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NMR DETERMINATION OF ABSOLUTE SITE-SPECIFIC NATURAL ISOTOPE RATIOS OF HYDROGEN IN ORGANIC MOLECULES. ANALYTICAL AND MECHANISTIC APPLICATIONS

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ABSTRACT

It has been shown that the "internal" isotope distribution within a given molecular species at the natural abundance level is accessible by a new method, SNIF-NMR, which is based on deuterium NMR Relative internal factors, $R_{i/j}$, have been defined which enable the isotope content of a given site, i, to be compared to that of another molecular site, j, taken as the reference Several referencing methods intended to provide direct access to relative externals, T_i, and absolute, $(D/H)_i$, site-specific parameters, are now discussed from both the theoretical and the experimental points of view In the intramolecular referencing method, which involves a time-consuming chemical transformation of the sample, the risk exists of more or less systematic errors resulting from discriminating fractionation effects However this technique offers, conversely, an interesting way of investigating kinetic isotope effects without the need for specific labelling In spite of its lower spectral precision the external referencing method has the advantage of being fast and less sensitive to systematic errors and may be used for direct rough routine determinations of the site-specific isotope contents More precise results can be obtained, at the price of contaminating the sample, when an intermolecular reference is added and signal heights are used, remembering however that the intensity parameters then have no strict physical meaning in terms of absolute isotope contents The site-specific parameters, T_i and $(D/H)_i$ thus accessible, provide new information on the mechanisms of the fractionation effects occurring in natural conditions and examples are considered

INTRODUCTION

The determination of the average isotope ratio $(\overline{D/H})$ of an organic molecule is usually carried out by mass spectrometry after combustion of the compound and reduction of the corresponding water [1, 2] This technique, developed during the last decade, has proved its efficiency for the characterization of the origin of natural products and for detecting the adulteration of food and beverages However, the main drawback of isotope ratio mass spectrometry lies in the requirement of burning the molecule which, in turn, usually entails the loss of all site specific information Using ^2H NMR spectroscopy at the natural abundance level we have introduced a new method (SNIF-NMR) for measuring the "internal distribution" of deuterium within a chemical molecule [3, 4] and this approach has led to a number of interesting developments in the sphere of natural compounds ethanols [5], essential oils [4,6], vanillin, etc [7,8] In this method the "internal" distribution has been characterized by typical parameters, R, which can be obtained with satisfactory accuracy but are only of relative significance since they are related to the ratios of the isotope contents in the different sites, i, with respect to that of a given site, j, taken as the reference We shall now consider the conditions of a direct access to the various absolute site-specific isotope ratios $(D/H)_i$, which are expected to provide further important information for mechanistic or analytical purposes For example when using such parameters for investigating the fate of the hydrogen isotopes in a fermentation process[7], the proportions of

(a) Permanent address : Institute of photographic chemistry Academia Sinica Beijing (People Republic of China) deuterium coming from glucose and from medium water in the molecules CH_2DCH_2OH (I) and CH_3CHDOH (II) can be quantitatively determined. Moreover the access to the absolute $(D/H)_i$ ratios of sites I and II in ethanols is expected to provide very useful information for the identification of beverages such as wines and beers which result from the fermentation of mixtures of glucides and water. From a more fundamental point of view the direct determination of absolute site-specific isotope ratios should provide a new way for investigating selective chemical or enzymatic isotope effects In addition, the method may be the source of new complementary data in the climatological[10] or physiological[11,1] investigations usually performed by mass spectrometry on tree cellulose or plants.

The main aim of this work is therefore to present a methodology which meets the challenge of a direct determination of absolute site-specific isotope ratios and to illustrate the potential of the method using several practical applications. Although these applications are chosen in the field of alcoholic fermentation , the SNIF-NMR method may have valuable developments in the various domains of chemistry where natural tracers are used. The method offers a new way of measuring kinetic isotope effects which may be of interest to the organic chemist since it avoids the need for labelling experiments.

I THEORETICAL SECTION

1) Definition of the site specific isotope ratios $(D/H)_i$

Denoting D and H the number of deuterium and hydrogen atoms in a given molecular species and N_D and N_H the number of monodeuterated and fully protonated molecules respectively, we may write

$$D \simeq N_D \text{ and } H \simeq P\,N_H + (P-1)N_D \qquad (1)$$

where P is the stoechiometric number of hydrogens in the molecule. The average isotope ratio $(\overline{D/H})$, usually determined by mass spectrometry, is therefore given by

$$(\overline{D/H}) \simeq N_D/PN_H \qquad (2)$$

If P_i is the number of equivalent positions of the hydrogen atoms in each diastereotopic site and D_i the number of deuterium atoms in site i, which is nearly equal to the number of monodeuterated molecules of type i, then :

$$P = \sum_{i=1}^{n} P_i \text{ and } D = \sum_{i=1}^{n} D_i \qquad (3)$$

We may define a statistical, F_i , and a true, f_i , molar fraction of the monodeuterated species i as

$$F_i = P_i/P \quad \text{and} \quad f_i = D_i/D \qquad (4)$$

Moreover it is convenient to breakdown the total number of hydrogen atoms H into n parts, H_i, corresponding to the n diastereotopic molecular sites i

$$H = \sum_{i=1}^{n} H_i \quad \text{with} \quad H_i \simeq P_i\,N_H \qquad (5)$$

On this basis we may introduce the site specific isotope ratio $(D/H)_i$

$$(D/H)_i \simeq D_i/(P_i N_H) \qquad (6)$$

Previously the "internal" molecular distribution has been characterized[3] by the relative parameter $R_{i/j}$ which is shown to satisfy equation (7)

$$R_{i/j} = P_j\,(D_i/D_j) = P_i\ (D/H)_i/(D/H)_j \qquad (7)$$

where the site j is taken as an internal molecular standard. These parameters make use either of signal heights (R_h) or of signal area (R_S). It should be emphasized that the R_h ratios are usually determined with a better accuracy than the R_S ratios but are devoid of physical meaning in terms of true isotope contents, and are only of relative significance[3]

2) NMR determination of the site-specific isotope ratios by the intermolecular referencing method

In this case, the determination of $(D/H)_i$ makes use of a working standard (WS). This standard must be the same for the whole series of molecules to be compared and its $\overline{(D/H)}$ value must be precisely known on the V-SMOW/SLAP scale[12] This scale involves a comparison of the relative deuterium content of the unknown with those of the international water standards V.SMOW and SLAP for which the absolute value of the (D/H) ratio has been determined (155 76 ppm and 89 05 ppm respectively) WS may be either contained in a sealed cell coaxial to the sample cell, or weighted and dissolved in the sample

Since the intensity, I_i, of a resonance signal, i, in an $^2H\{^1H\}$NMR spectrum is directly proportional to D_i, equation (8) holds

$$I_i \propto D_i = (D_i/N_H)N_H = (D/H)_i \; P_i N_H \qquad (8)$$

with

$$N_H = (V\,d)/M \qquad (9)$$

d and M are the specific weight and molecular weight of the investigated compound When the isotope ratios of different samples are to be compared, the active volume V for molecules i in the receiver coil should remain constant or should be taken into account Considering equation (8), we may compare the signal intensity I_i^A of site i in compound A to that of the working standard WS in order to determine $(D/H)_i^A$

$$(D/H)_i^A = \frac{I_i^A \; P^{WS} \; N_H^{WS}}{I^{WS} \; P_i^A \; N_H^A} \; (D/H)^{WS} \qquad (10)$$

$(D/H)^{WS}$ is the average isotope ratio of the working standard measured on the V-SMOW-SLAP scale[12], and P^{WS} its stoechiometric number of hydrogen atoms N_H^{WS} is the number of fully protonated molecules of WS which depends on its molar volume When the <u>coaxial arrangement</u> is used, it is not necessary to know $(D/H)^{WS}$, since it can be subsequently expressed in terms of $(D/H)^{SMOW}$ by referring I_i^A/I^{WS} to I^{SMOW}/I^{WS} Denoting T the ratio of the deuterium content of A, or SMOW, with respect to that of WS we have

$$T_i^A = \frac{D_i^A}{D^{WS}} = \frac{I_i^A}{I^{WS}} \text{ and } T^{SMOW} = \frac{D^{SMOW}}{D^{WS}} = \frac{I^{SMOW}}{I^{WS}} \qquad (11)$$

and

$$(D/H)_i = \frac{P^{SMOW} \; N_H^{SMOW} \; T_i^A}{P_i^A \; N_H^A \; T^{SMOW}} \; (D/H)^{SMOW} \qquad (12)$$

Using the same cell and pure samples, $V^A = V^{SMOW}$ and N_H^{SMOW}/N_H^A reduces to $d^{SMOW}M^A/d^A M^{SMOW}$ (at T = 293K)

When the working standard, WS, is <u>mixed</u> with sample A, the masses, m^{WS} and m^A, or the volumes, V^{WS} and V^A, must be very precisely known Then

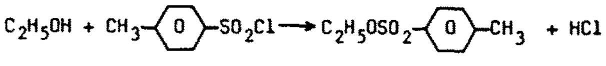
$$(D/H)_i^A = \frac{P^{WS} \; m^{WS} \; M^A \; T_i^A}{P^A \; m^A \; M^{WS} \; T^{WS}} \; (D/H)^{WS} \qquad (13)$$

3) NMR determination of the site-specific isotope ratios by the intramolecular reference method :

The external reference method is fast and readily operational However, it requires an external comparison of signal intensities which usually suffers from some lack of accuracy Therefore it seems valuable to consider the use of an <u>intramolecular standard</u> introduced into the investigated chemical species by an appropriate synthetic reaction Thus if the same reference substance, the $(D/H)_i^{ref}$ value of which has been determined beforehand, is reacted with a series of ethanols A, B M, the measured $(D/H)_i^{A,\,B \quad M}$ isotope ratios may be compared providing that no, or constant, isotope effect occurs during the synthesis of the derivatives[13]. For example, when ethanol is transformed into acetate, tosylate or silyl ether using the following reactions, the isotope contents in the ethyl fragments issued from ethanol can be referred to that of a reference group issued from the selected reactant.

$C_2H_5OH + (CH_3CO)_2O \longrightarrow C_2H_5OCOCH_3 + CH_3COOH$

$C_2H_5OH + ClSi(CH_3)_3 \longrightarrow C_2H_5OSi(CH_3)_3 + HCl$



Alternatively, if fractionation occurs the method can be applied to the determination of the <u>kinetic isotope effects</u> in the conditions of natural abundance. Indeed, it is possible to determine the ratio k_D/k_H involving the monodeuterated or the fully protonated species by measuring the $(D/H)_i$ ratio value of the starting reagent at t = 0 and that of the remaining reagent or of the products after partial completion of the reaction. Denoting $(D/H)_j^{ref}$ the isotope ratio of the internal reference, j, we measure now the ratio $T^A_{i/j}$ of site i with respect to site j ($T^A_{i/j} = I^A_i/I^A_j$) Then

$$(D/H)^A_i = \frac{P_j^{ref}}{P_i^A} T^A_{i/j} (D/H)^{ref}_j \qquad (14)$$

II EXPERIMENTAL SECTION

1 - NMR experiments

- <u>Spectrum acquisition</u>

The $^2H\{^1H\}$ NMR spectra were recorded at 61 37 MHz using a Brüker spectrometer. The acquisition time was 6.83 s or 13.66 s for a spectral width of 1200 Hz. A 90° flip angle was used ($55\ 10^{-6}$s) and a temperature of 302 ± 1K was maintained in the probe by the variable temperature unit The decoupling power was carefully controlled before each series of experiments. Good signal-to-noise ratios (S/N>100) were usually obtained for pure liquids or concentrated solutions within 10 min. (NS=100) to 30 min. (NS=250) without field-frequency locking . Usually 8 spectra of each sample are run in a sequence and the means and standard deviation of the various intensity ratios as well as of all other subsequent SNIF parameters defined in the theoretical section -are computed on a micro-computer (Digital P350) which also stores the results in a databank for comparison

With a view to optimizing the adjustment of the acquisition parameters, the ^2H T_1 relaxation times of the different molecules investigated were determined by the IRFT[14] method. Typically the following results were obtained •

Ethylacetates	CHD-O-	CH_2D-CO	CH_2D-CH_2-
T_1 (s) (σ)	1.6(0 13)	4.0(0 15)	1 35(0 2)
I_∞ calc (I_∞ exp)	8 6(8 5)	14.8(12 2)	10.8 (10 6)

Silylether	CHD-O	CH_2D-CH	CH_2D-Si -
T_1 (s.) (σ)	2.5(0.09)	2.3(0.2)	2.0(0.2)
I_∞ calc.(I_∞ exp)	1.65(1 64)	2.4(2.18)	7 9(8.0)

Since the procedure involves statistical determinations based on a large number of repetitive measurements it requires maximum automation in the estimation of the NMR parameters if it is to be of practical use. Programs have therefore been developed which directly utilize the intensity and surface parameters from successive Fourier transformations performed in <u>automatic standardized conditions</u> and no drawing of the spectra is exploited Consequently no attempt has been made to introduce particular adjustments or correcting treatments From the strict point of view of the quantitative measurements accuracy is therefore of a standard level and better results can obviously be obtained in special cases at the price of specific quantitative treatments of the spectra. For example, for an alcohol containing an intermolecular internal benzene reference, a series of 8 successive spectra treated in identical conditions leads to a confidence interval for 97 5% confidence which reaches about ± 0.004 - 0.006 for the site-specific parameters T_h derived from signal heights, and about ± 0 02 - 0 03 for the surface parameters T_S. When 20 spectra are successively recorded the corresponding confidence intervals for 99% confidence are usually better than ± 0.005 for the T_h parameters and ± 0.02 for the T_S parameters.

- <u>Intermolecular referencing method</u>

A sample of benzene having a $(\overline{D/H})$ value higher than those of the organic molecules studied here ($\overline{D/H}$ = 140 to 100 ppm) is introduced under a slight vacuum into a 5 mm OD cell adapted to fit the upper part of the 15 mm OD sample cell. The deuterium content of this working substance is determined with respect to that of a V.SMOW sample as described in the previous section. In practice the alcoholic grade in volume, t_V^A, of the ethanol sample must be taken into account when applying equation (12).

Alternatively, a known volume of working standard, carefully measured, can be introduced into the sample under study. For example, 5 ml (±0.05) of pure benzene (($\overline{D/H}$) ≈ 137 ppm) are mixed with 10 ml (±0 05) of an ethanol sample For an ethanol sample M with an alcoholic grade in weight, T^A_W, the mass m^A must be replaced in equation (13) by $t^A_W m^M$

2 - Synthesis of derivatives involving a reference substance

a) Ethyl acetates from standard acetic anhydride

In a typical reaction 26 5 ml of ethyl alcohol (0.33 mol) are introduced into a triple necked flask and mixed with 23 ml (0 27 mole, mol ratio 1 2 1) of acetic anhydride 0 3 ml of 1N H_2SO_4 (pH≈3) are added to catalyze the reaction and a mild reflux is maintained for 6 hrs The mixture is neutralized with a saturated solution of $NaHCO_3$ and finally, the acid-free mixture is washed twice with a saturated Na_2SO_4 solution to eliminate the unreacted alcohol After drying with anhydrous Na_2SO_4, ethylacetate is distilled and collected (Eb 76-77°C) When the starting ethyl alcohol is obtained by distillation of different wines or spirits the 95 % distillate is previously dessicated using molecular sieves (3A, activated at 550°C for 2 hrs) This method avoids isotope fractionation during dessication The experiments are repeated for different mole fractions of the reactants in order to investigate the influence of possible kinetic isotope effects

b) Trimethyl ethoxysilanes from standard trimethylchlorosilane

A solution of 50 ml (0 39 mole) of trimethylchlorosilane in 80 ml anhydrous ethyl ether is added very slowly over a period of 1 hour to a solution of 24 ml (0 39 mole) of ethyl alcohol and 40 ml of pyridine (0.43 mole) in 80 ml of anhydrous ethyl-ether A gentle reflux is maintained with stirring for 3 hrs. The salt formed between pyridine and HCl is removed by filtering and the resulting ethereal solution is washed three times with 250 ml H_2O The product is then dried over Na_2SO_4 Distillation gives a product boiling at 75.5 - 75 7°C

c) Ethyl tosylates from standard p-toluenesulfonylchloride

16 ml (0 275 mole) of ethyl alcohol treated as described previously, 80 ml (1 mole) of pyridine and 50 ml of anhydrous ethylether are introduced into a triple necked flask which is cooled by an ice-water bath A solution of 40 g (0 25 mole) of tosylchloride in 125 ml anhydrous ethyl ether is added very slowly to the cooled solution for 3 hours The reagents are stirred for 1 hr and the mixture is kept overnight in the refrigerator Then, the mixture is cooled down to 0°C and the pH is adjusted to a value of 2 with HCl (2N) The total volume reaches about 400 ml. The ethereal layer is separated and the aqueous layer is extracted twice by 200 ml ethyl ether All of the ethereal solution is dried over Na_2SO_4-K_2CO_3. Ethyl-ether is evaporated under a vacuum at room temperature and a solid product is obtained with a 62 % yield If the product does not crystallize spontaneously a crystal can be induced by scratching in a dry-acetone bath or by dissolving it in a small quantity of 30-60° petroleum ether and cooling it down to induce crystallization before filtering The precipitate can be transferred into a vacuum dessicator and dried at -or below- room temperature

III RESULTS AND DISCUSSION

A comparison of the two intermolecular referencing methods can be carried out on the basis of the $R_{i/j}$ (eq 7) and T_i (eq 11) parameters derived from measurements performed in the presence of external or mixed benzene (table 1) In order to investigate the influence of chemical transformations on the isotopic parameters and to check the potential reliability of an intramolecular referencing method 18 alcohols from different origins have been transformed into ethylacetates using standardized procedures (experimental section) The results in table 2 characterize 8 ethanols from the fermentation of the same kind of plant (grape) whereas the 10 other samples are alcohols from various natural or synthetic origins which are expected [3] to exhibit very different deuterium contents (table 3) In five cases the ethanol samples were also transformed into silylethers and tosylates in order to investigate the possible occurrence of fractionation effects in the course of the chemical reactions (table 4) The absolute site-specific parameters derived from these experiments are expected to provide complementary information on the influence of the origin of the glucose or fructose and on that of the water used by the plant for its photosynthetic cycle or involved in the fermentation process.

- The referencing method and the influence of the chemical tranformation

In a first step the reliability and the accuracy of the experimental methods can be checked by comparing the values of the internal parameters, R_h, R_S, determined in the different technical conditions Thus highly significant correlations are found between the $R_{II/I}$ values of the ethyl fragments (I and II denoting the methyl and methylene sites respectively) determi-

TABLE 1 - "Internal" fractionation factors, $R_{II/I}$, and site-specific isotope parameters, T_I, and T_{II}, of ethanol samples from different origins, determined in the presence of benzene either situated in a coaxial cell (a) or mixed with the sample (b)

			ISOTOPE FRACTIONATION FACTORS			SITE-SPECIFIC ISOTOPE PARAMETERS			
Nº	ORIGIN OF THE ETHANOL		Without ref	Without external ref		a		b	
				a	b	Site I	Site II	Site I	Site II
			R^o_S R^o_S	R^c_h R^c_S	R^m_h R^m_S	T^c_{hI}/t^A_V T^c_{SI}/t^A_V	T^c_{hII}/t^A_V T^c_{SII}/t^A_V	T^m_{hI}/k T^m_{SI}/k	T^m_{kII}/k T^m_{SII}/k
1	Maïze starch	c	2.317	2.320	2.347	0.968		0.641	0.502
			2.268	2.325	2.318	1.002	0.777	0.652	0.503
2		d	2.425	2.414	2.446	1.033	0.831	0.626	0.510
			2.359	2.342	2.393	0.981	0.766	0.658	0.522
3	Cane-sugar		2.121	2.098	2.121	0.956	0.669	0.650	0.460
			2.118	2.069	2.087	1.044	0.652	0.661	0.459
4	Grape	e	2.632	2.617	2.650	1.025	0.895	0.606	0.535
			2.606	2.576	2.643	0.960	0.824	0.615	0.542
5		f	2.491	2.499	2.517	0.980	0.816	0.618	0.519
			2.422	2.470	2.468	0.981	0.803	0.622	0.510
6	Beet-sugar	g	2.713	2.705	2.741	0.870	0.785	0.576	0.527
			2.691	2.693	2.737	0.867	0.771	0.582	0.531
7		h	2.748	2.740	2.766	0.935	0.854	0.549	0.494
			2.731	2.711	2.719	0.851	0.769	0.540	0.490
8		i	2.736	2.742	2.739	0.870	0.795	0.569	0.520
			2.664	2.674	2.682	0.921	0.826	0.596	0.536
9		j	2.706	2.725	2.730	0.840	0.762	0.558	0.506
			2.677	2.721	2.658	0.874	0.792	0.582	0.493

The internal ratios $R_{II/I}$ are defined[3] as $R_{II/I} = 3\ I_{II}/I_I$ where I_I and I_{II} denote either the heights (R_h) or the surfaces (R_S) of the methyl and methylene signals respectively. The site-specific parameters T^c_{hI}, T^c_{hII} derived from signal heights in the presence of an external reference (a) are given only for illustrative purposes but cannot be used for referencing (see text). In order to obtain site-specific parameters, T, which may be compared within the series, the experimental values have been corrected for the variations in the alcoholic grade t^A of the sample and in the relative weights of sample, m^M, and standard, m^{WS} ($k = m^{WS}/m^M\ t^a_W$). c, d and e, f, are derived from maïze and grape samples from France, g to j are produced from beet-roots grown in France during different years, and fermented in water having a relatively low deuterium content.

ned in the absence of reference, R^o, or in the presence of benzene, either mixed with the sample, R^m, or situated in a coaxial cell, R^c. For example considering the 9 ethanol samples <u>1</u> to <u>9</u> derived from different species of sugars (table 1) the following correlations are computed

	r	S_s	S_c	
$R_h^c = -\ 0\ 066 + 1.024\ R_h^o$	0.998	0 020	0.012	(15)
$R_S^c = -\ 0\ 070 + 1\ 014\ R_h^o$	0 990	0 055	0.034	(16)
$R_h^m = \ 0\ 005 + 1\ 009\ R_h^o$	0 998	0.018	0 011	(17)
$R_S^m = \ 0\ 018 + 0\ 999\ R_h^o$	0 992	0 046	0 029	(18)
$R_S^c = -\ 0\ 063 + 1\ 016\ R_S^o$	0 986	0 065	0 040	(19)

(r denotes the correlation coefficient and S_s and S_c are the standard deviations on the slope and on the estimation respectively)

Similarly for the 8 samples <u>10</u> to <u>17</u> from a given type of natural product, in this case wine, and therefore characterized by a more limited range of variations (table 2) :

$R_h^c = -\ 0\ 17 + 1\ 066\ R_h^o\ (r=0\ 989,\ S_s=0\ 065,\ S_c=0\ 009)$ (20)

The correlations are significantly improved when the mean values, R^M, over R^o and R^c, are considered Thus for the 17 samples <u>1</u> to <u>17</u> the slope of the correlation between the surface and height parameters approaches unity and the constant term is of the order of - 0 02

	r	S_s	S_c	
$R_h^c = -\ 0.071 + 1\ 027\ R_h^o$	0 998	0 016	0 011	(21)
$R_S^c = -\ 0\ 090 + 1\ 019\ R_h^o$	0 988	0 042	0 027	(22)
$R_S^M = -\ 0\ 019 + 0\ 991\ R_h^M$	0 992	0 032	0 021	(23)

The results confirm the better reliability of the comparative studies based on signal-height measurements with respect to those which make use of signal area Moreover it is observed that the standard deviation on the estimation of R^{ref} is of the same order of magnitude as that of the NMR determination itself It is concluded that the presence of an internal intermolecular reference or of an external reference has no effect on the reproducibility of the R measurement Satisfactory correlations are also obtained between the R_h^o parameter of the starting ethanols and the R values measured in the ethyl fragment of the corresponding acetates Thus for the 18 samples <u>10</u> to <u>27</u>

$R_h\ (acetate)=0\ 057 + 0\ 964\ R_h^o$ $(r=0\ 956\ ,\ S_s=0\ 074\ ,\ S_c=0\ 050)$ (24)

It is observed that both the relative order of the R_h parameters and the absolute magnitude of the R_S ratios are satisfactorily maintained in the course of the chemical transformation into acetates This behaviour indicates that, at the present state of accuracy, fractionation effects on the ethyl fragment may be roughly neglected, at least in the considered experimental conditions More scattered results are obtained when the ethanols are reacted to give silylethers and tosylates Thus for the five ethanols considered (table 4) the coefficients of the correlations between the R parameters of the chemical derivatives and R_h^o do not exceed 0 97 - 0 99 It should be noted that two unfavourable factors concur for limiting the accuracy in the case of silylethers i) the relatively high values of the relaxation times (see experimental section) render the line-widths and therefore the signal heights very sensitive to instrumental resolution and ii) the statistical weight of the reference signal is three times higher than that of site I, a situation which is detrimental to the precision of the intensity measurements

Although the standard deviation on the signal height parameters is usually less than ±0 066for a set of 8 successive experiments performed in the presence of <u>external benzene</u>, the site-specific parameters T_{hI}^c suffer from a lack of long-term repeatability as reflected by their poor correlations with the other T_h and T_S parameters (Table 1) By contrast when an <u>intermole-</u>

TABLE 2 - Relative natural distribution of deuterium and site-specific isotope ratios in ethanols extracted from 8 samples of wines of different origins

RELATIVE FRACTIONATION FACTORS				SITE-SPECIFIC ISOTOPE CONTENTS			
Ref	Without	External	Intramolecular	External Referencing		Intramolecular Referencing	
N°	R^o_h R^o_S	R^C_h R^C_S	R_h R_S	T^C_{SI}/t^A_V	T^C_{SII}/t^A_V	T_{hI} T_{SI}	T_{hII} T_{SII}
10	2 528	2 537	2 555			0 845	0 730
	2.464	2 484	2 493	0 986	0 816	0 884	0 734
11	2 617	2 629	2 609			0 828	0 731
	2 547	2 561	2 596	0 971	0 828	0 841	0 726
12	2 536	2 525	2 554			0 834	0 720
	2 492	2 481	2 627	0 998	0 824	0 884	0 742
13	2 617	2 613	2 617			0 823	0 733
	2 583	2 584	2.634	0 991	0 853	0 875	0 717
14	2 620	2 621	2 622			0 830	0 735
	2 585	2 582	2 576	0 996	0 856	0 843	0 723
15	2 470	2 458	2 480			0 859	0 706
	2 407	2.395	2 298	1 020	0.815	0 876	0 684
16	2 538	2 548	2 529			0 851	0 726
	2 490	2 485	2 462	1.016	0 842	0 825	0 722
17	2 561	2 560	2.555			0 862	0.733
	2 497	2 494	2 650	0 998	0 829	0 829	0 727

R_h and R_S are the relative internal II/I factors of the ethyl fragment (see caption to table 1) The site-specific parameters T_i have been computed as described in the theoretical part using as a reference either an external sample of benzene contained in a coaxial tube or the methyl site of a standard acetic anhydride sample reacted with the various ethanols in order to introduce an intramolecular reference The samples of wines have been selected with a view to benefit from a large range of isotope parameter values They are issued from different geographical origins and are not typical of the overall wine population *cular internal* reference is used the intensity parameter T^m_{hI} is satisfactorily correlated with T^c_{SI} (r=0.98) and T^m_{SI} (r=0.97). The precision reached on the T^m_{hI} and T^m_{hII} measurements in a series of successive spectra is in fact of the same order or magnitude as that on R_h as illustrated by the multidimensional correlation (n=9)

$$R^m_h = 1\ 65\ (1/T^m_{hI}) + 4.37\ T^m_{hII} - 2.43 \qquad (25)$$

which is characterized by a multiple correlation coefficient (r_{mult}) of 0.997, a standard deviation on the estimation of 0.005 and a Snedecor F value which is much higher than the theoretical one . F (2,6,0.99) = 10.9. The good three-dimensional correlation observed between the mean value $R^M_h = (R^a_h + R^c_h)/2$ and $1/T^m_{hI}$, T^m_{hII} (n=9)

$$R^M_h = 1.69\ (1/T^m_{hI}) + 4.19\ T^m_{hII} - 2.41 \qquad (r_{mult} = 0\ 998) \qquad (26)$$

also suggests a satisfactory long term repeatability. In terms of *relative* behaviours, therefore the signal-height parameters, T^m_h, are suitable for describing the variations in the site-specific deuterium contents However if absolute values are desired either allowance has to be made for correcting factors which account for line-width differences or less precise, but physically meaningful, surface parameters must be considered.

Although the relative orders of the site-specific isotope ratios are roughly maintained in the considered *chemical transformations*, the requirement for tedious preparation of the sample, which increases the risk of experimental errors, prevents the method from being routinely useful for absolute referencing

- The absolute isotope ratios

In order to calculate the *absolute isotope ratios* the different standards must be calibrated with respect to the international standard V.SMOW ((D/H) = 155.76 ppm [12]).The site-specific deuterium contents can then be determined as described in the theoretical and experimental sections. When equations 12, 13 and 14 are transformed, using the numerical values of the parameters involved, a general relation, $D/H=q_i T'_i$, is obtained where T'_i represents the site-specific parameter T_i corrected for the variations in the alcoholic grade and in the relative masses (table 1) and q_i takes the following values

Standard (j)	q_I	q_{II}	$q^c/q_{(j)}$
External (coaxial)	103.7	155.4	1.000
Internal (mixed) (a)	162.2	243 3	0.64
Intramolecular (b)	124.2	186.3	0.83

((a) the (D/H) value of benzene is 137 5 ppm (b) the D/H value of acetic anhydride is 124 2 ppm)

If we consider the data of table 1 the agreement between the two kinds of referencing (external coaxial and internal mixed) is found to be satisfactory. Thus the measured values of T^m_I / T^c_I and T^m_{II} / T^c_{II} which are respectively 0.65 (0.02) and 0.66 (0.03) are very close to the value expected on the basis of the ratio q^c/q^m given above

The results of table 2 may be used in order to check the intramolecular standard against the external one. The ratios T^i_{II} / T^c_{II} = 0.86 (0.03) and T^i_{II} / T^c_{II} = 0.87 (0.03) exhibit a slight but significant difference with the ratio q^c/q^j.This difference can be assigned to some systematic errors or fractionation effects intervening during the various chemical steps required by the intramolecular referencing procedure

From these results it is now possible to compute values of the *absolute site-specific parameters $(D/H)_i$* associated to ethanols issued from different kinds of plants or from oil. Thus the following ranges are estimated :

TABLE 3 - Isotope distribution in ethanols from natural and synthetic origins and in the acetates obtained by reacting these ethanols with a common pool of acetic anhydride

N°	ORIGIN OF THE ETHANOL	STARTING ETHANOLS		ETHYL ACETATES Internal Relative factors $R_{II/I}$		ETHYL ACETATES Site-specific parameters Site I		Site II	
		R^o_h	R^o_S	R_h	R_S	T_{hI}	T_{SI}	T_{hII}	T_{SII}
18	Beet-sugar France 80	2 711	2 655	2 650	—	0 794		0 680	
19	Beet-sugar France 82	2 758	2 697	2 682	2 679	0 793	0 783	0 697	0 693
20	Beet-sugar France 83	2 769	2 688	2 659	2 586	0 775	0 840	0 675	0.715
21	Maïze starch France 80	2 223	2 190	2 238	/	0 920		0.685	
22	Maïze starch France 82	2 317	2 268	2 294	2 235	0 926	0 940	0 683	0 681
23	Cane sugar Antillas 80	2 371	2.356	2 224	/	0 940		0.707	
24	Cane-sugar Antillas 82	2.352	2.269	2 280	2 179	0 938	0 951	0 688	0 662
25	Ethylene Germany 82	2 289	2 257	2.273	2 277	1 063	1 056	0.782	0 776
26	Ethylene France (a) 80	2 511	2 490	2 425	/	1 022		0 825	
27	Ethylene France (b) 80	2 311	2 210	2 256	/	1 075		0 808	

a) sulfuric acid process b) catalytic contact process

TABLE 4 - Influence of the chemical reaction on the isotopic parameters of the 5 ethanols from different origins considered in table 3

N°	SILYLETHERS R_h	R_S	$T_I(h)$	$T_I(S)$	$T_{II}(h)$	$T_{II}(S)$	TOSYLATES R_h	R_S	$T_I(h)$	$T_I(S)$	$T_{II}(h)$	$T_{II}(S)$
19	2 497	2 425	0 681	0.665	0 567	0 537	2 268	2 546	0.653	0 553	0 493	0.470
20	2 596	2 539	0 686	0 661	0 593	0 558	2 329	2 570	0 675	0.733	0.524	0 589
22	2 222	2.133	0 778	0 735	0 576	0 526	1.909	2 261	0.770	0 677	0.491	0.498
24	2 216	2 083	0 811	0.790	0 598	0 549	2.044	2 266	0.799	0 752	0 544	0.566
25	2 180	2.075	0 919	0 902	0 660	0 623	1 982	2 263	0 894	0.798	0 591	0 602

It should be noted that the site-specific parameters T_I and T_{II} cannot be compared in the corresponding acetates silylethers and tosylates since the molecular fragments used as an intramolecular reference, CH_3CO-, $(CH_3)_3Si$, and $CH_3-C_6H_4-SO_2-$ respectively, have different deuterium contents Moreover the slight variations exhibited by the ratios of the halft-height line-widths $\Delta\nu_{1/2}$ (II)/$\Delta\nu_{1/2}$ (I) must be taken into account when considering the R parameters Only vertical comparisons are therefore allowed in these series

		$(D/H)_I$	$(D/H)_{II}$
grapes	external	103 4 ± 1 6	129 4 ± 2 4
(table 2)	intramolecular	104 4 ± 1 5	135 4 ± 1 8 (a)
Beet-root	external	89 6 ± 2	121 9 ± 4
(table 1)	internal	88 2 ± 1 5	120 9 ± 1 2
ethylene	external	≃130 1	≃140 5
(table 3)	intramolecular	130 9 ± 2 5	149 0 ± 2 (a)

((a) the values derived from the intramolecular referencing are probably overestimated)

Again it should be kept in mind that such $(D/H)_i$ values are only illustrative of the methodological approach A valuable discussion of the natural dispersion should be based on well documented natural or synthetic samples since the $(D/H)_I$ and $(D/H)_{II}$ values closely depend on the physiological and technical conditions of the photosynthesis and of the fermentation, namely the deuterium content of the water

- The interpretation of the site-specific parameters

A number of overall deuterium contents have been determined by mass spectrometry but these values are usually difficult to interpret due to their lack of specificity and to the frequent absence of knowledge of the $\overline{D/H}$ ratios of the starting species (i e sugars) and of the fermentation water in particular Moreover in the case of ethanols these values include usually the isotope content of the hydroxyl site which is frequently deprived of intrinsic physical significance as a consequence of its sensitivity to fractionation effects caused by distillation and to exchange phenomena involving the aqueous medium From both the analytical and mechanistic points of view an important improvement has been brought about by the possibility of determining the relative internal parameters $R_{i/j}$[3] and a further step in the discriminating potential of the method is now provided by the site-specific isotope ratios, T_i , or $(D/H)_i$ Thus from the present results it appears, in particular, that the site I parameters of ethanol exhibit a larger range of variations and constitute better distinctive criteria of the nature of the plant than the parameters of site II The strong increase in the internal parameter $R_{II/I}$ observed[5] when comparing alcohols issued from plants having different metabolic pathways (C3 such as sugar-beet or C4 such as maïze) or different physiological characteristics (such as grape and beet-root) can be interpreted as resulting mainly from differences in the deuterium content of the methyl site The methylene site exhibits more limited variations, at least for ethanols produced by fermenting sweet juices issued from plants harvested in a specified country

When a given plant, such as grape, is considered the fact that two independent site-specific parameters are now available instead of the only relative factor, $R_{i/j}$, enables more subtle physiological or climatological effects to be investigated If we consider that most deuterium atoms found in the monodeuterated species I, CH_2DCH_2OH, arise from the sugar whereas the deuterium atoms in site II come predominantly from the water present in the fermentation step[9] a smaller value of the relative parameter $R_{II/I}$ can be interpreted as resulting either from an increase in the deuterium content of the sugar or from a decrease in the deuterium content of the juice water A conjunction of both effects may even occur if active leaf transpiration during the photosynthetic cycle has increased the $(\overline{D/H})$ content of the cell water and therefore favoured deuterium incorporation in site I whereas some weeks later heavy rains or flooding before harvesting may have produced a decrease in the deuterium content of the juice

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