

**Introduction**

Isotope ratio mass spectrometry (IRMS) is recognized as a sensitive and precise technique for determining isotopic contents at natural abundance. Since the fifties many raw materials, such as wood, or isolated molecular species, such as cellulose, have been investigated [1-7]. Thus the isotopic parameters of carbon, hydrogen, oxygen and nitrogen have become rich sources of information in domains as various as paleoclimatology, environment, plant metabolism, agronomy, nutrition, medicine and so on.

In IRMS the product is first burnt to give principally carbon dioxide whereas that of hydrogen is determined on the hydrogen gas obtained by reduction of water. Consequently the method gives access only to an overall isotope content averaged over the different molecular positions. The isotopic ratio corresponding to a given site, for instance the carbonyl or the methyl site of acetic acid, can be determined by IRMS [8] but at the price of lengthy degradations of the compound to isolate the atom under consideration. Obtaining the whole set of specific isotope contents of a given molecule by IRMS therefore requires appropriate chemical transformations in which possible fractionation effects are avoided or rigorously estimated.

In 1981 we showed that atomic discrimination due to chemical shift makes NMR a powerful instrument for investigating Site-specific Natural Isotope Fractionation (SNIF-NMR), specially in the case of hydrogen [9]. This approach has now been widely exploited either alone or combined with IRMS determinations.

**The different types of basic information provided by site-specific isotope ratios**

Initially proposed for solving an important problem both in France and in Europe, the detection of illegal enrichment of wines (chaptalization) [10], the SNIF-NMR method now provides new or improved information in a wide range of fundamental or applied areas:

- A simultaneous determination of primary and secondary kinetic isotope effects involving the whole set of observable isotopomers of a given molecular species is possible, without the need for selective enrichment [11-16]. The evolution of the isotope ratio at a given position $i$ of reactant $A$, $(D / H)_i$, as a function of the fraction of reaction, $\rho$, and of the primary, $k_{\rho}/k_D$, or secondary, $k_{\rho}/k_H^0$, kinetic isotope effects, is illustrated in figure 1 for a non-branched reaction. The fractionation factor can be obtained from the ratio of the values of $(D / H)_i^0$ at yield $\rho$ and at the beginning of the reaction [11]

$$\left(\frac{k_{\rho}}{k_D}\right)^{-1} = 1 + \frac{\ln \left[ (D / H)^0_i / (D / H)^i \right]}{\ln \left[ 1 - \rho \right]} \text{.}$$

Even in the case of small secondary isotope effect, $k_{\rho}/k_H^0$, the variation of $(D / H)^0_i$ may be large enough, specially at high yield of the reaction (Fig. 1), to ensure a satisfactory precision for the determination [11,16].

- The thermodynamic isotope effects occurring in systems at equilibrium can be determined with the same...
advantages as above. For instance hydroxylic exchanges in hydro-alcoholic mixtures, [17], or keto-enol interconversion in tautomeric equilibria [18] are isotopically characterized in a single experiment.

– All gas-liquid fractionation factors associated with the observable isotopomers (hydrogen, carbon or oxygen...) of a given molecule are accessible in the same set of distillation experiments [19].

– A matrix, [A], of isotopic coefficients, $a_{ij}$, connecting molecular sites $i$ of reactants to sites $j$ of products in chemical or biochemical reactions of any level of complexity can be defined [20]

$$[A] \vec{D}^3 = \vec{D}^0$$

(2)

where $\vec{D}^3$ and $\vec{D}^0$ are the column vectors of the site-specific isotope ratios of the starting molecules and end products respectively.

For instance, the isotopic transfer coefficients connecting sites $j$ of the glycolytic products, ethanol, water, and glycerol, to sites $i$ of the starting materials, glucose and water, in a fermentation reaction, are rich sources of information on the genealogy of the different hydrogens and consequently on the mechanistic pathway. In particular the $a_{ij}$ values provide information

• on the stereospecificity of the reduction step of dihydroxyacetone phosphate,
• on the relative contributions of the intra- and inter-molecular transfers of hydrogen 2 of glucose by phosphoglucoisomerase,
• on the relative rates of certain reaction steps etc. The influence of the reaction conditions (temperature, nature of the microorganism, composition of the medium...) on these coefficients enables possible modifications of the reaction mechanism to be detected and interpreted [21].

From a general point of view the natural abundance site-specific isotope ratios of a given product (ethanol for instance) are useful criteria for inferring many properties of the precursors (sugars), even when these precursors are fully exhausted or inaccessible.

The site-specific isotope ratios determined by NMR

The isotopic content at a specific molecular position $i$ may be described in terms of a natural abundance parameter $A_i$

$$A_i = \frac{[H_i]}{[H_i] + [L_i]}$$

(3)

where $[H_i]$ and $[L_i]$ are respectively the numbers of heavy and light isotopes at site $i$.

It may also be characterized by the isotope ratio $R_i$

$$R_i = \frac{[H_i]}{[L_i]}$$

(4)

This ratio is of the order of $1.5 \times 10^{-4}$ for hydrogen and $1.1 \times 10^{-2}$ for carbon for instance.

Alternatively a site-specific relative deviation, $\delta_i$, may be defined as

$$\delta_i(\%e) = \frac{R_i - R_{\text{ref}}}{R_{\text{ref}}} \times 1000$$

(5)

where $R_{\text{ref}}$ is the isotope ratio of an international standard [22,23] which is a carbonate (PDB) for carbon and mean ocean water (V.SMOW) for hydrogen.

At the high isotopic dilution of hydrogen the value of the isotopic abundance, $A_i$, is nearly equal to that of the isotope ratio, $R_i$, but the difference between $A_i$ and $R_i$ is no longer negligible in the case of carbon.

In NMR the signal areas measured in quantitative experimental conditions are simply proportional to the numbers of nuclei involved in the resonance signals. Consequently, as illustrated by the example of nicotine extracted from tobacco leaves, the mole fractions of the monodeuterated isotopomers can be directly computed from the $^2$H-NMR spectrum (Fig. 2). Taking as reference site 3 of the pyridine ring, the relative isotope ratios determined for positions 1 to 12 (order of decreasing chemical shift) have the following values for a sample from Thailand:

![Figure 1. Evolution of the isotope ratio at position $i$ (adjacent to the reacting site) of reactant $A$ involved in a non-branched reaction, as a function of the yield, $\rho$, and of the secondary isotope effect $k_H / k_D$ [11]. Relatively large variations in the deuterium content of site $i$, $(D/H)_i^0$, may occur at low residual concentrations of $A$, even for values of $k_H / k_D$ close to unity.](image-url)
This behaviour illustrates the existence of very strong deviations with respect to a statistical distribution of $^2$H which would lead to values uniformly equal to unity.

In the absence of an isotopic standard, the NMR spectrum gives direct access only to the relative distribution of the isotopes in the different sites of the molecule. By adding to the sample a known amount of an appropriate reference substance, the isotopic ratio of which has been previously calibrated (for instance tetramethylurea supplied by IRMM at Brussels), it is possible to determine the isotopic parameters $A_i$, $R_i$ or $\delta_i$ by using NMR spectroscopy alone. Alternatively the absolute isotope content can be computed from both the mole fractions $f_i$ obtained by NMR and the overall isotopic abundance of the molecule, $A_i$, measured by IRMS

$$A_i = \frac{f_i}{F_i}$$  \hspace{1cm} (6)

where $F_i$ are the statistical fractions.

In spite of its low natural abundance (of the order of $1.5 \times 10^{-4}$) and low intrinsic sensitivity ($\approx 10^{-2}$ with respect to $^1$H) deuterium is well suited to quantitative comparisons by NMR. Although more difficulties are encountered in the case of carbon, typical isotopic distributions can now be characterized with a reasonable level of confidence [25]. Thus it has been checked in the case of glycerol that the carbon iso-

topic pattern determined by NMR is in good agreement with site-specific IRMS data obtained by degrading the sample into hexamethylene tetramine (carbons 1, 3) and formate (carbon 2) [26].

The site-specific isotopic genealogy

In natural products the isotope contents at the different sites of a molecule are governed by the entire set of kinetic or thermodynamic fractionation effects resulting from physico-

chemical transformations (evapotranspiration in the leaves) or metabolic evolutions. These isotope fractionation effects depend on the biochemical mechanism and on the yields of the different reaction steps. For a given bioconversion, whatever its complexity, the isotopic coefficients $a_{ij}$ connecting sites $j$ in the products, $Q$, to sites $i$ in the reactants, $S$, (Eq. 2) can be estimated from the isotope ratios $(D/H)_{S_i}$ and $(D/H)_{Q_j}$ measured in a series of reactions conducted, in strictly identical conditions, with reactants characterized by different isotopic distributions. Thus in the case of glycerol obtained in fermentation reactions (Fig. 3) it is shown that the coefficients $a_{6\alpha}$, $a_{6\beta}$, $a_{\alpha\beta}$, $a_{\alpha\beta}$ connecting respectively sites 6$'$ of glucose to sites A(1S,3R) and B(1R,3S) of glycerol (Fig. 3) exhibit relatively high values [27]. In particular, the values of $a_{6\alpha}$ and $a_{6\beta}$ are not far from the theoretical value 0.25 which would represent a fully intramolecular transfer from the methylenic hydrogens at positions 6$'$ of glucose to the fourfold degenerated signals A and B of glycerol in conditions of complete conversion or negligible isotope effects. This behaviour characterizes a high degree of connectivity between hydrogens at positions $A_i = \frac{f_i}{F_i}$

Figure 2. Natural abundance $^2$H-NMR spectrum (76.7 MHz) of nicotine [24]. The solvent, $S$, is CH$_3$NO$_2$.

Figure 3. $^2$H-NMR spectrum (76.8 MHz) of the triacetate of a glycerol sample obtained by fermentation of maize glucose [27]. In spite of the symmetry degeneracy five isoto-

pomers are characterized (stereospecific numbering) since the origins of the hydrogen atoms on the two enantiotopic moieties (1S and 3R for instance) of glycerol are not the same. TMU identifies the signal of the isotopic reference, tetramethylurea.
1,2,3 and 3,6 (1,2,3 and 3,6) of glycerol and hydrogens at positions 2 and 6 of glucose. Consequently the isotopic pattern of glycerol is expected to provide a probe of the isotopic properties of its sugar precursors. As compared to ethanol, the widely exploited probe for sugars [10], glycerol exhibits similar but not fully identical connectivities. Its isotopic pattern may therefore provide some complementary information.

This genealogical approach is specially efficient to estimate the relative contribution of hydrogen transfer from water to specific positions in the products. It is obvious that when the percentage of intermolecular transfer to a given site is very high, the site-specific isotope ratio \(\frac{D}{H}\) no longer characterizes properties of the plant carbohydrates but reflects the behaviour of the aqueous medium. In this case \(\frac{D}{H}\) is likely to have stored information on the evapotranspiration processes undergone by the plant precursor.

### Origin inference and authenticity criteria

Since, as discussed above, the isotopic pattern of a molecule is governed by the mechanistic pathway and the environmental conditions of the synthesis it may be very helpful for answering a number of questions concerning the origin of the product. Thus the natural or synthetic origin of the molecule is usually very easily identified. Moreover, for natural products, the isotopic fingerprint is frequently typical of the metabolic pathway of the plant precursor (C₃, C₄, CAM…). For a given metabolic origin the isotopic distribution may enable the botanical species to be identified and for a given plant species the geographical origin may often be ascertained [11]. Thus, ethanol and water obtained in standardized fermentations of sugars can be used as a common isotopic probe for characterizing these sugars whatever their molecular structure (monosaccharides, disaccharides, polymeric structures). Figure 4 illustrates the hierarchical clustering ability of the hydrogen and carbon isotopic parameters to infer the botanical nature of the plant precursor [28]. In certain cases it is even possible, for a given molecule extracted from a given plant species grown in a given region, to get information on the year of harvest of the plant.

### Conclusion

Exploited alone, or more often in combination with overall carbon isotope ratios measured by IRMS (see other articles in this document), the SNIF-NMR fingerprint has already greatly contributed to establishing the notion of authenticity of food and beverages on well defined quantitative criteria. The method has now been adopted, and is currently used, by official regulating organisms in Europe and the United States. Further examples, in particular in the field of aroma components, can be found in references 29 to 38. Many IRMS studies in other domains such as agronomy [6], nutrition, medicine [39-40], will certainly benefit from SNIF-NMR development when appropriate isotopic probes become more firmly established.

### References

Isotopic analysis