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A simplified protocol for measurement of Ca isotopes in biological samples†

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We describe a chemical separation protocol of calcium from biological materials for isotopic measurement by multiple collector inductively coupled plasma mass spectrometry (MC-ICPMS). The method was tested using elution profiles along with HCl and HNO_3 acids only, on human urine, sheep serum and red blood cells (RBC), seawater and herbaceous plants. It allows the elimination of all interfering species (including K, Sr, Mg) and the remaining matrix (including Fe, P, Na and S) beyond required levels. In order to further test this protocol and better understand the Ca isotopic signatures of mammalian fluids and organs, we purified and analyzed a wide range of materials from sheep, *i.e.* serum, RBC, muscle, liver, kidneys, enamel, bone, urine and feces. The data show a wide range of variations, expressed as δ, over 1‰ per amu, with a precision of 0.1‰ or better, spanning most of the variability reported so far. Red blood cells appeared to be heavier than serum by 0.3‰ per amu. This isotopic difference between serum and red blood cells was not taken into account in previous studies and it provides further information on Ca isotopic cycling in organisms. The Ca isotopic compositions of organs are correlated with concentrations, bone and RBC representing the two end-members, bone being Ca rich and ^{44}Ca-depleted and RBC Ca poor and ^{44}Ca-enriched. The trend is compatible with a distillation process by which Ca is extruded from cells along with a kinetic fractionation process favoring lighter Ca isotopes.

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Introduction

The understanding of calcium (Ca) isotopic variations in biological systems constitutes a wide field of research with a growing interest in several disciplines. This encompasses Ca cycle studies on interactions between vegetation, soils and riverine systems,[1–8] present-day and fossil trophic level studies,[9–15] and biomedical research mainly focused on the role of bone in whole body Ca homeostasis.[10,12,16–19] This last topic is gaining importance because isotopic variations provide information on Ca homeostasis in healthy and pathological conditions.

Several studies on blood or urine Ca isotopic compositions were published, highlighting the variations of the bone mineral balance (BMB), defined as the bone mass gain to loss ratio.[16–19] The current box models linking Ca isotopic composition of blood or urine to the BMB are simplified. In these models, the Ca isotope compositions in mammalian tissues are mainly controlled by a significant isotopic fractionation, thought to occur during skeletal bioapatite precipitation, inducing a ^{44}Ca-depletion of the forming bone whereas the loss of Ca from bone is supposed to occur without sizeable fractionation.[9,10,12,16] Calcium isotopic mixing and fractionation occurring between the soft tissues of an organism are still poorly characterized and could affect the Ca isotopic composition of blood and urine. Moreover, bone is considered the main reservoir, and soft tissues are either described as an entire compartment,[10] or divided into several reservoirs: blood, kidneys and remaining soft tissues.[18,19] Clearly, efforts concerning the possible influence of other sub-reservoirs are needed to better understand the Ca cycling in mammals.

Using multiple collector inductively coupled plasma mass spectrometry (MC-ICPMS), we analyzed soft and mineralized tissues of sheep, including urine, blood (serum and red blood cells), muscle, liver, kidneys, bone, enamel, diet and feces. An important feature of biological materials is that Ca interfering species (such as K, Mg or Sr) show strong variations of concentrations and ratios to Ca from one sample type to the other. We tested their efficient elimination using a number of elution profiles.

Material and methods

Samples

Chemical purification of Ca was tested on seawater, freeze-dried human urine, sheep serum and red blood cells (RBC) and herbaceous plants. Seawater, SRM915b, bone meal NIST SRM1486, cave bear and two sheep enamel samples (CBE, E1634, E9646 respectively) were chosen to assess precision and

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fractionation mass-dependency during measurement sessions on MC-ICPMS. These standards were chosen because of their varying major element concentrations and their large range of Ca isotopic compositions.

Tissues and materials sampled during feeding experiment on sheep are serum, RBC, jawbone, enamel, liver, kidneys, muscle, urine, feces and diet based on herbaceous plants. The sheep were part of a feeding experiment fully described by Zazzo *et al.*,[20] and by Balter *et al*.[21] The experiment was carried out with the approval of the Teagasc, the Irish Agriculture and Food Development authority.

Digestion and sample dissolutions

Seawater, human and sheep urine samples (0.5 to 2 mL) were digested on a hotplate using 2 mL concentrated distilled HNO_3. Vials were heated at 120 °C for 2 h and regularly degassed. A volume of 2 mL Suprapur 30% H_2O_2 was then added on cooled samples and the vials were sealed, and regularly degassed at ambient temperature. Finally, vials were sealed and heated on a hotplate at 100 °C for 2 h and evaporated to dryness. Bone and enamel samples or standards (about 1 mg) were digested by adding 3 mL of distilled 3 N HNO_3. Vials were then sealed and heated at 100 °C on a hotplate for 3 to 5 h with repeated degassing, and evaporated to dryness. All other biological samples (100–400 mg) were digested by adding 10 mL of concentrated distilled HNO_3 in clean microwave bombs. Bombs were then sealed and introduced in a Milestone® Ethos microwave set to ramp to 150 °C in 20 min and to remain at 150 °C for another 20 min. Samples were then evaporated to dryness.

Calcium separation

The use of MC-ICPMS imposes efficient separation of Ca from samples because of isobaric interferences.[22,23] Strontium, potassium and to a lesser extent magnesium from samples have to be eliminated to avoid any bias in the measurement of $^{42}Ca^+$ (interfering with $^{84}Sr^{2+}$, $^{41}K^1H^+$ and $^{25}Mg^{16}O^+$), $^{43}Ca^+$($^{86}Sr^{2+}$) and $^{44}Ca^+$($^{88}Sr^{2+}$, $^{26}Mg^{18}O^+$). The elimination of the remaining matrix components is also required to avoid any bias due to matrix effects.

The first elution aims at discarding K and the majority of matrix elements. Once taken up in 1 N HCl, samples were processed on 0.76 cm internal diameter Teflon chromatography columns, filled with 2 mL of Biorad AG50W-X12 cationic resin, 200–400 mesh. The exact elution procedure is shown in Table 1. The AG50W-X12 columns were reused maximum 5 times, in order to avoid any aging of resin.

When Fe levels in samples were high compared to Ca, samples were processed on 2 mL Biorad columns, filled with 2 mL Biorad AG1-X8 resin (100–200 mesh). Iron is not a direct interfering species but has to be eliminated to avoid any matrix effects. The elution protocol followed for Fe elimination is a classical Fe extraction protocol (Table 1). Strontium was finally eliminated by processing samples on 0.7 mL Eichrom Sr-specific resin, packed in 2 mL Eichrom columns, following a classical Sr elimination protocol (Table 1).

Table 1 Elution steps of Ca purification[a]

Step	Eluent	Vol. (mL)
1. Matrix elimination		
AG50W-X12 resin (200–400 mesh) ~ 2 mL		
Condition	1 N HCl	10
Load	1 N HCl	2 + 1
Elution (matrix)	1 N HCl	55
Ca elution (Ca, Sr, Fe)	**6 N HCl**	**10**
2. Fe elimination if necessary		
AG1-X8 resin (100–200 mesh) ~ 2 mL		
Condition	6 N HCl	10
Load	**6 N HCl**	**1 + 1**
Elution (Ca, Sr)	**6 N HCl**	**6**
Remaining on resin: Fe (Zn, Cu)		
3. Sr elimination		
Sr-Specific resin (Eichrom) ~ 0.7 mL		
Condition	3 N HNO_3	5
Load	**3 N HNO_3**	**0.5 + 0.5**
Elution (Ca)	**3 N HNO_3**	**6**
Remaining on resin: Sr		

[a] Fractions marked in bold contain Ca and were recovered.

Concentrations, blanks and yields were measured using inductively coupled plasma atomic emission spectrometry (iCAP 6000 Series, Thermo Scientific Corporation) and quadrupole inductively coupled plasma mass spectrometry (7500 CX quadrupole mass spectrometer, Agilent Technologies).

MC-ICPMS measurement

Ca isotopic compositions were measured at the ENS-Lyon, using a Neptune plus MC-ICPMS (Thermo Scientific, Bremen, Germany). Standard and sample solutions were prepared to reach a 3 mg L^{-1} concentration in 0.05 N HNO_3 medium. Calcium solution was introduced as a dry aerosol with a Cetac Aridus II desolvating system allowing reduction of hydride and oxide formation. The Aridus desolvating system was used with Ar sweep gas flow and an additional N_2 gas flow. Aerosols were introduced in a 1200 W plasma with an uptake rate of 100 to 150 μL min^{-1}. The optimized MC-ICPMS instrument operating parameters were: cool gas (15 L min^{-1}), auxiliary gas (0.7–0.8 L min^{-1}) and sample gas (1–1.2 L min^{-1}). In this study, a sampler Jet Cone was used in combination with a skimmer X-cone in order to increase the sensitivity.[24] This combination allows a minimum 5-fold increase in transmission, compared to H sampler and skimmer cones. This is particularly important considering the low abundances of ^{42}Ca and ^{43}Ca, being respectively 0.647 and 0.135% of total Ca. Faraday cups were set to measure the $^{42}Ca^+$ signal on the L4 cup, $^{43}Ca^+$ on L2 and $^{44}Ca^+$ on the central cup. The use of these three isotopes is sufficient for mass-dependent stable isotope composition measurements in biological materials. $^{46}Ca^+$ and $^{48}Ca^+$ could also be measured, however these isotopes present low abundances and Ti induces strong interferences ($^{46}Ti^+$ and $^{48}Ti^+$), further complicating their measurement.[22–24] The L1 cup was used to monitor the $^{87}Sr^{2+}$

corresponding to $m/z = 43.5$. The 42 and 44 ion beam signals were measured with a 10^{11} Ω resistance on the faraday cup and 43 signal was measured with 10^{12} Ω resistance, because of the low abundance of ^{43}Ca (0.135% of total Ca). Calcium concentrations were adjusted to be within 10% of the fixed 3 mg L^{-1} concentration. This prevents significant changes of instrumental mass bias drift.[24] For a 3 mg L^{-1} Ca concentration, typical signal intensities yielded values of 15 V for $^{44}Ca^+$, 4 V for $^{42}Ca^+$ and 0.9 V for $^{43}Ca^+$. Medium mass resolution was sufficient to resolve polyatomic interferences: including $^{40}Ar^1H_2{}^+$, $^{12}C^{16}O_2{}^+$ and $^{14}N_3{}^+$ (Fig. S1 in the ESI†). As described by Wieser *et al.*,[22] we measured uninterfered Ca ion beam intensities on the low-mass flat shoulders of the Ca ion intensity peaks (on the left side of peaks, see Fig. S1†). Each sample analysis was preceded by 300 s washout pumping in 0.5 N HNO_3 to reach a well stabilized and minimal signal (2 to 4×10^{-3} V on ^{44}Ca) and 300 s in 0.05 N HNO_3 for conditioning of the desolvating system. Each analysis consisted of 40 measurements of 4.2 s integrations on m/z ratios 42, 43, 44 and 43.5. Even if Sr levels were very low due to the specific separation on the Sr-Spec resin, we corrected signals for Sr double charge interferences on Ca isotopes ($^{88}Sr^{2+}$ on $^{44}Ca^+$, $^{86}Sr^{2+}$ on $^{43}Ca^+$, and $^{84}Sr^{2+}$ on $^{42}Ca^+$) using the 43.5 signal corresponding to $^{87}Sr^{2+}$. We applied an exponential law using NBS 987 ratios as reference values ($^{88}Sr/^{87}Sr = 11.7952$, $^{86}Sr/^{87}Sr = 1.4078$, and $^{84}Sr/^{87}Sr = 0.0796$). The instrumental mass bias on Sr isotopes was estimated by measuring NBS 987 standard under the same instrumental conditions, and yielded a mass discrimination f value of 1.5. For the $^{44}Ca^+$ signal, the correction thus corresponds to $V_{44\text{-c}} = V_{44} - V_{43.5} \times 11.7952 \times (M_{88}/M_{87})^{1.5}$, where subscripts 44-c, 44 and 43.5 denote the corrected $^{44}Ca^+$, measured $^{44}Ca^+$ and $^{87}Sr^{2+}$ signals respectively, and where M_{88}/M_{87} represents the ^{88}Sr to ^{87}Sr isotope mass ratios. Corrected and uncorrected ratios were calculated for each measurement and values higher than 1SD from average were excluded. Instrumental mass bias was corrected by standard-sample-standard bracketing. A Specpure calcium plasma standard solution (Alfa Aesar), noted ICP Ca Standard below, was used as reference and bracketing in-house standard. ICP Ca Standard required purification because of the presence of Sr traces responsible for significant interferences on the three measured isotopes. A stock of ICP Ca Standard was thus purified through Sr-specific resin, using the same protocol as described above. Calcium isotopic compositions are reported using δ notation with respect to the in-house ICP Ca Standard for measured isotopic ratios: $\delta^{44/42}Ca$, $\delta^{43/42}Ca$ and $\delta^{44/43}Ca$. Mass-dependency of measured isotopic compositions was assessed by comparing $\delta^{44/42}Ca$ to $\delta^{43/42}Ca$ or $\delta^{44/43}Ca$. This allowed checking that no isobaric interference significantly affects the measured isotopic ratios. Long-term spectrometric reproducibility was evaluated by running international and secondary standards several times during a whole analytical session. This encompasses SRM915b, SRM1486 and seawater international standards as well as Cave Bear Enamel (CBE), E1634 and E9646 sheep enamel secondary standards. SRM915b was processed through Sr-specific resin in order to avoid Sr induced interferences. All other standards were processed on AG50W-X12 cationic resin and Sr-specific resin. For all standards, the reproducibility varied between 0.03 and 0.10‰ per amu for $\delta^{44/42}Ca$ (2SD). Accuracy of measured isotopic compositions was monitored during each session by comparing measured $\delta^{44/42}Ca$ of SRM1486 and seawater to literature values (Table 2). The measured isotopic composition of SRM915b, expressed with respect to the ICP Ca Standard, was used to transpose the isotopic compositions of standard literature values relatively to ICP Ca Standard (Table 2).

Results

Calcium elution

The first elution, on cationic resin, aims at discarding the sample matrix, irrespective of sample type. Elution curves are presented in Fig. 1 for human urine, seawater, red blood cells and herbaceous plants of the sheep diet (elution curve for serum is presented in Fig.S2 in the ESI†). All of these samples present different ratios of Ca to other elements, including P, Na, K and Mg (Table 3). Curves of Fig. 1 show that for all these matrices, P, Na and both K and Mg are successively and totally discarded in the first elution stage. Each peak of the elements is quite sharp and particularly reproducible from one matrix to the other. Potassium is efficiently removed from all kinds of matrices, even for materials with very low Ca/K ratios, such as urine. K is an interfering species, which can affect the measurement of $^{42}Ca^+$ by forming $^{41}K^1H^+$. It has been shown that there might also be matrix effects of K hydrides during measurement and that Ca/K ratios must be greater than 10 to avoid any bias during Ca isotopic measurement.[23] Using this protocol, Ca/K, Ca/Mg and Ca/S ratios after Ca separation through cationic resin usually exceed 70, 150 and 70, respectively. Iron is efficiently discarded on anionic resin, with a Ca/Fe ratio greater than 150 after processing of Fe rich samples. Only urine, bones and enamel were not treated on this resin, due to their high Ca/Fe ratios (>800 at minimum).

Chemical procedural yields of all samples for the entire Ca separation protocol exceeded or equaled 95% of recovered Ca, which is greater than the 85% threshold described by Morgan *et al.*,[23] for chromatography induced fractionation. Blanks for the whole procedure did not exceed 100 ng Ca. This is 400 times smaller than processed Ca (about 40 μg), and could not affect the measured isotopic compositions beyond the measurement precision.

Calcium isotopic ratios and concentrations

All measured isotopic compositions of sheep organs and standards are presented in Table S1 in the ESI,† together with 43.5/44 ratios. Mass-dependent fractionation of isotopic measurements of standards (Fig. 2A) and samples (Fig. 2B) was assessed by comparing the measured $\delta^{44/42}Ca$ to $\delta^{43/42}Ca$. The slope of the regression line is theoretically 0.5 following the linear approximation of mass-dependency. The 0.484 ± 0.011 and 0.500 ± 0.020 slopes for standards and samples respectively, together with squared correlation coefficients of 0.976 and 0.963, are in agreement with the mass-dependent fractionation of biological materials.

Table 2 Measured isotopic compositions of standards and comparison to literature values[a]

Material	$\delta^{44/42}$Ca (‰ p. amu)	Study
SRM915b	−0.06 ± 0.04 (2SD, n = 11)	This study (MC-ICPMS)
SRM1486	−0.48 ± 0.07 (2SD, n = 17)	This study (MC-ICPMS)
	−0.48 ± 0.02 (2SD)	Heuser *et al.*, 2011 (TIMS)
	−0.49 ± 0.01 (2SD, n = 142)	Heuser and Eisenhauer 2008 (TIMS)
Seawater	0.21 ± 0.03 (2SD, n = 2)	This study (MC-ICPMS)
	0.23 ± 0.04 (2SE)	Hippler *et al.*, 2003 (TIMS)
	0.20 ± 0.06 (2SD, n = 54)	Wieser *et al.*, 2004 (TIMS)
CBE	−0.52 ± 0.03 (2SD, n = 9)	This study (MC-ICPMS)
E1634	−0.56 ± 0.03 (2SD, n = 5)	This study (MC-ICPMS)
E9646	−0.46 ± 0.10 (2SD, n = 8)	This study (MC-ICPMS)

[a] $\delta^{44/42}$Ca values are given relatively to in-house ICP Ca Standard. Standard literature values relatively to SRM915b were calculated using a $\delta^{44/42}$Ca value of SRM915b measured by TIMS of 0.355‰ per amu, relatively to SRM915a.[25,29] Literature values of standards were then transposed relatively to ICP Ca Standard using the SRM915b measured isotopic composition of −0.06‰ per amu relatively to ICP Ca Standard. Uncertainties (2SD or 2SE) are given together with n number of measurements when available. Measurement techniques are indicated together with literature references.

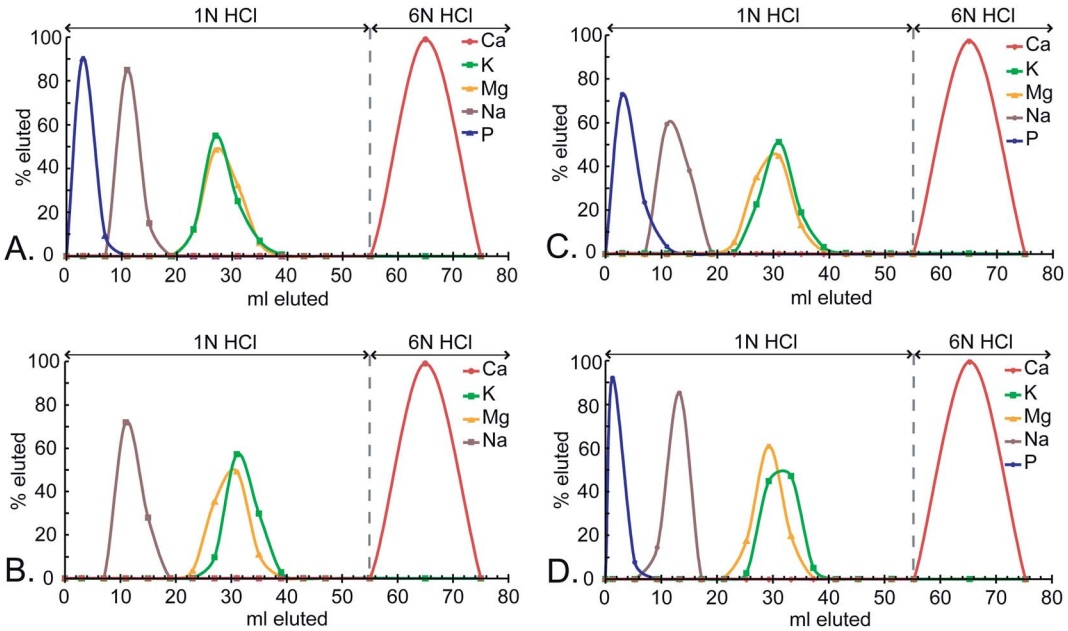

Fig. 1 Elution profiles of major mineral constituents on AG50W-X12 columns for urine (A), seawater (B), red blood cells (C) and herbaceous plants (D). Each curve represents the mass percentage of the eluted element as a function of introduced eluent volume.

Elution on AG50W-X12 induces no measurable change in isotopic composition processed samples. SRM1486 and seawater, that were processed on both cationic and Sr-specific resins, have indistinguishable isotopic compositions when compared to literature values (Table 2). Briefly, SRM1486 was measured at −0.48 ± 0.07‰ per amu, Heuser and Eisenhauer[25] and Heuser *et al.*,[15] reported a value of −0.49 ± 0.01‰ per amu and −0.48 ± 0.02‰ per amu, respectively. Seawater was at 0.21 ± 0.03‰ per amu, Hippler *et al.*,[26] and Wieser *et al.*,[22] reported 0.23 ± 0.04‰ per amu and 0.20 ± 0.06‰ per amu, respectively. These measurements are thus in accordance with other published results.

Results for sheep are plotted in Fig. 3. All $\delta^{44/42}$Ca isotopic compositions are reported in ‰ per amu, if not otherwise stated. For samples with 43.5/44 ratios greater than 10^{-5}, the difference between corrected and uncorrected $\delta^{44/42}$Ca exceeded the standard reproducibility. Below this threshold value, no significant difference was observed. All isotopic data presented here are thus corrected for Sr isotopes. Ca isotopic compositions of sheep samples vary between −0.6 and +0.5‰. This 1.1‰ overall variability spans most of the variability yet reported for mineral and biologic isotopic compositions, characterized by a typical 1.3‰ range.[13] Three samples of dietary ratio, sampled at different times during experiment, give a $\delta^{44/42}$Ca average value of −0.10 ± 0.08‰, while feces compositions yield an average of −0.13 ± 0.07‰, which makes feces and diet isotopically undistinguishable. RBC and urine have heavier isotopic compositions than average diet, by up to about 0.35‰ relative to diet mean, whereas serum, enamel, bone and kidneys are ^{44}Ca depleted, by up to −0.5‰ relative to diet. Muscle and liver

Table 3 Ca yields for first step purification columns and elemental compositions of urine, seawater, RBC, serum and herbaceous plants[a]

Material		Ca (ppm)	Fe (ppm)	K (ppm)	Mg (ppm)	Na (ppm)	P (ppm)	Sr (ppm)	Yields (%)
Human urine	Dry	2660	—	61319	862	63542	8398	—	101.6
Serum (#9646)	Dry	1003	41	2672	308	31105	138	—	100.2
RBC (#9646)	Dry	88	2411	1378	75	7466	75	—	96.9
Plants (FM1)	Dry	5379	80	6955	2058	7492	3434	34	101.0
Seawater	Wet	420	—	378	1223	9870	—	—	99.0

[a] Concentrations are given for freeze-dried samples except for seawater. When concentrations were below the detection limit of ICP-AES, no value is indicated. Uncertainties on concentration values are typically 5% and 2 to 3% for yield values.

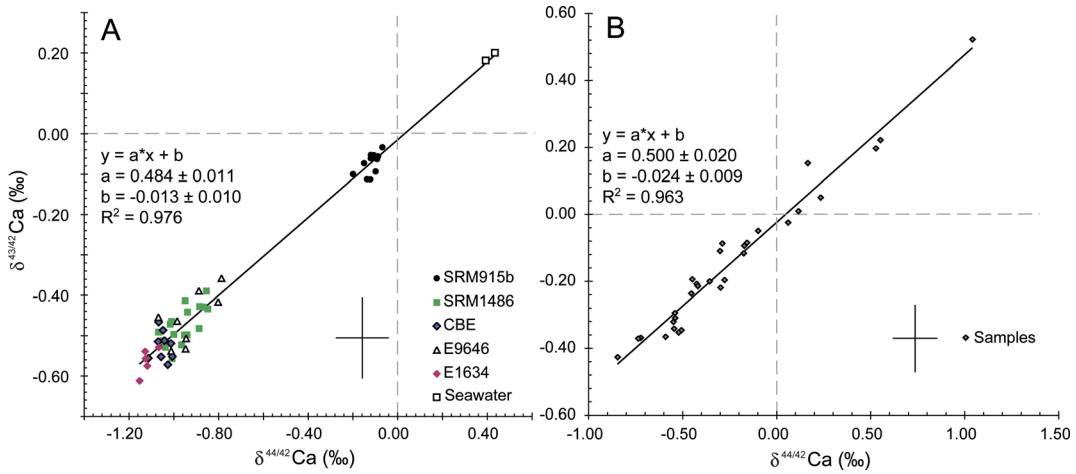

Fig. 2 $\delta^{43/42}$Ca *vs.* $\delta^{44/42}$Ca for standards (A) and samples (B). Best-fit lines are presented together with line equations and squared correlation coefficient of linear regressions. δCa values are expressed relatively to ICP Ca Standard. The slopes of regression lines are 0.484 and 0.500, which is in general agreement with the 0.5 slope predicted by the linear approximation of mass-dependent fractionation. Error bars represent the average of external reproducibility of standard measurements.

display significant inter-individual variability, respectively varying between −0.2 and +0.1‰ and −0.2 and +0.5‰.

Discussion

The main purification step consists in removing the matrix using a cationic resin in diluted HCl medium. This approach has been used in several studies.[17,22,24,27–29] In 2011, Morgan *et al.*[23] proposed a protocol for Ca isotopic measurement in urine, involving purification on cationic resin using HF and HBr acids. We show here that efficient purification of Ca from biological materials of mammals, including urine, serum, RBC and organs, is possible using only HCl acid on cationic resin, avoiding the additional use of dangerous HF and expensive HBr acids.

Bone tends to have heavier Ca isotopic compositions than enamel, by 0.15‰ on average. Two hypotheses can be proposed, which are not mutually exclusive. The first one involves the influence of a suckling signal in the enamel Ca isotope composition. The low Ca isotope composition of milk (about −0.2‰ per amu relative to diet[13]) could have influenced that of enamel during tooth formation. The second one implies that the supposed ^{44}Ca-depletion during bone formation[9,10,12,16] is very efficient during enamel formation. The formation of the mineral phase of bone and teeth, called carbonate hydroxylapatite (CHA), is the result of two different successive mineralization phases, *i.e.* the matrix deposition stage and the maturation stage.[30] Enamel CHA is much more crystallized than bone CHA, because the maturation stage is more intense and lasts longer. It is therefore tempting to conclude that the more intense the CHA maturation phase is, the stronger is the ^{44}Ca-depletion process. However, this does not definitely rule out that the Ca isotopic difference between enamel and bone can be due to different efficiencies of Ca translocation through ameloblast and osteoblast during the enamel and bone matrix deposition stage,[31] respectively.

The isotopic composition of Ca for each sample was measured along with its concentration (Table S1 in the ESI†). A plot (Fig. 4) reveals that the $\delta^{44/42}$Ca value of the bone, serum, muscle, kidneys and RBC are very significantly correlated with that of 1/[Ca] ($r^2 = 0.78$; $p < 10^{-4}$). Bone and RBC are the two end-members, bone being Ca rich and ^{44}Ca-depleted and RBC Ca poor and ^{44}Ca-enriched. Close to bone are the values of serum. The trend between serum and RBC is compatible with a distillation process by which Ca is extruded from cells along with a kinetic fractionation process favoring lighter Ca isotopes. The poorer the Ca content of a cell, the higher is its $\delta^{44/42}$Ca value. Extracting Ca from the cytosol is vital for all eukaryotic

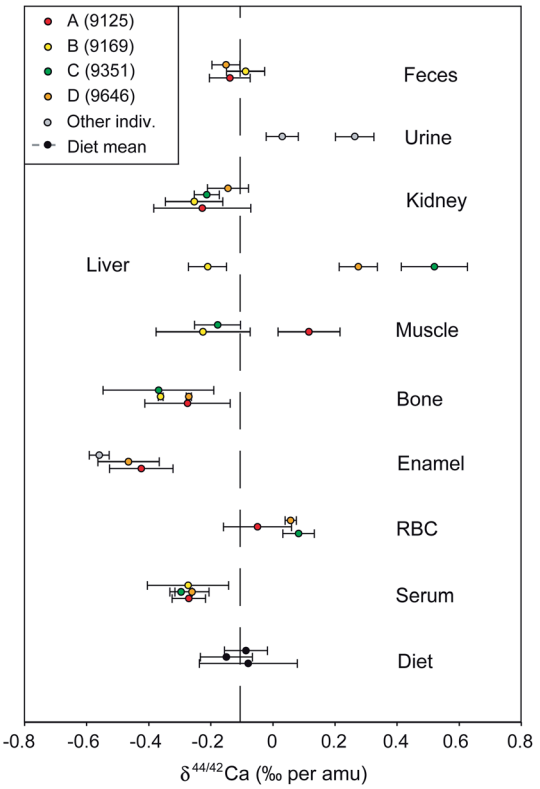

Fig. 3 $\delta^{44/42}$Ca values for sheep sampling, expressed relatively to ICP Ca. Standard colored circles represent different individuals. The grey ones indicate other individuals contributing to the dataset. For each sample, error bars represent the 2SD external reproducibility. The dotted line corresponds to the mean isotopic composition of diet.

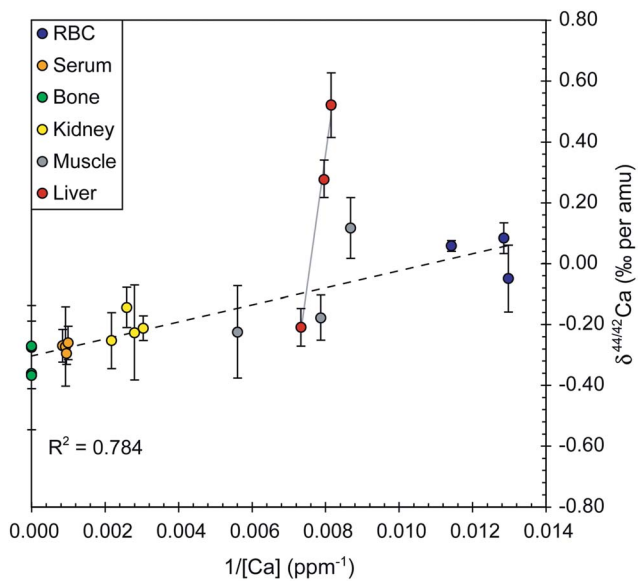

Fig. 4 $\delta^{44/42}$Ca *vs.* 1/[Ca] of bones and soft tissues for sheep individuals. The black dotted line is the linear regression applied on all materials, liver excepted. Note that the squared correlation coefficient is 0.784. $\delta^{44/42}$Ca is expressed relatively to ICP Ca Standard and error bars are 2SD uncertainties.

cell types because a very low intracellular Ca level is necessary to sustain Ca as an efficient secondary messenger. This is achieved with the plasma membrane ATPase pump (PMCA) and Na^+/Ca^{2+} exchanger.[32] This seems to hold for liver too, but in a more efficient way than for other cell types (Fig. 4). In hepatocytes, the rate at which Ca is extruded seems to be accompanied by a very strong, and cell-specific ^{40}Ca-depletion. The extent by which Ca is extruded from cells is probably the driving process by which the Ca isotope composition of soft tissues is fractionated in living organisms.

Past and present-day trophic level studies rely on the assumption that the $\Delta_{\text{diet–bone}}$ offset, defined as $\delta^{44/42}\text{Ca}_{\text{bone}} - \delta^{44/42}\text{Ca}_{\text{diet}}$, is constant and is about −0.30 to −0.35‰ per amu.[10,18] We measured a $\Delta_{\text{diet–bone}}$ value of about −0.2‰ per amu, close to the previous observations. However, in studies focusing on biomedical issues, soft tissues are assumed to have the same Ca isotope composition as diet, *i.e.* $\delta^{44/42}\text{Ca}_{\text{soft}} = \delta^{44/42}\text{Ca}_{\text{diet}}$,[10,19] the same holding true between blood and soft tissues, *i.e.* $\delta^{44/42}\text{Ca}_{\text{soft}} = \delta^{44/42}\text{Ca}_{\text{blood}}$.[15,18] Considered together, these assumptions imply that $\delta^{44/42}\text{Ca}_{\text{diet}} = \delta^{44/42}\text{Ca}_{\text{blood}}$. We measured in the present study $\delta^{44/42}$Ca values for serum, which accounts for more than 99% of total blood Ca, of −0.27 ± 0.03‰ and diet $\delta^{44/42}$Ca values of −0.10 ± 0.08‰. When considering the mean $\delta^{44/42}\text{Ca}_{\text{RBC}}$ value of 0.03 ± 0.14‰, total blood isotopic composition remains unchanged, with a $\delta^{44/42}\text{Ca}_{\text{blood}}$ value of −0.27 ± 0.03‰. Blood serum from sheep was sampled on healthy steady-state individuals and the four specimens have particularly close $\delta^{44/42}$Ca values. Our measured $\Delta_{\text{diet–blood}}$ offset of −0.2‰ is therefore in contradiction with the previous assumption that $\delta^{44/42}\text{Ca}_{\text{diet}} = \delta^{44/42}\text{Ca}_{\text{blood}}$. Instead we found that $\delta^{44/42}\text{Ca}_{\text{bone}}$ is similar to $\delta^{44/42}\text{Ca}_{\text{serum}}$, and therefore to $\delta^{44/42}\text{Ca}_{\text{blood}}$. We demonstrate that including the latter equality into the model of Skulan and DePaolo[10] which was further developed by Heuser and Eisenhauer[18] yields null solution. Taking eqn (3) and (4) of Heuser and Eisenhauer[18] into account gives:

$$\delta^{44/42}\text{Ca}_{\text{bl}} = \delta^{44/42}\text{Ca}_{\text{di}} + \frac{V_{\text{l}}\left(\delta^{44/42}\text{Ca}_{\text{bo}} - \delta^{44/42}\text{Ca}_{\text{di}}\right) - V_{\text{g}}\Delta_{\text{bo–bl}}}{V_{\text{d}} + V_{\text{l}}} \quad (1)$$

where the subscripts bo, bl and di denote the Ca isotope compositions of bone, blood and diet, respectively, V_{l}, V_{g} and V_{d} represent Ca fluxes from bone loss, bone gain and diet, respectively, and Δ the Ca isotopic fractionation between two compartments. Rearranging eqn (1) to express V_{l} as a function of V_g gives:

$$V_{\text{l}} = \frac{-V_{\text{g}}\Delta_{\text{bo–bl}} - V_{\text{d}}\left(\delta^{44/42}\text{Ca}_{\text{bl}} - \delta^{44/42}\text{Ca}_{\text{di}}\right)}{\delta^{44/42}\text{Ca}_{\text{bl}} - \delta^{44/42}\text{Ca}_{\text{bo}}} \quad (2)$$

where the denominator has a null value. Therefore, our Ca isotopic values do not seem to match the structure of the current model of bone mineral balance (BMB) based on Ca isotopic variations in blood[10,18] and urine.[19] Clearly, our results call for more experimental studies to understand the Ca isotopic cycling in mammals, first in the context of normal conditions, and then in that of pathological imbalances.

Conclusion

We propose a simplified Ca separation protocol for a wide range of biological materials involving the use of HCl and HNO_3 only, while maintaining quality requirements, *i.e.* elimination of all interfering species and the remaining matrix beyond required levels.

We demonstrate that the Ca isotopic compositions in a suite of organs of sheep raised with a controlled diet are inversely correlated with Ca concentrations. This correlation is compatible with a distillation process by which Ca is extruded from cells along with a kinetic fractionation process favoring lighter Ca isotopes. We also demonstrate that the Ca isotope compositions of serum and RBC are significantly different, and more importantly, that the Ca isotope compositions of serum and bone are similar. This suggests that it is not possible to assume systematically that diet, soft tissues and blood have identical Ca isotope compositions. Calcium isotope cycling needs to be further investigated, at the scale of an organism, by focusing on soft tissues and fluids in healthy and pathological conditions and at a cellular or molecular scale, by studying the effect of free and transmembrane Ca-binding proteins on the Ca isotopic variations.

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