JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 279−284 **279**

Study of the Influence of Alcoholic Fermentation and Distillation on the Oxygen-18/Oxygen-16 Isotope Ratio of Ethanol

CLAUDIA MONSALLIER-BITEA,† ERIC JAMIN,*,† MICHÈLE LEES,† BEN-LI ZHANG,§ AND GÉRARD J. MARTIN†

EUROFINS Rue Pierre Adolphe Bobierre, B.P. 42301, F-44323 Nantes Cedex 3, France, and LAIEM - UMR CNRS 6006, Université de Nantes, 2 Rue de la Houssinière, F-44322 Nantes Cedex 3, France

A laboratory procedure for the analysis of the oxygen-18/oxygen-16 isotope ratios of ethanol derived from sugars and fruit juices by pyrolysis−isotope ratio mass spectrometry (IRMS) has been applied to the study of isotopic fractionation induced by the isotope effects of fermentation and distillation. For both processes, an experimental model has been established to describe and explain the observed fractionation phenomena. It is shown that reproducible results can be obtained when appropriate analytical conditions are used. Moreover, the ability of ethanol to act as a reliable indicator of the $^{18}O/^{16}O$ ratio of sugars in orange juice (and therefore to be used as an internal reference for detecting water addition) is demonstrated both in theory and in practice.

KEYWORDS: Oxygen-18; exchange; water; ethanol; sugar; authentication; isotopic fractionation

INTRODUCTION

Measuring the isotope ratios of oxygen ($^{18}O/^{16}O$) and hydrogen ($^{2}H/^{1}H$) in water by isotope ratio mass spectrometry (IRMS) is routinely used for the detection of water addition in fruit juices and wines. Due to isotopic fractionation phenomena that occur during plant transpiration, these isotope ratios are usually higher in plants than in meteoric water, so that the addition of water results in a lowering of the ratios measured on authentic fruit juice or wine samples. Expected minimum values for these ratios in different fruit juices are provided as guidelines by the Association of the Industry of Juices and Nectars from fruits and vegetables of the European Union (AIJN) in their widely recognized Code of Practice (*1*). The analytical methods used to measure isotope ratios have gained official recognition from the Comité Européen de Normalisation (CEN), from the Office International de la Vigne et du Vin (OIN), and from the European Community (*2*−*4*).

The main drawback of this technique, however, is that the isotope ratios of fruit water depend on the geographical origin and harvesting period of the fruit, so that interpreting the results requires fairly extensive and up-to-date databases (*5*). This approach is further limited when the country of origin of a fruit juice is not known or specified, a situation that occurs frequently. In this case, the range of acceptable values is very broad, and as a consequence the detection limit of added water is relatively high.

To improve the detection of water addition in fruit juices, the $^{18}O/^{16}O$ ratio of the extracted sugars has been proposed as an internal reference (*6*). However, the ^{18}O-IRMS analysis of sugars is poorly reproducible, as described previously (*7*). We recently introduced an alternative method that consists of using the ethanol isolated in the course of the routine procedure for the isotopic analysis of site-specific $^{2}H/^{1}H$ ratios by SNIF-NMR and providing a higher reproducibility than sugars (*7*). The use of the $^{18}O/^{16}O$ ratio of ethanol as an additional authenticity criterion was also recently proposed in the case of wines and spirits (*8*). Although the feasibility of this technique has already been established, a more detailed evaluation of the isotopic behavior of oxygen during the analytical steps involved was required to provide a better understanding of the results and to strengthen the theoretical background of this analytical method.

The distillation step in any isotopic methodology must always be carefully monitored because thermodynamic isotope effects result in significant fractionation as a function of the extraction rate of the product considered. For example, Majoube (*9*) showed that water distillate is depleted in deuterium and in oxygen-18 and precisely determined the corresponding fractionation factors. In the case of ethanol, Moussa et al. (*10*) observed similar isotopic fractionation for hydrogen and carbon, but, interestingly, the distillates are enriched in the heavy isotopes of all the ethanol isotopomers with the exception of C_2H_5OD. It is therefore likely that the oxygen atoms in ethanol will also undergo isotopic fractionation during distillation, so that this point needs to be carefully measured and taken into account in all analytical procedures involving a distillation step.

Enzyme-catalyzed hydrogen exchange has been shown to occur during the fermentation of sugars with yeasts, and the

* Author to whom correponsdence should be addressed [telephone +33 (0)2 51 83 21 00; fax +33 (0)2 51 83 21 11; e-mail ericjamin@eurofins.com].
† EUROFINS.
§ LAIEM - UMR CNRS 6006.

10.1021/jf0516686 CCC: $33.50 © 2006 American Chemical Society
Published on Web 12/24/2005

isotopic abundances of isotopomers CH_3CHDOH and, to a lesser extent, of isotopomers CH_2DCH_2OH are directly proportional to the isotopic abundance of water; this dependency has been used to calculate the enzymatic fractionation factors of yeasts (*11*, *12*). The exchange of methylene hydrogen occurs in the oxidoreduction pathway of ethanol and acetaldehyde mediated by NAD^+/NADH. It is also very likely that ^{18}O of acetaldehyde exchanges with water through hydration, although previous results tend to indicate only a partial exchange (*13*). Therefore, to ensure the robustness of an analytical strategy using the measurement of the $^{18}O/^{16}O$ isotope ratio of fermentation ethanol, the extent of such isotopic exchange must be determined. This paper presents, from a series of experimental results, the isotopic fractionation phenomena associated with alcoholic fermentation and distillation and defines suitable analytical conditions to obtain meaningful $^{18}O/^{16}O$ measurements of ethanol.

MATERIALS AND METHODS

Sample Description. Beet sugar and orange juice concentrate were obtained from industrial suppliers and used as starting materials for the fermentation. The experiments were carried out using Nantes tap water (NTW), unless specified otherwise.

Fermentation, Distillation, and Dehydration of Ethanol. The procedures used in this study to quantitatively transform the sugars into ethanol and to isolate the ethanol for the subsequent IRMS measurement were adapted from the SNIF-NMR method AOAC 995.17 (*14*). Using *Saccharomyces cerevisiae* yeast ("Val Oeno", Vallet, France) at a concentration of 5 g/L, beet sugar solutions and orange juices (from concentrate) were completely fermented to ethanol, which was then extracted by high-yield automated distillation, using a Cadiot spinning band distillation column. The residual water in the distillate was trapped by storing for at least 24 h on molecular sieve (2 mm beads, UOP type 3A, Fluka Chemie GmbH, Buchs, Swizerland). The efficiency of the water removal after fermentation by high-yield distillation (>96%) followed by residual water trapping using a molecular sieve has been validated previously (*7*).

Isotopic Determinations. The $^{18}O/^{16}O$ isotope ratios of water were measured on CO_2 previously equilibrated with the juice samples according to the routine procedure described in the corresponding official method of fruit juice analyses (*3*). The CO_2 resulting from the equilibration step was introduced via a manifold (GV Instruments, Manchester, U.K.) into the source of an isotope ratio mass spectrometer (OPTIMA from GV Instruments).

The mass spectrometric determinations of the oxygen isotope ratios of ethanol were carried out by on-line analysis using an elemental analyzer (NA 1500 series, Fisons Instrument SpA, Milano, Italy) fitted to the same isotope ratio mass spectrometer. Ethanol samples are first placed in silver capsules as carefully as possible to avoid contamination by water or organic matter (in particular, the pipet and containers must be totally clean and dry, and once filled the containers must be sealed as quickly as possible with pliers). The samples are dropped into the elemental analyzer, where the pyrolysis takes place at 1060 °C. The furnace used in this study is a quartz tube filled successively with carbon fiber, nickel wool, and nickelized carbon. All of the carbon from the sample is degraded into carbon monoxide gas. A helium flow carries the pyrolysis gas into a GC column to separate carbon monoxide from any other gas generated by the pyrolysis. Carbon monoxide is then brought by the helium flow into the mass spectrometer.

The oxygen isotope ratio of carbon monoxide used as reference gas is calibrated against Vienna Standard Mean Ocean Water (VSMOW; IAEA, Vienna, Austria) by analyzing VSMOW and Standard Light Antarctic Precipitation (SLAP; IAEA) water samples. In addition, two working standards are used in each series of measurements: a 99.5% pure crystallized glucose and a 99% pure ethanol (SIGMA-Aldrich, Saint-Quentin Fallavier, France).

The results can be expressed on the δ ‰ scale with respect to the international standard VSMOW according to the relationship

$$\delta^{18}O_{sample}\ (‰) = (R_{sample}/R_{VSMOW} - 1) \times 1000 \qquad (1)$$

where $R = {}^{18}O/{}^{16}O$. However, the calculations to establish the models in this paper have been based on isotope ratios $^{18}O/^{16}O$ (noted as $R^{18}O$), to avoid numerical inaccuracies. The internal reproducibility of $R^{18}O$ for the methods used in our laboratory is estimated, based on daily measurements of working standards for several years, at 0.6 ppm for water and 1.0 ppm for ethanol, corresponding to 0.3 and 0.5‰, respectively, for the $\delta^{18}O$ of water and of ethanol.

Karl Fischer Titration and Density Measurements of Water−Ethanol Mixtures. These determinations were performed according to the procedures described in the SNIF-NMR method (*14*). The content of water in ethanol samples was measured using a Karl Fischer titrator type Mettler DL18 (Mettler Toledo S.A., France) and an analytical balance Mettler AE100 (linearity of 0.02 mg). The internal reproducibility of measurements is estimated at 0.05%. The results are expressed as alcoholic grade tD (w/w) of ethanol.

Study of the Isotopic Effects of Distillation. To complete previous work on carbon and hydrogen isotope ratios (*10*), a study of the liquid−vapor fractionation of oxygen during the distillation of ethanol was carried out. Several distillation experiments ($N = 1-10$) were performed with a Cadiot spinning band distillation column, using commercial absolute ethanol 99.5% (Technisolv, VWR International, Fontenay-sous-Bois, France) in which we measured an alcoholic grade tD = 99.99% (w/w) and an oxygen isotope ratio $R^{18}O = 2028.8$ ppm. The experiments were performed on the same spinning band column and under strictly identical conditions with the same starting quantity of ethanol (100 g). The distillations were stopped at different yields of ethanol extraction ρ. Isotope ratios of oxygen in the distillate R^D and the distillation residue R^L were measured by IRMS.

Study of the Oxygen Isotope Filiation and Exchange in the Alcoholic Fermentation. For a better understanding of isotopic exchange of oxygen during the fermentation, that is, to establish the proportion of ^{18}O in ethanol originating from sugar and from water, respectively, six fermentation experiments were carried out in three waters having three slightly different $^{18}O/^{16}O$ ratios. The initial water ($\delta^{18}O = -7.0$‰) was obtained by using an Ultrapure water system (Nanopure, Barnstead, Hayward, CA). Two batches of slightly enriched water ($\delta^{18}O = 1.7$ and 10.2‰, respectively) were obtained by evaporation of the previous deionized water at 70 °C under vacuum for different times using a Rotavapor Büchi R-114 (Büchi Labortechnik AG, Flawil, Switzerland). The fermentations were performed with orange juice concentrate (65° Brix) and crystallized beet sugar, respectively. The ethanol obtained was isolated by high-yield distillation using a Cadiot spinning band distillation column followed by residual water trapping on molecular sieve, as described above. The $^{18}O/^{16}O$ isotope ratios were measured in the fermentation medium (orange juice concentrate or beet sugar diluted in water), on the ethanol, as well as on the distillation residue. The $^{18}O/^{16}O$ isotope ratios of the initial sugars were also measured after extraction according to the method of Jamin et al. (*15*) in the case of the orange juice.

RESULTS AND DISCUSSION

Study of the Isotopic Effects of Distillation. The oxygen isotope ratios in the distillate and in the distillation residue for the different yields of ethanol extraction are given in **Table 1** and plotted on the graph in **Figure 1**.

The unit isotopic fractionation factor α was calculated by an exponential curve fitting using models (eqs 2a and 2b) derived from the Rayleigh equations (*10*):

$$R^L/R^Q = (1 - \rho)^{(\alpha-1)} \qquad (2a)$$

$$R^D/R^Q = [1 - (1 - \rho)^{\alpha}]/\rho \qquad (2b)$$

Table 1. Results of the Experimental Distillation Model[a]

			distillate				distillation residue				
sample	ρ	duration (min)	mass (g)	tD^D % (w/w)	R^D (ppm)	$\delta^{18}O^D$‰	mass (g)	tD^L % (w/w)	R^L (ppm)	$\delta^{18}O^D$‰	mass losses[b] (g)
1	0.0454	12	4.54	99.98	1991.8	–6.7	95.46	99.97	2029.6	12.2	0.0
2	0.1024	34	10.24	99.91	1992.8	–6.2	89.67	99.99	2032.1	13.4	0.10
3	0.1543	51	15.43	99.95	1995.9	–4.7	84.70	99.99	2034.0	14.4	–0.06
4	0.2004	69	20.04	99.98	1995.2	–5.0	79.90	99.99	2033.6	14.2	0.08
5	0.3900	138	39.00	99.50	1999.9	–2.7	60.73	100.00	2046.1	20.4	0.29
6	0.6142	211	61.42	100.00	2005.2	0.0	38.32	99.97	2064.0	29.3	0.27
7	0.7940	283	79.40	99.98	2012.0	3.4	20.08	100.00	2090.7	42.6	0.56
8	0.8379	331	83.79	99.99	2014.8	4.8	15.49	99.99	2101.4	48.0	0.73
9	0.8907	425	89.07	99.99	2017.7	6.2	10.31	99.98	2121.1	57.8	0.66
10	0.9434	375	94.34	99.99	2022.7	8.7	5.17	99.97	2145.3	69.9	0.50

[a] ρ is the yield of ethanol extraction. tD, R, and $\delta^{18}O$ are defined under Materials and Methods. [b] Mass losses during the distillation experiments.

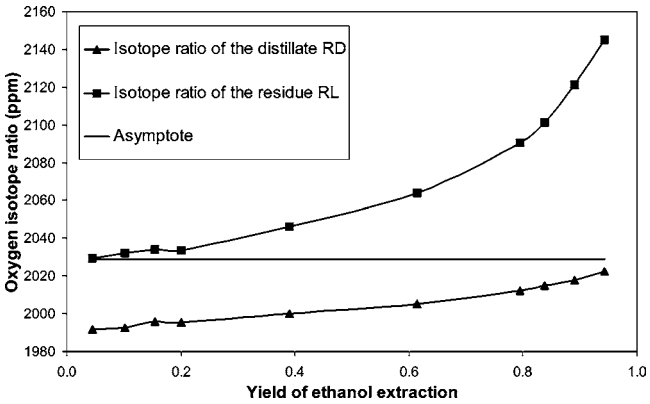

Figure 1. Behavior of the isotopic fractionation of oxygen observed during the distillation of ethanol, as a function of the extraction rate.

Table 2. Unit Fractionation Factors of Deuterium ($^2\alpha$), Carbon 13 ($^{13}\alpha$), and Oxygen 18 ($^{18}\alpha$) in Water and Ethanol, Measured with a Spinning Band Column at Boiling Point[a]

	temp (°C)	$^2\alpha$	$^{13}\alpha$	$^{18}\alpha$
water	100	0.903		0.978
ethanol	78.4	0.986	1.0037	0.981

[a] The data concerning water and ^2H and ^{13}C in ethanol are extracted from ref *10*. In each case α values correspond to the whole molecule.

Table 2 summarizes the fractionation factors of the different atoms found in the water and ethanol molecules.

Knowledge of the unit fractionation factors of ethanol is useful for standardizing analytical procedures that involve a distillation with a spinning band column. These values should be corrected for the properties of the column to give the true values of the equilibrium liquid−vapor fractionation factors of ethanol. The liquid−vapor equilibrium fractionation factor α_e can be estimated from the unit fractionation factor α considering that the number of plates of the rotating band column used is equal to 8 (*10*). The resulting α_e of ^{18}O in ethanol (at boiling point) is on the order of 0.997, which is quite consistent with that of ^{18}O in water (0.995).

The vapor pressure of $C_2H_6{}^{16}O$ is greater than that of $C_2H_6{}^{18}$), and therefore the distillate is enriched in light isotope ^{16}O, whereas the distillation residue is enriched in heavy isotope ^{18}O. Experimentally, the $^{18}O/^{16}O$ isotope ratios of both the distillate and distillation residue increase with increasing yield of ethanol extraction. However, for each distillation step, the ratio R^L is greater than R^D, indicating an enrichment in heavy isotope ^{18}O in the distillation residue and its simultaneous depletion in the distillate. For yields of ethanol extraction tending to 1, the $^{18}O/^{16}O$ isotope ratio of the distillate approaches the initial $^{18}O/^{16}O$ isotope ratio of the ethanol (see **Figure 1**), which is the condition required to avoid bias in isotopic analysis. Contrary to the inverse isotopic effects exhibited by the carbon and hydrogen isotopomers (with the exception of the hydroxyl group) in the case of ethanol distillation (*10*), normal isotope effects have been observed in the case of oxygen isotopomers.

Potential variations in oxygen isotope ratios in ethanol due to physical transformations occurring during distillation on Cadiot columns can be estimated from the calculated isotopic fractionation factors. This fractionation effect becomes negligible when the yield of ethanol extraction is >95%, which can be obtained under suitable operating conditions.

Study of the Isotope Filiation and Exchange of the Alcoholic Fermentation. The mass balance of the fermentation experiments performed in waters bearing three slightly different $^{18}O/^{16}O$ ratios is presented in **Table 3**. **Table 4** presents the $^{18}O/^{16}O$ ratios and the isotopic deviations of the start and end products.

According to the metabolic pathway of the alcoholic fermentation using baker's yeast, a transfer of the oxygen atoms from positions 2 and 5 of the glucose or fructose molecule is expected (*16*). Therefore, if the isotope exchange was not significant, the oxygen isotope composition of ethanol should reflect the initial isotope ratios of these positions in the sugar. A partial equilibrium of metabolic intermediates with the water of the fermentation medium has been postulated on the basis of literature values for sugar and ethanol, but until now no precise measurement of this exchange has been performed. Therefore, one of our goals was to estimate the proportion of oxygen transferred from sugars and water, respectively, into ethanol.

It has been shown that the hydrogen atoms of ethanol are readily exchanged with those of the fermentation medium (*11*, *12*), namely, by a substantial scrambling of the two moieties of fructose diphosphate resulting from the reactions catalyzed by triose phosphate isomerase and aldolase. On the other hand, pure ethanol dissolved in water exchanges readily when yeast is introduced in the mixture. The magnitude of the exchange depends on the incubation period and the quantity of yeast added (*17*). Because of the reversible transformation of acetaldehyde to acetaldehyde hydrate via water addition, it can also be postulated that the ^{18}O of ethanol would equilibrate with water, providing the enzymatic properties of the medium and the duration of the experiment are correctly adjusted. The purpose of this work was to determine the extent of the exchange in the usual conditions of fermentation and distillation of fruit juices.

Table 3. Analytical Conditions of the Experimental Fermentation Model[a]

			masses (g)						
sample	nature[b]	°Brix	fermentation medium	OC/BS	distilled juice	distillate	residue	loss	tD % (w/w)
14	OC + W1	12.0	488.13	110.55	348.26	15.24	331.78	1.24	90.41
15	OC + W2	12.0	491.14	110.93	349.05	15.65	332.70	0.70	90.92
16	OC + W3	11.9	495.86	111.43	348.96	15.64	332.72	0.60	91.66
17	BS + W1	12.0	527.39	71.89	345.48	22.40	322.67	0.41	91.62
18	BS + W2	11.9	534.95	72.49	345.79	21.81	323.53	0.45	92.08
19	BS + W3	12.0	529.15	72.09	345.26	22.12	322.73	0.41	92.27

[a] The fermentation medium is the solution obtained after dilution of orange juice concentrate or beet sugar in water to the °Brix indicated in the third column. The duration of fermentation for experiments 14–16 (OC) and 17–19 (BS) was 48 and 144 h, respectively. [b] OC, orange juice concentrate; BS, beet sugar; W, water.

Table 4. Isotopic Results of the Experimental Fermentation Model

		$R^{18}O$ values (ppm)				$\delta^{18}O$ values (‰)			
sample	nature	water	fermentation medium	ethanol	distillation residue	water	fermentation medium	ethanol	distillation residue
14	OC + W1	1991.3	1995.0	2061.8	1995.7	–7.0	–5.1	28.2	–4.7
15	OC + W2	2008.6	2009.3	2069.8	2010.7	1.7	2.0	32.2	2.7
16	OC + W3	2025.6	2025.5	2079.2	2025.6	10.2	10.1	36.9	10.2
17	BS + W1	1991.3	1991.1	2044.0	1991.5	–7.0	–7.0	19.3	–6.8
18	BS + W2	2008.6	2008.1	2057.5	2007.4	1.7	1.5	26.1	1.1
19	BS + W3	2025.6	2024.7	2069.-	2023.5	10.2	9.7	31.8	0.1

According to the mechanism involved in glycolysis and alcohol fermentation, acetaldehyde is the precursor of ethanol and the acetaldehyde carbonyl oxygen atoms come from C-2 and C-5 positions of glucose or fructose. Before the formation of acetaldehyde from glucose or fructose, there is very little exchange. However, the acetaldehyde can exchange its oxygen with water throughout the formation of acetaldehyde hydrate. For free acetaldehyde in water, this exchange is very significant. However, as shown by our results, for acetaldehyde as a biotransformation intermediate, this exchange is, fortunately, quite limited in the experimental conditions applied.

In a pure natural fruit juice, the oxygen atoms in the sugars are closely correlated with those of the juice water (*6*)

$$R_{\text{sugar}} = aR_{\text{juice water}} + b \qquad (3)$$

with $R = {}^{18}O/{}^{16}O$. The intercept b is the contribution of atmospheric CO_2 oxygen coming from the photosynthesis.

The ethanol produced through alcoholic fermentation of the fruit sugars contains oxygen atoms from both the fruit sugars and the sample water:

$$R_{\text{ethanol}} = a''R_{\text{sample water}} + a'R_{\text{sugar}}$$
$$= a''R_{\text{sample water}} + a'(aR_{\text{juice water}} + b) \qquad (4)$$

a'' is the slope of the equation, whereas $a'R_{\text{sugar}}$ or $a'(aR_{\text{juice water}} + b)$ is its intercept, which represents the sugar oxygen contribution.

On the basis of the results obtained in fermentations of a given sugar in waters of different $R_{\text{sample water}}$ values (**Table 4**), both the slope and intercept values of the equation (the responses of ethanol to the dilution water) can be calculated and are shown in **Table 5**. If we compare the two sets of experiments, the slope obtained with beet sugar is greater than that obtained with orange juice, whereas the situation is the contrary for the intercepts. These results show that the contribution of water oxygen is higher for ethanol derived from beet sugar than for ethanol derived from orange juice, which means that the integration of water oxygen is more significant in the former case. The results for both sets of fermentation show that the contribution of nonexchangeable oxygen from sugar in the ethanol is significant because the intercept values are large, especially for orange juice. This therefore demonstrates the validity of the method that uses the ethanol derived from juice sugars as internal reference.

Table 5. Linear Correlations between the $^{18}O/^{16}O$ Ratio of the Initial Waters and the $^{18}O/^{16}O$ Ratio of Ethanol Resulting from the Fermentation of Orange Juice Concentrate and Beet Sugar, Respectively, in These Waters[a]

	orange juice	beet sugar
slope (a'')	0.507	0.728
SD	0.026	0.032
intercept ($a'R_{\text{sugar}}$)	1051	595
correlation coefficient	0.997	0.998

[a] The symbols used refer to eq 4. SD corresponds to standard deviation of the slope.

It is also interesting to compare the data concerning oxygen exchange (**Table 5**) with those discussed previously (*11*, *12*, *17*) in the case of hydrogen. With regard to the fermentation of pure sucrose in water, the slopes of the dependence of the isotope ratio of ethanol versus the initial water are nearly identical (0.74) for oxygen and hydrogen, suggesting an isotope effect equal to ≈1.4. When the medium is enriched in nutrients present in fruit juices, the fermentation is accelerated and the rather slow exchange does not reach equilibrium (the slope is only 0.51). However, there are other possible explanations for the difference in behavior between fruit juices and pure sucrose. In the sucrose disaccharide, the anomeric carbons of both glucose and fructose are linked through an oxygen bridge. Before glycolysis, sucrose is converted to monosaccharides (glucose and fructose) through enzyme-catalyzed hydrolysis during which the *O*-glycoside link is broken and a water molecule is added. The water oxygen thus enters fructose on C-2 or glucose on C-1 after the disaccharide inversion. Orange sugars contain a limited proportion of sucrose (together with glucose and fructose), whereas beet sugar is pure sucrose, which might also explain why the proportion of oxygen atoms from

Table 6. Results of the Spiking Experiments

		masses (g)						
sample	nature[a]	distilled juice	distillate	residue	loss	tD % (w/w)	$\delta^{18}O_{juice\ water}$ (‰)	$\delta^{18}O_{ethanol}$ (‰)
20	100% NFC (0% FC)	349.3	16.27	332.14	0.89	91.49	2.5	29.7
21	80% NFC + 20% FC	298.50	13.69	284.45	0.36	91.63	0.8	29.1
22	60% NFC + 40% FC	299.03	13.93	284.68	0.42	89.35	–0.8	28.2
23	40% NFC + 60% FC	298.52	13.30	284.75	0.47	91.70	–2.4	28.0
24	20% NFC + 80% FC	298.45	12.93	285.06	0.46	92.08	–4.0	27.4
25	100% FC	348.95	15.52	332.66	0.77	91.80	–5.6	26.5

[a] NFC, orange juice not from concentrate; FC, orange juice from concentrate.

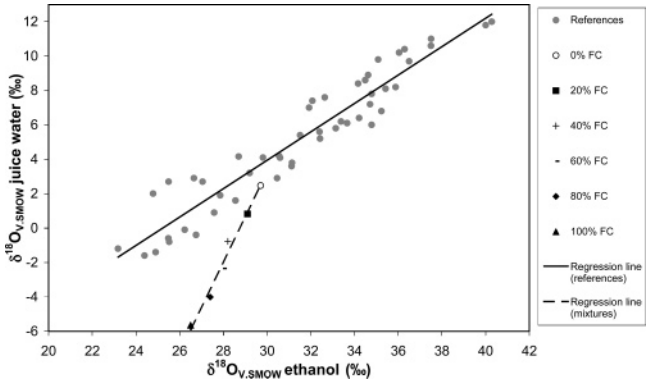

Figure 2. Relationship between the isotopic deviations of juice water and ethanol in authentic orange juice mixtures ("references") and in various blends of not from concentrate (NFC) and from concentrate (FC) orange juices ("mixtures" containing 0–100% FC juice). Values for "references" were taken from ref *7*; values for "mixtures" were taken from **Table 6**.

water is higher in ethanol derived from beet sugar (than that derived from orange juice). It is also likely that, as for the isotopes of other elements such as hydrogen and carbon, the oxygen isotope distribution in the six hydroxy groups of hexoses is nonstatistical. Thus, the site-specific oxygen isotope ratio difference at positions C-2 and C-5 between orange and beet sugars may also be, to a certain degree, responsible for the difference observed for the ethanol issued from the two different samples.

Application to Authenticity Testing of Not From Concentrate (NFC) Orange Juice. To check the usefulness of the relationship between the $^{18}O/^{16}O$ ratios of water and ethanol for testing NFC orange juice authenticity, several spiked solutions made up of mixtures of known composition of NFC orange juice with orange juice from concentrate were investigated.

The complete fermentation of samples 20−25 described in **Table 6** was carried out, and the resulting ethanol was analyzed as described above. In **Figure 2**, the oxygen isotopic deviations of the water $\delta^{18}O_{juice\ water}$ as a function of the isotopic deviations of ethanol $\delta^{18}O_{ethanol}$ for the various mixtures of NFC and FC orange juices prepared in this study are compared to previously published results obtained using the same method for authentic orange juices from a large number of origins worldwide (references) (*7*).

This graph clearly illustrates the possibility of lowering the detection limit of such adulteration by taking into account these two parameters simultaneously. Because the $^{18}O/^{16}O$ ratio of ethanol is only partially influenced by the isotopic $^{18}O/^{16}O$ ratio of the juice, as demonstrated in this study, and offers a better analytical precision than other internal references such as sugars, as shown previously (*7*), ethanol produced in standardized fermentation conditions acts as a reliable internal reference for the detection of water addition.

When the sample is an authentic juice, $R_{juice\ water} = R_{sample\ water}$, and eq 4 can be rewritten as

$$R_{sample\ water} = (R_{ethanol} - b)/[a'(a + a'')] \quad (4a)$$

When the sample has been modified by added water, $R_{juice\ water} \neq R_{sample\ water}$ and eq 4 can be written as

$$R_{sample\ water} = [R_{ethanol} - a'(aR_{juice\ water} + b)]/a'' \quad (4b)$$

The slope of eq 4b ($1/a''$) is larger than the slope of eq 4a [$1/[a'(a + a'')]$]. This difference can be seen in **Figure 2** ($\delta^{18}O$ is proportional to R) and explains why the samples with added water deviate from the authentic sample correlation. This correlation enables the detection of water addition at lower levels than the single-parameter approach based on water only.

In conclusion, further to the laboratory experiments performed in this study, results describing the isotopic fractionation of oxygen during the alcoholic fermentation and ethanol distillation are available for the first time. A practical application to the detection of water addition in fruit juices using ethanol as an internal isotopic reference has been validated using this theoretical background.

More generally, this work provides some additional knowledge for the interpretation of oxygen-18 measurements performed in various alcoholic matrices (especially wine and spirits). Although ethanol is a convenient probe for oxygen isotope measurements, the technological effects of fermentation and distillation of such products must be taken into account when using this parameter for authenticity control.

LITERATURE CITED

(1) AIJN. Code of Practice; accessible on-line at http://www.aijn.org.
(2) EC Regulation 822/97 amending EC Regulation 2676/90. Annex 43: determination of the isotopic ratio $^{18}O/^{16}O$ of the water in wine. *Off. J. Eur. Communities* **1997**, *L117/10*.
(3) CEN. Determination of the stable oxygen isotope ratio ($^{18}O/^{16}O$) of water from fruit juices; 1996.
(4) OIV. Resolution ENO 2/96. Watering: detection method. *Bull. O.I.V.* **1997**, 44−65.
(5) Rossmann, A.; Reniero, F.; Moussa, I.; Schmidt, H.-L.; Versini, G.; Merle, M. H. Stable oxygen isotope content of water of EU data-bank wines from Italy, France and Germany. *Z. Lebensm. Unters. Forsch.* **1999**, *208*, 400−407.
(6) Houerou, G.; Kelly, S.; Dennis, M. Determination of the oxygen-18/oxygen-16 isotope ratios of sugar, citric acid and water from single strength orange juice. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1257−1262.
(7) Jamin, E.; Guerin, R.; Retif, M.; Lees, M.; Martin, G. J. Improved detection of added water in orange juice by simultaneous determination of the oxygen-18/oxygen-16 isotope ratios of water and ethanol derived from sugars. *J. Agric. Food Chem.* **2003**, *51*, 5202−5206.

(8) Calderone, G. Application des modernes techniques isotopiques pour la caractérisation de produits alimentaires et de boissons. Ph.D. Thesis, University of Nantes, France, 2005.
(9) Majoube, M. Fractionnement en oxygène-18 et en deuterium entre l'eau et sa vapeur. *J. Chim. Phys.* **1971**, *2* (*18*), 1423−1436.
(10) Moussa, I.; Naulet, N.; Martin, M. L.; Martin, G. J. A site-specific and multielement approach to the determination of liquid−vapor isotope fractionation parameters. The case of alcohols. *J. Phys. Chem.* **1990**, *94*, 8303−8309.
(11) Martin, G. J.; Zhang, B. L.; Naulet, N.; Martin, M. L. Deuterium transfer in the bioconversion of glucose to ethanol studied by specific isotope labeling at the natural abundance level. *J. Am. Chem. Soc.* **1986**, *108*, 5116−5122.
(12) Zhang, B. L.; Yunianta; Martin, M. L. Site-specific isotope fractionation in the characterisation of biochemical mechanisms. *J. Biol. Chem.* **1995**, *270*, 16023−16029.
(13) Schmidt, H.-L.; Werner, R. A.; Rossmann, A. ^{18}O pattern and biosynthesis of natural plant products. *Phytochemistry* **2001**, *58*, 9−32.
(14) AOAC. *Official Methods of Analysis*, 17th ed.; AOAC International: Gaithersburg, MD, 2003; Vol. II.
(15) Jamin, E.; Gonzalez, J.; Bengoechea, I.; Kerneur, G.; Remaud, G.; Naulet, N.; Martin, G. G. Measurement of $^{13}C/^{12}C$ ratios of sugars, malic acid and citric acid as authenticity probes of citrus juices and concentrates. *J. AOAC Int.* **1998**, *81*, 604−609.
(16) Rossmann, A.; Butzenlechner, M.; Schmidt, H. L. Evidence for a nonstatistical carbon isotope distribution in natural glucose. *Plant Physiol.* **1991**, 609−614.
(17) Rabiller, C.; Mesbahi, M.; Martin, M. L. ^{2}H-NMR resolution of the methylenic isotopomers of ethanol applied to the study of stereospecific enzyme-catalysed exchange. *Chirality* **1990**, *2*, 85−89.

Received for review July 12, 2005. Revised manuscript received November 18, 2005. Accepted November 23, 2005.

JF0516686