Video Article

High Precision Zinc Isotopic Measurements Applied to Mouse Organs

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Abstract

We present a procedure to measure with high precision zinc isotope ratios in mouse organs. Zinc is composed of 5 stable isotopes (^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn and ^{70}Zn) which are naturally fractionated between mouse organs. We first show how to dissolve the different organs in order to free the Zn atoms; this step is realized by a mixture of HNO_3 and H_2O_2. We then purify the zinc atoms from all the other elements, in particular from isobaric interferences (*e.g.*, Ni), by anion-exchange chromatography in a dilute HBr/HNO_3 medium. These first two steps are performed in a clean laboratory using high purity chemicals. Finally, the isotope ratios are measured by using a multi-collector inductively-coupled-plasma mass-spectrometer, in low resolution. The samples are injected using a spray chamber and the isotopic fractionation induced by the mass-spectrometer is corrected by comparing the ratio of the samples to the ratio of a standard (standard bracketing technique). This full typical procedure produces an isotope ratio with a 50 ppm (2 s.d.) reproducibility.

Video Link

The video component of this article can be found at http://www.jove.com/video/52479/

Introduction

The measurement of high-precision (better than 100 ppm/atomic mass unit) zinc stable isotope composition has only been possible for about 15 years thanks to the development of multi-collector plasma-source mass-spectrometers and has since been mostly applied in Earth and planetary sciences. The applications to the medical field are novel and have a strong potential as biomarkers for diseases that modify the metabolism of zinc (*e.g.,* Alzheimer disease). This paper reports a method to measure with high precision the natural stable isotope ratios of zinc in various mouse organs. The same would be applicable to human samples. The method consists of the dissolution of the organs, the chemical purification of zinc from the rest of the atoms, and then the analysis of the isotope ratio on a mass-spectrometer.

The quality of Zn isotopic measurements is dependent on the quality of the chemical purification (purity of Zn, low blank compared to the amount of Zn present in the sample, high chemical yield of the procedure) and on the control of the instrumental bias. The high purity of the final Zn fraction is needed to remove both isobaric interferences and non-isobaric interference that create a matrix effect. Isobaric nuclides create direct interferences (*e.g.,* ^{64}Ni). Non-isobaric interferences generate the so-called “matrix” effect and alter the analytical precision of the measurements by changing the condition of the ionization compared to the pure zinc standard to which the samples are compared to[1]. A low blank (< 10 ng) indicates that there is no contamination of the samples by external Zn that would bias the measured isotopic composition. As Zn isotopes can be fractionated during ion-exchange chromatography[2], the collection of all the Zn atoms ensures that no isotopic fractionation occurs, which implies that the chemical procedure should have a full yield. Finally, the correction of the instrumental isotopic fractionation during the mass-spectrometry measurement is done via the “standard bracketing” method.

Therefore, the main difficulties to obtain precise measurements are controlling the external contamination (*i.e.,* low blank), producing a full yield chemical purification that is clean of any other atoms or molecules, and correcting the instrumental isotopic fractionation on the mass-spectrometer. In this paper we will describe our analytical protocol to separate Zn from the mouse organs as well as the mass-spectrometry measurements.

The extraction is done using a low quantity of diluted acids (HBr/HNO_3 media) on micro-columns (0.5 µl and 0.1 µl) of anion-exchange resin. It has a full yield and the measurements have an external reproducibility better than 50 ppm on the ^{66}Zn/^{64}Zn ratio. Another advantage of the method is that it is very fast. The method is therefore very well adapted to medical sciences, in which one needs to analyze a large number of samples compared to geosciences, where these analytical methods were developed.

Protocol

NOTE: Procedures involving animals have been approved by the Institutional Animal Care and Use Committee (IACUC) at the Université Paris Diderot.

1. Preparation of Materials

1. Sub-boil distill 1 L of the acids (HNO_3, HBr) in order to purify them from impurity.
2. Clean the beakers and tip adaptor in a hot (~ 100 °C) concentrated HNO_3 acid bath for at least two days.
3. Wash the pipette tips in a cold 3 N HNO_3 bath for several days and rinse individually three times with de-ionized water.

2. Sample Preparation

1. Anesthetize the mice by intraperitoneal injection of ketamine and xylazine. Assess anesthesia by the toe pinch method.
2. Collect the blood by a cardiac puncture in the presence of heparin in 1.5 ml tubes.
3. Separate the plasma from blood cells by centrifugation (10 min, 1,500 x g) and transfer the plasma to polypropylene cryogenic vials using polypropylene tips.
4. Remove the remaining blood from organs by cutting the hepatic vein and injecting DPBS through the heart. Assess death of the mouse by cervical dislocation.
5. Harvest the organs with sterile stainless steel instruments, free them of surrounding fat if any, and snap-freeze them in polypropylene cryogenic vials.

3. Chemical Purification

1. First, dissolve the samples in a mix of ~ 1 ml of concentrated (30%) H_2O_2 and ~ 1 ml of concentrated (~ 15 M) HNO_3. Do all these steps inside a fume hood.
    1. Place the whole organ of interest into a 15 ml Teflon beaker. Then, add the H_2O_2/HNO_3 to the beaker[5]. Keep the beaker open for a few minutes in order to avoid splashes due to the reaction of oxidation of the organic matter and the release of CO_2.
    2. Finally, put the beaker on a hot plate at about 100 °C for a couple of hours or until the solution is perfectly clear.
2. Open the beaker and dry the solution on a hot plate at about 100 °C.
3. Once the sample is dry, add 1 ml of 1.5 N HBr to the samples; close the beaker and let it dissolve on a hot plate at 100 °C for a couple of hours.
4. Meanwhile prepare the 500 µl columns.
    1. Add 500 µl of the AG1X8 200-400 mesh resin to the column and put it on the column rack with a trash beaker below it. Wash the resin by alternating: 5 ml of 18.2 MΩ # cm water, 5 ml of 0.5 N HNO_3, 5 ml of water, 5 ml of 0.5 N HNO_3, and then 5 ml of water. Condition the resin with 5 ml of 1.5 N HBr.
5. Remove the beakers from the hot plate and put them in an ultrasonic bath for about 30 min, and then let the beakers cool down to RT.
6. Once the beaker is cooled and the resin is washed, open the beaker. Put the tip adaptor to the syringe, add a pipette tip; pipette the 1 ml of sample and load it onto the resin (very slowly in order not to agitate the resin).
7. Once all the liquid passes through the column, add 5 ml of 1.5 N HBr.
8. Once the 5 ml of 1.5 N HBr pass through the column, replace the trash beaker with a clean 15 ml beaker.
9. Add 5 ml of 0.5 N HNO_3 2.5 ml at a time. At this stage the Zn is eluted from the resin.
10. Once 5 ml of HNO_3 passes through the column, remove the beaker and place it on a hot plate at 100 °C until dried.
11. Remove the column from the column holder; trash the resin (use a new resin for each sample).
12. Once the sample is dry, repeat the protocol with the same volume of acids on a smaller column (100 µl) and then place it on a hot plate until dried. The sample is now ready for mass-spectrometry.

4. Mass-spectrometry Measurement

1. Analyze the Zn isotopic composition on a multi collector-inductively coupled plasma-mass spectrometer (MC-ICP-MS).
    1. Use the machine parameters summarized in **Table 1**.
2. Position the Faraday cups to collect at mass (m/z) of ^{62}Ni, ^{63}Cu, ^{64}Zn, ^{65}Cu, ^{66}Zn, ^{67}Zn and ^{68}Zn.
3. Prepare a solution containing 500 ppb Zn in 0.1 M HNO_3 for isotopic analysis.
4. Analyse the 500 ppb solution of Zn by using a spray chamber combined with a 100 µl/min teflon nebulizer. For each sample, measure 30 scans (1 block of 30 cycles) in which the integration time of each scan is 8.389 sec.
5. Correct the background by subtracting the on-peak zero intensities from a blank solution (the 0.1 M HNO_3 solution used to re-dissolve the samples).
6. Control and correct possible ^{64}Ni isobaric interference by measuring the intensity of the ^{62}Ni peak. Assume that the $^{64}Ni/^{62}Ni$ ratio is natural (0.2548), correct this value from the instrumental mass bias, and then remove the ^{64}Ni on the mass 64 as:
$^{64}Zn_{real} = {}^{64}Zn_{measured} - {}^{64}Ni = {}^{64}Zn_{measured} - (^{64}Ni/^{62}Ni)_{natural} \times {}^{62}Ni_{measured}$.
7. Correct the instrumental mass bias by bracketing each of the samples with a 500 ppb standard solution of the JMC Lyon Zn standard (or another available standard such as IRMM-3702). Perform the standard bracketing by dividing the $^{66}Zn/^{64}Zn$ ratio of the sample by the

average of the $^{66}Zn/^{64}Zn$ ratio of the two standards measured before and after the sample minus 1 and multiplied by 1,000 (see **Equation 1**). Typical external precision on the JMC Lyon Zn standard is 0.05 permil/amu (2 standard deviation, 2 s.d.).

Representative Results

In 1.5 N HBr, the main zinc species (ZnBr3-) forms very strong complexes with the anion-exchange resin, while most other elements do not interact with the resin. Zinc is then recovered by changing the medium to diluted HNO_3, changing the speciation of Zn to Zn^{2+} which is released from the resin[6,7].

Isotope ratios are typically expressed as parts per 1,000 deviations relative to a standard:

$$\delta^{x}Zn\ (‰) = \left(\frac{\left({}^{x}Zn/{}^{64}Zn \right)_{sample}}{\left({}^{x}Zn/{}^{64}Zn \right)_{standard}} - 1 \right) \times 1000 \qquad (1)$$

with x = 66 or 68. The reference material used is the Zn "Lyon" standard JMC 3-0749 L[1]. The "Lyon" standard is the most broadly used reference material to normalize Zn isotope data. All the isotopic results reported are therefore relative. Using this reference the isotopic composition of the Earth for $\delta^{66}Zn$ is 0.28 ± 0.05[8]. Since the JMC-Lyon standard is not easily available, in the absence of this standard the alternative is to use the standard IRMM-3702 as a reference during the measurements and convert the results using reference [9] as: $^{66}Zn_{JMC\text{-}Lyon} = {}^{66}Zn_{IRMM\text{-}3702}+0.29$. The typical blank is < 10ng.

Typical results obtained with this method are represented in the **Figure 1**, as a three-isotope plot ($\delta^{68}Zn$ vs $\delta^{66}Zn$) for different mouse organs. **Table 2** and **3** report results of replicated experiments of a typical terrestrial rock (a Hawaiian basalt) and of mouse red blood cells.

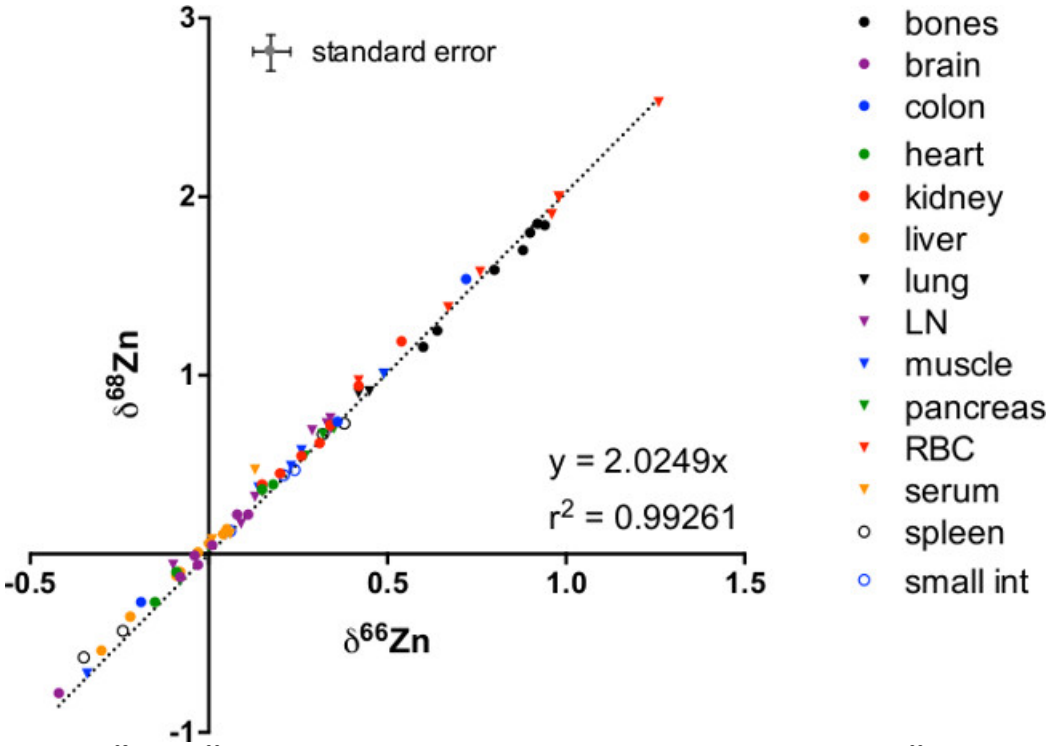

Figure 1. $\delta^{68}Zn$ vs $\delta^{66}Zn$ for different mouse organs. The typical error bar is 0.07 permil for $\delta^{66}Zn$ and 0.15 for $\delta^{68}Zn$ is shown on the figure. Data from reference [15].

MC-ICP-MS settings	Neptune
RF power (W)	1,300
Acceleration potential (V)	10,000
Gas flow rates	
Ar coolant (l/min)	18
Ar auxiliary (l/min)	1
Ar sample (l/min)	1-1.2
Solution uptake rate (molution	100
Analysis parameters	
Number of blocks	1
Number of measurements per block	30
Integration time (s)	8.389
Typical Zn concentration of samples and standard (ppb)	500
Typical transmission efficiency V/ppm	25

Table 1: MC-ICP-MS settings for the Zn isotope measurements at the Institut de Physique du Globe de Paris.

Samples	$\delta^{66}Zn$	2se	$\delta^{68}Zn$	2se	n[a]
replicate 1	0.34	0.01	0.68	0.04	4
replicate 2	0.34	0.01	0.68	0.01	3
replicate 3	0.34	0.02	0.67	0.02	4
replicate 4	0.36	0.06	0.7	0.09	4
replicate 5	0.31	0.02	0.65	0.06	4
replicate 6	0.33	0.01	0.68	0.02	3
replicate 7	0.32	0.06	0.63	0.1	6
Average	0.33	0.03	0.67	0.05	7
2SD	0.04		0.05		
[a] n=number of repeat measurement by MC-ICP-MS					

Table 2: Zn isotopic composition of the Hawaii basalt K179-1R1-170.9. Each replicate represents a full chemical purification and the average of several independent mass-spectrometer measurements. Data from reference [8].

Mouse number	$\delta^{66}Zn$	$\delta^{68}Zn$
11	0.82	1.6
12	0.79	1.55
13	0.84	1.65
14	0.87	1.72
Average	0.83	1.63
2SD	0.07	0.15

Table 3: Zn isotopic composition of bones of mice. Each replicate represents a full chemical purification. Data from reference [15].

Discussion

The reproducibility of the measurements is evaluated through replicated analyses of the same samples carried out during different analytical sessions. For example[6], we have replicated the same terrestrial rock 7 times and we obtained the results reported in the **Table 2**.

As expected from the theory of isotopic fractionation[10] and as measured in any solar system material so far (*e.g.*, meteorite[11-13], plants[3-5], deep-sea sediments[14], animals[15-17]), the results follow a mass-dependent law (see **Figure 1**). $\delta^{68}Zn$ is about twice $\delta^{66}Zn$ (**Figure 1**), because the mass difference between ^{68}Zn and ^{64}Zn is twice the difference between ^{66}Zn and ^{64}Zn. This shows that our measurements are free of isobaric interferences (that would drive the data out of the straight-line) and that zinc isotopes are fractionated out of the same isotopic pool.

For mouse organs, the limited amount of Zn in each organ has prevented us from performing many replicates of a single organ[15]. However, we can estimate a higher limit for the reproducibility by comparing data for the same tissue for different mice of the same age and same strain (for example for the bones of 16 week old mice, **Table 3**). This reproducibility is larger (0.04 vs 0.07 for the δ^{66}Zn) than what was estimated from basaltic rocks, which is not surprising because it includes the heterogeneity of the samples as well as the isotopic variability between the different mice. It is therefore an overestimation of the reproducibility, and we believe that the precision on each individual organ would be similar to what we had determined on basaltic rocks. We can safely assume reproducibility better than 0.10 for the δ^{66}Zn (2 s.d.) which represents a precision 10 times larger than the variability reported between certain organs (see **Figure 1** and reference [15]).

Measuring the stable isotope composition of Zn will be used in the future as a diagnostic tool for diseases that modify the Zn balance of the body. For example, the zinc-rich plaques associated with the Alzheimer disease change the concentration of zinc in the serum and since the brain and the serum have different isotopic composition[15] Zn isotopes could be used to detect early stage of the disease.

Most alternative methods to measure the Zn isotopic composition by MC-ICP-MS involve chemical purification in concentrated HCl media on larger columns than the one used here[1-4]. Our method based on micro-columns and diluted acids has low blanks and produces data which are two times more precise (50 ppm vs 100 ppm 2 s.d.). In addition, our method is very fast (due to the small size of the columns and the little amount of acid used) and is very well suited to analyze large amount of samples (as usually needed in clinical studies). The simplicity of the method would be well suited to be used in an automatic chemical purification system which would allow the measurements of a large numbers of samples.

One limitation of this approach is that only large bulk samples can be analyzed (the procedure uses ~ 1 µg of Zn). Reducing the size of the samples is crucial when dealing with precious clinical samples. This method is also limited to bulk measurements, while for some applications *in situ* analyses may be needed. Future improvement on the technique should be in relation with improving *in situ* isotopic measurements by combining a laser-ablation system with the plasma mass-spectrometer (LA-MC-ICP-MS). This would allow the measurements of spatially small samples without prior chemical purification (which tends to contaminate the samples). In addition, *in situ* measurements will allow the measurement of the Zn isotopic composition on living tissues. To our knowledge there has only been one attempt to measure zinc isotope ratios using such a technique[18] and the method is still not precise enough, however, measurements of high-precision isotope ratio by LA-MC-ICP-MS has been done for Fe[19] and B[20] and refining of the technique using modern lasers may lead to a major breakthrough.

Disclosures

The authors have nothing to disclose.

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