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Botanical and Geographical Origin Identification of Industrial Ethanol by Stable Isotope Analyses of C, H, and O

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The isotope ratios of carbon, hydrogen, and oxygen of rectified alcohols were determined to distinguish their botanical and geographical origins by continuous flow-isotope ratio mass spectrometry (CF-IRMS). The $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios of 27 fermented alcohols with known origins showed clusters derived from each botanical origin, *viz.* corn, sugarcane, wheat, and tapioca. C3 and C4 plants were easily distinguishable by the $^{13}C/^{12}C$ ratio. Sugarcane and corn are both C4 plants, and they showed small differences in isotope ratios. The combination plots of the D/H and $^{18}O/^{16}O$ ratios enabled us to designate the geographical origins of alcohol derived from the same kind of crop, such as Chinese or American corn. The chemically synthetic and fermented alcohols were clearly distinguished by D/H and $^{18}O/^{16}O$ ratios. Isotope ratios were useful for origin identification of alcohol. We plan to construct a database of alcohol isotope ratios to determine the origins of raw materials in alcohol.

Key words: isotope ratio mass spectrometry; ethanol; origin identification

Alcohol is consumed for potable and industrial use in Japan. The New Energy and Industrial Technology Development Organization (NEDO) Alcohol Enterprise Head Office is only permitted to sell the industrial alcohol. Industrial alcohol is made from ethylene (chemically synthesized alcohol) and carbohydrates (fermented alcohol). NEDO produces fermented rectified alcohol. The alcohol is used as a food preservative, disinfectant, material for vinegar fermentation, washing liquid for the food-processing machinery, and solvent for cosmetics, perfume, medicine, *etc.* Fermented rectified alcohol is mostly used in the food-manufacturing field. Recently, BSE problems, mislabeling of genetically modified organisms (GMO), allergens in manufactured foods, and adulterations in food have occurred in Japan. As a result, quality confirmation and the traceability of food are of much concern to consumers. Fermented rectified alcohol is also required to clarify the material origin and use of GMO.

In Europe, wine made by 'chapterlisation' with cane sugar is detected by the carbon isotope ratio ($^{13}C/^{12}C$ ratio) using isotope ratio mass spectrometry (IRMS).[1)] The addition of beet sugar to wine is also detected by analysis of the D/H ratio of the methylene site against that of the methyl site in ethanol by ^{2}H-NMR.[2)] The botanical origin of sugars made from sugarcane (C4 plant) and sugar beet (C3 plant) has been distinguished by IRMS. The geographical origin of wines has been identified by isotope ratio analysis.[3–6)] Whisky is usually made from malt (barley). It is also often made from corn blended with malt. Since barley is a C3 plant and corn is a C4 plant, the blending percentage of corn alcohol is determined by IRMS.[7)] Brazilian brandies are made from mixtures of grapes and cane sugar, and the mixing ratio can be determined by the $^{13}C/^{12}C$ ratio.[8)] Recently, tequila authenticity was assessed by headspace SPME-HRGC-IRMS.[9)]

Discrimination of alcohol made from the same C3 or C4 plants is possible only by D/H-ratio analysis by NMR. It was impossible to determine plant origins by carbon isotope ratios using IRMS.[10)] There is only one report on the origins of rectified fermented alcohol, but the data are too sparse to assess the discrimination of rectified alcohols by IRMS.[11)] Recently, analytical development using continuous flow-isotope ratio mass

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spectrometry (CF-IRMS) allows us to carry out routine analysis of the hydrogen and oxygen isotope ratios. We are trying to distinguish the plant origins of rectified fermented alcohol by multi-isotope ratio analyses employing not only the $^{13}C/^{12}C$, but also the D/H and $^{18}O/^{16}O$ ratios. We intend to collect data from authentic alcohol samples with known origins and plant-growing regions, and determine and confirm the origins and plant-growing regions of materials of rectified fermented alcohol. In this case, the preliminary data from rectified fermented alcohol will be shown along with the probability of origin confirmation by the CF-IRMS.

Materials and Methods

The isotope ratios in this paper were compared with international standards. The $^{13}C/^{12}C$ ratio was analyzed with reference to a standard ethanol with a known isotope ratio based on the VPDB (Vienna Pee Dee Belemnite) scale. On the other hand, the D/H or $^{18}O/^{16}O$ ratios were analyzed with reference to two standard waters with known isotope ratios based on the VSMOW (Vienna Standard Mean Ocean Water) scale.

The $^{13}C/^{12}C$ ratio was expressed in the δ notation as the relative deviation compared with the VPDB, as follows:

$$\delta^{13}C_{VPDB} = (R_{sample}/R_{VPDB} - 1) \cdot 1000$$

where R denotes the $^{13}C/^{12}C$ ratios. The $\delta^{13}C$ values were calibrated against National Bureau of Standard NBS-19, which has $\delta^{13}C = +1.95‰$ versus VPDB. A check of daily analytical stability was carried out by measuring an ethanol sample (NEDO, Japan) as a laboratory standard. The $^{13}C/^{12}C$ ratio of the standard ethanol was determined beforehand by IRMS with conventional off-line preparation.[12,13] The $\delta^{13}C$ value of the standard ethanol was $-12.56‰$. The D/H and $^{18}O/^{16}O$ ratios were expressed in the δ notation as the relative deviation compared with the VSMOW-SLAP (Standard Light Antarctic Precipitation) normalization.[14] The δD and $\delta^{18}O$ values of ethanol samples were directly calibrated against H_2 and CO gas derived from two standard waters with known isotope ratios based on the VSMOW scale, as follows:

$$\delta_{sample/VSMOW\text{-}SLAP\text{-}water} = [(\delta_{ws2/VSMOW\text{-}SLAP} - \delta_{ws1/VSMOW\text{-}SLAP})/(\delta_{ws2/refgas} - \delta_{ws1/refgas})] \cdot (\delta_{sample/refgas} - \delta_{ws1/refgas}) + \delta_{ws1/VSMOW\text{-}SLAP}$$ [15]

where $\delta_{sample/VSMOW\text{-}SLAP\text{-}water}$ is the oxygen or hydrogen isotope ratio of the ethanol sample, and ws1 and ws2 represent laboratory working standards of water. The δD and $\delta^{18}O$ values of the VSMOW are both 0‰; those of the SLAP are $\delta D = -428‰$ and $\delta^{18}O = -55.5‰$. The δD and $\delta^{18}O$ values of the laboratory standard waters were determined by IRMS with a water equilibration method[16] slightly modified. The δD and $\delta^{18}O$ values of the two standard waters were $\delta D = -1.5‰$ and $\delta^{18}O = -0.32‰$, and $\delta D = -396.6‰$ and $\delta^{18}O = -51.4‰$, respectively. A check of daily analytical stability was carried out by measuring another standard water ($\delta D = -62.1‰$ and $\delta^{18}O = -9.28‰$).

The purity of ethanol samples ranged from 95 vol % to 99 vol %. All samples were used without further purification.

The instrument used for $^{13}C/^{12}C$ ratio analysis consisted of an elemental analyzer (ThermoFinnigan Flash EA 1112, Thermo Electron, Bremen, Germany) and an isotope ratio mass spectrometer (ThermoFinnigan DELTA^{plus} XP, Thermo Electron) with an open split (ThermoFinnigan ConFlo III, Thermo Electron). Combustion into CO_2 was performed at 1,000 °C (oxidative furnace) and 680 °C (reductive furnace) using ultra-pure helium (> 99.99995%) as the carrier gas, in the oxidative furnace (quartz tube, packed with Cr_2O_3, Co_3O_4/Ag, 450 mm × 16 mm i.d.) followed by conversion in the reductive furnace (quartz tube, packed with CuO and Cu reduced wires, 450 mm × 16 mm i.d.) with a dehydration column. Subsequently, generated CO_2 gas was diluted with ultra-pure helium in the ConFlo III prior to IRMS. For analysis of D/H and $^{18}O/^{16}O$, a pyrolysis interface (ThermoFinnigan TC/EA, Thermo Electron) was used instead of the Flash EA. Pyrolysis of alcohol to H_2 and CO was performed at 1,400 °C[17,18] using ultra-pure helium (> 99.99995%) as the carrier gas, in a pyrolysis furnace [ceramic tube (Al_2O_3), furnished with an inner glassy carbon reactor packed with glassy carbon granules, 470 mm × 17 mm i.d.]. Two µl of each alcohol sample was packed into a tin container and introduced into the oxidative furnace in the Flash EA. Tin containers have no effect on the value under the condition of helium dilution. On the other hand, 0.12 µl of alcohol sample was injected directly into the pyrolysis furnace using a micro syringe in the TC/EA.

For interlaboratory comparison, IRMS coupled with a gas chromatograph-combustion-IRMS (GC-C-IRMS) method was done in the laboratory A. Headspace sampling and GC-C-IRMS or GC-pyrolysis-IRMS (GC-Py-IRMS) was performed in the laboratory B. Elemental analyzer-IRMS was performed in the laboratory C.

Statistical analysis of correlation coefficients and confidence limits were done by Student's *t*-test (Excel, Microsoft, Redmond, WA, U.S.A.).

Results and Discussion

Interlaboratory comparison in alcohol analyses

The same 16 samples of crude alcohol were analyzed in four independent laboratories in different ways, and the values were somewhat different from each other. One alcohol sample was measured twice or three times in our $\delta^{13}C$ analyses of 16 alcohol samples. In order to determine the precision in repetitive measurements by each laboratory, the range and the average of the

Table 1. Comparison of Analytical Precision of Four Laboratories

	NEDO lab.		Lab. A		Lab. B		Lab. C	
	Range of S.D.[a]	Average of S.D.[a]	Range of S.D.[b]	Average of S.D.[b]	Range of S.D.[c]	Average of S.D.[c]	Range of S.D.[d]	Average of S.D.[d]
$\delta^{13}C$	0.00–0.40	0.05	0.05–0.43	0.21	0.01–0.15	0.07	0.03–0.34	0.15
δD	0.0–2.1	0.7	0.1–5.6	2.3	0.1–2.7	0.9	—	—
$\delta^{18}O$	0.07–0.30	0.17	0.13–2.41	0.77	0.14–0.43	0.29	—	—

The range and average of standard deviation (S.D.) of 16 ethanol samples are shown. Thirteen ethanol samples were analyzed in Laboratory C. Each S.D. was calculated from repetitive measurements (a, n = 2–3; b, n = 1–6; c, n = 3; d, n = 2–3) of a sample. The times of repetitive measurements were restricted by the experimental equipment of each laboratory. In this case, the results of sample number n = 2 are included as the S.D. The results of n = 1 sample were not included in the calculation. —, Not analyzed. Elemental analyzer-IRMS was performed in the NEDO laboratory and Laboratory C. IRMS coupled with a gas chromatograph-combustion-IRMS (GC-C-IRMS) or GC-pyrolysis-IRMS (GC-Py-IRMS) method was done in Laboratory A. Headspace sampling and GC-C-IRMS or GC-Py-IRMS was performed in Laboratory B.

standard deviations for samples are compared in Table 1. The standard deviations of repetitive measurements ranged from 0.00 to 0.40 in the 16 alcohol samples (average of standard deviations, 0.05). Similarly, the range of the standard deviations was 0.0 to 2.1 (average, 0.7) in the δD analyses, and 0.07 to 0.30 (average, 0.17) in the $\delta^{18}O$ analyses (Table 1).

The high precision of our analytical system was confirmed by interlaboratory comparison. CF-IRMS coupled with Flash EA or TC/EA was valid for alcohol analysis.

It is a generally accepted procedure to calibrate the hydrogen and oxygen isotope ratios with international standards by complete conversion to hydrogen and carbon monoxide according to the chemical reactions in a vacuum tube (called the off-line method), but an international standard for alcohol is not available. Furthermore, complete conversion by the off-line method on the hydrogen and oxygen isotope is complicated and dangerous. Hence, we measured hydrogen and oxygen isotope ratios of alcohol by TC/EA-IRMS directly using standard waters, which have isotope ratios based on the international standard waters (VSMOW and SLAP). The values obtained by this method are designated the VSMOW-SLAP-water scale instead of the VSMOW scale, as explained in Materials and Methods.

The strong points of the VSMOW-SLAP-water scale measured by the TC/EA-IRMS are summarized as follows:

1) Considering the present research stage in isotope ratio analysis of alcohol, it appears to be a practical and reproducible method.
2) Raw data are easily calibrated by two-point calibration using the VSMOW-SLAP-water scale.[14] The international standard waters from IAEA are readily available. VSMOW-SLAP-water scale shows the difference between two standard waters with separate isotope ratios. The VSMOW-SLAP-water normalization eliminates biases derived from machine-specific and systematic differences based on international standards compared with one-point calibration by the VSMOW scale of a general off-line method.[14]
3) Since the results obtained by TC/EA-IRMS with international standard waters are feasible for other laboratories, the TC/EA-IRMS employed in our study is expected to be broadly applicable in the measurement of alcohol isotope ratios.

Although the VSMOW-SLAP-water normalization method is useful for practical measurement, there is no evidence of complete pyrolysis and the occurrence of the same isotope fractionation in water and alcohol. A project of international calibration of ethanol isotope ratios is currently being undertaken by the EU Joint Research Center. The international comparison of ethanol isotope ratios is expected to clarify variations in isotope ratios among many laboratories.

IRMS analysis of alcohols with known origins

The $\delta^{13}C$ and $\delta^{18}O$ values of 27 fermented alcohols with known botanical and geographical origins showed plots making clusters derived from each origin: corn (n = 9), $\delta^{13}C = -10.71 \pm 0.31‰$ and $\delta^{18}O = 12.21 \pm 4.61‰$; sugarcane (n = 11), $\delta^{13}C = -12.81 \pm 0.51‰$ and $\delta^{18}O = 12.65 \pm 4.14‰$; wheat (n = 3), $\delta^{13}C = -25.08 \pm 2.18‰$ and $\delta^{18}O = 16.12 \pm 8.26‰$; tapioca (n = 3), $\delta^{13}C = -27.74 \pm 0.35‰$ and $\delta^{18}O = 11.09 \pm 1.82‰$ (Fig. 1). The δD values of 27 fermented alcohol samples with known botanical and geographical origins ranged from −273.5‰ to −162.7‰, and the $\delta^{18}O$ values ranged from 7.13‰ to 25.46‰. On the other hand, those of synthetic alcohol samples ranged from −132.6‰ to −124.7‰ (δD), and from −2.14‰ to −0.98‰ ($\delta^{18}O$), respectively (Fig. 2). The combination plots of δD and $\delta^{18}O$ showed parallel lines of C3 and C4 plants (Fig. 2). The regression formula and coefficient were $y = 3.8 \times -310.7$, $R = 0.921$ (C3 plant), and $y = 5.1 \times -276.6$, $R = 0.861$ (C4 plant), respectively. Both correlation and coefficient were statistically significant ($p < 0.01$). Confidence limits of the two slops were 3.8 ± 1.4 and 5.1 ± 1.5 at a 95% confidence level.

In nature, all of the major organic bio-elements (C, H, N, O, and S) are a mixture of two or more stable isotopes. The isotope ratios of a given molecule vary depending on those origins.[10] The diversity is caused by isotope enrichment or depletion during physical, chemical, and metabolic processes. This phenomenon is called

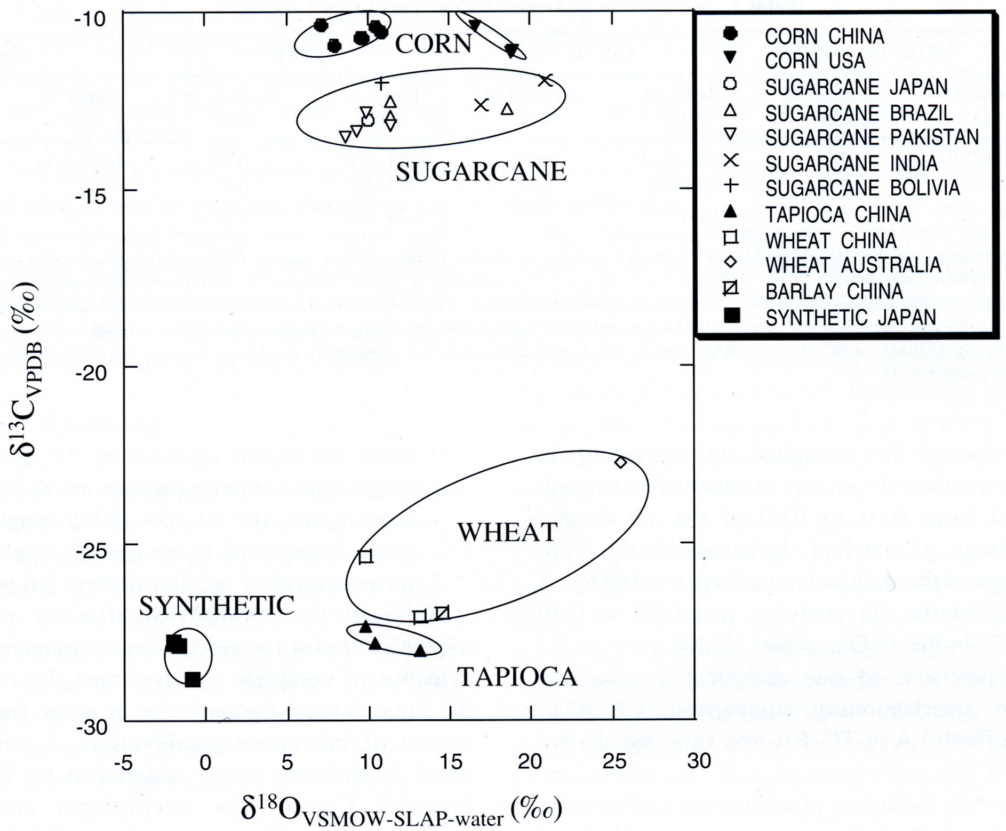

Fig. 1. Combined Analysis of $\delta^{13}C$ and $\delta^{18}O$ on Rectified Alcohols with Known Origins.
Thirty-one alcohol samples with known origins were analyzed by the IRMS method. Symbols are shown in the right-hand box, which contains origin information, plant, and cultivated regions of the raw materials.

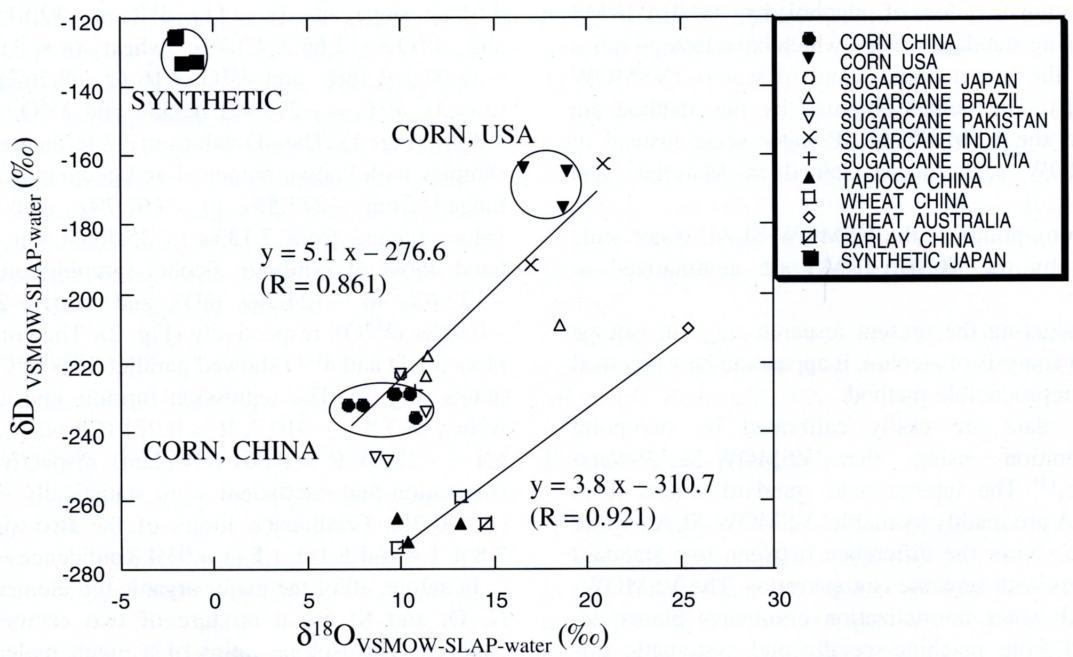

Fig. 2. Combined Analysis of δD and $\delta^{18}O$ on Rectified Alcohols with Known Origins.
Thirty-one alcohol samples with known origins were analyzed by the IRMS method. Symbols are shown in the right-hand box, which contains origin information, plant, and cultivated regions of the raw materials.

"isotope effects" or "isotope fractionations". C3 plants fix atmospheric CO_2 by ribulose 1,5-bisphospate carboxylase-oxygenase, which is accompanied with a strong ^{12}C isotope effect causing large depletion in the ^{13}C content of the plant. The carbohydrate $\delta^{13}C$ values of these plants range from $-28‰$ to $-23‰$.[19] On the other hand, C4 plants fix CO_2 by phosphoenolpyrvate carboxylase, which shows almost no isotope fractionation with respect to the ^{13}C content of atmospheric CO_2.[19] Therefore, products derived from C4 plants show higher ^{13}C contents than those from C3 plants. Only the diffusion of CO_2 into the intracellular space exhibits a small isotope fractionation of about 4‰. The carbohydrate $\delta^{13}C$ values of C4 plants are generally about $-10‰$.[19] In our results, the alcohol origins of C3 and C4 plants were easily distinguished by the $\delta^{13}C$ value as in previous reports.[10,19] Since sugarcanes and corns both belong to C4 plants, they showed a small difference in isotope ratio. The high precision of Flash EA-IRMS and TC/EA-IRMS enabled us to detect small differences. Although further studies are necessary to determine the reason for these small differences, different environmental growth conditions and different types of plant species might contribute to it. A two-dimensional plot of $\delta^{13}C$ and $\delta^{18}O$ values provided information to identify the botanical origins of alcohol.

The water derived from atmospheric vapor is called "meteoric water". In the meteoric water cycle, a well-known isotope fractionation takes place during the evaporation of the water from the oceans, where depletion in heavy isotopes is observed in the vapor. As the water with a higher content of heavy isotopes is successively removed from the vapor as precipitation, the residual vapor shows a lower content of heavy isotopes. Consequently, precipitation from the residual vapor shows depletion of heavy isotopes. Actually, the precipitation in a tropical area at low latitude shows enrichment of heavy isotopes. On the other hand, snow and ice samples from the Arctic and Antarctic show large depletion of heavy isotopes.[20] Moreover, Craig[21] showed that there is a linear correlation between δD and $\delta^{18}O$ in natural meteoric water from many parts of the world, referred to as the Meteoric Water Line. Our ethanol regression lines in the δD and $\delta^{18}O$ diagram might be due to the reflection of meteoric waters. Furthermore, it is necessary to take into consideration the processes of isotope fractionation in generating carbohydrates from plant water and alcohol from carbohydrates. The combination plots of δD and $\delta^{18}O$ enabled us to designate cultivated regions of the same crops. For example, Chinese corn (n = 6), $\delta D = -231.3 \pm 2.7‰$ and $\delta^{18}O = 9.26 \pm 1.43‰$; American corn (n = 3), $\delta D = -167.7 \pm 6.5‰$ and $\delta^{18}O = 18.10 \pm 1.28‰$. A combination of δD and $\delta^{18}O$ values provided the information to identify its geographical origin. The parallel lines of C3 and C4 plants might give us a new discrimination method between C3 and C4 plants. The synthetic alcohol was clearly distinguished from fermented alcohols by their high D/H and low $^{18}O/^{16}O$ isotope ratios. We constructed a small database of alcohol isotope ratios to determine the botanical and geographical origins of the raw material in alcohol.

IRMS analysis of commercial crude alcohols

The $\delta^{13}C$ and $\delta^{18}O$ values of 55 commercial crude alcohols were determined (Fig. 3). Values of most samples were fitted to our database, but the isotope ratios of three samples labeled with corn origin were out of our database.

The raw material of one sample ($\delta^{13}C = -26.6‰$, $\delta^{18}O = 13.88‰$) appeared to be tapioca or wheat. Another sample ($\delta^{13}C = -13.95‰$, $\delta^{18}O = 8.07‰$) was estimated to be sugarcane alcohol or a mixture of sugarcane and a little C3 plant alcohol. The other one ($\delta^{13}C = -21.18‰$, $\delta^{18}O = 11.41‰$) was deduced to be a mixture of C3 and C4 plant alcohol. Our alcohol database constructed with known origins suggested the possibility of detection of mislabeling in commercial rectified alcohol.

Conclusions

The isotope ratios of C, H, and O of rectified fermented alcohol were determined to distinguish the botanical and geographical origins of raw materials. Employing isotope ratio mass spectrometry (IRMS) coupled with a continuous flow elemental analyzer (EA-IRMS) or a continuous flow pyrolysis unit (Py-IRMS), measurements of the $^{13}C/^{12}C$, D/H, and $^{18}O/^{16}O$ isotope ratios were carried out to a level of high precision by comparison with interlaboratory data. The $\delta^{13}C$, δD, and $\delta^{18}O$ values of 27 fermented and four synthetic alcohols with known origins were measured, and a small alcohol database was constructed. The $\delta^{13}C$ and $\delta^{18}O$ values showed clusters derived from corn, sugarcane, wheat, and tapioca. A combination of the $\delta^{13}C$ and $\delta^{18}O$ values can provide information on the botanical origins of rectified alcohol. The difference between fermented and chemically synthetic alcohol was clearly shown by the values of δD and $\delta^{18}O$ recorded for authentic samples. The values of δD and $\delta^{18}O$ can designate cultivated regions of the same kind of crops. The mislabeling of commercial alcohols can be detected with a database constructed using authentic alcohols. It can be a powerful tool for checking and confirming the traceability of industrial fermented alcohol.

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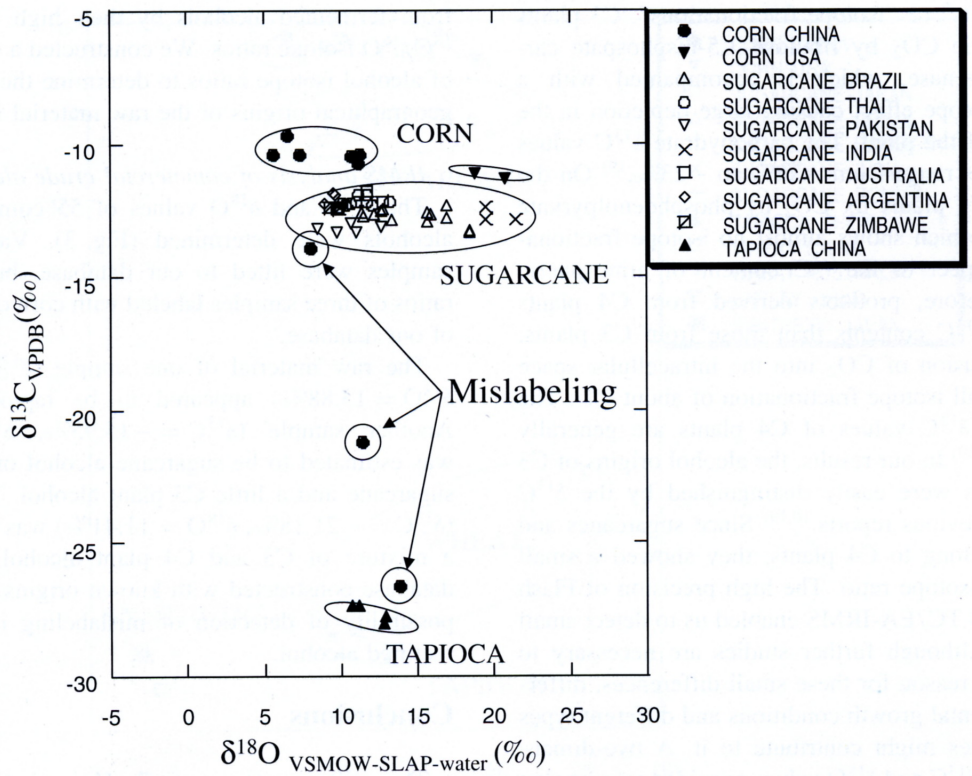

Fig. 3. Combined Analysis of $\delta^{13}C$ and $\delta^{18}O$ on Commercial Rectified Alcohols.
Fifty-five commercial crude alcohols were analyzed by the IRMS method. Symbols are shown in the right-hand box, which contains the labeled origin information.

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