analytical chemistry

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High-Precision Measurement of Variations in Calcium Isotope Ratios in Urine by Multiple Collector Inductively Coupled Plasma Mass Spectrometry

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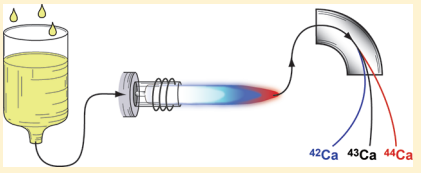
Supporting Information

ABSTRACT: We describe a new chemical separation method to isolate Ca from other matrix elements in biological samples, developed with the long-term goal of making high-precision measurement of natural stable Ca isotope variations a clinically applicable tool to assess bone mineral balance. A new two-column procedure utilizing HBr achieves the purity required to accurately and precisely measure two Ca isotope ratios ($^{44}Ca/^{42}Ca$ and $^{44}Ca/^{43}Ca$) on a Neptune multiple collector inductively coupled plasma mass spectrometer (MC-ICPMS) in urine. Purification requirements for Sr, Ti, and K (Ca/Sr > 10 000; Ca/Ti > 10 000 000; and Ca/K > 10) were determined by addition of these elements to Ca standards of known isotopic composition. Accuracy was determined by (1) comparing Ca isotope results for samples and standards to published data obtained using thermal ionization mass spectrometry (TIMS), (2) adding a Ca standard of known isotopic composition to a urine sample purified of Ca, and (3) analyzing mixtures of urine samples and standards in varying proportions. The accuracy and precision of $\delta^{44/42}Ca$ measurements of purified samples containing 25 μg of Ca can be determined with typical errors less than ±0.2‰ (2σ).



Calcium isotope variations in urine are emerging as a new technique to assess changes in bone mineral balance (BMB) in humans,[1,2] raising the possibility that Ca isotopic analyses in urine or blood could be employed clinically to detect and assess treatments for diseases that affect BMB. Such diseases include osteoporosis and multiple myeloma. X-ray bone scans or histological biopsies are the only clinical methods of measuring BMB currently in widespread use. Unlike these methods, a natural Ca isotopic biomarker would pose no radiological hazard, be noninvasive, and could detect changes BMB before reduction in bone density results in clinical outcomes such as fracture.[1,3,4] Bone formation and resorption can be measured separately using biochemical markers but, unlike the natural Ca isotope technique, these markers do not directly measure net BMB.[5,6]

The Ca isotope technique is based on small but measurable differences in the partitioning of Ca isotopes between bone and soft tissues. During bone formation, light isotopes of Ca are preferentially incorporated into bone.[9,1,7,8] This isotopic fractionation leaves soft tissue with an excess of heavy Ca isotopes relative to dietary Ca. Bone resorption involves bulk dissolution of small volumes of bone, so there is no isotope fractionation during this process. Therefore, bone resorption releases the isotopically light Ca that was stored in bone back into soft tissue. When the rates of bone formation and resorption are equal, these two effects balance so that soft tissue and excreted Ca have the same isotope composition as dietary inputs. When the rates of bone formation and resorption are not in balance, the Ca isotope composition in soft tissues shifts toward isotopically heavier values (in the case of net bone formation) or toward isotopically lighter values (in the case of net bone resorption).[9,7,8] As Ca is removed from the body primarily via urine, these natural variations in the Ca isotope composition of soft tissues are reflected in the Ca isotope composition of urine, and presumably of blood, which can be quantified by precise mass spectrometric measurements. The relationship between the Ca isotope composition of urine and BMB has been empirically confirmed.[1,2] However, the research needed to establish the clinical utility of the Ca isotope technique in specific medical conditions will require efficient analyses of large numbers of samples. This requirement leads to the need for a better chemical purification and mass spectrometric methodology than available previously.

Traditionally, Ca isotope compositions are measured using thermal ionization mass spectrometry (TIMS). Sample throughput with TIMS is not high, typically <8−10 samples per day, so it is not ideal for large sample suites. Ca isotope analysis by TIMS also requires the use of an isotopic double spike to correct for instrumental mass bias, which complicates sample preparation and analysis.[7,10]

In comparison, multiple collector inductively coupled plasma mass spectrometry (MC-ICPMS) offers the potential for higher rates of sample throughput than TIMS. For projects involving

Received: February 10, 2011
Accepted: July 8, 2011
Published: July 08, 2011

Table 1. Potential Isobaric Interferences and Their Abundances (%)[20−22]

^{40}Ca(96.941)	^{42}Ca(0.647)	^{43}Ca(0.135)	^{44}Ca(2.086)	^{46}Ca(0.004)	^{48}Ca(0.187)
$^{40}K^{+}$(0.01)	$^{84}Sr^{++}$(9.8)	$^{86}Sr^{++}$(7.0)	$^{88}Sr^{++}$(82.6)		
$^{39}K(93.3)^{1}H^{+}$(99.9)	$^{41}K(6.7)^{1}H^{+}$(99.9)	$^{27}Al(100)^{16}O^{+}$(99.7)	$^{26}Mg(11.0)^{18}O^{+}$(0.2)	$^{46}Ti^{+}$(8.0)	$^{48}Ti^{+}$(73.8)
$^{24}Mg(78.99)^{16}O^{+}$(99.7)	$^{25}Mg(10)^{16}O^{+}$(99.7)		$^{28}Si(92.2)^{16}O^{+}$(99.7)	$^{30}Si(3.1)^{16}O^{+}$(99.7)	$^{24}Mg(78.9)^{24}Mg^{+}$(78.9)
$^{40}Ar^{+}$(99.6)	$^{30}Si(3.1)^{12}C^{+}$(98.9)		$^{30}Si(3.1)^{14}N^{+}$(99.6)	$^{14}N(99.6)^{16}O_2{}^{+}$(99.7)	$^{36}Ar(0.337)^{12}C^{+}$(98.9)
	$^{28}Si(92.2)^{14}N^{+}$(99.6)		$^{12}C(98.9)^{16}O_2{}^{+}$(99.7)	$^{92}Mo^{++}$(14.8)	$^{32}S(95.0)^{16}O^{+}$(99.7)
	$^{40}Ar(99.6)^{1}H_2{}^{+}$(99.9)		$^{32}S(95.0)^{12}C^{+}$(98.9)		$^{96}Mo^{++}$(16.7)
	$^{40}Ar(99.6)^{2}H^{+}$(0.01)				

routine analyses of large numbers of samples, MC-ICPMS could be mated to an HPLC sample purification apparatus to automate the entire analytical process. An automated purification system has been used previously to prepare samples for Ca isotope measurement by TIMS,[11] but it did not substantially increase the sample throughput because the limiting factor was the rate of sample measurement by TIMS.

An important challenge for Ca isotope analyses by MC-ICPMS is the formation of isobaric interferences in the plasma itself (Table 1). Most important is interference from $^{40}Ar^{+}$ at $^{40}Ca^{+}$, a consequence of the Ar plasma used for ionization. As this interference cannot be resolved with existing technology, it is not possible to measure ^{40}Ca by MC-ICPMS using an Ar plasma. The plasma also generates polyatomic isobaric interferences (Table 1). Some of these interferences, such as from $^{40}ArH_2{}^{+}$, can be resolved using high- resolution MC-ICPMS, employing methodology described in Weiser et al.[12]

MC-ICPMS has been successfully used by geoscientists for high-precision Ca isotope determinations in calcium carbonate materials,[13,12−15] in which the abundances of interfering elements relative to Ca are low. However, few stable Ca isotope studies in biological materials have employed MC-ICPMS.[13,16,17] This is because the abundances relative to Ca of elements that can cause interferences are significantly higher in biological materials than in Ca-rich rocks like carbonates. Therefore, MC-ICPMS measurement of Ca isotope variations requires highly efficient separation of elements in the sample matrix that generate isobaric and polyatomic interferences. Critically, this separation must provide for a nearly quantitative yield of Ca because ion chromatography can fractionate Ca isotopes,[10,18,19] resulting in artificial isotope effects that are larger than the naturally occurring variations being measured.

Here we describe in detail a new liquid chromatography procedure that optimizes separation of Ca from matrix elements in urine samples sufficiently for accurate and precise determination of natural variations in Ca isotope compositions by MC-ICPMS. The same or similar methods should also produce accurate and precise $\delta^{44/42}$Ca values in blood and other biological materials.

METHODS

Samples. Our study focuses on developing a method suitable for Ca isotope determination in urine samples, because this is the sample type used in most biomedical Ca isotope studies to date. However, no international standard for urine is available. To assess the accuracy and precision of Ca isotope measurements in urine, we utilized (1) an in-house urine standard, (2) an inorganic mixture of major cations in concentrations and relative abundances similar to those in typical urine samples with a known Ca isotope composition ("Synthetic urine cation mixture", see Supplement Table S1 in the Supporting Information), (3) a series of mixtures of an unknown urine and known standards in varying proportions (described below), and (4) select urine samples were measured by TIMS using standard methods.[1] Ca standards and samples measured by double spike TIMS for this article and reported in the literature were processed and measured using our protocols to assess accuracy. The amount of sample or standard processed varied depending on the concentration of Ca; enough was processed to provide at least 25 μg of Ca for analysis. The amount of sample introduced to the mass spectrometer for a single analysis was typically <1 μg of Ca, but more material was processed for each sample measurement to allow multiple replicate analyses, to minimize any potential blank contributions and to permit more accurate yield measurements. We have not validated our analytical procedures on samples smaller than 5 μg.

Digestion Procedure. All samples and standards were processed in Class 10 laminar airflow exhaust hoods in a trace-metal-free clean lab certified to Biosafety Level 2. Acids used for chemical reagents were trace metal grade or better. Procedures were carried out in Teflon, polypropylene, and HDPE labware. All polypropylene and HDPE plastics were single-use and were cleaned by soaking at room temperature in 3.2 M reagent grade HNO_3 for 1 week, 2.4 M reagent grade HCl for 1 week, washed in 18 MΩ H_2O, and dried under a ultra low particulate air (ULPA) filter prior to use. Teflon vials were cleaned between uses in a heated 8 M HNO_3 bath for 24 h, followed by a heated 6 M HCl bath for 24 h, and then heated in 18 MΩ H_2O for 12 h.

For digestions, concentrated HNO_3 (2 mL) and ultra pure 30% H_2O_2 (2 mL) were added to weighed samples in Teflon microwave digestion vessels. A microwave system (MARS System, CEM Corporation) was set to ramp to 200 °C over 15 min and hold at 200 °C for 15 min. After cooling, the samples were transferred to Teflon reaction vessels and dried on a hot plate. Once dry, 1 mL of 16 M HNO_3 and 1 mL of 12 M HCl (modified aqua regia) were added and the vessels sealed and heated to 160 °C for 12 h. The sample solutions were dried, and 1 mL of 16 M HNO_3 and 1 mL of 30% H_2O_2 were added to the samples, heated to 160 °C for 12 h, and dried down. Alternating rounds of modified aqua regia and HNO_3/H_2O_2 digestions were performed until high aqueous surface tension in the droplet observed during dry down indicated the samples were free of significant dissolved organic material. The samples were then brought up in 4 mL of 2.5 M HCl and placed in HDPE screw-top bottles. Aliquots of the digested sample stocks equivalent to ~1 mL of urine were weighed and purified using ion exchange chromatography columns, as described below.

Column Chromatography. Once dissolved, samples and standards were processed using cation exchange resin (Biorad AG50-WX-12 200−400 mesh). Bio-Rad Econocolumns (0.7 cm × 30 cm) were filled with 2 mL of clean resin, to a height of 8.6 cm.

dx.doi.org/10.1021/ac200361t |Anal. Chem. 2011, 83, 6956–6962

Each column was equilibrated and stored in 2.5 M HCl. Samples were loaded onto a column with 1 mL of 2.5 M HCl, and an additional 1 mL of 2.5 M HCl was used to rinse the sample container. The following acids were added, in order, to rinse contaminant elements: 6 mL of 8 M HBr; 15 mL of 0.1 M HF; and 13 mL of 2.5 M HCl. Calcium and some Sr were then eluted in 25 mL of 6 M HCl (summarized in Supplement Table S2 in the Supporting Information). The eluate was dried down, brought up in 0.25 mL of 5 M HNO_3, and loaded onto 2 mm i.d. columns packed with 100 μL of Eichrom Sr-specific resin. In this HNO_3 molarity, Sr binds to the resin while Ca freely passes through. The column was rinsed three times with 0.25 mL of 5 M HNO_3 to ensure complete recovery of Ca. After the column protocols, samples were redigested in 1 mL of 16 M HNO_3 and 1 mL of 30% H_2O_2 to degrade residual organic material from the resin.

Between purifications, we reconditioned columns by cleaning them with 20 mL of 6 M HCl followed by equilibration with 20 mL of 2.5 M HCl. Variation in the measured isotope value of standards processed after five uses was within the measurement error. Hence, each column can be used for at least five sample purifications without significant changes to the position of the Ca elution peak, the resulting sample purity, or sample recovery.

Yields and elution curves, discussed below, were measured by quadrupole ICPMS (X Series, Thermo Scientific). Samples and calibration standard solutions were introduced in parallel with an internal standard solution containing Sc, Ge, Y, and In for normalization of instrumental sensitivity. Uncertainties for replicate Ca concentration measurements were approximately $\pm$10% (2σ).

MC-ICPMS Measurement. Calcium isotopes were measured using MC-ICPMS (Neptune, Thermo Scientific) following the methods of Wieser et al.[12] Briefly, samples were introduced into a 1200 W plasma with an uptake rate of either 50 or 100 μL per min. A desolvating system (Apex, CPI International) was used to reduce hydride and oxide formation and improve ion transmission to the plasma. The gas flow rate settings on the Neptune were 14.5 L/min for the cool gas, 0.8 L/min for the auxiliary gas, ~0.8 L/min for the sample gas, and 0.1 L/min for the N_2 additional gas for the Apex. Calcium concentrations for sample solutions varied from 1.5 to 5 ppm depending on daily instrumental sensitivity, resulting in ion beam intensities of 0.5−1.5 V on ^{42}Ca.

Medium and high-mass resolution modes on the Neptune were used to partially resolve some polyatomic interferences, such as ArH_2^+, CO_2^+, N_2O^+, and N_3^+. The polyatomic interferences cannot be completely resolved from Ca on the Neptune because this instrument lacks adjustable exit slits. Therefore, the ion beams were measured on the flat shoulders that correspond to the uninterfered $^{42}Ca^+$, $^{44}Ca^+$, and $^{46}Ca^+$ ion beams (see Wieser et. al, 2006, Figure 2). A similar approach on the same instrument is routinely used for the measurement of Fe isotopes.[23−25]

High-resolution mode on the Neptune was unable to resolve additional interferences compared to medium-resolution mode. High-resolution mode requires sample concentrations of ~20 ppm, which leads to more matrix effects and a buildup of sample on the cones that reduces sensitivity and requires more frequent cone cleaning. For these reasons, high-resolution mode was only used for a small number of analyses when the medium-resolution slits had degraded from use. However, data reported here that were obtained in high-resolution mode met all our quality control criteria (see below).

For each analysis, 30 measurement cycles integrated six ion beams (at ^{42}Ca, ^{43}Ca, ^{44}Ca, ^{46}Ca, ^{47}Ti, and ^{48}Ca) for 4.2 s. The intensity of ^{46}Ca (0.004%) was too low to produce reliable data. During each cycle, the ratios of the ion beams at ^{44}Ca/^{42}Ca, ^{44}Ca/^{43}Ca, ^{48}Ca/^{42}Ca, and ^{48}Ca/^{43}Ca were calculated. The ratios were averaged into a single measured value after automatic exclusion of replicates that deviated >2σ from the average. Uptake and washout times were 60 s, and data collection was 126 s.

Isotope values are reported using δ notation (eq 1):

$$\delta^{44/4x}Ca = \left[\frac{\left(\frac{^{44}Ca}{^{4x}Ca}\right)_{Sample} - \left(\frac{^{44}Ca}{^{4x}Ca}\right)_{Standard}}{\left(\frac{^{44}Ca}{^{4x}Ca}\right)_{Standard}} \right] \times 1000 \quad (1)$$

A 10 000 ppm ICP Ca concentration standard from NIST (lot no. X-10-39Ca; "ICP1") was used as the reference standard for the current work because there is no internationally certified Ca isotope standard, and NIST 915a used in other Ca isotope literature[9,13,8,12] is no longer commercially available. IAPSO was not used as the bracketing standard because it required chemical purification.

Agreement between $\delta^{44/42}$Ca and $\delta^{44/43}$Ca was used to verify mass dependent behavior as a check for isobaric interferences (see Supplement Figure S1A in the Supporting Information). If the two values differed by more than 0.11 ‰ per amu, the measurement was excluded. This quality control criterion was determined by monitoring the long-term accuracy and reproducibility of known standards; we observed empirically that by applying this criterion we were able to obtain long-term $\pm 2\sigma$ reproducibility of $\delta^{44/42}$Ca values typically better than $\pm$0.2‰ (see Supplement Figure S3 in the Supporting Information).

A major challenge in MC-ICPMS analyses is to correct for instrumental mass bias, such as that caused by space-charge effects, which leads to preferential transmissions of heavy isotopes.[18,26] There are several ways to correct for this problem.[27] Here, standard-sample-standard bracketing was chosen over a ^{42}Ca-^{48}Ca double spike because there are significant barriers to using a double spike in MC-ICPMS arising from ^{48}Ti interferences (see the Results and Discussion). Bracketing standards were measured immediately before and after each sample. Calcium concentrations in samples and standards were matched to within 10%.

Standard-sample-standard bracketing assumes mass bias drift, if it occurs, is linear over time and that natural standards behave similarly to standards. On the basis of multiple measurements of standards over the course of multiple analytical sessions, we find that this assumption generally is reasonable. However, mass bias instability cannot be excluded using only two bracketing standard measurements. Instability may result from, for example, temperature variations in the plasma and "memory effects" from prior samples, both conditions that may be more likely to develop if significant ionic or organic matrix remains in the sample solution. To minimize error caused by mass bias instability, we assumed that all of the observed difference between $\delta^{44/42}$Ca measured on bracketing standards was due to mass bias instability and discarded all measurements for which the difference between these measurements was outside of our typical precision. The $\delta^{44/42}$Ca value was calculated for bracketing standards,

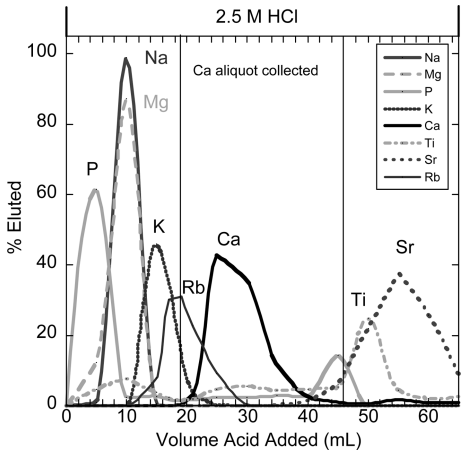


Figure 1. Elution curve of synthetic urine with published HCl Ca elution chemistry. Element concentrations were measured by Q-ICPMS in aliquots that eluted off the column. Vertical lines represent the Ca aliquot collected. Percent eluted was determined by dividing the amount of an element in an aliquot by the sum of that element in all aliquots.

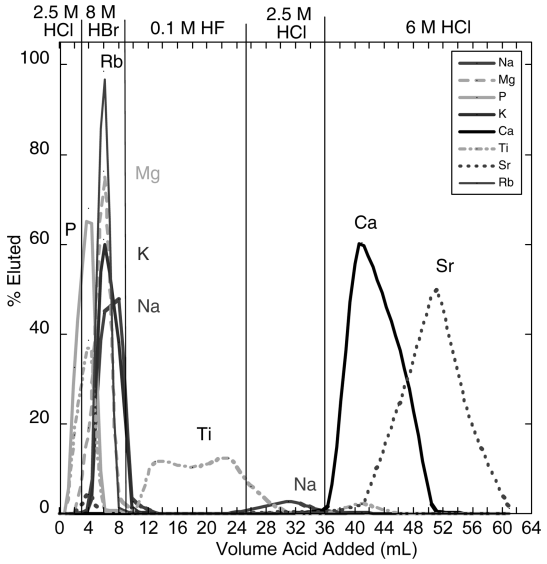


Figure 2. Elution curve of new HBr Ca elution chemistry. Element concentrations were measured by Q-ICPMS in aliquots that eluted off the column. Vertical lines represent acid changes. Percent eluted was determined by adding up all aliquots and dividing that value by the value in a single aliquot. Yields were verified by measuring the elemental composition of the sample before and after chemistry. All elements had at least 85% yield.

neglecting the intervening samples. If two standard deviations of the $\delta^{44/42}$Ca values of the standards bracketing a sample was >0.3‰, then the sample data was excluded. This highly conservative exclusion criterion limits possible error caused by unstable mass bias to values no greater than typical measurement error. However, relatively few measurements were excluded by this criterion (~10%).

Throughout each analytical session, we analyzed standards that had been processed through chemical purification to monitor instrumental performance (see Supplement Table S3 in the Supporting Information for measured and standard values). These standards included SRM 915a, SRM 915b, International Association for the Physical Sciences of the Ocean (IAPSO) seawater, and ICP1. For comparison to literature values, we measured the $\delta^{44/42}$Ca value for IAPSO seawater relative to ICP1 as 0.72 ± 0.21 ‰ (n = 61) and for NIST SRM 915a as −0.25 ± 0.12 ‰ (n = 188) (see Supplement Table S3 in the Supporting Information).

On the basis of replicate measurements (n > 3) of each secondary standard and sample, the external precision was typically no worse than ±0.2 ‰ ($\delta^{44/42}$Ca, ± 2σ, see Supplement Table S3 in the Supporting Information). Samples were routinely measured in multiple analytical sessions to ensure reproducibility.

RESULTS AND DISCUSSION

Improvements in Matrix Separation. Chromatography column procedures must be optimized for different sample types. The previously published chromatography column separation method for Ca isotope measurements uses a volumetric elution of HCl to separate Ca from other matrix elements in carbonate rocks.[12] However, the ratio of Ca to other elements in biological samples such as urine is significantly lower than that in carbonates. Figure 1 shows an elution curve using the published method on our "synthetic urine cation matrix" sample (see Supplement Table S1 in the Supporting Information). The collected Ca aliquot contains approximately 10% of the K, 30% of the Rb, 10% of the P, 10% of the Sr, and 10−20% of the Ti from the original sample. This degree of purification is insufficient for accurate Ca isotope measurements in biological samples such as urine. In addition to the overlapping elemental elution peaks, we found that when using the published method the Ca elution peak can shift by ±5 mL depending on the column height, sample concentration, and age of the resin, making it difficult to obtain reliable and quantitative yields in urine.

Our new purification protocol provides a higher degree of elemental separation between Ca and other elements than the previously published method.[13] Calcium elutes after an eluent molarity change, making the position and width of the Ca peak more predictable. The key to this protocol is the use of HBr, which removes most matrix elements and is more efficient than HCl at removing K (Figure 2). To reduce the impact of isobaric ^{48}Ti on ^{48}Ca, a 0.1 M HF eluent step removes additional Ti and 2.5 M HCl is used to remove the remaining Na.

The HBr-based chemistry presented here is more robust than the HCl chemistry over a variety of sample types. As the Ca elution peak does not contain any matrix elements other than Sr, the ratio of matrix elements to Ca in the initial sample can be higher without affecting the Ca purity after the column procedure. The new method is no more difficult than the HCl method. Both methods require an additional Sr-specific resin column to remove the Sr. In addition to obtaining more highly purified samples, the HBr-based chemistry achieves elution curves of consistent shape, which result in quantitative yields of Ca to within a measurement uncertainty of 10%.

Nonquantitative yields in chromatography can lead to isotope fractionation similar to that occurring in nature and so it is critical to assess the extent of fractionation that could arise from nonquantitative yields.[19,28,29] To determine how Ca isotopes fractionate during elution, Ca-containing aliquots were collected and their isotope compositions measured (see Supplement Figure S2 in the Supporting Information for the Ca isotope elution curve). In addition, standards with marginal yields

between 80% and 90% were analyzed. (see Supplement Figure S3 in the Supporting Information). For example, Ostrich (whole bone) had a yield of 82%, and chicken eggshell had a yield of 89% as measured by the Q-ICPMS. The measured isotope values for these two are within analytical error of published values (see Supplement Table S3 and Figure S3 in the Supporting Information). We find that Ca isotopes are not measurably fractionated by the new chemistry if yields are >85%.

Assessing the Effect of Isobaric Interferences. Reliable and efficient sample purification is essential because isobaric interferences can affect all isotopes of Ca (Table 1). Modifications to published chemical purification protocols were required because they were found to be insufficiently rigorous in purifying biological samples like urine. Some of these isobaric interferences, such as doubly charged interferences of Sr, isobaric interferences of Ti, and polyatomic interferences of K, cannot be resolved by current mass spectrometric techniques. Therefore, they require either mathematical correction by measurement of a different isotope of the interfering element or sufficient chemical separation to reduce the effect of the interfering isotope below the measurement error. In order to determine the effect of interferences from isotopes of Sr, Ti, and K, we spiked Ca standards with varying amounts of each potentially interfering element and compared the measured Ca isotope ratios in the spiked samples with the known values of the pure standard. These experiments allowed us to determine the threshold of Ca/elemental ratios required for the purification protocol to minimize the introduced error.

Sr^{2+} ions interfere on $^{42}Ca^{+}$, $^{43}Ca^{+}$, and $^{44}Ca^{+}$ due to the contributions of $^{84}Sr^{2+}$, $^{86}Sr^{2+}$, and $^{88}Sr^{2+}$, respectively. Offline correction of interfering Sr isotopes on the Ca isotope measurements can be made if the samples are not free of Sr;[16] this correction requires $^{87}Sr^{2+}$ (m/z 43.5 u) production to be monitored and corrections to be made on ^{42}Ca, ^{43}Ca, and ^{44}Ca for the contributions from Sr^{2+}. This Sr correction assumes that the $^{87}Sr/^{86}Sr$ ratio and Sr^{2+} production efficiencies are consistent between samples. To avoid this correction and the need to monitor $^{87}Sr^{2+}$, Ca is purified of Sr in the second column of the column purification protocol. To determine the threshold ratio of Ca/Sr that is required, we doped Ca standard with Sr to produce a range of Ca/Sr ratios and characterized $\delta^{44/42}Ca$ and $\delta^{44/43}Ca$ in these doped standards (Figure 3A, see Supplement Table S4 in the Supporting Information). For a Ca/Sr ratio of 50, the measured effect is -3.69 ± 0.04 ‰ per amu ($\delta^{44/43}Ca$) and 2.21 ± 0.08 ‰ per amu ($\delta^{44/42}Ca$), much larger than the natural variation of interest. We matched this effect by assuming Sr^{2+} production efficiencies of 0.003% and $^{87}Sr/^{86}Sr = 0.720$; the values were within error of the measurements (Figure 3A open and closed symbols, respectively), demonstrating that this large measured effect can be attributed dominantly to Sr^{2+} interferences. Results from these experiments also indicate that the Ca/Sr ratio needs to be >10 000 in order for the Sr^{2+} contribution to be smaller than analytical error of 0.1 ‰ per amu on $\delta^{44/42}Ca$ and $\delta^{44/43}Ca$ (Figure 3A, see Supplement Table S4 and Figure S1B in the Supporting Information). This threshold is easily achievable using Sr specific resin, which reliably produces Ca/Sr >100 000.

There is also an isobaric interference from ^{48}Ti on ^{48}Ca. In theory, this effect could be corrected by monitoring ^{47}Ti. However, this correction has a large error because the natural abundance of ^{48}Ti is 73.8%, the abundance of the monitored ^{47}Ti is only 7.44%, and the average abundance of ^{48}Ca to be corrected

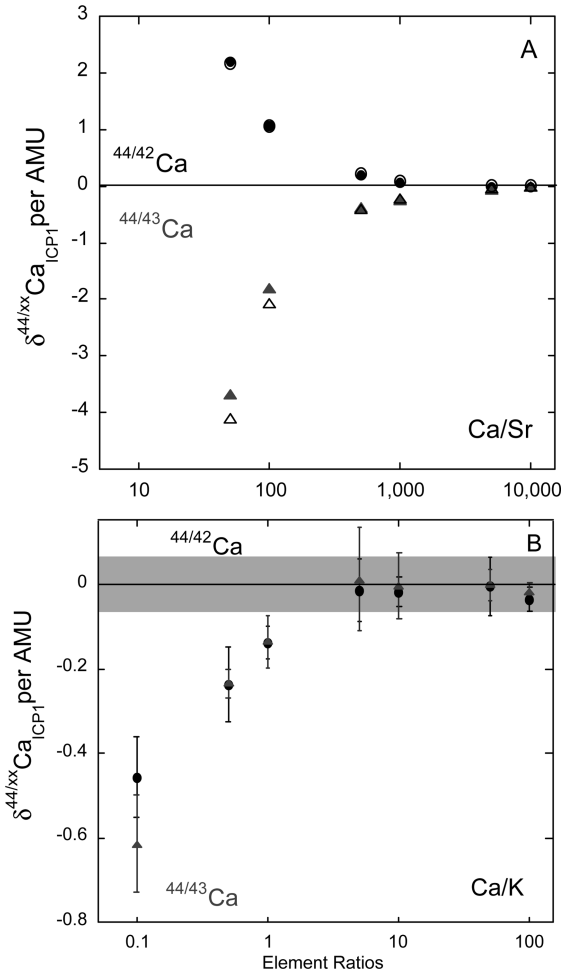


Figure 3. (A) $\delta^{44/4x}Ca$ per AMU as a function of decreasing amount of Sr in the Ca standard (NIST X-10-39Ca, ICP1) measured relative to ICP1 without Sr added. Error is 2σ on triplicate measurements. Closed symbols represent measured values while open symbols represent calculated values based on Sr^{2+} interference (see text for details). (B) $\delta^{44/4x}Ca$ per amu as a function of decreasing amount of K in the Ca standard (NIST X-10-39Ca, ICP1) measured relative to ICP1 without K added. Error is 2σ on triplicate measurements.

is only 0.187%. This means that even small amounts of Ti contamination could contribute significantly to the signal on ^{48}Ca. For example, even if the Ca/Ti is ~500, we find the uncorrected $\delta^{48/42}Ca$ and $\delta^{48/43}Ca$ offset by >150 ‰. The fractionation of Ti isotopes in the instrument may vary with time or sample matrix, introducing further uncertainty into the correction. In addition, we observe signals on mass 47 not attributable to Ti in natural samples, perhaps related to organic residues from the ion-exchange resin.

Although the HBr-based chemistry reliably obtains Ca/Ti > 10 000, far better purification is needed to reliably measure ^{48}Ca. Using the known isotope abundances and assuming 100% ionization efficiencies and normal instrumental sensitivities of Ca and Ti, we predict that Ca/Ti of >2 500 000 is required for the ^{48}Ti contribution to measured $\delta^{48/42}Ca$ and $\delta^{48/43}Ca$ to be less than the analytical error. This level of purity would be very difficult to achieve or even assess: A 5 ppm Ca solution would contain 2 ppt (parts per trillion) Ti, whereas the Ti detection limit by Q-ICPMS is typically 10 ppb due to high background

from polyatomic interferences. Therefore, it is not feasible to reliably measure isotope abundance ratios that include ^{48}Ca. For this reason we do not report ^{48}Ca ratios in our data. This is also a significant barrier to employing a ^{42}Ca-^{48}Ca double-spike.[8] In fact, the inability to reliably measure ^{48}Ca creates a challenge for the use of any double-spike technique. In order for the double spike data reduction routines to converge on accurate sample Ca isotope values, at least three ratios (four isotopes) need to be reliably measured. The major Ca isotope, ^{40}Ca (96.941%) cannot be measured by MC-ICPMS because of the Ar plasma and the overwhelming ^{40}Ar isotope interference on ^{40}Ca, while the very low abundance of ^{46}Ca (0.004%) makes accurately measuring this isotope difficult. Hence, with our existing sample type, cleaning protocols, and instrumentation, we are only able to measure two ratios accurately and precisely: $\delta^{44/42}$Ca and $\delta^{44/43}$Ca.

The other important matrix element that could affect Ca isotopic measurement is K, since $^{41}K^1H^+$ could interfere with ^{42}Ca (Table 1). When Ca/K in the samples is ≤ 1, $\delta^{44/42}$Ca and $\delta^{44/43}$Ca are offset from zero (Figure 3B, see Supplement Table S4 and Figure S1C in the Supporting Information). However, this interference does not explain the offset in $\delta^{44/43}$Ca. $^{41}K^1H^+$ is also restricted by the relatively low abundance of ^{41}K (6.7%) and the low efficiency of hydride formation. K-induced offsets in $\delta^{44/42}$Ca and $\delta^{44/43}$Ca are more likely the result of a matrix effect, such as might arise from different ionic strengths between samples and standards (due to different concentrations of K). Such differences may affect the instrumental mass bias in a manner that is not corrected by standard-sample-standard bracketing. Offsets of similar magnitude and direction are seen when Na and Mg are added to the Ca standard to produce Ca/Na and Ca/Mg ratios of ≤ 1. Therefore, for all alkali earth metals we require Ca/metal >10 (see Supplement Table S4 in the Supporting Information); the chemical purification method reported here reliably purifies samples to the requisite thresholds.

Assessing Accuracy. In addition to the precision of the analytical results reported in the Methods section above from replicate measurements of the same sample through chemistry ("external error"), the accuracy of the measurements was evaluated by measuring known samples. Samples independently measured by TIMS were processed and analyzed in our laboratory (Figure 4, see Supplement Table S3 in the Supporting Information). These included subsamples of the same bone and carbonate materials analyzed by Skulan et al.,[9] as well as new TIMS analyses of cow's milk and a suite of urine samples measured at the United States Geological Survey using established procedures.[1] For all sample types, our MC-ICPMS measurements are within error of TIMS values.

To further assess the accuracy of the isotopic compositions measured in a urine matrix, we collected the eluted matrix of a human urine sample purified of Ca using the HBr-based chemistry. This Ca-free matrix was then spiked with a Ca standard of known isotopic composition. This synthetic sample was reprocessed through Ca and Sr columns and analyzed on the MC-ICPMS. The measured Ca isotope value was within error of the standard value (Figure 4 [purified urine matrix spiked with ICP1], see Supplement Table S3 in the Supporting Information).

In a second experiment, we spiked a known standard (−0.01 ‰ ± 0.05, $\delta^{44/42}$Ca) with different amounts of a urine sample. Like the method of standard addition to determine concentration independent of matrix effects, this procedure can be used to determine the isotope composition of a urine

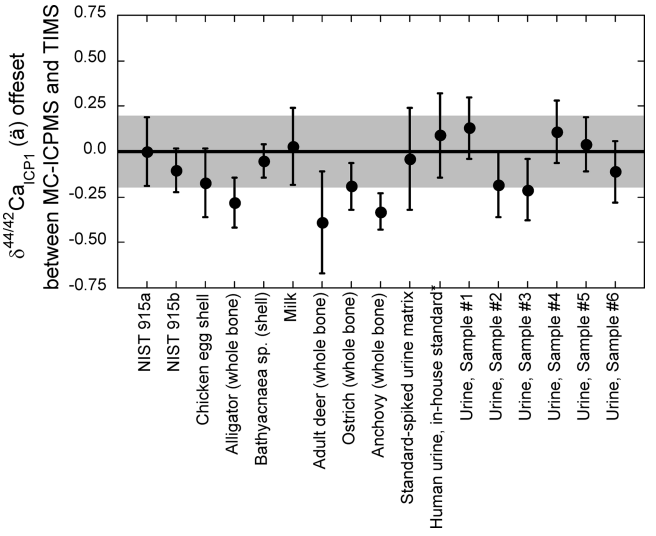


Figure 4. Offset of values measured by Neptune MC-ICPMS vs measurements by TIMS. Urine and milk samples were analyzed by TIMS as part of this study while all other TIMS measurements are taken from the literature. *Human urine, in-house standard" represents the average of seven replicate chemical purification procedures and MC-ICPMS measurements of the same material on multiple days; for all other data reported here, the material was chemically purified one time and analyzed n replicate times by MC-ICPMS (see Supplement Table S3). The gray bar represents our typical analytical uncertainty.

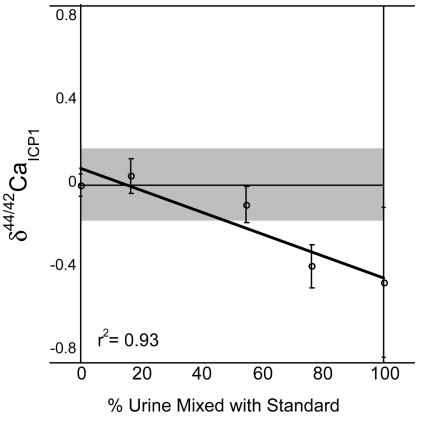


Figure 5. Standard urine mixture to determine urine measurement accuracy. A best-fit line $y = -0.0049(\pm 0.0009)x + 0.063(\pm 0.044)$, r^2 = 0.94 crosses the y-intercept and 100% urine sample within error of the measured value. The gray bar represents typical analytical uncertainty.

sample.[30] The y-intercept (0% urine sample) represents the isotope composition of the standard, while the value at y = 100% represents the isotopic composition of the pure urine sample. The urine-standard mixtures were processed and measured (Figure 5). A best-fit line was created for the urine-standard mixture samples ($y = -0.0049(\pm 0.0009)x + 0.063(\pm 0.044)$, $r^2 = 0.94$). The y-intercept (0% urine, 100% standard) is within error of the standard, 0.06‰ ± 0.04 ($\delta^{44/42}$Ca) and crosses y = 100% (100% urine, 0% standard) at −0.43‰ ± 0.04 ($\delta^{44/42}$Ca). Three separate aliquots of the pure urine sample were processed through column chemistry and measured, and the results at y = 100% averaged to be −0.43‰ ± 0.10 ($\delta^{44/42}$Ca) (Figure 5, see Supplement Table S5 in the Supporting Information). The fact

that the line crosses the *y*-intercept (ICP1 Ca standard) and that the extrapolated 100% urine value is within error of the measured value provides evidence that we are able to accurately measure Ca isotopes in complex matrix samples like urine.

CONCLUSIONS

We have demonstrated that Ca separation and purification using a new HBr-based protocol can be used to determine natural Ca isotope variations in urine samples using MC-ICPMS. In addition, we have quantified the degree of sample purity required to obtain accurate and precise Ca isotope values using MC-ICPMS. This study deals with human urine, but the elemental composition of urine is broadly similar to that of blood and serum, so the techniques developed to eliminate elements that interfere with MC-ICPMS analysis of urine also should work for other biomedical samples. Measurement of Ca isotope variations is a promising technique for detection of disorders affecting BMB and for assessing the effectiveness of treatments for those disorders. MC-ICPMS using the methods outlined here offers higher sample throughput than TIMS, and so our study lays the foundation for future research and applications of Ca isotope measurements in biomedicine.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

Funding provided by NASA's Human Research Project Grant Number NNX08AQ36G (A. D. Anbar). The authors thank Stephen Romaniello for helpful discussions about the fractionation pattern of Ca during elution. We thank Matt Fantle and an anonymous reviewer for their thoughtful comments, which greatly improved this manuscript.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on August 18, 2011 with the incorrect Synopsis and Abstract graphics. The corrected version was reposted on August 25, 2011.