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Method development for thermal ionization mass spectrometry in the frame of a biokinetic tracer study with enriched stable isotopes of zirconium

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ABSTRACT

Isotope dilution in thermal ionization mass spectrometry (ID-TIMS) is a precise method for quantification of isotope ratios in geological samples, or for trace elements analysis in geological and environmental samples. This work presents an optimized ID-TIMS method for application in the field of life sciences, more precisely in the frame of a human biokinetic study on zirconium with oral and intravenous administration of two stable isotope tracers. The method allows analyzing simultaneously different stable zirconium isotopes (two isotopically enriched artificial tracers and the natural background) in human blood plasma and urine. By optimization, tracer detection limits below 1 ng ml^{-1} could be achieved. The biological samples were prepared by microwave-assisted acidic pressure digestion followed by extraction chromatography. The purified zirconium from the samples was measured on carbon-coated rhenium single filaments. Multiple channel electron multipliers were used as detectors. Interferences were observed from molybdenum isotopes and were corrected for based on the ^{95}Mo count rate. Isotope ratios of zirconium were determined in ranges of 0.016–2.84 (^{91}Zr/^{90}Zr), 0.020–2.46 (^{92}Zr/^{90}Zr), 0.115–10.93 (^{94}Zr/^{90}Zr), and 0.004–5.70 (^{96}Zr/^{90}Zr). The respective relative uncertainties lay in the range of 0.03–4.2%. Typical relative uncertainties of tracer concentrations were 4%.

1. Introduction

Isotope ratios of inorganic forms of zirconium of terrestrial, lunar and meteoric origin have already been determined with thermal ionization mass spectrometry (TIMS) to high precision [1–3]. These measurements obtained relative uncertainties of isotope ratios in a range of 0.0018–0.2% with sample sizes of 1–15 μg and careful heating and measurement procedures over periods of up to several hours per sample.

TIMS has also successfully been used for the determination of isotopically enriched stable tracers of several other elements in biological samples for the purpose of biokinetic studies [4–8]. Studies with stable isotopes as tracers are a valuable tool to gather knowledge about the human metabolism of trace elements. Enriched stable isotopes are chemically similar to the naturally occurring isotopic mixture, yet physically distinguishable from it, and are not radiotoxic, in contrast to radioisotopes. If enough different stable isotopes of one element are available, i.e. at least as many as the number of different isotopic mixtures involved in the measurement, a single study can also employ two tracers simultaneously. In order to conduct such a study for zirconium, a suitable measurement method had to be developed first.

Zirconium is an element of interest in radiation protection, although it has no known essential biological function. Its radioisotopes, most notably ^{95}Zr, are generated by uranium fission with a high yield [9], as well as by neutron activation reactions in reactor components made from zirconium alloys ('Zircaloy'). In order to estimate the health effects after accidental exposure to radionuclides of zirconium, the internal radiation dose has to be known. However, the internal dose cannot be measured directly; it has to be calculated. For this purpose, the knowledge about the biokinetic behavior of zirconium in the human body plays a central role. Mathematical compartment models are used to describe these biokinetic processes. They are developed and published by the International Commission on Radiological Protection (ICRP). In its current state [10,11], the biokinetic model and the associated model parameters of zirconium are primarily based on animal experiments due to the scarcely available human data. Thus, it would be favorable to directly gather human metabolic data.

The measurement of zirconium isotopes in human biological samples at low concentrations poses a great challenge. When stable zirconium tracers are administered to the human body in a biokinetic study, the resulting concentration in blood should not exceed that of natural zirconium. This is important in order

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Table 1
Isotopic composition of zirconium types.

Type	Relative isotope abundance (at.%)				
	^{90}Zr	^{91}Zr	^{92}Zr	^{94}Zr	^{96}Zr
T-90[a]	**98.2**	0.6	0.6	0.5	0.1
T-91[a]	6.1	**88.7**	3.4	1.5	0.3
T-94-R[a]	3.8	1.5	2.5	**91.2**	1.0
T-96-H[a]	5.0	1.88	2.69	4.03	**86.4 ± 0.6**[d]
T-96-L[a]	19.8	5.3	8.2	8.4	**58.3**
Natural Zr[b]	51.45 ± 0.40	11.22 ± 0.05	17.15 ± 0.08	17.38 ± 0.28	2.80 ± 0.09
Zr standard[c]	51.49 ± 0.11	11.15 ± 0.02	17.29 ± 0.11	17.26 ± 0.12	2.82 ± 0.05

[a] Values given by the suppliers' certificates.
[b] IUPAC recommended representative natural composition [26].
[c] In-house reference measurements of a natural zirconium standard solution (PLZR2-2Y, SPEX CertiPrep, Metuchen, NJ, USA; diluted in 3% HNO_3; 2 ng per sample). The values given are the mean ± 1 standard deviation from 21 samples.
[d] Isotope abundance uncertainties were included in the suppliers' certificates.

to maintain normal metabolic processes. However, the naturally occurring zirconium concentrations in human blood and urine samples are very low. Zirconium concentrations reported in recent publications are 0.02–0.038 ng ml^{-1} in urine [12], 1.4 ± 0.3 ng ml^{-1} in plasma [13], and 0.09–0.16 ng ml^{-1} [12] or 0.9–7.4 ng ml^{-1} [14] in serum. Reports on concentrations in whole blood are highly variable with lower values found more recently: 9.6 ± 5.8 ng ml^{-1} [15], 5.0 ± 1.1 ng ml^{-1} [13], and <0.014–0.5 ng ml^{-1} [16]. There are only few suitable, isotope-sensitive measurement methods under these circumstances. A short comparison of two possible methods, TIMS and proton nuclear activation analysis (PNA), has been published recently [17]. The current work presents a detailed description of TIMS as the method of choice.

With a sample size of a few ml of blood plasma or urine, the expected zirconium amount of ~1 ng would be far below the amounts for which TIMS sample preparation and measurement routines are published. For example, the aforementioned precision measurements of isotope ratios were performed with sample amounts of up to several μg of zirconium. Even though the ion current in TIMS is not proportional to the sample amount, a large reduction in sample size could lead to an unstable ion beam, limit the measurement duration due to sample consumption, or necessitate the use of different ion detection technology.

A new TIMS measurement method for the determination of zirconium tracer concentrations in biological samples must address two further challenges. First, the natural and tracer zirconium must be separated from the biological matrix, because TIMS typically needs pure elemental samples. Second, isotope dilution quantifies a sample amount of known isotopic composition by a spike of the same element with known but different isotopic composition. In such a measurement, a single isotope ratio can be sufficient for the quantification. In contrast, the samples of a biokinetic tracer study can contain several different isotopic mixtures, e.g. two isotopically enriched tracers as well as natural zirconium, plus the spike. In this case, the measurement uncertainty of at least three isotope ratios, including potential interference corrections, must be low enough to allow calculating the tracer concentrations.

The focus of the presented work therefore lies on the adaptation of TIMS to the precise and accurate determination of stable isotope ratios of small amounts of zirconium in biological samples. The aim is to use TIMS for concentration measurements of enriched stable isotopes of zirconium as tracers in biokinetic studies with zirconium, especially in human blood plasma and urine samples.

2. Materials and methods

2.1. Biokinetic study

The presented method of ID-TIMS of stable zirconium isotopes was developed for the determination of tracer concentrations in the samples of a specific biokinetic tracer study. This section presents information on this study to characterize the framework of the method development.

The study was a human volunteer study with a double tracer setup to investigate the gastrointestinal uptake, plasma clearance and urinary excretion of stable isotopes of zirconium. The data were used to test and evolve biokinetic models of zirconium for application in radiation protection to calculate the internal dose after uptake of radionuclides of zirconium. The study was approved by the ethics committee of the medical faculty of the Technical University Munich (TUM), Germany. It was planned and conducted according to the World Medical Association's Declaration of Helsinki. Details on the study and its results will be published elsewhere.

Each investigation in the study employed two distinct stable isotopic mixtures (hereafter denoted as 'types') from the list of T-90, T-91, T-96-H, and T-96-L in Table 1. One of the tracers was injected intravenously (i.v.), the other administered orally (p.o.) at the same time. In this administration scheme, the i.v. tracer served as a reference for the clearance from blood plasma, distribution within the body, and excretion in urine. The time courses of the plasma concentrations of both tracers were used to quantify the fractional uptake of the p.o. tracer and to model the uptake process. This concept proved to be effective in prior studies with other elements conducted previously [7,18]. The fractional uptake is an especially important parameter in radiation protection, mainly for long-lived radioisotopes, because it determines how much activity remains in the metabolism after an ingested substance has passed the alimentary tract.

The measured sample materials were blood plasma and urine. Plasma samples were collected in increasing intervals from 5 min after tracer administration until 100 days later; urine was collected over 12 or 24 h sampling intervals during the first 7 days, as well as on days 30 and 100 after tracer administration. For both fluids, a blank sample was taken before the start of each investigation. Each investigation thus produced up to 15 plasma and 11 urine samples. The study included 7 female and 8 male volunteers in 16 double tracer and 7 further investigations.

Overall, approximately 600 biological samples were analyzed with TIMS, including repetitions for control purposes. In these samples, the maximal measured tracer amounts were 48.3 ng in plasma and 8.6 ng in urine. Median measured tracer amounts above detection limits were 9 ng in plasma and 0.5 ng in urine, typically with higher amounts of the i.v. tracer and lower amounts of the p.o. tracer. The minimal amount of natural zirconium measured in these samples was 1.2 ng. The presence of natural zirconium in a similar or larger amount than the two tracers necessitated the analysis of at least four isotopes in order to be able to distinguish between these types and the added spike.

Table 2
Relative standard uncertainties of tracer concentration measurements with ID-TIMS.

Basic quantities (type mass determination)	Estimated or expected relative uncertainty (%)	Range (%)
Tracer isotope ratios	0.39 (median)	0.07–2.6
Sample isotope ratios (R_{ji})	0.11 (median)	0.03–4.2
Relative atomic masses	$<10^{-5}$	
Reference tracer concentration	3.88 or 1.79[a]	

[a] Determined by repeated ID-TIMS measurements for the diluted solution used for spiking the samples (5.3 mg l^{-1}) in comparison to a natural standard; includes pipetting uncertainty; values apply to two different batches of solution.

2.2. Tracer concentration determination by isotope dilution

The concentrations of distinct types in samples were determined by isotope dilution (ID) [19]. By using a known amount of one type as reference, it is possible to quantify the concentration of other types in the same sample, if the relative isotope abundances or isotope ratios are known. For a single unknown type '*x*' and a reference type '0' (the spike), the mass m_x in a sample can be calculated as

$$m_x = m_0 \cdot \frac{AW_x}{AW_0} \cdot \frac{a_{j,0} - a_{i,0}R_{ji}}{a_{i,x}R_{ji} - a_{j,x}} \quad (1)$$

with *AW* being the mean atomic weights of the types, *a* the relative abundances of the isotopes *i* and *j* in type *x* or *0*, respectively, and R_{ji} the isotope ratio *j*/*i* in the sample. This equation was extended to four types, namely the reference spike, two different tracers, and natural zirconium present as background in the measured samples; the relative abundances were expressed by the measured isotope ratios. The type codes and relative isotope abundances are listed in Table 1.

Since zirconium has five stable isotopes, yet only four were needed for the above calculation, those isotopes were selected which had the highest abundance in at least one of the types present in the respective sample. If the choice was arbitrary (e.g. for both T-90 and natural zirconium present in the same sample), end results were determined as uncertainty-weighted means from the possible combinations.

2.3. Uncertainty calculation

Combined measurement uncertainties were calculated according to recommendations of the International Organization of Standardization [20]. The uncertainty evaluation was based on a first-order Taylor series approximation of the extended isotope dilution equations describing the mass of each type in a sample. Correlation terms were only taken into account (a) for the measured isotope ratios, since these were highly correlated in some measurements (correlation coefficients >0.95), or (b) for repeated pipetting processes. A coverage factor of 1 was used throughout the work. As far as possible, 'type A' standard uncertainties were used for the individual quantities; these were the respective standard deviations of the mean of repeated measurements of the reference tracer concentration, or of several measurement cycles for isotope ratios. In case no statistic data was available (type B), uncertainties were adopted or estimated from manufacturers' certificates and literature data. The latter was the case for pipetting uncertainties and relative atomic masses. A more detailed description can be found in [21]. The quantities taken into account are presented in Table 2.

3. Results and discussion

3.1. Development of sample preparation

The first necessary optimization during the method development concerned the sample preparation. Three main aspects are discussed due to their importance for ion yield and beam stability in TIMS measurements. These aspects are the degradation of organic compounds in biological samples, extraction and purification of zirconium from the resulting mixture of trace elements, and optimal deposition of zirconium on the TIMS sample carriers.

Table 3a
Sample digestion in Kürner microwave system.

Sample	500 μl blood plasma	1000 μl urine
Add spike	5 μl 91.2% enriched ^{94}Zr in 3% HNO_3/traces HF	
Incubate	8 h at 37 °C	
Add reagent	1 ml conc. HNO_3 (sub-boiling distilled)	
Digest	10 min at 600 W, no temperature control; 2 samples at a time in quartz vessels	
Evaporate to dryness	4 h at 120 °C in 10 ml Teflon vessels	

3.1.1. Digestion of biological sample material

Microwave-assisted acidic pressure digestion, the first step in sample preparation, has the objective to decompose blood plasma and urine samples by oxidizing all organic components. Two alternative schemes were used as presented in Tables 3a and 3b. The scheme change from the older Kürner microwave system (Kürner Analysentechnik, Rosenheim, Germany) to the newer system (MWS-2, Berghof Products + Instruments GmbH, Eningen, Germany) was necessary due to a defect in the former. Although the systems differed in the material of the pressure vessel (quartz vs. Teflon), vessel volume, minimal solution volume, and heating scheme due to pressure constraints, both digestion schemes delivered completely digested samples without visible residues.

The reagents used were 'suprapure' grade acids from Merck KGaA, Darmstadt, Germany. HNO_3 and HCl were further purified by sub-boiling distillation ('distillacid BSB-939-IR', Berghof, Eningen, Germany). For dilution of acids and rinsing of Teflon parts, deionized 'Milli-Q' water was used (Millipore GmbH, Schwalbach, Germany). All enriched zirconium tracers, including the reference spike (T-94-R in Table 1), were obtained from Campro Scientific GmbH, Berlin, Germany and Chemotrade Chemiehandelsgesellschaft mbH, Leipzig, Germany.

3.1.2. Purification of zirconium

In order to extract pure zirconium from the digested samples and remove possible interfering elements, column chromatography was employed. The procedure which was found to be ideal is presented in Table 4. It is a result based on preliminary experiments and personal communication from Pascale Louvat (Institut de Physique du Globe de Paris, France), as well as on the work of Münker et al. [22]. The column material for zirconium separation was EICHROM Ln-Spec resin, which consists of a HDEHP (di(2-ethylhexyl)phosphoric acid) coating on an inert polymeric carrier (Amberchrom CG71), with a particle size of 100–150 μm.

The sample recovery from the digestion and chromatography procedure was assessed by measuring two plasma and urine sam-

Table 3b
Sample digestion in Berghof MWS-2 microwave system.

Sample	500 μl blood plasma	1000 μl urine
Add spike	5 μl 91.2% enriched ^{94}Zr in 3% HNO_3/traces HF	
Incubate	8 h at 37 °C	
Add reagent	6 ml conc. HNO_3	4.5 ml conc. HNO_3, 0.5 ml H_2O_2
Digest	15 min at 140 °C, 30 min at 220 °C, 10 min at 230 °C	10 min at 145 °C, 10 min at 160 °C, 20 min at 190 °C
	in 60 ml Teflon vessels, 10 samples at a time	
Add reagent	0.4 ml H_2O_2	
Evaporate to dryness	6 h at 120 °C in 10 ml Teflon vessels	

Table 4
Extraction chromatography.

	Ln resin (volume 1 ml, column diameter ~8 mm)	
Step	Volume (ml)	Solution
Rinse prior to first use	5	6 M HCl
	5	2 M HF
	15	0.1 M HNO_3
Dissolve sample	1	0.5 M HNO_3/0.01 M HF
Precondition column	5	0.5 M HNO_3/0.01 M HF
Load sample	1	0.5 M HNO_3/0.01 M HF
Rinse matrix	5	4 M HCl
Rinse HREE[a]	20	6 M HCl
Elute Zr	12[b]	3 M HCl/0.1 M HF
Evaporate sample to dryness	–	–
Regenerate column	5	6 M HCl
	5	2 M HF
	5	6 M HCl
	5	2 M HF
	15	0.1 M HNO_3
Store at 3 °C		0.1 M HNO_3

[a] HREE: heavy rare earth elements.
[b] The first 4 ml and the last 2 ml were discarded for routine samples.

ples respectively, each spiked with 10 ng of isotopically enriched zirconium, which was a tracer amount also expected in biokinetic study samples. For each sample, elution profiles for natural and tracer zirconium were determined in successive 3 ml fractions of the eluted solution. The range of tracer recovery rates found in the four samples was 22–34% in a total elution volume of 12 ml HCL/HF. The fractional recovery of the tracer, i.e. the recovered amount in each 3 ml fraction, peaked at about three quarters of the 12 ml and declined slightly afterwards. This suggests that the tracer elution was still not complete after 12 ml, and that a larger collected volume would have lead to a higher recovery rate. However, in contrast to the tracer, the fractional concentration of eluted natural zirconium featured a continued increase with the collected volume. Therefore, an increase of the eluted and collected volume would also have lowered the ratio of tracer to natural zirconium in the measured samples, thereby leading to large uncertainties in the isotope dilution measurements. In order to lower the uncertainties by optimizing the ratio of tracer concentration to natural zirconium, the first 4 ml and the last 2 ml of the 12 ml elution volume were discarded.

The continuing increase of the concentration of natural zirconium during elution was interpreted as contamination of the acid with natural zirconium. The range of natural zirconium found in measured samples was 30.1–204 ng (median value of 81.3 ng) when using 'suprapure' grade HCl. Its concentration could be effectively reduced by further purification; for elution with sub-boiling distilled HCl, which was later used during routine analysis, the range of natural zirconium was 1.2–99 ng, with a median value of 3.7 ng.

Potential memory effects from the repeated use of chromatography columns were assessed by alternating loadings of a new column with pure 10 ng samples of enriched ^{92}Zr (88.6%) and ^{94}Zr (92.7%). Within 10 runs with intermediate regeneration according to Table 4, no cross-contamination was observed. A repetition of this experiment with samples of 100 ng confirmed the previous result. As consequence, chromatography columns were used up to 10 times for experimental samples.

All Teflon beakers in contact with the samples were intensively cleaned prior to use and reuse by dissolving contaminations with conc. HNO_3 in an ultrasonic bath, soaking each beaker over 48 h in conc. HNO_3, subsequent evaporation with hot nitric acid in a steaming equipment, and finally rinsing with deionized water. A detailed description of the cleaning procedure can be found in [21].

3.1.3. Sample carrier preparation

TIMS allows different ion source specifications based on the number and type of filaments and the sample deposition procedure, all of which can have a substantial impact on ion yield and beam stability. The suitability of different procedures is dependent on the element of interest due to its ionization energy. Possible alternatives range from simply transferring and drying a droplet of acidic sample solution [5] over layered deposition schemes with additives (e.g. silica gel and boric acid [23]) to electroplating from a fluid source [24].

TIMS filaments were prepared onsite from filament holders (Thermo Fisher Scientific GmbH, Bremen, Germany) and zone-refined rhenium ribbon of 99.995% purity, 0.7 mm width and 38 μm thickness (Rhenium Alloys Inc., Elyria, OH, USA). Assembled sample carriers were washed with 99.9% denatured ethanol in an ultrasonic bath and successively baked out in high vacuum over 30 min at 4.5 A, corresponding to a temperature of ~1600 °C, to remove superficial contamination.

Initial tests of the ionization yield of natural zirconium samples of 1 ng to 1 μg were performed with the mass spectrometer's standard setup of double rhenium filaments. These samples were directly deposited onto rhenium filaments from an HNO_3 solution. The tests showed unstable ion beams with very low intensity, which was expected due to the high primary ionization energy of zirconium, but would have been unsuitable for routine measurements of small samples. Similarly small samples (≥17 ng) were analyzed by Boswell and Elderfield [25], who found an enhanced ionization yield of zirconium when coating rhenium single filaments with colloidal graphite. This finding inspired the current filament preparation and allowed optimizing the ionization yield and beam stability through a series of measurements with mixtures of zirconium solution and a colloidal graphite suspension of variable concentration ('Aquadag' from Acheson Colloids Company, Port Huron, MI, USA; diluted with Milli-Q water). We obtained the best results concerning beam stability, beam current, and filament temperature with a mixture of 1 part Aquadag and 15 parts water and a layered deposition scheme: First, a droplet of 1 μl of diluted graphite suspension was deposited on the rhenium filament surface and dried at 1.2 A. Second, the standard solution (or later a sample redissolved in concentrated HNO_3) was consecutively transferred to the filament and dried at 1.2 A, followed by 1 μl of diluted graphite suspension to form a covering layer. After drying, the heating current was increased to 1.5 A for ~10 s. All of the coating steps were performed in air in a fume hood.

Apart from a small transient signal during the heating phase, no additional interferences from the graphite suspension were detected in comparison to directly deposited or silica gel-covered samples. Molybdenum isobaric correction (cf. Section 3.2.2) was generally lower than in uncovered zirconium samples, hinting at an improved ionization efficiency of zirconium in addition to the more stable beam at similar filament temperature.

3.2. Development of sample measurement

This subsection covers aspects of instrumental sample measurement, i.e. regulation of the ion beam, detector properties, and systematic corrections to the measured count rates. The latter are of special relevance to the current application of TIMS due to the low sample amount and the presence of more than two types of zirconium in each sample.

All measurements were carried out on a TRITON mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany) controlled by the supplier's software suite. The TRITON is a magnetic sector field mass spectrometer capable of multicollector analysis. Besides the Faraday cups typical for similar instruments and a single centered secondary electron multiplier, it is equipped with mul-

ticollector channel electron multiplier (CEM) packages mounted onto the outer two low-mass Faraday cups. In initial tests, the ion current obtained from zirconium natural standard samples was too low for reliable measurements with the Faraday cups. Thus, all results reported in the current work were obtained with a set of six of the more sensitive CEM detectors in ion counting mode.

3.2.1. Ion beam control

TIMS filaments must be heated to a temperature sufficient for evaporation and ionization of the sample element. If the heating current is increased too fast, parts of the deposited sample may break off the metal filament which deforms during the temperature increase; if the final temperature is too high, the sample will be evaporated too fast for a complete measurement routine. Careful heating schemes as reported in other zirconium isotope ratio measurements can take up to several hours [2,3,25], which would be unsuitable for routine measurement of a large number of samples. Therefore, a faster heating scheme was introduced as follows, taking ~13–20 min:

- 500 mA min^{-1} up to 2500 mA;
- 350 mA min^{-1} up to 3500 mA;
- 200 mA min^{-1} up to 4500 mA;
- 100 mA min^{-1}, until a signal of ^{90}Zr or ^{94}Zr sufficient to focus the ion beam could be observed (~100 s^{-1}).

This scheme showed no evident sample loss during the heating phase and typically resulted in slowly increasing or decreasing ion count rates during the subsequent measurements for most samples.

After the heating phase, the ion beam was focused by an automated optimization routine included in the software and by manual fine tuning of the filament position, starting from standard settings. The combination of both operations allowed improvements of the ion count rates from a few percent up to several orders of magnitude for some samples. Good focusing was an essential step for measurements in order to obtain suitable count rates, while still keeping the heating current and the corresponding sample consumption low enough to perform the complete measurement routine for samples of a few ng of zirconium. For typical measurements, the heating current was then adjusted by 50–100 mA min^{-1} until the total count rate of zirconium isotopes fell in the range of 10^4–10^5 s^{-1}.

When the ion beam was sufficiently intense and stable, the measurement routine was started. It was based on manufacturer recommendations and started with reference measurements for centering the beam on the detectors, taking 5–10 min. The subsequent ion count rate measurement (~25 min) consisted of 50 measurement cycles, grouped in 5 blocks of 10 cycles. In each cycle, the count rates of the masses 90, 91, 92, 94, 95, and 96 u were recorded for 16.7 s simultaneously by the CEM detectors, followed by successive measurements of the ^{90}Zr count rate on all detectors for 2 s each, in order to correct for yield deviations. The measurement routine calculated all count rate ratios relative to ^{90}Zr, taking into account relative detector yields, molybdenum isobaric correction, detector dead time and dark noise, as well as temporal change of the count rates ('dynamic evaluation' mode). The total operational time needed for typical samples was in the range of 45–60 min.

3.2.2. Interference correction

Potential isobaric interferences on chemically purified zirconium isotopes are limited to stable isotopes of other elements, i.e. ^{92}Mo, ^{94}Mo, ^{96}Mo, and ^{96}Ru, if they are present as impurities in the sample carrier filament material or acidic solvents. Doubly charged ions of the high-purity sample carrier material rhenium do not affect zirconium isotope count rates, since rhenium only has stable isotopes at odd mass numbers (^{185}Re and ^{187}Re), therefore producing signals at mass numbers 92.5 and 93.5. Other interferences, e.g. molecular ions, are unlikely due to the chemical separation of zirconium during sample preparation. Furthermore, sample evaporation and ionization take place in vacuum, avoiding reactions with gas molecules.

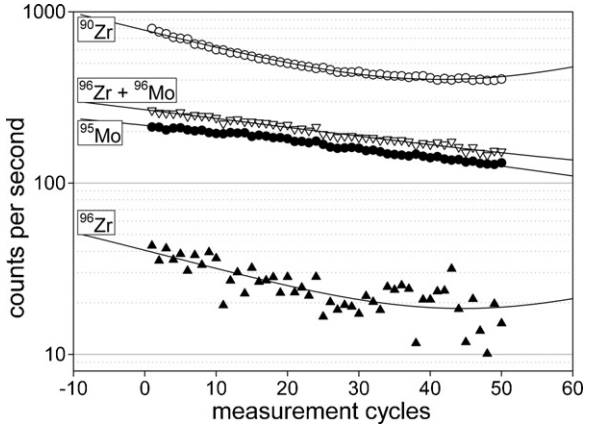

Fig. 1. Example for large isobaric interference correction. Symbols represent measured signals; only the full triangles (^{96}Zr) are interference-corrected count rates. The lines are the corresponding second-order polynomial fits, which are extrapolated for demonstrative purpose. The large (~8:1) isobaric interference of ^{96}Mo on ^{96}Zr causes high noise in the corrected ^{96}Zr signal, but the latter is still well-correlated to ^{90}Zr, demonstrating the effectiveness of the correction even in very unfavorable cases.

Initial tests with zirconium samples of known isotopic composition showed that only masses 92, 94, and 96 were affected by interferences. The magnitude of natural isobaric interferences on these masses can be quantified by the elemental isotopic compositions recommended by the International Union of Pure and Applied Chemistry [26] and the ion signals of ^{95}Mo and ^{99}Ru, which are the only stable isotopes of any element on their respective mass numbers. No correction for ruthenium isobaric interference was necessary with the given setup and running conditions, since no signal on mass 99 was observed. In contrast, molybdenum signals were present in all measurements of small samples, as well as for pure standard samples and even blank rhenium filaments, suggesting that the main source of this interference indeed was the filament material. An extended baking time of 60 min during the filament pre-treatment did not lead to a noticeable further reduction of molybdenum interference. Therefore, molybdenum interference correction for ^{92}Zr, ^{94}Zr, and ^{96}Zr was performed for every sample.

The interference correction was most demanding for the isotope ratio $^{96}Zr/^{90}Zr$, both because this ratio featured the lowest values and because the molybdenum interference was highest on ^{96}Zr. The ranges (and median values) of the corrected isotope ratios were 0.016–2.84 (0.210) for $^{91}Zr/^{90}Zr$, 0.020–2.46 (0.318) for $^{92}Zr/^{90}Zr$, 0.115–10.93 (1.16) for $^{94}Zr/^{90}Zr$, and 0.004–5.70 (0.094) for $^{96}Zr/^{90}Zr$. The ranges (and median values) of the ratio of molybdenum interference to corrected zirconium count rate on each affected mass were 0.003–5.9 (0.26), 0.003–1.0 (0.05), and 0.01–29.2 (0.50) for ^{92}Zr, ^{94}Zr, and ^{96}Zr, respectively. Especially in samples with both low abundance of ^{96}Zr and large Mo interference, the correction resulted in highly variable results for the corrected isotope ratio $^{96}Zr/^{90}Zr$. If a different temporal trend of molybdenum and zirconium signals was present in such samples, however, the corrected values of ^{96}Zr correlated well with ^{90}Zr even if the uncorrected values were clearly dominated by the molybdenum signal (cf. Fig. 1 for an extreme example). The resulting isotope ratio $^{96}Zr/^{90}Zr$ has an uncertainty of up to a few percent, which would not be sufficient for high-precision measurements, but is acceptable in view of the expected overall uncertainty of biological

tracer determination. The latter can exceed several tens of percent, e.g. due to potential contaminations, or issues related to sampling and storage [27].

3.2.3. Detector properties

The useful dynamic range of the CEM detectors can be a factor limiting the attainable uncertainty of the measurement procedure. Deviations from the expected linearity could be caused by several main aspects, i.e. detector dead time at high count rates, dark noise at low count rates, and changes in detector yield not corrected by the internal calibration during each measurement. Reference measurements and count rate control were employed to keep detector effects on the measurement results in an acceptable range.

Detector dead time effects and fast changes in detector yield were avoided by limiting the heating current in a way that the most abundant isotope caused a count rate below $10^5\ s^{-1}$. A slow change in detector yield over time, i.e. over several weeks, was observed during the peak centering phase of the measurements. Following reference measurements, all CEM amplifying voltages were changed several times during the study to adapt to detector ageing effects. This was especially necessary for the CEMs on masses 90 and 94 u, since ^{90}Zr and ^{94}Zr were the most abundant isotopes in most samples, resulting in consistently high count rates.

Repeated detector dark noise measurements revealed that the maximum permitted CEM dark noise of $0.1\ s^{-1}$ was not reached during regular operation. Whether this limit is low enough to prevent relevant dark noise effects on the measurement results can be shown by a consideration of worst-case count rates. The most extreme measured ratio of any combination of stable zirconium isotopes, i.e. the most demanding situation concerning detector dynamic range in the biological samples processed during the tracer study, was 244:1. Setting at $1000\ s^{-1}$ the minimum count rate level for the most abundant isotope, the minimal count rate of the least abundant isotope would be $4\ s^{-1}$ for the latter ratio. The maximum permitted CEM dark noise of $0.1\ s^{-1}$ thus would account for 2.5% of the signal in the worst case. The actual effect of detector dark noise is likely lower due to the software dark noise correction. In typical measurements, count rates $>10^4\ s^{-1}$ were attained for the most abundant isotopes, and the median ratio between the most and least abundant zirconium isotopes was 19:1, thus detector dark noise can be neglected as source of errors despite the small sample amounts of few ng of zirconium.

3.2.4. Instrumental stability

Reference measurements of 36 samples of natural zirconium showed no evidence of device-dependant drift of the isotope ratios $^{91}Zr/^{90}Zr$, $^{92}Zr/^{90}Zr$, and $^{96}Zr/^{90}Zr$ during the study. The long-term variability (relative standard deviation) was higher by a factor of 1.8–2.5 in comparison to the relative standard deviation of 21 preceding, closely consecutive measurements, which defined the in-house reference values (Table 1). For $^{94}Zr/^{90}Zr$, and consequentially for the relative isotope abundance of ^{94}Zr, an average relative increase of ~2% above the in-house reference value could be discerned during the last third of the study (after July 2007 in Fig. 2). This could be a sign for instrumental memory effects due to the use of enriched ^{94}Zr as reference tracer in all investigation samples (T-94-R in Table 1). However, the drift was still of the same magnitude as the variability between consecutive reference measurements, thus no correction for tracer concentration calculation was employed.

3.2.5. Mass fractionation

No correction for instrumental mass fractionation [28] was performed, since there was no fixed zirconium isotope ratio which could have been used to monitor instrumental mass fractionation. The reason for this was that all zirconium types used in the current work contained all stable isotopes of zirconium in non-negligible amounts.

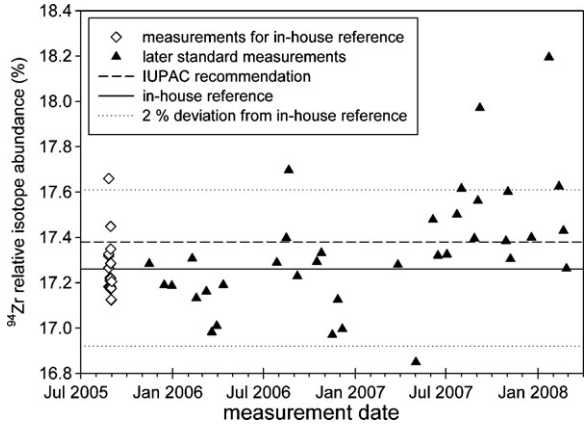

Fig. 2. Long-term drift of the relative isotope abundance of ^{94}Zr measured in reference samples. The in-house reference value (solid line at 17.26%) is based on measurements in August/September 2005 and is consistent with the IUPAC recommendation for naturally occurring zirconium (dashed line; cf. Table 1 for uncertainties).

However, the results of the reference standard measurements with natural zirconium samples of 2 ng performed throughout the study are in accordance with the IUPAC-recommended isotopic composition of natural zirconium (Table 1). Furthermore, these standard measurements have shown that the slow change of isotope ratios during a measurement due to mass fractionation usually was smaller than the random fluctuations of the isotope ratios, even for the isotope ratio $^{96}Zr/^{90}Zr$. The combination of both observations suggests that the effect of mass fractionation is negligible for the measurement procedure in its current application.

All samples were measured in a similar manner concerning sample consumption during pre-heating and measurement, with an estimated consumed fraction of 10–40%. Thus, the bias introduced by mass fractionation is assumed to be acceptably small for the purpose of tracer concentration measurements in biological studies.

3.3. Measurement uncertainty and detection limits

In contrast to precision isotope ratio measurements, comparatively large uncertainties of isotope ratios are acceptable in the application of tracer concentration measurement, as long as their effect on the overall uncertainty does not mask the temporal trend of the tracer concentration. The tolerable level depends on the expected behavior of the investigated element during the intended duration of a study, as well as other sources of uncertainty not directly related to measurement, e.g. variability of each volunteer's dietary habits, fluid uptake, or urinary excretion, or issues related to sampling and sample storage.

The tracer detection limits are very important to the application since they can effectively limit the duration of a tracer study due to the typically declining tracer concentrations with increasing time after tracer administration.

3.3.1. Isotope ratios

Zirconium isotope ratios measured in investigation samples had type A relative standard uncertainties in the range of 0.03–4.2% (standard deviation of the mean of 50 cycles), with a median value of 0.11%. The minimal absolute standard uncertainty was 1.3×10^{-5}.

3.3.2. Isotope dilution uncertainty

Relative combined uncertainties of tracer masses above detection limit were in the range of 1.79–53% (median value 3.90%);

Table 5
Blank sample measurements and detection limits.

Tracer (enrichment)	T-90 (98.2%)	T-91 (88.7%)	T-96-L (58.3%)	T-96-H (86.4%)
Number of blank measurements[a]	54	23	22	39
Number of outliers[b]	2	1	3	–
Detection limit (ng sample^{-1}[c])	0.23	0.06	0.08	0.07

[a] Without counting outliers;
[b] Defined as 10× the interquartile range below or above the median.
[c] 0.5 ml plasma or 1.0 ml urine.

absolute uncertainties lay between 2 pg and 3 ng. Large relative uncertainties were typically related to samples with small tracer concentrations in any sample material. Relative uncertainties were slightly larger on average for urine samples (median 3.95%) than for plasma samples (median 3.89%), which was interpreted as an effect of tracer concentration since that was typically lower in urine samples. The combined uncertainty was dominated by the standard uncertainty of the reference tracer concentration of 3.9% and 1.6% in the first and second batch of tracer solution, respectively. The difference between tracer types was small.

3.3.3. Tracer detection limits

Tracer detection limits L_D were calculated based on the definition of L. Currie [29], with probabilities for errors of the first (α) and second kind (β) of 0.05 each, and assuming a normal distribution of the results of repeated measurements:

$$L_D = 2.33 \cdot \sigma_0 + 2.33 \cdot \sigma_D \quad (2)$$

with σ_0 being the standard deviation of the mean of n repeated background measurements from samples without tracer, and σ_D being the estimated tracer concentration uncertainty at the concentration value of L_D. Although the observed distribution of tracer concentrations in blank samples was not strictly normal in the current work, the 95th percentile values were roughly consistent with those of a normal distribution; hence the value of 2.33 in the latter equation was adopted. From all tracer concentration measurements in a concentration range of <0.01–48.3 ng per sample, a typical relative uncertainty of 4% was determined and used to estimate σ_D:

$$\sigma_D^2 = \sigma_0^2 + (0.04 \cdot L_D)^2 \quad (3)$$

resulting in

$$L_D \approx 4.70 \cdot \sigma_0 \quad (4)$$

For the determination of σ_0 and L_D, 67 samples were evaluated concerning the concentration of one or more tracers, which were known to be absent from the respective samples. A conservative removal of outliers was performed, excluding only results more than 10 times the interquartile range apart from the median blank concentration of each tracer. The number of analyzed results, outliers, and the detection limits for the tracers are listed in Table 5.

The detection limit of T-90 is worse than those of all other tracers by a factor of 3–4. This is likely a consequence of the background of natural zirconium in each sample. In ID, for any pair of isotopes, the detection limit depends on the difference of the selected isotope ratio between spike and sample [30]. The isotope dilution equations for more than two types and isotopes are more complicated, but rely on the sample principle, i.e. types can be better distinguished from each other if their isotopic compositions are less similar. In this respect, the most important isotopes of the types presented in Table 1 are the enriched isotopes, since the others feature similar ratios between each other in each type, presumably because the types are all derived from natural zirconium. The enrichment factor of ^{90}Zr of ∼1.9 between T-90 and natural zirconium is the lowest of all enriched isotopes of the presented types, thus these two types are the least distinguishable in ID. Since all samples include a variable amount of natural zirconium, often more than the respective T-types, this especially degrades the detection limit of T-90.

4. Conclusions

Thermal ionization mass spectrometry, employing a magnetic sector field and multiple channel electron multipliers for ion counting, was optimized for the determination of enriched stable isotopes of zirconium as tracers in human blood plasma and urine.

The presented methodology is well suited for the intended application to tracer studies, since it allows for the simultaneous concentration measurement of two different tracers below 1 ng ml^{-1}. Furthermore, the associated method uncertainty of ∼4% is clearly lower than the overall variability expected in human tracer studies.

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