Characterization of Glycerol from Different Origins by ^{2}H- and ^{13}C-NMR Studies of Site-Specific Natural Isotope Fractionation

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The site-specific natural isotope fractionation of carbon and hydrogen has been investigated by SNIF-NMR for glycerol samples, extracted from plant lipids, obtained in the fermentation of sugars, or from commercial sources. In the first two cases, several plant species from either C_3 or C_4 metabolic origin have been considered. Large deviations with respect to a statistical distribution of deuterium in the glycerol skeleton are observed and the dispersion ranges of the isotopic parameters are much larger than the precision of the measure. The NMR determination of the site-specific carbon isotopic parameters (positions 2 and 1,3 of glycerol) provides an easy method for distinguishing natural and synthetic samples. More generally, the discriminating potential of the carbon and hydrogen isotopic fingerprint of glycerol has been estimated on a statistical basis in terms of the nature of the raw material (plant oil or sugar fermentation) and, in each case, in terms of C_3 or C_4 metabolism of the precursor. Glycerol may be proposed as an isotopic probe for discriminating between olive and sunflower and for investigating the adulteration of wines.

Keywords: *Fermentation; edible oils; origin assessment; authenticity*

INTRODUCTION

As a structural constituent of triglycerides, the glycerol skeleton plays an important role in natural products. Moreover, it is frequently formed in the course of glycolytic processes, and its presence in wine, for instance, has a noticeable influence on the sensory parameters. It is therefore desirable from both mechanistic and quality points of view to be able to trace the origin of glycerol in natural products. The spectrometric determination of isotopes at natural abundance is particularly powerful for investigating this kind of problem (Raven, 1992; O'Leary et al., 1992; Martin and Martin, 1990). Recently, isotope ratio mass spectrometry (IRMS) has been applied successfully to the analysis of the carbon-13 contents of glycerol, and typical differences in the isotope ratios of the three carbon positions have been observed (Weber et al., 1997). The purpose of this work is to estimate the performances of the ^{2}H- and ^{13}C-NMR investigation of site-specific natural isotope fractionation (SNIF-NMR) (Martin and Martin, 1981) in the characterization of glycerol either extracted from different kinds of oils or obtained by fermentation or chemical synthesis. To this aim, it is necessary either to elaborate a nonfractionating analytical chain or to define standardized analytical procedures in which isotopic fractionation is strictly controlled. The discriminating potential of the isotopic parameters will be examined, and a statistical evaluation will be performed.

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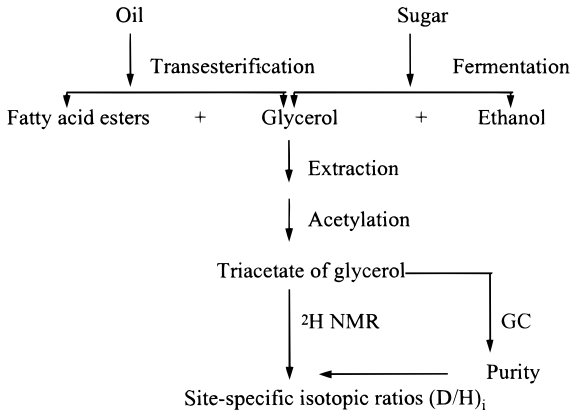
Scheme 1



EXPERIMENTAL PROCEDURES

The different steps carried out to determine the isotope ratios of glycerol are summarized in Scheme 1.

Fermentation. A normal fermentation experiment was carried out by adding 2 g of baker's yeast (*Saccharomyces cerevisiae*) in 800 mL of a sugar solution (150 g/L) in which no other additive was introduced. The yield of glycerol was equal to 4−5%.

An enhanced production of glycerol was obtained by using a fermentation medium with the following composition: sugar, 133 g/L; Na_2HPO_4, 0.5 g/L; $MgCl_2$, 60 mg/L; Na_2SO_3, 50 g/L. At the beginning 10 g of baker's yeast was added into 800 mL of the solution, and another 10-g portion was added after 24 h. The fermentation temperature was 30 °C. The yield of glycerol is equal to 15%.

Nantes tap water (NTW) characterized by a $(D/H)_w$ ratio of 150 ppm was used in all experiments.

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Extraction of Glycerol from the Sugar Fermentation Medium. *(a) Extraction from a Normal Fermentation Medium or from Wine.* Ethanol contained in the fermentation medium was extracted by fractional distillation. The residue was evaporated at 90 °C under a vacuum to remove water. When the volume was reduced to a third, the pH of the residue was adjusted to 9 by adding wet $Ca(OH)_2$. After filtration on a Büchner filter, the solution was again evaporated until a paste was obtained. The glycerol contained in the paste was extracted three times with an ethanol/ether (1:1 v/v) mixture. The extraction solutions were combined and filtered. After evaporation at 80 °C under vacuum, glycerol was obtained with a yield of 70%, computed from the quantity of glycerol found in situ by enzymatic measurement.

(b) Extraction from the Fermentation Medium Containing Added Na_2SO_3. When Na_2SO_3 was added, the fermentation solution could neither be distilled nor evaporated under vacuum because of the formation of a large quantity of foam. The evaporation was carried out by heating small portions of the solution added successively in a large beaker. The subsequent operations were similar to those used for normal fermentation media.

Extraction of Glycerol from Oil. Fifty grams of oil was transesterified with 20 mL of a methanolic solution of sodium hydroxide (0.4 mol/L) by heating for 30 min under reflux. The reaction mixture was then transferred into a separation funnel and settled overnight. Methanol in the glycerol phase was eliminated by vacuum evaporation. The residue was taken up in 40 mL of distilled water and acidified with 2 N hydrochloric acid until the fatty acid residue was separated. After filtration, the solution was washed twice with 30 mL of hexane and once with 20 mL of ether. The glycerol solution was filtered again when necessary and evaporated under a vacuum. About 5 g of raw glycerol was obtained (yield = 95%).

Synthesis of 1,2,3-Propanetriol Triacetate (Triacetin). Two grams (22 mmol) of glycerol, 10 mL of acetic anhydride (106 mmol), and 1.5 g of sodium acetate were mixed in a round-bottom flask and heated for 30 min with stirring under reflux. The reaction mixture was poured into 80 mL of ice water, and the solution was extracted three times with 30 mL of ether. The combined ether phases were washed successively with 30 mL of a sodium carbonate solution (15 w/v) and 20 mL of a semisaturated sodium chloride solution. After drying over sodium sulfate, the ether was evaporated and 4 g of triacetin was obtained (yield = 85%). To avoid isotopic fractionation, no further purification of the raw product was performed.

NMR Determinations. *(a) Deuterium Measurements.* The ^2H NMR spectra were recorded under broad-band proton decoupling using Bruker DRX 500 and DPX 400 spectrometers, both equipped with a fluorine lock device. The experimental conditions were the following for the DRX 500 spectrometer: recording frequency, 76.77 MHz; frequency window, 2400 Hz; memory size, 32K; exponential multiplication associated with a line broadening of 0.5 Hz; acquisition time, 3.4 s; delay time, 2 s; scan number, 5000. Three spectra were recorded for every sample, and an average $(D/H)_i$ value was calculated from the three measurements. In general, 2.5 g of sample, 0.17 g of TMU mixed with 50 mg of C_6F_6 (field-frequency locking material), and 1 mL of $CHCl_3$ (solvent) were introduced after filtration into a 10-mm NMR tube. The quantitative evaluation of the monodeuterated isotopomers was performed by using a new curve-fitting algorithm based on a complex least-squares treatment of the ^2H-NMR signal (Martin, 1994). This analysis involves automatic integrated management of all the experimental parameters, including the phases of the individual resonances and the baseline parameters (Eurospec program from Eurofins, Nantes, France).

The site-specific isotopic ratio $(D/H)_i$ is defined as

$$(D/H)_i = \frac{D_i}{H_i} = \frac{N_{D,i}}{P_i N_H} \quad (1)$$

where D_i and H_i are the numbers of deuterium and protium atoms at site i, $N_{D,i}$ is the number of monodeuterated isotopomers of type i, N_H is the number of fully protonated molecules, and P_i is the number of equivalent hydrogen positions at site i.

The $(D/H)_i$ ratios of glycerol (in the form of triacetin) were measured by ^2H-NMR spectroscopy using an external reference (TMU). The isotopic ratio of the reference $(D/H)_R$ was precisely calibrated on the V.SMOW scale (Hagemann et al., 1970). $(D/H)_i$ was calculated from eq 2 (Martin and Martin, 1990):

$$(D/H)_i = \frac{P_R m_R M_G S_i}{P_i m_G M_R S_R}(D/H)_R \quad (2)$$

where P_i and P_R are the stoichiometric numbers of hydrogens in site i and in the reference. M_G, m_G and m_R, M_R are, respectively the molecular weight and mass of the glycerol derivative and of the reference. $m_G = Qm_{sample}$, where m_{sample} is the mass of triacetin and Q its purity ($= m_{pure\ triacetin}/m_{sample}$) measured by gas chromatography. The standard deviation of repeatability of the isotope ratio determination involving both NMR and GC measurements is better than 2%.

(b) Carbon-13 Measurements. The quantitative ^{13}C-SNIF-NMR spectra were recorded using a Bruker DRX500 spectrometer fitted with a dual $^1H-^{13}C$ probe tuned carefully: recording frequency, 125.72 MHz; frequency window, 8000 Hz; memory size, 32K; line broadening, 1 Hz; acquisition time, 2s; delay time, 20s; $\pi/2$ pulse width, 4.5 μs; scan number (NS), 128; probe temperature, 308K. Since a crucial problem encountered in ^{13}C quantitative analysis is to maintain the decoupler instability below the required precision and to guarantee a long-term reproducibility, a specific experimental design was conducted. Four factors were varied: power and pulse width of the decoupling channel (B_2), number of scans, and overall pulse repetition time. Usually the signal-to-noise (S/N) is not a problem in ^{13}C-NMR. In the present case NS = 128 gives a high S/N value (3500), which leads to an excellent short-term precision (better than 1‰). By selecting a decoupling WALTZ mode (Shaka et al., 1985) with power and pulse width equal to 18 dB and 135×10^{-6} s, respectively, it is possible to reach a long-term standard deviation of reproducibility of 1‰. Since the ^{13}C relaxation times of the 1,3 and 2 carbon atoms are, respectively, on the order of 0.7−1.1 and 1.4−1.9 s, depending on the pH and dilution, the pulse repetition time chosen (22 s) ensures a complete recovery of all magnetizations. The signal intensities were extracted from the FID by using the Eurospec software (Martin, 1994). The ($^{13}C/^{12}C$) isotope ratios are reported in parts per thousand (‰) units with respect to PDB to be consistent with those obtained by IRMS (eq 3).

GC Measurements. The purity of the triacetin samples was determined by gas chromatography on a Hewlett-Packard HP DB-wax capillary column (diameter = 0.32 mm; length = 60 m; 0.25 micron film). Dimethyl malonate (Aldrich 99%) was used as an internal standard. The sample solutions were prepared by dissolving 0.2 g of triacetin and 0.2 g of the internal standard into 10 mL of methanol. One microliter of the solution was injected with a split 1:20. The working conditions were as follows: column head pressure, 3 bar; injection temperature, 220 °C; detector temperature, 220 °C; oven temperature programmed at 40 °C for 5 min and then increased at 20 °C/min from 40 to 180 °C. Every sample was measured five times, and every set of determinations was calibrated by carrying out two measurements with a reference solution containing pure triacetin and the internal standard. The flow rate of the helium carrier gas was equal to 40 mL/min.

Mass Spectrometric Determinations. The overall carbon isotope ratios of glycerol, sugars, and ethanol were measured using a Finnigan Delta E mass spectrometer coupled with a Carlo Erba NA 1500 elemental analyzer. The carbon

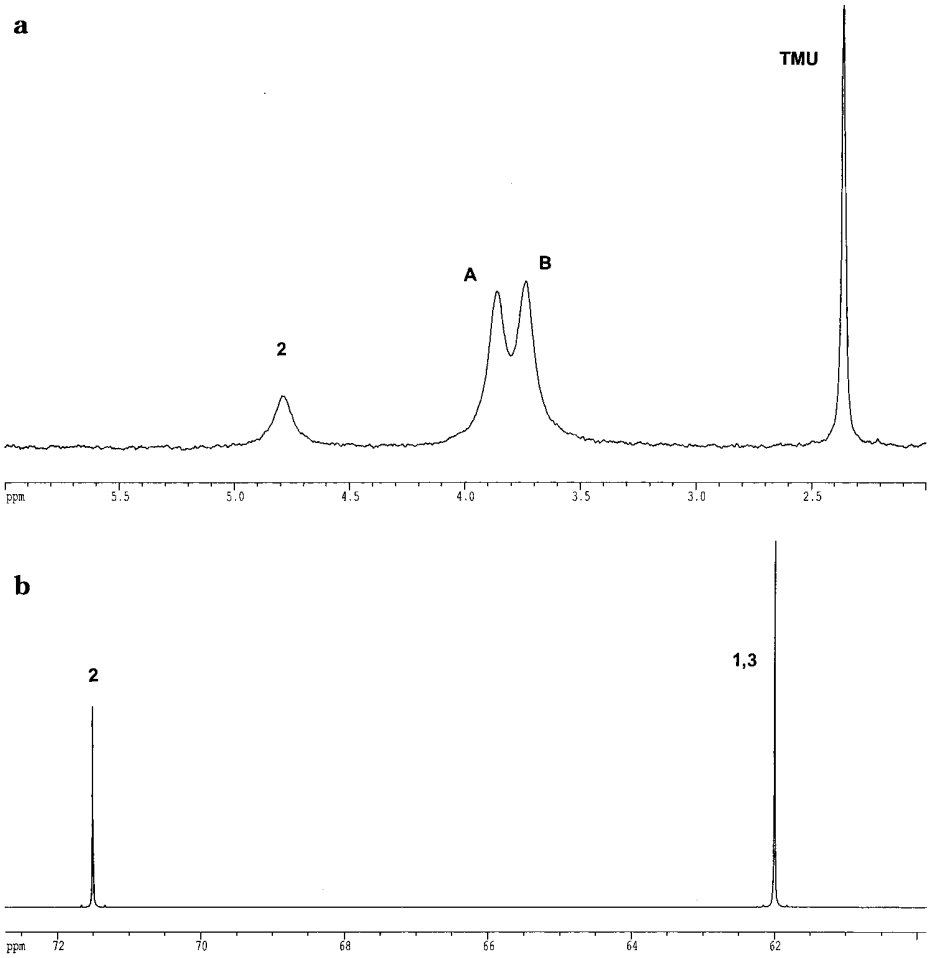


Figure 1. NMR spectra of glycerol: (a) 76.77-MHz deuterium spectrum of glycerol triacetate synthesized from glycerol obtained from sunflower [*N*,*N*-tetramethylurea (TMU) is used as an internal reference]; (b) 125.6-MHz ^{13}C spectrum of glycerol obtained from olive.

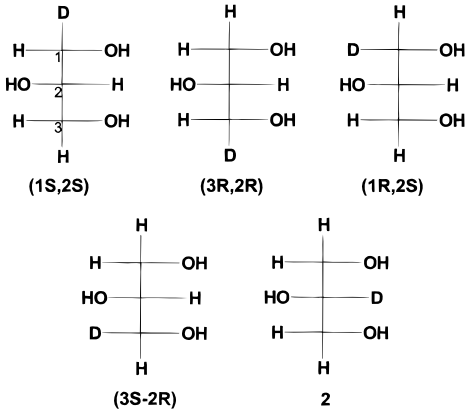
isotope content of a compound **A** is expressed on the relative δ scale:

$$\delta\mathbf{A}(‰) = \frac{(^{13}C/^{12}C)_{\mathbf{A}} - (^{13}C/^{12}C)_{PDB}}{(^{13}C/^{12}C)_{PDB}} \times 1000 \qquad (3)$$

where Pee Dee Belemnite (PDB) is a calcium carbonate adopted as an international reference. The standard deviation of reproducibility of the determinations, defined according to the ISO norm 5725, is equal to 0.25‰ (Koziet et al., 1993).

RESULTS AND DISCUSSION

Isotopic Criteria. Several sets of glycerol samples—resulting from the methanolysis of sweet corn and sunflower oils obtained by fermentation of beet root and cane sugars, directly extracted from wine, or prepared by chemical synthesis—have been investigated. Due to insufficient chemical shift resolution, glycerol itself is not a convenient probe for SNIF-NMR. Therefore, the isotope ratios of the carbon-bound hydrogens have been determined on the triacetate derivative. Since this chemical transformation involves only small secondary isotope effects and reaches a high yield, the results are preserved from spurious fractionation effects. Due to symmetry degeneracy, only three signals are observed for the five isotopomers monodeuterated on the carbon skeleton and denoted (stereospecific numbering) (1*S*,2*S*), (3*R*,2*R*), (1*R*,2*S*), (3*S*,2*R*), and 2.



The signals A and B (Figure 1) associated with the methylene groups are assigned, respectively, to the two pairs of isotopomers (1*S*,2*S*)/(3*R*,2*R*) and (1*R*,2*S*)/(3*S*,2*R*) (Kosugi et al., 1989). Consequently, they reflect only an average over the different hydrogen genealogies that lead from specific sites of glucose to the pro-*S* and pro-*R* positions on carbons 1 and 3 of glycerol.

The access to both the overall and site-specific isotope ratios by NMR requires that a known quantity of a calibrated reference product (*N*,*N*-tetramethylurea) be added to the sample. The values of the $(D/H)_i$ ratios associated with the three signals 2, A, and B are given

Table 1. Isotopic Parameters of Triglycerides Originating from Various Plants and of the Corresponding Glycerol Moieties[a]

		triglycerides	glycerol				
no.	botanical origin	$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)	$(D/H)_2$ (ppm)	$(D/H)_A$ (ppm)	$(D/H)_B$ (ppm)	$(D/H)_{tot}$ (ppm)
1	almonds	−27.9	−29.3	131.9	141.2	151.6	143.5
2	hazelnut	−28.1	−29.6	126.0	144.5	151.4	143.6
3	olive (mature)	−29.7	−31.7	130.9	144.5	150.8	144.3
4	olive (commercial)	−28.7	−31.1	134.4	145.3	148.9	144.6
5	olive (France)	−29.6	−31.5	128.5	139.7	142.8	138.7
6	olive (Greece)	−28.1	−30.3	121.3	142.5	140.6	137.5
7	olive (Greece)	−28.6	−30.6	138.5	146.7	149.1	146.0
8	olive (Greece)	−28.9	−30.5	126.8	141.1	145.5	140.0
9	olive (Greece)	−28.6	−29.7	121.9	143.0	145.8	139.9
10	olive (Italy)	−28.2	−30.9	131.8	141.5	146.4	141.5
11	olive (Spain)	−28.5	−30.6	126.5	143.4	145.8	141.0
12	palm	−29.7	−33.6	119.8	143.3	143.4	138.6
13	peanut	−27.6	−29.1	114.9	132.8	136.3	130.6
14	grape (seeds)	−27.8	−28.3	135.8	150.5	152.2	148.2
15	pistachio	−29.2	−31.2	123.7	146.3	151.1	143.7
16	rapeseed	−29.4	−32.4	122.6	138.1	138.1	135.0
17	sesame	−27.8	−30.1	117.4	145.2	141.8	138.3
18	sunflower	−28.8	−30.1	112.7	140.0	145.3	136.7
19	sunflower	−28.8	−30.2	107.7	142.3	139.2	134.1
20	sunflower	−28.7	−30.0	115.7	141.0	144.3	137.3
21	sunflower[b]	−29.4	−30.2	111.4	142.4	145.8	137.6
22	sunflower[c]	−28.7	−29.9	114.3	140.4	142.0	135.8
23	corn (sweet)[d]	−15.9	−15.8	130.2	144.8	154.7	145.8
24	corn (sweet)	−15.9	−15.8	138.5	147.7	161.0	151.2
25	corn (sweet)	−15.5	−15.2	132.4	147.8	160.1	149.6
26	corn (sweet)	−14.8	−14.5	132.4	149.4	159.3	150.0
27	corn (sweet)	−15.5	−15.4	136.5	150.9	166.7	154.3

[a] $\delta^{13}C$ was measured by IRMS and (D/H) by ^{2}H-SNIF-NMR. [b] Transgenic sunflower oil. [c] Mean of three series of data corresponding to three different extractions from the same sample. The standard deviations on the three determinations are 0.1‰ for $\delta^{13}C$ (glycerol), 2.3 ppm for $(D/H)_2$, 0.9 ppm for $(D/H)_A$, 3.2 ppm for $(D/H)_B$, and 2.1 ppm for $(D/H)_{total}$. [d] Mean of four series of data resulting from three different extractions from the same sample and two derivatizations in one case. The standard deviations on the four determinations are 0.1‰ for $\delta^{13}C$, 6.6 ppm for $(D/H)_2$, 2.1 ppm for $(D/H)_A$, 4.4 ppm for $(D/H)_B$, and 3.9 ppm for $(D/H)_{total}$.

Table 2. Isotopic Parameters of Glycerol Obtained in the Fermentation of Sugars or Obtained from Commercial Sources[a]

		sugar	ethanol	glycerol				
no.	botanical origin/product	$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)	$(D/H)_2$ (ppm)	$(D/H)_A$ (ppm)	$(D/H)_B$ (ppm)	$(D/H)_{tot}$ (ppm)
28	beet root sucrose[b]	−23.6	−25.6	−29.2	96.9	125.1	129.8	121.3
29	beet root sucrose[b]	−23.6	−25.6	−29.3	99.5	130.3	127.8	123.1
30	beet root sucrose[b]	−23.6	−25.6		99.9	130.3	130.1	124.1
31	beet root sucrose[c]	−23.6		−28.7	103.0	124.1	122.8	119.4
32	cane sucrose[b]	−10.7	−11.9	−17.3	82.6	132.1	144.9	127.3
33	cane sucrose[b]	−10.7	−11.9		85.4	131.1	147.1	128.4
34	cane sucrose[b]	−10.7	−11.9	−17.1	91.8	134.2	147.5	131.0
35	cane sucrose[c]	−10.7		−16.2	103.4	136.3	141.2	131.7
36	maize α-D-glucose	−10.6	−17.0	−17.0	96.5	139.5	150.1	135.1
37	grape (wine)		−28.7	−31.8	103.7	137.2	139.5	131.4
38	grape (wine)		−26.8	−30.5	108.8	139.3	142.3	134.4
39	grape (wine)		−27.3	−30.4	105.7	137.3	139.2	131.7
40	synthesized (fossil)[d]			−30.1	136.7	141.7	139.3	139.7
41	commercial, Merck[e]			−26.5	156.0	160.5	160.3	159.5
42	commercial, Prolabo[e]			−26.5	153.8	146.1	143.7	146.7
43	commercial, Acros[e]			−24.4	145.4	146.1	145.0	145.5

[a] $\delta^{13}C$ was measured by IRMS and (D/H) by ^{2}H-SNIF-NMR. [b] Data for 28−30, on the one hand, and 32−34 on the other hand, correspond to the same sugar precursor. 28 and 29/30 correspond to two fermentation experiments and the situation is the same for 32 and 33/34. 29 and 30 or 33 and 34 are associated with two different extractions from the same medium. Samples 29 and 30 have been derivatized in separate experiments. [c] Fermentation carried out in the presence of added Na_2SO_3. [d] Data for 40 correspond to the mean of two derivatizations of the same sample prepared by chemical synthesis. [e] Commercial samples 41−43 are from an unknown origin (probably animal).

in Table 1 for glycerol extracted from triglycerides and in Table 2 for glycerol obtained by fermentation.

To check the reliability of the analytical procedures several preparations of a given sample have been carried out. Thus, in the case of lipid raw materials (Table 1), several extractions and derivatizations have been performed on the same sunflower oil (entry 22) or sweet corn oil (entry 23). Although the repeatability of the whole procedure is lower than in the case of the ethanol probe (Zhang et al., 1995) the standard deviations, which remain <2−3 ppm, are still reasonable as compared to the intrinsic ranges of variation of the isotopic parameters. A similar situation has been observed for fermentation glycerol as detailed in Table 2 in the cases of beet sugar (entries 28−30) and cane sugar (entries 32−34) precursors.

Further isotopic characterization of glycerol rests on the determination of its overall carbon-13 content by

Table 3. Carbon SNIF-NMR Parameters Determined on Glycerol from Four Different Origins[a]

no.	botanical origin	*n*	*a*	SD(a)	δ^{13}C (IRMS) (‰)	*f*(1,3)	δ(1,3) (‰)	2SD(δ1,3) (‰)	*f*(2)	δ(2) (‰)	2SD(δ2) (‰)
43	synthesized (fossil)	11	1.9925	0.0010	−30.1	0.6658	−31.3	0.4	0.3342	−27.6	0.6
44	maize (oil)	8	1.9596	0.0011	−15.8	0.6621	−22.6	0.4	0.3379	−2.2	0.8
45	olive (oil)	8	1.9646	0.0033	−31.5	0.6627	−37.3	1.1	0.3373	−19.8	2.2
46	grape (fermented)	9	1.9732	0.0018	−30.0	0.6637	−34.4	0.6	0.3363	−21.2	1.2

[a] The parameters *a* and *f* represent, respectively, the ratio ^{13}C(1,3)/^{13}C(2) and the molar fraction of the monosubstituted molecules. SD is the standard deviation computed from *n* determinations of the parameters.

Table 4. Mean Values of δ^{13}C and of the Hydrogen Isotope Ratios of Sites 2, A, and B of Glycerol and Corresponding Standard Deviations (SD) for Sets of *n* Samples, Obtained either by Fermentation of Sugars or by Extraction from Oils, in the Case of C_3 or C_4 Plant Precursors[a]

metabolism	origin	statistics	δ^{13}C (‰)	$(D/H)_2$ (ppm)	$(D/H)_A$ (ppm)	$(D/H)_B$ (ppm)	R^a
C_3 plants	fermentation	mean	−29.9	102.5	131.9	133.1	0.77
	$n = 7$	SD	1.1	4.1	6.1	7.3	0.03
	triglycerides	mean	−30.5	123.4	142.5	145.4	0.86
	$n = 22$	SD	1.1	8.5	3.5	4.6	0.05
C_4 plants	fermentation	mean	−16.9	91.9	134.6	146.2	0.65
	$n = 5$	SD	0.4	8.4	3.4	3.3	0.06
	triglycerides	mean	−15.3	134.0	148.1	160.4	0.87
	$n = 5$	SD	0.5	3.4	2.3	4.3	0.02

[a] *R* is the relative parameter defined in eqs 4 and 5.

IRMS (Tables 1, 2, and 4) and of its site-specific carbon-13 contents by NMR (Table 3). To compare glycerol to its parent or brother products, the δ^{13}C parameters of the oil or sugar precursors and that of ethanol obtained in the same fermentation experiment have also been measured (Tables 1 and 2).

Overall Carbon Isotope Ratios and Metabolic Pathway. The well-known impoverishment in ^{13}C observed when going from C_4 to C_3 metabolism of the precursors is about the same in the sugar parent and in the glycerol and ethanol products. Thus, the differences between cane (C_4) and beet (C_3) are 12.9‰ for sugar, 12.1‰ for glycerol, and 13.7‰ for ethanol. In the case of wine (C_3) the differences with cane (C_4) are 13.7‰ for glycerol and 15.7‰ for ethanol. The same behavior is observed on glycerol from lipids since the depletion in sunflower (C_3) as compared to sweet corn (C_4) is 12.8‰ for the oil and 14.1‰ for glycerol. The mean values and their standard deviations for the whole sets of C_3 and C_4 glycerols are given in Table 4.

The ^{13}C impoverishment of glycerol with respect to ethanol derived from the same precursor (4−6‰) is in agreement with recent results (Weber et al., 1997). Whereas the two carbon sites of ethanol are largely protected from fractionation due to kinetic isotope effects by a high yield of the conversion of sugar into ethanol, the low yield of the side reaction renders glycerol very sensitive to isotope effects. Thus, the ^{13}C depletion with respect to the carbohydrate reactant reaches ∼6‰.

In contrast, as already observed in the case of olive oils (Bianchi et al., 1993), the overall ^{13}C content of the glycerol moiety extracted from triacylglycerides is very close to that of the whole oil source (Table 1). For the whole set of investigated C_3 samples a slight depletion is observed and the difference between the mean values δ^{13}C (glycerol) − δ^{13}C (oil) is −1.8‰ with a standard deviation of 0.8.

Site-Specific Carbon Isotopic Fingerprint Obtained by NMR. The site-specific parameters simultaneously accessible in the SNIF-NMR method (Table 3) corroborate the existence of large differences in the ^{13}C contents of the different molecular positions recently observed by IRMS on appropriate degradation derivatives of glycerol (Weber et al., 1997). Since we have restricted the investigation to the symmetric glycerol molecule, only the average δ^{13}C value of carbons 1 and 3, δ^{13}C (C_1, C_3), is measured.

Satisfactory agreement is found in particular between the differences $\Delta = \delta^{13}$C (C_2) − δ^{13}C (C_1 C_3) measured by IRMS (Weber et al., 1997) and SNIF-NMR. Thus, in the case of glycerol from olive oil the Δ values are 13.4‰ (IRMS) and 17.6‰ (NMR). They are 11.6‰ (IRMS) and 13.3‰ (NMR) for wine glycerol. A high Δ value (21‰) is also measured by NMR for corn oil, but much smaller differences are determined in synthetic products: $\Delta = -0.7$‰ (IRMS) and 5.5‰ (NMR). Both methods therefore confirm larger deviations with respect to a statistical distribution of ^{13}C in natural as compared to synthetic glycerols.

Although the occurrence of some systematic deviations cannot be excluded in the present state of the technique, it may be concluded that NMR provides a fast and reliable method for characterizing different kinds of glycerol on the basis of the site-specific δ^{13}C profile.

Site-Specific Hydrogen Isotope Characterization. Due to partial overlap of the relatively broad A and B signals, the accuracy of the isotopic determinations is usually on the order of only 2−3 ppm. However, since very large differences in the isotope ratios associated with different origins are observed (Tables 1 and 2), the $(D/H)_i$ parameters provide efficient characterization criteria. Thus, the $(D/H)_2$ values extend over a range of 56 ppm, whereas the methylenic parameters exhibit ranges of 27 ppm for $(D/H)_A$ and 44 ppm for $(D/H)_B$. It is convenient to define, as in the case of the ethanol probe (Martin and Martin, 1990), a relative parameter, *R*, which can be obtained without the need for adding to the sample and isotopic reference.

$$R = 4S_2/(S_A + S_B) \quad (4)$$

where S_2, S_A, and S_B are, respectively, the areas of the 2, A, and B signals in the ^{2}H-NMR spectrum.

This parameter represents the number of deuterium atoms in site 2 in a situation where the methylenic hydrogens groups are arbitrarily given the probability

Table 5. Discriminating Potential of the Site-Specific Hydrogen Isotope Parameters of Glycerol in Terms of Biosynthetic Pathways for the Two Kinds of Photosynthetic Metabolisms C_3 and C_4 Estimated by a One-Factor Analysis of Variance[a]

metabolism	differentiation of origin	statistics	$\delta^{13}C$	$(D/H)_2$ (ppm)	$(D/H)_A$ (ppm)	$(D/H)_B$ (ppm)	R^b
C_3	fermentation and triglycerides $P(d^2M)\% = 100.0$	$P(F)\%$	70.4	99.99	99.99	99.98	99.85
		LSD	1.63	11.2	6.1	7.6	0.06
		OD	*c*	20.9	10.6	12.3	0.08
C_4	fermentation and triglycerides $P(d^2M)\% = 100.0$	$P(F)\%$	99.91	99.99	99.99	99.96	99.99
		LSD	1.05	13.6	6.1	8.2	0.09
		OD	1.60	42.1	13.5	14.2	0.21

[a] $P(F)\%$ is the level of confidence for the differentiation of means computed from the F value of ANOVA, and $P(d^2M)\%$ is the level of confidence for the Mahalanobis distance d^2M between the two centroids; LSD is the least significant difference between means at the 99% confidence level, and OD is the observed difference. The values of LSD and OD are in ‰ for the $\delta^{13}C$ parameter and in ppm for the $(D/H)_i$ variables. [b] R is defined in eqs 4 and 5. [c] Difference not significant at the 99% confidence level.

factor 4. It is simply equal to the ratio of the isotopic contents in positions 2 and 1,3 of glycerol.

$$R = (D/H)_2/0.5[(D/H)_A + (D/H)_B] \quad (5)$$

Mean values of the site-specific hydrogen isotope ratios calculated over numbers of samples going from 5 to 22 are gathered in Table 4. For both C_3 and C_4 metabolisms the $(D/H)_i$ values are higher for glycerols extracted from triglycerides than for fermentation glycerols. This behavior is particularly marked for $(D/H)_2$. The internal distribution of deuterium is also typical. Although, in all cases, position 2 is significantly depleted with respect to positions 1,3, ($R \ll 1$), the depletion is less pronounced for the oil origin.

Discriminating Potential of the Isotopic Parameters. The high discriminating potential of the overall carbon isotope parameter in terms of C_3 or C_4 metabolism of the precursor is well established (O'Leary et al., 1992). It is further corroborated in the case of glycerol by the mean values estimated in Table 4. The carbon isotope parameter is less efficient for distinguishing fermentation and oil products pertaining to a given metabolic origin. It may be useful to resort to the internal ^{13}C distribution to improve the discriminating aptitude and in particular to identify synthetic and natural products (Table 3).

A one-factor analysis of variance was carried out on the hydrogen isotope ratios of glycerol to select the most discriminating parameters for recognition of the oil or fermentation origin of the products. The Fisher statistics variable, F, and the least significant difference (LSD) between C_3 and C_4 means were computed for the two sets of C_3 and C_4 compounds (Table 5). Efficient distinction is provided by nearly all variables and in particular by $(D/H)_2$.

It is interesting, especially in the case of oil products, to estimate the ability of the $(D/H)_i$ variables to differentiate glycerols issued from plants having the same photosynthetic metabolism. From this point of view, the example of olive and sunflower oils has been selected since it is of practical importance to have a tool for authenticating expensive olive oil (Bianchi et al., 1993; Lai et al., 1995) against other competitors such as sunflower oil which may be found now as a transgenic species. The results summarized in Table 6 are very promising. Although the number of investigated samples should be increased to better appraise the effect of geographical dispersion, it may be concluded that R and $(D/H)_2$ are efficient criteria for detecting and possibly quantifying the adulteration of olive oil by sunflower oil. When glycerol is produced in a glycolytic pathway, signals A and B contain important contributions from,

Table 6. Isotopic Identification of Glycerols Extracted from Triglycerides Produced by Plants with the Same Metabolism (C_3): Olive and Sunflower

C_3 plants	statistics	$\delta^{13}C$ (‰)	$(D/H)_2$ (ppm)	$(D/H)_A$ (ppm)	$(D/H)_B$ (ppm)	R^a
olive	mean	−30.8	129.0	143.1	146.2	0.89
$n = 9$	SD	0.6	5.6	2.2	3.2	0.03
sunflower	mean	−30.1	112.4	141.2	143.3	0.79
$n = 5$	SD	0.1	1.4	1.1	2.7	0.02
ANOVA	F^b	6.1	36.7	3.1	2.9	44.6
	LSD	*c*	8.2	*c*	*c*	0.05
	OD	0.7	16.1	*c*	*c*	0.10

[a] R is defined in eqs 4 and 5. [b] The critical value of the Fisher variable F is equal to 8.86 at the 99% confidence level. SD is the standard deviation calculated over the n investigated samples. LSD is the least significant difference between means at the 99% confidence level. OD is the observed difference. The values of mean, SD, LSD, and OD are in ‰ for the $\delta^{13}C$ parameter and in ppm for the $(D/H)_i$ variables. [c] Difference not significant at the 99% confidence level.

Table 7. Aptitude of the Isotopic Parameters of Glycerol To Discriminate the Three Pairs of Precursors: Grape, Beet Sugar, Commercial Glycerol

origin	statistics	$\delta^{13}C$ (‰)	$(D/H)_2$ (ppm)	$(D/H)_A$ (ppm)	$(D/H)_B$ (ppm)	R
commercial	mean	−26.9	148.0	148.6	147.1	1.00
$n = 4$	SD	2.4	8.8	8.2	9.1	0.04
beet sugar	mean	−29.1	99.8	127.5	127.6	0.78
$n = 4$	SD	0.3	2.5	3.3	3.4	0.07
grape	mean	−30.9	106.1	137.9	140.3	0.76
$n = 3$	SD	0.8	2.6	1.2	1.7	0.02
	F^a	5.5	70.7	13.2	9.3	51.0
commercial/	LSD	4.0	15.2	14.4	15.9	0.09
beet	OD	2.2[b]	48.1	21.1	19.5	0.22
oil/grape	LSD	4.3	16.4	15.6	17.2	0.10
	OD	4.0[b]	41.9	10.7[b]	6.7[b]	0.24
beet/grape[c]	LSD	4.3	16.4	15.6	17.2	0.10
	OD	1.8[b]	6.2[b]	10.5[b]	12.9[b]	0.02[b]

[a] The Fisher test F equals 9.55 at the 99% confidence level. [b] Difference not significant at the 99% confidence level. [c] Better discriminating performances are obtained if the results concerning beet sugar fermented in the presence of added Na_2SO_3 are excluded from the comparison.

respectively, sites 6 pro-*R* and 2 or sites 6 pro-*S* and 1 of glucose, which are expected to be isotopically typical of the plant precursor (Zhang et al., 1995). In principle, glycerol is therefore a good candidate as a tool for characterizing the botanical source of the carbohydrate.

An example of practical interest of the glycerol probe occurs in the field of wine. It concerns the identification of the origin of the glycerol component which either

results from fermentation of grape—or of added beet or cane sugar—or has been prepared by chemical synthesis from fossil sources or has been extracted from lipids (probably of animal origin) (Table 2). In the absence of a sufficient number of commercial samples from known origin, we have performed one-factor variance analyses of the data concerning beet or grape fermentation glycerol against the average parameters of the four commercial samples probably derived from fossil and animal sources (Table 2). In both cases the discriminating potential of some parameters makes the isotopic fingerprint of glycerol a useful criterion for detecting the adulteration of wines by commercial products.

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