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From stable isotope ecology to forensic isotope ecology - isotopes' tales

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Highlights

- Potential and caveats of isotopic analytical approaches to wildlife forensics are being discussed.
- Provides information on why differences in stable isotopic composition of a given compound result in measurable differences in its chemical and physical properties.

Abstract

Stable isotope ecology and forensic isotope ecology are not only linked by name. More often than not, knowledge and insights gained through the former serve as a springboard for application focused work of the latter. This review aims to offer a glimpse into the fascinating world of both though with more emphasis on forensic isotope ecology. To this end a selection of past and recent published work is presented and discussed to highlight both potential and limitations of isotopic analytical approaches to the detection of illegal trade in plants and animals.

Keywords: barn swallow; birds; bomb-curve; breeding; butterfly; C-13; C-14; caviar; CITES; cocaine; cotton; cycad; DNA-microsatellites; diet; food web; H-2; isoscape; isotopologue; ivory; lizards; N-15; migration; O-18; poaching; reptiles; S-34; snake skin; stable isotope; strontium; sturgeon; timber; trace elements; water; wood

1. Introduction

Ecology, and its underpinning environmental studies, are two areas within natural and life sciences with a long history of applying stable isotope chemistry and biochemistry to gain
insights into, for example, food webs, life cycles, or migration. It was only a matter of time before the methods that were developed, and the insights gained during ecological studies, were extended to wildlife forensics.

For the appreciation of as wide an audience as possible, in addition to highlighting the wealth of information that stable isotope abundance signatures can provide, in section 8 this review also provides some background information on the nature of stable isotopes, the terminology used, and why their relative abundance varies in the natural world. In-depth treatises on the subject of stable isotope chemistry in the context of ecological, geochemical, and forensic sciences, have been published in the form of several textbooks [1-5].

2. Water; one of the prerequisites for life as we know it

Most hydrogen in nature is bound in the hydrosphere, which is often called the "water sphere" as it includes all the earth's water that is found in streams, lakes, oceans, ice, the soil, groundwater, and in the air. The hydrological cycle traces the movement of water and energy between the various water stores and earth’s spheres, namely, the lithosphere, atmosphere, biosphere, and hydrosphere. Ocean water can be looked on as the starting point of the hydrological cycle. Water is transported from the oceans to the continents by clouds formed from evaporation of ocean water. Mass discrimination, resulting from different chemical and physical properties of $^1$H$^16$O, $^1$H$^18$O and $^2$H$^16$O (see Table 1, for example, leads to isotopic fractionation during evaporation, condensation, and precipitation, of meteoric water. Ultimately, this results in water taken up by plants, either through roots or leaf stomata, having different isotopic composition depending on geo-location. By the same token, isotopic composition of water consumed by animals will depend on geo-location. Depending upon latitude, longitude, altitude, temperature, and distance to the open seas, observed $\delta^2$H values of precipitation and, hence fresh water, can range from +20 to −270 ‰ across the world with the ‘heavier’ or less negative $\delta^2$H values being typical of coastal / near equatorial regions, and the ‘lighter’ or more negative $\delta^2$H values being typical of inland / high altitude / high latitude regions. This spatial or geographic variability in isotopic composition of water is illustrated in Figure 1 showing an isotopic map of freshwater across Scotland.

Geographical information system (GIS) maps and contour maps of meteoric $^2$H and $^{18}$O isotope abundance across the globe are in the public domain, and can be accessed via the Internet from resources such as http://www.waterisotopes.org, which is maintained by Gabriel Bowen at the University of Utah or the hydrogeology section of the International Atomic Energy Agency (IAEA) http://isohis.iaea.org/userupate/waterloo/index.html.

3. Isoscapes
During a conference in 2008, participants were asked to indicate their areas of research interest in which they do or would apply spatial representation of the variability of isotopic composition in different matrices and their geographic distribution. This survey returned ecology, climate change, biogeochemistry, hydrology, and forensics as the top five areas of research interest, and this clearly demonstrated the common ground shared by a wide spectrum of scientific disciplines through the study of stable isotope ratios, their variability at natural abundance level, and the underlying processes they reflect.

Thanks to the pioneering efforts of two scientists, Gabriel J. Bowen and Jason B. West, during the last 15 years, huge strides have been taken in the development and generation of a wide variety of matrix-specific isotope landscapes or "isoscapes" that illustrate in an impressive way the spatial (even temporal) variation in stable isotope abundance of compounds, and materials of interest such as feathers, leaf water, precipitation, or vegetation [6-16]. The term isoscape, coined by Jason West in 2005 intimates that isotopic landscapes are more than just maps showing contour lines obtained by (simply) connecting dots or points of equal stable isotope abundance values, as is the case in topographical maps that show contour lines representing points of equal altitude above sea level. Isoscapes of δ2H or δ18O values in precipitation, for example, are graphical representations of spatial variability of modelled stable isotope abundance values which are interpolated from a data-set of measured values by a detrended, latitude- and elevation-explicit algorithm [17].

Although isoscapes are powerful and useful, researchers and other end-users need to be aware of the underlying assumptions that are made, and how well these assumptions are being met [18, 19]. For example, using 2H composition of feathers or fur makes the assumption that any animals studied, actually recruit most of the hydrogen incorporated from water they drink (obligate drinkers) rather than satisfying most, if not all, their water requirement through the food they consume. Carnivorous cats for example can satisfy their water requirements by drinking the blood and urine of their prey. This weakens the otherwise strong correlation between δ2H value of a regional water source and the δ2H value of animal tissue, and results for example, in the hair of the North American puma (bobcat) not following the pattern expected from δ2H isoscapes of precipitation [20]. Conversely, in fish which live in, and obtain oxygen from, water,, isotopic fractionation associated with metabolism, bio-synthesis, and position in the food web, can have a significant effect on tissue δ2H values [21, 22].

4. Stable Isotope Ecology

Ecological sciences have benefitted greatly from applied stable isotope techniques [23, 24]. Either on their own, or in combination with genetic data, these techniques have made it possible to answer ecological questions about terrestrial and marine food webs, or diet change, and migration patterns of wild birds that otherwise would have been difficult if not impossible to obtain due to logistical challenges [8-10, 25-34]. Correlation equations (models) become
meaningful even though the processes occurring between input (precursor) and output (product; the measureable entity) are essentially a "black box". Provided these models are based on large data-sets (i.e. yielding a representative picture of a given population), algorithms can be developed that model the influence of metabolic pools and fluxes. Based on the resulting correlation equation, input values for, say, a food source or geographic origin, can be calculated on the measured value of a given output sample, such as animal tissue, with a high degree of confidence (Figure 2). One, if not the most impressive example, of where this was achieved successfully was the elucidation of the life cycle and migration of the Monarch butterfly by Keith Hobson and Len Wassenaar [35].

The study of the dietary and migration patterns of elephants by Ture Cerling and co-workers is another good example [36, 37]. Cerling's projects used sequential (growth-rate related time-resolved) $^{13}$C and $^{15}$N isotope analysis of elephants’ tail hair to generate a chronological history of the elephants’ eating habits, and even their feeding locations. Averaged $\delta^{13}$C and $\delta^{15}$N-values from all elephants in the study showed gradual changes from season to season. The results also showed that isotopic make-up of an individual elephant’s hair could be significantly different from that of the control group if the elephant in question were involved in night-time crop raiding.

Using a multivariate isotope analytical approach, carbon, nitrogen, and strontium isotope composition of bone samples collected from the mandibular symphysis of elephants in Amboseli Park, Kenya, were measured to examine changes in diet and habitat use since the 1960s [38]. Much more recently, in 2016, Coutu et al. published the results of a study of elephant ivory samples from known East African habitats which were analysed for their $^{13}$C/$^{12}$C, $^{15}$N/$^{14}$N, $^{18}$O/$^{16}$O, and $^{87}$Sr/$^{86}$Sr isotope ratios [39].

5. Forensic Isotope Ecology

With mounting concerns about declining populations of protected wildlife, either because of loss of habitat due to illegal logging, or illegal trapping and poaching, it was a logical step for techniques developed, and insights gained, from stable isotope ecology, to be applied in a forensic context. Putting in another way, when it comes to isotope wildlife forensics, the ecological aspects, for example, tracing the origin of plants or animals, is the flip-side of the stable isotope ecology coin where the aim is, amongst others, to elucidate food webs, the relationship between plants and their environment, and the migration patterns of wild animals [13, 40, 41]. Insights gained from studying geographic movement and food web status can also be exploited to determine the provenance of poached ivory. Another application is when a bird is claimed to have been bred in captivity by a controlled and licensed breeder but was, in fact, illegally caught in the wild and illegally imported; here, isotopic analysis can be used to detect violations of the Convention on International Trade in Endangered Species of Wild
Fauna and Flora (CITES) [42]. The international wildlife trade in animals or animal parts from animals allegedly bred in captivity has globally increased during recent years, while the legitimate origin of the animals concerned frequently remains questionable. Worldwide, authorities experience strong challenges effectively to control the international trade in CITES-listed species, and are struggling to detect and prove fraudulent claims concerning source and/or origin of traded animals and plants.

**Plants**

Cycads, which resemble spiky palm trees and bear pineapple-shaped seed cones, can be traced back at least 280 million years. They are the world’s most threatened plant group, according to the International Union for the Conservation of Nature. South Africa’s cycads, most of which are found nowhere else in the world, are especially threatened, despite laws regulating the trade in them. Slow-growing adult specimens, some hundreds of years old, sell for tens of thousands of dollars. Of South Africa’s 38 cycad species (37 species of *Encephalartos* and one species of *Strangeria*), 12 are critically endangered, four are endangered, nine are vulnerable, seven are near threatened and three are extinct in the wild. In South Africa, the indigenous *Encephalartos* cycad species is protected under provincial legislation and/or the National Environmental Management: Biodiversity Act 10 of 2004. Part of the difficulty in stopping the illegal trade is species identification. Stripped of leaves for transport, it is difficult to distinguish the trunk of an illicitly harvested endangered species from one that is legal to sell. A DNA database has helped with identification. In addition, stable isotope analysis in combination with radiocarbon dating has been tested to tell if a plant were harvested from the wild [43]. To this end, relocated and wild specimens from the African genus *Encephalartos* (*E. lebomboensis* and *E. arenarius*) were sampled. Results from $^{14}$C analysis indicated that a chronology with a $\pm$ 30-year uncertainty envelope could be reliably obtained. For *E. arenarius*, pre-relocation tissue was consistent with a wild origin, whereas tissue grown post-relocation was isotopically distinct from the wild for $^{87}$Sr/$^{86}$Sr ratio and $\delta^{15}$N values. For *E. lebomboensis*, $^{87}$Sr/$^{86}$Sr ratio as well as $\delta^{18}$O and $\delta^{34}$S values were different between relocated and control plants; these were consistent with the $>30$-year time window since relocation. The findings of this study demonstrated the potential for a forensic isotope approach to identify illegally extracted cycads [43].

**Plant products**

Detection and prosecution of illegal logging is hampered by a lack of available forensic methods that are able to prove or disprove conclusively the geographic origin of wood. This is important, not just as a means to detect illegally-sourced wood, but also as a means for the timber trade, for auditing and monitoring their supply for legal compliance. Studies by Keppler and co-workers have yielded encouraging results for $\delta^2$H signatures of lignin-bound methoxy groups by showing close correlation between methoxy $\delta^2$H values and $^2$H composition of precipitation, thus offering an indication of geographic origin [44]. Subsequent work extended $^2$H analysis of lignin methoxy groups to their $^{13}$C analysis and combined these data with $\delta^2$H, $\delta^{13}$C and $\delta^{18}$O of cellulose and whole wood of a single species, *Picea abies*, from one particular region in the Italian Alps [45]. Chemometric analysis of the
data showed strong correlation between $\delta^{2}H$ and $\delta^{18}O$ values of whole wood and temperature, but no significant correlation between isotope proxies and precipitation. These findings for wood from a relatively small geographic area were echoed by a study published in 2018 that looked at both stable isotope and DNA microsatellite markers [46]. While it was not possible to link geographic origin to stable isotope signatures, Bayesian clustering analysis of microsatellite markers correctly assigned 92% of samples analysed in a blinded fashion, including those of neighbouring areas only 14 km apart. The authors, however, stressed their observations merely pointed out the limitations of stable isotope analysis for geographic origin assignment of timber grown on a small spatial scale. Differentiation based on stable isotope signatures may still be possible at larger spatial scales or with stronger climatic or topographic variation. The fact that scientists at the US Geological Survey developed three whole wood reference materials specifically for stable isotope analysis of wood is testament to the interest in, and appreciations of, the potential of stable isotope analysis in this field of forensic ecology [47].

Being able to determine geographic origin of plant products is not just confined to detection of illegal trade of protected species but is also of interest where origin is either associated with consumer perception of quality or has consequences for international trade. An example that involves both these aspects is the origin of cotton. Here, wrongful declaration of origin may serve fraudulently to assert a desired product (Egyptian cotton) or to circumvent or reduce import duty. Ever more stringent product labelling requirements demand the supply chain to furnish proof of geographic provenance beyond merely paper-based audit trails. Using hierarchical cluster analysis (HCA) of multivariate stable isotope abundance data, a study comparing 17 US cotton and 15 non-US cotton samples was able to cluster 15 of the 17 US cotton samples in one group [48]. However, HCA was not able to resolve cotton from countries as far as apart as, for example, Pakistan and Senegal. Principal component analysis (PCA) of $\delta^{2}H$, $\delta^{3}C$ and $\delta^{18}O$ values from a subset of samples of cotton from 10 different countries also showed signs of separation but still with considerable overlap between some countries (Figure 3, top). Yet, combining $\delta^{2}H$, $\delta^{3}C$ and $\delta^{18}O$ values with abundance values of seven trace elements (Ca, Cu, Fe, K, Mg, Mn and Zn), resulted in cluster separation of all but one country (Figure 3, bottom). The combination of stable isotope and trace element abundance values shows the great potential that a synergistic approach, applying a combination of independent methodologies, will have to generate robust forensic scientific data in support of prevention, and prosecution of illegal trade in plants and plant products.

Snakes and lizards Many exotic reptiles and amphibians are protected under CITES which is an international agreement initiated in 1973 and is currently signed by 182 countries and the European Union (also known as Parties). It regulates international trade in more than 35,000 wild animal and plant species, including their parts, products, and derivatives. CITES Appendix I includes species threatened with extinction and provides the greatest level of
protection, including restrictions on commercial trade, while Appendix II includes species that, although currently not threatened with extinction, may become so without trade controls. A study published in 2017 examined the usefulness of isotopic signatures and elemental abundance data as a means of determining the source and origin of python skins entering international trade [49]. To this end the authors compared stable isotope abundance values of $^2$H, $^{13}$C and $^{15}$N as well as elemental composition of skin from wild and captive-bred pythons which had been raised under different diet regimes in Indonesia and Vietnam. Analysis of elemental markers focused in particular on abundance of V, Mn, Co, Ni, Cu, Zn, Al, Sn and As. Not only were both isotopic and elemental markers significantly different between wild and captive-bred snakes, significant differences were also found between different geographic origins. Ironically, concentration of transition, post transition, and heavy metals was much higher in skins of wild Burmese pythons as compared to skins from captive animals fed on pork, chicken, fish or rats. The authors claimed combinations of both techniques were able to discriminate between dietary treatments and geographic origins with up to 100% accuracy. The authors concluded that analysis of isotope abundance and elemental markers offered a powerful tool for verifying provenance of traded reptile skins. However, they also qualified their findings, suggesting the most likely to succeed applications of these methods would be in the case of species with small populations of genuine conservation concern.

Another example of a study to develop a forensic analytical method to investigate and detect wildlife crime also involved a reptile species from Vietnam namely the CITES listed crocodile lizard, *Shinisaurus crocodilurus*. The crocodile lizard is listed as endangered on the IUCN Red List mainly due to habitat loss and unsustainable exploitation for the international pet trade. In their study, van Schingen and co-workers investigated the application of measuring $\delta^{13}$C and $\delta^{15}$N values to discriminate between captive and wild crocodile lizards from Vietnam on the basis of their diet [50]. The results of this study showed significant differences in both $\delta^{13}$C and $\delta^{15}$N values between captive and wild individuals. Relative isotope abundance values of skin samples from captive specimens showed significantly higher $\delta^{13}$C and $\delta^{15}$N values as compared to specimens from the wild. The authors also used weighted k-Nearest Neighbour classifier to assign simulated samples back to their alleged place of origin and demonstrated that captive bred individuals could be distinguished with a high degree of accuracy from specimens that were not born in captivity. It was concluded that a combination of $^{13}$C and $^{15}$N abundance analysis showed great potential as a forensic tool to reduce the trade of wild-caught lizards that were temporarily kept in breeding farms with the aim of “disguising” them as captivity-bred animals. However, the authors acknowledged this potential might be limited to range-restricted or ecologically specialised species.

**Elephant ivory** Determining the provenance of ivory is another example of stable isotope techniques applied to fundamental ecological research questions serving as springboard for forensic ecology. Once it was established that stable isotope signatures recorded in elephant hair could provide insights on their feeding habits and feeding locations, it was only a matter
of time for multivariate (multi-element) stable isotope signatures of pulverised ivory to be studied to evaluate their potential and limitations as forensic technique in determining the provenance of ivory of unknown origin. A study published in 2016 did just that. The authors of this study reported a multivariate stable isotope data-set of $\delta^2$H, $\delta^{13}$C, $\delta^{15}$N, $\delta^{18}$O and $\delta^{34}$S values from 507 ivory samples [51]. Using a data-set of African reference samples from 208 different sites as a training set, the authors applied a k-nearest neighbour classification algorithm to infer accuracy of sample assignment to their correct provenance. The authors were able to assign 50% of all samples within 381 km thereby demonstrating stable isotope profiling of African elephant ivory works on a regional scale.

In addition to stable isotope abundance analysis as a tool for forensic ecology, particularly for wildlife forensics, bomb curve $^{14}$C dating has been proposed as method to determine the age of objects carved from ivory [52]. Above-ground thermonuclear weapons testing from 1952 through 1962 nearly doubled the concentration of $^{14}$C in the atmosphere. As a result, organic material formed during or after this period may be radiocarbon-dated using the abrupt rise and steady fall of the atmospheric $^{14}$C concentration; this is known as the bomb-curve. Tissues formed after 1955 are dated to within 0.3 to 1.3 years of formation, depending on the tissue type, whereas dating of tissues formed prior to 1955 have high age uncertainties (>17 years) due to the Suess effect. Bomb-curve $^{14}$C dating of confiscated animal tissues such as objects carved from ivory can, on account of their age, be used to determine whether trade of the item is legal. Many of the CITES restrictions are based on tissue age. Radiocarbon dating and, in particular, bomb-curve $^{14}$C dating can therefore serve as a powerful forensic tool to combat illegal trade in animal parts.

**Narcotic drugs**  Last but not least, the fight against organised crime is another fascinating aspect of forensic isotope ecology, as the following example in the trafficking of illicit drugs illustrates (Figure 4). Owing to the high level of accuracy and precision with which stable isotope abundance values can be measured by continuous-flow isotope ratio mass spectrometry (CF-IRMS), one can exploit even subtle differences in stable isotopic composition of a natural drug such as cocaine. Since environmental conditions such as light, moisture, or temperature, under which plants are grown, influence stable isotopic composition of secondary metabolites or plant products such as sugars, lipids, cellulose, or indeed, alkaloids [48, 53-55], $\delta$H, $\delta^{13}$C, $\delta^{15}$N and $\delta^{18}$O values are highly correlated with geographic location of sites where plants are cultivated. The discriminatory power of stable isotope signatures and resulting isoscapes increases with the number of independent variables, i.e. isotopes of different chemical elements being probed. While Figure 4 only shows the isoscapes of the geographical variation in the $^2$H and $^{15}$N composition of 336 authentic samples of Columbian cocaine, these two isoscapes already give an impressive illustration of the power of isoscapes in the fight against illegal drugs. In total, Jennifer Mallette and colleagues at the US Drug Enforcement Agency (DEA), have analysed 529 authentic cocaine samples from 19 known South American coca-growing
regions for their $^2$H, $^{13}$C, $^{15}$N and $^{18}$O composition. They thus established a multivariate data-set based on four independent variables as well as four isoscapes showing the spatial distribution of cocaine $\delta^2$H, $\delta^{13}$C, $\delta^{15}$N and $\delta^{18}$O values [56]. By supplementing this data-set and corresponding cocaine isoscapes, with data-sets and alkaloid landscapes of three minor alkaloids, Mallette et al. developed a framework model able to classify geographic origin of unknown illicit cocaine samples with an accuracy of approximately 96%.

6. Caveats - a fishy example

With publications of substrate- or tissue-specific isoscapes on the increase, applying stable isotope analysis to questions of forensic ecology or wildlife forensics has become deceptively easy. However, despite the great potential, it is important to remind ourselves that a high correlation coefficient $R$, or high coefficient of determination $R^2$, do not necessarily represent a high degree of direct causality. One must always remember that animal and plant physiology and metabolism represent a large black box containing an often complex system of pools and fluxes. In most cases, we have only access to, and can only analyse, the isotopic composition of precursor pool(s) and final product, and then link the two using correlation equations or model-based algorithms. In addition, a sound knowledge of the various systems and factors influencing them is required to avoid falling into the trap of interpreting a subject or object characteristic A linked to an analytical finding B in a bi-directional way. In other words, while characteristic A may be linked to finding B, it does not necessarily follow that finding B is inevitably linked to characteristic A - and only characteristic A. There is a danger of making or applying the assumption of bi-directionality if ethical or legal frameworks place constraints on a study. An example of this conundrum is the CITES protected Caspian Sea sturgeon. The question is whether any caviar on sale is being sourced from farmed sturgeon, or being sourced from illegally-caught wild sturgeon.

A survey carried out in 2011/2012 of 27 caviar samples from Romania and Bulgaria, used analysis of mitochondrial DNA sequences of the cytochrome b gene and DNA microsatellites for identification of species [57]. In ten cases, species identification matched the species code stated on the CITES labels. However, four tins were mislabelled, six tins contained counterfeit produce, and another four tins were sold as being of "wild" origin, in spite of existing bans on fishing for sturgeon in the wild. Another study, aiming to develop a forensic method to distinguish between caviar from wild and farmed sturgeon in the USA, analysed sturgeon roe for its fatty acid and trace element composition [58]. Statistically significant differences were found for concentrations of unsaturated fatty acid 18:2n6 (higher in farmed) and selenium (higher in wild). While both these studies clearly underline the potential of methods analysing for several, ideally independent, markers, each was based on a limited number of samples from a limited geographic region, and neither established a conclusive link between sample and geographic origin.
For obvious reasons, other than by virtue of samples seized on the spot by law enforcement personnel, it is nigh impossible to obtain authentic Caspian caviar from a protected species even for the purpose of populating a forensic reference data-base. On the other hand, compiling a data-base of stable isotope signatures of caviar from sturgeon farmed under controlled licensed conditions, and thus of known sample history, may be enough to convince a court of law that a sample not matching any entry in such a data-base, automatically represents caviar not harvested from farmed sturgeon, but rather from an illegal source and representing a CITES violation. Linking caviar $\delta^{13}C$ and $\delta^{15}N$ signatures to farmed status vs. illegally-obtained status may work provided (a) feed used for farmed sturgeon and food consumed by wild sturgeon are isotopically different and (b) farm feed is monitored for its isotopic composition on a regular basis. Correlating $\delta^2H$ and $\delta^{18}O$ signatures of caviar with the $^2H$ and $^{18}O$ composition of the water in which sturgeon are found theoretically offers even greater promise since the water of official operating breeding farms can be isotopically characterised. Furthermore, $\delta^2H$ and $\delta^{18}O$ signatures of the majority of fish farm water should be easily distinguishable from that of the Caspian Sea, unless, of course, a farm is located in the Caspian Sea. Water $\delta^2H$ and $\delta^{18}O$ values of farms breeding and raising sturgeon in on-shore, freshwater lakes, reservoirs, ponds, or even tanks in indoor facilities, (a) can be measured and (b) will be comparable to annually-averaged $\delta^2H$ and $\delta^{18}O$ values of local precipitation for which modelled data are available from the Online Isotopes in Precipitation Calculator (OIPC) [59]. For inland locations in countries such as Bulgaria, France, Germany, Italy, or the USA (California), modelled annually-averaged $\delta^2H$ and $\delta^{18}O$ values for precipitation, and actually measured freshwater $\delta^2H$ and $\delta^{18}O$ values, will be very similar, even almost identical. However, for off-shore locations around the coast line of the Caspian Sea, using modelled precipitation $\delta^2H$ and $\delta^{18}O$ values will not work. Even though modelled precipitation $\delta^2H$ and $\delta^{18}O$ values will most likely be numerically close to actually measured $\delta^2H$ and $\delta^{18}O$ values of precipitation, either of these will be significantly different from $\delta^2H$ and $\delta^{18}O$ values of Caspian Sea water. Due to its size and rate of recharge, water in the Caspian Sea shows relatively uniform $\delta^2H$ and $\delta^{18}O$ values across its entire expanse with $\delta^2H$ values ranging from -29.7 ‰ in the Northern Basin to -18.2 ‰ in the Southern Basin. The corresponding $\delta^{18}O$ values range from -2.76 to -1.32 ‰. For the Central Basin, $\delta^2H$ and $\delta^{18}O$ values are -21.7 and -1.83 ‰ respectively [60]. These $\delta^2H$ and $\delta^{18}O$ values are all but identical to $\delta^2H$ and $\delta^{18}O$ values of water in the Black Sea which are on average -23.03 and -2.84 ‰ respectively [61, 62]. These similarities of Caspian Sea and Black Sea $\delta^2H$ and $\delta^{18}O$ values is not really surprising given their similar size, volume and rate of recharge. Similar $\delta^2H$ and $\delta^{18}O$ values for precipitation are only observed in coastal, near equatorial regions of the world [17]. In other words, even if measured $\delta^2H$ and $\delta^{18}O$ values of caviar would seem consistent with $\delta^2H$ and $\delta^{18}O$ values of -67 and -9.0 ‰ for precipitation in the Northern Basin of the Caspian Sea along the coast lines of Russia or Kazakhstan, it cannot be concluded that such samples have come from sturgeon illegally caught in, or originating from, the Caspian Sea.
Only water isotopes and correlated caviar δ2H and δ8O values from inland locations would seem to offer a platform on which to base at least a presumptive test to decide if a suspect sample is farmed or, indeed, illegally sourced from wild sturgeon. However, based on a multivariate data-set of measured stable isotope abundance values of the two precursor pools, water (δ2H, δ8O) and feed (δ13C, δ15N, δ34S), as well as a multivariate data-set of measured abundance values of the product caviar (δ2H, δ8O, δ34S, δ34S and selected trace elements), it should be possible to generate a powerful set of exclusion criteria that samples would have to pass to be declared CITES compliant.

7. Conclusions

1. Stable isotope ecology yields insights into animal food webs and animal migration that are impossible to gain from traditional techniques, or difficult to obtain by observation or tagging.

2. Owing to growing data-sets and models based on them, stable isotope techniques correlating plant product or animal tissue with environmental conditions, geographic location, or feeding habit, can offer powerful information beyond mere comparison of evidence to determine geographic origin.

3. Stable isotopes in ecology, and in the forensic context, clearly demonstrate why robust data require funding. This is needed for the collation and compilation of large data-sets to ensure the required statistical support and for underpinning the conclusions drawn.

4. Large data-sets enable use of statistical tools and/or global information software to create maps showing spatial even temporal distribution of stable isotope signatures, as evidenced by δ2H or δ34S isoscapes of precursor pools such as meteoric water or vegetation respectively. These can then be interrogated or correlated with isoscapes of animal tissue or plant products [63]. Combining or overlaying element or isotope, specific landscapes may ultimately lead to probability or Likelihood Ratio maps.

5. Last, but not least, as examples discussed above have already shown, combining stable isotope data with those from independent techniques such as trace element analysis, trace element isotope ratio analysis, biochemical markers or DNA microsatellite analysis, to name but a few, will increase discriminatory power and thus the accuracy of source and origin assignment in cases of suspected misdeclaration or illegal trade [64, 65].

8. The science bit: isotopologues, chemically identical yet not the same

With the singular exception of phosphorous (P), all light elements essential to bio-organic chemistry of all living organisms exist in two or three stable isotopic forms that are not radioactive and, hence, do not decay. Coining the word isotope has been attributed to Dr
Margaret Todd, a family friend of Prof. Frederick Soddy who adopted the word to describe his discovery that different elements with different atomic weight but identical chemical characteristics might be assigned to the same chemical space. The word isotope borrows its origin from the two Greek words “isos” meaning “equal in quantity or quality” and “topos” meaning “place” or “position”, with isotope hence meaning “in the same position” (of the periodic table of the chemical elements).

Using the simplest of chemical elements as an example, hydrogen (H) in its most abundant isotopic form has a nucleus comprised of a single proton and therefore has the atomic mass of 1 (in atomic mass units or [amu]). The atomic mass or mass number A is indicated by a superscript prefix to the element letter, for example ¹H. The less abundant, by 1 neutron heavier hydrogen isotope has an atomic mass of 2 amu and is therefore denoted as ²H. Similarly, the less abundant, by 1 neutron heavier stable isotope of the most abundant form of carbon (¹²C) is denoted as ¹³C. Because either of these less abundant heavier isotopes contains in its nucleus the same number of protons as their corresponding more abundant lighter isotopes, their atoms also comprise the same number of electrons. As a consequence, there are no differences with regards to chemical character, i.e. the number of covalent bonds that isotopes of a given chemical element can form. Isotopes of a given chemical element are, as far as their atomic mass is concerned, not identical but are the same from a chemical point of view.

This situation however, changes the moment one looks at chemical and physical properties of isotopologues. Isotopologues are molecules of the same elemental composition as given by their chemical formula but are of different isotopic composition. Water without which life on earth would be impossible may serve as one example. Water molecules comprising either ²H or ¹⁸O or both are isotopologues of the most abundant form of water, ¹H₂¹⁶O. The difference in atomic mass between the lighter isotope ¹H and the heavier isotope ²H affects both strength and length of the covalent bond between hydrogen and oxygen which results in significant and measureable differences of chemical and physical properties between the various isotopologues of water. These include different dissociation constants, different melting points, different vapour pressures and, hence, different boiling points (Table 1).

Carbon dioxide, the precursor pool from which all plant life derives the carbon required to synthesise carbohydrates, lipids, and proteins, may serve as another example. Carbon dioxide molecules comprising either ¹³C or ¹⁸O or both are isotopologues of the major abundant form ¹²C¹⁶O₂. The difference in bond length and bond strength or bond energy between ¹²C-¹⁶O and ¹³C-¹⁶O is large enough to be measurable by the shift in near-infrared and mid-infrared absorption frequencies of CO₂. The difference in bond energy results in mass discriminatory effects during CO₂ fixation in plants using the Calvin-Benson cycle (C₃ plants) as compared to the Hatch-Slack cycle (C₄ plants). Differences in ¹³C abundance in plant products such as carbohydrates or lipids have been known since the 1960 owing to the pioneering work by Steven Epstein and co-workers [66-69]. For example, sugar made by sugar cane (a C₄ plant)
contains $^{13}$C at an abundance of approximately 1.0986 atom % while sugar made by sugar beet (a C$_3$ plant) contains $^{13}$C at an abundance level of approximately 1.0827 atom %.

To express these figures in a more convenient way, the International Union of Pure and Applied Chemistry (IUPAC) have defined the $\delta$-value. By virtue of its definition given in equation 1, the $\delta$-value is the numerical expression of the stable isotopic abundance of a given isotope $^h$E in a sample S relative to the stable isotopic abundance of a the same isotope $^h$E in a standard STD [70, 71].

$$\delta E_{S/STD} = \left( \frac{R_S - R_{STD}}{R_{STD}} \right) = \left( \frac{R_S}{R_{STD}} - 1 \right)$$  \hspace{1cm} \text{Equation 1}

In this equation, R is the measured isotope ratio of the heavier isotope $^h$ of a given chemical element E over the lighter isotope I of the same element E (e.g. $^{13}$C/$^{12}$C or $^2$H/$^1$H). $R_S$ and $R_{STD}$ denote the isotope ratios of the sample S and the chosen standard STD respectively. Since $\delta$-values derived on the basis of Equation 1 are numerically less than zero with significant numbers in the 2nd or 3rd decimal, they may be stated as $\%_o$ values as a representation of the scientific notation of presenting such numbers as multiples of $10^{-3}$. However, reporting $\delta$-values in this way should not cause the $\%_o$ sign to be confused with an SI unit of measurement, since a ratio of two ratios $\delta$-values does not have an SI unit of measurement. Similar to the % symbol, the $\%_o$ symbol is not a unit (of measurement) but merely a convenient way to express small numbers.

Example: with $R_S = 0.01101296$ and $R_{STD} = 0.0112372$,

$$\delta E_{S/STD} = (0.980045 - 1) = -0.01995.$$  

This $\delta$-value can be written as

$$\delta E_{S/STD} = -0.01995 = -19.95 \times 10^{-3} = -19.95 \%_o.$$  

In this example the minus sign signifies that the isotopic abundance of $^h$E in the sample S is less than the isotopic abundance of $^h$E in the chosen reference material STD. Conversely, $\delta E_{S/STD}$ values with a positive sign signify an isotopic abundance of $^h$E in the sample S that is higher than that in the scale defining standard STD. Given the $\delta$-value as defined by equation 1 represents an abundance value relative to a scale defining standard, it is of paramount importance for measurement of stable isotopic abundance and reporting its results to comply with IUPCA guidelines and recommendations [70-72]. Measured $\delta$-values must be scale calibrated using internationally recognised reference materials to ensure reported $\delta E_{S/STD}$ values are traceable, externally reproducible and, most importantly, internationally comparable [73-76].
Acknowledgements

The author is indebted to Cosme López Calderón (University of Sevilla, Spain) for his generous contribution of Figure 2 to this review.
References


Figure 1 (Scotland freshwater isoscapes) approx. here

Figure 2 (barn swallow migration) by Keith Hobson approx. here

Figure 3 (cotton PCAs) approx. here

Figure 4 (cocaine isoscapes) approx. here

Figure legends

Figure 1: Isoscapes of $\delta^2$H$_{VSMOW}$ (left) and $\delta^{18}$O$_{VSMOW}$ values (right) of water in freshwater lakes and reservoirs in Scotland. Red dots mark sampling locations. X and Y axes are degrees Longitude West and degrees Latitude North. Reproduced with permission of John Wiley & Sons Ltd, from “Stable Isotope Forensics - Methods and Forensic Applications of Stable Isotope Analysis 2ed.”, Meier-Augenstein, W., © 2018 [4].
Figure 2: Summary of the full process developed to identify the wintering areas of barn swallows breeding in Denmark. Figures A, B and C represent $\delta^2\text{H}$, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ feather isoscapes, respectively. Fig. D represents breeding (red circles) and wintering (blue circles) locations for a given individual breeding between 5°E and 15°E longitude (du Feu, C.R., Joys, A.C., Clark, J.A., Fiedler, W., Downie, I.S., van Noordwijk, A.J., Spina, F., Wassenaar, R. & Baillie, S.R; EURING Data Bank geographical index 2009; http://www.euring.org/edb). Figure E represents the population likely origin of sample individuals based only on the multivariate assignment of $\delta^2\text{H}$, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Figure F represents the likely origin population of our sample of individuals after incorporating the prior surface into the multivariate assignment of $\delta^2\text{H}$, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Source: Courtesy of Cosme Lopez (University of Sevilla, Spain).
Figure 3;  Top: Principal component analysis (PCA) of stable isotope abundance values ($\delta^2$H_{non-ex}, $\delta^{13}$C and $\delta^{18}$O) of raw cotton from 10 different countries.

Bottom: PCA of elemental (Ca, Cu, Fe, K, Mg, Mn and Zn) and stable isotopic abundance values showing improved differentiation between raw cotton from 10 different countries; US = black, China = red, Mexico = orange, Brazil = yellow, Guadalupe = dark green, India = light green, Senegal = blue, Pakistan = teal, Tanzania = purple and Zimbabwe = pink.
Figure 4: Isoscapes of $\delta^2$H (left) and $\delta^{15}$N (right) values of Columbian cocaine based on 336 authentic samples prepared from coca leaves. Reproduced under Creative Commons License CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/) from Mallette, J.R. et al. (2016) [60].
**Table 1:** Influence of isotopic composition on physical and chemical properties of H₂O and its isotopologues.

<table>
<thead>
<tr>
<th>Property</th>
<th>$^{1}\text{H}_2\text{^{16}}\text{O}$ FW = 18 g/mol</th>
<th>$^{1}\text{H}_2\text{^{18}}\text{O}$ FW = 20 g/mol</th>
<th>$^{2}\text{H}_2\text{^{16}}\text{O}$ FW = 20 g/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced mass $\mu$ of H-O bond [amu]</td>
<td>0.9412</td>
<td>0.9474</td>
<td>1.7778</td>
</tr>
<tr>
<td>Dissociation constant $pK_w$ at 25 °C</td>
<td>14</td>
<td>(14.008)$^{a)}$</td>
<td>14.957</td>
</tr>
<tr>
<td>Melting point [°C] at 101.33 kPa</td>
<td>0</td>
<td>0.28</td>
<td>3.82</td>
</tr>
<tr>
<td>Boiling point [°C] at 101.33 kPa</td>
<td>100</td>
<td>100.15</td>
<td>101.4</td>
</tr>
<tr>
<td>Vapour pressure at 20°C in [kPa]</td>
<td>2.3379</td>
<td>2.3161</td>
<td>2.0265</td>
</tr>
</tbody>
</table>

$^{a)}$ No documented value available. Value in bracket is an interpolated approximation.