SuperPure AA-10 (TAMA Chemicals, Kawasaki, Japan). Hydrochloric acid was of electric (EL) grade (Mitsubishi Chemicals, Tokyo, Japan). Deionized water was prepared by a Milli-Q SP ICP-MS Spec. system (Millipore, Billerica, USA). The level of Fe from the hypodermic needle and tube was 20 ng. Analytical Fe blanks from the PTFE apparatus and ion chromatograph column were 70 ng and 190 ng, respectively. The total blank from the present chemical procedures was < 300 ng for Fe. Since the typical amounts of Fe collected from 1 ml RBC samples were approximately 1 mg, the contributions of the chemical blank (0.03%) were negligible.

Table 2 Repeatability of $^{56}Fe/^{54}Fe$ and $^{57}Fe/^{54}Fe$ measurements JMC Fe relative to IRMM-014

Date	δ^{56}Fe, ‰	δ^{57}Fe, ‰
Run 1	0.39	0.59
Run 2	0.31	0.46
Run 3	0.31	0.41
Run 4	0.42	0.50
Run 5	0.48	0.55
Run 6	0.43	0.60
Run 7	0.34	0.65
Run 8	0.42	0.57
Run 9	0.43	0.39
Run 10	0.50	0.67
Run 11	0.43	0.38
Run 12	0.43	0.55
Mean (2σ)	0.41 ± 0.12	0.53 ± 0.20

Delta values with respect to IRMM-014.

Normalization and uncertainty of the measurement

The mass-discrimination effect was corrected by a sample-standard bracketing technique. In this study, IRMM-014 (Institute for Reference Materials and Measurements, Belgium) was used as a reference standard, and the $^{56}Fe/^{54}Fe$ and $^{57}Fe/^{54}Fe$ ratios of samples were expressed as a relative deviation from the same ratios for IRMM-014:

$$\delta^{56}Fe = [(^{56}Fe/^{54}Fe)_{sample}/(^{56}Fe/^{54}Fe)_{IRMM\text{-}014} - 1] \times 1000, \quad (1)$$

$$\delta^{57}Fe = [(^{57}Fe/^{54}Fe)_{sample}/(^{57}Fe/^{54}Fe)_{IRMM\text{-}014} - 1] \times 1000. \quad (2)$$

The external precision (reproducibility) of the isotopic ratio measurements was evaluated by the repeated analysis of an Fe solution prepared by the dissolution of a high-purity Fe reagent (Johnson-Matthey Chemicals) over a period of 5 months (Table 2). The resulting δ^{56}Fe and δ^{57}Fe values for the JMC Fe solution were 0.41 ± 0.12‰ (2σ) and 0.53± 0.20‰ (2σ), respectively. We have taken this value as the typical uncertainty of the Fe isotopic ratio measurement.

Results and Discussion

Matrix effect

As previously mentioned, the typical mass-discrimination effect on Fe was 3.5%/amu, the correction of mass bias factor being very important in obtaining precise and accurate Fe isotopic ratio data. More importantly, the level of the mass-discrimination effect is strongly dependent upon the concentrations of co-existing elements (*i.e.*, matrix effect). Therefore, great care must be taken concerning the concentrations of co-existing components for precise Fe isotopic analysis using the standard-sample bracketing technique. In fact, the measured $^{56}Fe/^{54}Fe$ and $^{57}Fe/^{54}Fe$ ratios varied significantly from the true ratio when the amount of co-existing elements or organic components were higher than ~500 μg/g level. To test the effect of the matrix effect on the mass-discrimination effect, we measured the Fe isotopic ratios for two different analytical solutions prepared by different chemical separation procedures. The first sample was prepared by a single-step solvent-extraction technique. The decomposed sample was dissolved in 10 ml of 6 M HCl, and agitated for 1

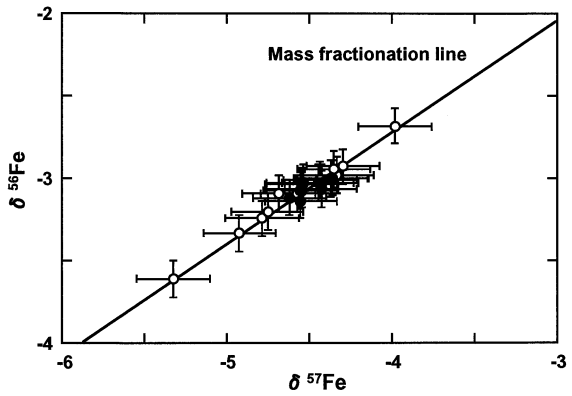

Fig. 2 Presence of co-existing elements or organic compounds, which causes changes in the degree of the mass-discrimination effect. The open symbol expresses an Fe analytical solution prepared by a simple solvent-extraction technique; the solid symbol represents an Fe solution prepared by the present separation technique using anion-exchange chromatography.

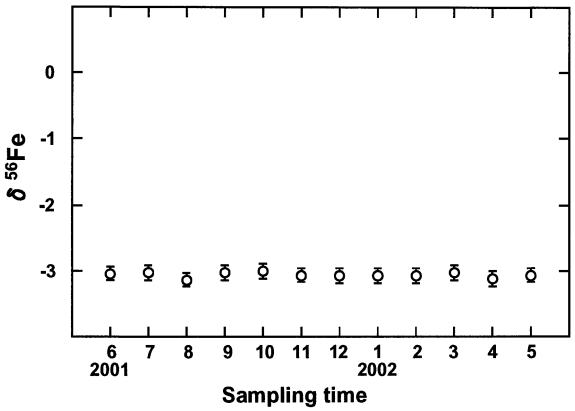

Fig. 3 δ^{56}Fe data for a series of 12 RBC samples collected from one person.

min with 10 ml of 4-methyl-2-pentanone (MIBK). The Fe was back-extracted to *ca*. 1 M HCl. The resulting solution was brownish-colored, indicative of the presence of a high concentration of matrix elements or organic components. The second analytical solution was prepared by the present ion-chromatography separation, as explained above. The resulting analytical solution had a clear-color, suggesting the lower contents of the co-existing elements and organic components. Figure 2 illustrates the resulting Fe isotopic data plotted on the δ^{56}Fe and δ^{57}Fe three-isotopes diagram. The Fe isotopic data obtained for the solution prepared by the simple solvent-extraction technique varied measurably (> 1‰), indicating that the degree of the mass-discrimination effect changed significantly due to the matrix effect. This is shown by the open symbol. The solid symbol denotes the Fe isotopic data obtained from the analytical solution prepared by anion-exchange chromatography. The overall ranges in resulting Fe isotopic ratios data were about 0.2‰, clearly demonstrating that the precision of the measurement could be improved remarkably by the present chemical-separation procedure using ion chromatography. Thus, for a better reproducibility in Fe isotopic ratio measurements, the ion-chromatography technique was used throughout this study.

Table 3 Fe isotopic composition of RBC sample (ID_No. 1) over a period of one year

Sample	δ^{56}Fe, ‰	δ^{57}Fe, ‰
June 2001	–3.04	–4.55
July 2001	–3.03	–4.45
August 2001	–3.13	–4.55
September 2001	–3.03	–4.42
October 2001	–3.00	–4.37
November 2001	–3.07	–4.55
December 2001	–3.07	–4.56
January 2002	–3.07	–4.43
February 2002	–3.07	–4.54
March 2002	–3.02	–4.54
April 2002	–3.12	–4.62
May 2002	–3.06	–4.54

Delta values with respect to IRMM-014. The analytical uncertainty is ± 0.12‰ and 0.20‰.

Seasonal change in Fe isotopes

In order to investigate the possible seasonal change in Fe isotopic ratios, a series of 12 RBC samples collected from one person through monthly-based sampling over a one-year period was used for the analysis. The resulting Fe isotopic data obtained for all 12 samples is summarized in Table 3 and plotted against the sampling month (Fig. 3). There were no significant changes in the resulting δ^{56}Fe and δ^{57}Fe values, indicative of no seasonal change in the Fe isotopic composition of RBC in the human body over a period of one year. The lack in seasonal variations in the Fe isotopic ratios can be due to a small contribution of net-dietary absorption of Fe (1 – 2 mg per day) into the large amount of Fe contents in a human body (2 – 4 g).[9,18] This result suggests that Fe isotopic data for human RBC samples can provide information about the dietary conditions over a long-term period. The averaged δ^{56}Fe and δ^{57}Fe values were –3.06‰ and –4.51‰, respectively, indicating that the Fe isotopic composition of the human RBC sample was largely fractionated from the Fe isotopic ratios of natural Fe-bearing ore (IRMM-014). It was reported that the Fe isotopic compositions of plants showed δ^{56}Fe = –1.5 – –0.1‰ and animal products, such as beef muscle, chicken muscle or shrimp muscle showed, δ^{56}Fe = –2.5 – –0.5‰.[9] This data demonstrate that the Fe isotopic ratios could be systematically fractionated through a preferential uptake of lighter Fe isotopes at a higher pyramid of the food chain.

Difference in the Fe isotopic ratios among individuals

In this section we examine the possible change in the Fe isotopic ratios. The basic question is whether the Fe isotopic composition is different among individuals. To test this, the δ^{56}Fe and δ^{57}Fe values for RBC samples collected from five different individuals were measured (Table 4). The resulting δ^{56}Fe and δ^{57}Fe values did not vary significantly in samples obtained from the four males. In contrast, the δ^{56}Fe and δ^{57}Fe values from the female clearly showed a higher value (0.3‰/amu) than the mean value of the males (Fig. 4). This result is consistent with previously reported data.[9] The difference in the Fe isotopic ratios among the individuals could be attributed to possible differences in the uptake or metabolic efficiencies through dietary processes. In order to discuss this more quantitatively, further analyses on Fe isotopes are highly desired.

Table 4 Fe isotopic composition of RBC samples collected from four males and one female

Sample	δ^{56}Fe, ‰	δ^{57}Fe, ‰
Male		
ID_No. 1[a]	–3.06	–4.51
ID_No. 2	–3.04	–4.41
ID_No. 3	–2.96	–4.27
ID_No. 4	–3.15	–4.71
Female		
ID_No. 5	–2.55	–3.77

Delta values with respect to IRMM-014. The analytical uncertainty is ± 0.12‰ and 0.20‰.
a. The mean of Fe isotopic composition of RBC samples over a period of one year.

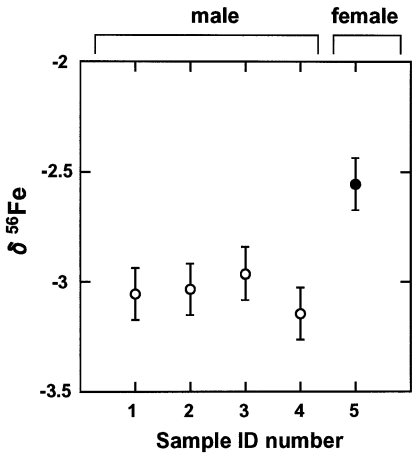

Fig. 4 δ^{56}Fe data for RBC samples collected from four males and one female.

Conclusion

A new chemical separation procedure using anion-exchange chromatography was developed for the Fe isotopic analysis of human RBC samples using MC-ICPMS. The resulting precision of ^{56}Fe/^{54}Fe and ^{57}Fe/^{54}Fe ratio measurements were 0.12‰ and 0.20‰ (2SD), respectively, and the level of analytical precision achieved was sufficient to detect the variation in the Fe isotopic ratios for human RBC samples. The Fe isotopic data for a series of 12 RBC samples collected from a person over a one-year did not vary measurably, suggesting that the contribution of daily-dietary Fe was relatively small. The Fe isotopic ratios for RBC samples were 1.3 – 1.6‰/amu lighter than those for natural Fe-bearing ore (IRMM-014), which could be due to a preferential uptake of Fe-isotopes through dietary processes. The Fe isotopic ratios for the female RBC sample were 0.3‰/amu higher than the mean value of the male's RBC samples. This might be due to an inherent difference in the uptake efficiency of Fe through dietary processes. The data obtained here clearly demonstrates that the Fe isotopic ratios can provide new information about the uptake or metabolic usage of trace metallic elements in the human body.

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