Part I
How it Works

Chapter I.1

What are Stable Isotopes?

Of the 92 natural chemical elements, almost all occur in more than one isotopic form, with the vast majority being stable isotopes, which do not decay, unlike radioisotopes, which are not stable and hence undergo radioactive decay. To put it another way, 61 of these 92 natural chemical elements appear in two or more stable isotopic forms. So, in this context "almost all" means with the exception of 20 stable chemical elements, including fluorine, sodium and phosphorus, which are mono-isotopic. Making up the difference between 81 and 92 are 11 naturally occurring radioactive chemical elements, including radon and technetium. The word isotope was coined by Professor Frederick Soddy at the University of Glasgow and borrows its origin from the two Greek words *isos* (ισος) meaning "equal in quantity or quality" and *topos* (τοπος) meaning "place or position", with isotope hence meaning "in an equal position" (of the Periodic Table of the Elements). Incidentally, Frederick Soddy was awarded the Nobel Prize in Chemistry in 1921 for his work on the origin and nature of isotopes. By coining this term he referred to the fact that isotopes of a given chemical element occupy the same position in the Periodic Table of the Elements since they share the same number of protons and electrons, but have a different number of neutrons. The word isotope therefore does not denote radioactivity, as is so often mistakenly thought. As mentioned above, radioactive isotopes have their own name, radioisotope. Non-radioactive or stable isotopes of a given chemical element share the same chemical character and only differ in atomic mass (or mass number *A*), which is the sum of protons and neutrons in the nucleus.

Moving from the smallest entity upwards, atoms are composed of positively charged protons and neutral neutrons, which make up an atom's nucleus, and negatively charged electrons, which make up an atom's shell or electron cloud. Due to charge balance constraints, the number of protons is matched by the number of electrons. A chemical element and its position in the Periodic Table of the Elements is determined by the number of protons in its nucleus. The number of protons determines the number of electrons in the electron cloud and the configuration of this electron cloud in turn determines chemical characteristics such

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as electronegativity and the number of covalent chemical bonds a given element can form. Owing to this link, the number of protons in the atomic nucleus of a given chemical element is always the same and is denoted by the atomic number Z, while the number of neutrons (in its nucleus) may vary. Since the number of neutrons (N) has no effect on the number of electrons in the electron cloud surrounding an atom the overall chemical properties of an element are not affected. In other words, a chemical element like carbon will always behave like carbon irrespective of whether the number of neutrons in its nucleus is N or $N+1$. However, differences in mass-dependent properties can cause compounds containing different amounts of carbon with N or $N+1$ neutrons or at different positions to behave subtly differently, both chemically and physically.

Mass number A ($=Z+N$) and atomic number Z (= number of protons) are denoted as whole numbers in superscript and subscript, respectively, to the left of the element symbol. So carbon-12, comprising six protons and six neutrons is written as ${}^{12}_{6}\mathrm{C}$ while carbon-13, which comprises six protons and seven neutrons, is written as ${}^{13}_{6}\mathrm{C}$. In general practice different isotopes of the same chemical element are denoted by mass number and chemical symbol only, for example $^{2}\mathrm{H}$ or $^{13}\mathrm{C}$.

The simplest of chemical elements, hydrogen (H), in its most abundant isotopic form has a nucleus comprising a single proton and therefore has the atomic weight of 1 (in atomic mass units, amu) and this is indicated by adding a superscript prefix to the element letter, that is, $^{1}\mathrm{H}$. The less abundant and 1 neutron heavier hydrogen isotope is therefore denoted as $^{2}\mathrm{H}$, although one will also find the symbol D being used since this stable hydrogen isotope has been given the name deuterium. The discovery of this isotope won Harold C. Urey the Nobel Prize in Chemistry in 1934, and today Urey is regarded as one, if not *the* father of modern stable isotope chemistry.

Staying with hydrogen as example, one could say $^{1}\mathrm{H}$ and its sibling $^{2}\mathrm{H}$ are identical twins that have different weights and different abundances. In the case of hydrogen, the weight difference between the more abundant $^{1}\mathrm{H}$ and the less abundant $^{2}\mathrm{H}$ is one atomic mass unit. The same is true for the carbon twins. Here sibling carbon-13 ($^{13}\mathrm{C}$) is the heavier twin, weighing 1 amu more than its sibling carbon-12 ($^{12}\mathrm{C}$), and as is the case for the two hydrogen isotopes the heavier $^{13}\mathrm{C}$ is again the less abundant of the two stable carbon isotopes. However, in the case of carbon the actual weight difference of 1 amu amounts to a relative weight difference of only 8.33 % for $^{13}\mathrm{C}$ relative to $^{12}\mathrm{C}$ (cf. Table I.1) while in the case of hydrogen the weight difference of 1 amu means relative to $^{1}\mathrm{H}$ the less abundant isotope $^{2}\mathrm{H}$ is 100 % heavier than its twin, the more abundant isotope $^{1}\mathrm{H}$. Where the normal weight versus overweight twin analogy has its limitations is in the matter of abundance or occurrence, but only for as long as we stay with the example of two complete twins. We will revisit the twin example in the following chapter after a brief excursion through the natural abundance of stable isotope and natural abundance level variations.

Chapter I.2

Natural Abundance Variation of Stable Isotopes

The global, or perhaps better described as the mean, stable isotope abundances of all non-radioactive elements were set when the Earth was formed and, on a global or globally averaged scale have not changed significantly since. On the basis of globally averaged isotope abundances many a generation of students was taught that the stable isotope abundances of chemical elements were fixed constants and traditional analytical techniques did nothing to dispel this incorrectly held perception. While infrared spectroscopy, nuclear magnetic resonance spectroscopy and mass spectrometry are able to detect the presence of ^{13}C in a given organic compound, none of these techniques are sensitive enough to accurately detect let alone quantify subtle variations in stable isotope abundance at a level of 0.1 atom% or below. When I studied chemistry in the 1980s we were still taught that the natural abundance of ^{13}C was a constant 1.11 atom% even though the seminal work by Epstein and co-workers in the 1960s and 1970s had already demonstrated carbon isotopic fractionation and associated differences in ^{13}C composition of bio-organic compounds as a result of kinetic isotope effects during biochemical reactions (Deniro and Epstein, 1977; Park and Epstein 1960, 1961; Smith and Epstein, 1971).

The figures usually quoted in chemistry textbooks for isotope abundance refer to the globally averaged values, that is, when considering the entire carbon mass of the Earth system the natural abundances of ^{12}C and the one neutron heavier ^{13}C are 98.89 and 1.11 atom%, respectively (cf. Table I.1). However, what tends to be overlooked when referring to these abundance values and, hence, not be taught to students in chemistry classes is the fact that the compartmental isotope abundance of light elements is not fixed, but is in a continuous state of flux due to the mass discriminatory effects of biological, biochemical, chemical and physical processes. For instance, when looking at individual carbon pools one finds some with a higher abundance of ^{13}C, such as marine carbonate sediments, whereas others are more depleted in ^{13}C, such as the hydrocarbons found in crude oil.

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Table I.1 Key figures for stable isotopes of light elements.

Chemical element	Major abundant isotope[a]	First minor abundant isotope[a]	Second minor abundant isotope	Relative atomic mass difference First minor/major	Isotope ratio First minor/major for scale reference point
Hydrogen	^{1}H	^{2}H		100 %	0.00015576
	99.985 atom%	0.015 atom%			VSMOW
Carbon	^{12}C	^{13}C		8.33 %	0.0112372
	98.89 atom%	1.11 atom%			VPDB
Nitrogen	^{14}N	^{15}N		7.14 %	0.0036765
	99.63 atom%	0.37 atom%			Air
Oxygen	^{16}O	^{18}O	^{17}O	12.5 %	0.0020052
	99.76 atom%	0.20 atom%	0.04 atom%		VSMOW
Sulfur	^{32}S	^{34}S	^{33}S	6.25 %	0.0450045
	95.02 atom%	4.22 atom%	0.76 atom%		VCDT

[a]Note that listed isotope abundance values in atom% are global mean values on which atomic weights given in the old-style Periodic Table of the Elements are based (e.g. 1.00797 for H or 12.0112 for C); they are not identical and should thus not be confused with isotope abundance values or isotope ratios of the materials chosen as scale reference points.

The fact that atomic fractions or mole fractions of isotopes varied in normal naturally occurring materials was recognized in 1967 by the International Union of Pure and Applied Chemistry (IUPAC) Commission on Isotopic Abundances and Atomic Weights (CIAAW) or the Commission on Atomic Weights as it then was. Tables of Standard Atomic Weights published by CIAAW prior to 2009 listed only a single atomic weight for each element with at least one minor stable isotope. As of 2009, CIAAW started to report atomic weight intervals for some elements with the upper and lower boundaries of the atomic weight for a given chemical element, defining the interval within which the atomic weight value for this element in a given normal compound or material might be encountered. Standard atomic weight values for each chemical element are reviewed by CIAAW on a regular basis and the results of these reviews are reported to IUPAC and subsequently published as IUPAC Technical Reports in the journal *Pure and Applied Chemistry*. In the 2011 Table of Standard Atomic Weights the atomic weight interval for carbon was given as [12.0096, 12.0116] (Wieser *et al*., 2013). This change in reporting the atomic weights of chemical elements as atomic weight intervals is now also reflected in a new IUPAC Periodic Table of the Isotopes (Holden *et al*., 2011) a reproduction of which can be found in Figure I.1. It can be downloaded as a PDF from the CIAAW website (http://www.ciaaw.org/pubs/Periodic_Table_Isotopes.pdf).

Expressed in units of atom% and staying with the example of ^{13}C, these differences are very small, with the range covered amounting to approximately 0.11 atom%. To express these minute variations, the δ notation has been adopted to report changes in isotopic abundance compared to a designated isotopic standard

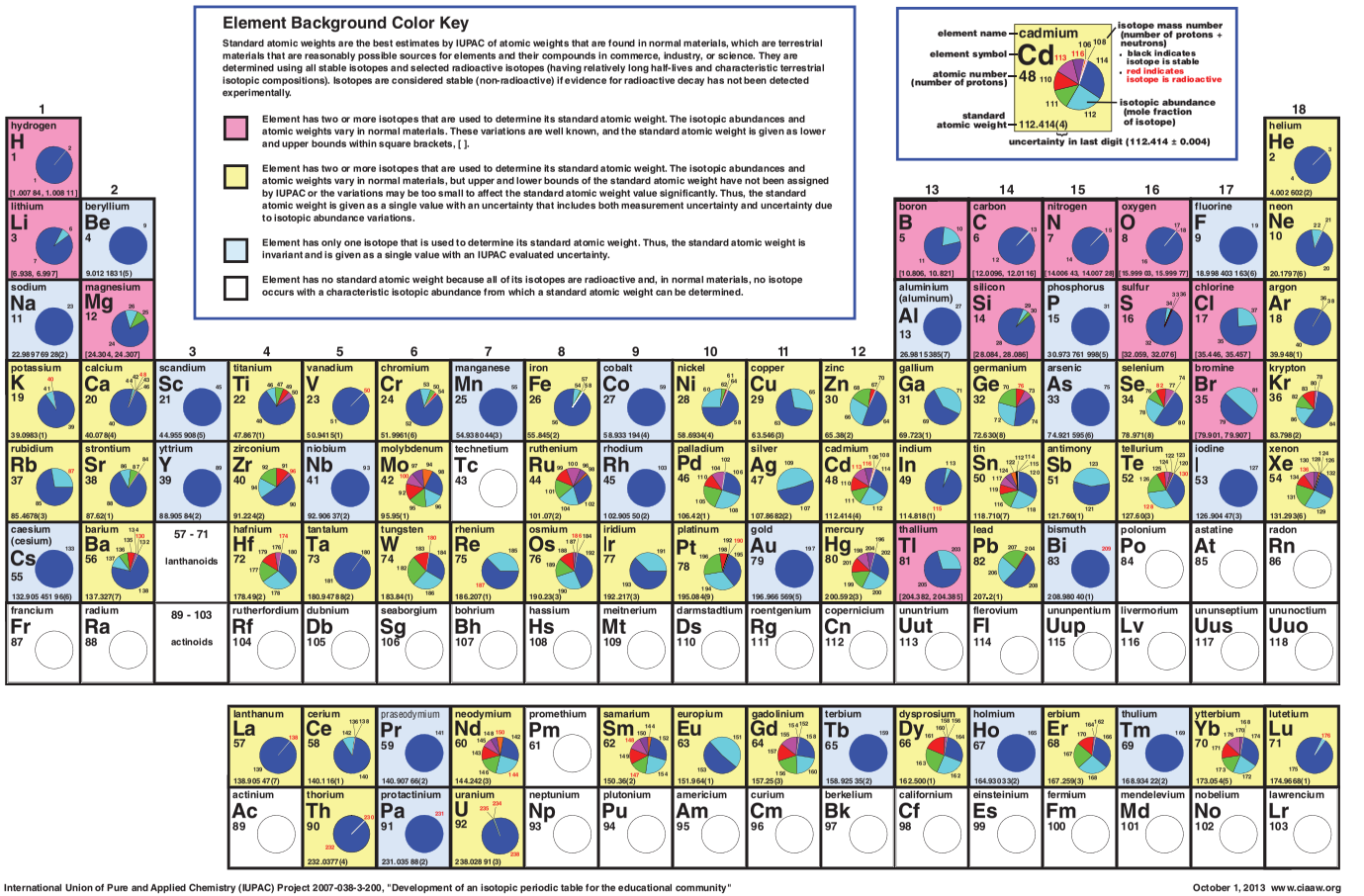

Figure I.1 New Period Table of Elements showing isotope abundance ranges. *Source:* Holden *et al.* (2011). Reproduced with permission of CIAAW.

(Equation I.1). In other words, δ values are relative isotopic abundance values suited for comparison, which is why it is crucially important to provide concise information on which δ value(s) and the nature of the scale anchor(s) whose δ values were employed to generate reported, scale normalized δ values (cf. Part II, Chapter II.3). In the most recent guidelines and recommended terms of stable isotope ratio measurements and reporting results thereof issued by IUPAC the δ value has been defined as follows (Coplen, 2011):

$$\delta^{\mathrm{h}}\mathrm{E}_{\mathrm{S}/\mathrm{STD}} = ([R_{\mathrm{S}} - R_{\mathrm{STD}}]/R_{\mathrm{STD}}) = ((R_{\mathrm{S}}/R_{\mathrm{STD}}) - 1) \tag{I.1}$$

In this equation, R_{S} is the measured isotope ratio of the heavier isotope h of a given chemical element E in a sample S over the lighter isotope l of the same element E (e.g. $^{13}\mathrm{C}/^{12}\mathrm{C}$ or $^{2}\mathrm{H}/^{1}\mathrm{H}$) and R_{STD} is the contemporaneously measured isotope ratio for the chosen standard (e.g. NBS 19 or Vienna Standard Mean Ocean Water, VSMOW). It should be noted the 2011 IUPAC definition of the δ value as stated in Equation I.1 no longer contains the extraneous factor of 1000. Since δ values derived on the basis of Equation I.1 are numerically less than zero with significant numbers to the second or third decimal place, they may be stated as ‰ values as a representation of the scientific notation of presenting such numbers as multiples of 10^{-3}. However, reporting δ values in this way should not lead the ‰ sign to be confused with an SI unit of measurement. As a ratio of two ratios δ values do not have an SI unit of measurement. Similar to the % symbol the ‰ symbol is not a unit (of measurement) but merely a convenient way to express small numbers.

For example, with $R_{\mathrm{S}} = 0.01101296$ and $R_{\mathrm{STD}} = 0.0112372$,

$$\delta^{\mathrm{h}}\mathrm{E}_{\mathrm{S}/\mathrm{STD}} = (0.980045 - 1) = -0.01995.$$

This δ value can be written as

$$\delta^{\mathrm{h}}\mathrm{E}_{\mathrm{S/STD}} = -0.01995 = -19.95 \times 10^{-3} = -19.95‰.$$

In this example the minus sign signifies that the isotopic abundance of $^{\mathrm{h}}\mathrm{E}$ in the sample S is less than the isotopic abundance of $^{\mathrm{h}}\mathrm{E}$ in the chosen reference material STD. Conversely, $\delta^{\mathrm{h}}\mathrm{E}_{\mathrm{S}/\mathrm{STD}}$ values with a positive sign signify an isotopic abundance of $^{\mathrm{h}}\mathrm{E}$ in the sample S that is higher than that in the reference material STD.

Using the δ notation, a difference in $^{13}\mathrm{C}$ abundance of 0.011 atom% corresponds to a change in $\delta^{13}\mathrm{C}$ value of 10 ‰. For example, a change in $^{13}\mathrm{C}$ abundance from 1.0893 atom% down to 1.0783 atom% corresponds to a change in $\delta^{13}\mathrm{C}$ value from −19.96 ‰ to −29.96 ‰ on the Vienna Pee Dee Belemnite (VPDB) scale. Owing to how accurately and precisely the $^{13}\mathrm{C}$ composition of homogeneous materials at the natural abundance level can be measured by modern analytical instruments, for organic materials measured differences > 0.3 ‰ are statistically significant. By comparison, the same difference of 0.011 atom% in the $^{2}\mathrm{H}$ abundance of a given compound corresponds to a change in $\delta^{2}\mathrm{H}$ value of approximately 706 ‰.

For example, a change in ^{2}H abundance from 0.016 atom% down to 0.005 atom% corresponds to a change in $\delta^{2}H$ value from +27.4 ‰ to −679.0 ‰ on the VSMOW scale. Yet a difference in ^{2}H abundance of 0.001 atom% corresponds to a change in $\delta^{2}H$ value of approximately 64 ‰ and not 70.6 ‰ as one might have expected.

The above examples illustrate that the δ notation or δ value is not a linear function of isotopic abundance since isotopic abundance is an atomic fraction (or a mole fraction). However, at near natural abundance levels where, for example, the ^{13}C atomic fraction ranges from 0.010 to 0.012 the deviation of $\delta^{13}C$ values from linearity when plotted against ^{13}C atomic fractions is virtually non-existent. Conversely, when dealing with samples enriched in ^{13}C and their ^{13}C atomic fraction exceeds 0.1, this non-linearity cannot be ignored. Using $\delta^{13}C$ values for, for example, isotope mass balance calculations as an approximation of isotopic abundance for atomic fractions in excess of 0.1 would introduce significant errors (Fry, 2006).

The δ notation or δ value as defined in Equation I.1 is a ratio and thus a derived quantity without a unit of measurement. Following tradition, in the majority of publications δ values are expressed in "units" of parts per thousand and communicated as per mil values (symbol "‰"). The "‰" symbol is set here in quotation marks to stress the point already made above that symbols such as the per cent sign (%) or the per mil sign (‰) are not units of measurement as defined in the International System of Units (SI) like, for example, meter (m), joule (J) or Pascal (Pa). To bring the δ notation or δ value in line with the International System of Units, in 2012 CIAAW members Willi Brand and Tyler Coplen proposed the introduction of a new unit for this derived quantity defined through Equation I.1 (Brand and Coplen, 2012). They proposed this new SI unit could have the name Urey (symbol Ur), in recognition of Harold C. Urey, who in 1934 received the Nobel Prize in Chemistry for his discovery of the hydrogen isotope ^{2}H. Their argument was that this new unit would follow the example set by the SI unit for pressure, the Pascal, unit symbol Pa, named after Blaise Pascal. The new Urey unit could be combined with any SI prefix used to express fractions or multiples of 10 of an SI unit. A δ value of 1 ‰ would thus become a δ value of 1 milliurey (symbol mUr).

Various isotope scale reference points are used for reporting the relative isotopic abundance of the light elements hydrogen, carbon, nitrogen, oxygen and sulfur. By virtue of Equation I.1, the δ values of each of these scale reference points are by definition 0. Carbon stable isotope ratios were originally reported relative to the reference material Pee Dee Belemnite (PDB), which also served as scale reference point. Since this reference material became exhausted, VPDB has become the new international scale reference point for the ^{13}C scale. Oxygen stable isotope abundance values of carbonates and calcites are also commonly expressed relative to VPDB. However, unlike the old scale anchor PDB the new scale anchor VPDB is a virtual reference material that does not exist in a material form. Instead NBS 19, a limestone material composed largely of calcite, is used to underpin the VPDB scale (cf. Table I.2). Sulfur stable isotope abundance values are scale anchored in a similar way. Originally the ^{34}S scale was anchored by Troilite (FeS) from the

Table I.2 Scale reference points and their defining scale anchors for stable isotopes of light elements.

Stable isotope	Scale reference point	Scale anchors	Chemical compound	Relative stable isotope abundance value as ‰
Hydrogen	VSMOW	VSMOW2	Water	0.0
		SLAP2	Water	−427.5
Carbon	VPDB	NBS 19	Calcite	+1.95
		LSVEC	Li_2CO_3	−46.60
Nitrogen	Air	IAEA-N-1[a]	$(NH_4)_2SO_4$	+0.43
		USGS32[a,b]	KNO_3	+180.0
Oxygen	VSMOW	VSMOW2	Water	0.00
		SLAP2	Water	−55.50
Sulfur	VCDT	IAEA-S-1	Ag_2S	−0.3
		IAEA-S-2	Ag_2S	+22.62

[a]Cf. page 439 of the IUPAC technical report by W.A. Brand *et al*. (2014) *Pure and Applied Chemistry*, **86**(3), 425–467.
[b]Recommendation that is still subject to confirmation by IUPAC.

Cañon Diablo meteorite (CDT) until questions arose about variability of its $\delta^{34}S$ values and the cause for this variability. In 1997 the decision was therefore taken to henceforth express $\delta^{34}S$ values on the Vienna Cañon Diablo Troilite (VCDT) scale, with IAEA-S-1 (silver sulfide) being used to underpin the VCDT scale (Krouse and Coplen, 1997). Like VPDB, VCDT does not exist but is a virtual isotopic reference material anchored by IAEA-S-1 (cf. Table I.2).

Stable oxygen and hydrogen isotopic abundance values are reported relative to VSMOW (Vienna Standard Mean Ocean Water) which is anchored by two scale anchors, VSMOW and SLAP (Standard Light Antarctic Precipitation). Nitrogen isotope abundance values are reported relative to Air (for nitrogen in atmospheric air).

The use of VSMOW and VPDB as scale reference points when reporting δ values means that measurements have been normalized according to International Atomic Energy Agency (IAEA) and IUPAC guidelines for expression of δ values relative to traceable reference materials on internationally agreed reference scales (Coplen *et al*., 2006a,b; Coplen, 2011). Table I.3 lists a number of international reference materials recommended for use as scale anchors and/or quality control materials. Scale normalization of measured δ values will be discussed in detail in Part II, Chapter II.3. The international reference materials listed in Table I.3 are available from the IAEA,[1] the National Institute of Standards and Technology (NIST),[2] and the United States Geological Survey (USGS).[3]

[1] https://nucleus.iaea.org/rpst/referenceproducts/referencematerials/Stable_Isotopes/index.htm.
[2] https://www-s.nist.gov/srmors/viewTableV.cfm?tableid=42.
[3] http://isotopes.usgs.gov/lab/referencematerials.html.

Table I.3 A representative but not exhaustive list of international reference materials for stable isotope ratio mass spectrometry together with their stable isotope abundance values as published by the Commission on Isotopic Abundances and Atomic Weights (CIAAW; http://www.ciaaw.org/reference-materials.htm).

International Reference Material (IRM)	Code	$\delta^{13}C_{VPDB}$ as ‰	$\delta^{15}N_{AIR}$ as ‰	$\delta^{2}H_{VSMOW}$ as ‰	$\delta^{18}O_{VSMOW}$ as ‰
TS-limestone	NBS 19	+1.95			+28.65[a]
Carbonatite	NBS 18	−5.01			+7.20[a]
Lithium carbonate	LSVEC	−46.6			+3.69[a]
Oil	NBS 22	−30.03		−116.9	
Sucrose	IAEA-CH-6	−10.45			
Polyethylene foil	IAEA-CH-7	−32.15		−100.3	
Caffeine	IAEA-600	−27.77	+0.91		−3.48
L-glutamic acid	USGS40	−26.39	−4.52		
L-glutamic acid	USGS41a	+36.55	+47.55		
Cellulose	IAEA-CH-3	−24.72			
Ammonium sulfate	IAEA-N-1		+0.43		
Ammonium sulfate	IAEA-N-2		+20.35		
Potassium nitrate	USGS32		+180.0		+25.4
Potassium nitrate	USGS34		−1.8		−27.78
Water	VSMOW			0	0
Water	VSMOW2			0	0
Water	GISP			−189.7	−24.78
Water	GISP2			−258.3	−33.43
Water	SLAP			−428.0	−55.5
Water	SLAP2			−427.5	−55.5
Water	IAEA-604			+799.9	−5.86
Benzoic acid	IAEA-601	−28.81			+23.14
Benzoic acid	IAEA-602	−28.85			+71.28

[a]Traditionally, $\delta^{18}O$ values for carbonates are reported on the VPDB scale. The $\delta^{18}O_{VPDB}$ values for NBS 19, NBS 18 and LSVEC are +2.20, −23.01 and −26.41 ‰, respectively.

Let us now revisit the twin analogy to picture what natural abundance means in praxis. Obviously, the abundance ratio of any given pair of twins is 1:1 or 50 %:50 %, that is, when meeting any one twin in a crowd where both are known to be present, one has an even chance of speaking either to twin A or twin B. However, if we consider a hypothetical case where both twins were victims of a major explosion, the probability of any given body part belonging to either twin now becomes a function of the number of pieces each body has been divided into. The same in a way is true for chemical elements and their "overweight" twins. If one took apart a lump of sugar to its molecular level, one would find that, depending on circumstances (in this case which plant had produced the sugar), one would have a 98.9617 % or a

98.9015 % chance of finding ^{12}C if the sugar was beet sugar or cane sugar, respectively. Similarly, one would have a 1.0833 % or 1.0985 % chance of finding ^{13}C in carbon from beet sugar and cane sugar, respectively. So, generally speaking, one always has a better chance of encountering ^{12}C than ^{13}C, meaning ^{12}C has a higher abundance than its heavier isotope ^{13}C. However, on a case-by-case basis one finds that chemically identical substances such as sugar may exhibit different isotopic compositions where a change in ^{12}C abundance is accompanied by a proportionate yet opposite change in ^{13}C. For example, beet sugar contains more ^{12}C and less ^{13}C than cane sugar while conversely cane sugar contains more ^{13}C and less ^{12}C than beet sugar (Hobbie and Werner, 2004; Meier-Augenstein, 1999; Rossmann *et al*., 1997). The chemical and physicochemical reasons behind these differences will be discussed in Chapters I.3 and I.4.

Chapter I.3

Chemically Identical and Yet Not the Same

The analytical methods traditionally applied in forensic science laboratories establish a degree of commonality between one substance and another by identifying their constituent elements and functional groups, and elucidating their chemical structures. Thus, for two samples of sugar all of the aforementioned data will correspond and it can be concluded that they are chemically indistinguishable, they are indeed both sugar. However, it can be argued that although the two substances in question are chemically indistinguishable they may not be the same, for example they may have come from different sources or be of different origin. Attention is drawn to the following: whenever we speak of the source and origin of a natural product such as sugar, by source we mean from which particular plant was the sugar sourced (i.e. ultimately made) whereas by origin we mean its geographic origin (i.e. where the plant was grown and harvested). In other words by differentiating between source and/or origin the distinction is being made that if two substances do not share the same provenance they are not truly identical even if chemically they are indistinguishable. This assertion can be contested by stable isotope analysis either to protect people from being convicted of a crime they have not committed, such as drug trafficking, or, staying with the example of drugs, to convict people who may be prepared to admit to the lesser offence of possession for personal use while in fact they are drug dealers or drug traffickers.

How is this possible? For reasons we will touch upon in this chapter and again in Chapter I.4, two chemically indistinguishable compounds will be isotopically distinguishable if they do not share the same compound history, that is, if the processes involved in their synthesis differ, which for biogenic natural compounds could mean they were derived from a different source (plant or organism) or even from an identical source but with a different geographic origin. Using the case of sugar as example, traditionally the two main sources of household sugar are sugar cane and sugar beet. With the help of stable isotope signatures it is perfectly straightforward to determine

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Table I.4 Select examples of ^{13}C and ^{2}H abundance values of sugar from different plant sources and of different geographic origin.

Sample	$\delta^{13}C_{VPDB}$ as ‰	$\delta^{2}H_{VSMOW}$ as ‰
Sugar (sugar beet, Poland)	−25.42	−71.0
Sugar (sugar beet, Sweden)	−26.84	−93.4
Sugar (sugar cane, Brazil)	−11.76	−21.4
Sugar (sugar cane, South Africa)	−11.10	−6.7

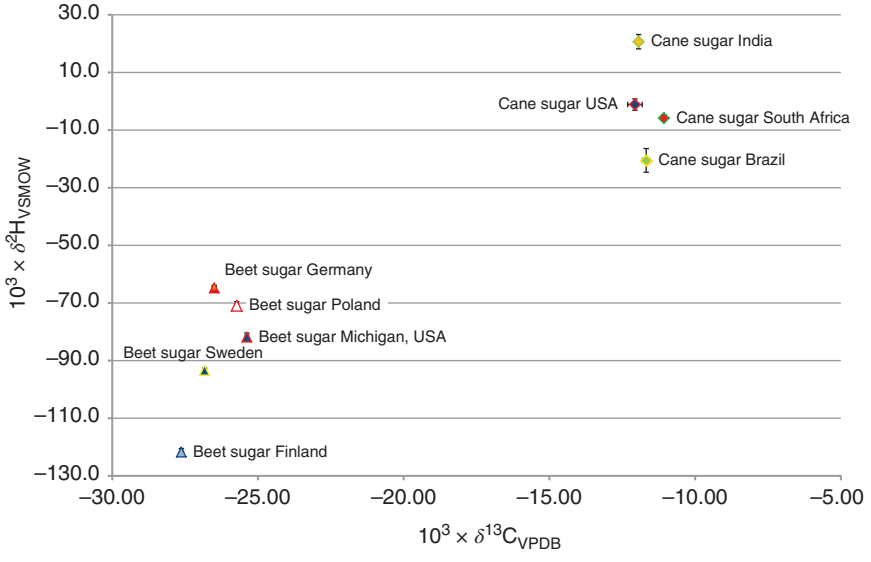

Figure I.2 $\delta^{2}H$ and $\delta^{13}C$ values of beet sugar and cane sugar of different geographic origin.

if a given sugar sample is either cane sugar or beet sugar. In addition, it is even possible to say where approximately in the world the sugar cane or sugar beet was grown and cultivated (cf. Table I.4 and Figure I.2).

The differences in isotopic composition between cane sugar and beet sugar are ultimately caused by differences in biochemical reactions where chemical bonds are broken and formed, and differences in the isotopic compositions of precursor pools. The latter can be the result of physicochemical processes. Even though these processes do not involve the formation or break-up of chemical bonds they nonetheless reflect differences in bond length and bond strength that can result in significant differences in compound properties such as melting point or boiling point. The isotopologues of water are an excellent example of this phenomenon

Table I.5 Influence of isotopic composition on physical properties of H_2O and its isotopologues.

Property	$^1H_2{}^{16}O$ FW = 18 g/mole	$^1H_2{}^{18}O$ FW = 20 g/mole	$^2H_2{}^{16}O$ FW = 20 g/mole
Reduced mass μ of H–O bond (amu)	0.9412	0.9474	1.7778
Melting point (°C) at 101.33 kPa	0	0.28	3.82
Boiling point (°C) at 101.33 kPa	100	100.15	101.4
Vapour pressure at 20 °C in kPa	2.3379	2.3161	2.0265
Heat of vaporization (kJ/mole) at 100 °C	40.657	(40.664)[a]	41.521
Neutral pH at 25 °C	7	(7.004)[a]	7.47
Dissociation constant pK_w ($mole^2/kg^2$) at 25 °C	14	(14.008)[a]	14.957
O–H bond length (Å)	0.990	(0.9899)[a]	0.9846

[a]No official values are available. Values in brackets are interpolated approximations.

(cf. Table I.5). In Chapter I.4 we will learn about why isotopologues, that is, molecules of the same compound but of different isotopic composition (Sharp, 2007), behave and react in subtly different ways even though on an atomic level the heavy and light isotopes of the same chemical element behave in the same way.

Chapter I.4

Isotope Effects, Mass Discrimination and Isotopic Fractionation

I.4.1 Physical Chemistry Background

If for a given compound a non-quantitative chemical reaction or a physicochemical process such as vaporization has taken place, this will be subject to mass discrimination (or associated with an isotope effect), which will cause a change in isotope abundance and hence result in isotopic fractionation. In principle two different types of isotope effects can cause isotopic fractionation: kinetic isotope effects (kinetic as in chemical reaction kinetics) and thermodynamic isotope effects. In general, mass discrimination is caused by differences in the vibration energy levels of bonds involving heavier isotopes as compared to bonds involving lighter isotopes.

Differences in the zero-point energy of chemical bonds containing one heavy isotope and one light isotope relative to bonds containing two light isotopes are reflected by differences in the rates of cleavage of these bonds because differences in zero-point energy results in differences in bond energy. For example, for hydrogen gas the bond strengths of ^{1}H–^{1}H, ^{1}H–^{2}H and ^{2}H–^{2}H are 436.0, 439.4 and 443.5 kJ/mole, respectively. Thus, ^{2}H–^{2}H bonds are broken at a slower rate than ^{1}H–^{2}H bonds, which in turn are broken at a slower rate than ^{1}H–^{1}H bonds. It is usually observed that the product of a chemical reaction involving bond cleavage will be isotopically lighter in the element(s) forming that bond compared to the corresponding isotopic composition of the initial precursor or source substrate.

It might be useful at this point to revisit some basic principles of physics and a mainstay of analytical chemistry instrumentation, infrared (IR) spectroscopy, to illustrate the relation between the reduced mass of a chemical two-atom system, bond length and bond strength aka bond energy, which are ultimately responsible

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for the mass discrimination that leads to the wide range of isotopic composition of natural and man-made compounds.

The rotational (or vibrational) kinetic energy E_{vib} of a rigid body can be expressed in terms of its moment of inertia I and its angular velocity ω:

$$E_{vib} = \frac{1}{2} I\omega^2 \tag{I.2}$$

The (scalar) moment of inertia I of a point mass m rotating about a known axis r is defined by

$$I = mr^2 \tag{I.3}$$

For a system comprising two masses (or two atoms) m_1 and m_2 joined by, say, a spring (or chemical bond) of the length r, and if this system rotates around an axis intersecting a point on that spring (or bond), the mass term m in Equation I.3 is replaced by the reduced mass μ of this system, which is given by

$$\mu = \frac{m_1 m_2}{m_1 + m_2} \tag{I.4}$$

The vibrational or rotational energy of a molecule can be measured by its IR absorbance. In the world of quantum physics where rotating or vibrating systems assume discreet energy levels, the associated discreet packets of energy differences ΔE can be expressed by the rotational constant B, the difference between two IR absorption bands:

$$\Delta E_{\Delta J=1} = 2B = \frac{h}{4\pi^2 cI} \tag{I.5}$$

Here h is the Planck constant and c is the speed of light.

As mentioned in Chapter I.3, the different isotopologues of water are a good example of how differences in bond strength affect the physicochemical properties of a compound (cf. Table I.5). IR spectrometry-based isotope analysers manufactured and marketed by companies such as Los Gatos, Picarro or Thermo Fisher Scientific exploit differences in IR active vibrational energy states between isotopologues of CO_2 to conveniently measure the abundance of, for example, atmospheric $^{13}CO_2$ as a tool for greenhouse gas monitoring. These laser-based systems operate either in the near-IR or the mid-IR part of the IR spectrum. In the near-IR, distance in units of wavenumbers (cm^{-1}) between absorption lines of the different isotopologues are quite small, for example <1 cm^{-1}, with absorption lines for $^{12}CO_2$ and $^{13}CO_2$ being found at 6251.75 cm^{-1} and 6251.30 cm^{-1} respectively. Moving into the mid-IR these differences become more pronounced, for example >20 cm^{-1}, with absorption lines for $^{12}CO_2$ and $^{13}CO_2$ found at 2325.58 cm^{-1} and 2247.19 cm^{-1}, respectively.

The IR spectra of gaseous hydrochloric acid (HCl) are another fine example of how differences in isotopic make-up and therefore differences in μ and r, and hence in I, result in differences in ΔE between neighbouring IR absorption bands for, for example, ^{1}H–^{35}Cl and ^{2}H–^{35}Cl. Since we are able to measure B and can calculate μ it is possible to calculate r, the bond length for the different HCl isotopologues.

Question I.1 Given that values for B for ^{1}H–^{35}Cl and ^{2}H–^{35}Cl have been measured to be 10.44 cm^{-1} and 5.39 cm^{-1}, respectively, what bond lengths would one calculate for a ^{1}H–^{35}Cl molecule and a ^{2}H–^{35}Cl molecule? Note: To calculate the reduced mass μ of one molecule one has to divide the reduced mass term (g/mole) by Avogadro's number (molecules/mol). For the Planck constant h use 6.62×10^{-34} Js and for the speed of light c use 3×10^{8} m s^{-1}.

The values for B for ^{1}H–^{37}Cl and ^{2}H–^{37}Cl are 10.42 cm^{-1} and 5.38 cm^{-1}, respectively.

I.4.2 Fractionation Factor α and Enrichment Factor ε

The difference in bond length and hence bond strength between bonds involving different isotopes of the same chemical element that already results in measurable differences in spectroscopic characteristics also leads to different reaction rates for a bond when different isotopes of the same element are involved (Melander and Saunders, 1980). The most significant isotope effect is the kinetic or primary isotope effect, whereby a bond containing the chemical elements under consideration is broken or formed in the rate-determining step of the reaction (Rieley, 1994), for example the reaction between two amino acids leading to the formation of the peptide bond R–CO–NH–R′ involving the carboxyl carbon of amino acid R and the amino nitrogen of amino acid R′.

The second type of isotope effect is associated with differences in physicochemical properties such as IR absorption, molar volume, vapour pressure, boiling point and melting point. Of course, these properties are all linked to the same parameters as those mentioned for the kinetic isotope effect, that is, bond strength, reduced mass and hence vibration energy levels. However, to set it apart from the kinetic isotope effect, this effect is referred to as *thermodynamic isotope effect* (Meier-Augenstein, 1999) because it manifests itself in processes where chemical bonds are neither broken nor formed. Typical examples for such processes in which the results of thermodynamic isotope effects can be observed are IR spectroscopy and any kind of two-phase partitioning (e.g. liquid/liquid extraction) or phase transition (e.g. liquid to gas, i.e. distillation or vaporisation). The thermodynamic isotope effect, or physicochemical isotope effect, is the reason for the higher IR absorption of $^{13}CO_2$ as compared to $^{12}CO_2$, for the vaporization of ocean surface water resulting in clouds (= water vapour) being depleted in both ^{2}H and ^{18}O compared to ocean surface water, and for the isotopic fractionation observed during chromatographic separations.

Another way of describing any isotope effect is to say that the reaction rate constant or equilibrium constant k of a given reaction or transformation

$$\text{Precursor (or Source)} \xrightarrow{k} \text{Product}$$

is in fact composed of two subtly different reaction rate constants k_L and k_H for the light (L) and heavy (H) isotope-containing molecules or isotopologues (Sharp, 2007), respectively, that make up the precursor or source compound. The ratio of these reaction rate constants yields the fractionation factor α:

$$\alpha = k_H/k_L \tag{I.6}$$

Since a molecule with a light isotope at the bond involved in the reaction usually reacts slightly faster (because breaking this light isotope bond requires slightly less energy) than a molecule with a heavy isotope in the same position (because breaking this heavy isotope bond requires slightly more energy), the ratio α of k_H over k_L is normally less than 1. For example, a reaction rate constant k_L that is 2 % faster than the corresponding k_H translates into a fractionation factor α of 0.98, thus already indicating that the product will be isotopically lighter compared to the precursor. In other words, in respect to the heavier isotope under consideration the δ value of the product will be lower than the δ value of the precursor or source.

Enrichment factors ε or fractionation values derived from α values by $\varepsilon = (\alpha - 1)$ show this difference immediately as δ values:

$$\varepsilon = (\alpha - 1) \tag{I.7}$$

Using the above example of an α value of 0.98 one can easily calculate an ε value of -0.02 or -20 ‰ for this hypothetical reaction, meaning the δ value of the product will be 20 ‰ lower than the δ value of the precursor or *Source:* $\delta_{Product} = \delta_{Source} - 20$ ‰. In most cases the straightforward difference between $\delta_{Product}$ and δ_{Source} yields an enrichment factor very similar to that calculated from α values. However, since it is quite simple to calculate α values from measured δ values (Equation I.8a), determination of enrichment factors from α values is recommended:

$$\alpha = \frac{\delta_{Product} + 1}{\delta_{Source} + 1} \tag{I.8a}$$

Note that Equation I.8a is based on the definition of the δ values given by Equation I.1. For δ values already expressed in the form of ‰ values (cf. Chapter I.2), Equation I.8b has to be used:

$$\alpha = \frac{\delta_{Product} + 1000}{\delta_{Source} + 1000} \tag{I.8b}$$

It should be noted that in some scientific disciplines, such as biology and ecology, the fractionation factor $\alpha_{L/H}$ is defined by the ratio of the reaction rates of the lighter isotopologue over the heavier:

$$\alpha_{L/H} = k_L/k_H \tag{I.9}$$

Adopting this convention leads to positive fractionation values Δ for reactions or transformations in which the lighter isotopologue L reacts faster than the heavier

isotopologue H. Since both conventions essentially describe the same phenomenon using the same principles, converting α into $\alpha_{L/H}$ and ε into Δ is quite simple: $\alpha = (1/\alpha_{L/H})$ and $\varepsilon = -\Delta$.

In Chapter I.6 we will have a look at some of the aforementioned scenarios in more detail to develop an appreciation for how and why we can exploit the different ways in which isotopic fractionation plays out in the context of forensic science.

I.4.3 Isotopic Fractionation in Rayleigh Processes

One of the most important isotopic fractionation processes is the change in isotopic composition of a reservoir because of the removal of an increasing fraction of its contents. A typical example for such a removal of a compound (= sink) from a reservoir without an additional input we can find in any chemical laboratory is when a solvent or another suitably volatile man-made or natural compound is purified by distillation. Another example would be the evaporation of water from a lake in an arid region during the dry season (i.e. no additional water input from a river or through rainfall). This particular type of Rayleigh process is therefore also referred to as Rayleigh distillation.

At a given time t a given reservoir comprises a total number of molecules N and a ratio of the rare (e.g. ^{2}H or ^{13}C) to the abundant (e.g. ^{1}H or ^{12}C) molecular concentration R, that is, the isotope ratio. In other words, $N/(1 + R)$ is the number of most abundant isotopic molecules and $RN/(1 + R)$ is the number of rare isotopic molecules. If we remove dN molecules with an accompanying fractionation factor α, the mass balance for the rare isotope can be written as follows.

$$\frac{R}{1+R}N = \frac{R+\mathrm{d}R}{1+R+\mathrm{d}R}(N+\mathrm{d}N) - \frac{\alpha R}{1+\alpha R}\mathrm{d}N \tag{I.10}$$

To simplify Equation I.10 somewhat we make the following approximations: (i) the total number of molecules is equal to the number of most abundant isotopic molecules and (ii) all denominators in Equation I.10 are taken to equal $(1 + R)$. The mass balance for the rare isotope hence simplifies to:

$$RN = (R+\mathrm{d}R)(N+\mathrm{d}N) - \alpha R\mathrm{d}N \tag{I.11}$$

By neglecting the product of differentials and separating the variables, the mass balance for the rare isotope becomes:

$$\frac{\mathrm{d}R}{R} = \frac{(\alpha-1)\mathrm{d}N}{N} \tag{I.12}$$

Integration of Equation I.12 while applying the boundary conditions for time $t = 0$ of $R = R_0$ when $N = N_0$ yields:

$$\frac{R}{R_0} = \left(\frac{N}{N_0}\right)^{\alpha-1} \tag{I.13}$$

We can write Equation I.13 using δ values with respect to a standardized reference.

$$\delta = (1 + \delta_0)\left(\frac{N}{N_0}\right)^{\epsilon} - 1 \tag{I.14}$$

Here N/N_0 represents the remaining fraction of the original reservoir while R_0 and δ_0 refer to the original isotopic composition. We also need to remember that ε $(= \alpha - 1)$ is a very small number, for instance $\varepsilon = 0.00938$ if the fractionation factor $\alpha = 1.00938$, as in the case of the ^{18}O equilibrium fractionation between water in the liquid phase and water in the vapour phase at a temperature T of 25 °C.

I.4.3.1 Isotopic Fractionation Summary

Appreciation of the phenomenon that is isotopic fractionation is crucial for developing an understanding of what we can detect and measure, the potential magnitude of the isotope effect we may be able to exploit and, last not but least, how it may impact on results due to the potential of fractionation during sample preparation. For this reason, we should summarize at this point the key facts about isotopic fractionation (Box I.1).

Box I.1 Equilibrium isotopic fractionation

- Equilibrium fractionation describes isotopic exchange reactions that occur between two different phases of a compound at a rate that maintains equilibrium, as with the transformation of water vapour to liquid precipitation.

Isotopic fractionation during physicochemical processes

- Mass differences give rise to fractionation during physicochemical processes (diffusion, evaporation, two-phase partitioning, molecular spectroscopy).
- Fractionation during physicochemical processes is again the result of differences ultimately influenced by the reduced mass of a system such as different velocities of isotopic molecules of the same compound in the gas phase (kinetic energy) or differences in absorption of energy, for example IR radiation (vibrational energy).
- Associated isotope effects are called secondary isotope effects or thermodynamic isotope effects since chemical bonds are neither formed nor broken during these processes.

Note, some textbooks refer to mass discrimination during evaporation as kinetic effect, although from a chemistry point of view this is incorrect because evaporation is not a chemical reaction.

Isotopic fractionation during (bio-)chemical processes

- Mass differences give rise to fractionation during chemical processes, whereby a bond containing the atom or its isotope is broken or formed in the rate-determining step of the reaction.
- Fractionation during chemical processes is again the result of differences ultimately influenced by the reduced mass of a system, primarily bond strength (energy) and bond length.
- Associated isotope effects are called primary isotope effects or kinetic isotope effects since the degree of fractionation is determined by the reaction kinetics of the particular chemical reaction.

Note, if a partner in a chemical reaction is fully consumed, that is, it reacts completely (quantitatively), no mass discrimination/isotopic fractionation is observed in respect of this compound.

Chapter I.5

Stable Isotopic Distribution and Isotopic Fractionation of Light Elements in Nature

I.5.1 Hydrogen

The vast majority of hydrogen in nature is found 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, soil, groundwater and air. The total volume of water on Earth amounts to $1{,}385{,}984 \times 10^3$ km^3 and 96.54 % of this volume is ocean water, making seawater the main reservoir of hydrogen in nature. Not surprisingly, standard mean ocean water (SMOW) as it was then called is the standard reference point for hydrogen (and oxygen) stable isotope analysis.

Hydrogen occurs in nature in two stable isotope varieties, ^{1}H and ^{2}H. The stable but less abundant hydrogen isotope ^{2}H was discovered by Harold Urey in 1931 (Urey, Brickwedde and Murphy, 1932) and in 1933 Urey's PhD supervisor, Gilbert Lewis, succeeded in isolating pure $^2H_2{}^{16}O$ by electrolysis (Lewis and MacDonald, 1933). Incidentally, as of 2005 IUPAC's guidelines and recommendations for the nomenclature of inorganic chemistry state that heavy isotopes of hydrogen should be written as ^{2}H and ^{3}H rather than as D and T, respectively (Connelly *et al.*, 2005). Accordingly, throughout this book the minor, less abundant hydrogen stable isotope is referred to as ^{2}H or hydrogen-2. The global or average natural abundances on Earth in atom% for ^{1}H and ^{2}H are 99.9844 and 0.01557, respectively (Sharp. 2007). From these natural abundance values the average terrestrial ^{2}H/^{1}H isotope ratio can be easily calculated as 0.00015572. The ^{2}H/^{1}H isotope ratio for the primary standard of the ^{2}H scale, VSMOW, is given as 0.00015576, so one can see why VSMOW was chosen to anchor the ^{2}H scale. Incidentally, the IAEA, who administer and distribute calibration materials (CM) and reference materials (RM) for stable isotope analysis strongly suggests only CM should be referred to as primary standards.

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The *hydrologic cycle* traces the movement of water and energy between the various water stores and Earth's spheres, that is, the lithosphere, atmosphere, biosphere and hydrosphere. Ocean water can be looked at as the starting point of the hydrologic cycle since clouds formed from evaporating ocean water transport water from the oceans to the continents. Isotopically speaking, the hydrologic cycle is one big Rayleigh process or a combination of Rayleigh processes.

Isotopic fractionation associated with Rayleigh processes (see Chapter I.4.3) such as evaporation, condensation and precipitation of meteoric water, ultimately results in drinking water having different isotopic composition depending on geo-location. Depending upon latitude, altitude, temperature and distance to the open seas, observed δ^2H values of meteoric water (precipitation) and hence fresh water can range from +20 to −270 ‰ across the world with the "heavier" or less negative δ^2H values being typical of coastal/near-equatorial regions and the "lighter" or more negative δ^2H values being typical of inland/high-altitude/high-latitude regions. Geographical information system (GIS) maps and contour maps of meteoric ^{2}H and ^{18}O isotope abundance 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 IAEA (http://isohis.iaea.org/userupate/waterloo/index.html), while information on ^{18}O isotope abundance in global seawater can be found on the web site of NASA's Goddard Institute for Space Studies (http://www.giss.nasa.gov/data/o18data).

To understand this phenomenon let us apply some of what we have learned in the preceding chapters. The isotopic composition of water vapour, that is, a cloud forming over seawater with seawater near the surface having relative isotope abundances of $\delta^2\text{H} = \delta^{18}\text{O} = 0$ ‰ versus VSMOW is somewhat lighter than a strictly theoretical calculation from isotopic equilibrium with the water would predict since, strictly speaking, evaporation of seawater is a non-equilibrium process. However, once a cloud with a given vapour composition has formed, the vapour and its resultant precipitation remain in isotopic equilibrium because the formation of precipitation occurs from saturated vapour. Consequently δ^2H and δ^{18}O values of precipitation both change in unison as dictated by the ratio of their respective fractionation values $^2\varepsilon$ and $^{18}\varepsilon$ (Mook, 2000).

Let us assume the ^{2}H isotopic composition of water vapour in a cloud formed above the ocean near the equator has a $\delta^2\text{H}_\text{v}$ value of −84.44 ‰, the temperature is a balmy 25 °C and the first precipitation out of this cloud occurs. The fractionation factor α for ^{2}H between water vapour and liquid water in equilibrium at this temperature is 1.0793 (Mook, 2000; Majoube, 1971). Rearranging Equation I.8 we can calculate the $\delta^2\text{H}_\text{l}$ value of this precipitation as:

$$\delta^2\text{H}_\text{l} = 1.0793(-84.44 + 10^3) - 10^3 = -11.84\ ‰$$

For ^{18}O the fractionation factor α between water vapour and liquid water in equilibrium at 25 °C is 1.00938. The typical ^{18}O isotopic composition of equatorial atmospheric vapour corresponds to a $\delta^{18}\text{O}_\text{v}$ value of −12.03 ‰. Inserting these

values into Equation I.8 and solving for $\delta^{18}O$ of the water falling as precipitation yields a $\delta^{18}O_l$ value of −2.76 ‰:

$$\delta^{18}O_l = 1.00938(-12.03 + 10^3) - 10^3 = -2.76\,‰$$

Assuming for reasons of simplicity that evaporation and condensation in nature occur in isotopic equilibrium, the relation between δ^2H and $\delta^{18}O$ values of precipitation would be determined solely by their equilibrium fractionations ${}^2\varepsilon$ and ${}^{18}\varepsilon$ (cf. Figure I.3). While values for ${}^2\varepsilon$ and ${}^{18}\varepsilon$ change with temperature, the ratio ${}^2\varepsilon/{}^{18}\varepsilon$ is virtually constant over a temperature range spanning 35 °C. The ${}^2\varepsilon/{}^{18}\varepsilon$ ratio for ${}^2\varepsilon$ and ${}^{18}\varepsilon$ values at 25 °C yields a value of 7.9. For ${}^2\varepsilon$ and ${}^{18}\varepsilon$ values covering the temperature range from −5 to +30 °C the ${}^2\varepsilon/{}^{18}\varepsilon$ ratio yields an average value of 8.2 (Mook, 2000).

While studying the isotopic composition of precipitation from different parts of the world Craig (1961) and Dansgaard (1964) found a relation between δ^2H and $\delta^{18}O$ values of precipitation (Craig, 1961). The correlation equation describing this relation is referred to as the Global Meteoric Water Line (GMWL):

$$\delta^2H = 8 \times \delta^{18}O + 10 \tag{I.15}$$

In the context of what we have learned thus far, the slope of 8 for the global meteoric water line (Equation I.15) is obviously not a random number but can be

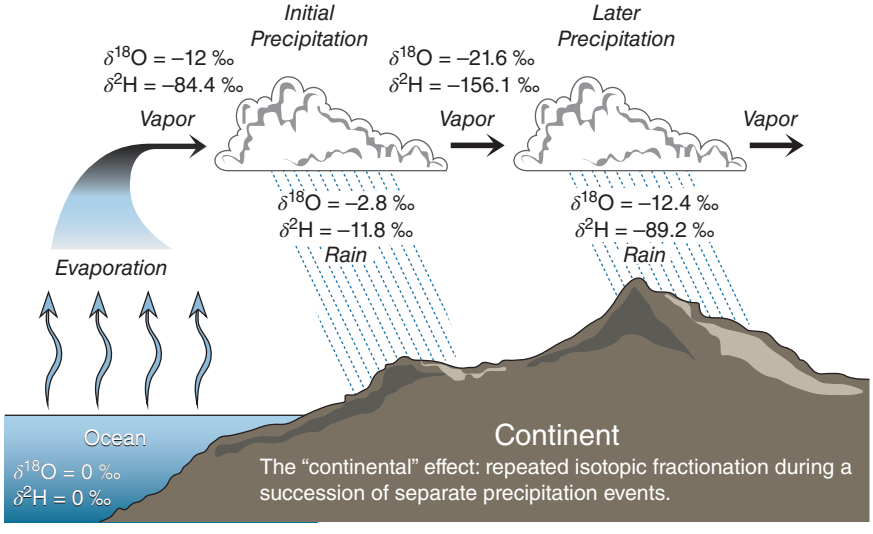

Figure I.3 Schematic representation of changing δ^2H and $\delta^{18}O$-values of meteoric water as a result of repeated fractional precipitation. *Source:* Adapted from Meier-Augenstein and Kemp 2012. Reproduced with permission of John Wiley & Sons.

explained by the ratio of the equilibrium fractionations $^{2}\varepsilon$ and $^{18}\varepsilon$ for the formation of precipitation.

For plants, water is the only available hydrogen precursor pool for the biosynthesis of carbohydrates, lipids and proteins. While the biosynthesis of lipids seems to be associated with a considerable isotopic fractionation of about −200 ‰ compared to the δ^2H value of source water (Fry, 2007), δ^2H values of carbohydrates such as sugars and cellulose seem to be largely unaffected, showing a very strong correlation with δ^2H values of source water (see Figure I.4). With knowledge of the underlying fractionation processes associated with evapotranspiration in plant leaves (the capillary flow of soil water through the xylem does not incur isotopic fractionation) and biosynthetic pathways, it is possible to use the δ^2H of plant material as an indicator of source water, that is, local precipitation and, hence, provenance (Tipple *et al.*, 2013).

Another and more direct source of information regarding the ^{2}H isotopic composition of source water and hence the geographic provenance of highly popular fruit such as blackberries, raspberries, red or black currants and strawberries is the water contained in or stored by the fruit itself. Almost all fruit, but soft fruit in particular, contain between 85 and 91 % water, so fruit water δ^2H values of freshly pressed or pulped fruit should be very closely correlated with δ^2H values of source water. Pilot data from a preliminary study carried out in our laboratory seem to support this hypothesis (see Figure I.5). Of course, only further studies will show if the correlation really is as strong as these pilot data seem to suggest, and if analysing for

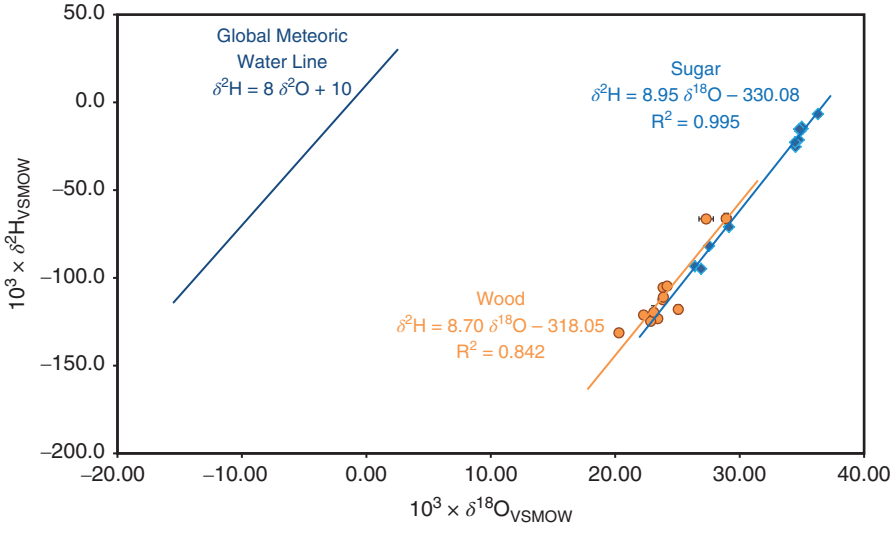

Figure I.4 δ^2H and δ^{18}O values of whole wood and plant sugars (beet as well as cane sugar) in the framework of the global meteoric water line (GMWL).

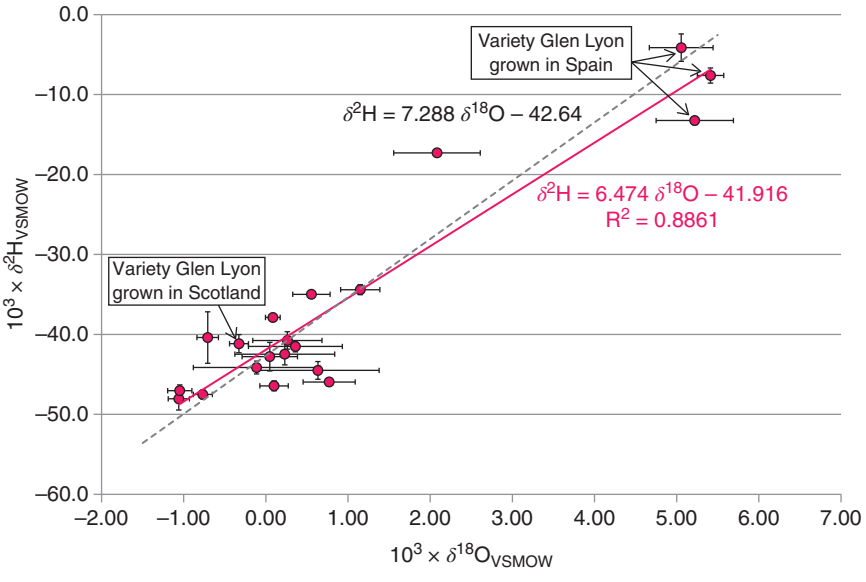

Figure I.5 Correlation plot of $\delta^{18}O$ values versus δ^2H values of fruit water pressed from fresh raspberries grown in Spain and in Scotland. Error bars are ± 1 σ. Solid trend line is based on least squares regression while the hashed trend lines is based on orthogonal regression.

δ^2H values of fruit water may provide a "forensic" tool to detect mislabelling of, for example, cheap imports as highly priced local or regional produce (cf. Section I.6.1).

In contrast to plants, for most animals, but particularly for land-living mammals and humans, the body's hydrogen pool is fuelled by three precursor pools: drinking water (including water in beverages in the case of human beings), water stored in food and water hydrogen chemically bound in food, although in most cases this can be simplified to water and water hydrogen chemically bound in food. This is an important point to remember since it has a bearing on our ability to ascertain geographic origin and geographic movement from ^2H isotope analysis of non-remodelling body tissue such as hair and nail.

I.5.2 Oxygen

Oxygen as O_2 comprises approximately 21 % by volume of the Earth's atmosphere and in the form of water it is at 88.8 % by mass the major component of the world's oceans. In fact, by mass oxygen is the Earth's most abundant light chemical element.

Oxygen exists in the form of three stable isotopes in nature, ^{16}O, ^{17}O and ^{18}O, with relative abundances of 99.7621, 0.0379 and 0.20004 atom%, respectively (Sharp, 2007). Given its low abundance, other than in a few specialist subject areas,

analysis of ^{17}O isotope abundance receives much less attention than ^{18}O in studies exploiting oxygen isotopes as natural tracers although the link between ^{17}O and ^{18}O abundance plays an important role when correcting the contribution of CO_2 isotopologue $^{12}C^{17}O^{16}O$ to mass 45 when analysing ^{13}C isotope abundance based on the abundance of CO_2 isotopologue $^{13}C^{16}O_2$ (Craig, 1961). As with hydrogen, the hydrologic cycle and hence water control most of the processes and fluxes involving oxygen, its transfer and its incorporation into organic and inorganic materials. However, atmospheric oxygen that as O_2 comprises approximately 21 % of the Earth's atmosphere also plays an important role as a precursor pool for many biochemical reactions. Due to its volume and gaseous nature atmospheric O_2 is regarded as a constant pool with a constant $\delta^{18}O$ value of +23.5 ‰ versus VSMOW.

The precursor pools for oxygen incorporated into plant products such as sugars, lipids or phenolic compounds encompasses water (H_2O), carbon dioxide (CO_2) and oxygen (O_2). Incorporation of oxygen into various biochemical compounds from these different precursor pools involves pool mixing and different biochemical pathways (reactions) which will be associated with different degrees of mass discrimination and thus different rates of isotopic fractionation. However, due to the complexity of these biosynthetic pathways typically only net isotopic fractionation factors can be determined or postulated (Schmidt, Werner, and Rossmann, 2001).

Biochemical reactions or transformations using atmospheric O_2 as a precursor pool are hydroxylation reactions such as converting proline units in pro-collagen fibres into hydroxyproline or the metabolic oxidation/hydroxylation catalysed by monoaminoxidase (MAO) and cytochrome P450 enzymes. In plants, O_2 is the precursor pool used by mono-oxygenases in conjunction with $NADH/H^+$ to introduce hydroxyl groups to aromatic carboxylic acids to yield hydroxy aromatic carboxylic acids of the hydroxycinnamic acid type, such as ferulic acid or caffeic acid. Hydroxycinnamic acids are produced by the shikimate pathway and are used by plants to form lignin.

Pool mixing and isotopic fractionation during biochemical processes are also the reason why plant sugars, including starch (flour), wood and cellulose fibres typically exhibit $\delta^{18}O$ values that are 25 to 45 ‰ higher compared to source water $\delta^{18}O$ values (see Figures I.4 and I.6).

I.5.3 Carbon

Going from the simplest organic compounds comprising only one carbon atom (C_1 bodies), such as methane or plant-derived methyl halides such as methyl chloride (Keppler *et al*., 2004, 2006, 2007, 2008; Keppler and Rockmann, 2007), to sediments or marine carbonate, the variation in natural abundance of ^{13}C covers approximately 0.11 atom% or 110 ‰ (Fry, 2006). This wide range reflects the varying degree of mass discrimination associated with the different photosynthetic pathways used by plants for carbon assimilation and fixation. As we have seen previously (cf. Table I.4

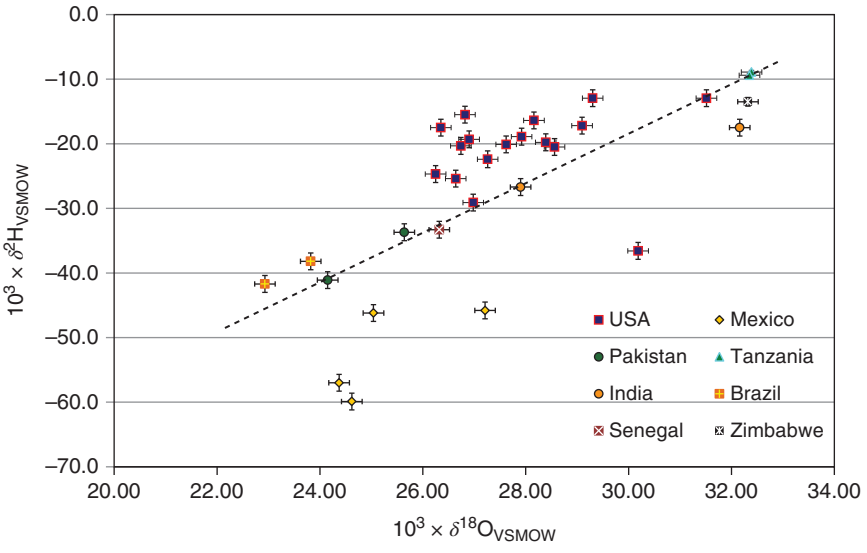

Figure I.6 Correlation plot of true, H-exchange corrected δ^2H_{VSMOW} values and $\delta^{18}O_{VSMOW}$ values of raw cotton from eight different countries. Error bars are $\pm$ 1 σ. Trend line is $\delta^2H = 3.83\ \delta^{18}O - 133.13$. *Source:* Meier-Augenstein *et al*. (2014). Reproduced with permission of John Wiley & Sons.

and Figure 1.2) in terms of ^{13}C isotopic abundance, beet sugar is not the same as cane sugar. In sugar beet, carbon dioxide (CO_2) fixation results in the formation of a C_3 body, 3-phosphoglycerate (3-PGA), an organic compound comprising three carbon atoms. This photosynthetic pathway of CO_2 fixation mediated by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is known as the Calvin–Benson cycle. Plants using the 3-PGA pathway for CO_2 fixation, such as sugar beet, are commonly called C_3 plants. However, in an adaptive response to hot climatic conditions some plants make use of a different pathway to increase the rate of glucose production. Here, CO_2 fixation by RuBisCO is compartmentalized and fuelled with CO_2 from a preceding carbon fixation step using the enzyme phosphoenolpyruvate carboxylase (PEPc). The first key product of this process yields a C_4-dicarboxylic acid, oxaloacetate, hence the term C_4 plants. The C_4-dicarboxylic acid pathway is also known as the Hatch–Slack cycle. The products of these two different pathways are characterized by their different ^{13}C isotopic compositions. Glucose in leaves of C_3 plants has a $\delta^{13}C$ value of about -28 ‰ whereas leaf glucose derived from C_4 plants exhibits a more positive $\delta^{13}C$ value of about -13 ‰. Since both plant types utilize atmospheric CO_2 with a $\delta^{13}C$ value of approximately -8 ‰ for glucose production this is equivalent to a net isotopic fractionation of only -5 ‰ for the Hatch–Slack cycle while the corresponding net isotopic fractionation for the Calvin–Benson cycle is about -20 ‰ (Fry, 2006). The most important C_4

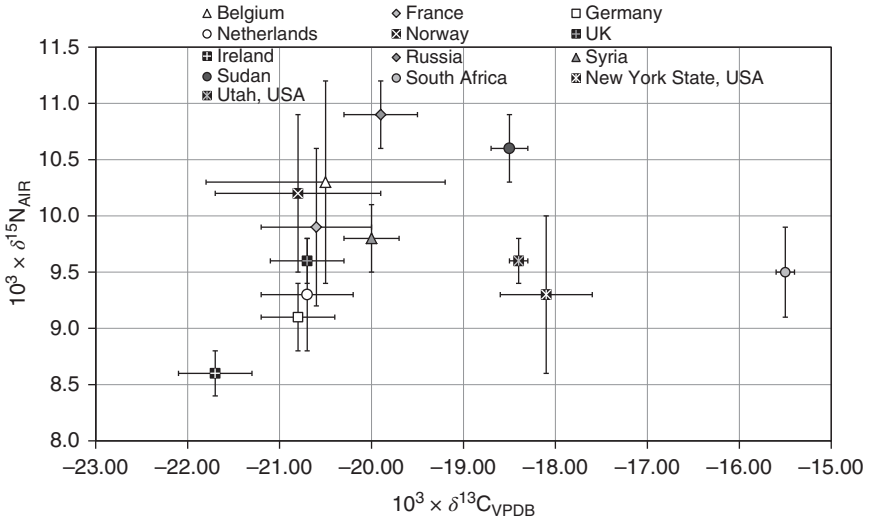

Figure I.7 Bivariate graph plotting δ^{15}N versus δ^{13}C-values of scalp hair samples volunteered by residents in different countries reflecting their regionally different diet. Error bars are $\pm$ 1 σ of groups comprising 4 to 10 individuals per region. *Source:* Data from Dr Isla Fraser 2008.

plants in terms of impact on dietary intake of ^{13}C by domestic animals and (directly or indirectly) by humans are sugar cane, maize (sweet corn), sorghum and millet. Owing to the extent to which C_4 plants pervade their staple diet, δ^{13}C values of scalp hair and human breath from North Americans and South Africans, for example, are more positive as compared to corresponding δ^{13}C values for Central Europeans, for example, with δ^{13}C values ranging from -18.5 to -15.5 ‰ (cf. Figure I.7).

The other major carbon fixation process in nature is the dissolution of CO_2 in seawater. Remembering our chemistry lessons we know the dissolution of CO_2 in water is pH dependent. Seawater has a pH of 8 and under these alkaline conditions over 99 % of the CO_2 dissolved in seawater is present in the form of HCO_3^-, with the remainder being present as CO_3^{2-}. As a result the oceans contain about 50 times more CO_2 than the atmosphere, thus making the oceans a major CO_2 sink. The net isotopic fractionation between atmospheric CO_2 ($\delta^{13}C = -8$ ‰) and total dissolved CO_2 in seawater ($\delta^{13}C = +1$ ‰) is +9 ‰, which ultimately results in marine particulate organic matter (POM), on which virtually all life in the oceans is based one way or another, with δ^{13}C values of about -22 ‰, thus displaying an apparent net fractionation of -23 ‰ between dissolved seawater CO_2 and marine POM. In contrast, dissolved CO_2 in freshwater lakes with pH values of about 6.2 is an equilibrium mixture of dissolved CO_2 (written as H_2CO_3) and HCO_3^-. Total dissolved CO_2 in freshwater lakes has a δ^{13}C value of approximately -15 ‰, which corresponds to a net isotopic fractionation of -7 ‰ against atmospheric CO_2. Freshwater POM exhibits a δ^{13}C value of about -35 ‰, that is, 20 ‰ more

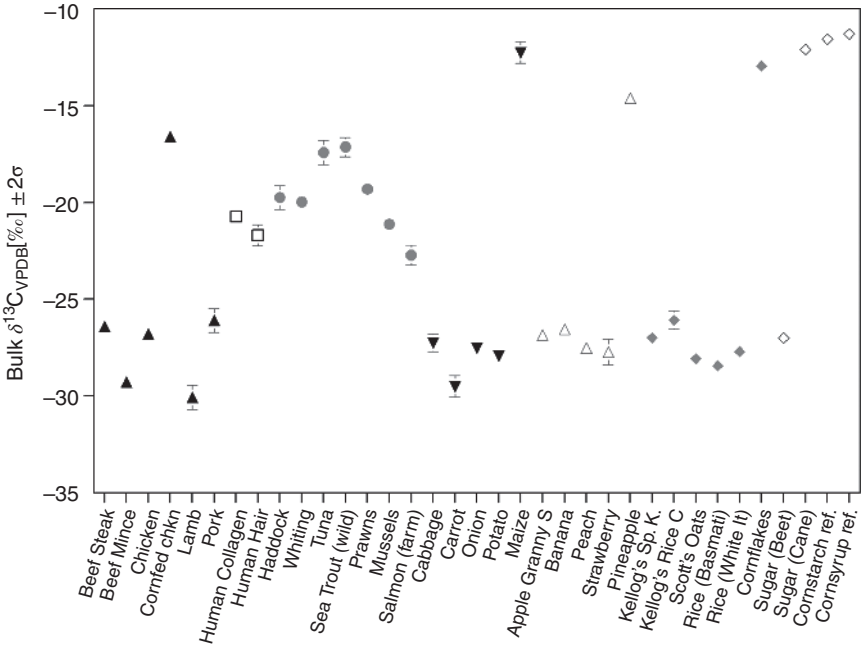

Figure I.8 ^{13}C isotopic composition of selected fruit, meat, fish and vegetables. Please note, human collagen and human hair $\delta^{13}C$-values included here are typical for people with a terrestrial C_3-plant dominated diet. *Source:* Data from Morrison *et al*. (2000) as well as author's own data.

negative than the CO_2 dissolved in freshwater. This pronounced difference in ^{13}C isotopic composition between freshwater POM and marine POM is the reason why the muscle tissue of marine plankton feeders such as mussels, prawns and whiting shows $\delta^{13}C$ values ranging from −21 to −19 ‰ (cf. Figure I.8). This leads us nicely on to the subject of isotopic fractionation associated with trophic ecology, that is, food chains and food webs. We will have a closer look at this phenomenon, which is known as trophic level (isotopic) shift, in Section III.5.1. In the meantime, let us proceed with our studies of the major light elements by finding out a bit more about nitrogen.

I.5.4 Nitrogen

Unlike for oxygen, the main biosphere reservoir for nitrogen in nature is the Earth's atmosphere, with nitrogen (as N_2) being its major constituent by volume, namely 78 %. Given that atmospheric N_2 is evenly distributed, the $^{15}N/^{14}N$ ratio of nitrogen in air is deemed to be a constant 0.0036765 and air nitrogen (Air) is therefore used as our standard scale reference point for reporting $\delta^{15}N$ values. However,

atmospheric nitrogen constitutes only 2 % of all nitrogen on Earth. The vast proportion of nitrogen is bound in rocks, accounting for 97.98 % of all nitrogen on Earth. Only 0.001 % of all nitrogen is bound in organic matter. Again unlike oxygen, the nitrogen contained in the Earth's atmosphere is not directly bio-available to most organisms, with the exception of a few nitrogen fixating soil bacteria. The nitrogen cycle through the biosphere is driven by five major processes: nitrogen fixation, nitrogen uptake (through growing organisms), nitrogen mineralization (decay), nitrification and denitrification. Microorganisms, in particular bacteria, are the major players in all of the aforementioned nitrogen transformation processes. For this reason ^{15}N abundance in the biosphere typically ranges from -20 to $+30$ ‰ for nitrogen bound as NH_4^+ or in animal waste, respectively (Sharp, 2007).

One reason for this relatively narrow range is that isotope fractionation associated with nitrogen fixation and mineralization (ammonification) of organic nitrogen to soil NH_4^+ is generally quite small. Due to natural variations associated with the pathways for these process the ε values for isotope fractionation reported for nitrogen fixation range from -3 to $+3.7$ ‰ but are typically taken to be $+1$ ‰ while breakdown of organic matter to ammonium is given as 0 ± 1 ‰ (Fry, 2006; Sharp, 2007). The combination of nitrogen fixation, ammonification and nitrification (i.e. the oxidation of NH_4^+ to NO_2^- and ultimately NO_3^-) results in plants exhibiting $\delta^{15}N$ values that can range from -10 to $+10$ ‰, although typically $\delta^{15}N$ values of -6 to $+6$ ‰ are observed while $\delta^{15}N$ values reported for soil can range from -10 to $+20$ ‰ (Hoefs, 2009), with $\delta^{15}N$ values of soil reflecting the ^{15}N isotopic composition of plant litter and overlying vegetation (Handley and Scrimgeour, 1997). It should be noted that even though some nitrification and ammonification processes can be associated with large isotope effects, the corresponding reactions often progress in a quantitative fashion due to slow rates of nitrogen supply and limited amounts of nitrogen substrate, which means all available nitrogen is converted and no overall net fractionation is observed.

However, there are two factors influencing the $\delta^{15}N$ values of materials we may encounter in the course of a criminal investigation requiring forensic stable isotope analysis: trophic ecology and anthropogenic activity. We have encountered the term trophic ecology before in Section I.5.3 and given its relevance to interpreting stable isotope signatures of human tissue we will have a closer look at the associated phenomenon of tropic level shift fractionation in Section III.5.1.1. For the moment it is sufficient to know that a primary consumer such as cattle feeding on a primary producer such as grass will increase its body ^{15}N isotopic composition by $+3$ to $+4$ ‰ as compared to the ^{15}N signature of grass by excreting urine that is ^{15}N depleted and comprises isotopically light, that is, ^{14}N-enriched ammonia (NH_3) and urea (OCN_2H_4) (Senbayram *et al.*, 2008). As a result of these processes and staying with the example of cattle, proteinous cattle tissue, even milk, typically exhibits $\delta^{15}N$ values of around $+6$ to $+7$ ‰ (cf. Figure I.9). Moving up in the food chain from one trophic level to the next, for example from primary producer (e.g. fruit and vegetables) to primary consumer (e.g. herbivore) or from primary

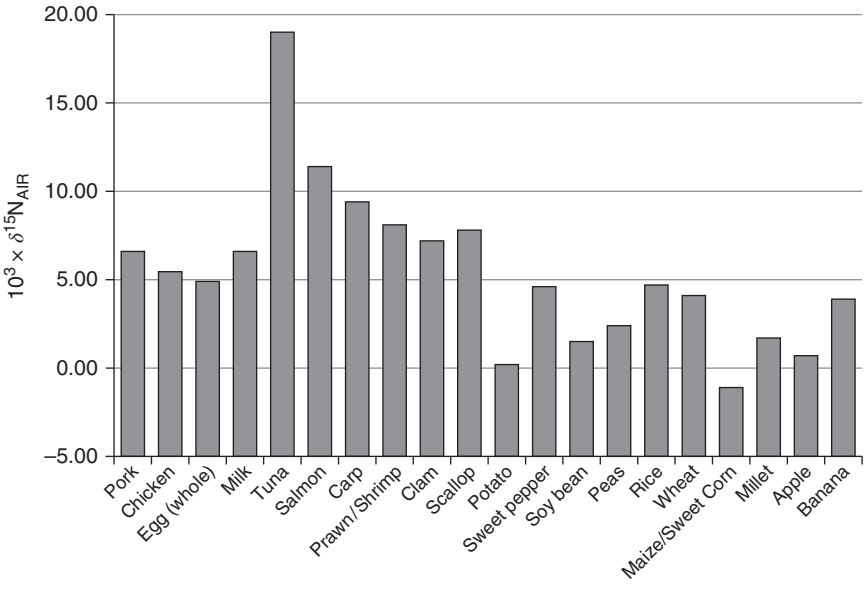

Figure I.9 ^{15}N isotopic composition isotopic composition of selected fruit, meat, fish and vegetables. Please, note, $\delta^{15}N$ values of fruit and vegetable are not fixed constants but vary depending on farming practice and type of fertilizer usage.

consumer (shrimp) to secondary consumer (carnivore, e.g. salmon), $\delta^{15}N$ values of proteinogenous tissue will shift upwards by +3 to +4 ‰ (cf. Figures I.9 and I.10).

Anthropogenic influences can be divided into two types of activity. One is human agricultural activity and its influence on the $\delta^{15}N$ values of cultivated soils through soil disturbance, but chiefly through the application of fertilizers, both synthetic and organic. The addition of organic (animal waste) fertilizer increases soil $\delta^{15}N$ values while the addition of synthetic nitrogenous fertilizer lowers soil $\delta^{15}N$ values since synthetic inorganic fertilizers tend to have $\delta^{15}N$ values ranging from −5 to +5 ‰. Synthetic fertilizers in turn are the result of the second type of anthropogenic activity, namely manufactured materials. Depending on the synthetic route and process, isotopic fractionation of −14 ‰ can be observed between precursor and product $\delta^{15}N$ values (Lock and Meier-Augenstein, 2008), with $\delta^{15}N$ values for materials of forensic interest such as MDMA or nylon fibres ranging from −18 to +35 ‰.

I.5.5 Sulfur

In comparison to the application involving stable isotopes of bio-relevant light elements such as carbon, hydrogen, nitrogen and oxygen, applications involving sulfur,

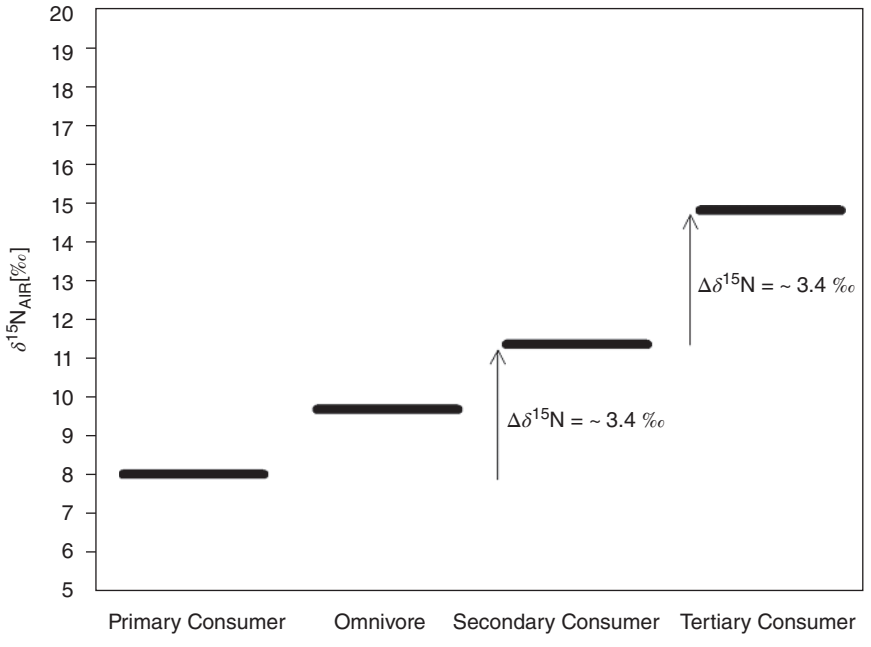

Figure I.10 Simplified schematic representation of typical δ^{15}N-values for proteinogenous tissue of terrestrial mammals in relation to their trophic level in the food web.

that is, its stable isotopes ^{32}S and ^{34}S, are rather under-represented in the scientific literature. This, however, is not necessarily a reflection on its usefulness but can rather be attributed to the fact that sulfur stable isotope analysis requires (i) a relatively large sample amount due to its low relative abundance in organic compounds, (ii) great analytical skill, and (iii) ideally an instrumental set-up dedicated to sulfur isotope analysis. One could argue the latter two points are more relevant to continuous flow isotope ratio mass spectrometry (CF-IRMS) due to the "viscous" and hygroscopic nature of SO_2, the gas into which samples have to be converted for sulfur isotope analysis by IRMS. A study published in 2009 addressed the issue of sulfur abundance in biogenic compounds by establishing a set of sample composition requirements or quality criteria for bone collagen preparations from archaeological bone samples (Nehlich and Richards, 2009).

Of all bio-relevant light elements the major sulfur stable isotope ^{34}S has the highest natural abundance of 4.215 atom%. The corresponding isotope ratio of 0.0430023 is quite close to that of primordial sulfur, which is represented by the $^{34}S/^{32}S$ ratio of 0.0450045 of the previous international primary isotope scale anchor Cañon Diablo Troilite (CDT) meteorite, a meteoritic troilite (FeS) found in a meteor crater near Flagstaff, Arizona. However, as mentioned already in Chapter I.2, in 1997 the decision was taken to henceforth express δ^{34}S values on the VCDT

(Vienna Cañon Diablo Troilite) scale, with IAEA-S-1 (silver sulfide, Ag_2S) being used as the primary scale anchor to underpin the VCDT scale. This move to the virtual scale reference point VCDT was intended to overcome problems associated with observed variability of $\delta^{34}S$ values of CDT (Krouse and Coplen, 1997). Dissolved sulfate (SO_4^{2-}), which is the chief component of the sulfur reservoir in the Earth's oceans, has a ^{34}S isotopic composition of about +21 ‰ on the VCDT scale. In a reflection of global-scale fluctuations in sulfate reduction activities over geological time scales $\delta^{34}S$ values of sulfate dissolved in river or sea water range from about +10 to +33 ‰. Continental uplift and preservation of marine sediments containing sulfur in different oxidation states, which can range from –II to +VI, have produced on land something like insular sulfur environments, each associated with different $\delta^{34}S$ values for sulfur in bedrock. Despite these large ranges observed for $\delta^{34}S$ values in general, $\delta^{34}S$ values for continental vegetation typically seem to range from +2 to +6 ‰ over large areas. The $\delta^{34}S$ values for terrestrial plants are, however, quite distinctly different from the more ^{34}S-rich values of +17 to +21 ‰ found in marine plankton and seaweeds.

As discussed for carbon, hydrogen, nitrogen and oxygen, sulfur isotope signatures of animal or human tissue can be regarded as a reflection of the sulfur isotopic composition of the consumed diet (Macko *et al*., 1999; Richards *et al*., 2003; Richards, Fuller, and Hedges, 2001). In turn the ^{34}S isotopic composition of diet is a reflection of ^{34}S isotope abundance in a given environment, incorporated into plant and animal tissue from sulfur sources such as bedrock weathering, atmospheric deposition and microbiological activity (Richards *et al.*, 2003). From a human provenancing point of view, tissue with a high collagen or keratin content has the highest potential to yield meaningful information given the relatively high sulfur content of either protein compared to other biogenic compounds. In a study of archaeological samples for paleodietary habits, bone collagen $\delta^{34}S$ values from inland sites in the Ukraine ranged from +5.2 to +7.9 ‰ while bone collagen $\delta^{34}S$ values from coastal sites in Scotland and Greece ranged from +15.0 to +22.2 ‰ (Richards, Fuller and Hedges, 2001).

Given the aforementioned differences between marine, freshwater and terrestrial $\delta^{34}S$ values, with the help of sulfur isotope analysis of animal or human tissue it is potentially possible to distinguish between freshwater/terrestrial or marine dietary sources (Privat, O'Connell, and Hedges, 2007; Richards *et al*., 2001, 2003). However, similar to considerations mentioned for drawing conclusions on human dietary habits based on $\delta^{15}N$ values, $\delta^{34}S$ values should be interpreted in the context of $\delta^{15}N$ and $\delta^{13}C$ values obtained from the same sample material. Presumed to be marine diet-derived ^{34}S signatures may actually be the result of consuming plants and animals cultivated and reared in coastal regions. One possible explanation for this phenomenon of artefact marine ^{34}S signatures is the use of seaweed and kelp as natural fertilizer or indeed feed stock for domestic animals such as sheep and cattle (Balasse, Tresset, and Ambrose, 2006). The other explanation is referred to as the "sea spray effect", that is, a result of airborne deposition of marine sulfur

particles from the ocean to the coast, although the extent of this mode of marine sulfur transfer onto coastal regions has not been yet been established. Having said that, the "sea spray effect" may permit plausible interpretation of $\delta^{34}S$ values in terms of geographic provenance with relatively high ^{34}S signatures being taken to intimate coastal residency, even when $\delta^{13}C$ and $\delta^{15}N$ values indicate a terrestrial- rather than marine-based diet (Richards, Fuller and Hedges, 2001). One should be equally careful when it comes to interpreting $\delta^{34}S$ values of tissue from modern-day people. These $\delta^{34}S$ values may also reflect the potential influence of the ^{34}S isotopic composition of fossil fuels or from other anthropogenic sources contained in atmospheric emission and from there incorporated into food sources as well as into human tissue directly.

A comparative study of scalp hair collected from 35 UK residents and visitors to the UK reported $\delta^{34}S$ values of $+2.30 \pm 0.14$ ‰ (Canada and USA, $n = 2$), $+5.41 \pm 0.97$ ‰ (UK and Germany, $n = 22$), 10.70 ‰ (Australia, $n = 1$) and $+10.30$ ‰ (Chile, $n = 1$) (Bol, Marsh, and Heaton, 2007). Given these encouraging ^{34}S data from human scalp hair I suggested in 2007 that it should be possible to extract useful information on the recent geographic movement and whereabouts of a person suspected to be involved in serious and organized crime, including terrorism, from as little as a single strand of hair using laser ablation coupled to a multi-collector inductive coupled plasma mass spectrometer (LA-MC-ICP-MS) since recent advances in MC-ICP-MS instrumentation have made it possible to measure ^{34}S isotope ratios at natural abundance level either by direct laser ablation or by direct introduction of a sample liquid into the plasma via a nebulizer (Santamaria-Fernandez and Hearn, 2008; Santamaria-Fernandez, Hearn, and Wolff, 2008). Indeed, in 2009 an LA-MC-ICP-MS method was reported for the measurement of chronological variations in the ^{34}S composition of single human hair strands from two UK residents and one person who had travelled to three different countries within the six months prior to sample collection. While time-corresponding hair strands from the two UK residents showed little or no variation in ^{34}S abundance, $\delta^{34}S$ values from a strand of hair collected from the frequent traveller varied between +5.5 and +10.5 ‰ (Santamaria-Fernandez *et al.*, 2009).

I.5.6 Isoscapes

The idea behind the contents of Chapters I.1 through I.5.5 was two-fold: (i) to provide the reader with an abridged yet solid foundation to appreciate and understand what will be discussed in the subsequent chapters and parts of this book, and (ii) to prepare the reader for and to appreciate the fact that the stable isotope ratio or stable isotope abundance of a given element is not a fixed natural constant no matter the circumstances but may depend on a number of different factors influencing the processes involved in making a given compound or material such as source

(made by whom) and origin (made where). However, it is likely some readers are still not entirely convinced that stable isotope abundances can and frequently do vary, and because of this measured stable isotope signatures can tell us something useful, something we did not know before and, in particular, something that would be unobtainable from or through traditional analytical techniques such as mass spectrometry. After all, a mass spectrum of a given compound tells us what the compound is, it identifies this compound. The intensity of the $[M+1]^+$ satellite peak in the mass spectrum of a given organic always seems to be 1 % of the molar peak $[M]^+$, suggesting a constant or fixed $^{13}C/^{12}C$ ratio. What evidence is there to prove the variable nature of stable isotope ratios if mass spectra seemingly tell us otherwise? What more is there to know?

Figures I.1 and I.2 may already provide some indication as to what the answers to these questions may be. Thanks to the pioneering efforts of two scientists, Gabriel J. Bowen and Jason B. West, the last 10 years have seen huge strides being taken in the development or 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 or materials of interest, such as feathers, human scalp hair, human teeth enamel, leaf water, olive oil, precipitation and vegetation (Bowen, 2008; Bowen and Revenaugh, 2003; Bowen and Wilkinson, 2002; Chiocchini, *et al*., 2016; Ehleringer *et al*., 2008a, 2010; Hobson *et al*., 2004, 2009; Suits *et al*., 2005; West, Sobek and Ehleringer, 2008). 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, similar to topographical maps that show contour lines representing points of equal altitude above sea level. Isoscapes are graphical representations of modelled stable isotope abundance values interpolated from a data set of measured values by a detrended, latitude- and elevation-explicit algorithm (Bowen and Revenaugh, 2003; Bowen and Wilkinson, 2002).

In 2010, a book called *Isoscapes* was published (West *et al.*, 2010). This book comprises 20 chapters based on the presentations given by invited speakers at the Isoscapes 2008 conference. During the conference, participants were asked to indicate their areas of research interest in which they do or would apply isoscapes. This survey returned ecology, climate change, biogeochemistry, hydrology and forensics as the top five areas of research interest, clearly demonstrating the common ground shared by a wide spectrum of science areas through the study of stable isotope ratios, their variability at natural abundance level, and the underlying processes they reflect. In line with the old adage "a picture is worth a thousand words" the readers' attention is drawn to Figures I.11 to I.13.

Let us for a moment assume the impression given by 20th century versions of the periodic table of elements is correct and isotope abundances and thus isotope ratios are fixed constants irrespective of chemical matrix, environmental conditions or location. In the case of hydrogen and using water as example, this would mean any water sample would have the same isotopic composition of 99.985 atom% and

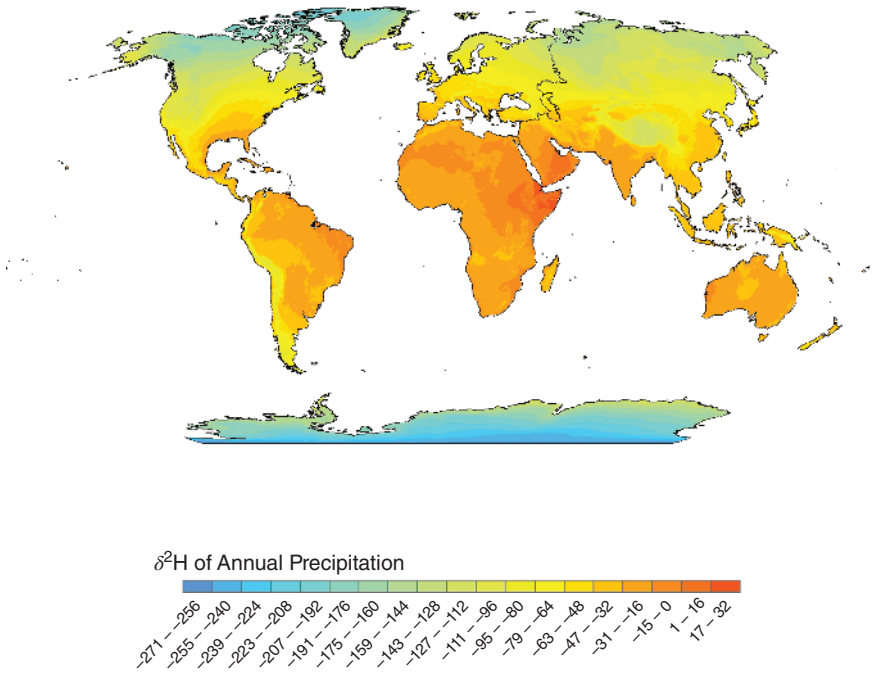

Figure I.11 Global δ^2H isoscape of ^{2}H abundance in annual precipitation. Courtesy: Reproduced with kind permission of Gabriel J. Bowen 2015.

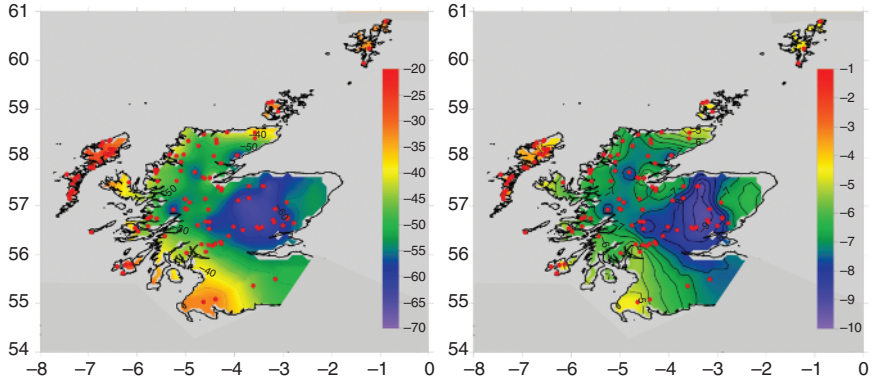

Figure I.12 Isoscapes of ^{2}H (left) and ^{18}O abundance (right) in freshwater lakes and reservoirs in Scotland. Red dots mark sampling locations. X and Y axes are degrees Longitude West and degrees Latitude North. *Source:* Data from Meier-Augenstein *et al*. (2013).

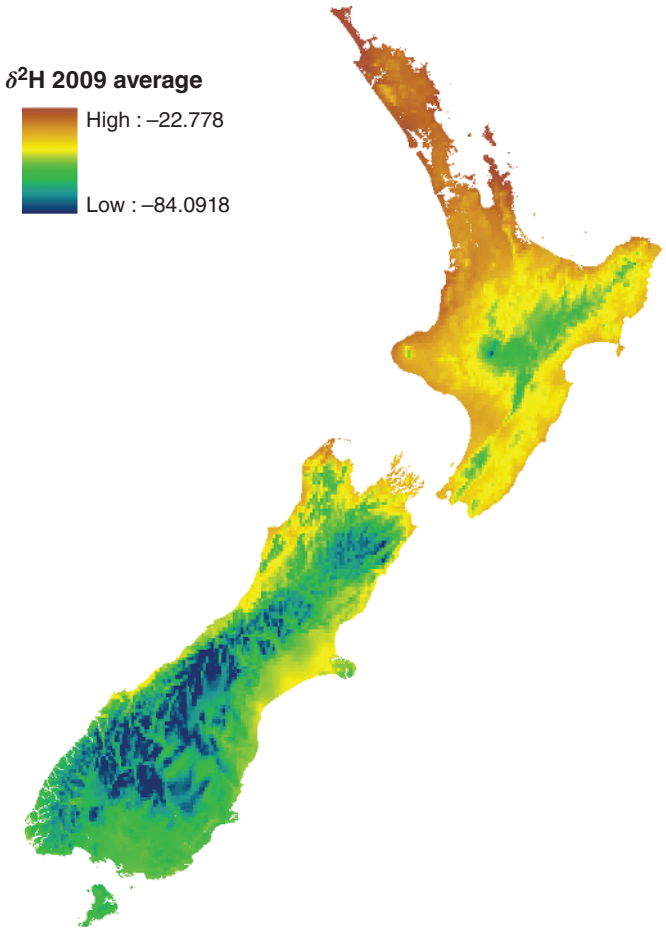

Figure I.13 δ^2H isoscape of ^{2}H abundance in annual precipitation of 2009 in New Zealand. Courtesy: Reproduced with kind permission of Russell D. Frew 2015.

0.015 atom% for ^{1}H and ^{2}H, respectively. The corresponding isotope ratio of ^{2}H/^{1}H would be 0.0001500225, which expressed as a relative abundance value on the VSMOW scale is equivalent to a δ^2H_{VSMOW} value of −36.8 ‰. However, isoscapes of δ^2H_{VSMOW} values found in meteoric water (precipitation), freshwater (e.g. lakes and reservoirs) or tap water clearly show that δ^2H_{VSMOW} values vary widely across the globe even if discrete locations can be found where the ^{2}H composition of water does indeed assume a δ^2H_{VSMOW} value of −36.8 ‰ (cf. Figures I.11, I.12 and I.13). The annual averaged δ^2H_{VSMOW} values of global precipitation range from +20 to −270 ‰ (Bowen and Revenaugh, 2003). The annual averaged δ^2H_{VSMOW} values of precipitation in the USA cover a comparably wide range from −20 to −135 ‰.

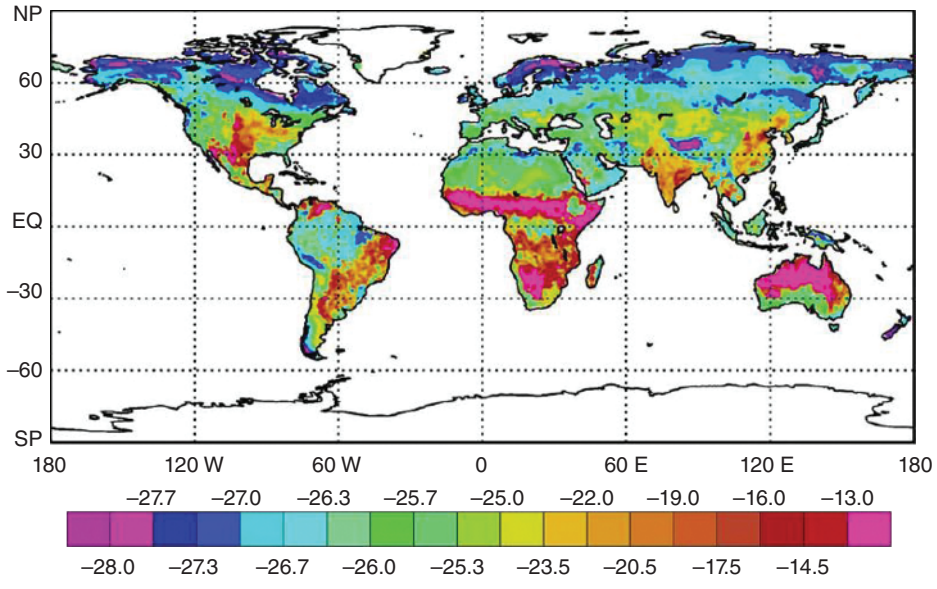

Figure I.14 Global $\delta^{13}C$ isoscape of mean annual ^{13}C abundance in plant carbon of terrestrial vegetation. *Source:* Suits *et al.* (2005). Reproduced with permission of American Geophysical Union.

This wide range in $\delta^{2}H_{VSMOW}$ values of precipitation is due to both the size and location of the North American continent (Bowen *et al.*, 2007), which but for the extremes of equatorial Africa, the Arctic and the Antarctic mirrors environmental conditions found elsewhere on Earth as they affect precipitation.

The variability of ^{13}C abundance in vegetation (or plant matter) across the globe provides another example (cf. Figure I.14). Based on the figures stated on 20th century versions of the Periodic Table of the Elements, any organic compound or material should comprise 98.89 atom% ^{12}C and 1.11 atom% ^{13}C, with an isotope abundance ratio of 0.0112246 corresponding to a $\delta^{13}C_{VPDB}$ value of -1.12 ‰. Yet, we have already learned in Section I.5.3 how ^{13}C is incorporated into plant matter and plant products such as sugar at different levels due to differences in isotopic fractionation associated with different pathways of CO_2 fixation. The three different pathways of CO_2 fixation, known as the Calvin–Benson cycle, the Hatch–Slack cycle and the crassulacean acid metabolism cycle, are evolutionary responses of plants to environmental, in particular climatic, conditions, and this is reflected in the global $\delta^{13}C$ isoscape of ^{13}C abundance in vegetation shown in Figure I.14 (Suits *et al.*, 2005).

Chapter I.6

Stable Isotope Forensics in Everyday Life

In contrast to the preceding occasionally somewhat dry chapters, the following chapters provide a compilation or review of stable isotope applications, albeit not always forensic applications in the true sense of the word. However, these are important applications of this analytical technique nonetheless, impacting as they do on our day-to-day life by making it directly or indirectly safer and more enjoyable through protection of our health and our environment.

In a report entitled *The Economic Impact of Counterfeiting and Piracy*, published in 2007,[4] the Organization for Economic Cooperation and Development (OECD)[5] stated that in 2005:

> *… the volume of tangible counterfeit and pirated products in international trade could be up to US$200 billion. This figure does not, however, include counterfeit and pirated products that are produced and consumed domestically, nor does it include the significant volume of pirated digital products that are being distributed via the Internet. If these items were added, the total magnitude of counterfeiting and piracy worldwide could well be several hundred billion dollars more.*

In the same report the OECD highlights the links between counterfeiting consumer goods and organized crime as well as international terrorism:

> *The groups involved in counterfeiting and piracy include mafias in Europe and the Americas and Asian "triads", which are also involved in heroin trafficking,*

[4] http://www.oecd.org/dataoecd/13/12/38707619.pdf; last accessed 14 November 2016.

[5] The OECD member countries are Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Japan, Korea, Luxembourg, Mexico, the Netherlands, New Zealand, Norway, Poland, Portugal, the Slovak Republic, Spain, Sweden, Switzerland, Turkey, the United Kingdom and the United States. The Commission of the European Communities takes part in the work of the OECD. URL: http://www.oecd.org/.

Stable Isotope Forensics: Methods and Forensic Applications of Stable Isotope Analysis, Second Edition.
Wolfram Meier-Augenstein.

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prostitution, gambling, extortion, money laundering and human trafficking. To address the situation, Interpol created an Intellectual Property Crime Action Group in July 2002, to help combat trans-national and organised intellectual property (IP) crime by facilitating and supporting cross-border operational partnerships. Some governments have also established bilateral operational partnerships in border enforcement and criminal investigations.

In addition to the established link between counterfeiting and piracy and organised crime, Interpol has highlighted a disturbing relationship of counterfeiting and piracy with terrorist financing, with IP crime said to be becoming the preferred method of financing for a number of terrorist groups. The links take two basic forms:

- Direct involvement*, where the terrorist group is implicated in the production or sale of counterfeit goods and remit a significant portion of those funds for the activities of the group. Terrorist organisations with direct involvement include groups which resemble or behave like organised crime groups.*
- Indirect involvement*, where sympathisers involved in IP crime provide financial support to terrorist groups via third parties.*[6]

Counterfeit products such as counterfeit pharmaceuticals are often substandard or even dangerous, posing health and safety risks to consumers that range from mild to life-threatening. Given the impact of counterfeit products on the economy as well as consumer health and safety, sadly very little about the impact applied stable isotope analysis has made on detecting and combating consumer good fraud seems to be known to the general public, which is surprising given that a review published in 2001 on the increasing importance stable isotope profiling has gained in the authenticity control of food and food ingredients lists 184 publications for this field alone (Rossmann, 2001). To a degree this is a sad indictment of the way real life benefits gained from advances in life sciences in general and from stable isotope research in particular are being disseminated to the public. Even more saddening is the knowledge that this failure to communicate state-of-the-art techniques as well as insights gained to the benefit of human everyday life as well as the advancement of science extends to schools, universities and colleges if the comments and opinions regarding stable isotope techniques voiced by a number of scientists and so-called expert advisers are anything to go by. Every reader, but in particular every student reader, of this book is therefore strongly encouraged to avail themselves of the positive aspects of the Internet and to search for further literature and publications on applied stable isotope analysis because even a specialist subject book such as this will never manage to capture all the published information out there (chiefly because behind this book is an author who is only human and, hence, fallible after all).

[6] *The Economic Impact of Counterfeiting and Piracy*, pp 15–16, © OECD 2008, http://www.oecd.org/sti/counterfeiting.

I.6.1 "Food Forensics"

It is a surprisingly little known fact that food products such as wine, certain spirits, high-quality single-seed vegetable oils, natural flavourings and honey are all subject to regular stable isotope analysis (more often than not in conjunction with other analytical techniques) to determine/verify product authenticity or to combat fraudulent labelling and misrepresentation of agricultural products (Ogrinc *et al*., 2003; Zhao *et al*., 2014). The UK Food Standards Agency (FSA) has supported the development of and applied stable isotope analytical techniques for food authentication since the mid-1990s and indeed for some applications stable isotope analysis has been declared the method of choice. Similarly, the European Office for Wine, Alcohol and Spirit Drinks (BEVAPS) has been using stable isotope analytical techniques and data to combat major fraud in the beverage sector since 1997, although stable isotope analysis of beverages was used as early as 1993 (Calderone, Guillou, and Naulet, 2003; Rossmann *et al*., 1996). BEVAPS was established by the European Union (EU) in 1993 and is now part of the Food Products Unit of the Institute for Health and Consumer Protection (IHCP) at the European Commission's Joint Research Centre (JRC) in Ispra (Italy).

I.6.1.1 Authenticity and Provenance of Single-Seed Vegetable Oils

In a way stable isotope profiling of food and food ingredients to detect adulteration or misrepresentation can be regarded as the first forensic application of this technology, although I have not managed to find any case reports published in a scientific journal describing the use of such data in conjunction with a legal case of fraudulent food adulteration or fraudulent misrepresentation. That being said, a single-seed vegetable oil survey of corn (maize) oil sold in the UK was carried out in 1995 on behalf of the FSA (or the Ministry of Agriculture, Fisheries and Food as it then was) and showed that 35 % of oils analysed for their ^{13}C isotopic composition were found to contain undeclared oils from different sources and were therefore not 100 % corn oil as declared on the label (Ministry of Agriculture, Fisheries and Food, 1995). Since maize is a C_4 plant, food products such as corn starch, corn syrup or corn oil show $\delta^{13}C$ values in the range of -14 to -10 ‰. C_3 plant-derived vegetable oils and oils comprising a mixture of corn oil and seed oil from a C_3 plant are therefore easily identified as such (cf. Figure I.15). These results were published and both retailers and producers were informed of the result of this survey. Given the legal framework at the time (Food Safety Act 1990) – the survey was carried out before the Food Labelling Regulations 1996 – this survey did not result in any legal action. However, a follow-up survey was commissioned by the FSA in 2001 in which 61 samples of corn oil were again analysed for five different parameters, including ^{13}C isotopic composition, and this time none of the samples were declared suspicious. The authenticity of the oils was assessed by comparing

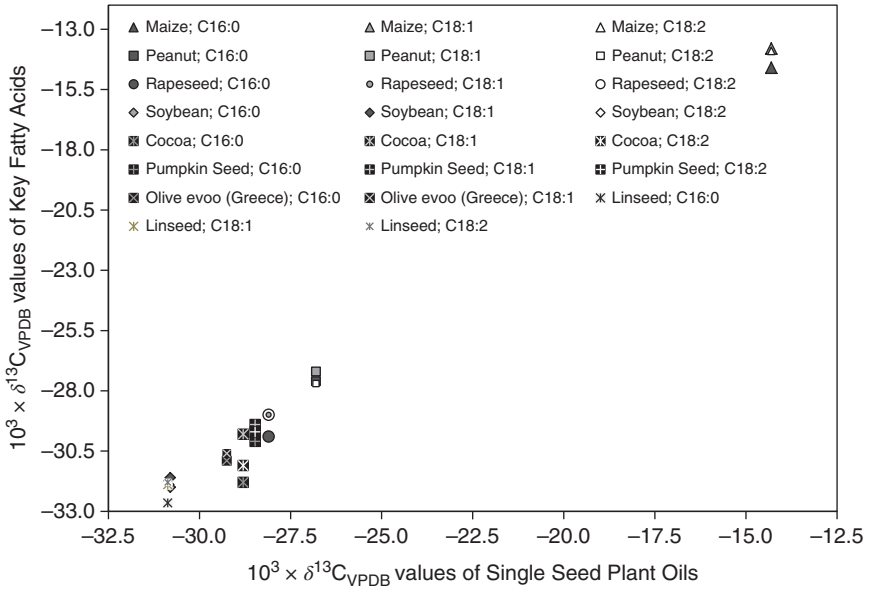

Figure I.15 Natural variation in ^{13}C isotopic composition of single seed vegetable oils and selected fatty acids isolated from these oils. *Source:* Based on data from Woodbury *et al*. (1998) as well as author's own data.

the results of analysis with a purity specification for corn oil. This specification is contained in the Codex Standard for Named Vegetable Oils, which was adopted formally by the Codex Alimentarius Commission in July 2001. In their report on the 2001 food authenticity survey the FSA concluded that corn oil now sold in the UK complied fully with the fatty acid and stable carbon isotope ratio specifications contained in the Codex standard and is named correctly on the label.

To detect adulteration of olive oils, Angerosa *et al*. compared $\delta^{13}C$ values of the aliphatic alcoholic oil fractions and found those of the adulterant pomace oil (oils of inferior quality, often pressed from fouled fruit or fruit already pressed once and subsequently cleaned up by water steam distillation) to be significantly more negative than those of virgin and refined olive oils (Angerosa *et al*., 1997). In a subsequent study, Angerosa *et al*. employed both ^{13}C and ^{18}O isotope analysis to determine the geographical origin of olive oils according to climatic regions from different Mediterranean countries such as Greece, Morocco and Spain (Angerosa *et al*., 1999). Apart from blending high-quality single-seed vegetable oils such as corn or virgin olive oil with lower cost oils such as rapeseed oil (Kelly *et al*., 1997; Kelly and Rhodes, 2002; Woodbury *et al*., 1995; Woodbury, Evershed and Rossell, 1998), thermally induced degradation due to deodorization or steam washing can alter the ^{13}C isotopic signature of the whole oil and its key fatty acid components (Spangenberg, Macko, and Hunziker, 1998). Other factors found to influence

both ^{13}C isotopic signature and relative fatty acid composition of olive oils are vintage (year of production), storage period (oxidation of unsaturated fatty acids), botanical species and maturity at harvest (Royer *et al*., 1999; Spangenberg and Ogrinc, 2001).

Several studies have used stable isotope data in conjunction with data from independent analytical techniques such as NMR or trace element analysis to obtain multivariate data sets for improved discrimination between olive oils from different regions of origin, between authentic and non-authentic virgin olive oils or between authentic and non-authentic Styrian pumpkin seed oils (Alonso-Salces *et al*., 2010; Aramendia *et al*., 2010; Camin *et al*., 2010; Meier-Augenstein, Kemp and Midwood, 2013). While olive oil is produced for export in several Mediterranean countries, such as Greece, Italy, Portugal and Spain, Styrian pumpkin seed oil is a premium single-seed vegetable oil that is uniquely linked to this specific geographic region of Austria, where it is grown and produced. In 1996, the strong regional ties of this typical Styrian speciality were recognized by the EU Commission, who declared Styrian Pumpkin Seed Oil P.G.I. (Steirisches Kürbiskernöl g.g.A.) to be a Protected Geographical Indication (article 5 VO(EWG) Nr. 2081/92). Despite the best efforts of the official association of Styrian pumpkin seed oil producers to control and document every step of production from farm to oil mill to bottling plant, there have been repeated claims that independent analyses of bottled Styrian pumpkin seed oil sporting g.g.A. seals have suggested these bottles contained either mixtures of Styrian and non-Styrian pumpkin seed oil or no Styrian pumpkin seed oil at all. Since keeping records of annual harvest amounts of pumpkin seeds would make it very difficult for an "over-production" by mixing or substitution of alien pumpkin seed oils to go unnoticed, it was suggested some of the red-flagged bottles might have been labelled with counterfeit g.g.A. seals and counterfeit serial numbers. A further hypothesis suggested using pesticide analysis as a method to detect non-authentic oils may also have contributed to the aforementioned claims since the presence of low levels of pesticides banned in the EU led to an adverse finding. However, the presence of low levels of certain banned pesticides could have been the result of cross-contamination introduced when milling Styrian pumpkin seeds in oil mills also processing imported oil seeds.

A pilot study analysing 11 pumpkin seed oils in a single blind fashion showed it should be possible to distinguish genuine Styrian pumpkin seed oils from other pumpkin seed oils by chemometric analysis of a multivariate data set comprising ^{2}H, ^{13}C and ^{18}O abundance values and concentration values of 17 trace elements (Meier-Augenstein, Kemp and Midwood, 2013). Of the 11 oils three were genuine Styrian pumpkin seed oils supplied by the producers' association (SPSO-ggA#), one oil was milled from Styrian pumpkin seeds by an independent oil mill (small to medium enterprise) not located in Austria (PSO-SPSs), three oils originated from China (two were from the same supplier but branded differently) while for the other four oils no information on geographic origin was available (PSO-X1 and PSO-Y1). Two of the oils of unknown origin were from two different batches but

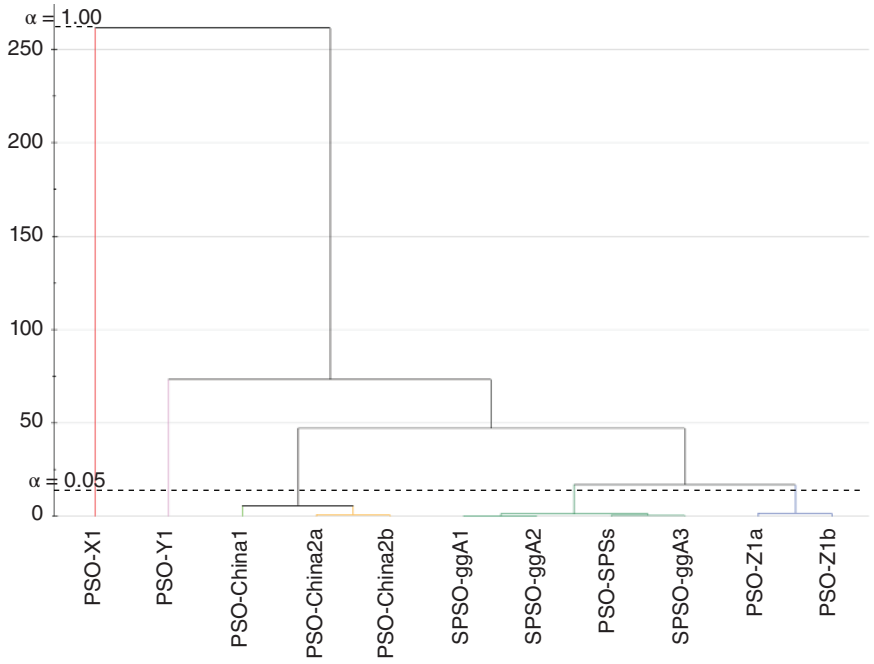

Figure I.16 Dendrogram of 11 pumpkin seed oils obtained from Hierarchical Cluster Analysis of a multivariate data set comprised of ^{2}H, ^{13}C and ^{18}O abundance values and concentration values of 17 trace elements.

the same supplier (PSO-Z1a and PSO-Z1b). At a cut-off level of 5 % similarity, hierarchical cluster analysis (HCA) grouped the genuine Styrian pumpkin seeds oils as well as the oil pressed from Styrian pumpkin seeds in one group (SPO-ggA# and PSO-SPSs), the three Chinese oils in a second group (PSO-China1, PSO-China2a and 2b) and the two oils from the same supplier were grouped together in a third group (cf. Figure I.16).

I.6.1.2 Authenticity and Provenance of Beverages

Another section of food products prone to misrepresentation, mislabelling and fraudulent use of adulterants are beverages. The seemingly nondescript term beverages is being used here deliberately since the spectrum covered by accidental or fraudulent misrepresentation up to the production and sale of counterfeit brand products ranges from sparkling water to fruit juices and from beer and wine to white spirits and whisky. Authentic sparkling beverages usually contain only the CO_2 generated by fermentation while authentic sparkling mineral waters only contain the CO_2 naturally present in the spring. A much cheaper and easier method for carbonation

is to saturate the beverage with industrial CO_2 from a gas tank or cylinder. Stable isotope analysis can detect the different modes of carbonation (Calderone *et al*., 2005, 2007) by comparing the carbon and oxygen isotopic composition of natural CO_2 from authentic sources against industrial CO_2, which typically exhibits $\delta^{13}C$ and $\delta^{18}O$ values of <-33 ‰ and <-36 ‰, respectively.

By equilibrating wine water from retail wines with CO_2 of known isotopic composition, $\delta^{18}O$ values of wine water were correlated with spatial climate and precipitation $\delta^{18}O$ patterns across the wine-grape growing regions of Washington, Oregon and California. A regression model was implemented spatially in a GIS with which it was possible to predict wine $\delta^{18}O$ values for all vintages and which generally reflected the consistent enrichment of wine from Napa relative to Livermore. GIS models of wine water $\delta^{18}O$ values could therefore become useful tools for independently verifying claims of regional origin and vintage (West, Ehleringer, and Cerling, 2007).

In fact, in 1997 the European Commission amended EC Regulation No. 2676/90 determining methods for the analysis of wines with EC Regulation No. 822/97[7] applying ^{18}O abundance analysis of wine water by IRMS to detect and quantify watering of wine in conjunction with comparison of such results with data obtained from genuine, that is, not manipulated, wines of the same geographic origin. The CO_2 equilibration method of the ^{18}O analysis of water as a way of determining water added to non-alcoholic beverages was adopted for the control of fruit juices to distinguish, for example, fresh juices from juices reconstituted from concentrate by the Comité Européen de Normalisation (CEN/TC174) as European pre-standard ENV 12141:1996[8] and by the then Association of Analytical Communities (AOAC Method 992.09). However, neither of these documents nor the underpinning study published in 1995 (Koziet *et al*., 1995) provides any information as to whether the process of equilibrating fruit juice (or wine) with CO_2 has been shown to proceed at the same rate as equilibrating pure water with CO_2. In other words, it would appear the assumption was made that the rate of the exchange process $CO_2 \leftrightarrow H_2O + CO_2 \leftrightarrow H_2CO_3$ and any associated mass discriminatory effects are the same in fruit juice (or wine) as in pure water so (i) measured $\delta^{18}O$ values could be scale normalized to VSMOW and (ii) this scale normalization could be quality controlled by equilibrating reference waters VSMOW, GISP and SLAP with CO_2 contemporaneously with fruit juice (or wine) samples on a like-for-like basis. Fruit juices such as apple or orange juice contain between 110 and 140 g of sugars per litre and approximately 11 g of organic acids, mainly malic acid and citric acid, per litre. Given the difference in pH between fruit juices and pure water alone, it is therefore not a foregone conclusion for the dissolution of CO_2 in fruit juices to take

[7] Commission Regulation (EC) No 822/97 of 6 May 1997 amending Regulation (EEC) No 2676/90 determining Community methods for the analysis of wines.

[8] European Prestandard ENV 12141, October 1996; Fruit and vegetable juices – Determination of the stable oxygen isotope ratio ($^{18}O/^{16}O$) of water from fruit juices – Method using isotope ratio mass spectrometry.

place at the same rate or the same extent as it would in pure water. We will return to this subject in Section I.6.1.3.

Stable isotope abundance analysis of a single element (e.g. oxygen) in a single beverage component (e.g. water) may not provide the discriminatory power required to come to an unambiguous conclusion about the quality and/or authenticity of a given product. Research carried out over the past 10 years has generated ample evidence for the need for a multi-isotope, even a multi-component, analytical approach when it comes to the food forensics analysis of beverages (Bat *et al*., 2012; Bontempo *et al*., 2014; Calderone and Guillou 2008; Camin *et al*., 2015; Ogrinc *et al*., 2001, 2009; Raco, *et al*., 2015). By analysing fruit juice water for both ^{2}H and ^{18}O abundance, fruit-specific $\delta^{18}O/\delta^{2}H$ correlation plots could be generated. Since meteoric water (as in precipitation water taken up by plants from the soil through their root systems) is the only water precursor pool available to plants, the ^{2}H and ^{18}O abundance values of fruit juice water should be strongly correlated. For example, work carried out in the author's laboratory determined the $\delta^{18}O/\delta^{2}H$ values of raspberry juice water could be plotted along a line given by $\delta^{2}H = (6.47 \times \delta^{18}O) - 41.92$ (cf. Figure I.5), while for apple juice water from apples grown in different regions of Slovenia a correlation equation of $\delta^{2}H = (6.73 \times \delta^{18}O) - 11.38$ has been reported (Bat *et al.*, 2012). Suspect fruit juice (or wine) samples could therefore be easily and conveniently assessed by checking how close their $\delta^{2}H$ and $\delta^{18}O$ data plot in relation to the fruit-specific correlation line and its particular uncertainty envelope.

It should also be noted that in instances where ^{2}H and ^{18}O analysis of water contained in beverages, including bottled water, is sufficient to determine the presence of added water, laser-based isotope analysers operating either in the near-IR or mid-IR may offer a more time- and cost-efficient alternative to IRMS (Chesson, Bowen and Ehleringer, 2010).

Detecting alien sugar, that is, natural sugar from a different plant source, in fruit juice and wine was a fairly simple task in the early days of food control using stable isotope analytical techniques since cheap corn syrup (*Zea mays* L.; a C_4 plant) was predominantly used to boost sugar and/or ethanol content (Brooks *et al*., 2002; Martinelli *et al*., 2003). Determining the $\delta^{13}C$ values of glucose or bulk carbon was sufficient to prove adulteration. However, addition of small amounts of C_4 plant sugars ($\leq$10 %) to C_3 plant products such as wine, fruit juice and honey, or the addition of sugars from other C_3 plants (sugar beet, concentrated and de-flavourized grape juice) could no longer be detected by these measurements and more sophisticated analytical techniques had to be developed based on high-precision compound-specific stable isotope analysis to detect the fraudulent addition of sugars (Dennis, Massey and Bigwood, 1994) and even vitamin C from alien sources (Schmidt *et al*., 1993). This second generation of "forensic" stable isotope analytical techniques exploited the fact that in authentic fruit juices $\delta^{13}C$ values of biogenetically related compounds such as L-ascorbic acid, L-malic acid and L-tartaric acid were strongly correlated with the $\delta^{13}C$ values of their parent sugars (Gensler, Rossmann and Schmidt, 1995; Gensler and Schmidt, 1994;

Jamin *et al*., 1997; Rossmann *et al.*, 1997; Weber *et al*., 1997; Weber, Gensler and Schmidt, 1997). For example, there is an isotopic fractionation of +4.8 ‰ between the $\delta^{13}C$ values of L-ascorbic acid and its precursor glucose. This enrichment is position specific, mainly located in the C-1 position of biogenetically authentic L-ascorbic acid, and would appear to be the result of plant-specific kinetic isotope effects during biosynthesis, whereas L-ascorbic acid of biotechnological origin preserves the ^{13}C signature and distribution of glucose.

The research group led by Schmidt discovered position-specific ^{13}C depletion at C-1 in glycerol originating from natural sources and they suggested this unique feature might be used as a means to test for illegal addition of synthetic glycerol to wines (Weber, Kexel and Schmidt, 1997). They also found a consistent $\Delta\delta^{13}C$ shift between ethanol and citric acid of +2.4 ‰ in addition to the known $\Delta\delta^{13}C$ shift between fermented sugar and ethanol of −1.7 ‰ (Weber *et al.*, 1997).

Using a single-isotope yet multi-component approach to compound-specific isotope analysis (CSIA) of a best-selling blended whisky, radar diagrams graphically representing $\delta^{13}C$ values of volatile whisky congeners acetaldehyde, ethyl acetate, *n*-propanol, isobutanol and amyl alcohol were constructed based on the analysis of eight product samples collected over two years of production to generate a profile and the variability range of the authentic product. On the basis of this multivariate approach it was possible to discriminate two other whisky samples from the authentic popular Scotch whisky brand (Parker *et al*., 1998).

The advent of on-line continuous-flow ^{2}H stable isotope analysis offered a new avenue for the control and authentication of food and food additives. In order to authenticate fruit juices the ^{2}H isotopic composition of citric acid was studied, comparing commercial citric acids with citric acid extracted from fruit juices. The developed method's ability to detect an addition of exogenous citric acid was successfully tested by identifying an orange juice sample that had been spiked with commercially produced citric acid (Jamin *et al*., 2005).

Analysing ethanol in wine for both ^{2}H and ^{13}C abundance can even detect the presence of alien grape sugar. A comparison of $\delta^{2}H$ and $\delta^{13}C$ values for ethanol in European wines from a particular year clearly showed a pronounced difference between a wine labelled as a vintage Austrian wine and wines from three other European countries. Given the similarities of the different regions with regard to northern latitude and climatic conditions, one would have expected $\delta^{2}H$ and $\delta^{13}C$ values for ethanol in a genuine Austrian wine to plot in close proximity to the values for the Hungarian wines. As it turned out, the wine of questionable provenance was the product of European, possibly Austrian, grape must to which deflavourized South African grape must had been added prior to fermentation.

The production of Scottish whiskies and in particular Scottish single malts is based on cereals grown in Scotland (barley for single malts) and Scottish water. While cereals (and barley) may be sourced from England in years of low harvest yields for Scottish-grown cereals, no such exception exists for the use of Scottish water. For this reason, all Scottish distilleries have their own local water supply

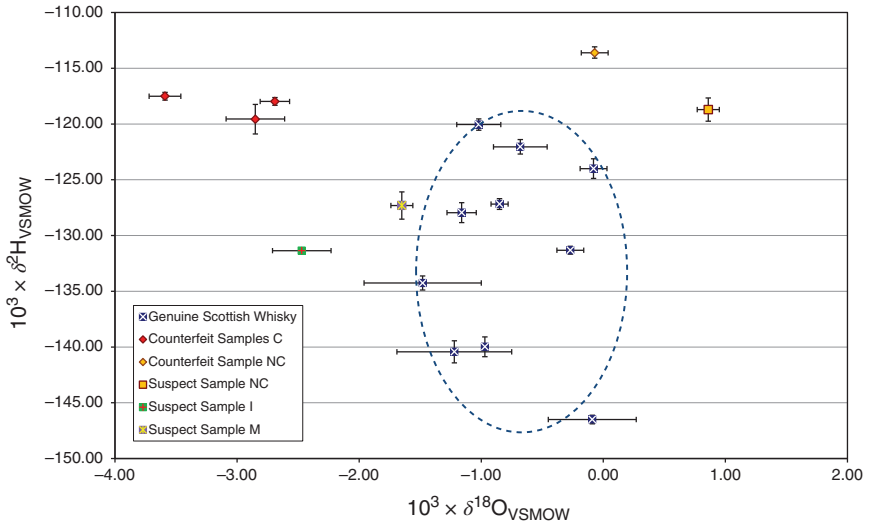

Figure I.17 Bivariate plot of δ^2H and δ^{18}O values of authentic Scottish whisky samples as well as whisky samples known or suspected to be counterfeit; *Source:* Based on data from Meier-Augenstein, Kemp and Hardie (2012).

which they use for mashing. A study published in 2012 sought to exploit this fact by analysing neat Scottish whiskies and the local source water used during whisky production for their ^{2}H and ^{18}O isotopic composition by high-temperature conversion-isotope ratio mass spectrometry (HTC/EA-IRMS). With coefficients of determination R^2 for δ^2H and δ^{18}O of 0.71 and 0.88, respectively, measured δ^2H and δ^{18}O values for the water used in the production of Scottish whisky were found to be well correlated with the δ^2H and δ^{18}O values observed for the corresponding whiskies (cf. Figure I.17). By plotting bulk ^{2}H against bulk ^{18}O isotope abundance data of authentic and counterfeit whisky samples it was possible to discriminate between samples of authentic whisky and samples of counterfeit whisky (Meier-Augenstein, Kemp and Hardie, 2012).

I.6.1.3 Caveats

As fascinating and promising as most, if not all, of the results are that have been described or referenced in the preceding three chapters, one must not lose sight of the fact most of the results reported provide information at the proof-of-concept level but would not meet the forensic requirements and standards of evidentiary reliability of data presented or given in evidence in court. In other words, while most of the stable isotope work published on food provenancing and food authentication shows great potential and great promise, most of the stable isotope data

presented fall short of forensic quality requirement because of a lack of validation and/or lack of traceability and international comparability. While pilot studies or proof-of-concept studies play an important role in the advancement of science and scientific methodology, data arising from such studies should neither be presented as or be confused with data of evidentiary reliability or data meeting international guidelines of traceability and comparability (Coleman and Meier-Augenstein, 2014; Coplen, 2011; Taylor, De Bievre and Valkiers, 2004). Sadly, examples of publications doing just that can be found even in high-impact scientific journals dedicated to food chemistry and food authenticity.

In the following we will look at an "official" method of detecting food adulteration, the results of which potentially may suffer from lack of proper method validation. This "official" method is concerned with the determination of added water to fruit juice labelled and sold as not from concentrate (NFC) using the CO_2 equilibration method to measure the ^{18}O composition of water as applied to discriminate between 100 % squeezed or pressed fruit juice and 100 % fruit juice albeit reconstituted from concentrate (Koziet *et al.*, 1995). While the CO_2 equilibration method has been adopted for the authentication of 100 % pressed fruit juice not from concentrate by organizations such as the EU Association of the Industry of Juices and Nectars (AIJN) there are no peer-reviewed publications validating the use of a method for ^{18}O abundance analysis of, for example, fruit juice that was originally conceived for ^{18}O abundance analysis of natural waters only. In adopting this method to ^{18}O analysis of fruit juice it is necessary to demonstrate that this method will yield true $\delta^{18}O$ values accurately reflecting the ^{18}O composition of fruit juice water, but will not yield artefact $\delta^{18}O$ values due to the pronounced differences in pH and osmolality between fruit juice and natural water. Fruit juices contain high levels of sugars and organic acids, such as malic acid, citric acid and oxalic acid, with levels of total dissolved sugar or total dissolved organic acid being as high as 180 or 49.2 g/l respectively (cf. Section I.6.1.2). Our initial CO_2 exchange experiments (as yet not submitted for publication) have shown differences of up to 0.5 ‰ in $\delta^{18}O$ values between pure waters and waters to which sugar and organic acids have been added when comparing deionized water as well as tap water with identical water samples to which sugar, malic acid and citric acid have been added at concentrations of 120, 5 and 6 g/l, respectively. This finding is in very good agreement with a study concerned with the effect of concentrating fruit juice by evaporation of water, which reported a difference in measured $\delta^{18}O$ values of 0.6 ‰ between pure water and water to which sucrose had been added at a concentration of 106.5 g/l (Yunianta *et al*., 1995). While the differences seen were small they were nonetheless statistically significant within the error of measurement and may well have a bearing as to whether individual cases should be declared a pass or a fail. Given the wide range of total sugar content (70–180 g/l) and total organic acid content (3.2–49.2 g/l) of juice depending on fruit, we will carry out further studies to test the hypothesis that the difference between observed and true $\delta^{18}O$ values for mock juice and pure water will depend on the actual amount of dissolved sugars and organic acids present in fruit juice.

The other potential problem with the standard operating procedure (SOP) for the CO_2 equilibration method to measure the ^{18}O composition of wine or fruit juice as given in regulation (EC) 822/97,[4] pre-standard method ENV 12141:1996[5] or method OIV-MA-AS2-12:R2009[9] relates to the equation stated explicitly in all of these method SOPs on the basis of which "the ^{18}O content of the sample with respect to the SMOW standard (on the SMOW/SLAP scale) is given as":

$$\delta^{18}O = \frac{(\delta'^{18}O - \delta'^{18}O_{SMOW})}{(\delta'^{18}O_{SMOW} - \delta'^{18}O_{SLAP})} \times 55.5 \quad \text{(I.16)}$$

In this equation, $\delta^{18}O$ refers to the corrected ^{18}O content of the sample while $\delta'^{18}O$ refers to the measured ^{18}O content of the sample and SMOW/SLAP standards.

However, Equation I.16 as stated in any of the aforementioned methods and thus forming the basis of these methods with regards to isotopic scale calibration is an abridged version of a proper scale normalization equation. Equation I.16 is valid if, and only if, SMOW (now VSMOW2) and SLAP (now SLAP2) have been analysed as standards (reference materials 1 and 2) alongside the samples under examination. The problem potentially arising from this instruction is the possibility of this particular equation as stated in the official methods being applied erroneously even when water standards other than SMOW and SLAP, but especially other than SMOW, are being used. A detailed discussion of the difference between proper scale normalization of measured $\delta^{18}O$ values to VSMOW (or SMOW as it then was) and this abridged normalization Equation I.16 can be found in Section II.3.3.3.

The exclusive applicability or dependency of Equation I.16 on the use of VSMOW and SLAP as standards or reference materials for sample analysis also creates a potential problem for employing official methods (EC) No. 822/97, ENV 12141:1996 or OIV-MA-AS2-12:R2009 in a forensic context if the method actually used to generate results would deviate significantly from any of the official methods stated in the report submitted to court as having been used. For example, in the UK expert evidence submitted to court either in response to a court order or as part of a witness statement must comply with rules and directions stated in part 19 of the UK's Criminal Procedure Rules 2015 (CPR 2015).[10] Part 3.5 of CPR 2015 states "if a party fails to comply with a rule or a direction then in some circumstances (a) the court may refuse to allow that party to introduce evidence; (b) evidence that that party want to introduce may not be admissible." In the USA, Federal Rule of Evidence 702 states "If scientific, technical, or other specialized knowledge will assist the trier of fact to understand the evidence or to determine a fact in issue, a witness qualified as an expert by knowledge, skill, experience, training, or education, may testify thereto in the form of an opinion

[9] Compendium of International Methods of Analysis – OIV, Isotope ratio of water; Method OIV-MA-AS2-12: Method for $^{18}O/^{16}O$ isotope ratio determination of water in wines and must (Resolution OIV-Oeno 353/2009).
[10] The Criminal Procedure Rules 2015, No. 1490 (L.18), Senior Courts of England and Wales. http://www.legislation.gov.uk/uksi/2015/1490/contents/made.

or otherwise, if (1) the testimony is based upon sufficient facts or data, (2) the testimony is the product of reliable principles and methods, and (3) the witness has applied the principles and methods reliably to the facts of the case." In short, for consideration of scientific evidence rule 702 puts emphasis on three criteria: relevancy, qualifications, and reliability. As a result, in a US court reliability is a, if not the, litmus test for admissibility. The potential problem with official methods (EC) No. 822/97, ENV 12141:1996 or OIV-MA-AS2-12:R2009 that may arise in a forensic context is reliability, namely the reliability of the principle and methods, and proper application of the principles and methods. An integral part of the proper application of the principles and methods is the quality of the standards controlling the technique's operation (or application) as well as standardization itself.

A deviation from any of the three aforementioned methods could already result from the need to use reference materials other than VSMOW and SLAP given the restriction on amounts available from suppliers such as the IAEA. Typically, only 20 ml of each can be ordered from the IAEA and repeat orders can only be placed every three years. However, the use of reference materials other than VSMOW and SLAP albeit out of necessity would not only result in a deviation from the reference materials stipulated in the aforementioned official methods but also, and more importantly, require the use of a correction equation that is significantly different to the correct equation as stated in all the official methods mentioned above (cf. Section II.3.3.3). A failure to recognize this fact and, hence, continuing to use Equation I.16 regardless would inevitably result in inaccurate $\delta^{18}O$ values being calculated (cf. Table I.6).

Related to this matter of Equation I.16 and normalizing measured stable isotope abundance values is the problem of many a publication found in the scientific literature presenting stable isotope abundance values that have not been scale normalized and thus do not meet fundamental scientific requirements regarding traceability, international comparability and thus inter-laboratory reproducibility (cf. Chapters II.3 and II.4). This means reported results cannot be reproduced independently by other laboratories and therefore cannot be confirmed. In a legal context, results that cannot be repeated or reproduced at the very least open the door to reasonable doubt or will be deemed inadmissible for lack of evidentiary quality. Regrettably, articles reporting such data slip through the peer-review process time and time again. Even more worryingly, it would seem indefensible practices such as single-point "calibration" are being used even by government laboratories whose very remits are food safety and consumer protection (cf. Chapters II.3 and II.6). Unfortunately, unwarranted and unsupported assertions, even to the point of incorrect or indeed false statements repeatedly being made and repeatedly succeeding in getting published unchallenged, eventually tend to assume a mantle of authority, albeit a false one. I can only urge readers to read reports on food forensics based on stable isotope analysis very carefully and to remember a fundamental principle of mathematics: ten times zero is still zero.

Table I.6 Impact of using reference materials (RM) other than VSMOW (RM1) and SLAP (RM2) on $\delta^{18}O$ values calculated using equation I.16.

Sample ID	$\delta^{18}O_{VSMOW}$ scale normalized using RM1 and RM2[a]	$\delta^{18}O$ calculated using Equation I.16[b]	$\delta^{18}O$ calculated using Equation I.16[c]
RM2	−51.24	−63.64	−64.42
RM1	4.96	0.01	0.01
Glacier water	−50.66	−62.98	−63.75
DI water	−7.34	−13.92	−14.09
Glacier water	−50.74	−63.07	−63.84
DI water	−7.32	−13.90	−14.07
Glacier water	−50.72	−63.05	−63.82
DI water	−7.33	−13.91	−14.08
RM2	−51.22	−0.01	−0.01
RM1	4.94	−63.61	−64.39

[a]Accepted $\delta^{18}O$ values for RM1 and RM2 are +4.95 and −51.23 ‰, respectively.
[b]Applying Equation I.16 with $\delta'^{18}O_{RM1}$ and $\delta'^{18}O_{RM2}$ replacing $\delta'^{18}O_{SMOW}$ and $\delta'^{18}O_{SLAP}$, respectively.
[c]Applying Equation I.16 as under (b) above but in addition with 55.5 ($\Delta\delta^{18}O_{VSMOW/SLAP}$) having been adjusted to the actual difference of 56.18 for $\Delta\delta^{18}O_{RM1/RM2}$ instead.

I.6.2 Authenticity and Provenance of other Premium Products

An area where compound-specific isotope profiling has become the method of choice is the authenticity control of flavours, fragrances and essential oils. Substituting synthetic or "nature identical" for natural flavours or fragrances is an all too easy way to defraud consumers by, for example, selling a product containing synthetic flavour or fragrance as the pure natural product or a product based on highly prized natural ingredients only. Natural lavender oil, for example, contains between 30 % and 60 % linalyl acetate and retails at about £450/l. Synthetic linalyl acetate (>97 %) costs £52.30/kg while natural linalyl acetate (∼80 %) costs £208/kg. Similarly an extract of natural γ-decalactone, a flavour compound contained in peach and apricots, costs approximately £500/kg while the synthetic compound only costs £70/kg. By combining CSIA via GC-IRMS with hyphenated techniques such as enantioselective capillary GC and multi-dimensional capillary GC it is possible to use stable isotope data for authenticity control of flavours, fragrances and essential oils (Juchelka *et al*., 1998; Mosandl, 1995, 1997; Mosandl *et al*., 1995; Nitz *et al*., 1991; Nitz, Weinreich, and Drawert, 1992). The analytical power of isotope analysis for discriminating between natural and nature-identical hemisynthetic or fully synthetic flavours and fragrance has resulted in their inclusion as part of the authentication process for a product to gain EU and/or

US Natural Certification. To maintain EU Natural Certified status as defined in European Communities (88/388/EEC), Article 9 (2), each new batch of a natural product has to undergo this analysis. The corresponding US Natural Certificate status is defined in the US Federal Food, Drug, and Cosmetic Act, and the US Code of Federal Regulations, Title 21, Sections 101.22(a) (3) and 101.22(i) (4).

Other fascinating examples of the power of multivariate analysis, including bulk and CSIA, are authenticity control and determining the origin of dietary supplements such as creatine-monohydrate (Huelsemann *et al*., 2011), provenancing the geographic origin of premium long-grain rice (Kelly *et al*., 2002), analysing vegetables and grains with the aim of distinguishing between conventional and organic methods of production (Schmidt *et al*., 2005), determining the geographic authenticity of Emmental cheese (Pillonel *et al*., 2003, 2005), revealing environmental and geographic history of fish based on the ^{2}H isotopic signature of fish muscle and otolith microchemistry (Whitledge, Johnson and Martinez, 2006), and determining the difference between farmed and wild caught fish such as salmon (Aursand, Mabon and Martin, 2000), sea bass (Bell *et al*., 2007) and sea bream (Morrison *et al*., 2007). Further information on the increasing use of natural abundance isotope variation and elemental concentrations as geographic "tracers" to determine the provenance of food, which reflects consumers' increasing concern about the origin of the foods they eat, can be found in a review article published in 2005 (Kelly, Heaton and Hoogewerff, 2005).

While also associated with a perception of particularly high quality (e.g. Egyptian cotton), cotton is a product that is often subject to incorrectly declared geographic provenance not just for reasons of perceived quality. Misrepresentation of the geographic origin of raw cotton or 100 % cotton fabrics happens chiefly for three reasons: (i) customers' perception of quality, (ii) country-of-origin-dependent import duty and (iii) farming practices or conditions of labour associated with certain countries. A study published in 2014 (Meier-Augenstein *et al*., 2014) showed that, on the basis of trivariate stable isotope signatures, US grown cotton could be distinguished from non-US cotton with a sensitivity of 93.75 % (cf. also Figure I.6).

I.6.3 Counterfeit Pharmaceuticals

The circulation of counterfeit pharmaceuticals is a problem affecting consumers as well as pharmaceutical companies world-wide. In 2005, after having discovered a counterfeit version of the drug, Pfizer UK issued a recall of 120,000 packs of its cholesterol-lowering drug Lipitor®.[11] They found approximately 60 % of all the returned packs were counterfeit. There is a general consensus this problem and associated risks for public health are escalating. Counterfeit drugs range from products with the correct ingredients but not manufactured to the standards of the

[11] http://www.in-pharmatechnologist.com/Drug-Delivery/Fake-Lipitor-lingers-in-supply-chain.

pharmaceutical industry such as Good Manufacturing Practice (GMP) to products with the wrong ingredients or not declared ingredients, products containing a lower dosage of and thus therapeutically insufficient amounts of the active pharmaceutical ingredient (API), products containing no API whatsoever or, worst of all, products containing toxic compounds. In 2010, the World Health Organization (WHO) estimated world-wide sales of counterfeit medicines would top US$75 billion, representing a rise of 90 % since 2005.[12]

The cost to patients' health resulting from receiving counterfeit pharmaceuticals instead of the genuine product is difficult to quantify but could be devastating. For example, in late 2008 counterfeit acetaminophen syrup containing diethylene glycol cost the lives of 109 children in Nigeria,[13] while in July 2009 counterfeit acetaminophen syrup was responsible for the death of 24 children in Bangladesh.[14] Even though not a case of counterfeit medicine, the case of 1235 Chinese babies suffering ill-health and eight babies having died from consuming milk powder feed to which melamine had been fraudulently added to increase nitrogen content, thus making the product appear to be rich in protein, is another stark example of the human cost counterfeit products extract from the public.

In their 2007 report the OECD had the following to say on the subject of counterfeit pharmaceuticals:[4]

> *In the case of* pharmaceuticals*, trademark-infringing products may include correct ingredients in incorrect quantities or may be composed according to a wrong formula. Products can furthermore contain non-active or even toxic ingredients. Ailments which could be remedied by genuine products may go untreated or worsen; in some cases this may lead to death. Most purchasers of counterfeit pharmaceuticals are likely to be completely unaware that they have been victimized.*

In other words, counterfeit pharmaceuticals can not only be substandard, they can even be dangerous, posing health and safety risks to consumers that range from mild to life-threatening.

In the light of the potential, yet unquantifiable, human cost and the quantifiable economical cost to the pharmaceutical industry it is a rather surprising finding that published articles on the subject of detecting and combating counterfeit pharmaceuticals are few and far between. However, the majority of publications on this subject agree on multivariate analysis, including stable isotope analysis, be it by IRMS or NMR, of one or more isotopes as the analysis method of choice (Brooker *et al*., 2014; Bussy *et al*., 2011; Deconinck *et al*., 2008; Gilevska, Gehre and Richnow, 2015; Jasper *et al*., 2004; Jasper, Weaner and Duffy, 2005; Remaud *et al*., 2013; Santamaria-Fernandez, Hearn and Wolff, 2008; Silvestre *et al*., 2009; Wokovich

[12] http://www.who.int/bulletin/volumes/88/4/10-020410/en/.

[13] http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5848a2.htm.

[14] https://www.gphf.org/images/downloads/brochure_counterfeit.pdf - last accessed 14 November 2016.

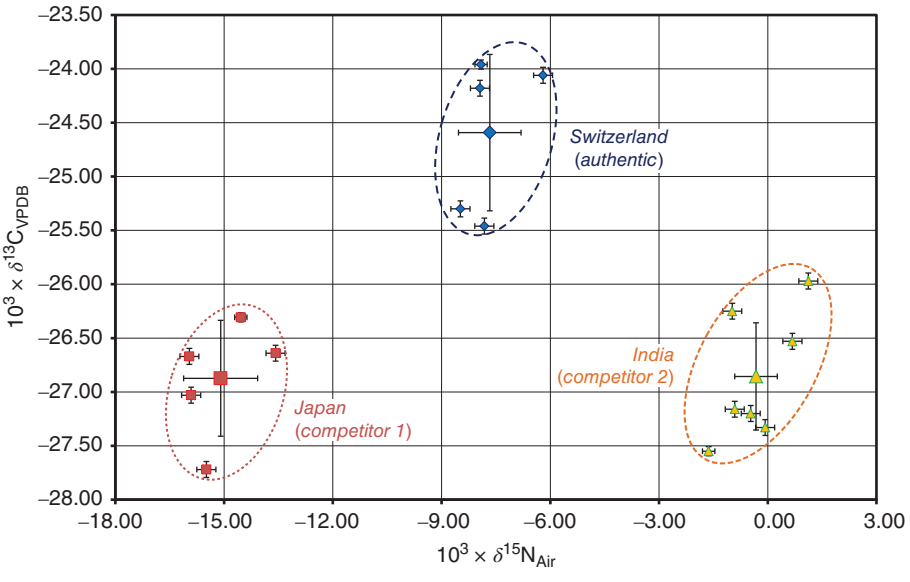

Figure I.18 An isotopic bivariate plot of $\delta^{13}C$ and $\delta^{15}N$-values of the API Folic Acid from three different manufacturers at three different locations; mean $\pm 1\,\sigma$ of each cluster is shown in the middle of the cluster. *Source:* Courtesy of J.P. Jasper, Nature's Fingerprint/MIT LLC, 2004.

et al., 2005). Using folic acid and the non-steroidal anti-inflammatory drug naproxen as examples, Figures I.18 and I.19 show the differences in two-dimensional stable isotope abundance plots between authentic and generic products.

Another example of a commercially successful drug targeted by counterfeiters is Viagra®. Since its first production in 1998, Viagra® has generated well in excess of $10 billion US dollars in sales. Until 11 December 2017, Pfizers' product will be the only FDA-approved sildenafil-based treatment option for sufferers of erectile dysfunction in the USA.[15] Until that date there will be no legally approved or officially licensed "generic Viagra" formulations available in the USA other than Revatio®, which is also made by Pfizer. As of 11 December 2017, the US pharmaceutical company Tera will be able to sell sildenafil citrate with FDA approval as a generic version of Viagra.[15] However, other formulations containing sildenafil citrate as the API are widely available over the Internet and often sold as "generic Viagra". However, the manufacturers' addresses printed on the back of blister packs, for example "S*** Industrial Estate B/H G*** Water Tank", strongly suggest that these cheaper alternatives are often manufactured under less than ideal conditions with little or no quality assurance or quality control procedures in place. Furthermore, a study published in 2012 showed 17 samples out of a total of 22 tested to be

[15] http://www.cbsnews.com/news/first-generic-drug-for-viagra-called-sildenafil-citrate-approved-by-fda/.

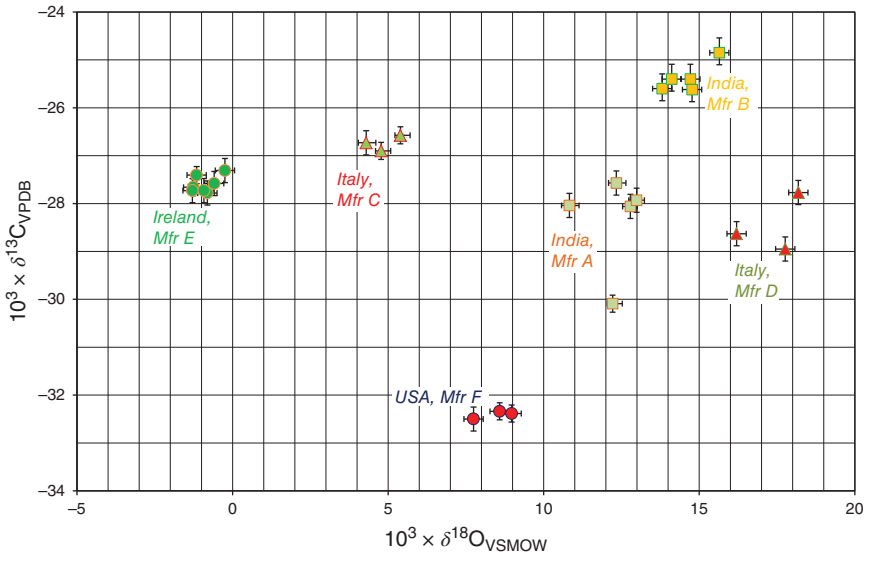

Figure I.19 An isotopic bivariate plot of $\delta^{13}C$ and $\delta^{18}O$-values of the API Naproxen from six different manufacturers (Mfr A to F) in four different countries. *Source:* Courtesy of J.P. Jasper, Nature's Fingerprint/MIT LLC, 2004.

counterfeit and to contain only between 30 % and 50 % of the API amount stated on the box or the label (Campbell *et al*., 2012). Conversely, as a study carried out in the author's laboratory has discovered, LC/MS analysis revealed some of the so-called generic products contained up to three times the stated amount of sildenafil citrate, probably in an attempt to create the impression of a superior product. However, in men suffering from heart conditions where use of sildenafil citrate would necessitate strict dosage control, ingesting a cheaper generic product containing three times the amount stated on the package might have severe consequences.

When we compared 23 samples of Viagra® and generic Viagra sourced via the Internet, based on the results of ^{2}H, ^{13}C, ^{15}N and ^{18}O analysis we found only five (samples V1 to V5 in Figure I.20) of the 22 samples to be the authentic product made by Pfizer (Kemp and Meier-Augenstein, 2013). In other words, 78.3 % of the samples analysed were either counterfeit or generic, a finding that is in good agreement with the figures reported in the 2012 study (Campbell *et al.*, 2012). From all appearances sample V6 looked like the genuine product too but its multivariate stable isotope signature and the outcome of a principal component analysis of all results prompted us to re-examine the box, information leaflet and blister pack more closely. Comparison with the genuine Pfizer product eventually revealed differences in how and where information was printed on the back of the blister packs, thus substantiating the isotope signature-based conclusion that sample V6 was a counterfeit (cf. Figures I.20 and I.21).

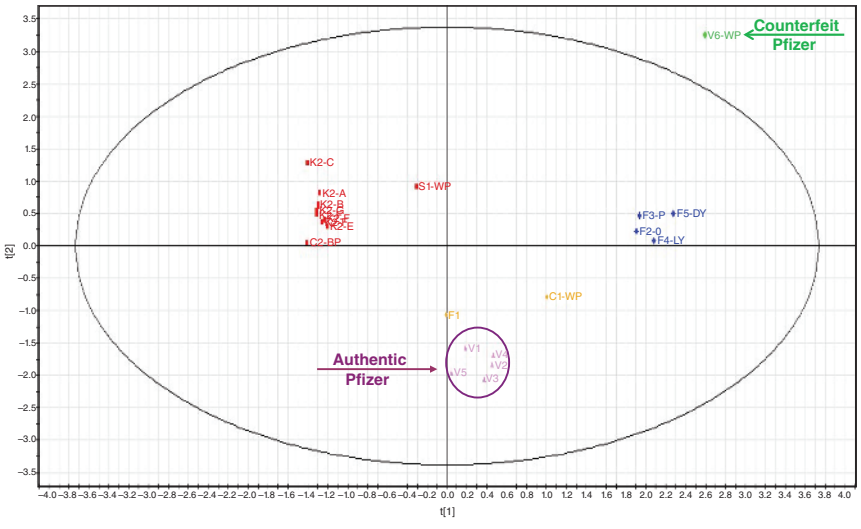

Figure I.20 Principal Component Plot from a principal component analysis of ^{2}H, ^{13}C, ^{15}N and ^{18}O abundance data of sildenafil citrate tablets from different manufacturers with first and second component explaining 48 % and 39 % respectively of all variability in the data. The large ellipse represents the 95 % confidence interval based on Hotelling's T^2.

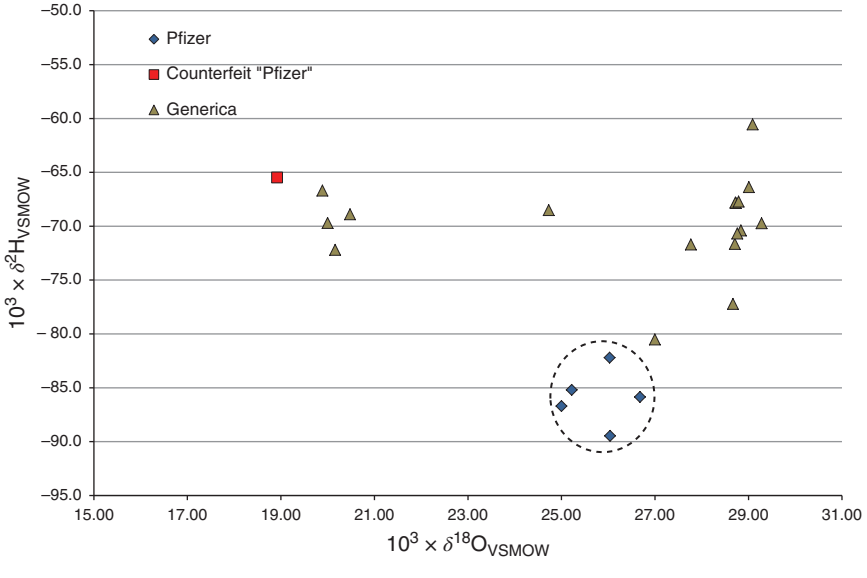

Figure I.21 ^{2}H and ^{18}O abundance data of sildenafil citrate tablets from different manufacturers including genuine Pfizer Viagra® (dashed circle).

Detection of counterfeit pharmaceuticals and authentication of genuine products are two sides of the same coin. While conventional analysis by GC/MS or LC/MS to check on purity and API content does have a role to play in the detection of counterfeit pharmaceuticals, as the above discussed example of Viagra® has shown, stable isotope abundance analysis does offer a time- and cost-effective way to screen suspect products. Product authentication seems a logical and integral part of combating fraud and counterfeit goods. It would also seem the logical thing to do for manufacturers of genuine pharmaceuticals to protect themselves from unjustified claims for damages caused by taking a counterfeit medicine that may match the genuine medicine in terms of ingredients, including the API, but may have been manufactured to lesser standards and hence may be contaminated. Given that both the EU and the USA have already introduced Natural Certification based on isotope analysis to guarantee the authenticity of natural food and cosmetics ingredients, it is likely that the pharmaceutical industry will eventually adopt a similar approach or scheme.

I.6.4 Environmental Forensics

Environmental forensics is another example of stable isotope forensics having a tangible impact on everyday life and public health by helping to enforce legislation such as the European Environmental Liability Directive 2004 (2004/35/EC), the first EC legislation to include the "polluter pays" principle. Of similar importance in this respect is its forerunner, the European Water Framework Directive (2000/60/EC), which came into force in December 2000 and became part of UK law in December 2003.[16]

Many of the methods employed today towards elucidating the chronology of an environmental pollution event, its point of origin and ultimately the polluter owe their origin to the desire of many a geochemist (and archaeologist) to extract as much information as possible from fossil biomarkers such as sedimentary long-chain fatty alcohols and sterols (Jones *et al*., 1991; Mudge, Belanger and Nielsen, 2008), triterpene-derived hydrocarbons (Hauke *et al*., 1992), neutral monosaccharides (Moers *et al*., 1993), long-chain alkanes (Bakel, Ostrom and Ostrom, 1994; Bjoroy, Hall and Moe, 1994; Ficken, Barber and Eglinton, 1998; Huang *et al*., 1996; Ishiwatari, Uzaki and Yamada, 1994), alkanes, polycyclic aromatic hydrocarbons (PAHs) and isoprenoids (Lichtfouse *et al*., 1997; Lichtfouse, 2000; Wilhelms, Larter and Hall, 1994). The common denominator for this explosive advancement in analytical method development to answer geochemical and environmental forensic questions was the commercial availability of continuous-flow IRMS systems coupled to a gas chromatograph via an on-line sample conversion interface (GC-IRMS), thus facilitating ^{2}H and/or ^{13}C CSIA of environmental protection relevant organic compounds present either in air or water (Herrero-Martin *et al*.,

[16] http://www.defra.gov.uk/ENVIRONMENT/water/wfd/.

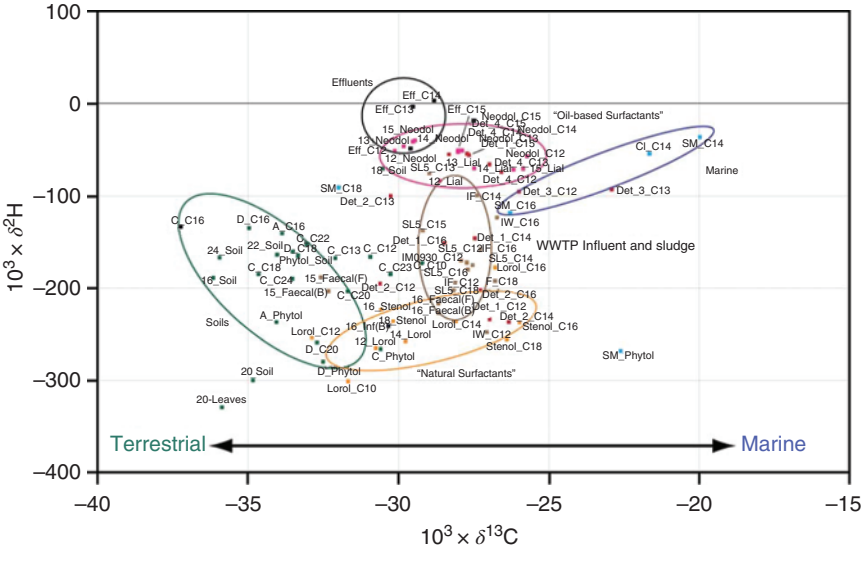

Figure I.22 Bivariate plot of corresponding δ^2H and δ^{13}C values of fatty alcohols showing differentiation according to source. *Source:* Mudge *et al*. (2010). Reproduced with permission of Royal Society of Chemistry.

2015; Shouakar-Stash and Drimmie, 2013), or as part of a complex sample matrix such petrol or diesel fuel (Muhammad *et al*., 2015). Compound-specific ^{2}H and ^{13}C analysis has also been used to determine the potential sources of long-chain fatty alcohols found in terrestrial soil and marine sediment as well as the water entering a waste-water treatment plant near the Menai Bridge in Wales (cf. Figure I.22). With the help of the resulting two-dimensional stable isotope signatures not only was it possible to distinguish between long-chain fatty alcohols from terrestrial and marine environments but long-chain fatty alcohols derived from natural surfactants or from petro-chemical surfactants could be distinguished too (Mudge *et al*., 2010).

Monitoring atmospheric gases such as methane, carbon monoxide and carbon dioxide linked to environmental and climate changes, and differentiating between natural (e.g. bacterial) and anthropogenic sources by measuring the δ^{13}C values of these gases has traditionally been carried out using dual-inlet IRMS systems. These measurements required time-consuming sample preparation steps involving large gas sample volumes. The higher abundance sensitivity of GC-IRMS compared to EA-IRMS in conjunction with low volumetric gas flow rates of 1–2 ml/min opened up the use of PLOT fused-silica capillary columns for routine GC analysis of highly volatile organic compounds (HVOCs) and permanent gases. Compound specific ^{13}C isotope analysis of HVOCs and permanent gases has now become the method of choice for scientists as air samples between 50 µl and 5 ml can be analysed on-line without any prior sample preparation (Archbold *et al*., 2005; Baylis, Hall

and Jumeau, 1994; Clayton *et al*., 1997; Davis *et al*., 2005; Harper *et al*., 2001, 2003; Kalin *et al*., 2001; Merritt, Hayes and Marias, 1995; Waldron *et al*., 1998; Zeng *et al*., 1994).

High-precision CSIA by GC-IRMS is now a standard analytical tool used in conjunction with traditional techniques such as GC-flame ionization detection and GC/MS to determine point of origin and identify sources of oil spills and oil pollution, including the accidental or otherwise release of petrol and diesel fuels (Boyd *et al*., 2006; Davis *et al.*, 2005; Hough *et al*., 2006; Mansuy, Philp and Allen, 1997; Oudijk, 2005; Philp, 2007; Philp, Allen and Kuder, 2002; Uzaki, Yamada and Ishiwatari, 1993), ocean-transported bitumen (Dowling *et al*., 1995), the characterization of refractory wastes at heavy-oil contaminated sites (Whittaker *et al*., 1996; Whittaker, Pollard and Fallick, 1995), and tracing the sources of PAHs in the environment (Mcrae *et al*., 1996; Murphy and Brown, 2005; Pies, Ternes and Hofmann, 2008; Saber, Mauro and Sirivedhin, 2005, 2006; Sun *et al*., 2003).

There is growing evidence that a chemical hailed as the saviour of the environment from lead pollution through car exhaust emissions of anti-knocking fuel additives will only create a new environmental problem instead. This chemical is methyl-*tert*-butyl ether (MTBE). Similar to benzene it is quite resilient to weathering and biotic breakdown, be it in soil or in ground water. The only remediation treatment that seems to work relatively efficiently is abiotic breakdown by a strong oxidizing agent such as potassium permanganate ($KMnO_4$). Not surprisingly therefore, MTBE has become the focus of environmental forensic research and environmental forensics application exploiting its ^{13}C signature and ^{13}C isotopic fractionation associated with its degradation to determine the Earliest Demonstrable Inception Date (EDID) and the Latest Possible Initiation Date (LPID) when investigating ground water plumes and environmental contamination resulting from accidental or deliberate fuel spills (Elsner *et al*., 2007; Gauchotte, C. *et al*., 2009; Oudijk, 2005, 2008; Schmidt, 2003; Schmidt *et al*., 2004; Smallwood *et al*., 2001; Smallwood, Philp, and Allen, 2002; Zwank *et al*., 2005).

Applying stable isotope techniques to a field of analytical science that deals with detection, identification, spatial distribution, ageing and natural attenuation as well as degradation of pollutants or xenobiotics in a complex setting, namely the natural environment, is of course not always straightforward and can be fraught with difficulties (Gauchotte-Lindsay *et al*., 2014). As mentioned in previous chapters, considering potential pitfalls and contextual interpretation of data are two of the most essential guiding principles when undertaking such work (Blessing, Jochmann and Schmidt, 2008; Ehleringer *et al*., 2008b; Schmidt *et al.*, 2004).

I.6.5 Wildlife Forensics

In a way stable isotope wildlife forensics, that is, tracing the origin of animals, is the flip-side of the stable isotope ecology coin whose aim is, amongst others, to

elucidate the food webs and migration patterns of wild animals (Fry, 2006; Hobson and Wassenaar, 1999; Thompson *et al*., 2005; West *et al*., 2006). Biological sciences have benefitted greatly from applied stable isotope techniques, and the study of the dietary and migration patterns of elephants by Cerling and co-workers is a good example (Cerling *et al*., 2006). This project used sequential, that is, 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 the isotopic make-up of an individual elephant's hair could be significantly different from the control group if the elephant in question was involved in night-time crop raiding. Obviously determining such differences is only possible if the crop's isotopic composition is significantly different from the control group's staple diet.

Staying with the example of elephants, using a multivariate isotope analytical approach the 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 (Koch *et al*., 1995). 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 (Coutu *et al*., 2016). This study clearly demonstrated the need for a multi-isotope approach, that is, multivariate data sets (i) to characterize different habitats and (ii) to explain the variation in the data set. Obviously, a tool enabling scientists to determine changes in diet and feeding locations based on measurable and quantifiable parameters rather than based on observational and anecdotal evidence alone can also be used be to determine if ivory comes from controlled and regulated sources or from poached animals. Similarly, stable isotope techniques applied to unravel the diet change and migration patterns of wild birds (Hobson *et al*., 2012; Hobson and Kardynal, 2016; Kelly, 2006; Kelly, Ruegg, and Smith, 2005; Studds *et al*., 2012) can equally be employed to determine if a bird sold as bred in captivity came from a controlled and licensed breeder or if it was illegally caught in the wild and illegally imported (Bearhop *et al*., 2003; Bowen, Wassenaar, and Hobson, 2005; Braune, Hobson and Malone, 2005; Fox and Bearhop, 2008; Hobson *et al.*, 2004; Hobson, 2005; Kelly, Thompson and Newton, 2009; Wassenaar and Hobson, 2000, 2006).

I.6.6 Anti-Doping Control

In most countries this area of applied stable isotope techniques does not really come under the forensic science umbrella since in most countries taking performance enhancing drugs is not a criminal offence. Furthermore, in challenges of failed doping tests the burden of proof rests with the athlete. In a reversal of the normal process of law, the World Anti-Doping Agency (WADA) presumes an athlete who has failed

a drug test to be guilty until proven innocent. Another problem arises from the fact that not all WADA accredited laboratories engaged in traditional and stable isotope analytical techniques to detect and provide evidence of a doping offence follow the same Standard Operating Procedures (SOPs) of analysis or indeed apply the same decision making factors on which basis to declare a failed test result. Furthermore, the data given in evidence by some of these laboratories does not conform to the level of standard of evidentiary reliability that forensic science service providers and their work have to comply with when presenting forensic evidence in a court of law. The "identification" of a compound derived peak in a GC/C-IRMS chromatogram by a mere comparison of peak retention time with a GC/MS chromatogram generated on a different instrument, even on a GC column of different polarity (Saudan *et al*., 2006) is just one example of how current practice in some WADA laboratories does not meet the forensic standard for unambiguous compound identification.

As of 2016, the situation described above will change in one country at least. On 10 December 2015 the German parliament (Bundestag) passed a law to combat doping offences in sport (Anti-Doping-Gesetz; AntiDopG). Prosecution of suspected doping offences will be carried out within the existing legal framework of the German Criminal Procedure Rules (Strafprozessordnung; StPO) which for the defendants means the presumption of innocence until proven guilty. Similarly, all evidence presented in court has to meet with the statutory requirements for evidentiary reliability. In the best interest of both sport and athletes, it can only be hoped other countries will follow the example set by Germany.

However much justifiable criticism one could voice concerning the methods employed by or practices in use in some anti-doping laboratories, one has to face the undeniable fact that use of performance enhancing drugs in most sports has been a problem for decades and continues to be so. As methods of cheating in competition and gaining an unfair advantage over honest, hard-working athletes who devote their lives to their chosen career and to representing their country in sporting events have become increasingly sophisticated, analytical techniques employed to detect doping offences must similarly develop. Since use of synthetic steroids became easier to detect due to increasing sensitivity of analytical instruments such as desk-top quadrupole and time-of-flight mass spectrometers, use of performance increasing substances moved on to natural or nature-identical steroids and hormones. Given what we have learned thus far from preceding chapters it will come as no surprise that compound specific ^{13}C isotope analysis was seen as the solution to the task of detecting natural or nature-identical steroid or hormone abuse (Abramson, Osborn and Teffera, 1996; Ueki, 1998, 2001; Ueki and Okano, 1999).

It was not until the 1998 Winter Olympic Games in Nagano and the 2000 Olympic Games in Sydney that stable isotope analysis as a method to establish the presence of exogenous testosterone was mentioned in anti-doping rules of sport and in fact applied. However, the suggestion that '^{13}C-CSIA using GC/C-IRMS could be used to detect doping with steroids goes back to 1994 (Becchi *et al*., 1994) and names

such as Aguilera, Becchi, Catlin, Flenker, Shackleton and Schänzer are firmly linked with research and development into doping control by means of ^{13}C CSIA and, equally as important, with population studies to determine confidence limits for $\delta^{13}C$ values of steroids and steroid metabolites reflecting intra- and inter-individual natural variability (Aguilera *et al*., 1996a,b, 1997, 1999, 2001; Aguilera, Chapman and Catlin, 2000; Bourgogne *et al*., 2000; Buisson *et al*., 2005; Flenker *et al*., 2007; Hebestreit *et al*., 2006). In the meantime advances in CSIA analytical techniques enabled Schänzer's group in Cologne to investigate the potential of improving on anti-doping control through ^{13}C-CSIA of testosterone metabolites by measuring differences in ^{2}H isotopic composition of testosterone metabolites caused by pathway specific isotope fractionation, particularly during the hydrogenation of testosterone to 5β-androstanediol and 5α-androstanediol. First reported differences between $\delta^{2}H$ values for testosterone metabolites (5β-androstanediol–5α-androstane-diol) and the subsequent metabolite pair (androsterone–etiocholanolone) were 12 and 21 ‰, respectively (Piper *et al*., 2009).

Chapter I.7

Summary of Part I

The sheer number of applications of stable isotope analytical techniques presented in the preceding chapters as well as their wide spectrum clearly demonstrates that this technique is a very powerful tool providing quantitative and qualitative information that cannot be obtained by any other means. Government agencies such as the UK Food Standards Agency (FSA), whose remit is the authenticity of food and food ingredients, the European Office for Wine, Alcohol and Spirit Drinks (BEVABS) at the IHCP of the European Commission JRC, which aims to ensure correct implementation of EU wine quality legislation and was set up to combat major frauds in this area, or sports bodies such as WADA have already declared stable isotope analysis a method of choice and an indispensible tool in combating counterfeit or mislabelled foodstuffs or combating doping in sports.

Since the first edition of this book, the need to identify counterfeit versions of agrochemicals such as the insecticide Fipronil in order to be able to prove violation of owned patent rights has prompted a major player in the field of industrial chemicals and agrochemicals (BASF, Ludwigshafen, Germany) to establish their own in-house stable isotope laboratory. Analysing more than 120 samples of authentic fluocyanobenpyrazole (aka Fipronil) for its ^{13}C, ^{15}N and ^{34}S composition, and comparing the data to results of stable isotope analysis of Fipronil samples from other sources by multivariate data analysis, scientists at BASF were able to differentiate between authentic (i.e. BASF made) Fipronil and generic products (Weller *et al*., 2011, 2012). In their report Weller *at al.* (2011) concluded that stable isotope signatures are a suitable method for authenticity control of active components in crop protection products. They anticipate that stable isotope analytical techniques will become a great asset in the fight against counterfeit and illegal parallel imports.

No matter what the application, however, one underlying principle of stable isotope forensics is already emerging. Similar to the tenet that meaningful forensic interpretation of evidence and analytical data has to be contextual and should be supported by corroborating information obtained from independent techniques, stable isotope forensic examination of evidence should also be based

Stable Isotope Forensics: Methods and Forensic Applications of Stable Isotope Analysis, Second Edition.
Wolfram Meier-Augenstein.

Companion website: www.wiley.com/go/meier_augenstein/sif

on a multivariate approach examining information based on several independent variables. This may already be achieved through multi-isotope profiling where single isotopic signatures are independent variables, which when combined will work like a multi-tumbler combination lock with only one solution in X^Y possible combinations, with X being the number of positions per tumbler and Y being the number of tumblers. However, given what we have learned about the drivers that account for the differences in isotopic composition of two otherwise chemically indistinguishable compounds it is clear that even multivariate stable isotope profiles should be combined with analytical data from independent analytical techniques to yield the maximum possible level of discrimination. That being said, based on what we have learned thus far it seems clear that multivariate stable isotope signatures of both natural compounds and synthetic materials hold the potential to serve as a powerful screening tool and to provide real-time forensic information to help direct resources in criminal investigations.

References Part I

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