

## 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

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A procedure for the analysis of the oxygen-18/oxygen-16 isotope ratio of ethanol derived from the sugars of orange juice using the preparation steps of the SNIF-NMR method followed by pyrolysis–isotope ratio mass spectrometry is presented. The isotopic fractionation induced by the isotope effects of fermentation and distillation have been investigated, and it is shown that reproducible results can be obtained when appropriate analytical conditions are used. It is also shown that the oxygen isotope distribution in the water and organic matter pools of fruits remains quite stable during the harvest period and is not altered by the precipitation rate within the last few days before the fruits are picked. Due to the robustness of the method and the fact that most of the oxygen-18 enrichment from the initial sugars is still present in the end-product, ethanol appears as a convenient internal reference to circumvent the spatial and temporal variability observed for the oxygen-18/oxygen-16 isotope ratio of water. A very strong correlation is observed between the isotopic deviations of ethanol and water, which is altered in the event of a water addition, even at a low level. Combining the information brought by these two parameters leads to a more efficient authenticity testing tool, which avoids false positive cases and provides a lower detection limit for added water in juices not made from concentrate, whatever the origin of the sample tested.

**KEYWORDS:** Orange juice; oxygen-18; water; ethanol; authentication

### INTRODUCTION

In the steadily growing fruit juice sector, the trend toward direct not-from-concentrate (NFC) juices is becoming more noticeable year after year. Often sold as short-shelf-life products available in supermarket chilled cabinets, NFC juices are to be found at the premium end of the market and respond to increasing consumer demand for natural, healthier products. This market situation can be incentive for mixing high-price NFC juice with orange juice made from concentrate (FC juice), which is impossible to detect on the basis of composition analyses only.

The detection of water addition in fruit juices and wines is routinely performed by measuring the isotopic ratios of oxygen ( $^{18}\text{O}/^{16}\text{O}$ ) and hydrogen ( $^2\text{H}/^1\text{H}$ ) by isotope ratio mass spectrometry (IRMS). Due to isotopic fractionation phenomena that occur during plant transpiration, these ratios are usually higher in plants than in groundwater, so that addition of water results in an impoverishment of the ratios observed in authentic fruit juice or wine samples. Guidelines regarding the minimum values for these ratios have been published 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 corresponding analytical methods have gained official recognition from the Comité Européen de Normalisation (CEN), from the Office International de la Vigne et du Vin (OIV), and from the European Community (EC) (2–4).

The main drawback of this technique, however, is that the isotopic ratios of fruit water depend on the geographical origin and harvest period of the fruit, so that large and up-to-date databases are necessary to reliably interpret the results (5). A further limitation to the use of the stable isotope ratios of water is that when the origin is not specified, for example, for an orange juice without its country of origin on the label, the range of acceptable values is rather broad and consequently the detection limit of added water is relatively high.

To improve the detection of water addition in fruit juices, the  $^{18}\text{O}$  deviations of water and organic compounds can be used simultaneously. With the recent development of techniques using on-line pyrolysis coupled to IRMS, the measurement of  $^{18}\text{O}/^{16}\text{O}$  ratios of organic compounds is now carried out routinely in several laboratories. The  $^{18}\text{O}$  contents of the sugars or citric acid extracted from orange juice have been proposed as internal references to detect water addition at lower levels than methods based on the water only (6). However, the use of this approach is limited by the fact that the oxygen atoms in carbonyl functions

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are readily exchangeable with water from the medium. Moreover, the sugars have to be crystallized without any trace of remaining water, which in practice is very difficult to achieve. The isolation of sucrose from the sugars would in principle avoid the presence of exchangeable oxygen and facilitate the removal of water, but on the other hand would add lengthy analytical steps and further risks of isotopic fractionation. The  $^{18}\text{O}$  analysis of cellulose from tree rings has been widely used as an environment indicator for past climatic conditions because of the tight correlation between cellulose and leaf water (7). Because cellulose is the main component of the pulp usually present in orange juices, it could also be used as an internal isotopic reference. However, we have shown in a previous study on carbon-13 analyses that the industrial conditions of pulp extraction and restoration alter the relationship observed in laboratory-squeezed fruits (8). Moreover, catering to consumer taste by adding extra pulp or cells, often from a different origin than of the fruit itself, is an increasingly frequent practice, which could be another drawback to the use of the cellulose as internal reference.

To get a more robust internal reference, we recently introduced a refinement of the method proposed by Houerou et al., which consists of using the ethanol isolated in the course of the procedure for the isotopic analysis of site-specific  $^2\text{H}/^1\text{H}$  ratios by SNIF-NMR (9). This paper appraises, from a series of experimental results, the isotopic fractionation phenomena associated with this new method and describes its practical application to the detection of water addition in the case of orange juice.

## MATERIALS AND METHODS

**Sample Description.** This work is based on the study of more than 100 different orange juice samples from different origins; 52 reference samples were obtained from fresh fruit representing a wide range of geographical origins (Brazil, Florida, Israel, Spain, South Africa, Australia, Greece, China, Costa Rica, and Egypt). All fruit samples were squeezed in the laboratory. In addition, 56 commercial samples from the market were tested.

**Equilibration of Water with  $\text{CO}_2$ .** The  $^{18}\text{O}$  deviations of water were measured on  $\text{CO}_2$  previously equilibrated with the juice samples according to the routine procedure described in the corresponding official method of fruit juices analyses (3).

**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 (9). Using *Saccharomyces cerevisiae* and *Saccharomyces bayanus* yeasts (Vitilevure B+C, Martin Vialatte Oenologie, Epernay, France), single-strength juices were completely fermented to ethanol, which was then extracted by high-yield automated distillation. The residual water in the distillate was trapped by storing for at least 24 h on a molecular sieve (2 mm beads, UOP type 3A, Fluka Chemie GmbH, Buchs, Switzerland).

**Isotopic Determinations.** The mass spectrometric determinations of the oxygen isotope ratios of water and ethanol were carried out by on-line analysis using an elemental analyzer (NA 1500 series, Fisons Instrument SpA, Rodano, Milano, Italy) fitted to an isotope ratio mass spectrometer (Optima from Micromass, Wythenshawe, Manchester, U.K.). The  $\text{CO}_2$  resulting from the equilibration step described above was introduced via a manifold (Micromass). The ethanol samples are first placed in silver containers as carefully as possible to avoid contamination by water or organic matter (especially 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 carbon monoxide used as reference gas is calibrated against Vienna Standard Mean Ocean Water (V.SMOW) by analyzing V.SMOW and Standard Light Antarctic Precipitation (SLAP) water samples. In addition, two working standards are used in each series of measurement: a 99.5% pure crystallized glucose and a 99% pure ethanol (Sigma-Aldrich, Saint-Quentin Fallavier, France).

The results are expressed on the  $\delta$  ‰ scale with respect to the international standard V.SMOW according to the relationship

$$\delta^{18}\text{O} (\text{‰}) = (R_{\text{product}}/R_{\text{standard}} - 1) \times 1000$$

where  $R = ^{18}\text{O}/^{16}\text{O}$ .

The internal reproducibility of the methods used in our laboratory is estimated at 0.3‰ for water and at 0.5‰ for ethanol (based on daily measurements of the ethanol working standard over more than one year).

**Karl Fischer Titration and Density Measurements of Water—Ethanol Mixtures.** These determinations were performed according to the procedures described in the SNIF-NMR method (9).

## RESULTS AND DISCUSSION

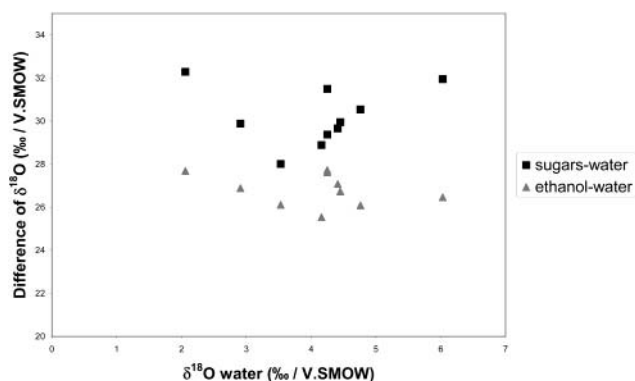
**Study of the Isotopic Effects of the Analytical Steps.** Some preliminary experiments were performed to evaluate the isotopic effects of the analytical steps described above and to define ways of circumventing them. These are presented in ascending order, from the last preparation step (water trapping) to the initial fermentation step.

The molecular sieve was tested by comparing the values measured on pure ethanol (purchased from Sigma, Saint-Quentin Fallavier, France) and on a simulated distillate prepared by adding 5% distilled water to the pure ethanol followed by the molecular sieve treatment described above. The residual water determined in both cases by Karl Fischer titration was found to be <0.5% w/w, which is negligible. Moreover, the  $\delta^{18}\text{O}$  values measured (respectively,  $-1.2$  and  $-1.1$ ‰) were not significantly different, thus demonstrating the absence of isotopic fractionation at this stage.

To study the isotopic fractionation of oxygen during distillation, a model solution of 5% v/v ethanol (same source as above) in distilled water was distilled using a high-yield automated distillation system (9). The distillation yield was 96.6% (in-line with the minimum of 96% required by the above method for SNIF-NMR determinations), and the resulting  $\delta^{18}\text{O}$  value ( $-1.4$ ‰) shows no significant shift from the initial  $^{18}\text{O}$  content of the ethanol.

This confirms previous findings regarding the behavior of the stable isotopes of carbon and hydrogen in the course of ethanol distillation (10): despite the stable isotope fractionation observed when the conversion is incomplete, these phenomena can be neglected when a sufficient distillation yield (>96%) is obtained.

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 molecule is expected (11). Therefore, if the fermentation is complete, the oxygen isotope composition of ethanol should partially reflect the initial isotopic ratios of these positions in the sugar. A partial equilibrium of metabolic intermediates with the water of the fermentation medium may also occur, although in most cases this exchange remains limited (12). There is a further risk of isotopic fractionation due to the potential effect of the slow exchange between the oxygen atoms of ethanol and water during



**Figure 1.** Plot of  $^{18}\text{O}$  enrichments (expressed as  $\delta^{18}\text{O}$  differences) of sugars versus water and of ethanol versus water in orange juice samples.

rehybridization of the  $\text{sp}_3$  carbon of ethanol into  $\text{sp}_2$  by the couple alcohol dehydrogenase— $\alpha$ -lipoyldehydrogenase.

Despite these isotopic effects, we have postulated that ethanol constitutes a potentially reliable internal isotopic reference, provided that reproducible results can be obtained in routine laboratory conditions. To evaluate the reproducibility of the fermentation step, the same orange juice was fermented in five independent experiments performed in reproducibility conditions (different dates and operators; in all cases the distillation was performed within 24 h after the end of fermentation). The standard deviation observed (0.25‰) is very close to the standard deviation of internal reproducibility of the IRMS measurement itself (0.18‰), which shows the good reproducibility of the process. In our experience, the precision and robustness of results obtained with ethanol are a considerable improvement over that which can be achieved with sugars in routine conditions. A comparison between the results obtained for total sugars, extracted according to the procedure described in a previous paper (13), and ethanol is presented in **Figure 1**. This figure displays the oxygen-18 shifts between water, sugar, and ethanol from a set of 10 orange juice samples. Although the number of results is limited and this must be interpreted with care, it appears that the differences measured between ethanol and water are more stable (the standard deviation of the difference is 0.8‰ instead of 1.4‰ for sugars). The rather small impoverishment (mean =  $-3.4\text{‰}$ ) observed between sugars and ethanol, which confirms that the isotopic exchanges during the fermentation are in fact very limited in conditions allowing a complete fermentation by yeast, is also worth noting.

**Study of the Influence of Rainfall on the Isotopic Composition of Water and Organic Matter.** The ability of the above procedure to provide relevant information is based on the hypothesis that the oxygen ratios of both water and ethanol reflect a long-term effect of environmental parameters, which assumes that the water and carbohydrate pools of oxygen in the fruit flesh change according to similar kinetics. It has been shown that the oxygen-18 deviation of water in fruits such as grape, in which there is considerable exchange between the berry and the atmosphere through the skin, can undergo dramatic changes depending on climatic conditions during the few weeks before harvest and even during the last days before picking (5). In the case of citrus juices, however, we anticipated that this effect would be much smaller due to the different fruit physiology and structure.

In a first experiment, some oranges were fixed on a metal support to mimic an orange tree and placed under a continuous water shower in the laboratory for 24 h at ambient temperature (20 °C). No change of the isotopic values was observed, which

**Table 1.** Apple Tree Experiment: Rainfall Levels (between Successive Samplings) and  $\delta^{18}\text{O}$  Values of Water and Ethanol Observed during a 2 Month Period

sampling date	rainfall (mm)	$\delta^{18}\text{O}$ water (‰)	$\delta^{18}\text{O}$ ethanol (‰)
Aug 29, 2002		-0.9	24.3
Sept 5, 2002	7	-1.2	23.9
Sept 12, 2002	31	-0.9	24.4
Sept 19, 2002	4	-1.3	23.9
Oct 4, 2002	15	-1.0	24.9
Oct 11, 2002	15	-1.4	24.5
Oct 21, 2002	35	-1.4	24.3

**Table 2.**  $\delta^{18}\text{O}$  Values of Water and Ethanol in Authentic Orange Juices Made from Laboratory-Squeezed Samples<sup>a</sup>

origin		$\delta^{18}\text{O}$ water (‰)	$\delta^{18}\text{O}$ ethanol (‰)
Australia	mean	11.0	37.8
	SD	0.9	2.1
Brazil	mean	3.3	29.3
	SD	3.9	4.8
Costa Rica	mean	-0.6	24.4
	SD	3.3	2.9
Europe (Spain, Italy, and Greece)	mean	4.7	31.1
	SD	3.5	4.0
Israel	mean	5.2	32.1
	SD	1.0	1.5
South Africa	mean	8.1	34.6
	SD	2.5	3.7
all origins	mean	5.0	31.3
	SD	4.2	4.8

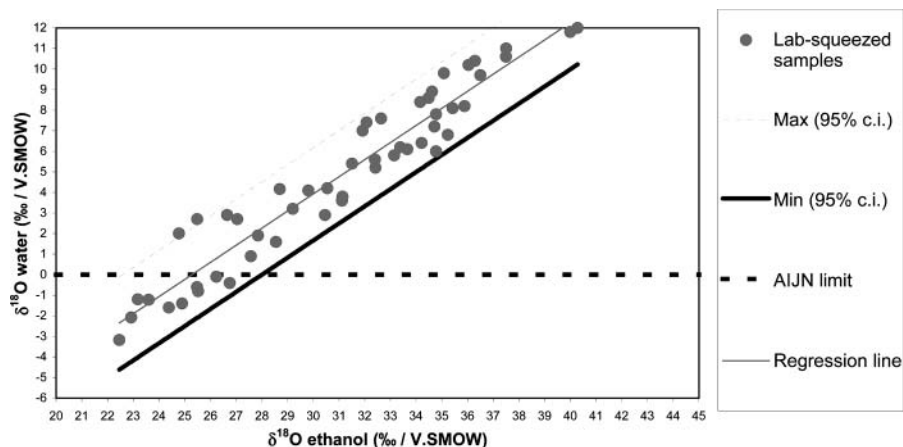
<sup>a</sup> When at least four samples of the same origin were available, the mean and standard deviation (SD) are given.

supports the assumption that very limited exchange occurs through the orange skin. We then examined the influence of rainfall levels in real conditions using fruits grown on a given tree. For convenience we used an apple tree as the model.

Approximately 4–5 kg of apples was collected each week from the beginning of September to the end of October, and the corresponding weekly rainfall was carefully recorded. The apples were crushed in the laboratory, and the resulting juice was submitted to the analytical procedure outlined above. **Table 1** displays the rainfall (between two successive samplings) and the  $\delta^{18}\text{O}$  values observed for water and ethanol during this period. Despite the variable rainfall rates observed from one week to another and the significant cumulated rainfall (107 mm), no truly significant trend is observed concerning the isotope ratios of water or organic matter. This should be all the more true in the case of citrus fruits, which have a thicker and less permeable skin. We have therefore concluded that the impact of rainfall is correctly taken into account, as are the other environmental sources of variability, through a widespread distribution of reference samples in space and time.

**Establishment of a Worldwide Database of Genuine Orange Juice.** Fifty-two reference samples of fresh fruits representing a wide range of geographical origins (Brazil, Florida, Israel, Spain, South Africa, Australia, Greece, China, Costa Rica, and Egypt) and harvest years (1994–2001) were squeezed in the laboratory, and the resulting authentic juices were submitted to the whole analytical procedure outlined above.

**Table 2** and **Figure 2** show the values measured for these samples. Interestingly, the slope of the regression line relative to water/ethanol is very similar to that in the work of Houerou et al. (6) relative to water/sugar. These lines differ by their intercept with the zero value of the  $\delta^{18}\text{O}$  of water (difference



**Figure 2.** Plot of authentic orange juice samples made from laboratory-squeezed samples in the plane of the  $\delta^{18}\text{O}$  values of ethanol and water ( $R = 0.96$ ,  $s = 1.1$ ).

of  $\sim 4\%$ ), which confirms the mean impoverishment calculated above from the data of **Figure 1**.

Due to the very broad range of origins involved, a very large spread of  $\delta^{18}\text{O}$  values is observed, with Australia usually at the highest end and Costa Rica usually at the lowest end. The AIJN minimum limit for the water in orange juice ( $\delta^{18}\text{O} = 0$ ) is also plotted on this graph, confirming that this limit is valid in most cases but also showing that the interpretation of  $\delta^{18}\text{O}$  values requires expert advice based on knowledge of the origin of the sample. The very low  $^{18}\text{O}$  content of the authentic samples of Costa Rican origin is a striking illustration of the risk of erroneous interpretation when the guideline is misused as an absolute limit. Some authors have published reference values for given origins and years (6, 14), which can be very useful whenever the information on the origin and harvest date are available. However, the fruit juice market is an increasingly global one, and the information regarding the fruit used is in most cases unavailable to the analyst or at best incomplete. It is also evident from **Table 2** and previously published results that even within a given country the range of  $\delta^{18}\text{O}$  values of water remains quite large.

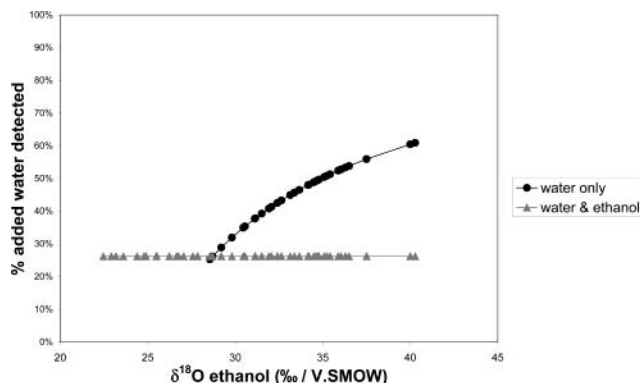
A more powerful interpretation is obtained by taking into account the very strong correlation observed between ethanol and water. A confidence interval can then be defined as a “conformity tunnel” around the observed correlation, calculated as the 95% confidence interval of the regression line from the following equation

$$y = ax + b \pm 2s \quad (1)$$

where  $y = ax + b$  is the linear regression model obtained from the 52 data points (the actual values are displayed in **Figure 2**) and  $s$  is the standard deviation of residues (difference between calculated and observed  $y$  values).

The use of the 95% confidence interval as an action limit for industrial samples has been justified previously on the basis of averaging effects associated with large volumes (15).

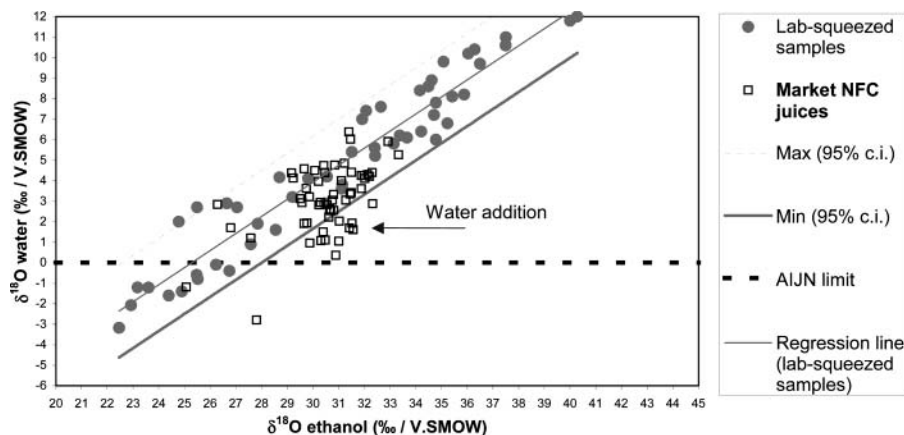
**Application for the Authenticity Control of Not from Concentrate Orange Juice.** Whenever a sample falls outside the conformity range, it indicates that the natural correlation between ethanol and water has been altered by the addition of water and/or sugar. Because the  $\delta^{18}\text{O}$  ranges of plant sugars (including orange and its usual adulterants beet/cane/cereals sugars) overlap (12), the presence of sugar is more efficiently checked using other methods. When these methods do not provide any evidence of sugar addition and the  $\delta^{18}\text{O}$  value of water falls below the lowest limit of the confidence interval, it



**Figure 3.** Model mixture of NFC and FC juices: plot of “average” detection limits as a function of the  $\delta^{18}\text{O}$  values of ethanol from reference NFC juice, based on the  $\delta^{18}\text{O}$  values of water only, or on the  $\delta^{18}\text{O}$  values of water and ethanol.

proves unambiguously that water has been added. This remains true even when the water  $\delta^{18}\text{O}$  value is over the AIJN minimum, which provides a significant improvement of the detection limit of added water.

This limit is still difficult to define because it will depend on the isotopic content of the water from the orange and that of the added water, both of which vary according to location, and because the  $\delta^{18}\text{O}$  value of ethanol is not strictly independent from the added water or juice from concentrate. However, the potential increase of water detection efficiency versus a single-parameter limit can be illustrated by an example. Considering the case of an addition of orange juice from concentrate (OJFC) bearing typical  $\delta^{18}\text{O}$  values of 26 and  $-8\%$  for ethanol and water, respectively, we have computed the detection limits of the single-parameter and two-parameter approaches for the model orange juices plotted in **Figure 2** as the regression line for genuine samples: see the resulting curves in **Figure 3**. The “water-only” detection limit was obtained as the ratio of the distances between the regression line and the AIJN minimum versus the distances between the regression line and the  $\delta^{18}\text{O}$  value of water in the OJFC. On the basis of laboratory trials, the “mixture curve” between the initial product and the adulterant in the plane of the  $\delta^{18}\text{O}$  values of ethanol and water was assumed to be a simple linear curve. The “water and ethanol” limit was obtained as the ratio of the distances between the regression line and the intercept of “minimum 95% curve” and “mixture curve” versus the distances between the regression line and the OJFC adulterant. As expected from its mathematical



**Figure 4.** Plot of authentic and market NFC orange juice samples in the plane of the  $\delta^{18}\text{O}$  values of ethanol and water.

properties, this ratio remains constant and corresponds in this example to the detection of ~25% or more OJFC, which would remain undetectable by classical analytical methods. As shown in **Figure 3**, the “water-only” detection limit varies with the  $\delta^{18}\text{O}$  value of organic matter and is in most cases much higher than the “water and ethanol” limit. As we have seen before, a  $\delta^{18}\text{O}$  value of water below the AIJN minimum but still within the conformity range defined above should not always be considered as evidence of adulteration. Therefore, the corresponding points have been withdrawn from the “water-only” curve in **Figure 3**.

Finally, market samples from various packers around the world underwent this new test, and results were compared to the authentic samples database (**Figure 4**). Whereas most of the samples are within the range defined previously, a significant number fall outside, which indicates added water, even if in some of the cases the values are still above the AIJN limit. Optimum testing of NFC orange juice samples should therefore include the analysis of the  $\delta^{18}\text{O}$  value of ethanol in addition to the usual stable isotope analyses. The multi-isotope analysis of ethanol, on the one hand (including SNIF-NMR, carbon-13, and oxygen-18 determinations), and the oxygen isotope analysis of water, on the other hand, provide an optimum control of potential sugar and water additions, which are still the most common adulterations of NFC juices. Future studies will involve the application of this approach to other fruit juices.

This study also contributes to a greater understanding of the behavior of oxygen isotopes in the orchard and during the alcoholic fermentation. A further improvement of this approach could be made in the future by normalizing the  $\delta^{18}\text{O}$  values of ethanol according to the  $^{18}\text{O}$  content of the fermentation medium.

#### ACKNOWLEDGMENT

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