Metal Stable Isotopes in the Human Body: A Tribute of Geochemistry to Medicine

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Metalloproteins play essential roles in biology and medicine. Calcium is a major component of bones, while electron and oxygen transport in the body relies on iron and copper. Isotope fractionation of metal stable elements uniquely reflects specific biochemical pathways. Variations in these isotope ratios from normal levels in body fluids can be used as reliable markers of pathological conditions. Metal stable isotope fractionation reflects the energetics of bonding, is amenable to theoretical calculations and is fast becoming a powerful medical diagnostic tool. Examples include how calcium isotopes can be used to monitor bone loss, how iron isotopes can react to genetic disorders, and how copper isotopes can help track cancer progression.

KEYWORDS: metallomics, stable isotope fractionation, medical isotopes, bone loss, cancer, biomarker

INTRODUCTION

Medical uses for the conventional stable isotope systems of C, H, O, N, and S, are very limited because these elements are ubiquitous and, as such, are not specific (diagnostic of) any particular biological process. However, elements such as the alkaline earth metals (Ca, Mg) and transition metals (e.g. Cu, Zn, Fe) appear more promising as medical diagnostic tools precisely because they *are* often more specific in biological functions and because their turnover rate in the body is relatively short. This is, for example, the case for iron in the heme molecule, which is a large heterocyclic organic ring porphyrin and a component of hemoglobin, which itself is a metalloprotein used by the body to shuttle oxygen and carbon dioxide around in the bloodstream. Likewise, copper plays a major role in moving iron from organs and tissues into the bloodstream. Calcium, as we saw in the previous issue of *Elements* (v11n3), is an essential component of bone apatite. Herein, I will review some appealing, and intriguing, applications of stable metal isotopes to medicine.

Whenever the word "isotope" is overheard in a medical context it often evokes thoughts of radioactive isotopes, such as technetium-99m (radioactive, metastable, Tc-99) or cobalt-60, used for radiotherapy. The public might also associate medical uses of isotopes with nutrition studies in which enriched stable isotopes are added to a diet to monitor the transit of a particular element. Both these uses of isotopes are invasive in the sense that they interfere with the natural metabolism of the patient, even if only to a trivial degree.

To understand stable isotopes in medicine and why their use differs from radioactive isotopes, we first need to establish a few concepts. The abundances of stable metal isotopes, which are *naturally* present in the body of humans and other organisms, tend to vary. This is known as the *isotope effect*, a term that describes the mass-dependent variations of natural isotope abundances for a particular element. The isotope effect is a consequence of the Heisenberg uncertainty principle on the distribution of energy levels of molecular vibrations. The energy of the lowermost vibrational energy state depends on the mass of the atoms involved in bonding, a characteristic that is at the heart of the isotopic variability of a given element between different parts of a biological system.

Isotope fractionation is the general term referring to differences in isotope abundances. It can be explained in a simple way: heavy isotopes vibrate more slowly than their lighter kin, and, because bond energy is proportional to vibrational frequencies, heavy isotopes tend to preferentially occupy the lowermost energy levels. At high temperatures, this tendency is opposed by the second law of thermodynamics, which works to randomize the distribution of isotopes across energy levels. At ambient temperatures, however, the total energy is minimized when heavy isotopes concentrate into the "stiffest" bonds, i.e. those with the lowest and, therefore, most stable energy levels (Bigeleisen and Mayer 1947). For a given element, the strength of a particular bond is expected to be higher for ions with higher charges and where bond energy is shared between fewer partners. Heavy isotopes will favor bonds that involve elements in high oxidation states (as for Fe^{3+}, Cu^{2+}) and that are in structural sites with small coordination numbers.

Kinetic effects may also play a role. The fact that lighter isotopes have smaller activation energies allows them to react faster: kinetic effects are thought to be a cause of biologically mediated isotope fractionation (Gussone et al. 2003), but they would require either non–steady state conditions or the existence of competing reaction pathways (FIG. 1).

Why has it taken so long for metal isotopes to be applied to biology and medicine? Calcium isotopes benefited from decades-long experience with thermal ionization mass spectrometry (TIMS) (Skulan et al. 1997). But it was only in the late 1990s that the development of multiple collector inductively coupled plasma mass spectrometry (MC–ICPMS) made it possible to precisely assess the isotopic variability of metals such as Cu, Zn, and Fe in biological material (Maréchal et al. 1999; Zhu et al. 2000). For example, using MC–ICPMS, isotope fractionation of Fe, Cu, and Zn was observed between body parts and fluids of

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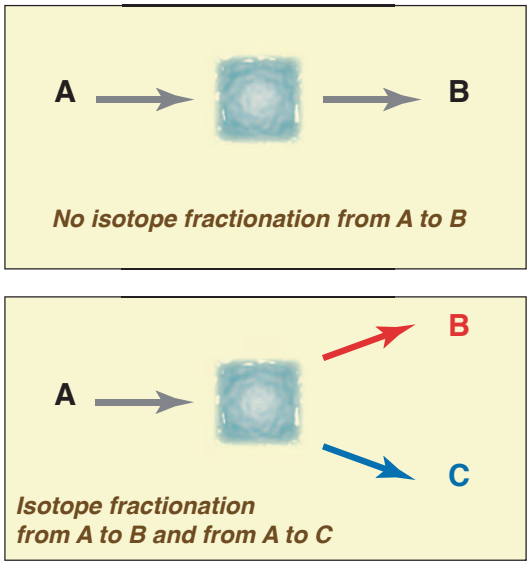

Figure 1 (**top**) At steady state, each individual isotope of each element is conserved during its transit, hence the cell or organ (represented by the blue square) is reduced to a single input and a single output that does not modify isotopic abundances. (**bottom**) When two pathways compete for an element, A to B and A to C, its individual isotopes become fractionated by the cell or organ.

humans, sheep, and mice at the per mil level (Walczyk and von Blanckenburg 2002; Balter et al. 2010, 2013; Moynier et al. 2013).

Let us try to find some guidelines on how to apply stable metal isotopes to medicine, a scientific field I suggest we refer to as *medical isotope metallomics*. The hassle of experiments makes it impractical to acquire, in the foreseeable future, the large amount of isotope fractionation data required for the very large number of medically relevant compounds. These data must, therefore, be computed by theoretical methods, typically by ab initio techniques such as density functional theory, which allows the electronic structure of atoms and molecules to be computed from first principles. The results are usually reported on the "ln β" scale, which may be thought of as ranking the relative appetite that different bonds have for heavy isotopes over their lighter relatives. The ligands that most frequently bond with metal ions involve certain inorganic anions (Cl^-, OH^-, HCO_3^-, SO_4^{2-}, PO_4^{3-}), functional O^- anions (such as lactates CH_3 CH (OH) COO^-), and covalently bound atoms (such as S in cysteine or N in histidine, two of the most common amino acids). The attraction of metals to charged particles may be conveniently illustrated by their ionization energy or by their electronegativity (Fig. 2): bonds with O (or OH) and N (or NH_2) are expected to be much stronger than bonds with S, whereas bonds with Zn should be stronger than equivalent bonds with Ca. Isotopic variations in body fluids and organs should, therefore, follow rather simple principles: heavy isotopes should prefer bonds with OH^-, HCO_3^-, PO_4^{3-} over Cl^-; histidine over cysteine; and Fe^{3+} and Cu^{2+} metalloproteins should be isotopically heavier than those containing Fe^{2+} and Cu^+, respectively.

CALCIUM STABLE ISOTOPES IN THE BODY: A MEDICAL DIAGNOSTIC TOOL FOR BONE DISEASES

Ninety-nine percent of calcium in our bodies resides in our bones in the form of hydroxylapatite, which is continually being renewed over our entire life. An average adult has a healthy balanced Ca diet of ~1 g per day (Matkovic and Heaney 1992), which corresponds to an overall bone turnover time of a few years. As a consequence, the much-feared condition of bone loss is a dynamic process. The bone-building cells (osteoblasts) produce collagen fibers on the outer part of new bone material. Alkaline phosphatase, an enzyme located in the membrane of osteoblasts, liberates phosphate from phosphate esters, such as ß-glycerophosphate (Chung et al. 1992), which allows apatite precipitation and mineralization. Bone demolishers (osteoclasts), on the other hand, are responsible for resorption, which returns Ca and phosphate to the bloodstream, a process regulated by parathyroid hormone. Lack of Ca regulation results in arterial calcification, a common disease of the elderly and of patients on dialysis. In the bloodstream, Ca^{2+} ions exist in three forms: free ions (50%); bound to albumin (40%); and bound to proteins (10%). Free Ca is one of the most tightly regulated parameters in the body, and parathyroid hormone regulates concentrations in the cell-free liquid fraction of the blood (serum) by stimulating bone resorption and Ca reabsorption by the kidney. The soft cysteine that is present in albumin, which would favor isotopically light Ca isotopes relative to the free-ion pool, does not seem to bind significantly with Ca^{2+} (Kragh-Hansen and Vorum 1993).

Calcium was the first non-conventional element for which variations of its six isotopes were investigated (Skulan et al. 1997). Calcium isotopes are routinely analyzed by two methods: thermal ionization mass spectrometry (TIMS) and multiple-collector inductively coupled plasma mass spectrometry (MC-ICPMS). Two main results have emerged from all the analyses. First, Ca becomes isotopically lighter as it moves through the food chain. Second, bone Ca is isotopically light (Skulan and DePaolo 1999; Reynard et al. 2010), which may come as a surprise because, as predicted from the electronegativity scale, heavy Ca isotopes should favor PO_4^{3-} over carboxylate and carbonyl groups. Bone deficit in heavy Ca isotopes inevitably reflects Ca binding with soft ligands, while the combination of Ca^{2+} with

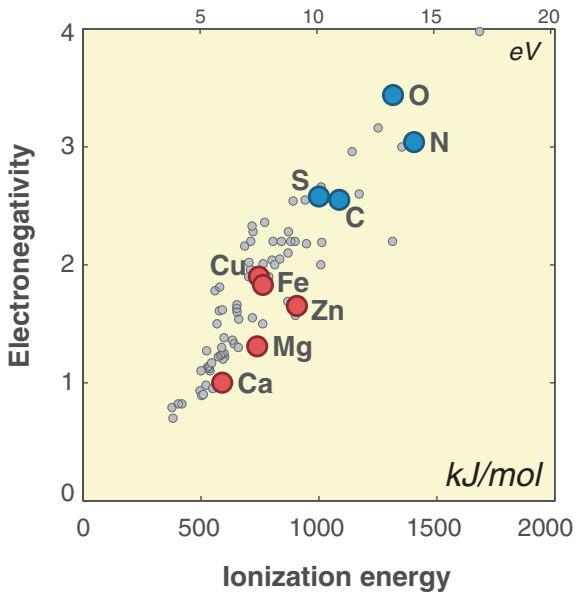

Figure 2 Graph of an element's electronegativity versus ionization energy. The strength of the bond can be roughly estimated using the energy of the first ionization potential or using the element's electronegativity: higher energy/potential = stronger bonds. Strong bonds preferentially incorporate heavy isotopes. This is the case for nitrogen, as in the amino acid histidine, and oxygen, as in lactate. In contrast, sulfide bonds, as in the amino acid cysteine, tend to preferentially incorporate light isotopes. Red dots represent metals for which isotope compositions are sought. Blue dots represent ligand-forming elements.

phosphate liberated by phosphate esters into the highly insoluble hydroxylapatite must be delayed until final delivery of P and Ca to the bone.

Bed-rest studies in which calcium isotopic variations were monitored (Heuser and Eisenhauer 2010; Morgan et al. 2012) were motivated by the frequent observation that astronauts suffer bone loss during space flight (Fig. 3). It was known that Ca in urine increases and becomes isotopically lighter with time, suggesting that Ca is being liberated by osteoclasts from the bones into the blood stream (Permyakov and Kretsinger 2011). Increased loss of isotopically light Ca among patients may reflect the way that bone is being mineralized as a response to changing proportions of free Ca to albumin-bound Ca. This loss may also be caused by an increased loss of bone Ca, which is recognized to result from enhanced expression of another calcium-binding protein, sclerostin, during bed rest (Spatz et al. 2012).

Calcium isotopes can be used to quantify the Ca fluxes in and out of the bones without having to resort to adding isotope tracers to a diet (Skullan and DePaolo 1999; Heuser and Eisenhauer 2010). This is also relevant to the study of Ca in osteoporosis, a condition particularly common in aging women.

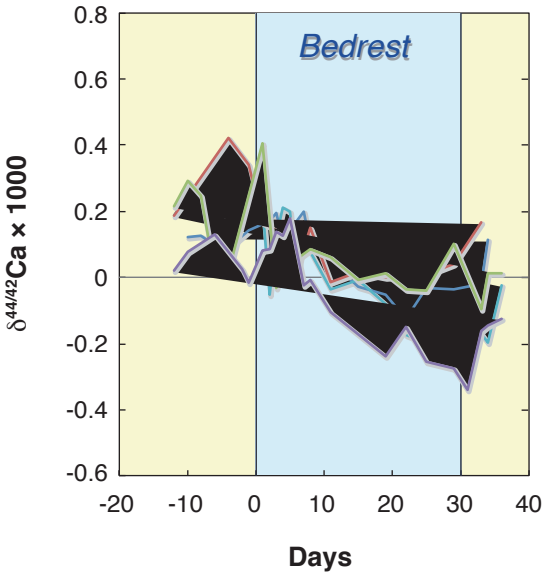

Figure 3 Calcium isotope evolution of the urine of volunteers (indicated by colored lines) confined to bed rest for a month (vertical blue band). Bone Ca is isotopically light relative to dietary Ca. Urine typically reflects dietary Ca. So, the observed changes in Ca isotopic composition of urine during bed rest indicates that bed rest enhances bone loss, which mimics what is observed in astronauts who have lived in space for a time (Morgan et al. 2012).

IRON STABLE ISOTOPES IN THE BODY: A TRACER OF HEREDITARY DISEASES

The role of iron in human biology is particularly important because Fe(II)-bearing hemoglobin is the prime carrier of oxygen in the blood. Other iron stores are present in the liver and the kidneys, largely as Fe(III) ferritin, a ferrihydrite analog wrapped in a protein shell.

Iron has two oxidation states: Fe(II), which is ferrous iron; and Fe(III), which is ferric iron. Ferric iron binds with many inorganic and organic ligands, and Fe(III) hydroxide is highly insoluble. Iron has three major isotopes—^{54}Fe, ^{56}Fe, and ^{57}Fe—and one minor isotope, ^{58}Fe. The pioneering study of Walczyck et al. (2002) demonstrated that there is a large variability in Fe isotope distributions between organs and body fluids. The important observation that blood Fe becomes isotopically heavier after bloodletting was interpreted to indicate that Fe is quickly retrieved from the liver and kidney to replace the lost blood iron (Hotz et al. 2012; Krayenbuehl et al. 2005).

The first disease studied from the viewpoint of the natural stable isotopes of iron was hereditary hemochromatosis (Krayenbuehl et al. 2005). This pathology is characterized by progressive iron overload of tissues due to a genetically disrupted function of the two critical proteins of hepcidin and ferroportin, which are involved in the control of intestinal iron absorption. This disruption leads to an ineffective control of intestinal iron absorption. Iron in the blood of patients with hemochromatosis is 0.2–0.4 per mil isotopically heavy compared to healthy individuals. This observation suggests that the blood of hemochromatosis patients uses the body's iron stores more heavily than is the case for people without the condition.

COPPER STABLE ISOTOPES IN THE BODY: A FLAG FOR POTENTIAL CANCER DIAGNOSIS

To understand how stable isotopes of copper can be used as a powerful medical tool for spotting cancer early, we first have to grapple with where copper resides in the body, what its function is, and what influence all this has on the distribution of its isotopes.

Copper plays both catalytic and structural roles in several essential enzymes: ceruloplasmin, which oxidizes Fe in human serum; cytochrome c oxidase in the mitochondrial membrane, which is the last courier in the respiratory electron chain; metallothionein, which is a major repository of intracellular Cu; and superoxide dismutase 1, which protects the cell and its DNA against reactive oxygen species, such as free oxygen ions. The Cu^{2+} in superoxide dismutase 1 binds with the nitrogen atoms of histidine, while some Cu^{2+} ions in ceruloplasmin bind to the sulfur atoms of cysteine and methionine. Due to its short bulk turnover time in the human body (~6 weeks; Goode 1991), Cu is a potentially valuable indicator of rapidly evolving diseases such as cancer. Anomalously high Cu levels are common in the serum of cancer patients; and, in experiments on mice, Ishida et al. (2013) demonstrated that increased levels of bioavailable copper actually promoted tumor growth. As a result, reducing body copper levels using the chelates tetrathiomolybdate and *D*-penicillamine (Brem et al. 2005; Brewer 2005) has now been approved for cancer treatment. Changes in copper concentrations in serum, however, do not remain amenable to quantitative predictions that are rooted in otherwise robust biochemical processes: isotope effects must also be examined.

The growth of centimeter-size cancer tumors is accompanied by pervasive neo-vascularization (the new growth of blood vessels), which secures the delivery of oxygen and nutrients to the tumor cells. Diffusion can only transport oxygen, carbon dioxide, and nutrients over a few hundred microns; therefore, tumors need to connect to the overall body's blood circulation system by growing blood vessels. Copper in cancer cells both facilitates vascularization (Carmeliet and Jain 2000) and plays a role in oxygen deficit (hypoxia), a hallmark of biological malignancies. Recent evidence suggests that hypoxia helps cancer stem cells survive in certain niches (Hill et al. 2009). Indeed, the overexpression of hypoxia-inducible factor 1 alpha, the protein that controls cellular oxygen levels, is associated with increased tumor growth, vascularization, and metastasis (Wilson and Hay 2011).

In order to explore the potential of Cu isotopes as a biomedical marker, a control dataset of healthy individuals first had to be established. Albarède et al. (2011) analyzed the copper isotope compositions of the serum and red blood cells of 50 young male and female blood donors

and found that ^{65}Cu is enriched in red blood cells with respect to serum. Copper is enriched in the light ^{63}Cu isotope in serum because of this isotope's soft bond with sulfur from cysteine in ceruloplasmin; copper is enriched in the heavy ^{65}Cu isotope in red blood cells, because this isotope strongly binds with nitrogen from the histidine-forming superoxide dismutase 1 (Albarède et al. 2011; Fujii et al. 2014).

Copper isotopes have the strong potential to be markers of cancer. Télouk et al. (2015) measured, using phenotype and molecular biomarker documentation (Fig. 4), the $^{65}Cu/^{63}Cu$ ratios in 140 serum samples from 8 patients with colorectal cancer and 20 patients with breast cancer. The $^{65}Cu/^{63}Cu$ ratio in the serum of the cancer group who had a negative prognosis (i.e. were likely to die from their condition) was lower than the control group. The $^{65}Cu/^{63}Cu$ ratio predicted, to a good degree, mortality in the colorectal cancer group and the ratio could discriminate, at a high confidence level, the group of breast-cancer patients from the group of control women. Furthermore, Balter et al. (2015) found that the $^{65}Cu/^{63}Cu$ ratio in the serum of liver-cancer patients was significantly lower relative to a control group of healthy blood donors; and, in a mirror image result, the $^{65}Cu/^{63}Cu$ ratio was higher in liver tumors relative to healthy tissue (Fig. 5).

The outstanding question is, therefore, "What is the mechanism responsible for lowering $^{65}Cu/^{63}Cu$ in the serum of cancer patients and for increasing it in tumor tissue when it is disabled in healthy individuals?" The mechanism is probably linked to copper chelation by lactate, a substance painfully familiar to athletes for accumulating in the muscles during prolonged strenuous anaerobic activity. This common substance is also observed in tumor cells.

As a biomedical marker, the longitudinal study of Télouk et al. (2015) showed that the variations of Cu isotope abundances precede by several months those of the more conventional molecular biomarkers, e.g. carcinoembryonic antigen (CEA) and carbohydrate antigen CA 19.9—both recommended markers for colorectal cancer—and CA 15.3, which is a recommended biomarker for breast cancer. As a process biomarker, the opposite shift of Cu isotope abundances between liver tissue and serum suggests that

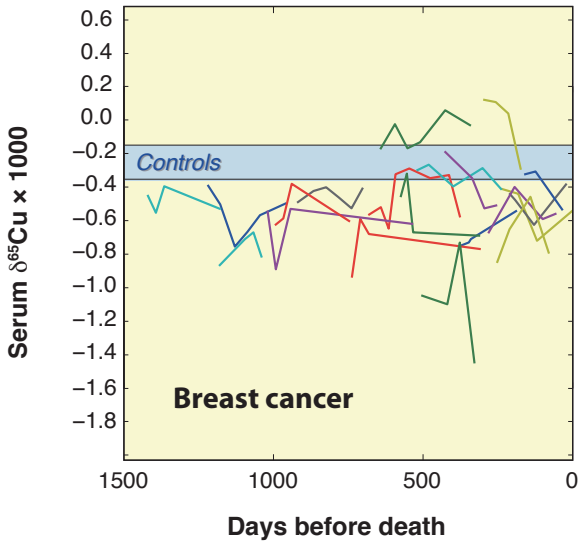

Figure 4 Evolution of serum ^{65}Cu in 140 samples taken from 20 breast cancer cases up to patient death. Each line represents a different patient, with color used for differentiation purposes. The blue band (controls) represents the range of ^{65}Cu in the serum of healthy donors. After Télouk et al. (2015).

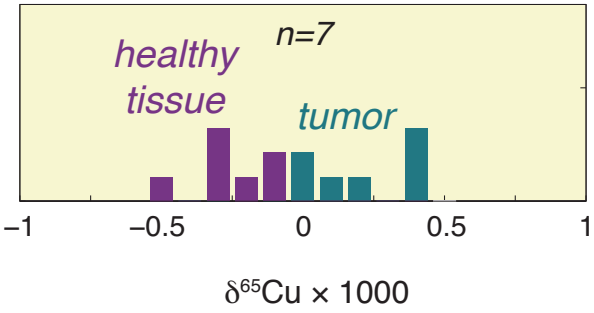

Figure 5 Graph of ^{65}Cu values in seven sample pairs of healthy tissue and of cancer tumor taken from the liver of the same patient. The higher ^{65}Cu values in the tumor relative to normal tissue is a mirror image of the lower ^{65}Cu of serum for patients with liver cancer relative to healthy individuals. Cu^{2+} is chelated by intracellular lactate in the tumor, which preferentially releases ^{65}Cu-depleted copper into serum. After Balter et al. (2015).

fractionation between chelated and non-chelated forms of Cu should play a role in the effectiveness of Cu isotopes as biomarkers.

Lactate generation in the body is linked to how a cell produces energy. The universal biological energy currency unit is adenosine triphosphate (ATP), which packs an enormous amount of energy in phosphate bonds. Glucose burning (glycolysis) is the primary fuel of ATP production, which is achieved by the attachment of on inorganic phosphate to adenosine diphosphate (ADP). In normal *aerobic glycolysis*, ATP is produced by a set of reactions also involving reduction of nicotinamide adenine dinucleotide (NAD^+), an electron acceptor, into NADH. The end product of *aerobic glycolysis* is a carboxylate ion known as pyruvate, which is transported into the mitochondrion to produce more ATP. NADH produced in the process is re-oxidized into NAD^+, which allows the transport of electrons into the mitochondrion while consuming protons in the process. Excess H^+ therefore remains limited.

In contrast, under oxygen-deficient conditions, pyruvate is used as electron acceptor instead of NAD^+, another carboxylate anion, lactate is now the end product, and aerobic glycolysis is replaced by *anaerobic glycolysis*. Excess protons are pumped out of the cell into the blood stream. Cancer cells mimic *anaerobic glycolysis,* and their tendency to produce lactate, despite the presence of adequate oxygen, is known as the Warburg effect. Lactate levels in the range of 10 mMol in the cytosol of tumor cells are observed after biopsy and correlate well with disease severity.

The strong signal of Cu isotopes appears to be related to Cu chelation by lactate, which binds Cu(II) in mono- and bi-dentate complexes, and is, therefore, associated with the Warburg effect. The Cu bond with the side hydroxyl of lactate is even stronger than the bond with histidine. Fujii et al. (2014) found that Cu mono- and bidentate lactate complexes are unusually stable and isotopically heavy. The extent of ^{65}Cu preference over ^{63}Cu for the lactate monodentate form with respect to analogous Cu isotopes that are bonded to cysteine is more than one per mil—which is very large compared to most common compounds—and for the lactate bidentate form it is about two per mil. Copper chelation begins to be significant at lactate concentrations of 0.1 mMol, but at 10 mMol, which is typical of tumor cells, 50–80% of the Cu is chelated by lactate. Such a process explains why tumor tissues rich in Cu^{2+}–lactate complexes are isotopically heavy, whereas Cu^+ excreted into the bloodstream is correlatively light.

How under these conditions would a biomarker based on Cu isotopes be useful? Serum lactate itself is tightly regulated by membrane transport and then metabolized

and is, consequentially, a poor marker of intracellular lactate. Copper isotopes in the bloodstream, by contrast, reflect the state of intracellular lactate metabolism and the extent to which this metabolism replaces normal glycolysis. The abundance of Cu isotopes in serum, therefore, appears to represent a potentially powerful biomarker of cancer growth and dissemination.

FUTURE DIRECTIONS FOR "MEDICAL ISOTOPE METALLOMICS"

Metal stable isotopes seem to have a bright future as biological markers, a field that I suggest be called "medical isotope metallomics" (metallomics being the study of metals as essential constituents in biological systems). The effect of specific ligands on isotope variability in body fluids can be predicted from first principles, which allows specific biological pathways to be identified. As epitomized by Ca isotopes as a determiner of the extent of bone loss, the observable extent of isotopic fractionation is a quantitative index of pathological evolution. Analyzing isotope compositions does not demand a stringent timetable, with the important consequence that many legacy samples can be analyzed years after they have been collected. Finally, different metals have different turnover times in the body, and, thus, isotope metallomics offers a broad spectrum of potential markers that are sensitive to the different timescales that characterize different types of disease. Nevertheless, it will be essential to assess whether isotopic drifts represent primary signals or whether they reflect the associated inflammatory conditions that normally accompany the evolution of a disease.

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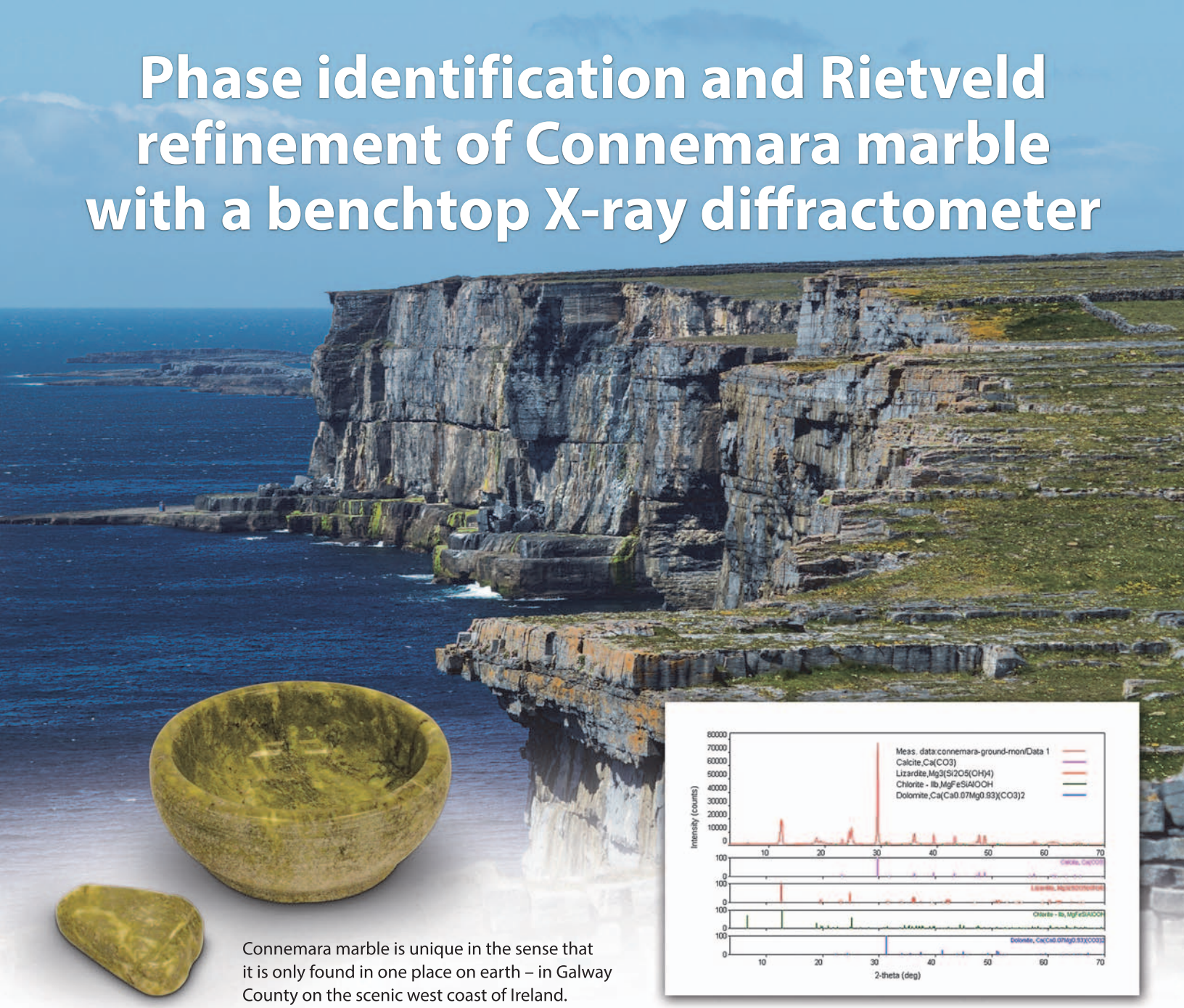
Phase identification and Rietveld refinement of Connemara marble with a benchtop X-ray diffractometer



Connemara marble is unique in the sense that it is only found in one place on earth – in Galway County on the scenic west coast of Ireland.

In addition to containing a limestone mineral (calcite), three other phases belonging to the serpentine mineral family are found in Connemara Marble. The main polymorphic forms are chrysotile, antigorite, and lizardite. X-ray diffraction is a viable technique to identify and pinpoint the exact phase of the serpentine family.

Mineral	Chemical Formula	Wt %
Lizardite	$Mg_3(Si_2O_5(OH)_4)$	38.2
Calcite	$CaCO_3$	44.5
Chlorite IIb	MgFeSiAlOOH	15.1
Dolomite	$CaMg(CO_3)_2$	2.2

Specimens of Connemara marble were pulverized and analyzed with the Rigaku MiniFlex benchtop XRD. A Rietveld analysis was performed using the model obtained from these phases.

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