Specific Natural Isotope Fractionation Studied by 2H Nuclear Magnetic Resonance (SNIF-NMR): History, Developments, and Applications

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Abstract:

Food authentication is the analytical process to verify that a food product is in compliance with its label description (Danezis et al., 2016). Food products that are of high value and undergo a number of processing steps are often target of fraudulent labeling .A powerful tool is isotopic analysis. A cutting edge in the authentication field was the introduction of quantitative ^{2}H NMR spectroscopy in early 80's. This analytical approach well-known as SNIF-NMR^{TM} protocol allows the determination of each ^{2}H isotopomer within a given molecule. Historically, the first application was the detection and quantification of the chaptalization process of wine. Then, as a generic methodology ^{2}H NMR could be used to tackle genuineness issues on any molecules as aromatics. The (geographical/botanical) origin inferring was also possible from the ^{2}H patterns of precursors. In particular, the 2D mapping by isotopic ratio measurements (irm-^{2}H) NMR in chiral oriented media allowed a key step in the fundamental understanding of enzymatic processes (stereoselectivity) of natural ^{2}H distribution in prochiral molecules.

Keywords: Deuterium, NMR, Isotope ratio, PSIA, Authentification, Isotopomers, Liquid crystals.

I. Introduction to the concept of isotopomers and isotopologues: what is measured

Definitions. In nature, isotopes exist in any compound and are generally defined as isotopomers or isotopologues as possessing the same constitution and same configuration but they differ in their isotope substitutions (Zhang et al. 2002a; Cohen et al. 2007). However their definition deserves first attention to avoid confusion. Within the frame of the present Handbook, we have adopted the following definitions. While isotopologue is the generic word for any molecule that that differs only in isotopic composition (number of isotopic substitutions), isotopomer of a molecule contains heavy isotope at different positions. There are many isotopologues and isotopomers of a compound. They can differ in: i) the elements substituted by its isotopes; ii) or/and isotopes; iii) or/and number of atoms of isotope; iv) or/and substituted sites. Consequently, every compound can be seen as a mixture of various isotopologues for each of which there may be several isotopomers. For example, the ethanol (CH_3CH_2OH) is composed of three chemical elements (C, H and O) of which each has three stable or radioactive isotopes (^1H, ^2H, ^3H; ^{12}C, ^{13}C, ^{14}C; ^{16}O, ^{17}O, ^{18}O) one, two or three of these elements can be substituted by their heavy isotopes. Hydrogen atoms are found at three molecular sites: three at methyl, two at methylene and one at hydroxyl. One or several hydrogen atoms can be replaced by their heavy isotopes. As a result, there are a lot of isotopologues and isotopomers of ethanol. But we could identified three ^2H isotopomers: $^2H^1H_2C\text{-}CH_2\text{-}O^1H$, $^1H_3C\text{-}^2H^1HC\text{-}O^1H$, $^1H_3C\text{-}^1H_2C\text{-}O^2H$. Notably, the different isotopologue and isotopomers of ethanol have the same chemical property (in a chemical reaction, all isotopologues and isotopomers undergo the same reaction) but may differ in physical and physico-chemical behavior (boiling point, spectra properties, chromatography retention time, evaporation, reaction rate constant and equilibrium constant, etc.).

At the natural abundance, the major isotopes of C, H, N and O are light isotopes, and hence most organic molecules composed of these elements contain only light isotopes. The probability of finding a given isotope of a given element in a molecule is proportional to the abundance of the isotope and to the number of atoms of the element in the molecule. The heavy isotopologues (molecules containing one or several heavy isotopes) of an organic compound exist only in very small amount. The majority of them are substituted only by one heavy isotope atom (isotopologue) of which the amount fraction represents the natural abundance of the heavy isotope at the substituted site. The probability of finding two or more heavy isotope atoms in one molecule is very small and can often (but not always) be neglected (see **Table 1**). Thus, the isotopologue containing two ^{13}C atoms of which the amount fraction is of order of 10^{-4} cannot be totally neglected (**Zhang et al.1999**).

Chirality generated by isotopic substitution: the case of ethanol. Chirality is an important geometric property in chemistry **(Eliel and Wilen. 1994)**. It defines a particular type of (rigid or flexible) molecules that have non-superposable mirror images. Ethanol and ethanol-1,1-d_2 are not chiral compounds (see **Figure 1a**), they are both defined as flexible prochiral compounds of C_s symmetry in

Table 1. Isotopic content for mono- and disubstituted isotopologue

	Content	
Isotope	**Monosubstituted isotopoloque**	**Disubstituted isotopologue**
^{2}H	$p_H \times 1.5\ 10^{-4}$	$p_H \times (p_H - 1) \times (1.5\ 10^{-4})^2$
^{13}C	$p_C \times 1.1\ 10^{-2}$	$p_C \times (p_C - 1) \times (1.1\ 10^{-2})^2$
^{2}H-^{13}C		$[p_H \times (1.5\ 10^{-4})] \times [p_C \times (1.1\ 10^{-2})]$

p_H: number of H atoms in the molecule, p_C : number of C atoms in the molecule. Unit: mole fraction. $1.5\ 10^{-4}$ and $1.1\ 10^{-2}$ are approximate mean values of ^{2}H and ^{13}C natural abundance.

average. Both of them possess enantiotopic atoms (^{1}H and ^{2}H atoms) exchangeable by a plane of symmetry (see **Figure 1b**). Associated internuclear directions ($^{12/13}C$-^{1}H or $^{12/13}C$-^{2}H) are noted pro-*R* or pro-*S,* accordingly with the CIP rules (see **Figure 1a**) (**Eliel and Wilen 1994, Moss 2016**). For chemists, two protons are called enantiotopic if replacing one of them by different group (as an heavier isotope) results in an enantiomeric pair. In other word, each of ^{1}H (^{2}H) atoms on the prostereogenic methylene site of ethanol is substituted by one ^{2}H (^{3}H) atom, a pair of isotopic enantiomers is obtained. This is called chirality by virtue of the isotopic substitution ($^{1}H/^{2}H$ or $^{2}H/^{3}H$). De facto, [1-$^{2}H_1$]ethanol at natural abundance level exists under the form of two monodeuterated enantio-isotopomers (*R* or *S*) that are mirror-images (see **Figure 1b**). Actually, in generic structures like "R_1-CH_2-R_2" with $R_1 \neq R_2$, the two methylene hydrogen atoms are enantiotopic, and hence their corresponding monodeuterated isotopomers are enantiomers (enantio-isotopomers). Typically, all methylene groups of saturated fatty acids (HOOC-$(CH_2)_n$-CH_3) provide *n* pairs of enantio-isotopomers (R-C*HD-R') associated to each inequivalent CH_2 group in the molecule (see below). Finally in a chiral molecule if the chiral carbon is bound to a prostereogenic carbon center (as a methylene group) on which there are two diastereotopic hydrogen atoms, the respective substitution of the hydrogen atoms by its heavy isotopes leads to two diastereoisomers, for example, 6-C methylene of glucose.

From the nuclear magnetic resonance (NMR) viewpoint, the analysis of all ^{2}H or ^{13}C isotopomers at natural abundance provides key data on the site-specific (also named position-specific) isotopic profile of the analyte, even when very low abundance isotopes such deuterons are considered. It should be emphasized, however, that in natural abundance, the different ^{2}H-{^{1}H} or ^{13}C-{^{1}H} NMR signals correspond to different molecules, while in ^{1}H NMR, different signals correspond to different hydrogen sites of the same molecule. No ^{2}H-^{2}H homonuclear scalar couplings, $J(^{2}H$-$^{2}H)$, can be observed on ^{2}H-{^{1}H} spectra due to the extremely low probability to have two deuterons on the same molecule ($1.5\ 10^{-2}$% × $1.5\ 10^{-2}$%). However, very small amount of bisubstituted isotopologue (1.1 % × 1.1%) leads to signals split by the homonuclear coupling, $J(^{13}C$-$^{13}C)$, between adjanecnt ^{13}C atoms in bisubstituted isotopomers, observable on natural abundance ^{13}C-{^{1}H} NMR spectra recorded with very high signal-to-noise ratios (SNR), **(Zhang et al. 1999)**. Besides if diastereoisomers are *a priori* spectrally discriminable, enantiomers can be only observed separately under special conditions, in particularly in

presence of enantiopur chiral moieties or chiral environments (**Eliel and Chisholm. 1994; Wenzel 2007; Wenzel et al. 2011**). This side aspect of isotope analysis by NMR and application will be described in sections "*Defining Biosynthesis Mechanisms: Interpretation of the Intramolecular ^{2}H Distribution*" and "*Recent Original ^{2}H NMR Developments*" of this chapter

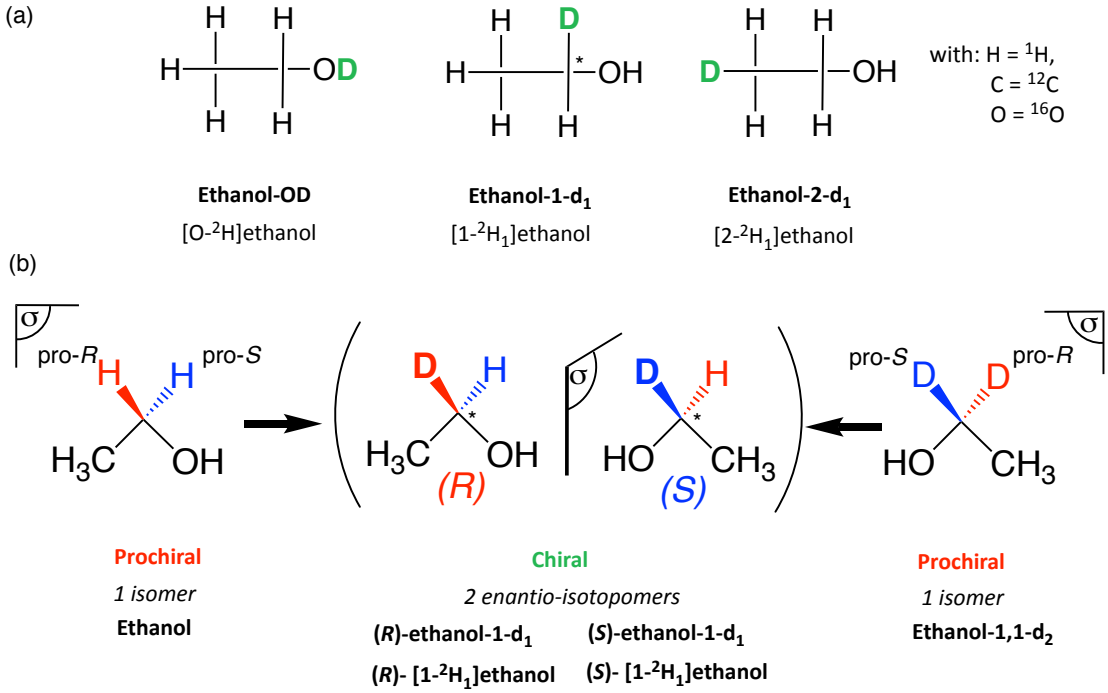

Figure 1. (a) Nomenclature of three families of monodeuterated isotopomers of ethanol. (b) Origin of the chirality generated by ($^{2}H/^{1}H$) isotope substitution.

In following parts of this chapter, we will describe how to study the ^{2}H isotopomers and measure the site-specific isotopic ratios by natural abundance ^{2}H NMR, its application in wine analysis and in origin identification of other compounds of interest. Then we will explain why we can distinguish ethanol molecule derived from sugars of different origins. Finally we will present the recent technical progress in the field.

II. The SNIF-NMR protocol: a success story for authentication issue!

The principle of NMR. In nature, many nuclei have spin $I \neq 0$, they are magnetically active. If an external magnetic field is applied, an energy transfer is possible between the base energy to a higher energy level. The energy transfer takes place at a "wavelength" that corresponds to radio frequency (resonance effect at Larmor frequency, $\nu_0 = -\gamma B_0/2\pi$), then spins return to their equilibrium state through various relaxation mechanisms (dipole-dipole, quadrupolar, …). The time NMR signal that matches this transfer is then measured and processed in order to yield a frequency NMR spectrum (after a Fourier transformation) for the nucleus concerned.

The pioneer experiment. Since long time, quality control and anti-falsification of wine have been a challenge for the authority of many countries. Isotope ratio monitoring by Mass Spectrometry (irm-MS, known also as IRMS: Isotope Ratio by Mass Spectrometry) allows distinguishing C_3 and C_4 products (and hence, cane or corn sugar addition to must) but not different plants within the same type (e.g. beet and grapewine, both C_3) hence new methods were required. In 1981, for the first time, Gérard J. Martin and coworkers explored and applied quantitative deuterium 1D-NMR (one dimensional NMR) technique to study intramolecular site-specific deuterium isotope content of organic molecules of interest, and in particular, the case of ethanol (**Martin et al. 1981, 1982, 1986, 1991**). In case of a stochastic distribution, the ratio of the abundance of two isotopomers is equal to their stoichiometric ratio, for example, $CH_2{}^{\mathbf{2}}\mathbf{H}CH_2OH/CH_3CH^{\mathbf{2}}\mathbf{H}OH$ = 3/2. In the first natural abundance ^2H NMR experiments, It was observed that natural phenomena lead to deviations from stochastic distribution, i.e., lead to isotopomers with different abundance, ($CH_2{}^{\mathbf{2}}\mathbf{H}CH_2OH/CH_3CH^{\mathbf{2}}\mathbf{H}OH \neq 3/2$). Thus, they observed the non-statistical distribution of hydrogen isotope at different molecular positions of a compound, meaning that the content of the different monodeuterated isotopomers depends on the deuterium-substituted position. The strength and sign of the deviation depends on the origin of the molecules, and can thus be used as a powerful tracer. They also showed that site-specific isotope fractionation depends on the origin of the analyte. The method was called Site-specific Natural Isotope Fractionation studied by Nuclear Magnetic Resonance (SNIF-NMR), and has been applied in a large range of applications, from the study of chemical and biochemical reaction mechanisms to the detection of the chaptalization practices as described below. From the success of the SNIF-NMR method initially explored and described by Martin et coll. [**Martin et Martin, 1981**] results the creation of the international Company “Eurofins Scientific”, which has developed an official analytical protocol associated the concept of SNIF-NMR^{TM} (TMU calibration, multi-laboratory ring tests, …).

Principles and features of the quantitative ^2H NMR. The principle of the SNIF-NMR protocol consists in recording the proton-decoupled Natural Abundance Deuterium (NAD) 1D-NMR spectra of the analyte in (achiral) isotropic solutions using quantitative acquisition conditions, namely when the recycling delay of NMR experiment is at least five time larger than the longest ^2H longitudinal relaxation time (analyte or calibration) ($T_{RD} > 5 \times T_1$). When an absolute quantification is required, the NAD spectrum must contain the signal of an internal chemical reference, such as TMU (tetramethyl urea) for which the $(^2H/^1H)_i$ ratio is known or a calibrated electronical signal as external reference (ERETIC method) (**Billault et al. 2002**).

For a given molecular hydrogen site (i), the site-specific ratio, $(^2H/^1H)_i$, expressed in parts per million (ppm) is defined as:

$$\left(\frac{^2H}{^1H}\right)_i = \left(\frac{N^{^2H_i}}{P_i.N^{^1H_i}}\right) \times 10^6 \qquad (1)$$

where $N^{^2H_i}$ is the number of mono-deuterated isotopomers with a deuterium nucleus at the site i, $N^{^1H_i}$ is

the number of fully isotopologues, (≈ number of all sample molecules in neglecting the minor isotopologues), and P_i is the stoichiometric number of equivalent hydrogen atoms at position i. Thus if the isotopic ratio ($^2H/^1H$) difference between two samples is 1 ppm, in delta notation, this difference is δ‰ 6.4 (or 6.4 mUr, milli Urey).

Since the area of an NMR signal is proportional to the number of nuclei resonating at the considered frequency, the isotopic data are directly accessible from peak area measurements. Thus, using the standard SNIF-NMR protocol and TMU as calibrated isotopic reference, **Equation 1** can be recasted as:

$$\left(\frac{^2H}{^1H}\right)_i = \left(\frac{^2H}{^1H}\right)_{TMU} \times \left(\frac{S_i^{anal}}{S_{TMU}}\right) \times \left(\frac{P_{TMU}}{P_i^{anal}}\right)\left(\frac{m_{TMU}}{m_{anal}} \times \frac{M_{anal}}{M_{TMU}}\right) \quad (2)$$

In **Equation 2**, P_i^{anal} and P_{TMU} are the stoichiometric numbers of equivalent hydrogen atoms in the site i (or position i) of the analyte and in TMU, M_{anal}, m_{anal} and M_{TMU}, m_{TMU} are the molecular weight and the mass used, respectively, and S_i^{anal} and S_{TMU} are the areas of the NAD signals at site i and TMU. If the analyte is not pure (for example, alcohol containing some water), its mass should be corrected with its purity as $m_{anal} = p_{anal} \times m'_{sample}$ where p_{anal} is the sample purity in mass fraction and m'_{sample} is the mass of sample used.

Since the first proof of concept, an intensive work has been carried out for the development of high-precision quantitative NMR technique aiming for accurate isotopic ratio measurements at natural abundance and its application. In the early years of the isotopic NMR method development, the research focused mainly on ^2H NMR for three reasons: i) with deuterium which exists in nearly all organic molecules as isotopic probe, there was a lot of applications that could be explored and exploited; ii) in practice, the realization of high-precision quantitative deuterium NMR is much less difficult than with ^{13}C NMR (**see Chapter 2 of this Section 6 of Handbook**) and the obtained results were more repeatable and reproducible because the variation of deuterium abundance is very large ($\Delta\delta$‰ can reach 500 and even more, but for ^{13}C it can only be about 50), and so less sensitive to measurement uncertainty (**Martin and Martin 1991**); iii) some interesting spectral specificities of ^2H nuclei. To illustrate this last point, **Table 2** compares the nuclear properties of three nuclei ^1H, ^2H and ^{13}C in NMR experiments.

From a spectral viewpoint, both the small gyromagnetic ratio (0.4107×10^8 rad.T^{-1}.s^{-1}), responsible for poor intrinsic sensitivity (9.6×10^{-3} with respect to ^1H), and the very low natural abundance of deuterium atoms (1.5×10^{-2}%) are the main difficulties for the study of NAD spectra. Besides, the intrinsic resolution of deuterium spectra is low since in a given magnetic field, the chemical shift discrimination expressed in frequency units is 6.5 times lower in ^2H-NMR as compared to ^1H NMR.

Table 2. Main NMR characteristics of ^{1}H, ^{2}H, ^{12}C and ^{13}C nuclei

Isotope	**^{1}H**	**^{2}H**	**^{12}C**	**^{13}C**
Spin	½	1	0	½
Mean natural abundance (%)	99.845	0.0155	98.9	1.1
Gyromagnetic ratio (10^{8} rad.T^{-1}.s^{-1})	2.675	0.4107	0	0.673
Relative sensibility[a]	1	0.0096	0	0.016
Larmor Frequency at 9.4 T (MHz)	400.0	61.4	0	100.0
Relative resolution[a]	1	1/6.5	0	1/4
Chemical shift range (ppm)	12	12	/	250

[a] ^{1}H nucleus is used as reference.

At the natural abundance, quantitative ^{2}H NMR is more difficult than routine ^{13}C NMR but easier than quantitative ^{13}C NMR which requires a much higher precision. Hence, historically, the quantitative NAD NMR was more favorable for the study of small molecules. However, with the advent of high-field modern NMR spectrometers (14 T or higher) equipped or not with sensitive deuterium cryogenic probe, NAD spectra are currently obtained with better SNR and spectral resolution, thus broadening the application domains. New NMR experiments and methods were designed and still in development, especially, anisotropic ^{2}H NMR (see **Section V** "*Recent Original 2H NMR Developments*" of this chapter) which offers a good means to overcome the resolution difficulty in 1D ^{2}H NMR study and open up prospects for the study of big molecules.

Due to its spin (I = 1), the relaxation of the deuterium nuclei is dominated by relatively efficient quadrupolar mechanisms. Consequently, nuclear Overhauser effects (nOe), which are mainly due to dipolar relaxation mechanisms between two interacting nuclei, may usually be neglected. In practice, nOe is associated to a change in the intensity of NMR resonances that occurs when: i) another nuclei is saturated in the molecule by irradiation with a radiofrequency field, ii) for two interacting nuclei close in space (< 4 -5 Å) **(Neuhaus and Williamson 2000).**

Thus it is not necessary to resort to time-consuming gated decoupling sequences aimed to eliminate Overhauser enhancements For simple organic molecules reorienting relatively rapidly (liquids states), the ^{2}H longitudinal, $T_1(^{2}H)$, and transversal, $T_2(^{2}H)$, relaxation times are generally equal and relatively short. Interestingly, short relaxation times are favorable from the sensitivity point of view since they authorize a fast signal accumulation. Nevertheless, the relaxation time is frequently higher than 0.1 s, and signals with moderate linewidth are obtained. It is easy to assign deuterium signals since the ^{2}H chemical shifts expressed in ppm are generally identical to the corresponding ^{1}H chemical shifts (**Lesot et al. 2009**). Another advantage of deuterium NMR compared with isotope ratio measured by mass spectrometry (irm-MS or IRMS) is that the exchangeable hydrogen atoms do not disturb the analysis while they should be removed before IRMS analysis. Finally, SNIF-NMR can also be applied to the analysis of a mixture of compounds (**Zhang et al. 2006**).

Authentication of wines by ^2H NMR. Nowadays, various types of frauds in various consumer sectors are practiced over the world. Consequently, fighting against illegal adulteration of drinks or foods, (high-commercial-value products) is of primary importance for economy, but also for human health. Historically, one of the best known practices is to add cane/beet sugar, before or during the fermentation process to increase the natural content of ethanol, and hence modify the wine quality proposed in the market. Chaptalization is strictly controlled in the European Union and generally only permitted in more northerly areas where grapes might not ripen enough. The amount of chaptalization allowed depends on the wine growing zone. According to the European Union regulations, chaptalization with sucrose is permitted only in some vine-growing regions, especially Zone A, B and parts of Zone C (see **Table 3**) (**Commission of the European Union, 2008**) Exceptional permits of chaptalization for compensation of ripening deficits caused by adverse meteorological situation have to be considered. The chaptalization practice should not only be detected qualitatively but also controlled quantitatively.

Table 3. Example of European regulations on chaptalization of wine in three different regions A, B and C[a] (Ia, Ib, II and III)

Area	**A**	**B**	**CIa**	**CIb**	**CII**	**CIII**
t%$^{min\ b}$	5	6	7.5	8	8.5	9
c%$^{max\ b}$	3.5	2.5	2	2	2	2

[a]With the exception of vineyards in Italy, Greece, Spain and Portugal and vineyards in the French departments under jurisdiction of the courts of appeal of: Aix-en-Provence, Nîmes, Montpellier, Toulouse, Agen, Pau, Bordeaux, Bastia. [b] t% and c% are expressed in v/v of ethanol in wine. t% for natural wine and c% is the increased percentage by added sugar.

Another type of wine adulteration is mixing high wines of various qualities, generally produced in different geographical regions or even countries, but more serious frauds impacting human health are the examples of adulteration with ethylene glycol (a noticeable poisonous antifreeze) with the aim to enhance the oenological quality of wine.

In order to guarantee the quality of marketed products, the adulteration of edible products can be detected using many analytical techniques such as HPLC, GC-MS, GC-FTIR, IRMS and NMR (**Cordela et al. 2002**) Among them, isotopic analyses using quantitative NMR spectroscopy (SNIF-NMR) and/or isotopic ratio mass spectrometry of (irm-MS) are becoming now official / standard methods in Europe and North America for routine uses. Based on the measurement of stable isotope global or site-specific content ($^2H/^1H$, $^{13}C/^{12}C$, $^{18}O/^{16}O$, …), either on the final product or one specific component of the product after isolation (such ethanol obtained by distillation in case of wines), both methods can provide reliable data for authentication issues.

Figure 2 presents a typical example of the ^2H SNIF-NMR 1D spectrum (proton decoupled) of distilled ethanol from which the ($^2H/^1H$) ratios at OH, CH_2 and CH_3 groups can be determined using **Equation 2** (**Guillou 1991**). In ethanol, the ($^2H/^1H$) ratios measured at the methyl and methylene sites (denoted $(^2H/^1H)_{CH_3}$ and $(^2H/^1H)_{CH2}$, respectively) provide various types of specific molecular

information. Thus, the $(^2H/^1H)_{CH_3}$ ratio is related to the nature of plant species which synthesized the natural sugar, while the $(^2H/^1H)_{CH_2}$ ratio is more related to the climatology of the place of production of the grapes (type of rain water and weather) and to a lesser extent the sugar concentration of the original must (**Pîrnău et al. 2013**). The typical values $(^2H/^1H)_{CH_3}$ of ethanol issued from a wine is 102 ppm. Due to difference of the geographical and climatological conditions, the $(^2H/^1H)_{CH_3}$ values may vary according to the region and production years. Hence it is necessary to establish a specific isotopic database that contains isotopic data of authentic and representative samples from different region and production years.

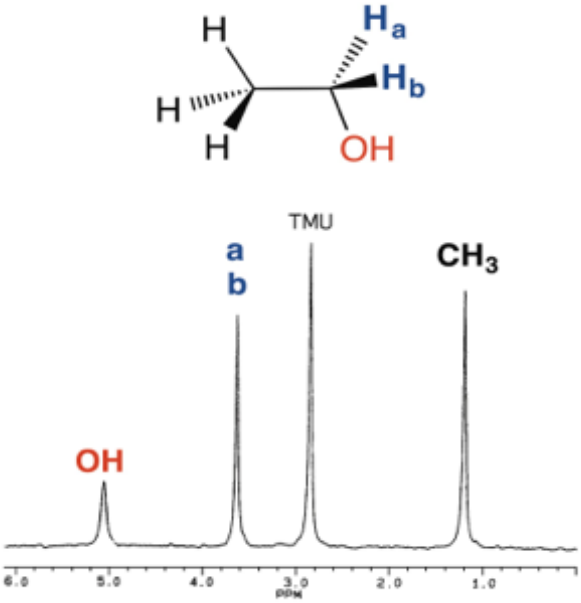

Figure 2. 61.4 MHz SNIF-NMR spectra of ethanol recorded using TMU as isotopically calibrated internal reference. Only three NAD signals are observed for ethanol since the enantio-isotopomers associated with the prostereogenic methylene group (H_a/H_b) are not discriminated in achiral liquids. Figure adapted from *Analusis*, 1991; 19:29, with permission from EDP Sciences.

Comparison of experimental isotope data with the authentic wine isotopic data in an official wine databank (EU- WineDB) at the Joint Research Centre (JRC) in Geel, Belgium, allows the wine origin to be identified. In order to compare the sample isotope data with the authentic wine isotopic data of the database, the isotopic data of wine should be measured in following strictly the official working protocols (OIV-MA-BS-23: R2009), including fermentation, alcohol extraction, sample preparation and isotopic analysis by NMR or IRMS. In sample preparation for isotopic analysis, if the analyte is not completely recovered in extraction and purification, isotopic fractionation may occur and can alter analysis results. The OIV protocol ensures a very high recovery rate (> 98%) of ethanol from wine and thus minimizes the isotopic fractionation. For the detection of chaptalization, we need specific isotopic ratio $(^2H/^1H)_{CH_3}$ of ethanol from the wine sample, that of authentic wine in the data bank and that derived from currently used beet or cane sugar. The percentage (%) of added sugar, c, in chaptalization process can be calculated by the following relation:

$$c(\%) = 100 \times \frac{\left[\left(^2H/^1H\right)_{CH_3}^{sample} - \left(^2H/^1H\right)_{CH_3}^{wine}\right]}{\left[\left(^2H/^1H\right)_{CH_3}^{sugar} - \left(^2H/^1H\right)_{CH_3}^{wine}\right]} \quad (3)$$

with $\left(^2H/^1H\right)_{CH_3}^{sample}$ is the ethanol specific isotopic ratio of the analyzed sample, $\left(^2H/^1H\right)_{CH_3}^{wine}$ is the ratio of the authentic wine and $\left(^2H/^1H\right)_{CH_3}^{sugar}$ is the ratio of the added sugar.

It should be noted that SNIF-NMR is very efficient in detection of C_3 (beet) or C_4 (cane) sugars added in wine, while the addition of a mixture of C_3 and C_4 sugar, the ^{13}C isotope IRMS analysis should be used to detect the chaptalization (see **Figure 3**) (**Christoph et al. 2015**).

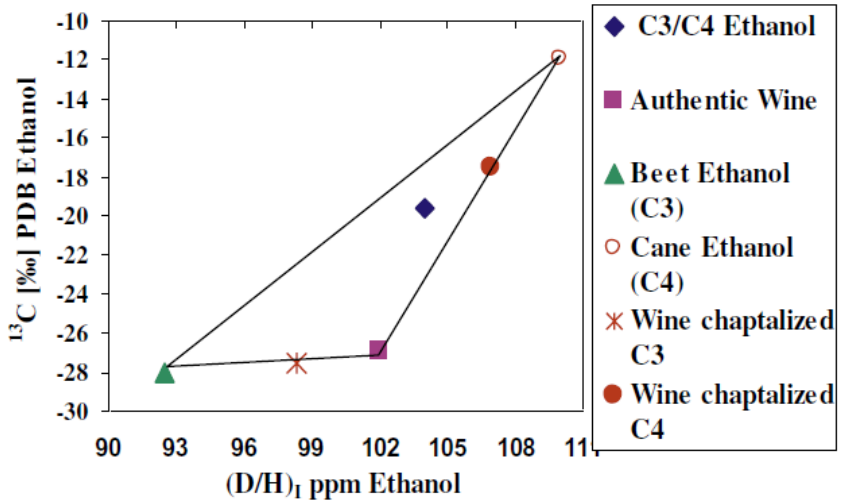

Figure 3. Typical isotopic analysis results of detection of chaptalization of a wine with beet (C_3), cane (C_4) sugar, and their mixtures (C_3/C_4) using SNIF-NMR and ^{13}C-IRMS analysis of ethanol. Here $(^2H/^1H)_I$ corresponds to the isotopic ratio of the methyl group. Figure extracted from Review and outlook, *BIO Web of Conferences* 2015; 5, 02020, with permission from EDP.

How to setup an international official method? After several years of effort, operational SNIF-NMR working protocols using ethanol as probe for the control of wine, spirits and fruit juice was established. In 1986, the repeatability and reproducibility of the ethanol measurement has been tested in an inter-laboratory collaborative study. Fifteen laboratories in Belgium, Denmark, France, Germany, Ireland, Netherland and the United States took part in the study. Three ethanol samples originating from beetroot, grape and maize were measured on sixteen different NMR spectrometers. As the coherence of the results were satisfactory (**Guillou et al. 1988**), the SNIF-NMR technique has been adopted as an official method for determining the enrichment of the wine with sugar by the International office of Vines and Wine (OIV) and European Union (EU).

Ethanol is also a reliable isotopic NMR probe of fruit juices (the sugars in juices should be converted to ethanol by fermentation and then the ethanol is isolated for analysis). The NMR method is capable to detect the addition of beet sugar (C_3 plant) to fruit juices while carbon isotope isotopic ratio analysis by IRMS can only detect added C_4 sugars (sugar cane, corn). A collaborative study published in 1996 on the SNIF-NMR method for detection of added beet sugar in fruit juice showed that the repeatability and reproducibility in the analysis is excellent (**Martin et al. 1996**). The SNIF-NMR method

for detection of added beet sugar in fruit juices has been adopted and validated by the AOAC International.

SNIF-NMR has not only been applied to authentication of food origin but also to the studies of reaction and isotopic fractionation mechanism in other natural science fields. The detemination of intramolecular site-specific ^{2}H isotope profile of natural compounds was also intensively used to investigate the primary and secondary ^{2}H KIEs. These effects provides key data on the ^{2}H enrichment or depletion effects by sites, and thereby on the understanding of enzymatic mechanisms producing biocompounds. New achievements and progresses are reported regularly in this domain.

III. Practical issues on the application of ^{2}H NMR for authentication purposes

After the success of SNIF-NMR for detecting chaptalization by using ethanol as a probe, ^{2}H NMR has been extended to characterize the origin of wine, fruit juice and other beverages, and then to series of matrices of (economic) interest, such as food flavors. Indeed if natural flavors are often seen as a strong marketing advantage, they are more expensive than artificial/synthetic ones, are prone to numerous adulterations. Reviews are regularly published that list the different applications of ^{2}H NMR (**Jamin and Thomas 2017**).

For analysis of new molecules unknown in routine operations, ^{1}H NMR spectrum of the sample should be recorded for evaluation. The resolution, solvent and internal reference should be considered. Since ^{2}H NMR resolution is only 1/6.515 of that of ^{1}H NMR and in both spectra, the chemical shift is nearly the same, the ^{2}H NMR analysis results can be estimated. The choice of solvent for solid sample is important. Not only the sample solubility in the solvent should be high, but also the solvent signal should be absent (for example CCl_4, CS_2, etc.) or very small and far from the sample signals. When possible, TMU is the preferable internal reference. Its added amount should be calculated so that its signal intensity is comparable with the sample signals. Otherwise, an occasional internal reference can be used as described below. The ^{2}H NMR acquisition parameters (acquisition time depending on the longest T_1 which should be measured, scan number, decoupling, etc.) should be carefully chosen. For quantitative analysis, curve-fitting software should be used in spectra treatment and intensity calculation.

Classically (historically), the ^{2}H-{^{1}H} NMR experimental parameters applied in ethanol analysis and using classical NMR probes (not cryogenic probes) are: i) a 10-mm o.d. NMR tube, ii) a sample temperature 28 - 29°C, iii) an acquisition time 6.8 s for 1200 Hz spectra width (16k data memory, i.e., ca 20 ppm at 61.4 MHz), iv) a 90° pulse, v) a delay time before acquisition must be the same as dwell time (quadrature detection), vi) an offset O_1 between $O^{2}H$ and $CH^{2}H$ signals of ethanol. The value offset (O_2) of ^{1}H decoupling is determined from ^{1}H spectrum obtained through decoupling ^{1}H coil on the same tube. O_2 is set to the middle of frequency interval existing between CH_3 and CH_2. Broadband or composite decoupling mode for complete decoupling from proton, scan number 200 for 9.4 T NMR spectrometer. Finally, to obtain a quantitative integration of ^{2}H-{^{1}H} signals, NMR spectra are recorded

with a recycling delay, T_{RD}, (total time of the experiment) equal (at least) 5 time the largest $T_1(^2H)$ relaxation times in the sample (either the analyte studied or TMU).

While the full recipe can be applied on the most targeted molecules typically as vanillin, limitations or difficulties may be encountered in some cases. Below are presented some selected examples of analytes which illustrate the issue, and the appropriate answer: i) international or secondary internal chemical reference; ii) no calibrated reference (^{2}H-profiling); iii) derivatization of the molecular probe; iv) how to work with very toxic and/or corrosive molecules?

Internal isotopic reference. The standard protocol for using SNIF-NMR consists in separation/purification of the molecule, and then addition of TMU (tetramethyl urea) as isotopically calibrated internal reference. This standard was developed during the normalization of ^{2}H NMR for the detection of chaptalization of wine. If TMU cannot be used with signal overlap when other molecules are analyzed, it is possible to choose another compound with only one NMR signal which was calibrated with TMU or by IRMS then is used as an occasional internal reference. Another typical illustration is that of vanillin which is the main molecule in vanilla aromas, one of the most famous ones used in food industry. Here

difference of price between synthetic and natural origin vanillin has generated numbers of types of frauds, and hence the necessity to experimentally discriminate between natural and synthetic vanillin, but also from biosynthesis **Figure 4** shows the main ways leading to vanillin: i) extraction from vanilla beans; ii) synthesis from guaiacol; iii) synthesis from lignin and iv) obtained from natural ferulic acid by a fermentation process (**Gallage and Lindberg Møller 2015**). Due to their different isotopic histories, SNIF-NMR spectra with TMU can be exploited to distinguish natural and synthetic vanillin (see **Figure 5a**) (**Remaud et al. 1997a**).

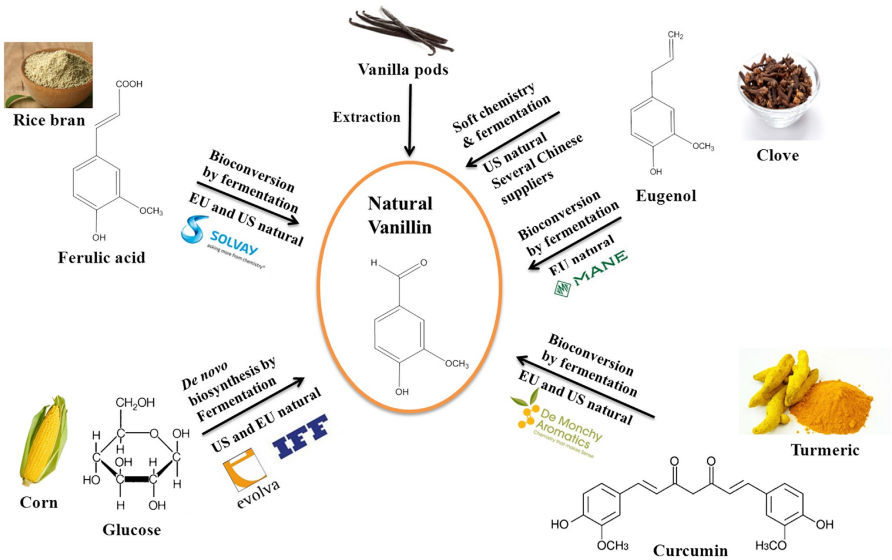

Figure 4. Principal sources for the production of vanillin. Figure extracted from *Molecular Plant*, 2015; 8:40–57, with permission from Elsevier.

For a set of vanillin samples, the canonical analysis of their specific deuterium isotopic data showed that products from three origins (guaiacol, lignin and natural bean) can be clearly distinguished (see **Figure 5b**). In recent years, much work has been devoted to the analysis of vanillin, new progress thanks to, for example, the development of quantitative ^{13}C NMR was reported (**Remaud and Akoka 2016**). Finally in 2018, another original analytical approach involving ^{2}H NMR in oriented solvents has permitted the spectral discrimination of the ^{2}H sites of aromatic ring, leading to a new possible tool for differentiating between the natural and synthetic vanillin (see below).

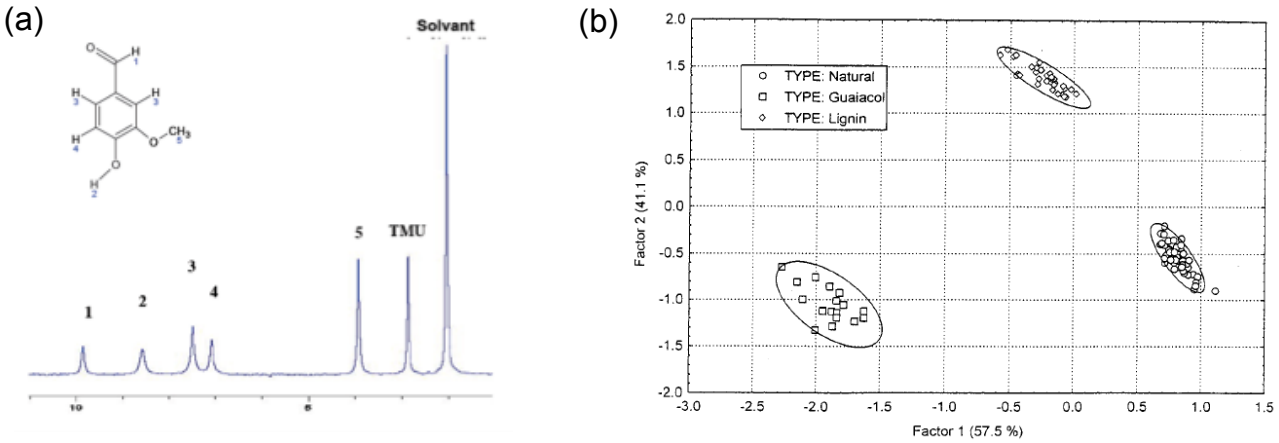

Figure 5. (a) 76.7 MHz SNIF-NMR 1D spectrum of vanillin with TMU. (b) Representation of the reference groups of vanillin (ex-beans, ex-lignin, and ex-guaiacol) projected in the plane of the canonical variables. $(^{2}H/^{1}H)_1$, $(^{2}H/^{1}H)_3$, $(^{2}H/^{1}H)_4$, and $(^{2}H/^{1}H)_5$ were the initial parameters. The ellipses drawn correspond to the 95% confidence interval. Figure 5b reprinted from *J Agric Food Chem.* 1997; 45:859, with permission from ACS.

Raspberry ketone, 4-(4-hydroxyphenyl)butan-2-one (**3**) is the key aroma component of raspberry fruit. It can be obtained in a process mimicking that of nature, by enzymatic saturation of the double bond of 4-hydroxybenzalacetone (**2**), prepared upon condensation of 4-hydroxybenzaldehyde (**1**) of extractive botanical origin with acetone produced by sugars fermentation, using several microorganism as depicted in **Figure 6a** (**Fronza et al. 1998**). Since the compound of "synthetic" origin (reactants of petrochemical) is cheaper, the adulterations of the

expensive "nature" materials with readily available "synthetic" products may occur. The measurement of the global ^{13}C and/or ^{14}C content may be a useful tool to verify the non- petrochemical origin of a substance. However, these methods do not guarantee against the transformation of natural precursors by "non-natural" synthetic methods while SNIF-NMR provided a robust mean to do it.

The determination of $(^{2}H/^{1}H)_i$ ratios required a reference but TMU could not be used since the ^{2}H signal of the CH_3 groups was overlapped with the methylene peaks of the raspberry ketone. The signal of the tert-butyl sulfide was used as internal chemical reference, this secondary reference being calibrated against the official standard TMU. As seen in **Figure 6b**, the graphical representation of the ratios $[(^{2}H/^{1}H)_3/(^{2}H/^{1}H)_2]$ *versus* $[(^{2}H/^{1}H)_5/(^{2}H/^{1}H)_4]$ shows two distinct regions allowing to distinguish between "natural" raspberry ketones and other raspberry ketone samples

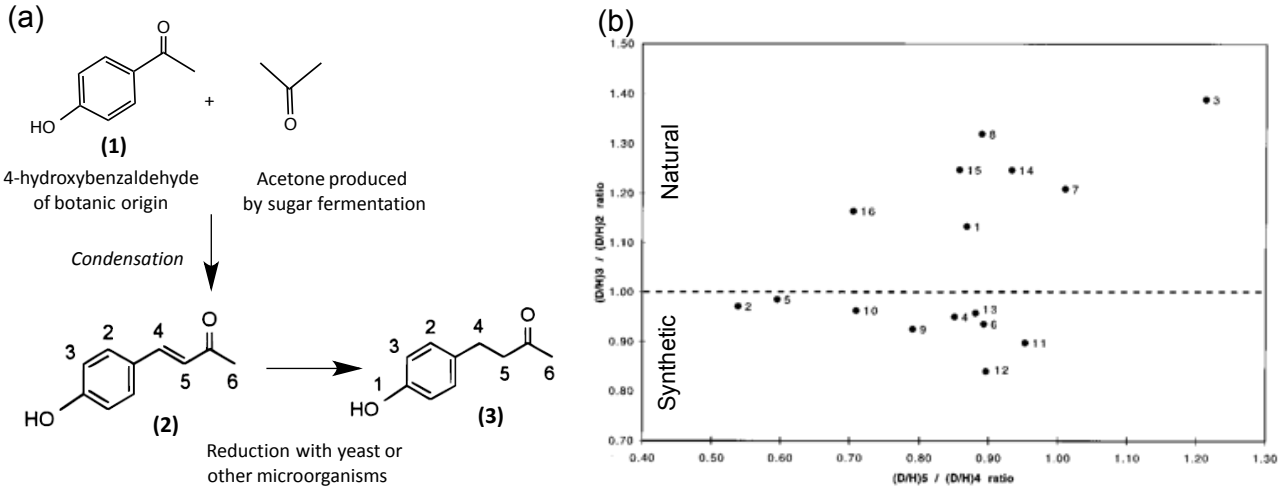

Figure 6. (a) Industrial production of natural raspberry ketone (**3**). (b) Graphical representation of the ratios $[(^2H/^1H)_3/(^2H/^1H)_2]$ *vs* $[(^2H/^1H)_5/(^2H/^1H)_4]$ showing two distinct regions for the natural samples and the synthetic samples. Other details can be found in the original article. Figures adapted from *J Agric Food Chem*. 1998;46:248–54, with permission from ACS.

obtained in different "non-natural" ways. The same problematic was met for the determination of the position-specific ^2H in citric acid. In that case the authors employed dioxane as secondary internal reference which was calibrated against the official primary reference TMU (**Gonzalez et al. 1998**).

In the early stage of the application of SNIF-NMR no official reference was available, discrimination was achieved using relative intramolecular ^2H profile calculated from the ^2H spectra as the relative $R_{i/j}$ ratios ($R_{i/j} = f_j \times [S_i/S_j]$ where S is the signal height), which represent the actual number of deuterium atoms in site i with respect to site j, which is arbitrarily given its stoichiometric number of hydrogens, f_j. This approach was efficient to discriminate the origins of trans-anethole, i.e. natural: anise, fennel, star anise, semi-synthetic: estragole from turpentine, synthetic: from anisole (**Martin et al. 1982**). Similarly, origins of benzaldehyde could be also separated (**Hagedorn 1992**). Nowadays, the concept of isotope profiles which compares the relative amounts of different isotopomers of a sample on is still pertinent when calibrated chemical reference cannot be used and when classification is the purpose of the study, not the isotope affiliation which describes isotope movement from the initial reactants to the final product in the history of the formation of a compound.

Derivatization of the target molecule: the case of cinnamaldehyde. Cinnamaldehyde molecule is the main component of cinnamon oils. In order to identify the origin of the oil and detect possible adulteration with synthetic products, SNIF-NMR was considered. Unfortunately the ^2H NMR spectrum of cinnamaldehyde showed overlapping signals leading to a restricted number of $(^2H/^1H)_i$. Since a source of synthetic cinnamaldehyde is benzaldehyde, the retroaldolization could transform efficiently the cinnamaldehyde into benzaldehyde that could then be the molecular probe (**Remaud et al. 1997b**). Interestingly, benzaldehyde is also the main compound of bitter almond oil, thus its authenticity could also be checked. Benzaldehyde can be mainly obtained in four ways (see **Figure 7**). Reaction 1 refers to the natural origin of benzaldehyde from hydrolysis of amygdalin extracted

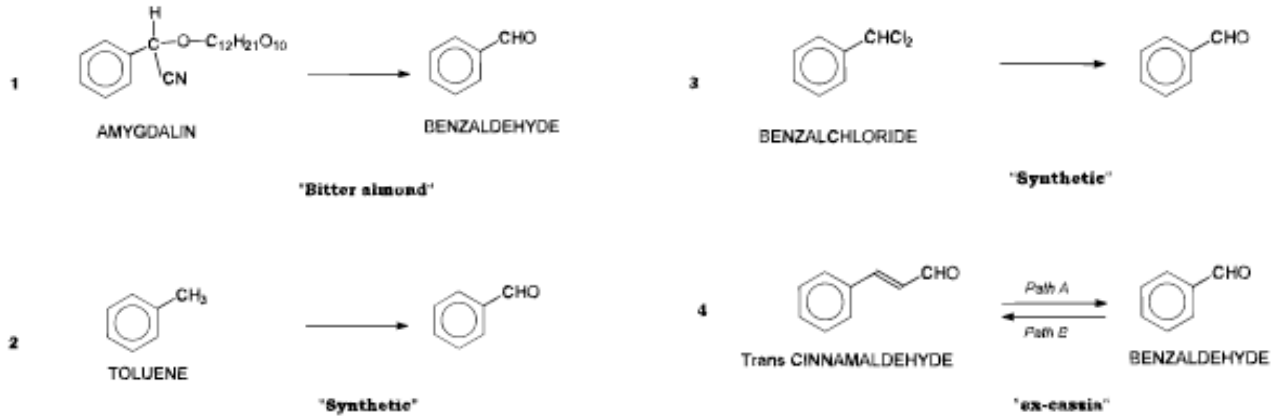

Figure 7. Main pathways for the production of "natural" and "synthetic" benzaldehyde. For pathway 4, the double arrow does not indicate a chemical equilibrium but only the possibility to produce benzaldehyde from *trans*-cinnamaldehyde and *vice-versa* (paths A and B). Figure adapted from *J Agric Food Chem.* 1997; 45:4042, with permission from ACS.

from bitter almonds. Reactions 2 and 3 are the two most important industrial processes for manufacturing benzaldehyde from toluene and benzalchloride, respectively. Benzaldehyde "ex-cassia" is obtained by reaction 4 (path A). In 1997, from the ^{2}H NMR measurement of a set of 65 samples, three specific isotope parameters were calculated: i) site-specific isotopic ratio $(^2H/^1H)_i$; ii) molar fractions of the monodeuterated isotopomers f_i, which describe the natural distribution of ^{2}H in the different molecular sites; and iii) the relative $R_{i/j}$ ratios. Adopting a principal component analysis (PCA) (see **Figure 8**), the samples of different origin can be distinguished clearly, thus demonstrating again that the ^{2}H-NMR is a powerful tool in term of authentication and quantification of adulteration (**Remaud et al. 1997b**). The study showed also that it was possible to detect as little as 10-15% of synthetic cinnamaldehyde in cinnamon oil. The obtained isotopic data of the natural samples can be used in the isotopic database.

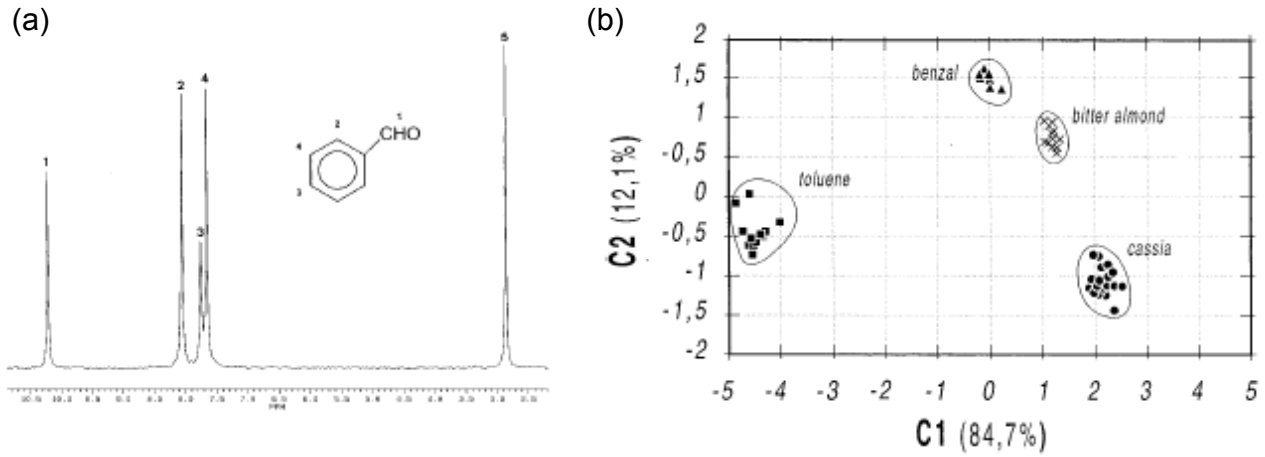

Figure 8. (a) Example of 76.7 MHz ^{2}H SNIF-NMR spectrum of benzaldehyde in presence of TMU (peak 5). (b) Results of PCA carried out on the 65 samples of benzaldehydes performed with molar fractions F_i and relative $R_{i/j}$ ratios ($R_{2/1}$, $R_{3/1}$, $R_{4/2}$, and $R_{4/3}$). Figures adapted from *J Agric Food Chem.* 1997; 45:4042, with permission from ACS.

How to handle very toxic and/or corrosive product: the example of allyl isothiocyanate?

Allyl isothiocyanate (H_2C=CH-CH_2-NCS) is the major component of the mustard oil (**Remaud et al. 1997c**). As vanillin, this molecule can be synthesized at a much lower price than its cost when extracted from mustard seeds. Adulteration of natural mustard oil by adding synthetic allyl isothiocyanate is therefore very profitable. This molecule (a liquid) is highly lachrymator land corrosive. As ^{2}H NMR requires relatively large amount > 1 mL caution should be taken for routine analysis for the protection of both the operator and the spectrometer in case of breaking NMR tube. A safe approach is to use of Teflon tube (Teflon liners) inserted in a classical 10-mm o.d. NMR tube. Interestingly this molecule contains four atomic elements leading to four pairs of stable isotopes ($^{2/1}$H, $^{13/12}$C, $^{15/14}$N and $^{34/33}$S) that can be exploited in an authentication workframe. Thus to detect such a fraud, quantitative ^{2}H NMR (see **Figure 9a**) or IRMS have been used (**Remaud et al. 1997c**). However the combination of SNIF-NMR and multi-isotope IRMS analysis leads to much more reliable results in terms of discrimination (natural/synthetic origin) as well as the origin of mustard seed (geographical determination). **Figure 9b** shows the results obtained by the principal component analysis performed on two analytical isotope parameters of a series of allyl isothiocyanates of natural origin (mustard seeds from Canada and India) or produced chemically: i) the standard parameters δ^{13}C, δ^{15}N and δ^{34}S determined as $\delta^{heavy}X$ (‰) = [($^{heavy}X/\ ^{light}X)_{sample}$/($^{heavy}X/\ ^{light}X)_{ref}$ - 1] × 1000) where $^{heavy}X$ and $^{light}X$ are the heavy and light isotopes of an element X, respectively; ii) the ratios $R_{2/1}$ and $R_{3/1}$ ($R_{i/j}$ = f_j × [S_i / S_j], see above) (**Remaud et al. 1997c**).

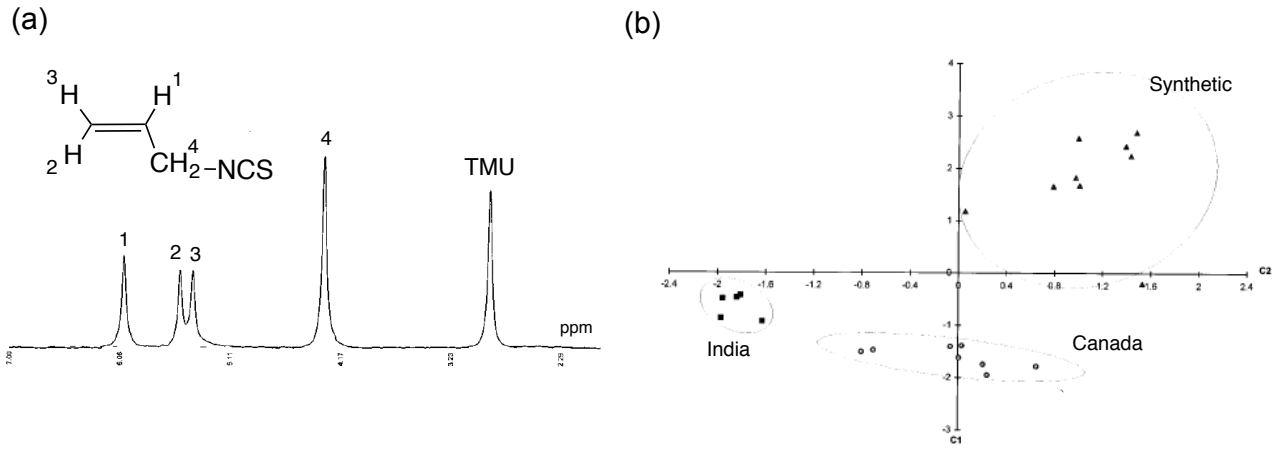

Figure 9. (a) 79.7 MHz ^{2}H SNIF-NMR spectrum of isothiocyanate in presence of TMU. (b) Principal component analysis performed on the analytical parameters δ^{13}C, δ^{15}N, δ^{34}S, $R_{2/1}$, and $R_{3/1}$ of allyl isothiocyanates of natural origin (mustard seeds from Canada and India) or produced chemically. The results are represented in the plane of the two main components, C1 and C2 (Principal Components Analysis). The ellipses represent the 99% bivariate confidence intervals. Figures reprinted from *J Agric Food Chem*. 1997; 45:1844, with permission from ACS.

IV. Defining biosynthesis mechanisms: interpretation of the intramolecular ^2H distribution

^2H affiliation from substrate to products. As described above, the detection of chaptalisation and identification of the origin of wine, beverage and fruit juice are based on the site-specific deuterium isotopic ratio, $(^2H/^1H)_i$ which is related to the site i isotopomer content of the ethanol derived from sugars (sucrose, glucose and fructose) of different vegetal and geographical origin. How can ethanol be a probe of the origin of its carbohydrate precursor? Understanding the isotope affiliation and fractionation mechanism in the biotransformation is essential for correct and better application of the SNIF-NMR method in food origin identification.

In the alcoholic fermentation of sugars by yeast, the main product is ethanol, of which the carbon source is the sugar, while the hydrogen sources are the carbon-bound unexchangeable hydrogen and the medium, which contains the hydrogen atoms of water and those of the hydroxyls of sugars. Since the natural abundance isotopic ratios of the unexchangeable hydrogen of sugars and of water depend on their physiological and environmental conditions of photosynthesis, there should be a connexion between the isotopic distribution in the alcoholic fermentation products and that of the carbohydrate precursors and fermentation medium (**Martin et al. 1986**).

The isotopic connexion between the substrate, the medium and the products in an alcoholic fermentation can be described by a mathematical model (**Martin et al. 1986**; **Zhang et al. 1995; Pionnier et al. 2003**). In a complex multi-step bio-organic transformation, the hydrogen (^1H, ^2H) atoms of site j of a reactant (substrate) and the reaction medium (m, water) are transferred to site i of a product. The hydrogen atoms at the prostereogenic positions of ethanol may be of different origin and have different isotopic ratios. Their isotopic data can be used in origin identification purpose. This was confirmed in the study. The two methylene monodeuterated enantiomers, (*R*)- and (*S*)-1-[$^2H_1$]-ethanol signals were separated and measured through transformation into two diastereoisomers.

A transfer matrix can be then defined that links the isotopic content $(^2H/^1H)_i$ at each position *i* of the product(s) to the isotopic content $(^2H/^1H)_j$ at each position *j* of the reactant (substrate) and to $(^2H/^1H)_m$, the isotopic content of the water of the fermentation medium as following:

$$(^2H/^1H)_i = a_{im}(^2H/^1H)_m + \sum_j a_{ij}(^2H/^1H)_j \qquad (4)$$

where $(^2H/^1H)_i$ is the isotopic ratio at position *i* of the product, $(^2H/^1H)_m$ is the isotopic ratio of the medium and $(^2H/^1H)_j$ is the isotopic ratio at site *j* of the substrate. The terms a_{im} and a_{ij} are isotope redistribution coefficients. **Equation 4** shows that $(^2H/^1H)_i$ is a linear function of the specific isotopic ratios of the carbon-bound hydrogen positions of the substrate and of the medium (see **Figure 10**).

The terms a_{im} and a_{ij} are isotope redistribution coefficients when hydrogen is transferred from the medium (m) and site j of the substrate molecule to site i of the product molecule, respectively. Index k

in **Equation 5** may be m or j. Since there are also several hydrogen sites in the product, the model can be expressed in matrix notation:

$$(^2H/^1H)_i = [A] \times (^2H/^1H)_k \quad (5)$$

where $(^2H/^1H)_i$ and $(^2H/^1H)_k$ are the column vectors of the site-specific natural isotopic ratios of the product and the substrate and medium, and [A] the redistribution matrix (**Martin et al. 1986; Pionnier et al. 2003**). The key for understanding the isotopic connection between the substrate and product molecules is the determination of the matrix [A] that characterizes the specific genealogies of the deuterium atoms. It also depends on the complex isotope effects during the biochemical reactions.

Establishing the matrix [A] involves a detailed study of the deuterium transfer mechanism in the considered bio-organic reaction. In the study, an experimental method of quantitative isotope tracing close to the natural abundance has been developed (**Zhang and Yunianta 1995; Pionnier et al. 2003**). As $(^2H/^1H)_i$ is a linear function of $(^2H/^1H)_j$ or $(^2H/^1H)_m$ when only m or a given j is considered (see **Equation 6**), isotope redistribution coefficients a_{ik} (a_{im} or a_{ij}) and the intercept b can be established using only two different $(^2H/^1H)_i$ values and their corresponding $(^2H/^1H)_j$ or $(^2H/^1H)_m$ values.

$$(^2H/^1H)_i = a_{ik}(^2H/^1H)_k + b \quad (6)$$

Then, by changing the labelling positions (m and j) of the starting materials in a set of experiments, the matrix [A] can be determined.

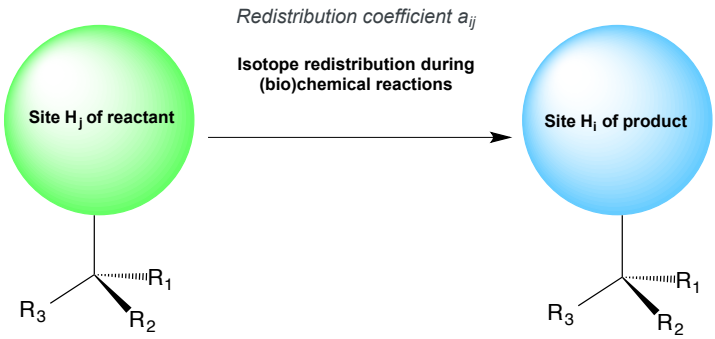

Figure 10. Schematic description of the principle of the isotope redistribution during a (bio)chemical reaction.

Isotope labelling near the natural abundance. Several sugars, such as glucose, fructose and sucrose can be used as substrate in the fermentation. Since fructose can be considered as an intermediate in glycolysis, of which the first step is the conversion of glucose 6-phosphate (G6P) to fructose 6-phosphate (F6P), and since sucrose contains both glucose and fructose, it is most useful to study the isotope behavior of glucose that has 7 unexchangeable hydrogen sites. In the experiment with substrate (glucose) labelling, a substrate containing isotope at natural abundance was used as reference. Its natural site-specific isotopic ratios should be known or not. The site-specific labelling of substrate was achieved by adding a very small amount of substrate deuterated at site j to the reference substrate. The addition of a site j deuterated substrate to the reference only increased the $(^2H/^1H)_j$ of this site, while the specific isotopic ratios of other sites remained unchanged. The variation of the

$(^2H/^1H)_j$ was evaluated on the basis of the mass of the added enriched substrate obtained by weighing, with a correction for its purity (see above). All reactions with the reference substrate and labelled substrates were performed in natural tap water (reference). In medium labelling experiments, only the reference substrate (glucose) was used and the reaction should be carried out in two water media of different deuterium contents. It is preferable to use deuterium-depleted water as reference since the intercept value can be determined more precisely. Natural tap water (or any water of which isotopic ratio is well known) can be used as reference too, while the enriched water was prepared by adding very small amount of D_2O into the tap water. $(^2H/^1H)_m$ of the medium should be known. The $(^2H/^1H)_i$ of product samples produced in the isotopically different mediums and substrates under controlled fermentation conditions were measured by ^2H NMR.

Analysis of the isotope redistribution coefficient a_{im}. The redistribution coefficient $a_{im(j)}$ contains mechanistic and isotope fractionation information. Indeed**,** **Equation 4** is equivalent to the following form:

$$\left(\frac{^2H_m + \sum_j {}^2H_j}{^1H_m + \sum_j {}^1H_j}\right)_i = \left(\frac{^1H_m}{^1H_m + \sum_j {}^1H_j}\right)_i f_m^{IE} \left(\frac{^2H_m}{^1H_m}\right) + \left(\frac{\sum_j {}^2H_j}{^1H_m + \sum_j {}^1H_j}\right)_i \quad (7)$$

where

$$\left(\frac{^2H_m + \sum_j {}^2H_j}{^1H_m + \sum_j {}^1H_j}\right)_i = (^2H/^1H)_i\,, \quad \left(\frac{^2H_m}{^1H_m}\right) = (^2H/^1H)_m\,, \quad \left(\frac{^1H_m}{^1H_m + \sum_j {}^1H_j}\right)_i f_m^{IE} = a_{im}$$

and

$$\left(\frac{\sum_j {}^2H_j}{^1H_m + \sum_j {}^1H_j}\right)_i = \sum_j a_{ij}(^2H/^1H)_j$$

In this equation, $\frac{^1H_m}{^1H_m + \sum_j {}^1H_j}$ is the hydrogen (≈ its major isotope: protium, ^1H) transfer mechanistic term and represents the percentage of medium ^1H atoms on site i of the product. Its value depends on the reaction pathway and f_m^{IE} is isotope effect term. This latter depends on the global equilibrium and/or kinetic deuterium isotope effects in the course of hydrogen transfer from the medium to site i of the product. f_m^{IE} is equal to 1 if there is no global isotope effect or the effect can be neglected. $f_m^{IE} < 1$ if the global isotope effect is normal. $f_m^{IE} > 1$ if the global isotope effect is inverse. As a reminder, in a reaction, the light isotopologue reacts faster or progresses better than the heavy isotopologue, the

isotope effect is normal. On the contrary, the effect is inverse. In fact, f_m^{IE} = [k(^{1}H) / k(^{2}H)]$^{-1}$ or [K(^{1}H) / K(^{2}H)]$^{-1}$. When site i of the product is exclusively constituted of the medium hydrogen, **Equation 4** becomes

$$(^2H/^1H)_i = a_{im}(^2H/^1H)_m + 0 \quad (8)$$

and **Equation 7** becomes

$$\left(\frac{^2H_m}{^1H_m}\right)_i = \left(\frac{^1H_m}{^1H_m}\right)_i f_m^{IE}\left(\frac{^2H_m}{^1H_m}\right) + 0 \quad (9)$$

and

$$a_{im} = \left(\frac{^1H_m}{^1H_m}\right)_i f_m^{IE} = f_m^{IE}$$

Since the amount of medium (water) is in large excess, f_m^{IE} = [k(^{1}H)/k(^{2}H)]$^{-1}$ or [K(^{1}H)/K(^{2}H)]$^{-1}$ where k is the rate constant and K is the equilibrium constant of the reaction in which the medium hydrogen atoms are transferred to site i of the product) (**Zhang et al. 2001**).

Analysis of the isotope redistribution coefficient, a_{ij}. When one site j of the reactant is considered, the other site are j'. **Equation 4** is equivalent to the following form:

$$\left(\frac{^2H_m + \sum_j {}^2H_j}{^1H_m + \sum_j {}^1H_j}\right)_i = \left(\frac{^1H_j}{^1H_m + \sum_j {}^1H_j}\right)_i f_j^{IE}\left(\frac{^2H_j}{^1H_j}\right) + \left(\frac{^2H_m + \sum_{j' \neq j} {}^2H_{j'}}{^1H_m + \sum_j {}^1H_j}\right)_i \quad (10)$$

where : $\left(\frac{^2H_m + \sum_j {}^2H_j}{^1H_m + \sum_j {}^1H_j}\right)_i = (^2H/^1H)_i$, $\left(\frac{^2H_j}{^1H_j}\right) = (^2H/^1H)_j$ and $\left(\frac{^1H_j}{^1H_m + \sum_j {}^1H_j}\right)_i f_j^{IE} = a_{ij}$

and with : $\left(\frac{^2H_m + \sum_{j' \neq j} {}^2H_{j'}}{^1H_m + \sum_j {}^1H_j}\right)_i = a_{im}(^2H/^1H)_m + \sum_{j' \neq j} a_{ij'}(^2H/^1H)_{j'}$.

The term $\left(\frac{^1H_j}{^1H_m + \sum_j {}^1H_j}\right)_i$ is the hydrogen (^{1}H) transfer mechanistic term and represents the percentage of the reactant site j ^{1}H atoms on site i of the product. Its value depends on the reaction pathway. f_j^{IE} is deuterium isotope effect term, expressed as follows:

$$f_j^{IE} = \frac{(^2H_j/^1H_j)_i}{(^2H/^1H)_j} = \frac{(^1H/^2H)_j}{(^1H_j/^2H_j)_i} = \alpha. \quad (11)$$

In **Equation 11**, α is the isotopic fractionation factor, (^{2}H$_j$/^{1}H$_j$)$_i$ is the isotopic ratio of hydrogen from site j of the substrate at site i of the product (this ratio cannot be determined except in special case) (^{2}H/^{1}H)$_j$

is the isotopic ratio at site j of the substrate. Ratio $(^2H_j/^1H_j)_i$ and $(^2H/^1H)_j$ may be different due to isotope effects.

Isotopic fractionation is often described as *"preferential enrichment of one isotope relative to another in a chemical or physical process"*. But the real situation is not so simple. In isotope study by NMR, we study isotopomers. Isotopic fractionation should be considered as a simplified description of "isotopologue/isotopomer fractionation", because in most cases, especially for organic molecules, isotopes do not exist as single atoms but in isotopologue and isotopomers. In isotopic fractionation of a system, not only the global isotope content changes but also the intramolecular isotopic distribution changes. Fractionation can be observed between all different isotopologue and isotopomers of a compound. Due to different isotope substitution, the isotopologue and isotopomers of a compound may exhibit different physical behavior causing isotopologue/isotopomer fractionation. The isotopologue/isotopomer behavior is not the behavior of an isotope atom but of the isotopomer molecule as a whole. For a compound, isotopologues of different masses or of the same mass (such as CH_3CH_2OH, $CH_2{}^2HCH_2OH$, CH_3CH^2HOH, $CH_3CH_2O^2H$, $^{13}CH_3CH_2OH$ and $CH_3{}^{13}CH_2OH$ of ethanol), may have different fractionation behaviors. Their fractionations are different in chemical, physical and biological process. It can always be observed that *"preferential enrichment of one* ***isotopologue****/isotopomer relative to another in a chemical, physical or biological process"*. For example, the isotopic fractionation factor, α, of different ^2H isotopomers of ethanol in liquid/vapor equilibrium is equal to 0.9967 (for $CH_2{}^2HCH_2OH$), 0.9977 (for CH_3CH^2HOH), and 1.0216 (for $CH_3CH_2O^2H$) **(Zhang et al. 2002a)**.

When all protium and deuterium atoms of site j of the reactant are transferred exclusively to site i of the product, there is no isotope fractionation, and hence f_j^{IE} = 1. When site i of the product is exclusively constituted by hydrogen of the reactant site j which only transfers part of its protium and deuterium atoms to site i, $a_{ij} = f_j^{IE}$.

From the data obtained in the study, the numerical form of **Equation 5** was established for ethanol biosynthesis in glucose fermentation (some a_{ij} values are the mean of that obtained in repeated work). (**Pionnier et al. 2003**):

$$\begin{pmatrix} 0.127 & 0.09 & 0 & 0 & 0 & 0.155 & 0.155 & 0.175 \\ 0 & 0 & 0 & 0.088 & 0 & 0 & 0 & 0.78 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0.78 \end{pmatrix} \begin{pmatrix} (^2\mathrm{H}/^1\mathrm{H})_1 \\ (^2\mathrm{H}/^1\mathrm{H})_2 \\ (^2\mathrm{H}/^1\mathrm{H})_3 \\ (^2\mathrm{H}/^1\mathrm{H})_4 \\ (^2\mathrm{H}/^1\mathrm{H})_5 \\ (^2\mathrm{H}/^1\mathrm{H})_{6\mathrm{pro}\text{-}R} \\ (^2\mathrm{H}/^1\mathrm{H})_{6\mathrm{pro}\text{-}S} \\ \\ (^2\mathrm{H}/^1\mathrm{H})_{\mathrm{m}} \end{pmatrix} = \begin{pmatrix} (^2\mathrm{H}/^1\mathrm{H})_{\mathrm{CH_3}} \\ (^2\mathrm{H}/^1\mathrm{H})_{\mathrm{CH_{pro}\text{-}R}} \\ (^2\mathrm{H}/^1\mathrm{H})_{\mathrm{CH_{pro}\text{-}S}} \end{pmatrix} \qquad (12)$$

The results are in good accordance with the glycolysis and ethanol formation mechanism (see **Figure 11**). Thus the hydrogen of the methyl site are strongly connected to the carbon bound hydrogens 1, 2,

6 and 6' of glucose, while those of the methylene site come mainly from fermentation water and site 4 of glucose.

Some isotopic fractionation information can be obtained from a_{im}. Unfortunately, with the available data it is difficult to evaluate all the kinetic or equilibrium isotope effects in hydrogen transfer from the medium to the methyl or methylene of ethanol. At position i of ethanol, there are deuterium atoms (^2H) and protium atoms (^1H) from the unexchangeable sites of glucose (s) and the medium (m)

$$(^2H/^1H)_i = \frac{^2H_i}{^1H_i} = \frac{^2H_{i,s} + ^2H_{i,m}}{^1H_{i,s}' + ^1H_{i,m}'} = \left(\frac{^2H_m + \sum_j {}^2H_j}{^1H_m + \sum_j {}^1H_j}\right) \quad (13)$$

If we characterize the complex isotope effects by a global isotopic fractionation factor (IFF = $1/f_m^{IE}$ = $k(^1H)/k(^2H)$ or $K(^1H)/K(^2H)$) during the hydrogen transfer from the medium to site i of the product. **Zhang and Pionner. 2001**)

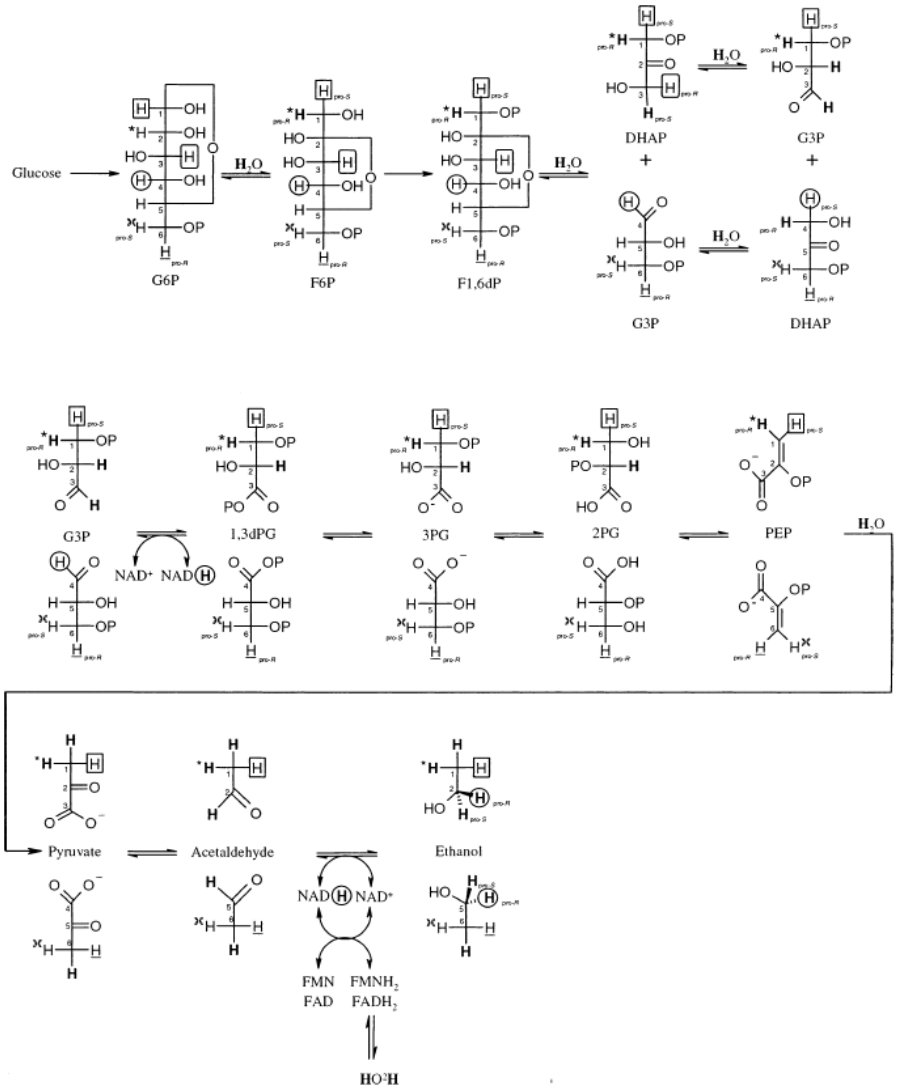

Figure 11. Hydrogen affiliation in biotransformation of glucose into ethanol. Figure reproduced from *J Agric Food Chem* 2003; 51:2076, with permission from ACS.

$$IFF_{i,m} = \frac{(^2H/^1H)_m}{(^2H/^1H)_{i,m}} = \frac{(^2H/^1H)_m}{^2H_{i,m}/^1H_{i,m}} \quad (14)$$

In the experiments with labelled water (medium), we have:

$$(^2H/^1H)_i = \frac{^2H_{i,s}+^2H_{i,m}}{^1H_{i,s}+^1H_{i,m}} = a_{im}\left(\frac{^2H}{^1H}\right)_m + \frac{^2H_{i,s}}{^1H_i} \quad (15)$$

with $^1H_i = {}^1H_{i,m} + {}^1H_{i,s}$, and $\frac{^2H_{i,s}}{^1H_i} = \left(\frac{\sum_j {}^2H_j}{^1H_m + \sum_j {}^1H_j}\right) = \sum_j a_{ij}\left(\frac{^2H}{^1H}\right)_j$.

After rearrangement, **Equation 15** becomes

$$\frac{^2H_{i,m}}{^1H_{i,s}+^1H_{i,m}} = a_{im}(^2H/^1H)_m \quad (16)$$

From **Equation 16**, we have

$$\frac{1}{a_{i,m}} = \frac{(^2H/^1H)_m}{^2H_{i,m}/(^1H_{i,s}+^1H_{i,m})} > \frac{(^2H/^1H)_m}{^2H_{i,m}/^1H_{i,m}} = IFF_{i,m} \quad (17)$$

Although $H_{i,m}$ and $H_{i,s}$ are unknown, we have:

$$IFF_{i,m} < 1/a_{im} \quad (18)$$

as in the case of i = $CH^{pro\text{-}R}$, on this site C-4 hydrogen of glucose was found and $IFF_{pro\text{-}RCH,m} = 1/f_m^{IE} < 1/0.78 = 1.28$. In contrast, whether $^2H_{i,s}$ and $^1H_{i,s} = 0$ (no hydrogen transferred from the substrate to i), for example, when i = $CH^{pro\text{-}S}$, **Equation 8** can be applied and $IFF_{i,m} = 1/f_m^{IE} = 1/a_{im} = 1/0.78 = 1.28$ (**Pionnier et al. 2003**). It can be concluded that the global isotope fractionation during the transfer of hydrogen from medium to the *S*-enantiomer is normal (protium is more easily transferred than deuterium) and is more significant than that of *R*-enantiomer formation.

The complete mathematical model representing the hydrogen isotope affiliation during alcoholic fermentation can provide not only information on the reaction mechanism but can also be used to estimate the isotopic data of the product, based on those of the substrate and the medium. For the commercial corn glucose and Nantes tap water used in the work as references, the calculation of the $(^2H/^1H)_i$ of ethanol and glycerol based on the $(^2H/^1H)_j$ of the glucose and $(^2H/^1H)_m$ are shown in **Tables 4** and **5**. Taking into account the uncertainty in determining $(^2H/^1H)_i$, $(^2H/^1H)_j$, $(^2H/^1H)_m$ and a_{ik}, the agreement between the calculated and experimental results is satisfactory. (**Pionnier et al. 2003**)

Table 4. Site-specific isotopic ratios, $(^2H/^1H)_j$, of a corn (C_4) glucose used for alcohol fermentation

Glucose site j	**1**	**2**	**3**	**4**	**5**	**6 (pro-*S*)**	**6(pro-*R*)**
$(^2H/^1H)_j$ (ppm)	173.8	156.7	146.4	140.3	145.4	151.7	142.7

Table 5. Calculated and experimental $(^2H/^1H)_i$ of the products in the fermentation of a corn glucose[a] in Nantes tap water[b]

	Ethanol				Glycerol	
$(^2H/^1H)_i$	$(^2H/^1H)_{CH_3}$	$(^2H/^1H)_{CH}^{pro\text{-}R}$	$(^2H/^1H)_{CH}^{pro\text{-}S}$	$(^2H/^1H)_A$	$(^2H/^1H)_B$	$(^2H/^1H)_C$
Calculated	108.0	128.9	116.5	144.9	151.8	94.7
Experimental	111.1	134.2	112.2	143.3	160.7	99.6

[a]For the site-specific isotopic ratios of the glucose, see **Table 4**. [b]In Nantes tap water $(^2H/^1H)_m$ = 149.4 ppm.

In fermentation, besides the main product ethanol (95-97%), there is a minor product, glycerol (3-5%) whose biosynthesis is also related to the glycolysis process. The corresponding matrix can be simply expressed as (**Zhang et al. 2000; Pionnier and Zhang. 2002**):

$$\begin{pmatrix} 0 & 0.156 & 0 & 0.03 & 0 & 0.27 & 0. & 0.52 \\ 0.22 & 0 & 0.075 & 0 & 0 & 0 & 0.243 & 0.44 \\ 0 & 0 & 0 & 0.1 & 0 & 0 & 0 & 0.54 \end{pmatrix} \begin{pmatrix} (^2H/^1H)_1 \\ (^2H/^1H)_2 \\ (^2H/^1H)_3 \\ (^2H/^1H)_4 \\ (^2H/^1H)_5 \\ (^2H/^1H)_{6pro\text{-}R} \\ (^2H/^1H)_{6pro\text{-}S} \end{pmatrix} = \begin{pmatrix} (^2H/^1H)_A \\ (^2H/^1H)_B \\ (^2H/^1H)_C \end{pmatrix} \quad (19)$$

For glycerol, three sites can be distinguished by ^2H NMR: A: (1*S*,2*S*)- and (1*R*,2*R*)-(1-$^2H_1$)glycerol, B: (1*R*,2*S*)- and (1*S*,2*R*)-(1-$^2H_1$)glycerol and C: (2-$^2H_1$)glycerol. In the two biosynthesis routes, the transfer pathways of 1,2 and the two 6 hydrogen sites are very similar. The 3 and 4 hydrogen sites are different. This is also in agreement with the reaction mechanism. The calculated and experimental isotope data are also in good accordance in this case.

In studying the hydrogen isotope balance in the biotransformation, the conversion rates of deuterated glucose molecules to ethanol can be evaluated using the a_{ij} parameter. In the fermentation, n_{RH} totally protiated glucose molecules are completely converted to $2n_{RH}$ ethanol molecules.

$$\underset{n_{RH}}{\text{Glucose } (^1H)} \longrightarrow \underset{2n_{RH}}{2 \text{ Ethanol } (^1H)}$$

n_{RD} site j monodeuterated glucose molecules were converted to xn_{RD} site i monodeuterated ethanols, where x is the conversion rate of site j deuterated glucose ($0 \le x \le 1$).

$$\underset{n_{RD}}{(j\text{-}^2H_1) \text{ Glucose}} \longrightarrow \underset{xn_{RD}}{x(i\text{-}^2H_1) \text{ Ethanol } (^2H_j)}$$

These deuterated ethanol molecules are denoted by ^2H_j. The other totally protiated moiety of the deuterated glucose may become ethanol. As their quantity is very small, it can be neglected. According

to the definition of $(^2H/^1H)_i = N_{Di}/(P_iN_H)$, for site j of glucose since $P_j = 1$ for j = 1, 2, 3, 4, $6^{pro\text{-}R}$ and $6^{pro\text{-}S}$, we have:

$$(^2H/^1H)_j = n_{RD} / n_{RH} \quad (20)$$

For site i of ethanol, we have:

$$(^2H_j/^1H)_i = xn_{RD} / (P_i.2n_{RH}). \quad (21)$$

It should be noted that $(^2H_j/^1H)_i \neq (^2H/^1H)_i$ because ^2H_j is produced only by site j deuterated glucose molecule, while the deuterium of site i of ethanol may also be of other origins. Dividing **Equation 21** by **Equation 20** gives thus:

$$(^2H_j/^1H)_i / (^2H/^1H)_j = x / (2P_i) \quad (22)$$

with $(^2H_j/^1H)_i = (^2H/^1H)_i$ - [the intercept of $(^2H/^1H)_i$ vs. $(^2H/^1H)_j$] = b in **Equation 6**, because the intercept is the specific isotopic ratio of site i of ethanol concerning only the deuterium of other sites k ≠ j of glucose and of the medium. Thus $(^2H_j/^1H)_i/(^2H/^1H)_j = a_{ij}$, and the conversion rate x of site j of deuterated glucose molecule to site i of deuterated ethanol molecule can be evaluated with:

$$x(\%) = 2P_ia_{ij} \times 100. \quad (23)$$

It should be emphasized that the site-specific conversion rates of deuterium may be different from that of protium due to isotope effects ($f_j^{IE} \neq 1$). Interestingly, only deuterium conversion rate can be evaluated while the exact protium conversion rate is unknown. Only when isotope effects are neglected, the two conversion rates can be considered as equivalent.

The conversion rates of deuterated glucose isotopomers were calculated (**Pionnier et al. 2003**) (see **Table 6**) The deuterium of the two C-6 hydrogen of glucose are near totally transferred to the ethanol methyl site. Most C-1 and about 50% of C-2 glucose deuterium atoms are also transferred to this site. Ethanol methylene deuterium mainly comes from the medium including exchangeable sites of glucose. These results explain why $(^2H/^1H)_{CH_3}$ should be used as the probe of carbohydrate precursor origin and not $(^2H/^1H)_{CH_2}$.

Table 6. Conversion rate of the deuterated glucose isotopomers in alcoholic fermentation

^{2}H site j of glucose	**^{2}H site i of ethanol**	**x (%)**
1	CH_3	84
2	CH_3	54
4	pro-R^{CH}	18
6 (pro-*R*)	CH_3	96
6 (pro-*S*)	CH_3	96

The order of $(^2H/^1H)_1$, $(^2H/^1H)_6^{pro\text{-}R}$ and $(^2H/^1H)_6^{pro\text{-}S}$ values is cane > grape > beet (see **Figure 12**) (**Zhang et al. 2002b; Zhou et al. 2018**). That is why we found that the $(^2H/^1H)_{CH_3}$ values of the corresponding ethanol are in the same order, namely cane (≈ 110 ppm) > wine (≈ 102 ppm) > beet (≈ 92 ppm).

The study proved in a detailed and quantitative way that the metabolites contained isotopic information about their precursor in a biotransformation and can be used for the identification of its origin. For example, $(^2H/^1H)_{CH_3}$ reflects mainly the isotopic data of sites 1, 6 and 6' of its precursor glucose. Hence when ethanol is used as probe, this is the main parameter in identification of sugar origin (**Martin et al 1986, 1996)**. The method of quantitative isotope tracing close to natural abundance established in the study of isotope affiliation can be applied to other chemical and biochemical reactions.

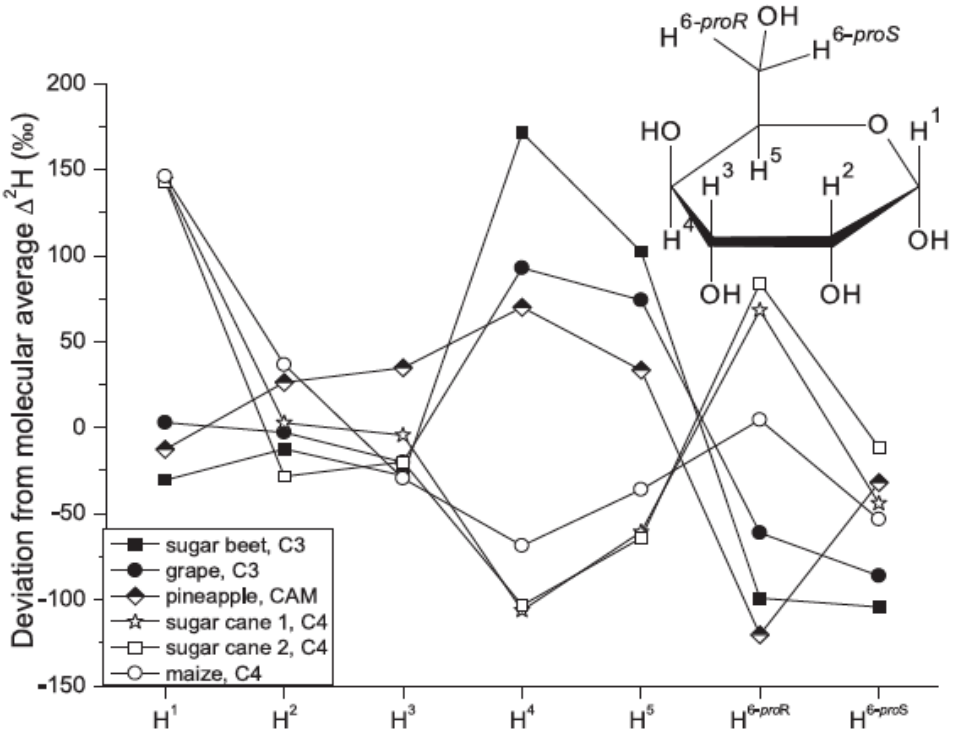

Figure 12. Relative site-specific hydrogen isotopic ratios of different glucose samples. The original $^2H/^1H$ data (in ppm) were converted to δ^2H using δ^2H (‰) = $[(^2H/^1H)_{sample}/(^2H/^1H)_{V\text{-}SMOW} - 1] \times 1000$, where $(^2H/^1H)_{V\text{-}SMOW}$ (Vienna-standard mean ocean water) is 155 ppm, and are expressed as deviation $\Delta(^2H)$ from the average of all seven C-H groups (carbon-bound hydrogen atoms) in glucose. Figure reprinted from *Phytochemistry,* 2018; 145:197, with permission from *Elsevier.*

V. Recent original ^2H NMR developments

Anisotropic irm-^2H 2D-NMR principles. As mentioned above, the analytical potential of isotropic irm-^2H 1D-NMR using routine liquid solvents ($CHCl_3$, DMF, …) is basically limited by two spectral specificities. First, the ^2H chemical shift dispersion (expressed in Hz) is 1/6.515 compared to ^1H, and so

can lead to a strong rate of peak overlaps even for middle-size molecules (**Lesot et al. 2004**). Increasing the strength of the magnetic field of NMR spectrometer, B_0, can reduce the overlaping effects, but cannot solve the case of fortuitously equivalent ^{2}H sites (namely without symmetry element) on the basis of ^{2}H chemical shifts, $\delta^{iso}(^2H)$. Second, the absence of chiral solvents (or chiral external entities) prevents the possibility to discriminate the ^{2}H NMR signal of enantiotopic C-H directions in CH_2 groups of prochiral molecules (C_s symmetry). Typically, this tool fails to determine the three (^{2}H/^{1}H) ratios of vanillin aromatic sites (**Texier-Bonniot et al. 2018**) or the two (^{2}H/^{1}H) ratios of the pro-*R*/pro-*S* enantiotopic hydrogen sites of ethanol (see above) (**Guillou 1991**) or BPTH (**Lesot et al. 2004)**. For more complex prochiral bioanalytes such as long-chain unsaturated fatty acid methyl ester (FAMEs) (**Lesot and Courtieu 2008**) or homogeneous triglycerides (**Lesot et al. 2012a**) only a limited number of deuteron sites are spectrally discriminating (even at 14.1 T) in isotropic solvents, thus significantly reducing the amount of isotopic information associated to each natural ^{2}H isotopomer. This lack of specific isotope data is clearly detrimental for a multi-factorial understanding of the stereoselectivity of enzymatic reactions (FAS and Desaturases) leading to this molecular family (**Baillif et al. 2009**).

In some cases, the ^{13}C ratio measured by NMR (irm-^{13}C NMR) for position-specific isotope analysis (PSIA-^{13}C) 1D-NMR can be used as complementary method for providing some additional isotopic information (**Remaud and Akoka 2016**). However, this tool does not give a direct access to the desired ^{2}H information that was missing. A chemical approach consisting to cleave the analyte into smaller molecular fragments is another option (**Lesot et al. 2004**). Nevertheless, this strategy can: i) be time-consuming or not always possible; ii) lead to loss of some isotope information or may also modify the natural isotopic fractionation, while the problem of the spectral discrimination of ^{2}H enantio-isotopomers associated with enantiotopic hydrogen sites remain not solve.

Another recent and original alternative consists in replacing the (classical) liquid solvents by lyotropic chiral liquid crystals (CLCs) such as organic solutions of polypeptide polymers, in order to detect all order-dependent (anisotropic) observables, such as the quadrupolar interaction that is specific for spin $I > ½$ (as deuterium) (**Lesot et al. 2009, 2020**). For ^{2}H nuclei, the quadupolar interaction induces a splitting of the single resonance observed in liquids (for a given deuteron) into two components (a deuterium quadrupolar doublet, noted ^{2}H-QD) This situation originates from a shift of energy levels when a ^{2}H nucleus is both submitted to Zeeman ($\boldsymbol{H}_Z$) and quadrupolar interaction ($\boldsymbol{H}_Q$) (non-averaged to zero in anisotropic environments, as oriented solvents), as shown in **Figure 13**.

Combined with QUOSY-class (QUadrupole Order SpectroscopY) 2D-NMR (two dimensional) experiments instead of single-pulse ^{2}H-{^{1}H} 1D sequences, simplifying the spectral analysis, we benefit of interesting analytical advantages (**Lesot and Courtieu 2009**). From the irm-^{2}H NMR viewpoint, this combination can be considered as a change of paradigm for two main reasons. First, the anisotropic natural abundance deuterium (ANAD) NMR spectra is a sum of independent ^{2}H quadrupolar doublets (^{2}H-QD) corresponding to each inequivalent ^{2}H site; the splitting between components of QD (noted Dn_Q) being equal to the ^{2}H residual quadrupolar coupling (^{2}H-RQC) (**Lesot and Courtieu 2009**). From

an instrumental point of view, the achievement of cryogenic NMR probes has contributed considerably to the development and application of ANAD NMR spectroscopy in various analytical domains."

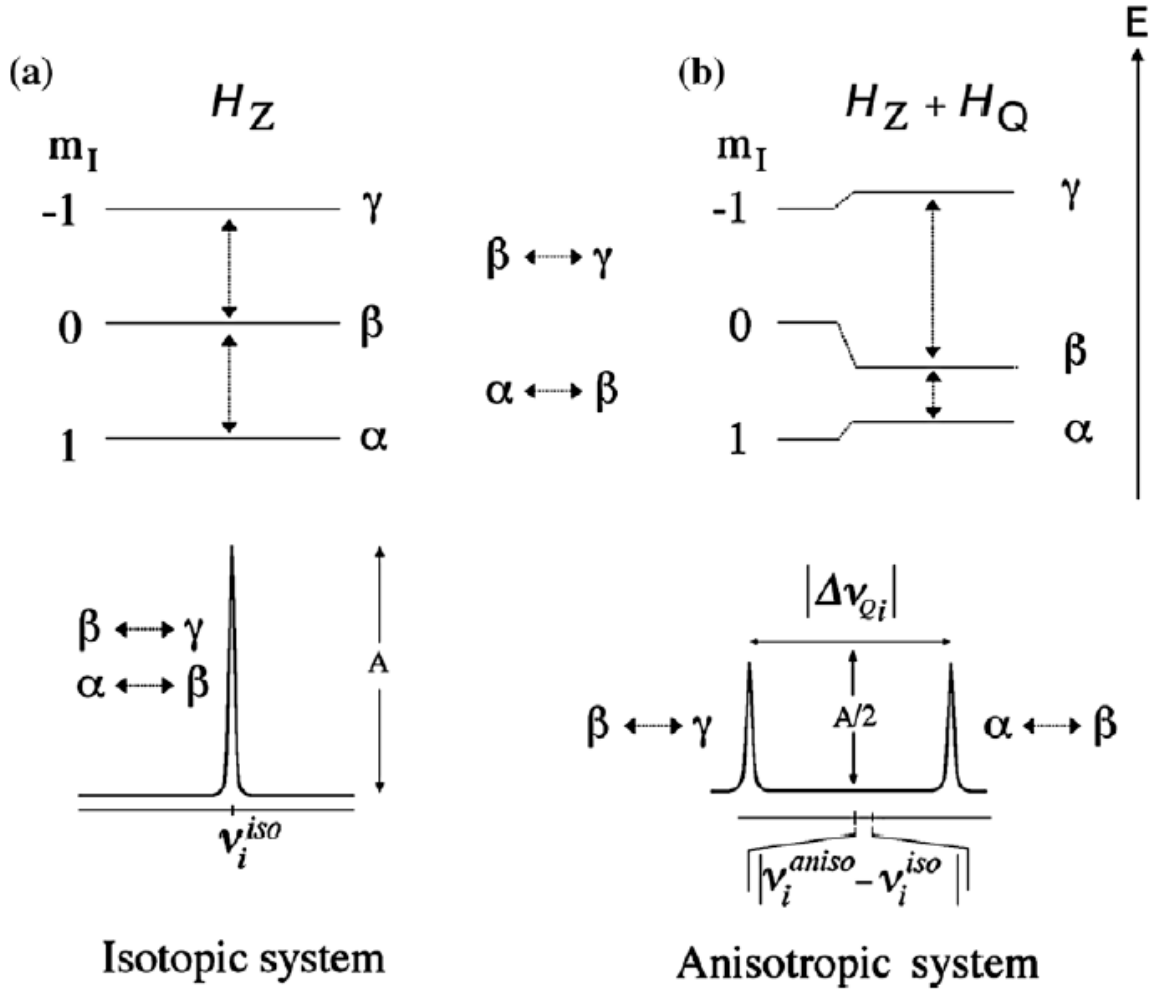

Figure 13. Variation of three energy levels ($I(^2H)$ = 1, with $m_I(^2H)$ = +1, 0, -1) of a single deuterium under the effect of (a) the Zeeman ($\boldsymbol{H}_Z$) in isotropic solvents and (b) the Zeeman and quadrupolar ($\boldsymbol{H}_Q$) interactions in anisotropic solvents. In oriented media, two distinct transitions existing in oriented solvents lead to two spectral components (a quadrupolar doublet) whose difference in frequency (Hz) is called quadrupolar splitting ($\Delta\nu_Q$). Figure reprinted from *Prog Nucl Magn Reson Spectrosc*. 2009; 55:128, with permission from Elsevier.

Interestingly, the variation of ^{2}H-RQCs and $\delta^{aniso}(^2H)$s for each site leads generally to a larger distribution of signals, thus reducing their ovelapping, while the analysis of 2D maps simplifies the identification/assignment of each QD. Second, in a CLC, the local order parameters (degree of alignment) of enantiotopic C–H directions in methylene groups are different, and imply that the natural monodeuterated (*R*/*S*)-enantio-isotopomers associated with pro-*R*/pro-*S* directions can be spectrally differentiated on the basis of ^{2}H-RQC ($\Delta\nu_Q^R \neq \Delta\nu_Q^S$), accordingly with

$$\Delta\nu_{Qi}^{R\text{ or }S} = \frac{3}{2} C_Q^{D_i} \times S_{C-D_i}^{R\text{ or }S} = \frac{3}{2}\left(\frac{eQ_{D_i} V_{C-D_i}}{h}\right) \times \left(\frac{1}{2}\left\langle 3\cos^2\left(\theta_{C-D_i}^{R\text{ or }S}\right) - 1\right\rangle\right). \quad (24)$$

where $C_Q^{D_i}$ is the ^{2}H-quadrupolar coupling constant and S_{C-D} is the order parameter of the C-^{2}H direction (value varying from 1 to -1/2), while Q_D is the ^{2}H nuclear electric quadrupole moment, V_{C-D} is the electric field gradient of deuteron (along the C-^{2}H internuclear direction) and θ_{C-D} is the angle between the magnetic field axis (B_0) and the C-^{2}H vector. Spectrally, the ordering difference between the C-D$^{R/S}$ directions leads to two ^{2}H-QDs mainly centered on the same chemical shift ($\delta^{aniso}(^2H)^R \approx \delta^{aniso}(^2H)^S \approx \delta^{iso}(^2H)^{R/S}$), as schematically depicted in **Figure 14** (**Lesot et al. 2011**).

Contrarily classical (isotropic) method, it is possible to control/optimize of the chiral mesophase parameters (degree of alignment and magnitude of enantiodiscriminations) by changing the composition and temperature of the sample, the nature of polypeptide or the polarity of organic co-

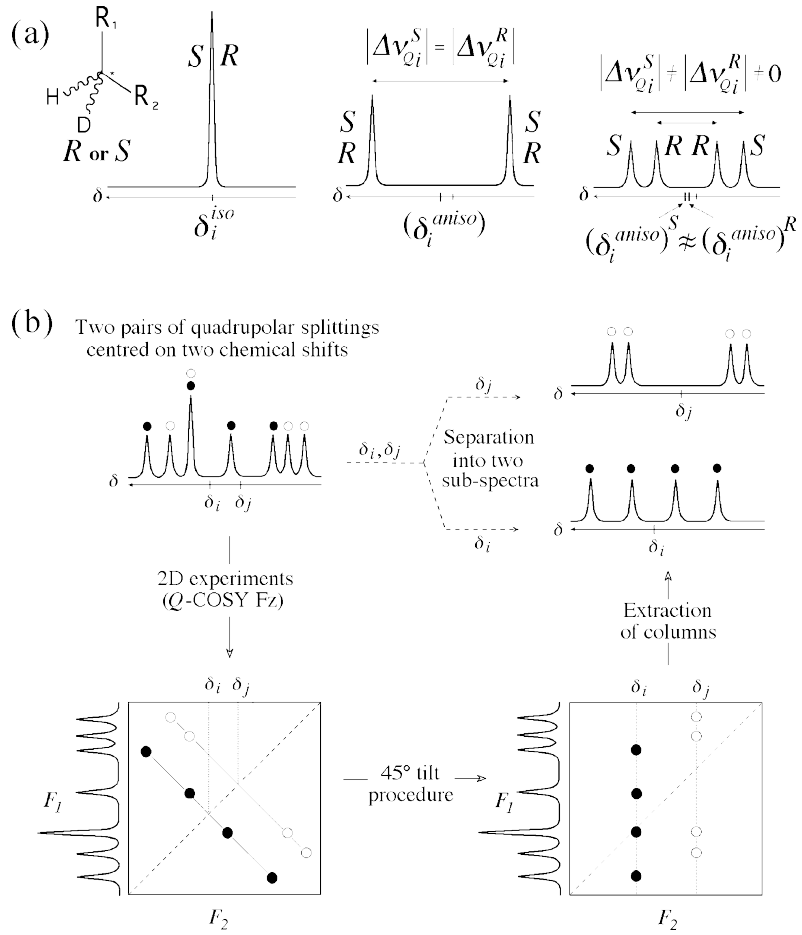

Figure 14. (a) Variations of proton-decoupled ^{2}H 1D spectra of two monodeuterated enantio-isotopomers (chiral molecule by virtue of the isotopic substitution) dissolved in achiral liquids (left), in achiral LC's (middle) and CLC's (right). The *R*/*S* stereodescriptors are arbitrary. (b) Principle of the separation of each ^{2}H-enantio-isotopomer 1D subspectrum using the NAD *Q*-COSY Fz 2D experiments (QUOSY type). Here two inequivalent deuterium sites, i and j, (with two chemical shifts, δ_i and δ_j) are considered. Figure reprinted from *Anal Bioanal Chem* 2011; 399:1187, with permission from Springer.

solvent (from weakly polar to polar ones), offering a broad adaptability and flexibility of the anisotropic irm-^{2}H NMR for a given molecule (**Baillif et al. 2006; Serhan et al. 2010**).). Finally, the use of cryogenic ^{2}H NMR probes (5-mm o.d.) instead of classical probes allows to compensate (partially) the loss of SNR due to the dispersion of ^{2}H signal on two components in an ALC (or four in a CLC) for each ^{2}H-QDs, while a single resonance was observed for each inequivalent ^{2}H site in isotropic NMR (see **Figure 14a**) The specificity of the probes (commercially available) is to reduce the thermal noise of electronic components of the NMR antenna as well as those of the preamplicator of spectrometer (**Kovacs et al. 2005**).

Application to the study of prochiral fatty acids. Historically, the analytical interest of irm-^{2}H NMR combined with CLCs was experimentally evidenced from 2004 by Philippe Lesot and coworkers, when analyzing the 1,1'-bis-(phenylthio)-hexane (BPTH), a prochiral fragment of an unsaturated essential FAME, the methyl linoleate (ML) (**Lesot et al. 2004**). In this pioneer example, the analysis of the 61.4 MHz ^{2}H QUOSY map obtained in the PBLG/$CHCl_3$ phase has provided the first information on the intramolecular isotope ratios of each enantiotopic hydrogen site. Interestingly, it has been have then revealed that the (*R*)-enantio-isotopomers in both methylene sites of BPTH were enriched in deuterons compared to the (*S)*-enantio-isotopomers (**Baillif et al. 2006**). Taking advantage of NMR spectrometer operating at higher field (14.1 T, i.e. 92.1 MHz for ^{2}H) equipped with a selective ^{2}H cryogenic probe, the method was successfully extended to the study of C-18 mono or poly unsaturated FAMEs (conjugated or not), such as methyl oleate, ML, methyl linolenate, methyl vernoleate, methyl punicate, ... (**Lesot et al. 2008; Lesot et al. 2011; Serhan et al. 2010**).

For illustrating the efficiency of the tool, **Figure 15b** presents the ^{2}H 1D subspectra (*Q*-COSY Fz map) of ML oriented in a PBLG/pyridine chiral phase (**Serhan et al. 2010**). Contrarily to isotropic ^{2}H 1D-NMR spectra where only 30% of inequivalent ^{2}H sites are clearly observed (see **Figure 15a**), all monodeuterated isotopomers and enantio-isotopomers of the mixture are spectrally differentiated. Combining quantitative isotropic/anisotropic irm-^{2}H NMR measurements, the evaluation of the isotopic fractionation of each enantio-isotopomer (*R* and *S*) was obtained with good accuracy (see **Figure 15c**). As it can be seen, large variations of $(^2H/^1H)_i$ ratios exist between each enantioisotopomeric pair (for instance the sites 15/15' and 17/17'), showing the variability of source of hydrogens. As new molecular information related to isotope fractionation, the discrimination of (*R*/*S*)-enantio-isotopomers in CLCs allows the determination of the bio enantio-isotopomeric excess*, eie (%)* for each CH_2 group of prochiral molecule, according to **Equation 25** (**Serhan et al. 2010**):

$$eie(\%) = \frac{\left|\left(^2\mathrm{H}/^1\mathrm{H}\right)_i^R - \left(^2\mathrm{H}/^1\mathrm{H}\right)_i^S\right|}{\left(^2\mathrm{H}/^1\mathrm{H}\right)_i^R + \left(^2\mathrm{H}/^1\mathrm{H}\right)_i^S} \times 100. \qquad (25)$$

In case of ML, it has been shown that *eie* at odd CH_2 positions are larger than those measured at even CH_2 groups all along the chain. This observation was explained by the different incorporation mechanisms of hydrogens into the chain during the elongation of fatty acids *via* the fatty acid synthetase (FAS) enzyme (**Baillif et al. 2009**).

The application of the anisotropic irm-^{2}H approach enabled to answer to two biochemical issues on the unsaturated FAMEs never solved, so far: i) some stereoselectivity aspects during the elongation (by FAS) and the desaturation steps (by the Δ^9 and Δ^{12} desaturase) leading to the biosynthesis of the ML in *Fusarium Lateritium,* (a fungus species) (**Baillif et al. 2009**); ii) the difference of bioconversion processes of linoleic acid to vernoleic acid in the case *Euphorbia lagascae* and *Vernonia galamensis*, plants that use different enzymatic systems (**Billault et al. 2012**). Unexpectedly, the analysis of results indicated that effects acting as the overall determinants of the final $(^2H/^1H)_i$ distribution in the final product are the encumbrance of the activesite pocket and constraints to conformational readjustment during the linoleate to vernoleate transformation. Finally in the same family of prochiral molecules, it has been also experimentally demonstrated that anisotropic NAD NMR could provide a possible means of accessing to a larger number of ^{2}H sites (compared to isotropic NMR) in the more complex cases of C-14 to C-18 saturated FAMEs, (myristate, palmitate or stearate) for which the spectral distribution of ^{2}H chemical shifts of methylene groups is very limited due to absence of double bonds (**Serhan et al. 2012**), but also in the exciting case of homogenous short-chain unsaturated triglycerides (**Lesot et al. 2012a**).

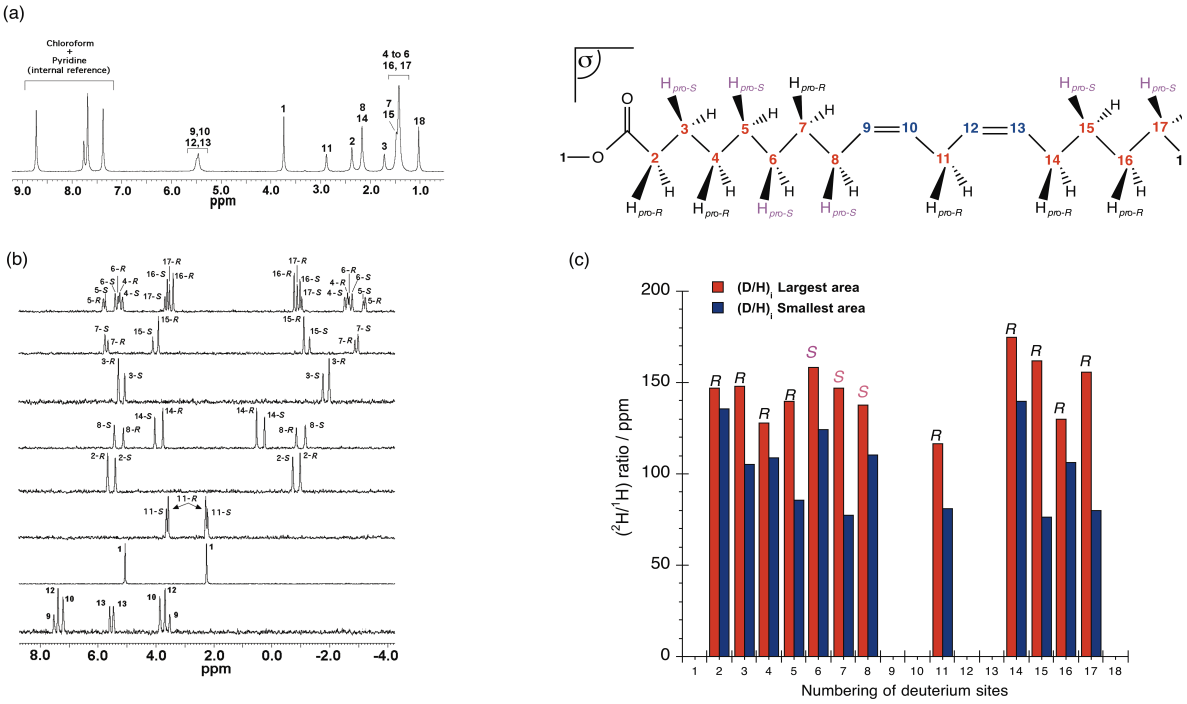

Figure 15. (a) Isotropic ^{2}H 1D spectrum of ML (T_{exp} = 5 h) in presence of pyridine used as external isotopic reference. Note the numerous overlaping of ^{2}H resonances. (b) Series of NAD 1D sub-spectra of ML in PBLG/pyridine extracted from the anisotropic ^{2}H tilted Q-COSY Fz map (T_{exp} = 16 h). The ^{2}H-QD of methyl 18 is not shown. (c) Variation of $(^2H/^1H)$ isotopic ratios of pair of (R/S)-enantio-isotopomers associated with each CH_2 group. Figure partially reprinted from *Chem Commun*. 2010; 46:6599, with permission from RSC.

Other analytical application domains. If anisotropic irm-^{2}H 2D-NMR can provide a great help to biochemists for answering bioanalytical issues, other disciplines such as Geosciences can be concerned. Indeed the determination of the site-specific $(^2H/^1H)_i$ ratio values in molecular biomarkers could not only provide key paleoenvironmental information (analysis of past climatic conditions based on the variation in the isotopic composition of hydrogen (global composition and/or site-specific composition) of molecular biomarkers stored in sedimentary archives, but also help to predict the impacts of diagenesis and to define the essential steps of the biosynthetic process.

To evaluate its analytical interest for geochemists, who use generally GC-irm-MS methods, the anisotropic irm-^{2}H NMR has been tested to study the intramolecular hydrogen isotope profile of miliacin in 2016. Miliacin is a chiral enantiopur triterpene, extracted from broomcorn millet (*Panicum miliaceum L*) (see **Figure 16a**) that can be used as a specific geological biomarker, which is potentially preserved in sedimentary archives (**Berdagué et al. 2016**). In this new example, the analysis of spectral data extracted from ANAD QUOSY 2D map enabled, for the first time, the ($^2H/^1H$) ratios on all methyl sites of this molecule to be determined (see **Figure 16a**). Interestingly, from the analysis of variations in ($^2H/^1H$) ratio values at endogenous methyl sites (23-30 sites) and tracking back of their origins in the various reaction steps leading to the miliacin, it has been experimentally assessed that the key biosynthetic step is a tail-to-tail coupling of two farnesyl pyrophosphate units that are then cyclized. Besides, it appears that this approach should also have the potential to predict fractionations during the pentacyclic triterpene diagenesis (**Berdagué et al. 2016**).

More recently, in 2018, the anisotropic irm-^{2}H 2D-NMR has been for the first time involved in a new challenging analytical domain associated with molecular authenticity/traceability investigations in food products. Among possible molecular targets, the case of the vanillin, the most widely-used aroma molecule and an important component of perfumery has been examined, in particular because ^{2}H isotopic composition of the aromatic core cannot be evaluated for sites H_a and H_b ($\delta(^2H_a) = \delta(^2H_b) \neq \delta(^2H_c)$ by the isotropic irm-^{2}H 1D-NMR (**Texier-Bonniot et al. 2018**). In contrast, in the optimized polypeptide oriented phase (PBLG/$CHCl_3$/CCl_4), the ^{2}H signal of both sites has been separated on the basis of a difference of ^{2}H-RQC magnitude ($|\Delta\nu_Q(C\text{-}^2H_a)| \neq |(\Delta\nu_Q(C\text{-}^2H_b)|$). Interestingly, the assignment for each ^{2}H-QDs was possible because that the C-^2H_a and C-^2H_c bonds are collinear in the vanillin ($S_{C\text{-}Ha} = S_{C\text{-}Hc}$) and lead to equal RQCs for both sites ($|\Delta\nu_Q(C\text{-}^2H_a)| = |\Delta\nu_Q(C\text{-}^2H_c|$).

As seen in **Figure 16b**, the variations of the relative distribution coefficients, $k'(H_{a,b,c})$ of deuterium of three aromatic sites, H_a, H_b and H_c, corresponding to a fraction of peak surfaces S, and defined as

$$k'_{^2H_{i\,(i=a,b,c)}}(\%) = 100 \times \frac{S_{^2H_i}}{\sum_{i=a,b,c} S_{^2H_i}} \qquad (26)$$

were found to be different between aromatic ring originating from natural and synthetic origin. In the

series of vanillin analyzed, it appeared that the ratios vary as $H_a < H_b < H_c$ for the samples of natural origin (bean or from a natural precursor (ferulic acid or lignin), whereas the two synthetic samples both show $H_a < H_b > H_c$. From these reference isotopic trends, it becomes possible to establish the origin of an unknown sample of vanillin by comparing its distribution coefficients on the three sites with the reference data obtained for known vanillin origin. From an applicative viewpoint, these results show that the evaluation of intramolecular ^2H isotopic composition of the aromatic ring should enable to discern between natural and synthetic origin, information that can be used in combination with isotopic data of non-aromatic sites of the molecule. From a theoretical viewpoint, the analysis of this isotopic information reveals that considerable biosynthetic data is available in the obtained results, and it has been shown how the ^2H distribution might relate to the biosynthesis of vanillin (**Texier-Bonniot et al. 2018**).

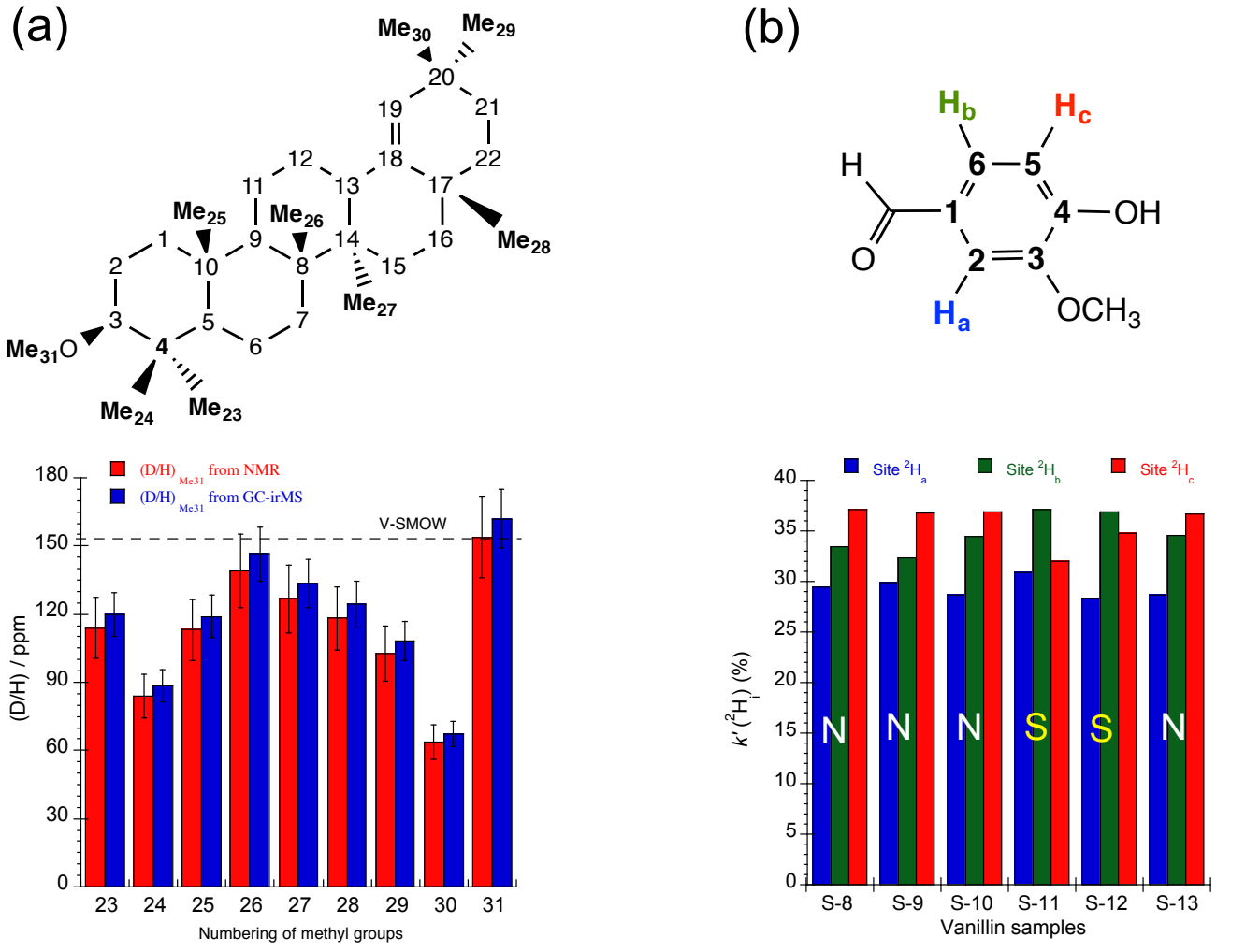

Figure 16. (a) Variations of the absolute ($^2H/^1H$) ratios (in ppm) for methyl groups of miliacin measured from a combinaison of anisotropic and isotropic irm-^2H NMR measurements (red) or a combinaison of anisotropic irm-^2H NMR measurements and GC irm-MS (gas chromatography coupled to isotopic ratio mass spectrometry) measurements (blue) to determine the absolute ($^2H/^1H$) values for each methyl group. (b) Variations of the relative distribution coefficients of deuterium for the three aromatic sites (H_a, H_b and H_c) for different vanillin samples with a natural aromatic core from lignin, ferulic, bean (noted N) and synthetic core from synthetic guaiacol, ferulic acid, (noted S). Sample 13 was of an unknown origin (N or S). Figures 16a and 16b adapted from *Geochim Cosmochim Acta* 2016; 173:337 with permission of Elsevier, and from *Flavour Fragr J*, 2018; 33: 217, with permission of Wiley, respectively.

VI. Conclusions and Perspectives

The ^{2}H NMR for the titration of ^{2}H isotopomers is based on the intrinsic property of NMR: separation of the signal and quantitative response. This quantitation can be on the absolute scale, *i.e.* calibration against a reference (internal or external) for which an international consensus on the ($^{2}H/^{1}H$) ratio has been obtained as for TMU (internal chemical reference) or on relative scale to generate intramolecular profiles. The choice between these two approaches depends on the purpose of the study, e.g. classification and also on the difficulty of introducing another chemical in the sample (NMR overlapping, chemical compatibility, etc.). The main limitation of NAD NMR is the sensitivity since relatively large amount of sample is required, often > 200 mg of pure product on common NMR spectrometers (9 – 11 T). Increasing the sensitivity of ^{2}H experiments can be obtained by operating with very high magnetic fields. The analysis of molecules of interest extracted from end-products can be prohibitive e.g. ^{2}H NMR on 10 mg of vanillin purified from ice cream, for instance, is not reasonably possible. In this context isotopic ^{13}C NMR appears as a possible alternative for authentication purpose (see **dedicated chapters of this section**).

A recent achievement around the determination of isotopic profiling overcoming the analytical limits of the historical SNIF-NMR protocol is the anisotropic irm-^{2}H 2D-NMR. Use as a complementary tool or as a full alternative, it can provide a robust solution to numerous isotope-related problems met by the bio/geo/chemists investigating natural middle-size (prochiral) molecules. Due to the specificity of NMR, anisotropic irm-^{2}H 2D-NMR also possesses its own limitations: i) the sensitivity that might impact the precision on isotope measurements; ii) the resolution of all signals on homonuclear QUOSY 2D map (**Lesot and Courtieu 2009**). Increasing the sensitivity of ANAD 2D-NMR can be obtained by operating with very high-field (> 21 T) spectrometers equipped with ^{2}H cryogenic probe (5-mm o.d.), or exploring a more challenging combination involving high-field NMR instruments (< 14 T) with promising techniques for the NMR signal enhancement such as SABRE approaches or the dissolution DNP techniques) (**Green et al. 2012; Griesinger et al. 2012**). Significant improvement of the distribution ^{2}H signals on QUOSY 2D maps can be obtained by correlating the ^{2}H and ^{13}C signals at the level of natural abundance, associated with each ^{13}C-^{2}H isotopomer and/or ^{13}C-^{2}H enantio-isotopomer of a mixture. Such a detection was experimentally demonstrated in 2012 in the case of a small prochiral molecule (benzylic alcohol) dissolved in a PBLG CLC (**Lesot and Lafon. 2012b; Lesot et al. 2014**). Although still very academic, these heteronuclear QUOSY 2D experiments entitled NASDAC (Natural Abundance Spectroscopy Deuterium and Carbon) open interesting perspectives to the so-called clumped isotopes application, consisting on the detection of multiply substituted isotopologues, with a notable application in paleothermometry (**Spencer et al. 2018**), and should therefore justify or motivate new methodological developments in the near future.

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