Alike as two water drops: distinguishing one source of the same substance from another

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Introduction

It is usually possible to identify a substance by using chemical or physical methods. However, when two substances are alike, physicochemical methods are unable to distinguish between different sources of that same substance. When the question is not "how much", but rather "where from", stable isotopes may be a helping tool: isotope ratio measurement is a tool useful in a large range of fields, frequently completely unrelated; not a science in itself.

In fact, every process of biogeochemical interest will involve substances that contain one or more of the light elements H, C, N, O and S, in different proportions: they are in the air we breath, the food and drink that we take, the environment that we live in … And the processes that determine the direction and magnitude of isotopic fractionation are fundamental ones, responding to basic thermodynamic principles that cannot be tampered with.

Definitions: Elements and Isotopes

Isotopes are atoms of the same element that have the same numbers of protons and electrons, but different numbers of neutrons. The difference in the number of neutrons between the various isotopes of an element means that these have similar charges, but different masses. For example, among the hydrogen isotopes, deuterium (denoted as D or ^{2}H) has one neutron and one proton. This is approximately twice the mass of protium (^{1}H), whereas tritium (^{3}H) has two neutrons and is approximately three times the mass of protium.

Isotope Terminology: the "δ" notation

Measuring an absolute isotope ratio or abundance is not a simple task, requiring sophisticated instrumentation. Further, measuring this ratio on a routine basis would lead to problems in comparing data sets from different laboratories. However, we are mainly interested in comparing the variations in stable isotope concentrations rather than actual abundance, and so a simpler approach is used. Rather than measuring a true ratio, an apparent ratio can easily be measured by gas source mass spectrometry. The apparent ratio differs from the true ratio due to operational variations (machine error, or m) and will not be constant between machines or laboratories, or even different days for the same machine. However, by measuring a known reference on the same machine at the same time, we can compare our sample to the reference.

$$\delta^{18}O_{Sam} = \frac{m\left(\frac{^{18}O}{^{16}O}\right)_{Sam} - m\left(\frac{^{18}O}{^{16}O}\right)_{\mathrm{Re}\,f}}{m\left(\frac{^{18}O}{^{16}O}\right)_{\mathrm{Re}\,f}}$$

Isotopic concentrations are thus expressed as the difference between the measured ratios of the sample and reference over the measured ratio of the reference. Mathematically, the error (m) between the apparent and true ratios is cancelled. This is expressed using the delta (δ) notation (with oxygen as an example, where *"Sam"* stands for the unknown sample, and *"Ref"* for the reference material). Since fractionation at the natural abundance levels is usually small, δ-values are expressed as the parts per thousand or "per mil" (‰) difference from the reference. This equation then becomes the more familiar form usually employed:

$$\delta^{18}O_{Sam} = \left(\frac{\left(\frac{^{18}O}{^{16}O}\right)_{Sam}}{\left(\frac{^{18}O}{^{16}O}\right)_{\mathrm{Re}\,f}} - 1 \right) \times 1000$$

The stable isotopic compositions of low-mass (light) elements such as hydrogen, carbon, nitrogen, oxygen, and sulphur are therefore normally reported as "delta" (δ) values in parts per thousand (denoted as ‰) enrichments or depletions relative to a standard of known composition.

Basic Principles: fractionation

Since the various isotopes of an element have different mass, their chemical and

physical properties are also slightly different. The isotopes of the light elements have mass differences that are large enough for many physical, chemical, and biological processes or reactions to "fractionate" or change the relative proportions of various isotopes. Two different types of processes - equilibrium and kinetic isotope effects - cause isotope fractionation. This fractionation may be indicative of the source of substances involved, or of the processes through which such substances went through.

Equilibrium isotope-exchange reactions involve the redistribution of isotopes of an element among various species or compounds. At equilibrium, the forward and backward reaction rates of any particular isotope are identical. Equilibrium isotope effects derive from the effect of atomic mass on bond energy. The bond energy consumed by molecules incorporating the heavy isotope is higher than bond energy of molecules formed by the light isotope. Bonds involving the light isotope are weaker, and therefore easier to break. Molecules incorporating the light isotopes are thus "more reactive" than molecules of the same substance, but formed by a higher proportion of the corresponding heavy isotope.

Kinetic isotope fractionations occur in systems out of isotopic equilibrium where forward and backward reaction rates are not identical. The reactions may, in fact, be unidirectional if the reaction products become physically isolated from the reactants. Reaction rates depend on the ratios of the masses of the isotopes and their vibrational energies; as a general rule, bonds between the lighter isotopes are broken more easily than the stronger bonds between the heavy isotopes. Hence, the lighter isotopes react more readily and become concentrated in the products, and the residual reactants become enriched in the heavy isotopes.

Biological processes are generally unidirectional and are excellent examples of "kinetic" isotope reactions. Organisms preferentially use the lighter isotopic species because of the lower energy "costs", resulting in significant fractionations between the substrate (heavier) and the biologically mediated product (lighter).

Measurement: Gas Source Mass Spectrometry (Isotope Ratio Mass Spectrometry; IRMS)

Although the first precise measurements of isotope abundance ratios had been done in 1936 by Alfred Nier, it was not until 1947 that he built the first dual inlet, double

collector gas source mass spectrometer. Since then, the IRMS has become the measurement technique of choice for most of the light elements (certainly so for H, C, N, O and S).

Main differences between IRMS currently derive from the inlet method and the on-line preparation systems available. Originally, gases were generated off-line, and admitted into the sample bellows of the dual inlet (DI) system. A second bellow would have the reference gas, and sample and reference would be alternatively admitted into the ion source by means of capillaries and a 4-way change-over valve. The variable bellows allow matching the pressures of sample and reference gases, to prevent fractionation.

Recently, continuous flow (CF) machines have become common. In CF instruments the dual inlet system is replaced by a carrier gas flow, commonly He. This type of design is particularly suitable for the use of on-line preparation systems, such as elemental analyzers, gas chromatographs or equilibrium devices.

Regardless of inlet method (DI or CF), on reaching the ion source, the gas is ionized by electrons emitted by a heated filament, that strip an electron off the molecule, creating positive ions. Ionization efficiency is very variable, commonly in the range 0.01 to 0.1% (850 to 2000 molecules required per ion formed). A high voltage accelerates these ions through a set of exit slits that focus the beam into the mass analyzer, where ions are separated as a function of mass. The resolved ion beam is collected on ion detectors (commonly Faraday Cups), the signal is electronically amplified, and the isotopic ratios are reported by the data system.

Sample gases fed to the DI systems are produced from the sample offline the mass spectrometer, in purpose built extraction lines. This is time consuming, and requires relatively large samples and specialized knowledge, but results in turn in highly precise measurements.

The two most important advantages of the continuous flow systems are the increase in sample throughput and the decrease in the amount of sample required, such that measurements in the nanomol range (10^{-9}M) are routine now.

All CF methods use a He flow to introduce the sample gas into the mass spectrometer. The sample gas is produced in a range of automated preparation systems, that can lead to “bulk” (BSIA) or “compound-specific” (CSIA) isotopic analysis.

Elemental Analyzers

An elemental analyzer coupled to a magnetic sector mass spectrometer (EA-IRMS) gives the bulk isotopic composition of the sample. Depending on the specific set up, it is possible to measure isotopic ratios of H, C, N, O and S in a range of solid and liquid matrices.

In combustion mode, C-, N- and S-containing materials are loaded into Sn capsules, which are dropped into a reactor packed with suitable catalists. O_2 is admitted into the reactor, resulting in flash combustion of the sample to a mixture of N_2, NO, CO_2, O_2, SO_2 and H_2O, depending on the nature of the substances combusted. This gas mix is carried by the He flow to a reduction reactor, where NO_x are reduced to N_2, and excess O_2 is eliminated. Water is eliminated in a chemical trap, and the gases of interest separated on a suitable chromatography column.

The pyrolysis mode is employed for the analysis of O and/or H. For oxygen, samples are loaded into Ag capsules and dropped into a ceramic reactor lined by a glassy carbon tube in the inside, and packed with nickelized carbon, where the sample is pyrolyzed to CO. As before, water is chemically trapped, and contaminant gases separated by chromatography. H from water samples is obtained by injecting 0.2-0.4 µl via a liquid autosampler onto a reactor packed with Cr.

GC-C-IRMS

CSIA is possible by coupling a GC to the mass spectrometer via a combustion interface. Initially, only C could be determined. Later on, however, modifications were made that allow measurement of N, O, and H isotope ratios.

In its simplest form, the interface is an oven packed with an oxidant (CuO, NiO, Pt, … depending on manufacturer). The compounds eluted from the chromatography column are carried by the He flow; combusted in the interface, and carried into the source of the mass spectrometer. H_2O generated during combustion is physically eliminated by membrane diffusion. If N is to be determined, an additional reduction oven is required. Precise $\delta^{15}N$ measurements depend on quantitative exclusion of CO_2 (since this fragments in the source into CO^+, which isobarically interferes with N_2 at masses 28 and 29). To this end, a cryogenic trap is placed before the inlet of the mass spectrometer. This requirement prevents C and N being determined on the same sample.

Something similar happens for H and O, which require different reactors, and that cannot, therefore, be measured from the same aliquot.

LC-IRMS

The main problem associated to GC-C-IRMS is that samples need to be soluble in a suitable volatile solvent. If they are not, they need to be derivatized to a suitable form. But derivatization can affect the isotopic results. Fractionation can occur either by kinetic effects or incomplete conversion, and the derivatizing agent adds extraneous C, and maybe other elements as well.

Coupling a liquid chromatograph to the IRMS may overcome some of these problems. However, LC-IRMS requires that the liquid phase be prevented from reaching the mass spectrometer, eliminating it either before or after oxidation of the sample of interest. Several solutions have been proposed to this end, such as nebulizing the LC effluent and reacting the sample in a plasma; placing the effluent in a moving wire or belt, evaporating the solvent and combusting the dry sample, or else oxidizing the sample to CO_2 while still dissolved, and separating the CO_2 afterwards.

Whatever the approach, the technique is useful to analyze high molecular weight compounds not well suited for GC-IRMS, such as sugars, aminoacids or active components of pharmaceuticals and drugs.

Applications and uses

Food and food fraud

Food and food-related products represent a large percentage of GDP. They also are a major component to be considered within the average family's domestic economy. Additionally, certain products are perceived as of higher quality on grounds such as provenance ("Denominación de Origen" –DO-; "Apellation d'Origine Contrôlée et Garantie" –AOCG-, …), growing and/or breeding method ("Organically Grown Product") or health benefits, and as such reach substantially higher market prices, warranting the need for objective quality and provenance control methods.

Since the physical, chemical or biochemical reasons underpinning isotope fractionation can be affected by factors such as geography (climate, altitude, latitude,…), species and variety, culture method, … etc., each product to be considered requires

establishing a suitable database to be used as reference. In Europe, this is done through the European Commission's Joint Research Centre (JRC), who establishes validated methods, analytical standards, and mutually recognized approaches to analytical quality assurance (Table 1).

TABLE 1. Official EU analytical methods employing isotope data, and current projects (Calderone et al., 2003).

Product	**Official method / Research Project**	**Application**
Wine	UE # 2676/90, 822/97, 440/2003, OIV ré. ENO 2/96, ENO 17/2001, Wine-DB, GLYCEROL	Chaptalization, Geographycal provenance, Watering.
Alcohol	OIV, BEVABS	Botanical origin, Ethanol
Spirits	OIV, BEVABS	Botanical origin
Sugar	SUGAR ^{18}O	Botanical origin
Honey	AOAC method 999.41	Addition of sugars
Perfumeand Fragance	GCC-IRMS	Botanical origin, anethol, vainillin
Dairy and cheese	MILK	Geographycal provenance
Fruit juice	CEN/TC #s. 108, 109, 110, AOAC method 995.17, SUGAR ^{18}O	Addition of sugars, Dilution
Oil and fat	MEDEO	Origin
Fish	COFAWS	Geographycal provenance; Wild / Cultured
Vinager	OIV	Botanical origin

The term "food" normally includes a complex and highly variable set of products, both natural and at various stages of processing, making it extremely difficult to define detailed chemical compositions that are common to a range of products. Stable isotope distribution, however, responds to fundamental processes, that are fixed at primary production, thus providing a suitable method to test for the authenticity of the items of interest.

As it is not commercially viable to circumvent isotopic detection, stable isotopes have provided a way to detect fraud in products as diverse as, among many others, honey, fruit juices, wine, milk and dairy, Iberian ham, vegetable oil, mineral waters, beverages, food additives and even animal feed (i.e., Rossmann, 2001).

An actual example of how stable isotopes can be used is that of Fig. 1, where the $\delta^{13}C$ and $\delta^{15}N$ of the solid extract of locally purchased beer has been plotted according to the ingredients declared in the label. All beers brewed according to the “Bavarian Purity Law” of 1516 have $\delta^{13}C < -25‰$, as do the wheat and most pure barley malt

beers. Some brands claiming to be "pure barley malt", however, are suspicious of misslabelling.

Other recent examples have to do with characterizing pork products. Traditional breeding of the Iberian pig depends on the "*Dehesa*", a distinctive ecosystem where the animals are free ranging, feeding on natural resources all year long, with a major contribution by acorns in the winter months. It is acorns that impart special characteristics, both in terms of taste and healt benefits, to this class of pork, with quantity and quality of the product mirroring the resources available. But modern market demands are decoupled from natural cycles, which results in a modification of traditional practices in order to satisfy demand, leading to an ever increasing number of animals being reared on non-natural resources; mostly on formulated feed.

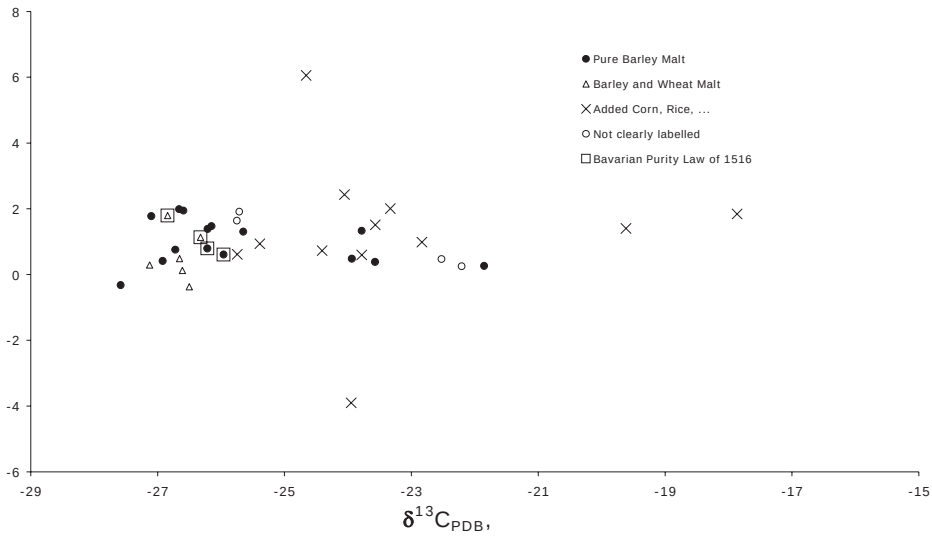

FIGURE 1. Binary plot of $\delta^{13}C$ vs $\delta^{15}N$ measured on the dry extract of beer. Note two well-separated groupings, mostly determined by C isotopic values. Some samples gave values that, a priori, do not correspond with the declared ingredients. Data from Cubides Castillo et al. (2007).

The high quality of Iberian pork products -and subsequent consumer appreciation and willingness to pay a corresponding high price- derives, however, from the traditional breeding and feeding system, so a method to test for substantial acorn consumption is

required. Fig. 2 shows that $\delta^{13}C$ of oleic fatty acid is a useful index to differentiate animals raised on grass + acorns from those fed formulated feed.

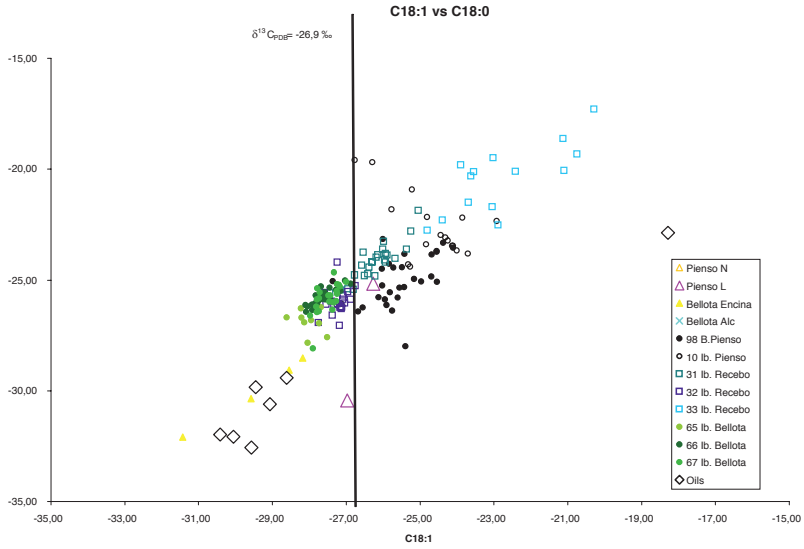

FIGURE 2. $\delta^{13}C_{VPDB}$ obtained for the methyl ester of Oleic Acid (C18:1) versus those of Estearic Acid (C18:0). No single sample classed as Bellota has $\delta^{13}C_{C18:1}$ > -26,88‰. Values of different formulated feeds, acorns and several vegetable oils have been included for reference. From Recio (2007).

Forensics

The analytical techniques commonly available to forensic scientists allow identification of the substances present, but cannot tell one source of the same substance from another. Stable isotope variation, however, responds to fundamental physical, chemical and biological processes, and can thus be used to differentiate among otherwise chemically identical substances. Stable isotopes can corroborate and confirm many evidential leads in the investigation of serious crime (Benson et al., 2006). One potential use is in geo-location, that is, the relationship between stable isotopes and location. Stable isotope ratios can also be used to provide information about the origin of counterfeit money by measuring the hydrogen and oxygen isotope ratios in the paper.

The source of the cellulose can be determined. Other fields of use include the investigation of drug-related crime, where it can assist in distinguishing between different countries of manufacture or different manufacturers of pharmaceutical drugs, and can be employed to determine the procedence of drugs such as heroin: differences in the growing regions of the drug plant will result in differences in the N, C and O isotopic ratios.

Explosives and Fire

All commonly used explosives (gunpowder: a mixture of sulphur, charcoal, and saltpeter -potassium nitrite, KNO_2-; Trinitrotoluene (TNT): $C_6H_2(NO_2)_3CH_3$; Nitroglycerine: $CH_2(ONO_2)$-$CH(ONO_2)$-$CH_2(ONO_2)$; RDX: $(CH_2$-N-$NO_2)_3$; PETN: $C(CH_2ONO_2)_4$, and the mixtures known as Plastic Explosives) have at least C and N into their formulation, and some also H, O and S; all of which have stable isotopes, whose isotopic ratios are amenable to determination by mass spectrometry..

TABLE 2.- Range of $\delta^{15}N$ and $\delta^{13}C$ observed for different explosives (Belanger, 2002).

Material	**$\delta^{15}N$**	**$\delta^{13}C$**	**No. of samples**
Ammonium Nitrate	Range = 5.4 ‰	-	7
Gunpowder	Range = 30.4 ‰	Range = 2.5 ‰	7
TNT	Range = 19.8 ‰	Range = 3.1 ‰	3
Plastic Explosive	Range = 14.0 ‰	Range = 10.1 ‰	4

Studies with PETN have shown that batches produced by the same manufacturer fall within a tight cluster of stable isotope ratio values, distinguishable from other manufacturers. Therefore, information can be obtained as to whether a suspect sample is consistent with being produced by a certain manufacturer. The analysis of samples of ammonium nitrate, gunpowder, TNT and plastic explosive confirmed that there was indeed a range of values for carbon and nitrogen isotope ratios and that the technique had the potential for the forensic analysis of explosives (Table 2; Fig.3).

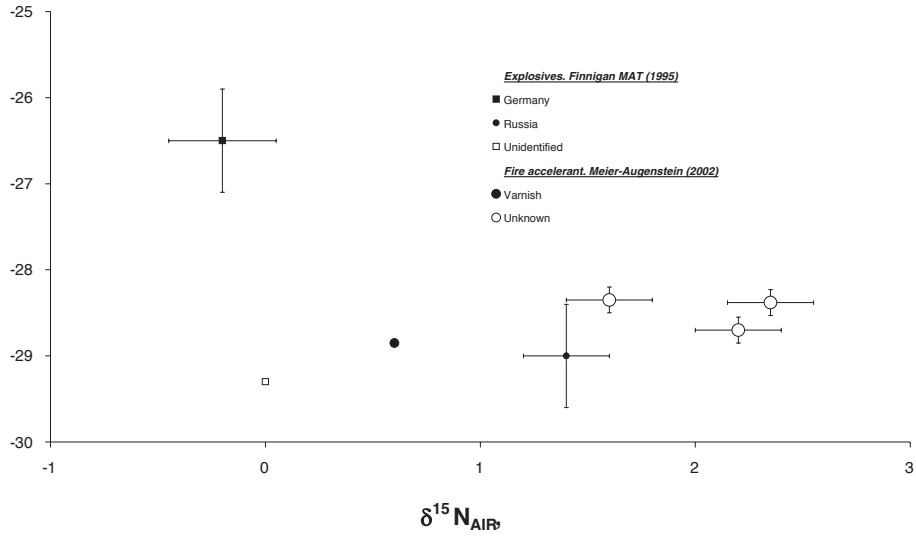

FIGURE 3. Values of $\delta^{15}N$ vs $\delta^{13}C$ measured for actual explosives (Finnigan MAT, 1995) and fire accelerants (Meier-Augenstein, 2002).

When investigating an intentional fire, its origin and how it propagated can frequently be established by physical means. But gathering unequivocal evidence that can be sustained on Court is not always an easy task (Jasper et al., 2002). Traditionally, arson residues are characterized by GC-MS that gives a "fingerprint" of the organic compounds of the accelerants employed. This fingerprint can then be used to identify specific classes of petroleum products by forensic chemists. But this just identifies the accelerant, not the individual that used it.

By using GC-C-IRMS, it is possible not only to identify the individual hydrocarbons composing the accelerant, but also the individual $\delta^{13}C$ of each of them. Commonly, accelerants may be formed by a mixture of up to 50-100 individual hydrocarbons that can be chromatographically separated. Obviously, after a fire, most of them will have degraded, but if we can recover say 4 useful compounds, our chances of unequivocally characterizing the accelerant used are high. GC-C-IRMS techniques can link together accelerant residues from the scene with partially evaporated residues on the arsonist or its environment, and with the remaining pristine accelerant in the container used (Jasper et al., 2002).

Drugs of Abuse and Pharmaceuticals

When fighting trafficking of drugs, an extremely useful piece of information for Authorities is to determine both the area of production and the possible routes followed by dealers. When a batch of heroin, cocaine or other drugs is seized, the drug itself and the impurities it contain keep a record of its origins and its distribution circuit.

Drugs suchs as heroin or cocaine derive from plant materials. The photosyntetic pathway of the plant (C_3 vs C_4), the soil nitrogen the plant had available for gowth, and environmental factors, such as climate and water availability, all impart an specific isotope signature to the final product (Desage et al., 1991; Ehleringer et al., 1999, 2000; Galimov et al., 2005). If adulterants used to "cut" the drug are also considered, the final result is that every batch is almost unique on its combined $\delta^{13}C$ and $\delta^{15}N$ signature (Fig. 4).

But not only batches can be characterized. Heroin is a highly addictive drug that is processed from morphine, a naturally occurring substance extracted from the seedpod of the Asian opium poppy *Papaver somniferum.* Once inside the body it is rapidly metabolised to morphine, which is then excreted in the urine. The presence of morphine in urine cannot alone be used as a marker for illicit heroin abuse since morphine and codeine (which is also metabolised to morphine) can be found in prescriptive medicines (treatments for pain, coughs and diarrhoea) and foods (pastries containing poppy seeds have also been shown to lead to the presence of morphine and codeine in the urine). However, the intermediate metabolite of heroin, 6-monoacetylmorphine (6-MAM), can

be used as a specific marker for heroin as it does not result from the metabolism of either morphine or codeine. In addition, acetylcodeine is a known impurity of illicit heroin synthesis and may be used to distinguish between the pharmacologically pure heroin that is used in heroin maintenance programs and illicit 'street' heroin. Stable isotopic analysis of such components, extracted from body fluids, can aid in distinguishing between prescribed and illicit drugs.

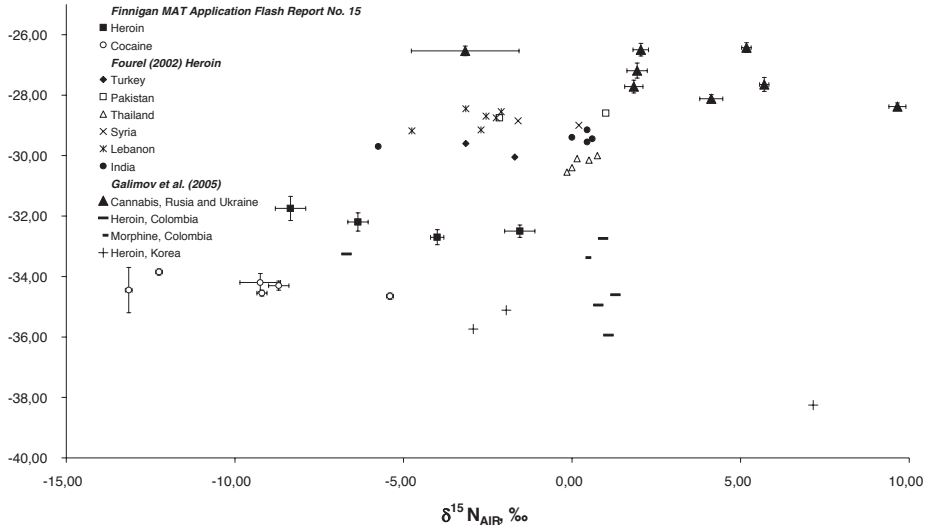

FIGURE 4. C and N isotopic values in four different seizures of heroin and and six shipments of cocaine. Variation of $\delta^{13}C$ is relatively small, but $\delta^{15}N$ clearly individualizes each batch (Finnigan MAT, 1995). Additional data on Heroin and Cannabis samples of known origin have been included for comparison (from Fourel, 2002; Galimov et al., 2005).

In contrast with heroin and cocaine, ecstasy is a synthetic drug, not derived from plant extracts. Ecstasy (3,4-methylenedioxymethylamphetamine; MDMA) is typically prepared from a number of cheap and readily available natural products, via conversion to 3,4-methylenedioxyphenylacetone (MDA) and reductive amination to form an N-substituted amine (Palhol et al., 2003, 2004; Carter, 2002).

According to the UK's Home Office statistics for 1999, 29% of 16-29 year olds have experienced hallucinogenic drugs. Police and HM Customs seized approximately 6.5 million ecstacy tablets, for an estimated supply of 26 million tablets. In France, with

about 25% of total ecstasy seizures of Europe, the number of tablets analyzed by Customs authorities increased 400% in 5 years (Carter et al., 2002).

Tablets sold as ecstasy are notorious for containing, in addition to MDMA, other drugs e.g. MDA, MDEA, amphetamine and caffeine. Target analysis of these and other amphetamines in biological samples is of great importance for clinical and forensic toxicologists alike. Plasma is traditionally one of the most commonly investigated specimens for the confirmation of illicit drug use.

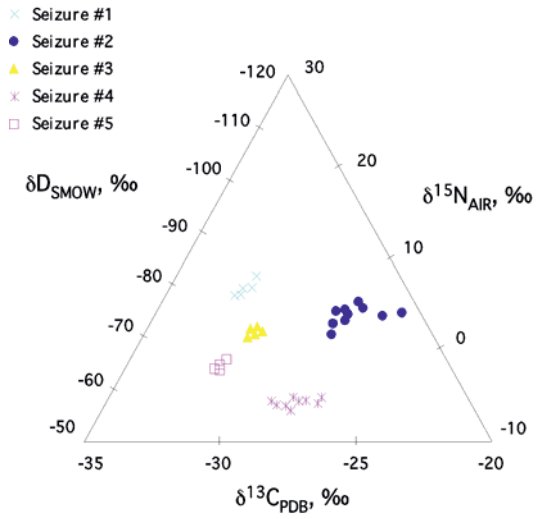

FIGURE 5. Combined use of H, C and N isotopic values allows characterisation of individual ectasy seizures. From Carter et al. (2002).

"Isotopic fingerprinting" can be used to distinguishing from batch to batch (Fig. 5); linking "trace" and "bulk" evidence; to trace drugs to a common source of manufacture or supply, and to determine synthetic method. $\delta^{13}C$ and $\delta^{15}N$ on ecstasy directly depend on the physicochemical environment from which they are derived, marking both precursors and method of synthesis, although cannot provide information on geographical procedence. Results to date indicate that $\delta^{15}N$ is a major discriminating factor, while δD and $\delta^{13}C$ are minor factors. The combined use of $\delta^{13}C$ and $\delta^{15}N$ reflects reductive amination, and δD reflects origin and solvent history.

Geolocation

Stable isotopes can be used to track the recent history not only of substances, but also that of people. C and N isotopes record diet (De Niro and Epstein, 1978, 1981; Bold and Pflieger, 2002; O'Connel and Hedges, 1999), and O and H isotopes track drinking habits (water, but also other usual drinks, such as beer) (Cerling et al, 2003; O'Brien and

Wooller, 2007; Ehleringer et al., 2008). These techniques are widely used to study animal migration, but it is only recently that they have been applied to forensic issues (O'Brien and Wooller, 2007). Several substrates are suitable for seeking evidence: human blood records both local drinking water and diet (Ehleringer, 2002). Breath CO_2 can also inform about recent locations. Bone and theet keep a longer, sometimes lifelong record, but the implications of obtaining such samples should be obvious. Non-invasive samples, such as hair, nails or even urine, have been proven useful to track medium-term travel (from a few days to a few months; Fraser and Meier-Augenstein, 2007; Bol and Pflieger, 2002; O'Connel and Hedges, 1999; Bowen et al., 2005; Fraser et al., 2008).

Interpreting δD and $\delta^{18}O$ values in terms of geographical location requires an understanding of the multiple sources of oxygen and hydrogen, and of the factors that can contribute to isotopic fractionation. Isotopic values of human hair keratin should be the result of the various contributions from drinking water, food water, dietary protein and atmospheric sources (Ehleringer et al., 2008). However, these various contributions can be successfully modelled (Ehleringer et al., 2008; O'Brien and Wooller, 2007; Fraser and Meier-Augenstein, 2007). Also, different target samples record different information and time periods. According to O'Brien and Wooller (2007), 90% turn over of body water, as recorded by urine, requires some 24 days. Hair and nails, on the other hand, are only "alive" at the root. From there on, no isotopic modification occurs (if properly cared for; see Bowen et al., 2005; Fraser et al., 2008), such that hair, for example, keeps a linear record into the past, whose duration depends on hair length: on average, hair grows at an average rate of 1 cm per month. Bleaching, dying, or coloring has only minor effects on the isotopic composition of hair (Cerling et al., 2003).

Doping in Sport

The analysis of performance enhancing doping agents in sport has become an important area of analytical toxicology. Specific methods of analysis are necessary to meet international regulatory thresholds in a variety of matrices, most commonly urine and blood, but also other biological matrices such as hair and sweat. In addition, new strategies are required to distinguish between exogenously administered hormones and their natural analogues. Quantitative measurement of doping agents in complex matrices

such as blood and urine to regulatory levels requires the high selectivity and sensitivity offered by GC/MS and LC/MS/MS. Gas Chromatography-Isotope Ratio Mass Spectrometry (GC-IRMS), on the other hand, is proving a powerful tool for the detection of drug abuse in both humans and animals, potentially applicable to the analysis of any organic metabolites. IRMS is now widely accepted as the only technique that can unambiguously distinguish between endogenously produced and synthetic, self-administered performance enhancing drugs. The measurement of deuterium isotopes provides additional evidence for sourcing drug metabolites.

This technique has been accepted by the IOC Medical Commission (1997) as a viable technique for distinguishing between exogenous and endogenous steroid metabolites. The World Anti-Doping Agency (WADA, 2004) requests that any T/E ratio above 4 be confirmed by means of stable C isotope analysis.

Testosterone

There is an increasing need in sport for new methodologies in proving testosterone misuse. Up to recently, GC-MS was employed to determine the excreted Testosterone / Epitestosterone ratio from urine. A value of greater than 4:1 was considered as confirmation of drug administration (WADA, 2004). This ratio was accepted because the distribution of world population fell below this figure. However, there are minorities whose ratio can be naturally higher (Table 3; see Sottas et al., 2008). Also, this ratio can be manipulated downwards by simultaneous administration of epitestosterone. The GC-MS test is therefore fallible, potentially giving both false positives and false negatives (Sottas et al.,

Study day	**V_1**	**V_2**	**V_3**	**V_4**	**V_5**
1	0.26	1.28	**4.69**	0.10	0.12
2	0.33	1.48	**5.26**	0.11	0.11
3	0.65	**8.77**	**29.30**	0.26	0.70
4	1.95	**24.20**	**36.90**	1.77	**6.69**
5	2.81	**45.80**	**41.80**	2.01	**5.91**
6	2.79	**33.20**	**61.80**	1.96	3.71
7	3.03	**15.40**	**54.90**	1.91	**4.24**
8	**4.13**	**36.40**	**57.70**	1.48	**5.95**
9	**5.30**	**30.30**	**39.70**	1.51	**5.78**
11	3.20	**26.50**	**33.00**	0.58	3.69
13	0.77	2.63	3.42	0.68	3.41
15	0.22	0.99	**4.37**	0.19	2.27
17	0.26	2.02	**4.80**	0.13	0.21

2008). There are methods for overcoming this; the administration of a drug called ketoconazole to an athlete with a naturally high T/E ratio results in a decrease of such ratio, but in athletes who have received exogenous testosterone it causes the ratio to increase.

This test could be used to determine innocence or guilt after a positive T/E test, but it involves administering a drug to detect a drug. Additionally, the athlete needs to be supervised for up to 8 hours for the drug to take affect, therefore being invasive and inconvenient for both the sampling officer and the athlete.

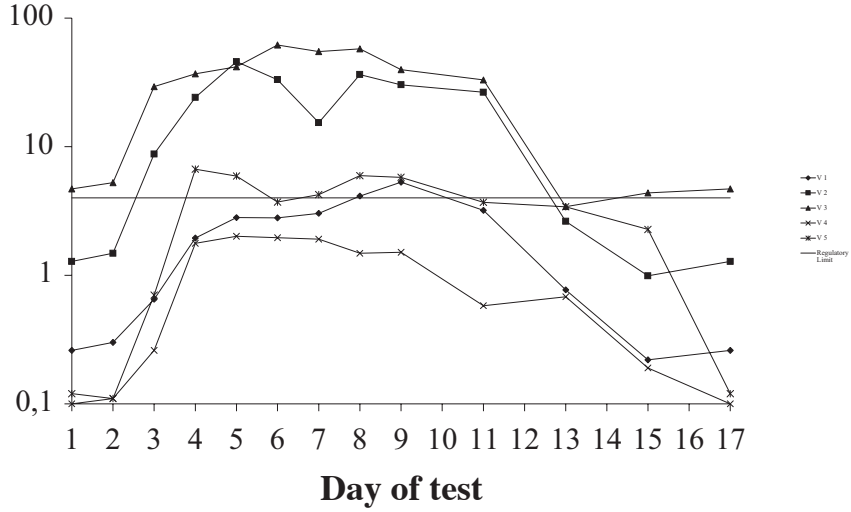

FIGURE 6. Evolution of the $\delta^{13}C$ values of Table 3. All but one volunteer test positive.

The use of Stable Isotope Ratio Mass Spectrometry, however, has shown that synthetic testosterone (derived from plants) has a lower $\delta^{13}C$ than the endogenous hormone (for example, Shackleton et al., 1997). The quantity of urine collectable from an athlete in events is minimum (50-100ml), and this has to be split to perform several analyses. Targeting the metabolites of testosterone, especially the androstanediols, which are naturally abundant and are at a higher concentration than testosterone, would make it easier to detect and monitor isotopically. The technique, therefore, uses a multistep, but

relatively simple procedure, consisting in separating two or three functional groups by LC; removing the pregnane metabolites by oxidation, and derivatising the steroids by acetylation. Only one chromatographic separation removes up to 75% of the unwanted steroids from the urine. The oxidation removes the majority of the complex side chains from the 17-hydroxypregnane steroid resulting in a cleaner sample for a simpler chromatogram. The derivatised samples are dissolved on cyclohexane and injected into the GC-C-IRMS, where they are analysed for their isotopic ratios.

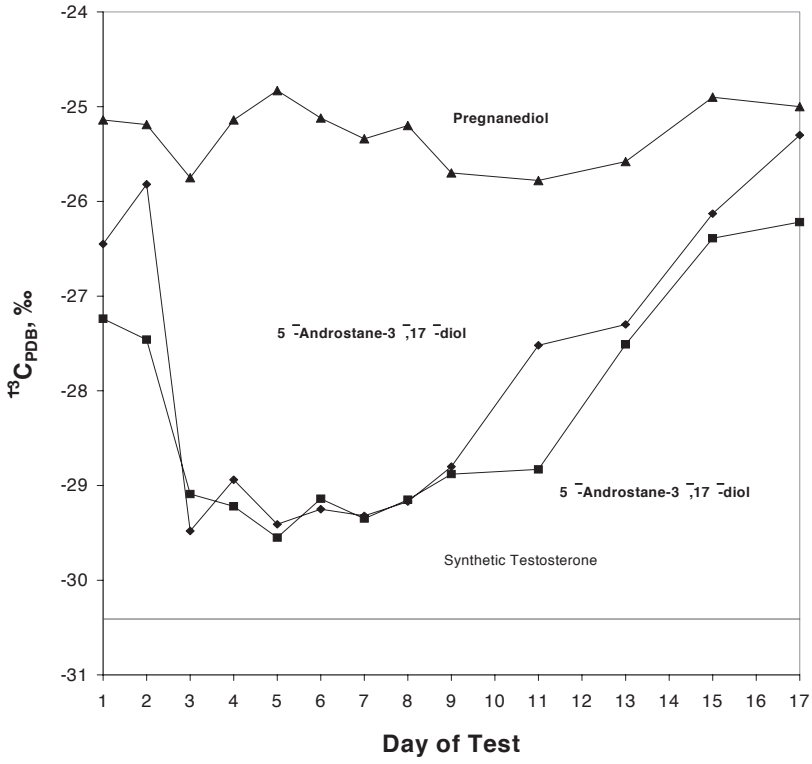

FIGURE 7. Evolution of the $\delta^{13}C$ value of 5α- and 5β-androstane-3 α,17β diols and pregnanediol after administration of testosterone at the end of day 2. Data from Shackleton et al. (1997) and Phillips (1999).

Experimental results (Fig. 7) show that $\delta^{13}C$ of pregnanediol does not change dramatically by administration of testosterone, while $\delta^{13}C$ of the 5α- and 5β-androstane-3α,17β diols become lighter after the administration, and only starts to recover after 6-7

days. The pregnanediol could be monitored as an internal reference to relate to diet, or can possibly be used to develop an indication ratio for abuse. Metabolites that are unaffected by testosterone could be used as the individual's baseline, and therefore regulatory values could be agreed upon for future doping tests.

This technique could also be applied to the testing of other anabolic reagents, such as DHT, DHEA and epitestosterone. Cawley et al. (2004) found that 3α,5-cyclo-5α-androstan-6β-ol-17-one ("3α,5-cyclo") is a natural byproduct of metabolism of dehydroepiandrosterone (DHEA), but whose concentrations are elevated (5-15 times) following DHEA administration. By considering average values from available databases, it was proposed that any sample with 3α,5-cyclo ≥ 140 ng/ml be tested by IRMS for significant ^{13}C depletion, since it was confirmed experimentally that $\delta^{13}C$ of 3α,5-cyclo change from –24.3‰ to –31.1‰ 9 hrs after administration of a single dose of DHEA, returning to baseline after 48 hrs. In case of continued administration of DHEA, $\delta^{13}C_{3\alpha,5\text{-cyclo}}$ reached even lower values at –33.9‰.

Nandrolone

Evidence is emerging that exercise and legal supplements may raise endogenous 19-norandrostereone (19Na) above 2 ng per ml of urine, the current IOC threshold for reporting a doping offence. As a result, a preliminary study was undertaken by Phillips et al. (2000) to establish the feasibility of using stable isotope ratio mass spectrometry (IRMS) to distinguish between high endogenous urinary excretion and high excretion due to administration of a nandrolone doping agent.

Six volunteers (3 male and 3 female) provided a single urine sample prior to steroid administration. The $^{13}C/^{12}C$ isotopic ratio of urinary pregnanediol (PD) and 19-norandrostereone (19Na) were then determined in a similar analytical manner to that described in Shackleton *et al*. (1997.a, b). The volunteers then self-administered 100 mg of 19-norandrostanedione, a legal "over the counter" food supplement. A second urine sample was taken 48 hours following administration and the $^{13}C/^{12}C$ isotopic ratio of urinary PD and 19Na was determined.

Prior to steroid administration the $\delta^{13}C$ value of PD (a nandrolone precursor) and 19Na (a nandrolone metabolite) were approximately equal (Fig. 8, top). 48 hours

following administration of 19-norandrostanedione the $\delta^{13}C$ value of urinary 19Na decreased relative to PD (Fig. 8, bottom). IRMS analysis of the doping agent returned a lower abundance of ^{13}C ($\delta^{13}C_{PDB}$ of 19-norandrostanedione equalled -32.8 ±0.3‰) than endogenous 19Na. As a result, it appears that oral administration of the doping agent altered the isotopic value of the steroid's metabolites, yet left the steroid precursors unchanged.

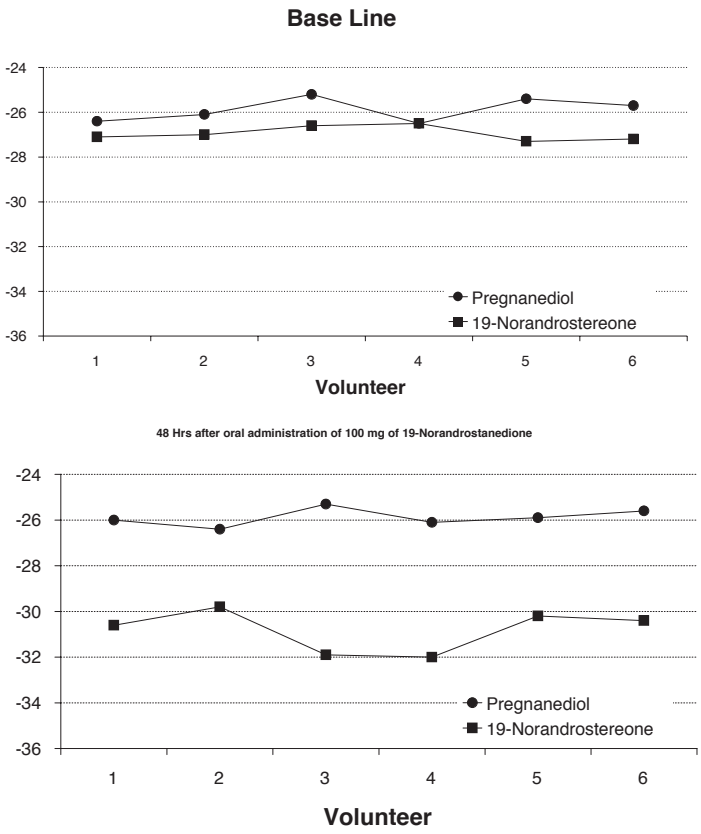

FIGURE 8. Evolution of the $\delta^{13}C$ value of urinary steroids before and 48 hrs. after administration of 19-norandrostanedione. Data from Phillips et al. (2000).

Urine from pregnant women in their third trimester were also analysed and the ^{13}C values were very much the same as those of the volunteers prior to nandrolone administration, yet it is known that at this stage in their pregnancy, women can show large increases in concentration of urinary 19Na. The data shows that the IRMS analysis

of nandrolone metabolites and nandrolone precursors could provide a feasible method for detecting nandrolone abuse. This is however a preliminary study and requires considerable work before a valid test can be applied to doping control.

Pollution / Environmental

Compound-specific isotope analysis (CSIA) is facilitating the application of isotopic tools at much finer spatial and temporal scales than was possible ever before, and opening up for detailed isotopic investigation of a whole new range of organic compounds typically present at ppm to ppb levels in the subsurface. CSIA has made a major impact in biogeochemical and environmental research. Basic and applied questions pertaining to pollutant fate and transport in near-surface environments can be addressed employing isotopic techniques, resulting in the ability to trace the sources and pathways of a wide variety of natural and anthropogenic organic compounds.

As with any tool applied to environmental research, there should always be recognition by practitioners of isotope geochemistry that, at best, we may hold a small piece to a grand environmental puzzle. Isotopic measurements are most useful when used in tandem with careful field observations and other geochemical, geophysical and geological constraints.

Non Point-Source Pollution

Preventing the pollution of soil and water requires characterising the contaminants, and elucidating their provenance. Among the many possible contaminants, the nitrates present in soil and water are of major concern. Conventional chemical methods suffer from multiple interferences that render them of only limited use at best. Nitrates can originate from a point source –industrial and urban sources: liquid waste and leachate of MSW landfill sites-, or from a non-point source –agricultural activities, for example-.

Agriculture –crop fertilizers and watering- represents a non-point source, and is the most important contributor to nitrates in continental waters, although point sources can be locally important (Perlmutter and Koch, 1972, Kreitler, 1975; Kreitler and Jones, 1975; Kreitler et al., 1978; Porter, 1980; Flipse et al., 1984; Flipse and Bonner, 1985). Natural fixation of nitrogen by the biosphere will only exceptionally result in anomalously high nitrate contents in soil and, eventually, water (Böhlke et al., 1997; Kreitler and Jones, 1975). High N and P contents result in water mass eutrophization,

and high N in drinking water has been linked to diseases such as metheglobinemia and cancer (Vigil et al., 1965). The issue is widely acknowledged by international organizations. In Europe, the "Nitrates Directive", 91/676/EEC, establishes mandatory maximum limits of 50 mg/l NO_3^- (11,3 mg/l N, although it recommends not to exceed 25 mg/l NO_3^- or its equivalent 5,6 mg/l N, as established in the "Drinking Water Directive", 80/778/EEC). These are very similar values to those recommended by the WHO[1], and the ones currently adopted by the US's EPA.

Process	*Reaction*	α, fractionation factor
N_2 fixation	N_2 ➔ NH_4^+ ➔ organic N	0.991 to 1.0041
N_2O reduction	N_2O ➔ NH_4^+ ➔ organic N	1.00343
Denitrification	NO_3^- ➔ N_2O	1.028; 1.033
NO_3^- assimilation	NO_3^- ➔ NH_4^+ ➔ organic N	1.0027 to 1.03
NH_4^+ assimilation	NH_4^+ ➔ organic N	1.0091 to 1.02
NO_2^- assimilation	NO_2^- ➔ NH_4^+ ➔ organic N	1.007
Nitrification	NH_4^+ ➔ NO_2^-	1.025 to 1.035
Diffusion	NH_4^+, NH_3, or NO_3^- in solution	~ 1.00
	NH_3 diffusion in gas phase	1.018

Table 4.- Nitrogen isotope fractionations during biologically-related reactions.From Handley and Raven (1992). Fractionation factors are composites from a variety of organisms cultured in laboratory or field experiments.

Transcription of the European Directive to Spanish national legislation (R.D. 261/1996, 16 February; B.O.E. # 61, 11/3/1996), keeps the concentration of nitrate mentioned, and includes the publication of the so called "Código de Buenas Prácticas Agrarias" (Good Agricultural Practice Code), that, among other things, regulates fertilizer application (quantity and season) and density of livestock per surface area, such that the nitrogen load is always lower than 170 $KgHa^{-1}yr^{-1}$. This represents between 367

[1] The WHO set an upper limit of 10 mg/l of N derived from NO_3^-, and 1 mg/l N derived from NO_2^- (equivalent to 44,3 and 3,3 mg/l of the respective ion in drinking water; World Health Organization, 1984, Guidelines for Drinking-Water Quality; WHO, Geneva)

and 2125 Kg/Ha/year of chemical fertilizer (depending on its nature; Orús et al., 2000), or a livestock density that varies between 580 hens and 2 cows per Ha and year (Regulation CEE 2092/91).

It is therefore evident from the above that control instruments must be sought in the search for possible links between source and sink. But cause-effect relationships are not enough; it is necessary to search for parameters that allow a firm association between source and pollutant, as well as quantifying relative contributions in the case of multiple sources.

Classical chemical methods can easily determine nitrate in water; however, they are not suited to identify the source of pollution. Whatever its origin, the different forms of soil N are subject to transformations that mask its origin. The stable isotopic ratios D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$, however, can fulfil the requirements. Different substances have characteristic isotopic ratios, that allow not only identification, but also quantification of individual pollutants (Kendall and McDonnell, 1998). Natural soil N derives ultimately from the atmosphere via biological fixation. Except in very particular locations, the geological materials are not a significant source of N. Anthropogenic nitrate or ammonium, however, can locally or regionally represent a noticeable contribution.

Atmospheric N_2 is also used to produce NH_3 by the Haber method, and this is the base to synthesise chemical fertilizers. It is estimated that by the year 2000, the amount of fertilizer-N already equalled the amount of N fixed in organic matter by natural processes. Once incorporated into the soil, organic matter is employed as substrate by multiple microbial reactions. Nitrates are highly soluble; they are not as reactive with organic complexes as ammonia, and can be easily transported by water percolating through the soil.

Each of the steps described has the potential for fractionating N isotopes. Except fixation and assimilation as a nutrient, other processes can result in fractionations between 10 to 30‰ (Table 4), or even larger (the product depleted in ^{15}N with respect to the reagent), as observed after fertilizer application.

Nitrate $\delta^{15}N$ is useful in tracing its provenance (for example, Aravena et al., 1993, Durka et al., 1994; Wassernaar, 1995; Ging et al., 1996; Kendall et al., 1997). However,

although $\delta^{15}N$ depends directly on the N source, exchange with soil-N can affect its isotopic signature (Komor and Anderson, 1993). An even larger effect is associated to ammonium volatilization (Mariotti et al., 1988; Bötcher et al., 1990). It is therefore necessary to establish the isotopic relationships within the context of the local conditions of the area under investigation. A multielement / multisotopic study will help in this regard. For example, the additional determination of $\delta^{18}O$ allows characterisation of possible processes that might potentially modify the concentration and the isotopic ratios of N in nitrates, such as denitrification (Amberger and Schmidt, 1987; Böttcher et al., 1990).

The few remaining uncertainties after N and O isotopic analysis can be clarified by the use of additional isotopic systems (S and O in sulphates; O in phosphates, … etc) and chemical data (for example, Böhlke and Denver, 1995). Typical $\delta^{15}N$ values are between –5 to +3,5‰ in soils where chemical fertilizers have been applied; natural soil organic matter has $\delta^{15}N \approx +3,5$ to +7,5‰; animal manure is between +10 to +20‰, and downstream of septic tanks values around $\delta^{15}N \approx +10$‰ (Aravena et al., 1993) to $\delta^{15}N \approx +8$‰ (Kreitler et al., 1978) have been measured.

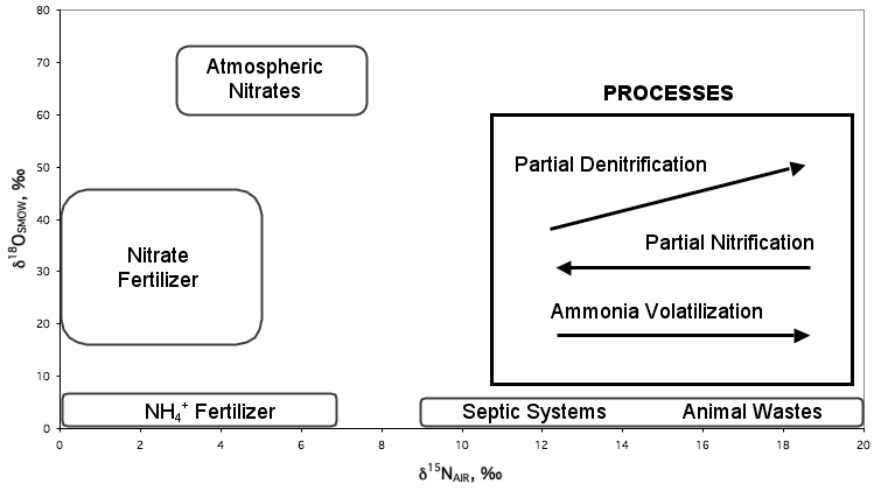

FIGURE 9. O and N isotope characterisation of various sources and processes that can contribute nitrate pollution to soil and water. From Bleifuss et al. (2001).

The combined use of $\delta^{15}N$ and $\delta^{18}O$ is useful for differentiating nitrates of varied provenance (see Fig. 9). O isotopes are particularly suited to identify atmospheric N, particularly in contrast with that derived from bacterial nitrification that occurs naturally in the soil. It is thus realtively easy to tell nitrate derived from chemical fertilizers from soil nitrate, or that derived from manure. Bacterially fixed nitrate varies regionally, since only one oxygen derives from gaseous O_2, while the remaining $^2/_3$ are derived from ambient water (Amberger and Schmidt, 1987; Durka et al., 1994; Kendall et al., 1997; Anderson and Hooper, 1983; Hollocher, 1984), whose $\delta^{18}O$ follows the meteoric water line (MWL).

Concluding Remarks

In former pages, we have taken a very quick tour around some of the multiple applications of stable isotopes, in fields as different as Food Science, Environmental Pollution, Forensic Science.... It never was my intention to do an exhaustive review of possible applications -an impossible task, anyway, for any single scientist in a reasonable time- but to provide a quick overview of the fundamentals underpinning the analysis and interpretation of stable isotopic ratios, and how "scientific common sense" can make use of such a tool to get answers to intricate scientific subjects, but also to down-to-Earth everyday problems.

Many fundamental fields of research have been left intentionally and unavoidably out. No mention has been made of the use of stable isotopes in Earth Sciences, the mother science from which stable isotope analysis was born. Biological Sciences at large -and particularly Ecology- have also been left aside, together with natural derivations such as Agricultural Sciences. The reason behind is that these fields have, in the course of time, created a body of science able to stand alone as important disciplines in their respective fields, and the interested reader should be able to find plenty of treatises and research articles which will address the use of stable isotopes in due depth. Other sectors of active research employing stable isotopes, such as climate change, oil exploration, medical and clinical applications, … to mention but a few, are subdisciplines of broader scientific areas, and normally of interest to the specialists only.

As mass spectrometry evolves, new technological developments, both in the mass

spectrometers themselves and in the preparation systems coupled to them, open up new perspectives for the use of stable isotopic ratios, but the fundamental principles remain unchanged. Current technology allows the analysis of isotopic ratios on specific molecules, and on samples on the nanogram size.

In terms of the traditional physicochemical techniques, to substances can be *"alike as two water drops"*. Stable isotopes, however, are a useful tool when in hands of a skilled researcher, able to *tell one source of the same substance from another*. It is only the imagination of the researcher that can place limits on the application of stable isotope techniques to the solution of an increasingly wider range of challenges.

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