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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · MARCH 1983

Impact Factor: 2.91 · DOI: 10.1021/jf00116a032

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A New Method for the Identification of the Origin of Ethanols in Grain and Fruit Spirits: High-Field Quantitative Deuterium Nuclear Magnetic Resonance at the Natural Abundance Level

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It is shown that very different internal natural distributions of the deuterium isotope exist in ethanol samples from various origins. Quantitative ^2H NMR at the natural abundance level provides a new and efficient tool for investigating these distributions, and original selective parameters are introduced. Thus, the R parameter varies from about 2.2 for gins and rums which are obtained from corn and sugar cane, respectively, to 2.7 for ethanols extracted from sugar beet, and bourbon whiskies are unambiguously differentiated from malt scotch whiskies. The method is also capable of providing the overall ^2H content obtainable by mass spectroscopy. Moreover, the selectivity of the NMR method enables information to be obtained on the biosynthetic mechanisms which intervene in the formation of the ethanol and on the influence of the climatological factors.

The identification of ethanol in spirits and beverages is always a challenge for the chemist concerned with analytical problems or with chemical and biochemical mechanisms. Until now, radioactivity methods based on the use of ^{14}C (Simon et al., 1975) or ^3H tracers (Simon and Medina, 1968) or mass spectrometry determinations of the ^{13}C and ^2H contents of ethanol (Bricout et al., 1975; Bricout, 1978) have been used to solve, at least in part, this question. However, a major drawback of these methods is that only the overall isotopic content of the molecule is directly available. In fact, although chemical transformation of ethanol prior to mass analysis (Rauschenbach et al., 1979) may give an indication of the isotope content of the methyl group, the interpretation of the results usually implies some assumptions concerning the biomechanisms of deuterium fractionation during the fermentation (Ponticorvo, 1968).

In the last few years the development in the technology of high-field nuclear magnetic resonance has made possible the recording of ^2H NMR spectra of samples at the natural abundance level and, recently, we have shown that substantial differences exist in the intramolecular deuterium distribution of various ethyl and vinyl (Martin and Martin, 1981a,b) derivatives. In the light of these results we develop here a new, nondestructive, and rapid method, for identifying the origin of ethanols in various spirits and beverages. This method which has been used to measure the quantity of sucrose added in wines (Martin and Martin, 1981c) may also be the source of valuable information on deuterium fractionation and redistribution in biochemical pathways, and we shall discuss the results of NMR in reference to the commonly accepted biomechanisms of ethanol fermentation.

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EXPERIMENTAL SECTION

Principle of the Method. The proton decoupled ^2H spectrum of ~95% ethanol consists of four lines which have the same chemical shifts as those of the signals observed in the corresponding proton spectrum (Figure 1). Due to the very small natural abundance of deuterium in organic molecules ($100–160 \times 10^{-6}$) the ^2H NMR spectrum of pure ethanol can be explained in terms of the relative contributions of the different monodeuterated molecules: CH_2DCH_2OH (I); CH_3CHDOH (II); CH_3CH_2OD (III). In order to compare the quantity of the deuterated species II to that of I, which can be normalized to the statistical value of 3, we define the parameter

$$R_s(i) = 3S_i/S_I \qquad (i = II, III) \qquad (1a)$$

where S denotes the integrated area of signal i. In practice the signal heights H_i are measured with a better precision than the area and it may be convenient to define the internal ratios

$$R_h(i) = 3H_i/H_I \qquad (1b)$$

These parameters represent the true value of the relative amount of resonating nuclei and are therefore equal to R_s only on the condition that the line widths are identical. Otherwise the R_h ratios must be considered as empirical parameters. In fact, if the ratio of the line widths of signals I and II, $\Delta\nu_{1/2}(II)/\Delta\nu_{1/2}(I)$, does not change significantly in a series of different samples, the $R_h(II)$ parameter offers a precise way of comparing the deuterium distribution in molecules I and II of different ethanol samples. Due to the large fluctuations in the line width of the OD signal

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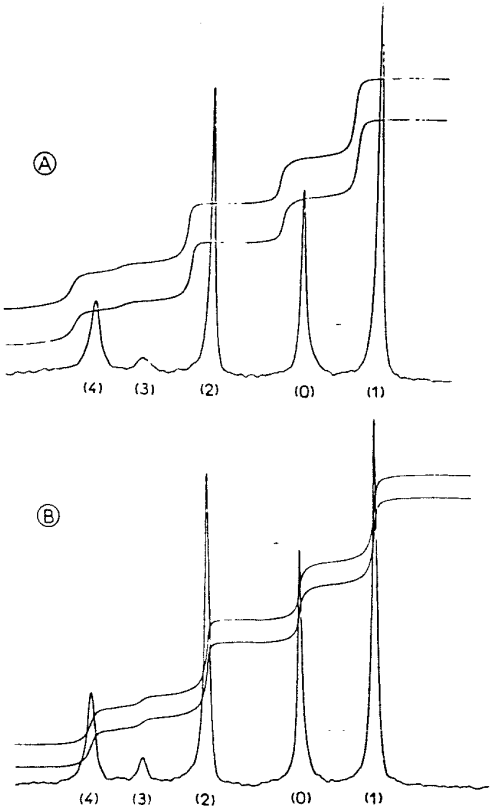

Figure 1. Typical ^2H NMR spectra of natural ethanols obtained (A) from corn and (B) from sugar beet. The signals (0), (1), (2), (3), and (4) refer respectively to the working standard (CH_3CN), CH_2DCH_2OH, CH_3CHDOH, HOD, and CH_3CH_2OD.

as a function of sample preparation only the R_s values will be determined in this case. We shall also calculate the mole fractions $f(i)$ of the monodeuterated molecules I, II, and III.

$$f(i) = S_i / \sum_{i=1}^{3} S_i \tag{2a}$$

[Note that $3f(II)/f(I) = R(II)$ and $3f(III)/f(I) = R(III)$.] The statistical distribution of deuterium in ethanols (I, II, and III) would be characterized by mole fractions $F(i)$

$$F(i) = P_i / \sum_{i=1}^{3} P_i \tag{2b}$$

where P_i is the number of equivalent atoms in each molecular site.

The internal distribution of ^2H, which was until now inaccessible, may therefore be represented by the ratios, R_h, R_s, or the mole fractions $f(i)$ which constitute new powerful parameters for characterizing different samples of ethanol. The average deuterium content $\overline{(D/H)}$ of an ethanol can be expressed as

$$\overline{D/H} = (D/H)_I F(I) + (D/H)_{II} F(II) + (D/H)_{III} F(III) \tag{3}$$

with

$$(D/H)_i = \frac{f(i)}{F(i)} \overline{(D/H)} \tag{4}$$

Since the exchange reaction between HOD and C_2H_5OD is fast with respect to the NMR time scale and since the equilibrium constant is nearly equal to unity, $(D/H)_{III} \sim (D/H)_{HOD}$. For ethanols prepared in France, this value referred to the V_SMOW/SLAP Scale (Gonfiantini, 1978) is on the order of 150–152 ppm.

In fact, when a series of identical chemical molecules is investigated, it is not necessary to refer the isotope ratios $\overline{(D/H)}$ to those of standard water samples (Craig, 1961; Hagemann et al., 1970). In this case, it is more convenient to compare the $\overline{(D/H)}$ value of an ethanol sample (x) to that $\overline{(D/H)}^0$ of a reference ethanol (0). Then, we may define a parameter ρ which represents the change of the $\overline{(D/H)}$ value within a series of different ethanols, whereas the R parameter (eq 1) expresses the dispersion of the deuterium distribution in a given ethanol:

$$\frac{(D/H)^x}{(D/H)^0} = \rho \tag{5a}$$

and

$$\frac{(D/H)_i^x}{(D/H)_i^0} = \rho_i \tag{5b}$$

ρ can be obtained either from mass spectrometry or from NMR measurements and ρ_i is deduced from the NMR data. Defining the experimental parameter T and taking eq 4 into account ρ and ρ_i are given by eq 7a and 7b:

$$T = \sum_{i=1}^{3} S_i / S_{WS} \tag{6}$$

$$\rho = \frac{T^x}{T^0} \tag{7a}$$

$$\rho_i = \frac{f(i)^x}{f(i)^0} \frac{T^x}{T^0} = \frac{f(i)}{f(i)^0} \rho \tag{7b}$$

S_{WS} is the signal intensity of a working standard (C_6H_6, CH_3CN, $H_2O + H^+$, ...) contained in a coaxial 5-mm OD tube assembled in the high-precision 15-mm OD NMR cell. When azeotropic ethanols are compared (water content ~ 4.4%) eq 6–7 can be considered as a good approximation.

However, the molar fractions $f(i)$ determined from peak areas suffer of a greater experimental error than the R values, obtained from peaks heights (see Materials and Techniques). It is then more accurate to deal with the parameter M

$$M_I^x = \frac{H_I^x}{H_{WS}} \Big/ \frac{H_I^0}{H_{WS}} \tag{8}$$

providing that the condition of identical line width ratios $\Delta\nu_{1/2}(I)/\Delta\nu_{1/2}(WS)$ in samples x and 0 is satisfied.

Thus, a two-dimensional representation of the deuterium content of ethanols in the plane (R, ρ) offers a powerful way of discriminating the origins of natural or synthetic products.

Materials and Techniques. The sample is used as an aliquot resulting from a simple distillation of the spirit followed by a rectification of the ethanol using a standardized column. It is advisable but not necessary to dry the ethanol samples by consecutive treatments with CO_3K_2 and CaH_2. However, this treatment may induce small but significative changes in the deuterium distribution.

Usually 8–10 mL of ~95% ethanol are introduced into a 15-mm OD NMR cell, but smaller quantities, of the order of 1 mL, can be handled providing that small-size cylindrical cells are used. It should be noted that field-frequency locking at the deuterium frequency is prevented when observing ^2H resonance using a conventional probe. However, this situation is not really a drawback with superconducting spectrometers which produce usually a very

Table I. ^2H NMR and Mass Spectrometry Data Concerning Some Typical Ethanols Obtained from Controlled Fermentation of Vegetals or Hydration of Ethylene[a]

identified industrial ethanols	NMR							MS[b]		
	M_I^x	s	R_h(II)	s	f(I)	f(II)	f(III)	D/H, ppm	s	ref
corn (starch)	1.00		2.22	0.04	0.457	0.338	0.205	122.1	2.0	*f, g*
sugar cane (sucrose)	0.98	0.03	2.29	0.05	0.451	0.346	0.203	123.2	2.5	*f*
wheat (starch)	0.94	0.02	2.47	0.02	0.433	0.358	0.209	119.5	*c*	*g*
barley (starch)	0.95	0.03	2.51	0.05	0.430	0.360	0.210	not available		
apple (fructose)	0.90	0.02	2.58	0.03	0.425	0.364	0.211	118.7	*c*	*f*
potato (starch)	0.87	0.02	2.71	0.03	0.414	0.372	0.214	117.6	4.0	*g*
sugar beet (sucrose)	0.85	0.02	2.72	0.05	0.410	0.372	0.218	114.7	1.0	*f*
ethylene[d]	1.10	0.01	2.31	0.03	0.460	0.353	0.187	133.9	*c*	*f, g*
ethylene[e]	1.08	*b*	2.51	*c*	0.444	0.372	0.184	136.0	*c*	*f, g*

[a] The parameter R_h represents the relative intramolecular distribution of deuterium in the ethyl fragment and M is the relative depletion (or enrichment) in deuterium of the methyl group in the series, the value measured for corn being taken as the reference (see the text for the definition). The reported standard deviation (s) indicated the dispersion on the R or $\overline{D/H}$ values measured on different samples. The standard deviation of the spectroscopic determination is smaller than 0.01. [b] The MS data were extracted from the literature and therefore do not correspond to the same samples of ethanol as those studied by NMR. The $\overline{D/H}$ values were computed from $\delta D‰$ values according to the formula $\overline{D/H} = 0.15576(\delta D‰ + 1000)$. [c] Only one value was available. [d] Contact process. [e] H_2SO_4 process. [f] Bricout (1978); Bricout et al. (1975). [g] Rauschenbach et al. (1979).

stable field. The spectrometer used in these experiments (Brüker WM 250) has a field drift of less than 0.1 Hz in 1 h. The homogeneity of the field should be carefully adjusted and that can be done, for example, by observing the ^1H spectrum of the sample through the decoupling coil. Since we are dealing with compounds presenting a small number of lines in the frequency domain, the free induction decay (FID) in the time domain has a simple and characteristic shape which can be used to monitor the shims in order to improve the resolution and to maintain it at a fixed level (Martin et al., 1980). The ^2H spectrum is recorded by using the following acquisition parameters: sweep width 1200 Hz, acquisition time 6.8 s; pulse width $\sim 115 \times 10^{-6}$ s (90° flip angle), ^1H broad-band decoupling. If we consider that the T_1 relaxation time values measured in a series of ethanols (Martin et al., 1982b) are on the order of 1 s, the pulse repetition time used in the experiment is long enough to recover 99.9% of the magnetization. Several runs are performed on the same sample with a number of transients sufficient to give a signal-to-noise ratio as high as ~150 in the transformed spectrum for the signal of molecules I. Usually 4000–6000 transients are stored on a magnetic disc in blocks of different sizes, according to the quality of the tuning of the probe. The FID's are weighted by using two different values of line broadening in order to minimize the effect of fluctuations in the line heights, and a 32K Fourier transform with zero filling is performed. The different frequency spectra are then processed in two ways, from the peak printouts given by the computer and from the paper records which also offer the possibility of checking the straightness of the base lines of the frequency spectrum and integral curve. A statistical treatment of about 18–24 intensity values automatically leads to the mean and standard deviation for R(i), f(i), and M_i. The standard deviation computed for R_h(i) which is usually on the order of 0.01 or less corresponds to a reproducibility better than 0.5%. In terms of limits of 99% confidence level, assuming an approximately normal distribution—the degree of freedom is large and the t value of the Student distribution is nearly equal to the z parameter of the normal one—we obtain for the mean $\bar{R} \pm 0.006$.

The mole fractions are less precisely known since the dispersion of integral measurements is greater by about 1 order of magnitude than that of the R_h determinations. The standard deviation of a mole fraction value f(I) or f(II) is on the order of 0.005 for the different samples studied and corresponds to a reproducibility of 1.5–2%. However, the confidence limits are larger since the number of available integral measurements is considerably smaller; for a t distribution we obtain $\bar{f} \pm 0.006$.

Table II. Internal Distribution of Deuterium in Ethanols Extracted from Different Commercial Spirits Obtained from Cereals[a]

nature		origin	sample	R
whiskies	bourbon	Canada	CW1	2.273
	bourbon	Canada	CW2	2.303
	bourbon	Canada	CW3	2.295
	bourbon	U.S.A.	AW1	2.371
	bourbon	U.S.A.	AW2	2.304
	bourbon	U.S.A.	AW3	2.320
	rye	U.S.A.	AW4	2.405
	b	Ireland	IW1	2.480
	b	Ireland	IW2	2.393
	b	Ireland	IW3	2.429
	pure malt	Scotland	SW1	2.462
	malt	Scotland	SW2	2.457
	blended	Scotland	SW3	2.405
	blended	Scotland	SW4	2.418
vodkas	pure grain	USSR	RV1	2.463
	b	USSR	RV2	2.627
	pure grain	Poland	PV1	2.546
	b	Poland	PV2	2.557
	pure grain	Finland	FV1	2.551
	pure grain	Finland	FV2	2.558
gins	*b*	England	EG1	2.220
	b	England	EG2	2.259
	b	England	EG3	2.217
	b	England	EG4	2.221

[a] The standard deviation of the NMR measurements is smaller than 0.01. [b] Unspecified on the label.

RESULTS AND DISCUSSION

A series of experiments has been performed on fully identified natural and synthetic alcohols. The natural ethanols were obtained from fermentation of sugar cane, sugar beet, and apple and from hydrolysis and fermentation of starches from various cereals (barley, corn, oat, rye, wheat) and also from potato. The synthetic ethanols were obtained from ethylene hydration processes based on either the contact or sulfuric acid method. The results corresponding to several test alcohols are collected in Table I and typical spectra of corn and sugar beet ethanols are shown on Figure 1.

Another series of measurements concerns commercial spirits. Table II gives the deuterium contents of ethanols extracted from different kinds of spirits produced in the fermentation of cereals (whishies, vodkas, gins) and Table

Table III. Internal Distribution of Deuterium in Ethanols Extracted from Different Commercial Rums or Fruit Spirits[a]

nature		origin	sample	R
rums:	molasses	West Indies	RR1	2.211
	b	West Indies	RR2	2.187
	b	West Indies	RR3	2.227
	b	West Indies	RR4	2.204
	molasses	Antillas	AR1	2.273
	molasses	Antillas	AR2	2.268
	molasses	Antillas	AR3	2.251
	b	Antillas	AR4	2.245
	cane juice	Antillas	AR5	2.272
	cane juice	Antillas	AR6	2.300
apple brandies		France	AB1	2.582
		France	AB2	2.576
		France	AB3	2.574
		France	AB4	2.538
plum brandies		France	PB1	2.611
		France	PB2	2.536
		France	PB3	2.631
		France	PB4	2.561
		Yugoslavia	PB5	2.614
		Yugoslavia	PB6	2.602

[a] The standard deviation of the NMR measurements is smaller than 0.01. [b] Unspecified on the label.

III shows the results concerning rums (sugar cane), apple brandies, and plum brandies. These commercial samples were manufactured from vegetals grown in various geographical regions.

In principle it is possible to check the origin of the ethanols contained in commercial beverages by resorting to the data obtained from standard ethanols, and the case of the reference alcohols will be discussed first.

Reference Ethanols. It is clear from the examination of Table I that the high-field quantitative ^2H NMR method provides a simple and rapid way for identifying ethanols obtained in the fermentation of different vegetals. Moreover, since the intramolecular distribution of deuterium may depend on the nature of the plant, on the biochemical pathway of fermentation and on environmental or climatological conditions, the new selective parameters which we have defined, along with the discussion of their relationship with the overall ($\overline{D/H}$) content determined either by NMR or by mass spectrometry, should provide useful information on the mechanisms of the bioconversion of fruits and cereals into alcohol.

A clear distinction is made between ethanol samples obtained from C3 (corn, sugar cane) and C4 plants. The difference in deuterium fractionation between the plants belonging to both groups can be understood in terms of the contents of water involved in the photosynthesis cycle and in terms of cellular permeation. In this respect experiments are in progress for getting more insight into the biomechanisms of isotopic fractionation which occur in the formation of the carbohydrates and in the fermentation processes.

From the analytical point of view which is considered here, it is important to emphasize that the different kinds of C3 vegetals lead to ethanols characterized by very different ^2H internal distributions. Note also that an increase in the R parameter corresponds to a greater depletion in deuterium of the methyl group (M). The regular decrease of M which accompanies that of the $\overline{D/H}$ values of glucoses (Bricout et al., 1975; Bricout, 1978) extracted from corn (157.5 ppm) sugar cane (154.7 ppm), and sugar beet (145.4 ppm) suggests a direct filiation of the methyl group of ethanol from the nonexchangeable sites of the saccharide. It should be noted, however, that small but significative changes in the R parameter may be introduced by the

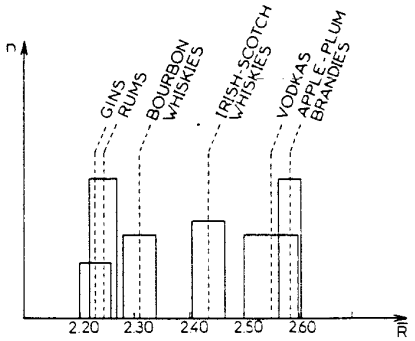

Figure 2. Statistical distribution of the population sample of various grain and fruit spirits. $\bar{R}$ is the sample mean of the selective ratio R. Its interval of confidence, represented by the width of the block corresponding to each spirit, was computed at the 95% level from the relations (s = standard deviation) (ti, ts) = $x - t(n-1, 0.975)s/n$, $x + t(n-1, 0.975)s/n$ (a Student t distribution was assumed for the samples; n is the number of independent observations). Note that the width of the block represents the dispersion of the population sample and not that of the NMR measurement which is significantly smaller ($\bar{R} \pm 0.006$ at the 99% confidence level).

industrial procedure used to ferment and to extract the spirit. Slight dependencies on rainfall and temperature (Martin et al., 1982b) are also the source of limited scattering in the R values. For a series of sugar beet ethanols grown in the North of France for example a relationship is found between R and both the temperature and the height of the rainfalls. Taking into account the fact that natural water has a higher deuterium content than ethanol, in which the methyl group is depleted with respect to the methylene group, it may be suggested than an increase in temperature favors the incorporation of deuterium into the carbohydrate producing a decrease in the R value. By contrast a higher quantity of water coming from rainfall appears to decrease the deuterium content in the methyl group. These phenomena constitute only second-order effects, which explain the dispersion observed in the R values ($R = 2.72 \pm 0.05$), the main factor remaining being the nature of the plant.

Commercial Spirits. Seven categories of commercial spirits have been investigated. The results are collected in Table II and III and Figure 2 allows the statistical distributions to be compared.

Grain Spirits (Table II). From the examination of Table II it is clear that as far as deuterium distribution is concerned, whiskies must be divided into two groups. A genuine bourbon whisky should be characterized by an R value lower than 2.31 (0.03) (the so-called AW1 bourbon may be suspected to result at least in part from C3 cereals). The three Canadian whiskies investigated probably contain a high proportion of corn spirit. It is also interesting to note that the rye whisky is clearly distinguishable from the corn spirits and from the other alcohols obtained from C3 cereals (wheat, barley, oat). Scotch whiskies also present interesting peculiarities. Clearly "single malt" and "malt" whiskies have greater R values than blended whiskies. Thus, if values $R = 2.48$ and $R = 2.23$ are retained respectively for a pure malt whisky and for a pure corn alcohol, the blended whiskies SW3 and SW4 are assumed to contain respectively about 30% and 25% of corn alcohol. Of course, since the number of perfectly identified samples is too small to have a statistical meaning, these figures should be considered as illustrative examples of the potentiality of the method and not as definitive standards of deuterium distribution. The average R value of Irish and Scotch whiskies is 2.435 (0.032); since the average R

value for the whole population of whiskies is 2.379 (0.069), Irish-Scotch whiskies are significantly different from bourbon whiskies (F test).

The four samples of gin investigated here offer good examples of pure corn alcohol since their average value $\bar{R}$ = 2.23 (0.02) is very close to the value of the standard corn ethanols (Table I).

Finally, the vodkas are characterized by high R values denoting either C3 cereals or mixtures of potatoes and cereals. It should be noted that the VR2 vodka is an exported spirit whereas the VR1 sample has been bought in the USSR. The Polish and Finnish vodkas either exported or sold in the country have roughly the same value (R = 2.56). The average values of the samples investigated is 2.55 (0.05).

Rums and Fruit Spirits (Table III). Like corn, sugar cane is a C4 plant and its photosynthetic cycle is responsible for a higher deuterium content and therefore a relatively small R value: R = 2.245 (0.036). The standard deviation corresponds to the population sample studied here (n = 10), but a more discriminative inspection of the results suggests two remarks. First, it appears that geographical and climatological conditions are second-order factors which play a role in the variations of the deuterium distributions since $\bar{R}$ = 2.21 (0.015) for West India's rums and $\bar{R}$ = 2.27 (0.02) for Antillas rums. Second, the procedure used in the manufacturing of the rums also affects the R value and slight but significative differences are observed between rums processed from sugar cane juices or molasses.

Apple and plum brandies display relatively high R values, and it seems that a significant difference exists between the two means of the population samples. It is also interesting to note that the average $\bar{R}$ value obtained for plum brandies is nearly equal to the R value measured for an ethanol extracted from a fermentation of pure fructose, $\bar{R}$ = 2.59 (0.035). The average value observed for apple brandies appears to be slightly smaller, $\bar{R}$ = 2.565 (0.02), and for the whole population of fruit spirits $\bar{R}$ = 2.585 (0.03).

CONCLUSION

We have therefore shown that very substantial differences exist in the distribution of ^2H within ethanol molecules according to their origin. We dispose now of new and very efficient parameters for identifying alcohols and investigating more deeply the biomechanisms which govern their formation.

ACKNOWLEDGMENT

We thank Bardinet S.A. and the Compagnie Métropolitaine des Rhums from which the samples of rums have been obtained.

Registry No. Deuterium, 7782-39-0; ethanol, 64-17-5.

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Received for review July 6, 1982. Accepted November 22, 1982.