

Hydrogen Isotopic Profile in the Characterization of Sugars. Influence of the Metabolic Pathway

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The site-specific natural hydrogen isotope ratios of plant metabolites determined by ^2H nuclear magnetic resonance (SNIF-NMR method) can provide powerful criteria for inferring mechanistic and environmental effects on biosynthetic pathways. This work examines the potential of isotopic profiles for the main constituents of carbohydrates, glucose and fructose, to distinguish different photosynthetic pathways. An appropriate analytical strategy, involving three suitable isotopic probes, has been elaborated with a view to measuring simultaneously, in conditions devoid of isotopic perturbations, all (or nearly all) of the carbon-bound hydrogen isotope ratios. It is shown that the type of photosynthetic metabolism, either C3 (sugar beet, orange, and grape), C4 (maize and sugar cane), or CAM (pineapple), and the physiological status of the precursor plant exert strong influences on the deuterium distribution in the sugar molecules. Consequently, this isotopic fingerprint may be a rich source of information for the comparison of mechanisms in metabolic pathways. In addition, it can provide complementary criteria to ethanol as a probe for the origin of sugars.

KEYWORDS: Glucose; fructose; plant metabolism; isotope ratio; deuterium; SNIF-NMR

INTRODUCTION

Despite the various exchange phenomena that are likely to intervene in the course of photosynthetic pathways, the hydrogen isotope distribution in glucose, the first stored molecule formed, is far from being homogeneous (1). The ^2H NMR investigation of site-specific natural isotope fractionation (SNIF-NMR) (2) is presently the only way to access directly and simultaneously the hydrogen isotope ratios, $(\text{D}/\text{H})_i$, associated with different positions, i , in a given molecule. Unfortunately, some compounds, such as polymeric and even simple carbohydrates, are not suitable for direct observation by ^2H NMR. In this context, ethanol, obtained by fermenting the sugars in strictly standardized conditions, offers a very attractive probe that enables carbohydrates with different molecular structures to be compared (3). By resorting to this common probe, it could be shown that plant physiological properties and environmental factors exert significant influence on the kinetic and thermodynamic isotope effects associated with hydrogen transfers intervening in different photosynthetic pathways (4). However, when the genealogy of the hydrogen isotopes was taken into account, it was concluded that results obtained on ethanol reflect properties of the glucose skeleton in which only sites 1, 2, pro-6R, and pro-

6S are concerned (5). In practice, although the botanical origin of some sugars may be inferred from the isotopic profile in ethanol derived by their fermentation, some overlap persists in a number of cases. In order simultaneously to observe a larger number of carbon-bound hydrogen atoms from the glucose moiety, several kinds of chemical derivatization have been carried out (1, 6). By using this approach, and on condition that possible perturbations due to isotope effects occurring in the course of the chemical transformations are avoided or strictly controlled, new isotopic data, complementing those obtained from ethanol, may now be obtained. To estimate and to compare the potential of the individual parameters pertaining to carbohydrate skeletons to discriminate between different botanical species, an appropriate analytical strategy must be developed. The aim of this work is to obtain reliable simultaneous determination of the maximum number of isotopic ratios associated with up to seven diastereotopic positions in glucose and fructose. The analytical chain involved in this isotopic characterization requires previous extraction and separation steps, which must be conducted in isotopically controlled conditions. This strategy has been applied to the isotopic characterization of compounds photosynthesized through the three main metabolic pathways, C3 (sugar beet, orange, and grape), C4 (sugar cane and maize), and CAM (pineapple). Sugar beet and sugar cane sugars principally contain sucrose, whereas maize glucose derived from the complete maize starch hydrolysis and orange and pineapple juices contain both glucose and fructose.

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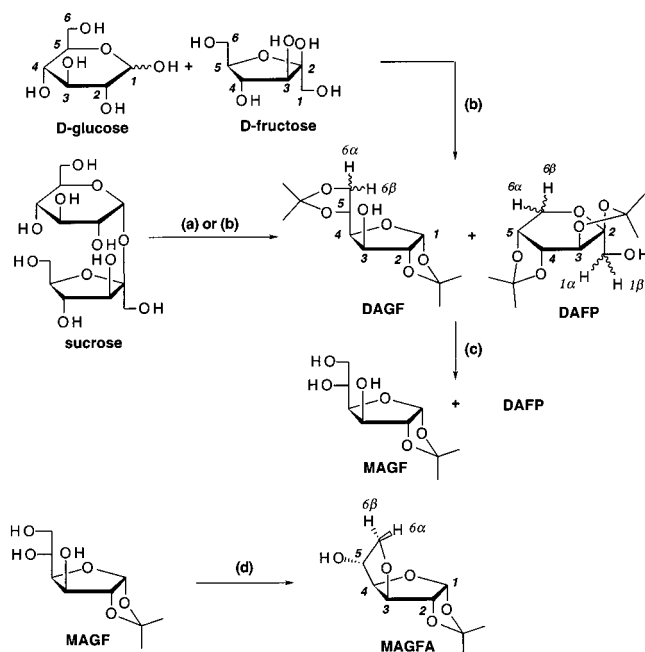


Figure 1. Scheme of the synthetic methods used for synthesizing derivatives of sugars from different origins: (a) method a, acetone, ZnCl₂; (b) method b, acetone, I₂; (c) AcOH aq 80%; (d) methyl carbonate, MeONa, dioxane.

MATERIALS AND METHODS

Carbohydrate Materials. The commercial samples of sucrose and D-glucose were from Prolabo or Lemaitre. Fruits were directly collected from the producers by Eurofins Scientific: oranges from Brazil, pineapples from the Caribbean, and the grapes from the Bordeaux region in France.

The commercial samples (sugar beet, sugar cane, and maize) were ground to give a powder that was dried over P₂O₅ in a vacuum desiccator. In the case of fruits, the carbohydrates were previously isolated by successive elutions on cation exchange (AG50-WX8 from Dowex) and anion exchange (AG1X8 from Bio-Rad) resins. The resulting syrups were successively coevaporated in 95 and 100% ethanol until a solid was obtained. They were then dried over P₂O₅. The percentages of glucose, fructose, and/or sucrose present in the orange and pineapple juices were estimated by HPLC. The ratio of D-glucose/D-fructose/sucrose = 19.0:28.7:52.3 and 28.2:38.6:33.2 in pineapple juice and orange juice, respectively.

Chemical Synthesis of the Carbohydrate Derivatives. Two methods, denoted a and b (Figure 1), have been used to synthesize isopropylidene derivatives of sucrose or of mixtures of D-glucose, D-fructose, and sucrose isolated from fruit juices. In both methods of two compounds, 1,2;5,6-di-*O*-isopropylidene- α -D-glucopyranose (DAGF) and 2,3;4,5-di-*O*-isopropylidene- β -D-fructopyranose (DAFP), was obtained. In method a, ZnCl₂ and H₃PO₄ in acetone were used (7), whereas method b was carried out in neutral medium by using iodine in acetone (8). Whichever method was used, equimolar amounts of DAGF and DAFP were produced when sucrose was used as starting material. The mixture of DAGF and DAFP was then treated (Figure 1, step c) with a solution of 80% glacial acetic acid (9), which selectively hydrolyzed DAGF to give 1,2-*O*-isopropylidene- α -D-glucopyranose (MAGF). Only traces of remaining glucose were observed, and DAFP remained unchanged. The selectivity of the hydrolysis step was independently checked by conducting the reaction separately on DAGF and on DAFP. DAFP was recovered by liquid-liquid extraction using successively chloroform and ethyl acetate, while MAGF remained in the aqueous phase. Purification by flash chromatography gave pure DAFP with a yield of 76–79% irrespective of whether method a or method b was used. MAGF was transformed (Figure 1, step d) into 3,6-anhydro-1,2-*O*-isopropylidene- α -D-glucopyranose (MAGFA) by using methyl carbonate and a solution of sodium methanolate in methanol

(6, 10). Following flash chromatography, the final yield of MAGFA was ~57% when MAGF was prepared via method a and 63–74% when method b was used.

Chromatographic Techniques. The purification of DAFP and MAGFA was performed by flash chromatography on silica columns (60–63 mesh, 200 μ m). The elution solvents were mixtures of light petroleum ether (40–65 °C) and diethyl ether.

The composition analysis of sugar mixtures was carried out by HPLC: equipment from Waters (model 510); aminopropyl column, Chrompack, Hypersil APS (250 mm, 3 mm, 5 μ m); flow, 1 mL/min; solvent, CH₃CN/H₂O (80:20); detection, differential refractometer (Waters 410).

Isotope Ratio Mass Spectrometry Measurements. The overall hydrogen isotope ratio of the sample of pyridine used as a quantitative reference in the NMR experiments was measured by isotope ratio mass spectrometry (IRMS). The sample was first combusted in a microanalyzer (Carlo Erba NA 1500). The resulting water was collected and reduced to H₂. The (D/H) ratio of H₂ was then measured on a VG SIRA 9 IRMS instrument. A value of 146.6 ppm was obtained.

SNIF-NMR Experiments. The ²H NMR experiments were performed on a DRX 500 Bruker spectrometer operating at 76.7 MHz and fitted with a ¹⁹F locking device. About 2.5 mL of sample containing ~0.7 g of the carbohydrate derivative was introduced into a 10 mm o.d. (Wilmad) NMR tube. Pyridine (0.1–0.2 g) was added as isotopic reference for quantitative determinations. Typical acquisition parameters were the following: sweep width, 1200 Hz; pulse width (90°), 11.5 μ s; broad-band proton decoupling; acquisition time, 3.8 s (DAGF and DAFP), 5.6 s (MAGFA); temperature, 312 K (DAGF), 315 K (DAFP), 320 K (MAGFA); number of scans, 12000–14000 except for DAGF (8000). Quantitative analyses of the ²H NMR spectra were carried out by means of a dedicated software (SNIF-NMR Concept) based on a complex least-squares curve-fitting algorithm that rigorously integrates all of the NMR variables (11).

The values of the chemical shifts, δ_{H} , and of the coupling constants, J_{HH} , were determined from rigorous analyses of the ¹H NMR spectra of the three compounds investigated in CDCl₃, taking into account second-order effects. In the following list of δ_i and J_{ij} values the atoms, i and j , are numbered as described in Figure 1. The chemical shifts are expressed in parts per million with respect to TMS, and the absolute values of the coupling constants are in hertz.

DAGF: δ_1 5.86; δ_2 4.45; δ_3 4.24; δ_4 3.99; δ_5 4.26; $\delta_{6\alpha}$ 4.09; $\delta_{6\beta}$ 3.92; $J_{12} = 3.6$; $J_{23} = 0.6$; $J_{34} = 2.8$; $J_{45} = 7.8$; $J_{56\alpha} = 6.2$; $J_{56\beta} = 5.3$; $J_{6\alpha 6\beta} = 8.7$.

MAGFA: δ_1 5.93; δ_2 4.62; δ_3 4.50; δ_4 4.77; δ_5 4.27; $\delta_{6\alpha}$ 3.93; $\delta_{6\beta}$ 3.49; $J_{12} = 3.5$; $J_{23} = 0.4$; $J_{34} = 3.8$; $J_{45} = 4.6$; $J_{56\alpha} = 6.5$; $J_{56\beta} = 7.4$; $J_{6\alpha 6\beta} = 8.9$; $J_{14} = 0.3$; $J_{24} = 0.3$; $J_{46\beta} = 0.5$.

DAFP: δ_1 3.65; $\delta_{1'}$ 3.68; δ_3 4.33; δ_4 4.60; δ_5 4.23; $\delta_{6\beta}$ 3.76; $\delta_{6\alpha}$ 3.90; $J_{1\alpha 1\beta} = 11.7$; $J_{34} = 2.6$; $J_{45} = 7.9$; $J_{56\beta} = 0.8$; $J_{56\alpha} = 2.0$; $J_{6\alpha 6\beta} = 13.0$.

Because, in a given medium, the chemical shifts of the monodeuterated isotopomers are the same as those of the corresponding protons, the ²H NMR spectra (Figure 2) were assigned on the basis of the ¹H analyses (taking into account solvent effects).

Determination of the Site-Specific Hydrogen Isotope Ratios. The site-specific isotopic ratios, (D/H)_{*i*}, were calculated (13) from the ²H NMR spectra carried out on samples containing known quantities of the isotopic reference, pyridine. Denoting (D/H)_R as the isotopic ratio of the reference signal, the (D/H)_{*i*} value was computed from

$$(D/H)_i = P_R m_R M_S S_i (D/H)_R / (P_i m_i M_R S_R) \quad (1)$$

where P_i and P_R are the stoichiometric numbers of hydrogens in site i and in the reference and M_S and M_R and m_S and m_R are, respectively, the molecular weight and mass of the sugar derivative and of the reference.

RESULTS AND DISCUSSION

Selection of Isotopic Probes. Principally due to relatively fast relaxation rates and insufficient chemical shift resolution, glucose, fructose, and sucrose are not convenient molecules for

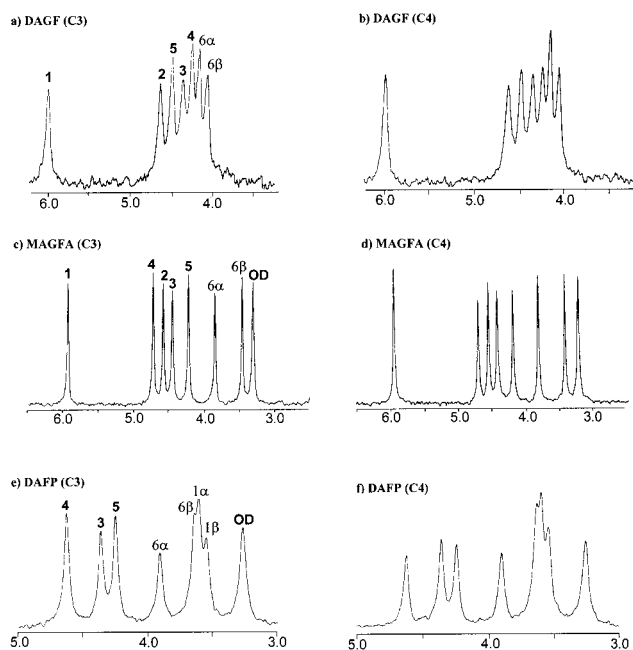


Figure 2. Natural abundance ^2H NMR spectra of DAGF (a, b), MAGFA (c, d), and DAFP (e, f) derived from glucose, sucrose, or fruit juices (for numbering of the atoms, see **Figure 1**). The isotopic profiles of samples derived from plants with different photosynthetic metabolic routes are compared. The sugar origins are sugar beet (spectra a and e) or grape (spectrum c) for the C3 plants and maize (spectrum b) or sugar cane (spectra d and f) for the C4 plants.

the investigation of site-specific natural isotope fractionation of hydrogen by means of ^2H NMR (SNIF-NMR) (2). Nevertheless, the ratios of the numbers of deuterium and hydrogen atoms at individual positions of the glucose skeleton, $(\text{D}/\text{H})_i$, can be derived from a combination of complementary data measured on appropriate chemical derivatives that exhibit partial chemical shift resolution (1). In this respect, it is desirable to observe the maximum number of isotopomers in a single derivative, because the requirement for tedious independent syntheses and spectral analyses of complementary derivatives introduces undesirable inaccuracy into the final computed results. As illustrated in **Figure 1**, the glucose derivative 1,2,5,6-di-*O*-isopropylidene- α -D-glucopyranose (DAGF), which is easily prepared when pure glucose is available, shows some discriminatory potential but is insufficient for the complete resolution of all seven carbon-bound hydrogens. In contrast, excellent chemical shift resolution is exhibited by a derivative of D-glucose, prepared in good yield through three chemical steps, 3,6-anhydro-1,2-*O*-isopropylidene- α -D-glucopyranose (MAGFA) (6).

As D-fructose is also a common carbohydrate of many plants, we have also investigated the isopropylidene derivative of D-fructose, 2,3,4,5-di-*O*-isopropylidene- β -D-fructopyranose (DAFP). Although the resolution of DAFP is inferior to that of MAGFA, it still enables four monodeuterated isotopomers to be separately observed. The 1-*O*-acetyl, 1-*O*-methyl, 1-*O*-trifluoroacetyl, or 1-*O*-[4-methylphenylsulfonyl] derivatives of 2,3,4,5-di-*O*-isopropylidene- β -D-fructopyranose have also been prepared (data not shown). These compounds offer some advantages for observing particular positions of the carbohydrate molecule. However, the resolution is not significantly improved with respect to DAFP and, in the present state of investigation, DAGF, MAGFA, and DAFP have been retained as the best choice for determining the maximum number of $(\text{D}/\text{H})_i$ ratios in a reasonable period of time. Two methods, denoted a and b

(**Figure 1**), have been used to synthesize isopropylidene derivatives of sucrose or mixtures of D-glucose and D-fructose. Although the monodeuterated isotopomers of MAGFA are sufficiently resolved in the ^2H NMR spectrum, this compound is insufficiently soluble to be a suitable probe for quantitative ^2H NMR measurements. However, an excellent probe is generated by the formation of its 3,6-anhydro derivative (**Figure 1**) (6, 10).

Inspection of the ^2H NMR spectra (**Figure 2**) shows that the $(\text{D}/\text{H})_i$ ratio is strongly influenced by the botanical nature of the plant from which the sugars were derived. It should be noted that in the case of sugars extracted from orange or pineapple the amounts of DAGF and DAFP recovered are reduced, probably due to the presence of inhibitory impurities.

Determination of the Isotopic Distribution. The ^2H NMR signals of the monodeuterated isotopomers (**Figure 2**) could be assigned on the basis of the chemical shifts and coupling constants computed from the ^1H NMR spectra of solutions of DAGF, MAGFA, and DAFP in CDCl_3 (see Materials and Methods) (12). Taking into account the angular dependency of the three-bond coupling constants, the J_{HH} values are in agreement with the values based on the expected stereochemistry of the molecules. In the case of MAGFA, the signals pertaining to the diastereotopic protons in position 6 can tentatively be assigned on the basis of both the angular dependence of $^3J_{\text{HH}}$ and the enhancement of long-range coupling associated with a zigzag geometry. The hydrogen atom labeled 6 α (**Figure 1** and **Tables 1–3**) corresponds to the pro-*R* position. This assignment has been corroborated by labeling experiments (6). It should be noted that the SNIF-NMR method may also be exploited for stereochemical assignments of ^1H and ^2H NMR signals. Once the pro-*R* and pro-*S* positions had been assigned in MAGFA, the 6 α and 6 β signals of DAFP could be tentatively assigned by comparing the values of the isotopic ratios in both compounds. It was observed that the (D/H) ratio of 6 α in the DAFP derivative obtained from sugar cane precursors is relatively close to that of the 6 β signal in MAGFA, which was attributed to the *S* monodeuterated isotopomer. Unfortunately, this assignment could not be corroborated by using the 6 β atom because its signal is not sufficiently resolved (**Figure 2**).

The isotopic profile of a given sugar can be represented by the set of molar fractions, f_i , of the seven isotopomers monodeuterated on the six carbon sites of the glucose skeleton. The determination of this relative distribution does not require the use of an isotopic reference. However, to compare the isotope content at specific molecular positions in carbohydrates extracted from different plant precursors, it is necessary to develop a referencing method. For this purpose, an appropriate quantity of a suitable reference compound in which the isotopic content has been previously characterized may be added to the sample (13). To ensure a relatively fast accumulation of moderately broad signals, the deuterium quadrupolar relaxation rates of such a reference must be in a convenient range. Moreover, its NMR signals should not interfere with those of the target molecule, and its volatility, chemical inertness, and solubility should be suitable. Despite having three signals, pyridine was selected because it satisfies most of these requirements. The hydrogen isotope ratio for each of its three signals was calibrated by determining the overall (D/H) by IRMS and the molar fractions of the isotopomers by ^2H NMR (13). Optimization of both the signal-to-noise ratio and the chemical shift separation of the carbon-bound deuterium atoms requires appropriate selection of the solvent and proper adjustment of the temperature and concentrations of all compounds contained in the NMR tube

Table 1. Reproducibility of the Isotopic Ratios of the Glucose Skeleton Measured on the MGFA Derivative

expt ^a	precursor	synthesis method ^b	(D/H) _{tot} , ^c ppm	(D/H) _i , ^d ppm (SD)						
				D-C 1	D-C 2	D-C 3	D-C 4	D-C 5	D-C 6 α	D-C 6 β
1	sugar beet	a	128.1	122.8 (1.6)	126.3 (1.2)	122.4 (1.5)	154.2 (1.6)	146.4 (2.0)	113.1 (3.0)	111.1 (1.5)
2		a	129.1	124.2 (2.2)	126.9 (1.6)	125.1 (2.2)	156.9 (3.3)	144.4 (1.7)	113.7 (1.2)	112.8 (1.3)
3		b	128.5	124.9 (2.2)	128.2 (1.6)	123.6 (2.2)	155.4 (3.3)	142.9 (1.7)	111.6 (1.2)	112.7 (1.3)
4		b	126.6	121.5 (2.0)	123.1 (2.4)	123.8 (1.8)	152.7 (2.4)	142.5 (1.9)	112.2 (1.4)	110.7 (1.4)
5	sugar cane 1	a	138.6	161.2 (2.7)	138.8 (2.3)	138.3 (1.7)	122.4 (1.9)	129.0 (1.6)	148.3 (1.8)	132.1 (3.1)
6		b	136.2	158.5 (3.0)	136.9 (2.3)	135.2 (1.8)	119.2 (2.3)	126.8 (2.8)	147.7 (1.8)	129.0 (2.0)
7	sugar cane 2	a	143.1	165.4 (2.7)	138.7 (1.6)	140.0 (1.3)	127.0 (2.3)	133.1 (3.9)	156.2 (1.2)	141.3 (4.1)
8	sugar beet +	a	134.3	144.8 (1.9)	133.9 (1.5)	129.5 (1.2)	139.6 (2.0)	137.3 (1.6)	133.6 (1.6)	121.7 (2.5)
9	sugar cane 2	calcd	135.5	144.1	132.5	131.2	140.6	139.7	134.6	126.2
10	sugar beet	b	126.4	120.7 (1.7)	122.7 (2.0)	124.1 (1.7)	152.4 (2.0)	141.7 (1.8)	112.1 (1.8)	111.3 (1.4)
11		b	126.9	122.4 (2.3)	123.5 (2.7)	123.6 (1.8)	153.1 (2.8)	143.3 (2.0)	112.3 (1.1)	110.2 (1.5)
12	grape	a	144.5	145.7 (2.0)	146.8 (5.2)	141.3 (2.4)	161.5 (2.6)	156.7 (2.7)	130.0 (2.4)	129.9 (1.6)
13		a	144.8	145.3 (2.6)	144.4 (1.6)	141.7 (2.0)	159.3 (1.6)	156.4 (1.6)	135.3 (1.7)	131.4 (2.2)

^a Twelve experiments have been performed on the MAGFA derivative. ^b MGFA has been synthesized either by method a or by method b (Figure 1). Experiments 1–2, 3–4, 10–11, and 12–13 are pairs of replicates. Pairs 1–2 and 3–4 have been performed by different operators. Two different preparations of the NMR tube have been examined in the case of sugar beet (pair 10–11) and grape (pair 12–13). The values obtained on an equimolecular mixture of sugar beet and sugar cane sugars, in experiment 8, are compared, in line 9, to predicted values (calcd) computed from the isotopic ratios of the individual components. ^c (D/H)_{tot} denotes the overall hydrogen isotope ratio (in ppm) of the seven carbon-bound positions of the glucose skeleton. ^d (D/H)_i refers to the site-specific parameters (in ppm) (numbering of the atoms is given in Figure 1); SD is the standard deviation.

Table 2. Influence of the Metabolic Pathway on the Isotopic Profile of the Glucose Skeleton Expressed in Terms of Molar Fractions

metabolism	precursor	f_i^a (SD)						
		D-C 1	D-C 2	D-C 3	D-C 4	D-C 5	D-C 6 α	D-C 6 β
C3	sugar beet	0.138 (0.003)	0.141 (0.002)	0.137 (0.002)	0.173 (0.003)	0.161 (0.002)	0.125 (0.002)	0.125 (0.002)
	orange	0.148 (0.002)	0.143 (0.002)	0.148 (0.003)	0.150 (0.001)	0.153 (0.001)	0.131 (0.002)	0.127 (0.002)
	grape	0.143 (0.002)	0.143 (0.001)	0.140 (0.001)	0.157 (0.002)	0.154 (0.001)	0.133 (0.001)	0.130 (0.003)
	mean	0.143 (0.005)	0.142 (0.001)	0.142 (0.006)	0.160 (0.012)	0.156 (0.004)	0.130 (0.004)	0.127 (0.003)
C4	sugar cane 1	0.166 (0.003)	0.143 (0.001)	0.142 (0.002)	0.126 (0.001)	0.133 (0.002)	0.154 (0.001)	0.136 (0.002)
	sugar cane 2	0.166 (0.002)	0.137 (0.001)	0.132 (0.001)	0.121 (0.002)	0.133 (0.001)	0.154 (0.002)	0.157 (0.002)
	maize	0.164 (0.002)	0.148 (0.002)	0.138 (0.002)	0.133 (0.004)	0.138 (0.003)	0.144 (0.002)	0.135 (0.002)
	mean	0.165 (0.001)	0.143 (0.006)	0.137 (0.005)	0.127 (0.006)	0.135 (0.003)	0.151 (0.006)	0.143 (0.012)
CAM	pineapple	0.141 (0.002)	0.147 (0.001)	0.148 (0.002)	0.153 (0.002)	0.148 (0.001)	0.125 (0.001)	0.138 (0.001)

^a f_i are the molar fractions (measured on the MAGFA derivative) of the monodeuterated isotopomers associated with the carbon-bound positions of the glucose skeleton (for numbering of the atoms, see Figure 1).

(solvent, solute, isotopic reference, and locking substance). Thus, at 320 or 325 K, partial overlap of the signals associated with the 6 α and hydroxyl position of MAGFA can be avoided by adjusting the concentration of pyridine. Unfortunately, even under optimal conditions, the NMR experiment remains relatively time-consuming. The quantitative isotopic results are

presented in Tables 1–4. They show that the molar fractions f_i and the (D/H)_i ratios of the investigated samples have very large intra- and intermolecular variabilities.

Reproducibility of the Analytical Procedure. To represent the sugar precursors faithfully, the isotopic ratios measured on the DAGF, MAGFA, and DAFP derivatives must be free of

Table 3. Influence of the Metabolic Pathway on the Isotope Ratios of the Carbon-Bound Hydrogens of the Glucose Skeleton

metabolism	precursor	(D/H) _{tot} ^{a,b} ppm	(D/H) _i ^{a,c} ppm (SD)						
			D-C 1	D-C 2	D-C 3	D-C 4	D-C 5	D-C 6 α	D-C 6 β
C3	sugar beet	128.1	123.3 (2.0)	126.1 (1.7)	123.7 (1.9)	154.8 (2.6)	144.0 (1.8)	112.6 (1.7)	111.8 (1.4)
	orange	146.2	151.2 (2.3)	146.3 (1.6)	151.2 (2.4)	153.7 (1.4)	156.5 (1.4)	134.0 (1.8)	130.5 (1.7)
	grape	144.8	145.3 (2.6)	144.4 (1.6)	141.7 (2.0)	159.3 (1.6)	156.4 (1.6)	135.3 (1.7)	131.4 (2.2)
C4	sugar cane 1	137.4	159.8 (2.8)	137.8 (2.3)	136.7 (1.8)	120.8 (2.1)	127.9 (2.2)	148.0 (1.8)	130.5 (2.5)
	sugar cane 2	143.1	165.4 (2.7)	138.7 (1.6)	140.0 (1.3)	127.0 (2.3)	133.1 (3.9)	156.2 (1.2)	141.3 (4.1)
	maize	151.0	173.8 (1.8)	156.7 (2.7)	146.4 (1.3)	140.3 (4.1)	145.4 (2.1)	151.7 (1.7)	142.7 (1.4)
CAM	pineapple	153.5	151.5 (1.8)	157.6 (1.8)	158.9 (2.5)	164.4 (2.2)	158.7 (1.8)	134.7 (1.5)	148.5 (1.7)

^a The isotopic ratios (in ppm) have been measured on the MGFA derivative. ^b (D/H)_{tot} is the overall isotope ratio of the carbon-bound hydrogens of the glucose moiety. ^c (D/H)_i denotes the site-specific isotope ratios (for numbering of the atoms, see Figure 1).

Table 4. Influence of the Metabolic Pathway on the Isotope Ratios of the Carbon-Bound Hydrogens of the Fructose Skeleton

precursor	(D/H) _{tot} ^{a,b} ppm	(D/H) _i ^{a,c} ppm (SD)				
		D-C 3	D-C 4	D-C 5	D-C 6 α	D-C 1 α , 1 β , 6 β
sugar beet	145.8	136.8	156.4 (4.4)	152.9 (3.1)	116.8 (4.5)	152.5 (1.4)
	133.5	136.8	166.4 (3.3)	147.1 (3.6)	110.9 (1.6)	124.4 (0.1)
	138.5	141.7	166.1 (6.0)	147.9 (5.7)	116.8 (2.9)	131.2 (0.7)
	137.4	147.9	157.7 (4.7)	148.9 (6.2)	112.2 (6.2)	131.6 (3.1)
sugar cane 1	154.9	154.9	162.8 (9.8)	127.4 (5.4)	133.9 (5.4)	143.4 (9.9)
	155.6	155.6	165.1 (10.4)	135.1 (4.8)	136.3 (6.4)	150.0 (7.2)
sugar cane 2	151.5	151.5	153.9 (8.2)	123.3 (4.1)	134.0 (4.5)	142.2 (2.8)

^a The isotopic ratios (in ppm) have been measured on the DAFP derivative. ^b (D/H)_{tot} is the overall isotope ratio of the carbon-bound hydrogens of the fructose moiety. ^c (D/H)_i denotes the site-specific isotope ratios (for numbering of the atoms, see Figure 1).

discriminating fractionation due to possible isotope effects introduced by the chemical reaction, the separation, and the purification steps. Because such fractionation effects would be avoided in fully quantitative transformations, efforts have been made to maximize the yields of the successive steps. Moreover, the relative significance of the isotopic trends has been tentatively preserved (even though systematic shifts in the absolute values of the isotope ratios due to residual fractionation effects cannot be excluded) by maintaining standardized experimental conditions. To estimate the reproducibility of the analytical procedure, two different operators have carried out several experiments on the same raw material. These experiments were conducted according to either method a or method b and were characterized by different overall yields. The data summarized in Table 1 (experiments 1–7) show that the results were not significantly influenced by the chemical procedure. The standard deviations remained of the same order of magnitude as those that characterized repetitions on the same sample of the NMR experiment alone (experiments 10–13). It was checked, in particular, that results measured on an equimolar mixture of sugar beet sucrose and sugar cane sucrose were in

satisfactory agreement with the mean values computed from the (D/H)_i parameters determined on the individual carbohydrates (Table 1, experiments 8 and 9). From a quantitative point of view the best results were obtained with the MAGFA probe. The primary DAGF derivative is useful to obtain an isotopic profile typical of the botanical precursor (Figure 2), and the DAFP derivative characterizes specifically the fructose moiety (Table 4).

Influence of the Photosynthetic Pathway on the Isotopic Distribution. Whereas the overall carbon isotope ratios of plant metabolites provide unambiguous criteria for distinguishing C3 and C4 photosynthetic pathways, the overall deuterium content is not discriminating in terms of metabolic type. In principle, the overall hydrogen isotope ratios can be measured by IRMS on the hydrogen gas obtained by reduction of water, itself resulting from combustion of the sample. However, because exchange phenomena involving hydroxyl groups are likely to perturb the deuterium contents, sugar molecules cannot be studied directly by IRMS. Synthesis of octanitrate derivatives has been used to determine the overall isotope ratio of the carbon-bound hydrogen positions, (D/H)_{tot} (14–17). The SNIF-NMR method with intermolecular referencing presently described offers an alternative to the IRMS-based method. The (D/H)_{tot} values obtained on sugars from orange, sugar beet, or sugar cane, for instance (Tables 1, 3, and 4), are in reasonable agreement with the IRMS literature values. The lack of metabolic discriminating potential of the overall (D/H)_{tot} parameter is further illustrated by the existence of larger differences among members of the same C3 family, such as orange and grape, on the one hand, and sugar beet, on the other hand, as compared to differences between species from different families, such as grape (C3) and sugar cane (C4), for instance (Table 3). In contrast, the isotopic profile represented by the molar fractions of the monodeuterated isotopomers, *f_i*, exhibits features that are common to members of the same photosynthetic type (Table 2). Thus, sites 4 and 5 of the glucose skeleton are relatively enriched in the C3 species as previously reported for bean and spinach sucrose (6). However, the C4 sugars differ markedly in that the highest deuterium contents are found in sites 1 and 6 (Table 2). Furthermore, the profile of maize starch strongly differs from that of bean endosperm starch (6) in that site 4 shows the lowest deuterium content (Table 2). Although it strongly differs from the others, the isotopic profile of the

CAM fruit, pineapple, is closer to that of C3 species than to that of the sugars investigated from C4 plants.

The discriminating behavior of the hydrogen isotope profile contrasts with that of the carbon-13 distribution, which is rather similar, in terms of molar fractions, for C3 and C4 glucose molecules (18). In this respect, the significant ¹³C enrichments observed, in both cases, at the 3- and 4-positions have been attributed to an equilibrium isotope effect associated with the aldolase reaction (19), which is also known to be responsible for secondary hydrogen isotope effects (20).

Despite the similarities observed in the isotopomeric contents of sugars from the same metabolic family, the site-specific ratios, (D/H)_i, may not be considered as the simple product of a constant metabolic profile and of an overall deuterium content depending on environmental factors. The fractionation phenomena that govern the relative distribution of deuterium among the molecular sites are also influenced by physiological features of the plant. Thus, within the C3 family, the relative deuterium enrichment at site 5 and at the C4-position, which originates from NADPH, is more accentuated in sugar beet than in fruits such as orange (Tables 2 and 3). Taking into account the existence of three different sources of triose phosphate precursors in C3 plants, variations in the level of photorespiration can be expected to be accompanied by significant modifications of the isotopic profile. In this respect, the present strategy provides an attractive approach for investigating physiological responses of plants to variations in growing conditions.

From a general point of view, the source water is enriched in deuterium in the warmer and drier countries where orange trees are grown. Moreover, not only will active evapotranspiration in leaves further enhance the D/H ratio of water entering the photosynthetic process but photorespiration plays an increasingly important role when C3 plants are grown in warmer regions. In this context, results obtained via the ethanol probe suggest that the hydrogen isotope parameters of sugars from grape vary coherently with environmental factors, such as temperature, humidity, and insolation (21). The present results show that differences in physiological characteristics of plants may be responsible for variations in the fractionation factors resulting mainly from kinetic isotope effects. Consequently, because the isotopic fractionation at every molecular site may be affected differently, a simple correlation of overall or site-specific deuterium contents with climatic parameters is possible only when the molecular probes compared have the same fractionation profile. This behavior must be kept in mind when isotopic data are used to infer the prevailing climate. In this respect, comparing the same molecule pertaining to the same organ of the same botanical species grown in variable climatic conditions (a situation that was approached in large scale comparisons of grape ethanol) meets the prerequisite of constant isotopic fractionation profile, thus providing a valid comparison (21).

Isotopic Affiliation between Sugars and Fermentation Ethanol. It has been shown that, in a complete chemical or biochemical transformation, the site-specific isotope ratios of products, (D/H)_j, and reactants, (D/H)_i, may be related by a matrix, [A], of transfer coefficients, a_{ji}, which characterizes the specific genealogies of the hydrogen atoms (22)

$$(\mathbf{D}/\mathbf{H})_j = [\mathbf{A}](\mathbf{D}/\mathbf{H})_i \quad (2)$$

where (D/H)_j and (D/H)_i are the vectors of the *j* and *i* isotope ratios.

In standardized experimental conditions this matrix is typical of the considered reaction, whatever its complexity. When such

Table 5. Calculated and Experimental Isotopic Values of Ethanol Derived from the Fermentation of Sugars from Different Metabolic Origins

metabolism	precursor	(D/H) _i , ^{a,b} ppm calcd	(D/H) _i , ^{a,c} ppm exptl
C3	sugar beet	90.4	90–94
	orange	101.3	107–112
	grape	100.8	98–106
C4	sugar cane 1	103.7	108–113
	sugar cane 2	107.3	108.5
	maize	109.3	111.1
CAM	pineapple	105.1	100–106

^a (D/H)_i is the isotopic ratio (in ppm) of the methyl site of ethanol. ^b The calculated values have been computed from eq 1 as described in the text. ^c The reported experimental data are either ranges of values measured on ethanol samples from the same botanical origin (3) or the single value measured on ethanol obtained by fermenting the specified starting sugar in standard conditions.

a matrix has been determined for a given reaction, it becomes possible to infer isotopic values of reactants from parameters measured on products and vice versa. Thus, coefficients a_{ji} that connect the hydrogen isotope ratios of ethanol and water resulting from complete fermentation to those of starting glucose and water have been estimated (5). On this basis, the consistency of the isotopic values determined on sugars produced via different metabolic pathways can be checked by estimating, using eq 2, the corresponding values for ethanol. Thus, the isotope ratio, (D/H)_I, of the methyl site, I, can be computed from the coefficients a_{Ii}, which connect it to site *i* = 1 (0.118), *i* = 2 (0.082), and *i* = 6R,S (0.300) of glucose and *i* = w of water (0.212). The value of the isotope ratio, (D/H)_{II}, of the methylene site, which is connected to the starting water (0.730) and to site 4 (0.05) of glucose, strongly depends on the origin of the aqueous medium. The calculated values of (D/H)_I (Table 5) are in satisfactory agreement with values measured on authentic samples from the same botanical origin (3).

Analytical Potential of the Individual Isotopic Parameters. Taking into account the favorable methodological performances, the accuracy and reliability of the results, the reasonable cost of the analyses, and the high discriminating potential of the (D/H)_I parameter, ethanol remains in many cases a privileged probe for characterizing sugars. However, in ambiguous situations, it may be helpful to resort to the present strategy in order to benefit from the simultaneous determination of up to seven isotopic parameters. Thus, the CAM fruit, pineapple, which exhibits δ¹³C and (D/H)_I values very close to those of sugar cane (a C4 species), is easily distinguished when the whole set of hydrogen isotope ratios of the glucose skeleton is considered (Tables 1–3). The aptitude of the method to quantify an addition of exogenous sugar to fruit juice, for instance, is illustrated by a comparison of the results obtained on an equimolar mixture of beet and sugar cane sugars to those predicted from the values measured on the individual components (Table 1). Although the precision of the determination based on every single parameter is inferior to that which would be reached by using ethanol as the probe, the reliability of the present approach is reinforced by the multiplicity of the accessible independent variables.

Conclusion. The described chemical methodology enables a simultaneous comparison of nearly all of the site-specific hydrogen isotope ratios of the glucose or fructose skeletons of sugars, which are pivotal molecules in plant physiology. This strategy can be applied not only to glucose (maize starch) but

to mixtures of glucose and fructose, to sucrose (sugar beet and sugar cane), and to mixtures of glucose, fructose, and sucrose (orange and pineapple). The existence of very large deviations with respect to a random distribution of deuterium and the strong variability observed make the hydrogen isotope profile a rich source of information on the photosynthetic pathways and on physiological responses of plants to environmental factors. Despite the complexity of sugar metabolism in plants, which involves a number of hydrogen exchange phenomena, isotopic signatures typical of the plant species may be observed. From an analytical point of view, the present approach provides complementary criteria with respect to ethanol as a probe for authenticating sugars from different origins and for detecting adulteration.

ABBREVIATIONS USED

DAFP, 2,3,4,5-di-*O*-isopropylidene- β -D-fructopyranose; DAGP, 1,2,5,6-di-*O*-isopropylidene- α -D-glucofuranose; IRMS, isotope ratio mass spectrometry; MAGF, 1,2-*O*-isopropylidene- α -D-glucofuranose; MAGFA, 3,6-anhydro-1,2-*O*-isopropylidene- α -D-glucofuranose; SNIF-NMR, site-specific natural isotope fractionation studied by nuclear magnetic resonance.

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