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ARTICLE

Improved Characterization of the Botanical Origin of Sugar by Carbon-13 SNIF-NMR Applied to Ethanol

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Until now, no analytical method, not even isotopic ones, had been able to differentiate between sugars coming from C4-metabolism plants (cane, maize, etc.) and some crassulacean acid metabolism plants (e.g., pineapple, agave) because in both cases the isotope distributions of the overall carbon-13/carbon-12 and site-specific deuterium/hydrogen isotope ratios are very similar. Following recent advances in the field of quantitative isotopic carbon-13 NMR measurements, a procedure for the analysis of the positional carbon-13/carbon-12 isotope ratios of ethanol derived from the sugars of pineapples and agave using the site-specific natural isotopic fractionation−nuclear magnetic resonance (SNIF-NMR) method is presented. It is shown that reproducible results can be obtained when appropriate analytical conditions are used. When applied to pineapple juice, this new method demonstrates a unique ability to detect cane and maize sugar, which are major potential adulterants, with a detection limit in the order of 15% of the total sugars, which provides an efficient mean of controlling the authenticity of juices made from this specific fruit. When applied to tequila products, this new method demonstrates a unique ability to unambiguously differentiate authentic 100% agave tequila, as well as misto tequila (made from at least 51% agave), from products made from a larger proportion of cane or maize sugar and therefore not complying with the legal definition of tequila.

KEYWORDS: Pineapple juice; tequila; isotopic ^{13}C NMR; SNIF-NMR; carbon-13; ethanol; authenticity

INTRODUCTION

The detection of sugar addition in fruit juices and spirits is routinely performed by measuring the isotopic ratios of carbon (^{13}C/^{12}C) by isotope ratio mass spectrometry (IRMS) and site-specific deuterium ratios by ^{2}H-SNIF-NMR on ethanol resulting from the fermentation of fruit juice sugars or distillation of wines/spirits alcohol. Checking the botanical origin of sugars and derived products (beverages and spirits) is routinely performed by official laboratories to enforce international regulations.

According to the definitions of the EC fruit juice directive 2001/112 and AIJN code of practice (*1*), pineapple juice must be made solely from the edible part of *Ananas comosus* (L.) Merr., and if exogenous sugar is added, this addition and proportion must be clearly labeled on the bottle. By definition, tequila is a spirit produced from the fermentation of agave sugar (either 100% or as a mixture with other plant sugar sources) and the distillation of the resulting alcohol. The product name "tequila" is restricted to spirits produced in this way in Mexico using the so-called "blue agave" variety *Agave tequilana* Weber var. *azul*. In its regulation NOM-006-SCFI-2005, the Mexican "Consejo Regulador" defines two major types: tequila 100% agave and misto tequila, which must by definition be made from at least 51% agave. There are no legal specifications regarding the type of adjunct sugar sources to be used in misto tequila manufacture, and theoretically any kind of fermentable sugar can be used, but in practice and from an economical point of view, only cane sugar and corn syrup are employed (*2*).

The isotopic analytical methods used to determine the origin of sugars have gained official recognition from the International Organisation of Vine and Wine (*3*), the European Union (*4*, *5*), and AOAC (*6*, *7*). Guidelines regarding the expected values for these parameters have been published for pineapple juice (*1*) and tequila (*8*) based on databases of authentic samples, which enable the detection of addition of C3-plant derived sugar (e.g., beet, cereals) but not those from C4-plant derived sugar (e.g., maize). In the case of pineapple juice, the use of multicomponents approaches (*9*, *10*) allows to lower the limit of detection, whatever the geographical origin of the fruits.

One major gap of this technique is that the global ^{13}C isotopic ratios (δg^{13}C) of pineapple fruit sugars overlap with those of cane and maize plants (*8*), therefore the addition of sugars from these botanical sources cannot be detected by the measurement of this isotope ratio. Complementary approaches such as the

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chromatographic analysis of oligosaccharides in pineapple juice (*11*) or of the volatile constituents in tequila (*8*) have been proposed, but the sensitivity of these methods is limited by the large variation in the natural levels of marker compounds.

Recently, the use of NMR technology for measuring isotope ratios has been extended to the measurement of site-specific ^{13}C isotope ratios: isotopic ^{13}C NMR spectrometry. This major breakthrough in the technology was achieved thanks to the improvement of NMR sequences (*12*, *13*) for homogeneous and robust ^{1}H decoupling. These progresses allowed reaching a sufficient precision level to observe the differences in carbon isotope intramolecular distribution in nature. The use of an appropriate relaxation agent (*14*) shortens considerably the acquisition time and allows the method to be used for routine control applications in several food and industry sectors: origins of ethanol (*13*, *15*−*17*), origins of vanillin (*18*), and counterfeiting pharmaceuticals (*19*). The method is also applicable to the studies of noncovalent isotope effects (*20*), biotransformation (*21*), equilibrium isotope effects (*22*), and metabolism (*23*). In the case of the alcoholic fermentation, producing sugars from ethanol, this new insight into site-specific distributions produced new and unexpected knowledge about carbon isotope distributions (*15*−*17*) and allowed going very far beyond preliminary IRMS investigations which required cumbersome procedures to break down the molecules (*24*).

From preliminary investigations regarding ethanol from various plants (*13*, *15*−*17*), it seems that the site-specific measurement of carbon-13 deviations does not discriminate better between C3 and C4 plants than the overall carbon-13−IRMS measurement. However, in the case of CAM plants such as agave (used to produce tequila) or pineapple, a large difference of carbon-13 content between the two sites was observed. Moreover, for ethanols from CAM plants, the pattern of ^{13}C distribution on the two sites is in the opposite direction to that observed for ethanols derived from C4. For the latter, both sites of the molecules are also much closer to each other (*13*). In this study, we have explored whether this phenomenon could be used to detect added cane or maize sugar in pineapple juice and to differentiate 100% pure tequila vs misto tequila. Therefore, we hope to transfer the capabilities of ^{2}H-SNIF-NMR to ^{13}C-SNIF-NMR for authenticating juices and beverages.

MATERIALS AND METHODS

Samples Description. Fifty-eight authentic samples of pineapple juice were obtained either from fresh fruit or juice concentrates of known origin representing a wide range of geographical origins (Cameroon, Costa Rica, Ghana, Guinea, Indonesia, La Réunion, Mauritius, Philippines, Porto Rico, South Africa, Thailand, and Vietnam). In addition, 81 commercial juice samples from the market were tested.

Fifty-one authentic samples of 100% agave musts or tequila and 19 "Misto" (>51% agave) tequilas were obtained through contacts with local producers. In addition, 24 commercial tequila samples from the European market were purchased and tested.

Finally, 46 sugars or ethanols of known botanical origin (26 from cane, 14 from beet, and 6 from maize) have also been included in this work.

Chemicals. [1,2-$^{13}C2$]-Ethanol (99 atom %) was purchased from CortectNet (78960 Voisins-Le-Bretonneux, France). Tris(2,4-pentadionato) chromium(III) (97%) ($Cr(Acac)_3$), acetone-d_6, and dimethylsulfoxide-d_6 (DMSO-d_6) were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesnes, BP 70101, 38297 St. Quentin Fallavier, France).

Extraction and Purification of Ethanol. The procedure used in this study to quantitatively transform the sugars into ethanol and to isolate the ethanol for the subsequent IRMS measurement was adapted from the SNIF-NMR method (*3*−*6*). Using *Saccharomyces cerevisiae* and *S. bayanus* yeasts (Loire Viniviti Distribution, Zone Artisanale Du Landreau, 49610 Moze sur Louet, France), single-strength juices or concentrates diluted to ca. 12°Brix were completely fermented to ethanol, which was then extracted by high yield automated distillation using Cadiot columns piloted by ADCS software (Automated Distillation Cadiot System, Eurofins, Nantes, France).

Isotope Ratio Mass Spectrometry: Determination of the Global ^{13}C Deviation. The mass spectrometric determinations of the carbon isotope ratios of ethanol were carried out by online analysis following the methods described in (*7*) using an elemental analyzer (FlashEA-1112), fitted to an isotope ratio mass spectrometer (Delta V Advantage), both from Thermo Fisher Scientific (Hanna-Kunath-Strasse 11, D-28199 Bremen, Germany).

The carbon dioxide used as reference gas is calibrated against V.PDB (Vienna Pee Dee Belemnite) by analyzing calibrated international reference materials (NBS-22 and IAEA-CH-6, purchased from IAEA, Vienna, Austria). In addition, a working standard is used in each series of measurement to correct the drift: a L-glutamic acid sample of >99% purity (purchased from Sigma-Aldrich, L'Isle d'Abeau Chesnes, BP 70101, 38297 St Quentin Fallavier, France).

The results are expressed on the δ‰ scale with respect to the international standard V.PDB according to the relation:

$$\delta^{13}C(‰) = (R_{product}/R_{standard} - 1) \times 1000 \quad \text{where } R = {}^{13}C/{}^{12}C$$

The internal reproducibility of the method used in our laboratory was estimated at 0.3‰ for ethanol. In this paper, the global $\delta\,^{13}C$ of ethanol is expressed as $\delta g\,^{13}C$.

^{13}C-SNIF-NMR: Determination of the Site-Specific ^{13}C Deviations. Quantitative ^{13}C-SNIF-NMR spectra were recorded using a Bruker DPX 400 spectrometer (Bruker Biospin, 67166 Wissembourg, France) fitted with a 5 mm id dual probe $^{13}C/^{1}H$ carefully tuned at the recording frequency of 100.62 MHz. The temperature of the probe was set at 303 K. The experimental parameters for ^{13}C NMR data acquisition were adapted from previously published methodology in (*6*−*8*): pulse width (P1) 7.4 μs (90°), acquisition time (AQ) 1 s, repetition time (D1) 17 s in order to allow complete relaxation; for precise quantification (order of magnitude 0.1‰), the sum AQ + D1 must be higher than 10 times the maximum longitudinal relaxation time T1 for ethanol, measured at 1.69 s in our conditions with the relaxation agent $Cr(Acac)_3$. The offsets for both ^{13}C and ^{1}H nuclei were set at the middle of the frequency range for each molecule (O1 = 4050 Hz and O2 = 1190 Hz). 120 scans are registered for each spectrum (37 min per spectrum) leading to signal-to-noise ratio higher than 2000, and 5 spectra are measured on each tube, leading to a total experiment time of about 3 h per sample. NMR sample tubes were prepared as follows: 1000 μL of ethanol and 100 μL of 0.1 M $Cr(Acac)_3$ solution in DMSO-d_6. Inverse-gated decoupling techniques were applied in order to avoid nuclear Overhauser effect (NOE). The decoupling sequence employed a cosine-adiabatic pulse with appropriate phase cycles, as previously described (*13*). A bilabeled ethanol sample tube (prepared with 600 μL of [1,2-$^{13}C2$]-ethanol, 200 μL of demineralized water, and 200 μL of acetone-d_6) is measured once a month to control if the decoupling is total for all the sites of ethanol.

The free induction decay (FID) was transformed using an exponential multiplication line broadening of 2 Hz. The curve fitting and calculations were performed using a Lorentzian mathematical model using Eurospec version 4.0 software (Eurofins, Nantes, France) (*25*).

The isotopic distribution in a molecule is characterized by the actual ^{13}C molar fractions f of a specific site i: $f_i = S_i/ST$ where S_i is the area of the ^{13}C NMR signal of i and ST is the sum of the areas of all the signals for the molecule. Each S_i is corrected in order to compensate for intensity losses due to satellite lines, which are assigned to the bilabeled $^{13}C-^{13}C$ isotopomers. In accordance with the ^{13}C natural mean abundance of 1.1%, areas were multiplied by $(1 + n0.011)$, where n was the number of carbons directly connected to carbon site i (for further information on these parameters, see AOAC Official Method 2004.1 (*7*)). Thus, the corrected ^{13}C molar fraction f_i for a specific site i is expressed as: $f_i(C) = (1 + n0.011) \times S_i/ST$. The statistical molar fraction for the distribution of ^{13}C in the molecule is designated as F_i (i.e., the molar fraction if no site-specific variation has been introduced). Hence, the deviation in the site-specific isotopic distribution from the statistical distribution (Δf_i) is the reduced molar fraction defined as: $\Delta f_i = f_i/F_i$. From (Δf_i) using the global value for $\delta^{13}C$ (‰) obtained by IRMS, the $\delta_i\,^{13}C$ (‰) for each peak can be calculated as follows: (i) the isotopic abundance ($^{13}C/(^{12}C+^{13}C)$) of each NMR peak (carbon) A_i is defined from the global abundance A_g: $A_i = \Delta f_i \times A_g$; (ii) A_g is determined from δg obtained by IRMS through: $R_g = ((\delta g/1000) + 1) \times R_{PDB}$, where R_g is the global isotope ratio $^{13}C/^{12}C$ and R_{PDB} is the isotope

ratio $^{13}C/^{12}C$ of the international reference PeeDee Belemnite (R_{PDB} = 0.0112372); (iii) $R_{PDB} = (A_{PDB})/(1 - A_{PDB})$ and A_{PDB} = 0.01111233; (iv) $A_g = R_g/(1 + R_g)$ and $R_i = A_i/(1 - A_i)$; then (v) the site-specific carbon content for each carbon i, $\delta i = ((R_i/R_{PDB}) - 1) \times 1000$ (‰). All these calculations steps have been automatized for ethanol molecule in Eurospec version 4.0 software.

In this paper, the positional $\delta^{13}C$ of the CH_2 and CH_3 sites of ethanol are expressed as $\delta^{13}C_{CH2}$ and $\delta^{13}C_{CH3}$, respectively.

RESULTS AND DISCUSSION

Method Validation. To ensure the correct interpretation of the results, the method should show appropriate accuracy, which is defined as the trueness and the precision (*26*, *27*).

Precision. The repeatability of the ^{13}C NMR measurements was for a long time the main drawback of the technique. As mentioned previously, a good long-term repeatability has been recently achieved by using adiabatic pulses sequences for ^{1}H decoupling (*13*). For any new application, especially for detecting frauds, this characteristic should be determined. For this purpose, we have set that one ^{13}C NMR measurement consists of the collection of five spectra. The standard deviation between CH_3 signals of ethanol in the spectra is calculated, and its average on all our experiments was sr = 0.2‰, which leads to a repeatability limit, expressed as 2.8sr, of 0.56‰. Routinely, a measurement is valid if the standard deviation between the five spectra is lower than 0.5‰.

In parallel, some duplicates of the same sample have been analyzed to evaluate the overall uncertainty (including preparation steps). Two bottles of the same batch of juice were collected in the market. The fermentation followed by distillation was realized on the first bottle. Ethanol obtained was then measured by ^{13}C-SNIF-NMR. The second bottle was kept in the refrigerator during one week and was then fermented and distilled and the ethanol was prepared for NMR measurement. The difference between the two results was calculated. The same process has been done on five bottled juices and five fresh-squeezed pineapple fruits from different countries. We obtained an overall internal reproducibility of 0.5‰, which is within the repeatability of the NMR measurement. It confirms that preparation steps (fermentation and distillation) are well controlled and do not add uncertainty to the final value.

As a quality control of the NMR measurement, a pineapple ethanol sample is measured on each analytical session and the value of the ^{13}C deviation of CH_2 and CH_3 ($\delta^{13}C_{CH2}$ and $\delta^{13}C_{CH3}$, respectively) are gathered in a quality control chart. An example of this control chart is given in **Figure 1**. The limits have been fixed using the internal reproducibility of 0.5‰. The long-term repeatability limit of the measurement is in accordance with previously published values (*13*, *14*).

Also, for one year, a monthly control orange juice sample was processed and analyzed from fermentation through the ^{13}C-SNIF-NMR analysis. **Figure 2** shows the control chart obtained with these measurements for the parameter $\delta^{13}C_{CH2}$. The standard deviation over this period was less than 0.5‰.

Trueness. To evaluate the interlaboratory reproducibility of the method, a peer-testing study (*16*) was performed on 38 ethanol samples, covering a wide range of botanical origins: rums (from C4 sugar cane), tequila (from CAM agave), whiskies from Scotland (from C3 barley) and USA (from C4 maize), sake (from C3 rice), wine (from C3 grape), beers (C3 and C4 mix), ethanol samples from fermentation of fruits, orange, apple (C3), pineapple (CAM), of C4 sugars, cane, corn isoglucose, palmsugar, of C3 sugars, beet, wheat, potato, of honeys from various floral origins, and of lactose (whey alcohol). These distillates have been measured on Bruker NMR spectrometers operating at different magnetic fields (400 and 500 MHz). Spectrometers acquisition

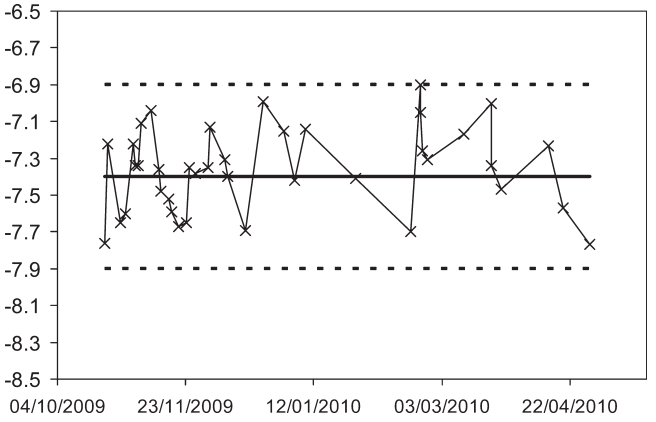

Figure 1. Quality control of the isotopic ^{13}C NMR measurement: long-term reproducibility of $\delta^{13}C_{CH2}$ (‰) of the control ethanol, measured once per session to validate the session.

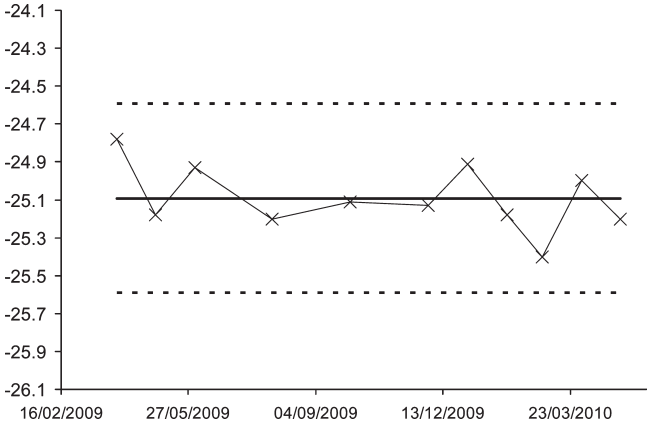

Figure 2. Quality control of the whole procedure (from the fermentation to the measurement): long-term reproducibility of $\delta^{13}C_{CH2}$ (‰) of the ethanol produced by fermentation of the sugars of a reference fruit juice, measured once per month.

parameters have been optimized, and the number of scans have been adapted in order to obtain a signal-to-noise ratio larger than 2000 (signal-to-noise ratio measured on the two sites with Topspin 1.3, Bruker Biospin, 67166 Wissemboug, France). With optimized conditions, the five spectra per sample last about 90 min for the 500 MHz spectrometer and about 270 min for the 400 MHz spectrometer. Results have been evaluated, and the difference between the two laboratories is in average on the 38 samples 0.07‰, with a standard deviation of 0.30‰. This difference is comparable with the repeatability of the measurement. It is clear that the method, as presented in this work, shows appropriate accuracy for analytical purposes.

To check the absence of bias from previously published data, the commercial ethanol sample used by Caytan et al (*13*) has also been analyzed. The results ($\delta^{13}C_{CH2} = -32.67$‰ ; $\delta^{13}C_{CH3} = -26.03$‰) were in accordance with the published values ($\delta^{13}C_{CH2} = -32.7$‰ ; $\delta^{13}C_{CH3} = -26.0$‰).

Application to the Detection of Added Sugar in Pineapple Juice. The site-specific carbon isotope deviations of ethanol measured on authentic pineapple juices, cane, maize, and beet sugars are displayed in **Figure 3** and summarized in **Table 1**. A clear-cut discrimination between the populations of pineapple juices and the various sources of sugars appears. In the case of beet sugar, the shift of the mean value of $\delta^{13}C_{CH2}$ versus pineapple is approximately 20‰, which results in a better separation than the one based on $\delta g^{13}C$ IRMS values, which is approximately 15‰

based on typical $\delta g^{13}C$ values of ethanol (*1*). Because the standard deviations of $\delta^{13}C_{CH2}$ and $\delta g^{13}C$, due to natural fractionation factors, are in the same order of magnitude, this creates a significant increase of the sensitivity to detect beet sugar (and by extension other C3 plants sugars) in pineapple. Moreover, in **Figure 3**, a clear discrimination is observed between the ethanols derived from C4 plants cane and maize, which both show similar carbon-13 distribution, and the ethanols derived from CAM plant pineapple. The latter are characterized by a very strong impoverishment of the CH_3 site as compared to the CH_2 one, thus creating a clear discrimination based on positional isotope deviations. A similar carbon-13 distribution pattern is also observed for ethanols derived from agave which is also a CAM plant. Although not yet fully elucidated, an isotopic fractionation mechanism specific to the particular metabolism and physiology of CAM plants can thus be hypothesized. Further works are in progress to understand this behavior. It is already clear that the internal ^{13}C distribution in glucose is very different according to the CO_2 assimilation metabolism (*23*).

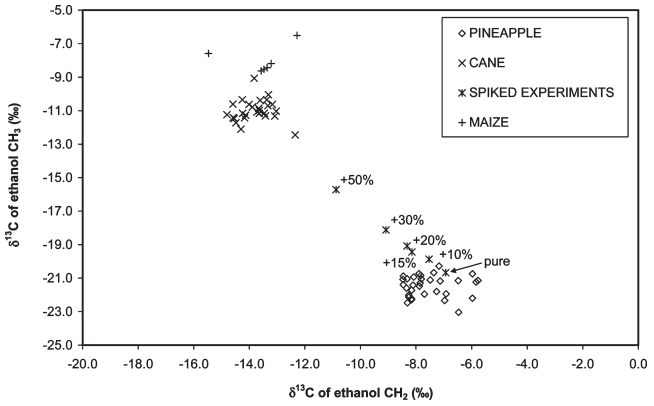

Figure 3. Two-dimensional plot of the site-specific ^{13}C deviations of ethanol fermented from authentic pineapple juices, cane and maize sugars, and pineapple samples spiked with cane sugar (the percentages correspond to the proportion of added cane sugar in total sugars).

Table 1. Means and Standard Deviations (SD) of the Global ^{13}C Content ($\delta g^{13}C$) and Site-Specific Carbon-13 Deviations of Ethanol ($\delta^{13}C_{CH2}$ and $\delta^{13}C_{CH3}$) Fermented from Pineapple, Beet, Cane, Maize, and Agave Sugars

		$\delta^{13}C_{CH2}$ (‰)		$\delta^{13}C_{CH3}$ (‰)		$\delta g^{13}C$ ethanol (‰)	
	no. of samples	mean	SD	mean	SD	mean	SD
pineapple	58	−7.4	0.7	−21.3	0.7	−14.4	0.5
beet	14	−27.9	1.4	−28.0	0.7	−28.0	0.9
cane	26	−13.8	0.6	−11.1	0.5	−12.4	0.4
maize	6	−13.6	1.0	−8.0	0.8	−10.8	0.7
agave	51	−7.3	0.8	−16.5	0.9	−11.9	0.5

The difference between the $\delta^{13}C_{CH3}$ of ethanols from pineapple and from C4 sugars leads to a theoretical detection limit of approximately 10% added sugar. This was checked experimentally by spiking a pineapple sample with increasing amounts of cane sugar, as shown in **Figure 3**. It is clearly shown that the drift of site-specific isotopic parameters caused by the addition of sugar is linear and that the theoretical detection limit of 15% is reached (when the spiked samples come out of the population of reference samples defined as the 95% confidence interval of the observed pineapple population).

A market survey was performed using these new isotopic parameters and the statistical limits defined as the 99.9% confidence intervals (means ±3 standard deviations), which showed three cases of C3 plant sugar addition and 15 cases of C4 plant sugar addition (**Table 2**). Cases 1, 2, and 3 have a global ^{13}C deviation of ethanol out of the usual range observed in authentic pineapple (*1*), but still in agreement with the lowest values observed in our laboratory for specific origins, so they would remain dubious without the ^{13}C-SNIF-NMR confirmation. In both cases, the positional measurement allows for more confidence for the proof of adulteration and for the quantification of exogenous sugar. Moreover, cases 4−17 would have been undetected without a positional ^{13}C measurement. The occurrence of such adulterations found on "real life" samples taken from the market highlights the sensitivity and ability of the method to pick-up new adulteration cases. Indeed, several adulteration cases found in the present work would remain undetected on the sole basis of IRMS measurements.

Application to Tequila. The site-specific carbon isotope deviations of ethanol measured on pure agave products are summarized in **Table 1**. The results obtained for 100% agave musts and tequilas, Misto (>51% agave) tequila, cane ethanol, and maize ethanol, are displayed in **Figure 4**. A clear differentiation between 100% agave and misto tequila appears, with especially a significant decrease for the $\delta^{13}C$ of ethanol CH_2 in misto products as compared to 100% agave tequila due to the presence of C4-plant sugars.

Some spiked samples have been prepared in order to evaluate the method. Three spirits prepared with different proportions of

Table 2. Real Cases of Pineapple Juices from the Market Judged as Being Adulterated by Sugar Addition

	$\delta^{13}C_{CH2}$ (‰)		$\delta^{13}C_{CH3}$ (‰)		$\delta g^{13}C$ ethanol (‰)		
	result	conformity	result	conformity	result	conformity	conclusion
case 1	−11.8	OUT	−21.6	IN	−16.7	OUT	22% added C3 sugar
case 2	−10.9	OUT	−20.9	IN	−15.9	OUT	17% added C3 sugar
case 3	−11.1	OUT	−19.9	IN	−15.5	OUT	18% added C3 sugar
case 4	−10.7	OUT	−18.2	OUT	−14.5	IN	31% cane or 24% maize addition
case 5	−10.7	OUT	−17.7	OUT	−14.2	IN	35% cane or 28% maize addition
case 6	−10.7	OUT	−17.7	OUT	−14.2	IN	35% cane or 28% maize addition
case 7	−10.3	OUT	−17.9	OUT	−14.1	IN	33% cane or 26% maize addition
case 8	−10.0	OUT	−18.1	OUT	−14.1	IN	30% cane or 25% maize addition
case 9	−10.4	OUT	−17.6	OUT	−14.0	IN	36% cane or 29% maize addition
case 10	−9.5	IN	−18.3	OUT	−13.9	IN	30% cane or 23% maize addition
case 11	−9.7	OUT	−18.2	OUT	−13.9	IN	31% cane or 24% maize addition
case 12	−9.8	OUT	−18.0	OUT	−13.9	IN	33% cane or 26% maize addition
case 13	−10.0	OUT	−17.8	OUT	−13.9	IN	35% cane or 27% maize addition
case 14	−10.2	OUT	−17.6	OUT	−13.9	IN	36% cane or 29% maize addition
case 15	−10.4	OUT	−17.5	OUT	−13.9	IN	38% cane or 30% maize addition
case 16	−8.3	IN	−18.9	OUT	−13.6	IN	24% cane or 19% maize addition
case 17	−10.6	OUT	−16.5	OUT	−13.6	IN	47% cane or 37% maize addition

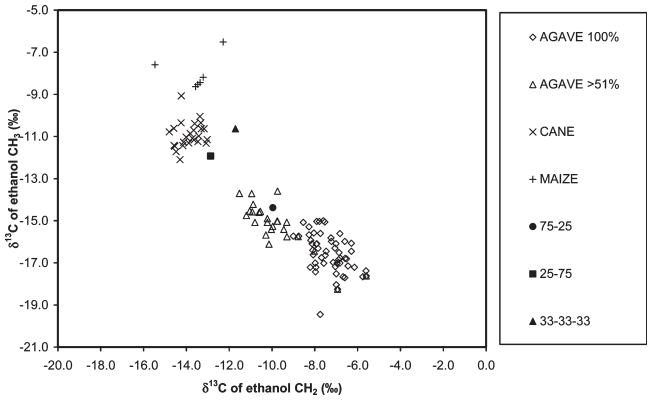

Figure 4. Two-dimensional plot of the site-specific ^{13}C deviations of ethanol from tequila (100% and misto), agave musts, cane and maize ethanol, and tequila samples spiked with cane and/or maize ethanol (the percentages correspond to the proportion of added ethanol in tequila, the first number represents the percentage of agave ethanol).

agave, cane, and/or maize sugar have been prepared in an industrial plant and analyzed with ^{13}C-SNIF-NMR. Samples are represented in the **Figure 4**. All samples are located correctly according to their sugar composition. It also appears that products made with an agave proportion lower than 51% are clearly evidenced by this method, which was hardly possible to detect until now.

The 24 commercial products analyzed in this study were found to have results in accordance with their labeling, so for the sake of clarity they have been incorporated in the tequila groups (100% and > 51% respectively) on the graph of **Figure 4**.

In conclusion, a new method for the optimum detection of all sources of added sugar (including the previously undetectable cane and maize sources, readily available in pineapple and tequila producing countries) has been established. On the basis of preliminary results obtained in our laboratory, it seems that the method will also be applicable to dragon fruit (*Hylocereus undatus*), which is also a CAM plant and becomes more and more popular in fruit beverages and food.

After a phase of dissemination within laboratories performing carbon-13 SNIF-NMR analyses, this new method could become a new official standard for the control of authenticity of fruit juices and spirits.

ABBREVIATIONS USED

AIJN, (European) Association of Fruit Juice Producers; AOAC, (International) Association of Official Analytical Chemists; EC, European Community; SNIF-NMR, site-specific natural isotope fractionation−nuclear magnetic resonance.

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Supporting Information Available: Reproducibility of the whole method: Results Table. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) AIJN *Code of Practice*, http://www.aijn.org (accessed September 2010).

(2) Cedeño Cruz, M.; Alvarez-Jacobs, J. In Production of Tequila from Agave: Historical Influences and Contemporary Processes. *Alcohol Textbook*, 4th ed.; Jacques, K. A., Lyons, T. P., Kelsall, D. R., Eds.; Nottingham University Press: Nottingham, UK, **2003**; Chapter 15.

(3) *Compendium of International Methods of Analysis of Wines and Musts of the International Organisation of Vine and Wine* (*OIV*); http://www.oiv.int, 2009; Chapters MA-AS-311-O5:R2009 and OIV AS-312−06:R2009 (accessed September 2010).

(4) Detecting Enrichment of Grape Musts, Concentrated Grape Musts, Rectified Concentrated Grape Musts and Wines by Application of Nuclear Magnetic Resonances of Deuterium. *Off. J. Eur. Communities: Legis.* **1990**, *L272*(33), 64−73, EC Regulation 2676/1990, Annex 8, 3 October **1990**.

(5) List and description of methods of analysis referred to in the first paragraph of Article 120g of Council Regulation (EC) no. 1234/2007—Annexes 8 and 10. *Off. J. Eur. Communities: Inf. Not.* **2010**, *C43*(53), 19 February **2010**.

(6) AOAC Official Method 995−17 Beet Sugar in Fruit Juices; Site-Specific Natural Isotope Fractionation Nuclear Magnetic Resonance (SNIF-NMR) Method. AOAC International, Official Methods of analysis, http://www.eoma.aoac.org (accessed September 2010)

(7) AOAC. . *Determination of the $^{13}C/^{12}C$ ratio of ethanol derived from fruit juices and maple syrups; isotope ratio mass spectrometry* (*IRMS*); *Official Methods of Analysis*; AOAC International: Gaithersburg, MD, 2005; Official Method 2004.1, http://www.eoma.aoac.org (accessed September 2010).

(8) Bauer-Christoph, C.; Christoph, N.; Aguilar-Cisneros, B. O.; Lopez, M. G.; Richling, E.; Rossmann, A.; Schreier, P. Authentication of tequila by gas-chromatography and stable isotope ratio analyses. *Eur. Food Res. Technol.* **2003**, *217*, 438−443.

(9) Jamin, E.; Gonzalez, J.; Remaud, G.; Naulet, N.; Martin, G. G. Detection of exogenous sugars or organic acids addition in pineapple juices and concentrates by ^{13}C IRMS analysis. *J. Agric. Food Chem.* **1997**, *45*, 3961−3967.

(10) Gonzalez, J.; Remaud, G.; Jamin, E.; Naulet, N.; Martin, G. G. Specific natural isotope profile studied by isotope ratio mass spectrometry (SNIP-IRMS): $^{13}C/^{12}C$ ratios of fructose, glucose and sucrose for improved detection of sugars addition to pineapple juices and concentrates. *J. Agric. Food Chem.* **1999**, *47*, 2316−2321.

(11) Low, N.; Brause, A.; Wilhelmsen, E. Normative data for commercial pineapple juice from concentrate. *J. Assoc. Off. Anal. Chem.* **1994**, *44*, 965−975.

(12) Tenailleau, E.; Akoka, S. Adiabatic ^{1}H decoupling scheme for very accurate intensity measurements in ^{13}C NMR. *J. Magn. Reson.* **2007**, *185*, 50−58.

(13) Caytan, E.; Botosoa, E.; Silvestre, V.; Robins, R. J.; Akoka, S.; Remaud, G. Accurate quantitative ^{13}C NMR spectroscopy: Repetability over time of site-specific ^{13}C isotope ratio determination. *Anal. Chem.* **2007**, *79*, 8266−8269.

(14) Caytan, E.; Remaud, G.; Tenailleau, E.; Akoka, S. Precise and accurate quantitative ^{13}C NMR with reduced experimental time. *Talanta* **2007**, *71*, 1016−1021.

(15) Remaud, G.; Gilbert, A.; Sivestre, V.; Botosoa, E.; Akoka, S.; Robins, R. J. Quantitative ^{13}C NMR spectrometry as a new tool for site-specific isotopic fractionation studies. Isotopes 2009 Congress, Cluj-Napoca, Romania, 2009.

(16) Silvestre, V.; Segebarth, N.; Remaud, G.; Guillou, C.; Akoka, S. Results of an interlaboratory comparison of site-specific isotope ratio in quantitative ^{13}C NMR. 4th International Symposium on Isotopomers Congress, Tokyo, Japan, 2008.

(17) Remaud G.; Caytan E.; Botosoa E.; Silvestre V.; Robins R. J.; Akoka S. ^{13}C-SNIF-NMR—a contribution to the authentication of alcoholic beverages? Developments and perspectives. In Proceedings: 3rd International Workshop on Alcoholic Beverages Authentication IWABA 2007, Stresa, Italy, 2007; EUR 23398 EN ISBN 978-92-79-09322-7.

(18) Botosoa, E. *Protocoles analytiques pour l'étude de la vanilline par RMN 13C isotopique en abondance naturelle: Reproductibilité méthodologique, purification, origines du fractionnement isotopique*. Ph.D. Thesis, University of Nantes, France, 2008.

(19) Silvestre, V.; Mboula, V.; Jouitteau, C.; Akoka, S.; Robins, R. J.; Remaud, G. Isotopic ^{13}C NMR spectrometry to assess counterfeiting of active pharmaceutical ingredients: site-specific ^{13}C content of aspirin and paracetamol. *J. Pharm. Biomed. Anal.* **2009**, *50*, 336−341.

(20) Botosoa, E.; Silvestre, V.; Robins, R. J.; Moreno Rojas, J. M.; Guillou, C.; Remaud, G. Evidence of ^{13}C noncovalent isotope effects obtained by quantitative ^{13}C nuclear magnetic resonance spectroscopy at natural abundance during normal phase liquid chromatography. *J. Chromatogr., A* **2009**, *1216*, 7043−7048.

(21) Botosoa, E.; Blumenstein, C.; MacKenzie, D. A.; Silvestre, V.; Remaud, G.; Kwiecień, R. A.; Robins, R. J. Quantitative isotopic ^{13}C nuclear magnetic resonance at natural abundance to probe enzyme reaction via site-specific isotope fractionation: the case of chain-shortening reaction for the bioconversion of ferulic acid to vanillin. *Anal. Biochem.* **2009**, *393*, 182−188.

(22) Botosoa, E.; Caytan, E.; Silvestre, V.; Robins, R. J.; Akoka, S.; Remaud, G. Unexpected fractionation in site-specific ^{13}C isotopic distribution detected by quantitative ^{13}C NMR at natural abundance. *J. Am. Chem. Soc.* **2008**, *130*, 414−415.

(23) Gilbert, A.; Silvestre, V.; Robins, R. J.; Remaud, G. Accurate quantitative isotopic ^{13}C NMR spectroscopy for the determination of the intramolecular distribution of ^{13}C in glucose at natural abundance. *Anal. Chem.* **2009**, *81*, 8978−8985.

(24) Rossmann, A.; Butzenlechner, M.; Schmidt, H.-L. Evidence for a nonstatistical carbon isotope distribution in natural glucose. *Plant Physiol.* **1991**, 609−614.

(25) Martin, Y. L. A global approach to accurate and automatic quantitative analysis of NMR spectra by complex least-squares curve fitting. *J. Magn. Reson., Ser. A* **1994**, *111*, 1−10.

(26) *Accuracy* (*Trueness and Precision*) *of Measurement Methods and Results—Part 1: General Principles and Definitions*; ISO 5725-1:1994, 1994; http://www.iso.org (accessed September 2010).

(27) Gustavo González, A.; Ángeles Herrador, M.; Asuero, A. G. Intralaboratory assessment of method accuracy (trueness and precision) by using validation standards. *Talanta* **2010**, *82*, 1995−1998.

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