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Improved Detection of Sugar Addition to Maple Syrup Using Malic Acid as Internal Standard and in ^{13}C Isotope Ratio Mass Spectrometry (IRMS)

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Stable carbon isotope ratio mass spectrometry ($\delta^{13}C$ IRMS) was used to detect maple syrup adulteration by exogenous sugar addition (beet and cane sugar). Malic acid present in maple syrup is proposed as an isotopic internal standard to improve actual adulteration detection levels. A lead precipitation method has been modified to isolate quantitatively malic acid from maple syrup using preparative reversed-phase liquid chromatography. The stable carbon isotopic ratio of malic acid isolated from this procedure shows an excellent accuracy and repeatability of 0.01 and 0.1‰ respectively, confirming that the modified lead precipitation method is an isotopic fractionation-free process. A new approach is proposed to detect adulteration based on the correlation existing between the $\delta^{13}C_{malic\ acid}$ and the $\delta^{13}C_{sugars} - \delta^{13}C_{malic\ acid}$ ($r = 0.704$). This technique has been tested on a set of 56 authentic maple syrup samples. Additionally, authentic samples were spiked with exogeneous sugars. The mean theoretical detection level was statistically lowered using this technique in comparison with the usual two-standard deviation approach, especially when maple syrup is adulterated with beet sugar : 24 ± 12% of adulteration detection versus 48 ± 20% (*t*-test, $p = 7.3 \times 10^{-15}$). The method was also applied to published data for pineapple juices and honey with the same improvement.

KEYWORDS: Maple syrup; sugars and malic acid; internal standard; authentification; stable isotope analysis; ^{13}C IRMS; pineapple juices; honey

INTRODUCTION

Maple syrup, which is produced from the xylem sap of *Acer saccharum* March, has recently become a luxury product in many countries. It is thus important to develop an effective and adequate method to detect low levels of adulteration. Maple syrup adulteration can be accomplished by adding inexpensive sugars (mainly sucrose) to maple sap or by simple dilution of maple sap with sugar syrup, in either case for economic gain. Several methods have been developed to detect adulteration (*1*−*5*). Most of them are based on the determination of the stable carbon isotope ratio of the dry maple syrup. This method is applicable because the $^{13}C/^{12}C$ ratio of a plant-derived material reflects the photosynthetic pathways employed by plants during atmospheric CO_2 fixation.

There exist three photosynthetic pathways or cycles: the Calvin cycle (*6*), the Hatch−Slack cycle (*7*) and the crassulacean acid metabolism (*8*). Plants using the Calvin cycle, such as the maple tree and sugar beets, are commonly called C_3-plants. This photosynthetic pathway is recognized to produce organic compounds depleted in ^{13}C (*9*) where the stable carbon isotopic ratio, $\delta^{13}C$, varies between −20 and −32‰ with a mean of −27‰ (relative to the international standard VPDB like all $\delta^{13}C$ presented here). Plants using the Hatch−Slack cycle, like cane sugar, are commonly called C_4-plant, and their $\delta^{13}C$ values range fall between −9 to −17‰ with a mean of −13‰. Finally, plants using the crassulacean acid metabolism are named CAM-plant and have an intermediate stable carbon isotopic composition with values varying between −10 and −20‰.

Adulteration of maple syrup (C_3-plant) by cane sugar (C_4-plant) or beet sugar (C_3-plant) can be detected by carrying out the stable carbon isotopic ratio of the dry maple syrup as shown by the current methods of adulteration detection (*1*−*5*). Their detection limits (varying from 10 to 50% of sugar addition) depend on several factors, particularly on the natural stable carbon isotopic distribution of the authentic product (maple syrup) and of the adulterant (cane or beet sugar). The adulteration detection limits are obtained from the standard deviation of the natural variation of the maple syrup isotopic composition. Lower adulteration detection levels may be achieved using an

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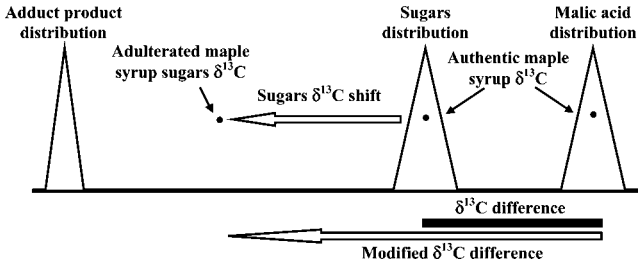

Figure 1. Schematic representation of authentic product adulteration and implication on the δ^{13}C values of the product and the internal standard and the δ^{13}C difference between them (product − internal standard).

internal standard, but this technique has not been applied to maple syrup. Therefore, there is a need for new isotopic internal standards to enhance the information provided by δ^{13}C of the dry maple syrup. Ideally, if the value of the stable carbon isotopic ratio of the original components of the mixture was known, this would be under the best experimental conditions to determine adulteration.

To achieve these conditions, several authors have proposed the use of an internal standard (*10*−, *11*) naturally present in the sample. This internal standard should have the same isotopic characteristics as the original product (information about photosynthetic origin and environmental and physiological behavior). **Figure 1** illustrates the δ^{13}C shift produced on the product and the isotopic value difference between the product and the internal standard when adulteration occurs. It also demonstrates the potential of internal standard utilization to obtain information about the original δ^{13}C value of the product if an isotopic correlation relationship exists between the internal standard and the product.

The ideal candidate for such an approach must be easy to isolate in sufficient quantity to be analyzed. Furthermore, it should not be commercially available at an attractive price for use on a large scale (*11*). For food adulteration detection, pulp (*10*, *13*), proteins (*12*, *14*), and malic and citric acid (*17*−*19*) have been used. From the maple syrup constituants (*20*), malic acid seems to be the most interesting internal standard because commercial sweeteners contain very little malic acid (*21*). There are already several methods for malic acid extraction in foods (*22*, *23*). The use of intermolecular stable carbon isotope correlation between sugars and malic acid in fruit juices has been suggested as a criterion for authenticity. The standard deviation of the natural isotopic variation of the internal standard or the isotopic difference between the internal standard and the sugars is used to determine the adulteration detection limits (*17*−*19*). However, maple syrup is produced from the thermal evaporation of the sap water. Complex chemical reactions occur, which may alter the isotopic composition of the sugars and the malic acid and therefore may lower the specificity normally associated with the internal standard procedure used for juice adulteration as juice is produced from a physical process like pressing, which does not produce isotopic fractionation. Moreover, the correlation usually found between the internal standard and the target adulteration specie, mainly sugars, should be used to determine the adulteration limits instead of using the standard deviation on a single isotopic population as used now.

We present herein the results obtained for maple syrup using malic acid as an internal standard. An optimized procedure is described for extraction and separation of malic acid from maple syrup involving a lead precipitation procedure. It will be examined how the isotopic correlation allows a better maple syrup adulteration detection, and a novel calculation approach will be introduced and compared with the current technique for adulteration detection of maple syrup. This proposed approach will also be tested on other foodstuffs using previously published data.

MATERIALS AND METHODS

Apparatus and Reagents. Potassium dihydrogen phosphate (KH_2PO_4) ≥ 99.5% and D-malic acid 99% were purchased from Sigma-Aldrich (Milwaukee, MI). Anhydrous oxalic acid ~97%, shikimic acid >97%, succinic acid >99.5%, citric acid >99.5%, fumaric acid >99.5%, and l(+)-tartaric acid >99.5% were purchased from Fluka (Milwaukee, MI). Sodium hydroxide (NaOH) 98+% A.C.S. was obtained from OMEGA (Levis, Quebec, Canada), lead nitrate ($Pb(NO_3)_2$) A.C.S. from Laboratoire MAT (Montréal, Quebec, Canada), hydrochloric acid (HCl) concentrate A.C.S. from Fisher (Nepean, Ontario, Canada), phosphoric acid (H_3PO_4) 85% from Mallinckrodt (Paris, KY), ethanol 95% from Commercial Alcohols (Montréal, Quebec, Canada), artificial maple flavor 187 M-11C from Richard Facey Ltd. (Rexdale, Ontario, Canada), cane sugar from Lactil (Toronto, Ontario, Canada), and water from a standard Millipore Milli-Q Plus Water system (Bedford, MA) were obtained.

The LC system consisted of a Waters Model 510 LC pump (Waters, Milford, MA), a Model 7725i Rheodyne injector (Supelco, Oakville, Ontario, Canada), a Perkin-Elmer Model LC-55 spectrophotometer (Perkin-Elmer, Nornalk, CT), and a HPChem data acquisition station (Agilent Technology, Montréal, Quebec, Canada). A 250 mm × 21.21 mm i.d., 5 μm Phenomenex Maxcil C_{18} column and a 30 mm × 4.60 mm i.d., 5 μm Phenomenex Maxcil C_{18} guard column were used for preparative chromatography (Phenomenex, Torrance, CA). A dual-inlet Model Prism VG Isotech (now GV Instruments, Manchester, UK) IRMS system was used.

Solutions Preparation. Dilute Maple Syrup Solution. In a centrifuge tube (50 mL), about 10 g of maple syrup was diluted with 30 mL of water and mixed well.

Artificial Maple Syrup Test Mixture. A total of −66.5 g of cane sugar and 0.24 g of malic acid were dissolved in water and diluted to 250 mL. The concentrations of cane sugar and malic acid correspond to real sample concentrations. To this solution, about 10 drops of artificial maple flavor was added to simulate the color of maple syrup.

Organic Acids Mixture. −A total of 0.01, 0.01, 0.05, 0.10, 0.10, 0.10, and 0.70 g of reagent grade shikimic, fumaric, oxalic, malic, citric, tartaric, and succinic acid, respectively, were dissolved in an LC mobile phase and diluted to 100 mL. To adjust the pH of the solutions, aqueous 14 M NaOH, aqueous 0.5 M NaOH, and aqueous 0.0036 M HCl were used.

Lead Nitrate Solution. A total of −49.7 g of $Pb(NO_3)_2$ was dissolved in water and diluted to 250 mL.

LC Mobile Phase. A total of 13.6 g of KH_2PO_4 was dissolved in water, diluted to 500 mL, and adjusted to pH 2.4 with concentrated H_3PO_4. The solution was filtered through a 0.45 μm filter, type HA (Millipore, Bedford, MA) and degassed.

Sample Selection. Samples were obtained from a wide range of geographical locations. Included were 231 samples from several areas in Quebec, Canada and 26 samples from the United States (Vermont, Maine, New Hampshire, and New York). All the samples were certified to be authentic. The δ^{13}C analysis of syrup was done to obtain the natural stable carbon isotopic distribution. The distribution normality was tested using the Kolmogorov−Smirnov one-sample test. All statistical analyses were performed using SYSTAT, version 10 (SPSS Science Marketing Department, Chicago, IL). The test showed a normal distribution with $p = 0.982$. The lower and higher values of this distribution were −24.75 and −22.82‰, respectively. Only 13% of the 231 Quebec maple syrup samples were selected to be analyzed for their malic acid stable carbon isotopic ratio. These 30 maple syrup samples were selected to represent the natural distribution of malic acid, which should follow that of the maple syrup, assuming that a correlation exists between these two variables. In addition, all the American maple syrup samples were selected to enhance the area of method application.

Malic Acid Isolation Procedure and δ^{13}C Determination. The organic acids contained in the maple syrup were precipated by three consecutive stages. The pH of the dilute maple syrup solution was adjusted to 10.0, and 2 mL of 0.6 M $Pb(NO_3)_2$ was added and mixed well. After 15 min of reaction, the solution was centrifuged at 2500 rotations per minute (rpm) for 10 min. The resulting liquid phase was carefully decanted, the pH was adjusted to 7.0, and 1 mL of 0.6 M $Pb(NO_3)_2$ was added and mixed well. After 15 min of reaction, the solution was centrifuged at 2500 rpm for 10 min. The pH of the decanted liquid phase was adjusted to 7.0, and 0.5 mL of 0.6 M $Pb(NO_3)_2$ was added and mixed well. After 15 min of reaction, the solution was centrifuged at 2500 rpm for 10 min. The liquid phase was discarded. The combined solid residues containing the organic acids were washed 3 times with 10 mL portions of ethanol by agitating for 20 s on a vortex mixer followed by centrifugation at 2500 rpm for 10 min and discarding of the liquid phase. Excess ethanol was removed from the remaining solid residue by being brought to dryness overnight under a fume hood.

The organic acids contained in the solid residue were extracted in four steps. To the residue, 5 mL of the LC mobile phase was added, mixed well, and shaken for 30 min with a lateral mixer. The solution was then centrifuged at 2500 rpm for 10 min, and the liquid phase was decanted and reserved. This procedure was repeated once at 30 min of agitation and twice more with 10 min of agitation. The four extracts containing the organic acids were combined to obtain a volume of about 20 mL.

The pH of this extract was adjusted to 2.0 with concentrated HCl and heated to 90 °C for 30 min. The solution should not be evaporated to dryness to avoid malic acid decarboxylation. After 30 min of reaction, the solution was rapidly cooled in a cold bath (water and ice mixture). Once at room temperature, the pH of the solution was adjusted to 2.4 with 14 M NaOH, and the LC mobile phase was added to obtain a final volume of 20 mL. The solution was now ready for preparative LC separation.

Prior to the injection of the sample into the preparative LC system, all solutions were filtered on a HAWP 0.45 μm filter (Millipore, Bedford, MA). The injection volume was 2 mL delivered by a 2 mL injection loop. The mobile phase flow was set at 9.9 mL min^{-1}. The column eluant was monitored with a UV detector set at 210 nm, and the malic acid retention time was 8.7 min. A home-built automated fraction collector was used to collect the malic acid fraction eluting between 6 and 9.5 min. No isotopic fractionation was noticed (data not shown). The mobile phase of the malic acid fraction was evaporated under a N_2 stream, and the solid residue, containing the malic acid, was dissolved in 0.5 mL of water and transferred quantitatively into a 6 mm i.d. by 9 mm o.d. quartz tube previously annealed at 800 °C. The water was then evaporated under a smooth air jet.

The malic acid solid residue combustion involves the oxidation of the organic compound in a pure oxygen environment at 800 °C. The CO_2 produced was then trapped and purified cryogenically from the other combustion gases (excess O_2 and H_2O) (*23*). The stable carbon isotope ratios were determined using a VG Prism dual-inlet mass spectrometer. All δ^{13}C values were related to the Vienna Pee Dee Belemnite (VPDB) carbonate standard. The analytical repeatability including the combustion, and the cryogenic distillation step was 0.01‰.

Maple Syrup δ^{13}C Determination. The δ^{13}C content of the maple syrup was determined on its dry residue obtained by placing about 7 mg of maple syrup in a vacuum oven at 1×10^{-2} mbar and 65 °C for about 1 h. The dry residue was then transferred into a 4 × 6 mm i.d. and o.d. quartz tube for the combustion and distillation steps.

RESULTS AND DISCUSSION

Reliability of the Malic Acid Isolation Procedure. The δ^{13}C of commercial malic acid was determined to see if the proposed method induced isotopic discrimination or contamination. Malic acid was purified by preparative LC before determining the δ^{13}C because fumaric acid was present as an impurity. To isolate malic acid, we increased the recovery time before and after the malic acid peak to avoid the isotopic fractionation that occurs across LC peaks (*22*). By this technique, pure malic acid was collected, and the δ^{13}C was determined by IRMS to be −29.86 ± 0.02‰ ($n = 7$).

Using this procedure, δ^{13}C of commercial malic acid isolated from a solution of organic acids was determined to evaluate if the other organic acids present in maple syrup and extracted by lead precipitation could interfere with malic acid. LC separation obtained a complete resolution between each chromatographic peak. Malic acid from this solution was isolated, and δ^{13}C was determined. The value found was −29.86 ± 0.04‰ ($n = 10$). These results showed no stable carbon isotopic contamination, and since the concentrations used were slightly higher than those found in typical maple syrup samples, with the exception of malic acid, any chromatographic separation with environmental concentrations allows complete malic acid isolation without isotopic contamination.

The complete organic acid separation procedure with lead was tested on the artificial syrup test mixture made from cane sugar, with added malic acid of known δ^{13}C values and artificial maple flavor. The δ^{13}C found for isolated malic acid was −29.80 ± 0.07‰ ($n = 9$). The mean value seems to be shifted by 0.06‰ from the original malic acid value. Moreover, the repeatability is higher (0.07 vs 0.04‰). This observation is possible as the analytical repeatability was 0.01‰, which allowed the detection of small isotopic ratio changes occurring by the malic acid isolation procedure.

Three organic sources were suspected to cause the observed behavior: the sucrose, the artificial flavor, and the ethanol used during the washing step. When the sugars were not added into the test maple syrup, the malic acid δ^{13}C obtained following its isolation was found to be −29.86 ± 0.04‰. Therefore, sucrose seems to be the source of isotopic contamination as the isotopic results show no isotopic shift and the repeatability was reduced to its original value. In fact, a very limited amount of mixing between residual sucrose and malic acid due to overlapping chromatographic peaks could significantly change the malic acid δ^{13}C. To test this hypothesis, a sucrose solution was injected into the preparative LC with the UV detector wavelength set at 190 nm, the maximum absorbance in the UV for carbohydrates. The chromatogram clearly confirmed the occurrence of interference by a sucrose tailing peak within the malic acid fraction. To avoid such an isotopic discrimination caused by sucrose tailing, a hydrolysis step (conversion of sucrose to glucose and fructose) was proposed since glucose and fructose test mixtures did not show any overlapping peaks with malic acid. Sucrose hydrolysis using enzymes, even if the reaction is quantitative and selective, cannot be used because Pb^{2+} is a strong invertase inhibitor (*25*). Acid hydrolysis of sucrose with HCl was found to be the most effective (*26*). Acid hydrolysis tests were then performed before LC separation, and the δ^{13}C value obtained for malic acid was −29.85 ± 0.05‰ ($n = 11$). By this technique, the overall repeatability matched that reported in the literature by several authors for malic and citric acid isolation in fruit juices, concentrates, and nectars (*17*−*19*).

The overall repeatability was determined by performing 12 independent analyses on the same authentic maple syrup sample. The δ^{13}C value obtained for malic acid was −26.81 ± 0.08‰ and −24.40 ± 0.04‰ for maple syrup sugars. In the literature, the overall repeatability with δ^{13}C values varying between 0.1 and 0.5‰ has been reported for equivalent procedures (*17*−*19*). A typical LC chromatogram is shown in **Figure 2**.

δ^{13}C Values of Sugars and Malic Acid from Authentic Maple Syrup. The stable carbon isotope ratios of sugars and

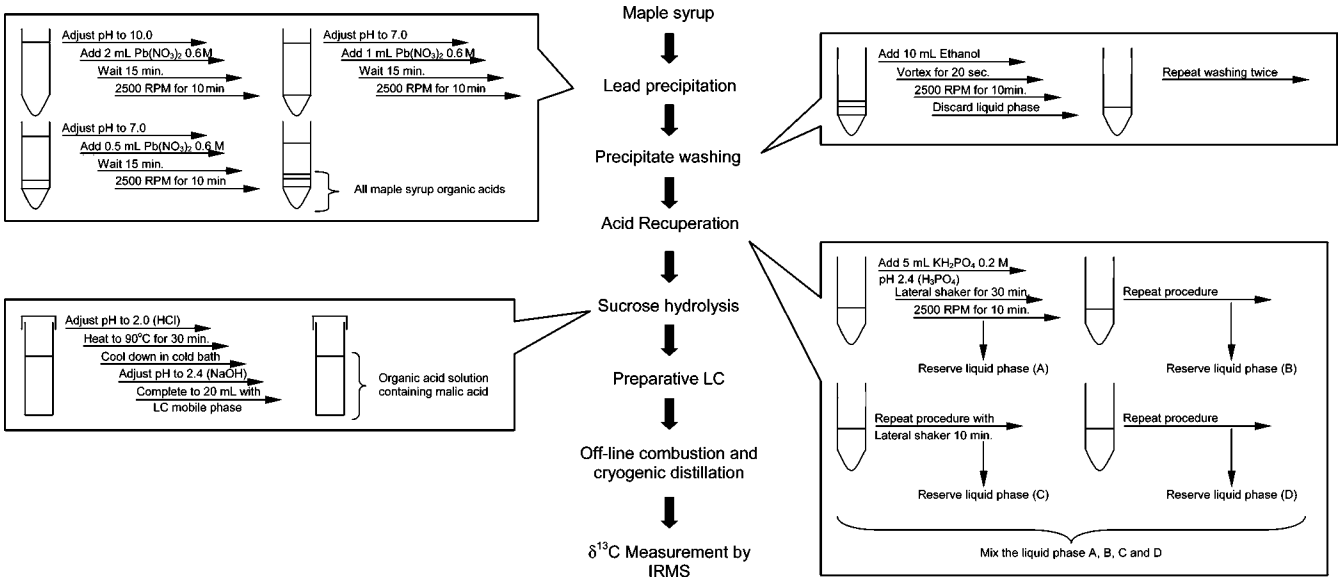

Figure 2. Flow chart of the analytical protocol used for the organic acids isolation from authentic maple syrup (each step is described in the text).

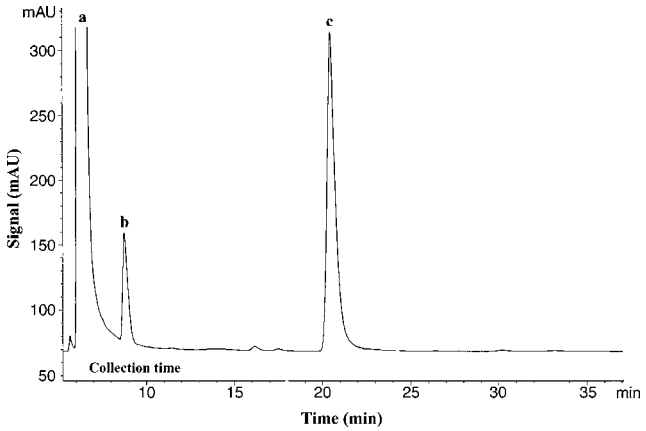

Figure 3. Typical LC chromatogram performed on organic acids extract from authentic maple syrup samples. The chromatographic peaks are (**a**) HNO_3, (**b**) malic acid, and (**c**) fumaric acid.

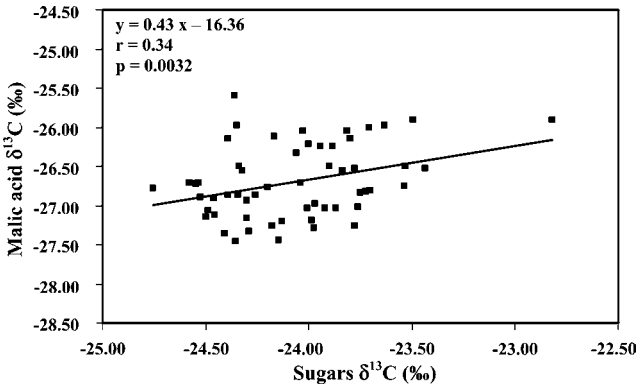

Figure 4. Correlation plot between $\delta^{13}C$ of sugars and malic acid from authentic maple syrup samples.

malic acid of 56 selected authentic maple syrup samples (30 from Quebec, Canada and 26 from the northeastern states) were determined, and the differences between these two values ($\Delta\delta^{13}C = \delta^{13}C_{sugars} - \delta^{13}C_{malic\ acid}$) were calculated from those quantities. The sugar and malic acid isotopic distributions passed the normality Kolmogorov−Smirnov one-sample test with p values of 0.711 and 0.304, respectively. A correlation plot between $\delta^{13}C$ values for sugars and malic acid is presented in **Figure 3**. The regression equation is $0.43x - 16.36$ with $r = 0.34$ ($p = 0.0032$). Higher correlation coefficients are reported in the literature (e.g., 0.75−0.92 between honey and proteins (*11*, *14*), 0.78 and 0.90 between sugars and malic acid in pineapple and apple juices (*17*−, *18*), and 0.89 between sugars and pulp from orange juice (*10*) since juice fabrication involves mechanical treatment only (pressing, centrifugation, and/or filtration)). Maple syrup is produced by the thermal evaporation of the maple sap, a process in which volatile organics evaporate and insoluble organics precipitate and are eliminated by filtration, potentially causing isotope discrimination or fractionation. However, with a p value of 0.0032, the correlation is statistically significant and confirms the isotopic relationship between sugars and malic acid (internal standard). It is surmised that adulteration of a batch of syrup by simultaneous addition of exogenous sugar and malic acid in such a manner to defy detection is sufficiently complex to discourage the practice, and more so if an internal standard is used to improve the detection level (*17*−*19*).

2-SD and Regression Line, RL, Methods to Detect Adulteration. Typically, the simultaneous $\delta^{13}C$ determination of sugars and the internal standard provides information that allows the threshold limits of adulteration detection to be lower (*19*). As the correlation between the carbon isotope ratios of sugars and malic acid has been established, threshold limits may be determined. Previous studies used a method based on the standard deviation, SD, of the difference of $\delta^{13}C$ values between the sugars and the malic acids, $\Delta\delta^{13}C$, to calculate two fixed detection limits for all samples (*19*). In this study, we propose instead a method based on the actual regression line, RL, existing between $\Delta\delta^{13}C$ and $\delta^{13}C_{malic\ acid}$ to generate detection limits specific to each individual sample. These methods are called the 2-SD and the RL methods, respectively.

In the 2-SD method, the limit values are calculated with a 95% confidence level that allows values within two standard deviations (2-SD) from the mean values of the normal distribution of $\delta^{13}C_{sugars}$ and $\delta^{13}C_{malic\ acid}$ values to be accepted, but lower detection limits are usually obtained when the limits are calculated from $\Delta\delta^{13}C$. Applied to our data, the 2-SD method generated the limits of 1.67 and 3.59‰ from $\Delta\delta^{13}C$. A schematic representation of this method is presented in **Figure 4**, where the two dotted lines represent the 2-SD fixed limits. If a sample $\Delta\delta^{13}C$ value does not lie between these limits, the sample is declared adulterated.

Table 1. Theoretical Adulteration Limits for Maple Syrup, Honey, and Pineapple Juice Adulterated with Cane, Beet Sugar, or HFCS Using Two Different Internal Standard Methods

	internal standard	adulterant (δ^{13}C ‰)	mean % adulteration 2-SD method (min to max)	mean % adulteration RL method (min to max)	*t*-test (*p* value)
maple syrup	malic acid	cane sugar (−12.3‰)	8.5 ± 3.7 (2–17)	4.9 ± 2.5 (1–10)	2.5×10^{-14}
		Beet sugar (−26.1‰)	48 ± 20 (1–84)	24 ± 12 (1–48)	7.3×10^{-15}
honey	proteins	HFCS[a] (−10.3‰)	10.8 ± 5.2 (4–33)	7.5 ± 4.5 (1–23)	8.0×10^{-15}
	malic acid	beet sugar (−26.1‰)	8.6 ± 3.9 (1–16)	3.7 ± 2.2 (1–8)	2.4×10^{-9}
		HFCS (−10.3‰)	59 ± 32 (8–100)	26 ± 20 (1–100)	3.1×10^{-8}
pineapple juice	Citric acid	beet sugar (−26.1‰)	7.7 ± 3.5 (1–15)	4.8 ± 2.7 (1–10)	6.1×10^{-8}
		HFCS (−10.3‰)	53 ± 27 (5–100)	33 ± 23 (1–100)	2.2×10^{-7}

[a] HFCS: high fructose corn syrup.

In the regression line method (RL method), a plot of the $\Delta\delta^{13}$C values against the $\delta^{13}C_{\text{malic acid}}$ values from authentic maple syrup samples was drawn (**Figure 4**) and demonstrated a good correlation ($r = 0.704$). The least-squares regression line obtained through the data is expressed by $\Delta\delta^{13}C = -0.70\delta^{13}C_{\text{malic acid}} - 16.01$. A virtual space can be obtained by drawing lines parallel to the regression line that encompasses 95% of all the data, being at a vertical distance corresponding to twice the covariance, s_{yx} (*26*). These lines from which $\Delta\delta^{13}$C limits can be calculated have the form

$$\Delta\delta^{13}C(\text{limits}) = -0.70\delta^{13}C_{\text{malic acid}} - 16.01 \pm 2s_{yx}, \text{ where } s_{yx} = 0.25 \quad (1)$$

The covariance term, s_{yx}, has the same statistical meaning as the standard deviation but was applied to both the *x* and the *y* correlation values. Therefore, as for the 2-SD method, the calculated limits from twice the covariance, $2s_{yx}$, include 95% (the confidence level) of all point based on a normal distribution of both variables.

By this alternative approach (RL method) consisting of deriving parallel lines on both sides of the regression line, we can calculate for a specific maple syrup sample the two values that establish for $\delta^{13}C_{\text{malic acid}}$ the domain of acceptable $\delta^{13}C_{\text{sugars}}$ values for authenticity. These $\delta^{13}C_{\text{sugars}}$ limit values are expressed from **Figure 4** as

$$\delta^{13}C_{\text{sugars}}(\text{limits}) = \delta^{13}C_{\text{malic acid}} + \Delta\delta^{13}C(\text{limits}) \quad (2)$$

For a reasonable security margin to minimize the error of finding added sugar when there is none, only the highest value of $\delta^{13}C_{\text{sugars}}$(limits) was used to calculate the amount of added C_4 sugars as follows:

$$\%\ \text{add} = \frac{100[\delta^{13}C_{\text{sugars}} - \delta^{13}C_{\text{sugars}}(\text{limits})]}{\delta^{13}C_{\text{adulterant}} - \delta^{13}C_{\text{sugars}}} \quad (3)$$

Improved Detection of Addition of a C_4 and C_3 Product in Maple Syrup Using the RL Methods. Adulteration was mathematically simulated for the set of 56 maple syrup samples. The effect of adding exogenous C_4 (beet) and C_3(cane) sugars, respectively, with δ^{13}C values of −12.3 and −26.1‰ were calculated by the following equation:

$$\delta^{13}C_{\text{aldulterated}} = \frac{(\delta^{13}C_{\text{add}}\%\ \text{add}) + [\delta^{13}C_{\text{initial}}(100(100 - \%\ \text{add}))]}{100} \quad (4)$$

where % add is the amount of C_4 and C_3 product added, $\delta^{13}C_{\text{add}}$ is the δ^{13}C value of the C_4 and C_3 product added, and $\delta^{13}C_{\text{initial}}$ is the initial δ^{13}C value of the maple syrup sugars.

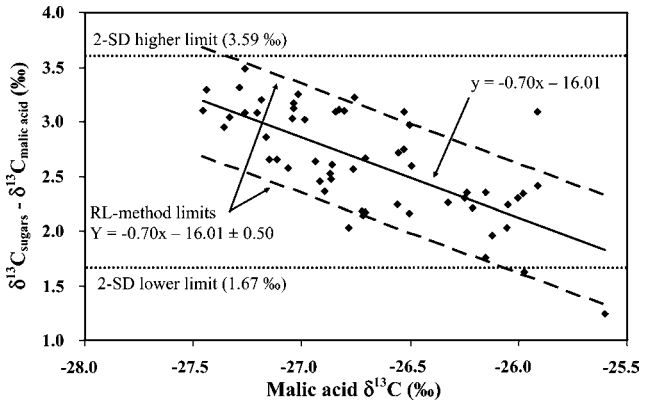

Figure 5. Correlation plot between δ^{13}C of malic acid and the isotopic ratio difference ($\delta^{13}C_{\text{sugars}} - \delta^{13}C_{\text{malic acid}}$) from authentic maple syrup samples. Upper and lower limits for the 2-SD and the RL method at 95% confidence level are drawn on the graph.

To set the expected adulteration detection limits for both methods, the $\delta^{13}C_{\text{alduterated}}$ was increased until its value was outside the domain of acceptable values for authenticity. For the RL method, eqs 1 and 2 were used to calculate each maple syrup limit, and for the 2-SD method, the fixed limit $\Delta\delta^{13}$C values of 1.67 and 3.59‰ (from $2.63 \pm 2 \times 0.48$‰) were used. The mean threshold adulteration detection limits for a C_4 sugar was found to be 4.9 ± 2.5% (range of adulteration from 1 to 10%) calculated from the RL method and 8.5 ± 3.7% (range of adulteration from 2 to 17%) calculated from the 2-SD method. A paired *t*-test ($p = 2.5$ E-14) confirmed that the RL-method gives a lower adulteration detection limit of 3.6 ± 2.7% as compared to the 2-SD method (**Table 1**). The RL-method also gives lower adulteration limits for C_3-sugars adulteration than the 2-SD method, 48 ± 20% with the 2-SD method in comparison with 24 ± 12% with the RL-method (p from paired *t*-test is 7.3×10^{-15}).

In **Figure 4**, two samples may be seen as outliers: sample A, $\delta^{13}C_{\text{sugars}}$ −24.36‰, $\delta^{13}C_{\text{malic acid}}$ − 25.60‰ and sample B, $\delta^{13}C_{\text{sugars}}$ −22.82‰, $\delta^{13}C_{\text{malic acid}}$ −25.91‰. According to the 2-SD method, sample A will be identified as adulterated ($\delta^{13}C_{\text{malic acid}}$ −25.60‰, $\Delta\delta^{13}$C 1.24‰) but will be identified as authentic by the RL method (**Figure 5**). Contrary, sample B will be identified as adulterated by the RL method ($\delta^{13}C_{\text{malic acid}}$ −25.91‰, $\Delta\delta^{13}$C 3.09‰) and authentic by the 2-SD method (**Figure 5**). As all maple syrup samples used in this study were certified as authentic by the producers, this observation brings to light the limitations of both methods (SD and RL). The statistics were performed at a confidence level of 95%, which opens the door to false positive interpretations. Regulatory bodies would use such results as an indicator of adulteration and proceed with additional confirmation analyses before making a final adulteration statement on a specific sample. Adulteration statements should never be based on a single

adulteration detection method as demonstrated by **Figure 5**. Higher confidence levels have been reported for honey adulteration where a 4-SD method was used to achieve a probability of error as low as 1 in 25 000 (*15*). This approach is unusual and seems specific to honey adulteration where for other foodstuffs, a confidence level of 95% is well accepted by the scientific community.

Improvement on Other Food Products Adulteration with the RL Methods. The potential application of this new technique to the adulteration of other food products was evaluated. We have used the isotopic data for honey (50 samples) published by White and Winters (*11*) where the proteins of honey were employed as internal standard and those from Jamin et al. (*17*) for pineapple juices (30 samples) where malic and citric acids were used as internal standards. The simulated adulteration involved the addition of high fructose corn syrup (HFCS $\delta^{13}C = -10.3‰$) to honey and HFCS and beet sugar to pineapple juices. The calculated adulteration limits are reported in **Table 1**. For all cases, the adulteration limit of the RL method was statistically better than the 2-SD method for honey adulteration. In the case of pineapple juices where malic acid was used as internal standard, the 2-SD method was better for one sample for beet sugar adulteration and two samples for HFCS adulteration. When citric acid was used, the 2-SD method was better for two samples either for beet sugar or HFCS adulteration. With these results, the RL technique can be applied to several foodstuffs where the technique of the internal standard is applied to detect adulteration, and most of the time, this technique is better to detect sugar adulteration than the 2-SD method.

C_3- versus C_4-Plants as Adulterant. We also observe that the RL method is especially powerful in the cases where the $\delta^{13}C$ values of the adulterant and the adulterated food are similar. In fact, an improvement of 24 ± 17% with values as high as 58% when beet sugar (C_3-plant, −26.1‰) was used in comparison to an improvement of 3.6 ± 2.7% when cane sugar (C_4-plant, −12.3) was used to simulate adulteration on maple syrup (C_3-plant) was observed (**Table 1**). The same trend was observed for adulteration simulation on pineapple juices (C_4-plant) with malic acid as internal standard where an improvement of 4.9 ± 3.1% was found when beet sugar was added as compared with 33 ± 24% when HFCS (C_4-plant) was added. With citric acid as an internal standard, the improvement for beet sugar detection was 2.8 ± 2.1% in comparison with 20 ± 16% for HFCS detection.

The $\delta^{13}C$ of maple syrup sugars and malic acid has been determined on 56 authentic maple syrup samples from Canada and the United States. The mean $\delta^{13}C$ of maple syrup sugars found is −24.07‰, and the mean $\delta^{13}C$ of malic acid found is −26.71‰, which corresponds to typical C_3-plant stable carbon isotopic ratios. Organic acids were isolated from maple syrup by lead precipitation using an optimized sample preparation procedure, and pure malic acid was separated from the other organic acids by preparative reversed-phase liquid chromatography. Good correlation between sugars and malic acid has been found ($r = 0.34$, $p = 0.0032$), defining malic acid as a suitable internal standard. The technique has shown no isotopic discrimination for sugars or malic acid $\delta^{13}C$ determination.

To improve the actual decision limit of maple syrup adulteration, an improved calculation technique was developed. This technique is based on the correlation existing between malic acid, the internal standard, and the isotopic difference between maple syrup sugars and malic acid ($\Delta\delta^{13}C = \delta^{13}C_{sugars} - \delta_{13}C_{malic\ acid}$). The regression line is used to determine for each individual maple syrup sample the authentic $\delta^{13}C_{sugars}$ range derived from the malic acid $\delta^{13}C$.

To validate this technique, simulated sugar spiking has been performed with cane (C_4-plant, $\delta^{13}C$ −12.3‰) or beet (C_3-plant, $\delta^{13}C = -26.1‰$) on the 56 authentic maple syrup samples, and the theoretical adulteration limit has been evaluated for both the RL and the 2-SD methods. A narrower detection limit of 3.6% is obtained for cane sugar adulteration and 24.6% for beet sugar adulteration with the RL method, which has also been applied to honey and pineapple juice adulteration by using data found in the literature. In those cases, beet sugar and HFCS (C_4-plant, $\delta^{13}C$ −10.3‰) were the target adulterants. In both cases, the RL method provided a narrower detection limit, making it suitable for use in food adulteration. This technique is all the more suitable when the photosynthetic origin of the food product and the adulterant is the same as shown by the results obtained for maple syrup adulteration by beet sugar and for pineapple juice adulteration by HFCS.

SAFETY

Lead nitrate is highly toxic if ingested and/or inhaled, and care must be exercised by handling in a chemical fume hood using adequate personal safety equipment such as impervious gloves, apron, or protective clothing. Lead nitrate should be stored in a cool, well-ventilated area (lower than 30 °C), away from sparks and flames. Waste lead material should be disposed of in accordance with applicable local, state, and federal regulations.

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Supporting Information Available: Stable carbon isotope ratios of sugars and malic acid from authentic maple syrup samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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