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RESEARCH ARTICLE

Determining sugar and molasses origin by non-exchangeable hydrogen stable isotope of ethanol and carbon isotope ratio mass spectrometry

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Abstract

This study explores the differentiation of sugar and molasses produced from sugar beet and cane, which are susceptible to fraudulent labeling due to differing production costs. The research aimed to authenticate these products by botanical origin using novel analytical techniques. Utilizing ethanol isotopic measurement–isotope ratio mass spectrometry (IRMS) for non-exchangeable hydrogen stable isotopes alongside carbon stable isotopes analysis through elemental analyzer–IRMS, the study accurately identified the origin of various sugar and molasses samples, pinpointed mislabeled goods, and determined the source of products with previously unknown provenance. These methods were also effective in revealing sugar and molasses adulteration and quantifying the extent of such fraud. The combined isotope analyses demonstrated their potential as robust tools for combating misrepresentation and adulteration in the sugar industry.

KEYWORDS
botanical origin, carbon isotopes, hydrogen isotopes, isotopic content, molasses

1 | INTRODUCTION

Table sugar, chemically known as sucrose, is a major transitional molecule of photosynthesis and a stable transportable material produced by plants in nature (Nelson et al., 2008). Sugar is one of the most important multifunctional ingredients for the food industry as it provides various functional properties to food like sweetness, aroma, nutritional value, texture, color, caramelization, and preservation (Asadi, 2007; Eggleston, 2018). Similarly, sugar molasses, which are produced as a byproduct during the extraction of sugar, also possess unique properties. It is a syrup composed of fermentable sugar and non-sugar substances and is a good raw material to produce ethanol, yeast, and citric acid. Mainly, sugar molasses is used as an energy supplement for the feed of livestock (Saric et al., 2016; Valli et al.,

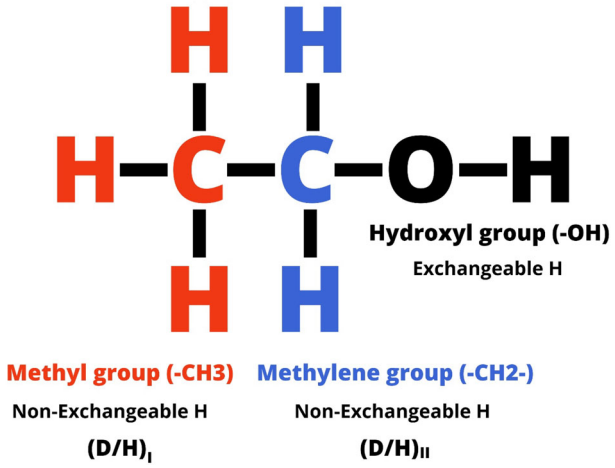

FIGURE 1 Ethanol molecule structure and its hydrogen type.

2012), but a small portion is also utilized as a sweetener in the food industry due to its nutritional and antioxidant properties for human consumption (Chen et al., 2015; Filipčev et al., 2010, 2012, 2016).

The industrial sugars and sugar molasses could be obtained from several plant sources, but at the industrial level, they are mainly obtained from sugar beet (*Beta vulgaris* L.) roots or sugar cane (*Saccharum officinarum* L.) stalks (Asadi, 2007; Eggleston, 2018; Palmonari et al., 2020; Srivastava & Rai, 2012). Sugar beet and sugar cane plants differ in their carbon dioxide (CO_2) fixation mechanisms, being C_3 and C_4 plants, respectively (Bubník et al., 1995; Evans, 2013; Wang et al., 2014). The C_3 plants follow the Calvin–Benson photosynthetic pathway, where three-carbon compound 3-phosphoglycerate acts as an intermediate for CO_2 fixation and integrates less ^{13}C isotope. However, the C_4 plants carry out the Hatch–Slack photosynthetic pathway to fix CO_2 and use the four-carbon compound oxaloacetate, which does fractionate more atmospheric carbon dioxide (Wang et al., 2014). Hence, the rate of carbon isotope fractionation differs between C_3 and C_4 plants.

IRMS (isotope ratio mass spectrometry) is an already known technique used to determine the carbon isotope ratio $\delta^{13}C$ (^{13}C:^{12}C) in C_3 and C_4 plants (Chartrand & Mester, 2019; Kelly, 2003; Muccio & Jackson, 2009), which is estimated around −23‰ to −30‰ in C_3 plants, whereas −9‰ to −16‰ in C_4 plants (Coplen & Shrestha, 2016). However, Birch et al. (2021) reported that in a close proximal range of $\delta^{13}C$ values, it will not be enough to determine the botanical origin by using only IRMS. Therefore, multi-isotopic analysis can be a useful alternative method (Rossmann, 2001). The alternate methods always provide more accurate and reliable results compared to traditional and standard methods. They can be used for cross validation and enhance the robustness and confidence in the findings.

The analysis of the hydrogen stable isotope ratio is another good alternative. Since, in carbohydrates and hydrocarbons, hydrogen exists in two forms: exchangeable hydrogen, which is attached to the oxygen atoms (O–H), and non-exchangeable hydrogen, which is attached to the carbon atoms (C–H) (Figure 1) (Lehmann et al., 2022). The measurement of hydrogen stable isotopes using IRMS in hydrogen gas depends on the exchangeable hydrogens. However, these exchangeable hydrogens are in equilibrium with the surrounding hydrogen, such as ambient water, which leads to changes and perturbations in the original isotopic composition. Thus, the sugar molecules cannot be analyzed by IRMS directly to obtain relevant information that reflects the original biochemical or environmental conditions of the samples (Zhang et al., 2002). Therefore, different methods, such as Site specific Natural Isotopic Fractionation by Nuclear Magnetic Resonance (SNIF-NMR) and Cumulative Screening-quantitative Nuclear Magnetic Resonance (CS-qNMR), have been developed to isolate non-exchangeable hydrogen to achieve a more accurate and precise determination of the isotopes in the sugar samples (Doner et al., 1987; Dunbar & Schmidt, 1984; Ivlev et al., 2019; Katerinopoulou et al., 2020; Lao et al., 2021; Mohammed et al., 2021; Schuler et al., 2022; Wassenaar et al., 2015).

Nuclear magnetic resonance (NMR)-based methods to obtain non-exchangeable hydrogen stable isotope information use different internal standards and require ^{19}F nucleus stabilization for the calculation of signal integration (Ivlev et al., 2019; Kalabin et al., 2018; Smajlović et al., 2023). Even though NMR-based methods can be used to authenticate the botanical and geographical origin and detect adulterations in various products, they are quite expensive in terms of equipment acquisition, method application, and maintenance, consequently giving considerably high analysis costs per sample.

Ethanol isotopic measurement (EIM)–IRMS is another alternative faster and cost-effective method developed by SG Isotech (Republic of Serbia), which is based on the quantitative intramolecular dehydration of ethanol. This method introduced a new $\delta(^2H/^1H)_n$ (δD_n) ethanol value analytical parameter that represents the deviation of the relative ratio of non-exchangeable hydrogen stable isotopes in ethanol $(D/H)_n$ in regard to relative ratio of non-exchangeable hydrogen stable isotopes in the AAWES—Afusali Authentic Wine Ethanol Standard, expressed in parts per 1000 (‰), whose value is traceable to Vienna Standard Mean Ocean Water (VSMOW) (Smajlovic, 2011). The δD_n value is very useful to obtain information about the botanical origin of the samples as the non-exchangeable hydrogen stable isotopes cannot be easily exchanged without enzymatic action, so their isotopic analysis provides significant and accurate information about the hydrological, climatic, or biochemical conditions of the samples (Lehmann et al., 2022). In this method, the ethanol molecule is used as an intermediary to analyze non-exchangeable hydrogen stable isotopes. During alcoholic fermentation, hydrogen and deuterium atoms from sugar (sites 1, 6, and 6′ of glucose) and surrounding water are redistributed to the methyl and methylene groups from ethanol, respectively. In this sense, the relative ratio of hydrogen stable isotopes from the methyl site of ethanol $(D/H)_I$ is informative about the origin of the sugar that was fermented into ethanol. In contrast, the relative proportion of hydrogen-stable isotopes of the methylene site of ethanol $(D/H)_{II}$ is in constant dynamic equilibrium with the surrounding water (water used in alcoholic fermentation) (Martin et al., 2001; Smajlović et al., 2013; Zhang et al., 2002). According to Perini et al. (2020), the $(D/H)_I$ value depends on the sugar source (C_3 or C_4 plant) and provides information about its botanical origin, whereas the $(D/H)_{II}$ values depend on the water used in the fermentation medium. Additionally, the combination of δD_n and $\delta^{13}C$ values of ethanol allowed for the differentiation of various ethanol sources present in wine (Smajlović et al.,

2013). A previously published interlaboratory study by four different laboratories showed the robustness of the EIM–IRMS method in determining the authenticity of wine (Smajlović et al., 2023). The EIM–IRMS showed more precise measurements than CS-qNMR, with a repeatability standard deviation of .17 ppm compared to 1 ppm, making it a more accurate method than its alternatives (SNIF-NMR, CS-qNMR, etc.). SG Isotech (2022) considered this method as a potential authenticity tool for wines, juice concentrates, honey, and other types of foodstuffs. According to Thermo Fisher Scientific (2008), analyzing the isotopes of ethanol is simple, low priced, and reproducible, and it provides very high precision, allowing for the analysis of a high number of samples per day.

Although the chemical composition of both cane and beet sugar is almost identical and completely sucrose based, different studies (Eggleston, 2004; Lu et al., 2017) reported some differences, mainly because of source and production processes. Moreover, according to Sturrock (2008), the production cost of cane sugar is significantly lower than that of beet sugar, which ultimately leads to unfair competition in the markets. This generates a great commercial opportunity for fraud practice, which is a problem for countries that seek to protect their domestic market by just producing beet sugar. Similarly, the nutritional composition of molasses is also different based on its source. Palmonari et al. (2020) used principal component analysis (PCA) applied on multiple analysis methods to differentiate molasses according to their botanical origin. The use of various methods is always time-consuming and costly. Therefore, it would be ideal to have a cost-effective and fast alternative analytical technique that provides all the information regarding the botanical origin and/or adulterations. In this study, we propose the implementation for a more accurate and reliable authenticity method of crystal sugars and molasses according to their botanical origin using δD_n non-exchangeable hydrogen stable isotopes of ethanol by EIM–IRMS in combination with the measurement of the relative ratio of stable isotopes of carbon by EA–IRMS.

2 | METHODOLOGY

2.1 | Samples collection

The 31 sugar samples were collected from different sugar brands in 12 different countries, where 13 samples were labeled as beet sugar, 16 as cane, and 2 samples of unknown botanical origin. Moreover, the mixtures of sugars from beet and cane were also prepared in different proportions of sugar beet: sugar cane (20:80; 40:60, 60:40, and 80:20); for this purpose, AL2205-0034 and AL2205-0037 samples were used. For molasses, two molasses samples were labeled as sugar cane, four as sugar beet, and two samples were unknown (Table 1).

2.2 | Samples preparation

The sugar samples were directly analyzed for $\delta^{13}C$ using the tin capsule sample introduction method (3.3 × 5 mm, IVA Analysentechnik). Each sample of 0.15–0.25 mg, weighed on an analytical balance (ABT 220-5DNM, KERN), was placed in tin capsules and sealed with tongs. For the remaining analyses, including $\delta^{13}C$ of molasses and δD_n of both sample types, a fermentation process was carried out. The 20 g of dry matter (DM) of each sugar sample, weighed using an analytical balance, were transferred to fermentation bottles and diluted with 200 mL of tap water. In case of molasses, they were quantitatively transferred with 200 mL of tap water, and 2 g of *Saccharomyces cerevisiae* var. *bayanus* yeast (LALVIN EC-1118, LALLEMAND) were added to each bottle. The bottles were closed, shaken, and left to ferment at room temperature (≈22°C) for 10 days. After the fermentation period, ethanol extraction was performed using the EIM-PADS (ethanol isotope measurement-preparation automated distillation system) distillation apparatus. The samples were transferred to 500 mL flasks and subjected to the distillation process in the EIM-PADS heating mantles. The ethanol extraction temperature was maintained at 78 ± 0.5°C (ethanol ebullition temperature). This method ensured distillation without isotopic fractionation (Smajlović et al., 2013). The resulting ethanol had an alcoholic strength of approximately 90%–94% (v/v) and recovery rates of over 96% (Table S1).

Furthermore, for δD_n analysis according to the patented protocol developed by Mr. Ivan Smajlović from SG Isotech, for samples containing fermentable sugars and with an increased content of DM over 65 Bx (honey samples, sugar syrups, or solid sugars), it is necessary to prepare a set of internal standard samples in order to determine the correction factor for recalculating the results in the tested samples and neutralizing the contribution from the methylene group of ethanol, on which water has the greatest influence. In parallel with the preparation of the set of tested samples, a set of internal standard samples is also prepared in the same way. An amount of 20 g DM of the internal standard sample is dissolved in 200 mL of water, with the fact that in this case a set of different water matrices with different isotopic profiles is used for preparation. *S. cerevisiae* is added, and alcoholic fermentation is carried out. After the complete fermentation, ethanol is quantitatively extracted from the sample as described earlier. This is necessary to make a subsequent correction for the contribution of the relative ratio of hydrogen stable isotopes on the methylene group of ethanol. By preparing a set of internal standard samples in water matrices with different isotopic profiles and correlating the obtained δD_n values from the extracted ethanol with the δD values of the water matrices that were used to prepare the set of internal standard samples, a linear water curve of the internal standard with the line equation is obtained. The principle of recalculation of the results in the tested samples is based on the correction of the obtained δD_n ethanol value of the tested sample for the correction factor of the internal standard for a certain specific δD value of the water matrix. The reference δD_n value of ethanol of the internal standard is the one that corresponds to the specific δD value of the water in which the ethanol was produced.

The internal standard is also prepared by using the same tap water as for all other samples and tested in the same way as described above. The correction factor is calculated as the difference in the δD_n values of ethanol from the internal standard obtained in tap water and the δD_n value of ethanol from the internal standard that corresponds to the specific δD value of water.

TABLE 1 Sugar and molasses samples information, including code, country, sugar types, and labeled botanical source.

No	Sample code	Sugar type	Labeled botanical source	Country of origin
1	AL2205-0007	White sugar	Sugar beet	Belarus
2	AL2205-0008	White sugar	Sugar beet	Belarus
3	AL2205-0009	White sugar	Unknown sample	Belarus
4	AL2205-0010	White sugar	Sugar beet	Belarus
5	AL2205-0011	White sugar	Sugar beet	Belarus
6	AL2205-0012	White sugar	Sugar beet	Belarus
7	AL2205-0013	Brown sugar	Sugar cane	Colombia
8	AL2205-0014	White sugar	Sugar beet	Germany
9	AL2205-0015	Brown sugar	Sugar cane	Germany
10	AL2205-0016	White sugar	Sugar cane	Italy
11	AL2205-0017	Brown sugar	Sugar cane	Italy
12	AL2205-0018	White sugar	Sugar cane	Pakistan
13	AL2205-0019	White sugar	Sugar cane	Pakistan
14	AL2205-0020	White sugar	Sugar cane	Pakistan
15	AL2205-0021	White sugar	Sugar cane	Pakistan
16	AL2205-0022	White sugar	Sugar cane	Pakistan
17	AL2205-0023	White sugar	Sugar cane	Pakistan
18	AL2205-0024	Brown sugar	Sugar cane	Pakistan
19	AL2205-0025	White sugar	Sugar cane	Pakistan
20	AL2205-0026	Brown sugar	Sugar cane	Philippine
21	AL2205-0027	White sugar	Sugar beet	Poland
22	AL2205-0028	White sugar	Sugar beet	Poland
23	AL2205-0029	Brown sugar	Sugar cane	Portugal
24	AL2205-0030	White sugar	Sugar cane	Portugal
25	AL2205-0031	White sugar	Unknown sample	Romania
26	AL2205-0032	White sugar	Sugar beet	Serbia
27	AL2205-0033	White sugar	Sugar beet	Serbia
28	AL2205-0034	White sugar	Sugar beet	Serbia
29	AL2205-0035	White sugar	Sugar beet	Serbia
30	AL2205-0036	White sugar	Sugar beet	Türkiye
31	AL2205-0037	White sugar	Sugar cane	UAE[a]
32	AL2205-0038	Mixture (20:80)	Beet:cane	Mixture
33	AL2205-0039	Mixture (40:60)	Beet:cane	Mixture
34	AL2205-0040	Mixture (60:40)	Beet:cane	Mixture
35	AL2205-0041	Mixture (80:20)	Beet:cane	Mixture
36	AL2209-0010		Unknown sample	Chile
37	AL2209-0012		Sugar beet	Türkiye
38	AL2209-0013		Sugar beet	Türkiye
39	AL2209-0014		Sugar beet	Türkiye
40	AL2209-0015		Unknown sample	Chile
41	AL2209-0016		Sugar beet	Chile
42	AL2209-0017		Sugar cane	Spain
43	AL2209-0018		Sugar cane	US[b]

[a]United Arab Emirates.
[b]United States.

All other samples from the sequence are corrected for this correction factor and thus placed in the same line, that is, as if they were all made in the same water matrix. In this way, the contribution from the methylene group of ethanol is blocked, and in this case, the differences in δD_n values of ethanol arise only based on differences in the relative ratio of hydrogen stable isotopes from the methyl group of ethanol, which is indicative for determining the botanical origin of ethanol and the sugar raw material from which it was derived (Smajlović et al., 2023).

2.3 | Isotopic measurements

The isotopic measurements of $\delta^{13}C$ and δD_n of non-exchangeable hydrogen stable isotopes were performed at ANA LAB laboratory in Pančevo, Republic of Serbia. $\delta^{13}C$ values were directly measured in sugar samples and from ethanol obtained by distillation of previously fermented molasses samples. $\delta^{13}C$ analysis, by mass spectrometry, is measured in CO_2 produced from excess oxygen combustion of injected sample. For measuring isotopic ratios of carbon ($^{13}C/^{12}C$), the technique of mass spectrometry with a double collector was used. Isotopic ratios were measured by the simultaneous measurement of three ion beams ($^{12}C^{16}O^{16}O^+$, $^{13}C^{16}O^{16}O^+$, and $^{13}C^{16}O^{18}O^+$) and by comparing the sample to a standard (Smajlović et al., 2023). For expressing the relative difference between isotopic ratios of sample and reference gas (standard), $\delta^{13}C$ is used, which is defined as

$$\delta^{13}C\ (‰) = \left[\frac{R_{Sample} - R_{Standard}}{R_{Sample}}\right] \times 10^3$$

where $R_{Standard}$ is the absolute isotopic ratio ($^{13}C/^{12}C$) of an international standard for carbon, and it represents $R = 0.0112372$ (Rossmann, 2001). By international convention, $\delta^{13}C$ is always expressed in relation to the value for the standard of calcium carbonate, known as PDB. This standard is a carbonate obtained from the *Belemnitella americana* fossil. The base of PDB scale is the value $\delta^{13}C = 0‰$ for this standard. $\delta^{13}C$ value indicates if the sample has a greater (+) or lower (–) $^{13}C/^{12}C$ ratio than PDB. The $\delta^{13}C$ values of measured isotopes were calibrated using reference standard Vienna PeeDee Belemnite (VPDB) on a scale defined by two working standards: Sorghum Flour Standard OAS—IVA33802159—CoA 257737 with certified $\delta^{13}C_{VPDB}$ value of −13.68 and uncertainty (± ‰) .19; and Wheat Flour Standard OAS Cat No. IVA33802157 certificate no. 114858, with certified $\delta^{13}C_{VPDB}$ value of −27.21 and uncertainty (± ‰) .13.

The measurement of the $^{13}C/^{12}C$ was performed using a continuous flow technique using a ECS 4010-CHNS-O elemental analyzer (Costech Analytical Technologies, Inc) directly interfaced to Finnigan Delta plus XP isotope ratio mass spectrometer (Thermo Electron Corporation) using an open split.

δD_n values were measured in ethanol obtained by distillation of previously fermented sugar and molasses samples. Continuous flow peripheral EIMPyro (SG Isotech DOO Pancevo), which contains dehydration and pyrolysis reactors and is over ConFlo III interface connected to Finnigan Delta plus XP isotope ratio mass spectrometer (Thermo Electron Corporation), was used for determination of δD_n values in previously extracted ethanol samples (Figure 2). The principle of EIMPyro-IRMS is based on the rapid and quantitative intramolecular dehydration of ethanol sample over custom made EIM catalyst, specifically designed by SG Isotech Company, at temperatures above 250°C and prior to pyrolysis and high precision isotope ratio measurement during a single analysis (SG Isotech, 2022).

The ethanol $\delta(^2H)_n(\delta D_n)$ value is related to the ethanol standard material AAWES also provided by the SG Isotech Company and expressed as deviation of the relative ratio of non-exchangeable deuterium and hydrogen atoms in working ethanol standard $(D/H)_n$ in regard to relative ratio of non-exchangeable deuterium and hydrogen atoms in the AAWES, expressed in parts per 1000 (‰), where AAWES has δD_n value of−211.89‰ in reference to VSMOW and expanded uncertainty (± ‰) 2.5.

2.4 | Determination of $\delta^{13}C$ values

The capsules of blanks, certified standards, and sugar samples were loaded to the automatic sampler of the elemental analyzer peripheral. In the case of molasses, 0.35 μL of ethanol was injected directly into the combustion reactor of the elemental analyzer with a microliter syringe. All the samples were measured twice.

2.5 | Determination of δD_n of non-exchangeable hydrogen stable isotopes values in ethanol

For δD_n analysis, the AAWES standard (Smajlović et al., 2023) was injected eight times at the start and at the end of the analysis. Regarding the samples, 0.2 μL of ethanol of each sample was directly injected six times by using an autosampler system (AI 1310, Thermo Fisher Scientific). Moreover, for the measurement quality control, a control wine ethanol sample with previously determined δD_n value was injected during the sequence to make sure that repeatability and reproducibility of results are achieved. Mean values were reported (Table 2).

2.6 | Data analysis

Different statistical parameters (e.g., mean, standard deviation, range, etc.) were analyzed using Statgraphics Centurion XVI software (Statgraphics Technologies, Inc.). A two-dimensional plot of δD_n and $\delta^{13}C$ values was performed to combine the isotopic data to differentiate the botanical origin of crystal sugars and molasses. In addition, a PCA was performed with both variables ($\delta^{13}C$ and δD_n), where the score plot was evaluated. Variables were previously auto scaled for PCA. To identify if there are significant differences or not in the $\delta^{13}C$ and δD_n values between C_3 and C_4 plants, a *t*-test was performed at 95% confidence. To analyze the normality of the data, the Shapiro–WilK test was applied at an $\alpha = .05$. To evaluate the predictive capacity of both variables, a linear discriminant analysis (LDA) was performed. For this purpose, certain samples were excluded and not used for model calibration (unknown samples and those samples that differed in their

TABLE 2 δD_n value of ethanol and $\delta^{13}C$ value of industrial sugars and molasses from cane (C), sugar beet (B), unknown source, and mixtures (B:C) between them.

Sample code	Botanical source	δD_n value of ethanol (‰ vs. AAWES)	St.dev.	$\delta^{13}C$ value (‰ vs. VPDB)	St.dev.
AL2205-0007	B	−267.19	2.36	−25.68	.19
AL2205-0008	B	−268.19	.69	−26.08	.14
AL2205-0009	unknown	−273.43	.77	−26.23	.26
AL2205-0010	B	-268.16	.84	−25.16	.01
AL2205-0011	B	−272.52	1.19	−25.24	.17
AL2205-0012	B	−271.2	.74	−25.25	.16
AL2205-0013	C	−232.91	1	−11.98	.04
AL2205-0014	B	−268.41	.98	−26.75	.1
AL2205-0015	C	−233.24	1.54	−12.32	.67
AL2205-0016	C	−215.24	.95	−11.28	.05
AL2205-0017	C	−214.72	.7	−11.74	.22
AL2205-0018	C	−210.03	1.09	−12.17	.03
AL2205-0019[a]	C	–	–	−11.42	.01
AL2205-0020	C	−211.23	1.51	−11.97	.01
AL2205-0021	C	−216.55	.58	−12.06	.01
AL2205-0022	C	−207.04	.64	−10.71	1.46
AL2205-0023	C	−207.78	.98	−11.58	.34
AL2205-0024	C	−206.89	.81	−11.65	.01
AL2205-0025	C	−207.06	.62	−12.17	.02
AL2205-0026	C	−217.35	.37	−11.23	.03
AL2205-0027	B	−259.57	1.72	−26.16	.14
AL2205-0028	B	–	–	−25.25	.76
AL2205-0029	C	−197.84	1.24	−11.5	.11
AL2205-0030	C	−207.63	1.11	−11.52	.26
AL2205-0031	Unknown	−258.84	1.21	−25.71	.01
AL2205-003	B	–	–	−25.44	.03
AL2205-0033	B	−261.45	.79	−24.73	1.04
AL2205-0034	B	−260.56	1.17	−25.53	.02
AL2205-0035	B	−266.26	.98	−25.54	.21
AL2205-0036	B	−265.65	.94	−23.58	.89
AL2205-0037	C	−208.3	.64	−11.8	.09
AL2205-0038	B:C	−215.96	.44	−14.16	–
AL2205-0039	B:C	−223.89	.96	−16.51	–
AL2205-0040	B:C	−234.47	1.32	−18.87	–
AL2205-0041	B:C	−246.36	.41	−21.22	–
AL2209-0010	Unknown	−208.42	.97	−19.00	.16
AL2209-0012	B	−242.93	.67	−26.93	.10
AL2209-0013	B	−248.80	.66	−24.48	.16
AL2209-0014	B	−251.05	.98	−26.80	.48
AL2209-0015	Unknown	−225.98	.80	−15.34	.00
AL2209-0016	B	−249.09	.71	−26.92	.00
AL2209-0017	C	−204.00	.41	−13.47	.10
AL2209-0018	C	−198.31	.70	−13.55	.03

Abbreviations: AAWES, Afusali Authentic Wine Ethanol Standard; St.dev, standard deviation; VPDB, Vienna PeeDee Belemnite.
[a]Samples not analyzed for ethanol δD_n value due to the limit of its quantity.

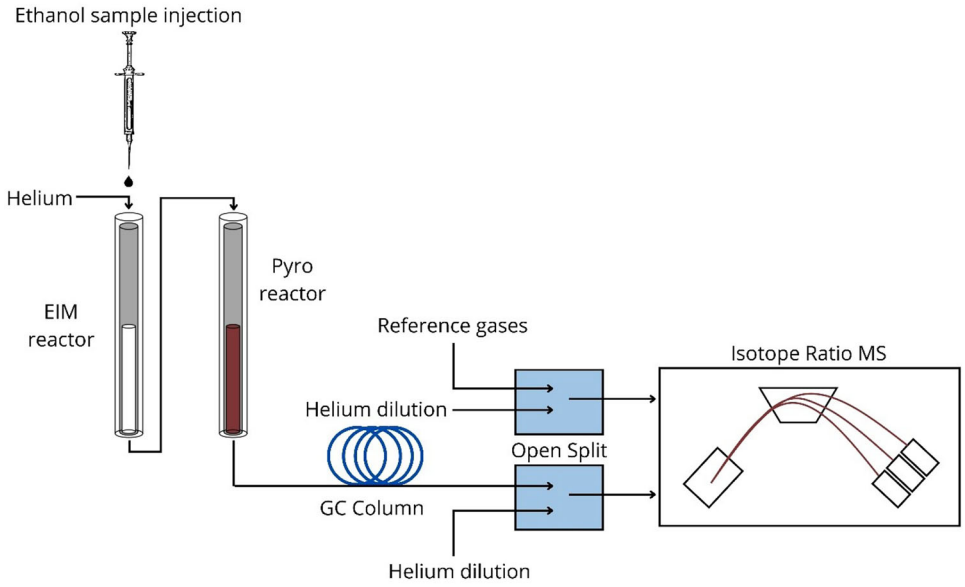

FIGURE 2 Ethanol isotopic measurement–isotope ratio mass spectrometry (EIM–IRMS) method scheme.

TABLE 3 Basic statistics of ethanol δD_n value (‰ vs. Afusali Authentic Wine Ethanol Standard [AAWES]) and $\delta^{13}C$ value (‰ vs. Vienna PeeDee Belemnite [VPDB]) for sugar crystals and molasses samples from sugar cane and sugar beet.

	Ethanol δD_n value (‰ vs. AAWES)		$\delta^{13}C$ value (‰ vs. VPDB)	
	Cane sugar	Beet sugar	Cane sugar	Beet sugar
Mean	−211.54	−261.40	−11.93	−25.66
St.dev	9.79	9.25	.72	.98
Min	−233.24	−272.52	−13.55	−26.93
Max	−197.84	−242.93	−10.71	−23.58

behavior in the PCA score plot). The botanical origin of these samples was predicted using the linear function. In the case of sample mixtures, two simple regression models were evaluated for the beet sugar versus cane proportions (B:C), where the independent variable for one model was the $\delta^{13}C$ value and for the other the ethanol δD_n value. The significance of the variables was evaluated at 95% confidence level. PLS-Toolbox version 9.0 (Eigenvector Research) MATLAB interface and Statgraphics Centurion XVI software (Statgraphics Technologies, Inc) were used for data analysis.

3 | RESULTS

3.1 | Analysis of $\delta^{13}C$ and δD_n of non-exchangeable hydrogen stable isotopes in ethanol values of crystal sugars and molasses

The results of the $\delta^{13}C$ and δD_n values obtained for each sample are shown in Table 2. According to the labeled information, the samples from the beet and cane sugar showed $\delta^{13}C$ values between −26.93‰ to −23.58‰ and −13.55‰ to −10.71‰, respectively (Table 3). Regarding δD_n values, samples labeled as cane sugar were found in the range of −233.24‰ to −197.84‰, and for beet sugar, −272.52‰ to −242.93‰.

3.2 | Combined analysis of $\delta^{13}C$ and δD_n values of ethanol obtained for sugar crystals and fermented molasses samples

A two-dimensional plot of the data is shown in Figure 3. This type of graph allowed us to observe clustering according to the botanical origin as well as the tentative limits for the δD_n values for C_3 and C_4 plants. It was also possible to evaluate the behavior of the unknown samples (AL2205-0009, AL2205-0031, AL2209-0010, and AL2209-0015). The obtained δD_n and $\delta^{13}C$ values of samples AL2205-0009 (δD_n: −273.43‰ and $\delta^{13}C$: −26.23‰) and AL2205-0031 (δD_n: −258.84‰ and $\delta^{13}C$: −25.71‰) showed that these samples belong to sugar beet. The ethanol δD_n and $\delta^{13}C$ values of the sample AL2209-0010 were −208.42‰ and −19.00‰, being closer to the cane group but outside the limits established for such class. As for the unknown

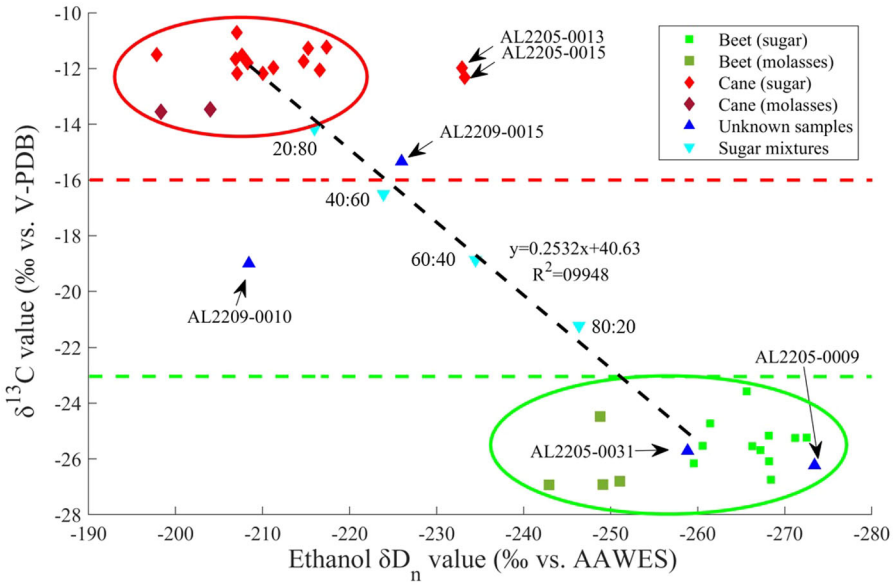

FIGURE 3 Two-dimensional plot of ethanol δD_n and $\delta\,^{13}C$ values of molasses and crystal sugars from sugar cane, sugar beet, and mixtures.

sample, AL2209-0015, $\delta^{13}C$ value (−15.34‰) indicated that it is a C_4 plant; however, its δD_n value is displaced from the other samples of the sugarcane group. At the same time, it is observed that samples AL2205-0013 and AL2205-0015 present more negative δD_n values, moving away from the other samples belonging to sugarcane. As for the mixture of sugars from C_3 and C_4 plants, in Figure 3, a linear relationship ($R^2 = .9984$) was observed between δD_n ethanol value and $\delta^{13}C$ values, indicating a high correlation between these isotopic values and the mixture proportion. The analysis carried out allowed us to distinguish samples according to the source as well as detect mixtures from C_3 and C_4 plants.

The PCA performed by using both variables ($\delta^{13}C$ and ethanol δD_n values) also revealed a separation between samples from different plants, specifically from different photosynthetic pathways (Figure 2). It divided cane and beet sugar samples into two different groups, which were separated by the first principal component (PC1), explained by 95.5% of the variance. The mixture samples were aligned between the groups with respect to their respective proportions. The unknown samples, AL2205-0009 and AL2205-0031, appeared to be beet sugars, whereas the sample AL2209-0010 was observed to be located above the PC1 area in which the cane sugar samples were found. However, it was outside the 95% confidence limit. The samples that were further away from the cane sugar class (AL2205-0013, AL2205-0015, and AL2209-0015 [unknown samples]) (Figure 3) showed similar behavior. The samples AL2205-0013 and AL2205-0015 were positioned separately from the groups as in the biplot analysis. The samples AL2209-0015 and AL2209-0010 were indicated to be a mixture of different botanical origins due to the shifts in the values of both isotopes analyzed.

It was observed in δD_n values that the samples AL2205-0013 and AL2205-0015 demonstrated particularly different behavior; therefore, they were treated as unknown samples. The data followed a normal distribution (p-value > .05) (Table S2). As the data followed a normal distribution (p-value > .05) (Table S2), means were compared using a t-test ($\alpha = .05$) for equal variances. The differences between the means were statistically significant with a confidence level of 95% ($t = 43.07$; p-value = 0). The δD_n values for ethanol also showed significant differences, with a confidence level of 95% ($t = 18.64$; p-value = 0). Thus, both isotope values show significant differences between C_3 and C_4 plants.

3.3 | Linear discriminant analysis (LDA)

In pre-transformation scrutiny, the unknown samples as well as the samples AL2205-0013 and AL2205-0015 that showed a different behavior (Figures 3 and 4) were excluded from the analysis. A discriminant function with a p-value of less than .05 (p-value = .0000) was obtained, making it statistically significant at a confidence level of 95%. The applied standardized discriminant function was $1.02809\delta^{13}C + 0.723945\delta D_n$. Among the 30 samples used to fit the model, 30 (100%) were correctly classified (Table S3). To evaluate and validate the model, the samples previously excluded (AL2205-0009, AL2205-0013, AL2205-0015, AL2205-0031, AL2209-0010, and AL2209-0015) were subjected to the model to classify them according to their botanical origin (Table S4). By building the LDA model with only ethanol δD_n values in both the calibration and validation of the model, 100% of the samples were correctly assigned; however, by using only

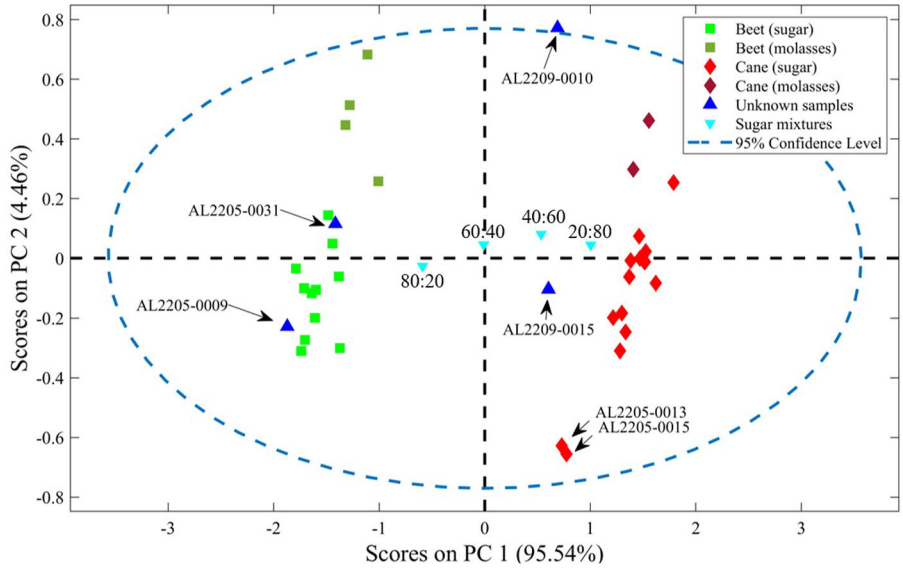

FIGURE 4 Scores plot of principal component analysis performed with ethanol δD_n and $\delta^{13}C$ values of molasses and crystalline sugars from sugar cane, sugar beet, and mixtures.

TABLE 4 Analysis of variance of simple regression using $\delta^{13}C$ value and δD_n value.

Source	Sum of squares	DF	Mean square	*F*-ratio	*p*-Value
For $\delta^{13}C$ value					
Model	6896.83	1	6896.83	267.39	.0001*
Residue	103.17	4	25.79		
Total (Corr.)	7000.0	5			
For δD_n value					
Model	6895.73	1	6895.73	264.55	.0001*
Residue	104.27	4	26.07		
Total (Corr.)	7000.0	5			

* indicates significant differences.

$\delta^{13}C$ values, one validation sample (AL2209-0010) was misclassified (data not shown).

3.4 | Analysis of crystal sugar mixtures through a regression model

The simple regression model to predict the proportion of sugar adulteration with different origins presented an equation as follows: B:C = −84.772–7.4811$\delta^{13}C$, whose R^2 was 98.53%. For the ANOVA, a *p*-value < .05 was obtained, showing a statistically significant relationship between B:C and $\delta^{13}C$ value (Table 4). A standard error of the estimate of 5.08 and a correlation coefficient of −.99 was obtained, indicating a relatively strong relationship (Table 5). Figure 5A shows the plot of the observed versus predicted value of B:C from the $\delta^{13}C$ regression model. On the other hand, for the simple regression model with ethanol δD_n values, the equation obtained was B:C = −389.843–1.89923δD_n. A statistically significant relationship between B:C and ethanol δD_n value was determined (*p*-value < .05) (Table 4). This model presented an R^2 of 98.12%, a standard error of the estimate of 5.11, and a correlation coefficient of −.99, also indicating a relatively strong relationship (Table 5). In Figure 5B, the observed versus predicted value of B:C plot obtained from the simple regression model of δD_n value is observed.

TABLE 5 Coefficients and parameters obtained for simple linear regression using ethanol δD_n value (‰ vs. Afusali Authentic Wine Ethanol Standard [AAWES]) and $\delta^{13}C$ value (‰ vs. Vienna PeeDee Belemnite [VPDB]).

	$\delta^{13}C$ value (‰ vs. VPDB)	Ethanol δD_n value (‰ vs. AAWES)
Correlation coefficient	−.99	−.99
R-squared (%)	98.53	98.51
Standard error of the estimate	5.08	5.11

4 | DISCUSSION

The ranges of $\delta^{13}C$ values for C_3 and C_4 plants reported in this research agree with those previously reported by different authors, with ranges between −23‰ to −30‰ and −9‰ to −16‰, respectively (Coplen & Shrestha, 2016; O'Leary, 1988; Troughton et al., 1974). This is consistent with the fact that C_3 plants have a higher $^{13}C/^{12}C$ fractionation than C_4 plants (Adami et al., 2010). The analysis of C isotope ratios has been extensively studied, reporting significant differences between the $\delta^{13}C$ values of plants with different carbon metabolism (Farquhar et al., 1989; Monson & Rawsthorne, 2000). In contrast, to date, no

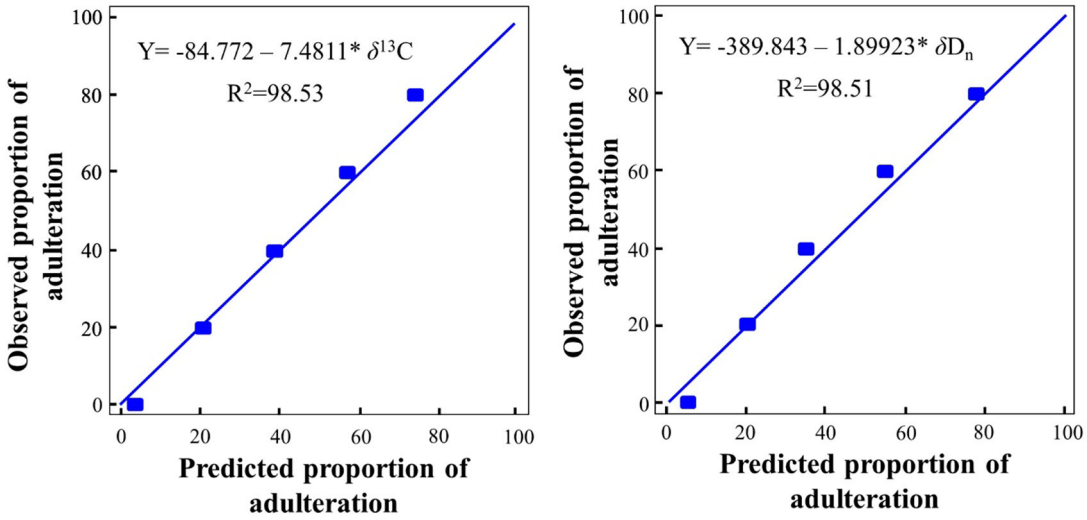

FIGURE 5 Observed versus predicted values of beet sugar versus cane sugar proportion by simple regression model using the $\delta^{13}C$ value (A) and ethanol δD_n value (B).

δD_n value ranges have been reported for C_3 and C_4 plants, being the present study the first report on δD_n versus AAWES value ranges associated with both types of plants from sugar and molasses. It was determined that the values δD_n obtained from sugar beet ethanol samples present more negative values in comparison with those from sugar cane (average ethanol δD_n of beet = −261.40‰ vs. average ethanol δD_n of cane = −212.34‰), which agrees with reports by Smajlović et al. (2013) on the adulteration of wines.

However, the carbon isotope ratio analysis only provides information on whether the sample corresponds to a C_3 or C_4 plant but does not allow us to differentiate between different C_3 and/or C_4 species. For instance, it is possible to determine that a sugar sample comes from a C_3 plant, but it is not possible to determine whether it comes from sugar beet, rice, or another C_3 species (Moore et al., 1995). In this sense, as Rossmann (2001) indicated a multi-isotopic analysis could give more accurate results, so the two isotopes were analyzed together. The results from the two-dimensional analysis and PCA were totally concordant, allowing the separation of the samples into two clusters according to botanical origin as well as observing the behavior of the sugar mixtures between these two clusters. Regarding the samples of unknown origin, both results allowed the identification of their source of origin. Samples AL2205-0031 and AL2205-0009 were found to be sugar beet samples. On the other hand, AL2205-0013 and AL2205-0015 samples showed a different behavior, their $\delta^{13}C$ values indicate that these samples come from a C_4 plant, but their δD_n values were more negative than the other samples coming from sugarcane. Recent analyses developed by the ANA Lab DOO laboratory have reported that corn, C_4 plant, presents more negative δD_n values with values around −230‰ versus AAWES (unpublished results). Schuler et al. (2022) noted significant differences in non-exchangeable hydrogen isotope ratio (δ^2H_{ne} or δD) values between the samples of C_3 and C_4 plants, as well as between C_3 and CAM plants. Furthermore, these variations in δD values correspond to the "natural range" of these both botanical sources. In other words, as indicated by Abrahim et al. (2020), natural variations in δ values of non-exchangeable hydrogen are attributed to varietal, geographical, climatic, and environmental conditions. Thus, these variations are the result of internal physiological differences connected to the location from which the C_3 or C_4 plant originates and its climate and environment conditions in which these plants were grown. So, the analysis of δ values of non-exchangeable hydrogen in sugars allows the study of processes and environmental conditions, which are responsible for the D/H fractionation of carbohydrates. As for the AL2209-0015 sample, a particular behavior was also observed, moving away from the cane cluster, presenting more negative $\delta^{13}C$ and δD_n values, possibly coming not from cane, but from another C_4 plant. However, there is a possibility that this sample is a mixture between C_3 and C_4 plants, as it shows similar behavior to the mixtures analyzed. At last, sample AL2209-0010 showed a totally different behavior in terms of its $\delta^{13}C$ values (−19.00‰), so according to these results it would not come from a C_4 or C_3 plant. There is a third type of plant with crassulacean acid metabolism, mainly known as CAM plants, which temporarily separates their chemical reactions between day and night. This strategy increases water use efficiency by modulating stomatal conductance (O'Leary, 1981). Some studies have reported that CAM plants have $\delta^{13}C$ values between −10‰ and −20‰ (O'Leary, 1988). Griffiths and Smith (1983), Winter (1979), and Holtum and Winter (1999) have reported $\delta^{13}C$ values of −19.8‰, −21.2‰, and −22.6‰ for different species with CAM metabolism. As mentioned before, CAM plants have a temporal separation, so during the day they behave as a C_4 plant, with $\delta^{13}C$ values around 11‰ and at night as a C_3 plant with values around −28‰ (O'Leary, 1988; Winter, 1979).

So far, the analyses of δD_n and $\delta^{13}C$ values were extremely useful to obtain information about the samples, noting that $\delta^{13}C$ allows to clearly determine if the samples correspond to C_3, C_4, or even CAM plants, whereas δD_n values deliver apart extra information that could, for example, give a base to differentiate between species with the same type of metabolism. Therefore, both isotope ratio values would be very useful for developing botanical origin prediction tools, which could be

used as authentication and certification tools at a commercial level. The results obtained with the LDA model with both isotope ratio values agree 100% with the two-dimensional and scores plot (Figures 3 and 2). In the model validation, samples AL2205-0009 and AL2205-0031 were classified as sugar beet and the remaining four samples of unknown origin (AL2205-0013, AL2205-0015, AL2209-0010, and AL2209-0015) as sugar cane. The same results were also obtained when only δD_n values were used in the model, but not when only $\delta^{13}C$ values were used, where sample AL2209-0010 was misclassified. In this sense, to classify and predict the botanical origin of sugar samples (sugar crystals and molasses) using only the $\delta^{13}C$ value is not the most appropriate and can lead to misclassification errors. On the other hand, our results support the approach of Rossmann (2001), where multi-isotopic analysis gives more accurate results. As for the adulterated samples (mixtures between botanical origins), the results show that when simple linear regression models are developed with $\delta^{13}C$ or δD_n values, very similar results are obtained, with practically equal errors, allowing both isotopes to predict the proportion of sugar adulteration.

In this research, it was concluded that the use of non-exchangeable hydrogen stable isotopes of ethanol by EIM–IRMS in combination with the relative ratio of carbon isotopes by EA–IRMS is a potential method to differentiate industrial sugars and molasses according to their botanical origin. The multi-isotopic approach allows for more information about the samples. With the information from both isotopes, it is possible to distinguish between cane and beet sugar as well as to determine the botanical origin of the unknown samples. The use of both isotopes gave better results in terms of LDA models compared to the results obtained when the model was developed only with $\delta^{13}C$ values. It was observed that analyzing only $\delta^{13}C$ values could lead to errors; therefore, a combined analysis with the values of δD_n is suggested. However, when using only the δD_n values in this case, no errors were identified. At the same time, it was determined that the δD_n value of ethanol allows to identify the adulteration ratio of sugar crystals with a standard error of the estimate of 5, the same error obtained when the simple regression model was performed with the $\delta^{13}C$ value. In both cases, high correlations with the adulteration ratio were found. Moreover, it was also observed that the range difference between the δD_n ethanol values of C_3 and C_4 plants was much higher than the $\delta^{13}C$ values. For $\delta^{13}C$ values, the difference between C_3 and C_4 plants was around 13‰, whereas the δD_n ethanol values showed a difference of approximately 30‰, which gives possibility of better differentiation and additional proof of mixed sample from different botanical origin. Therefore, the use of both tools together provides adequate, accurate, and reliable chemical information that can be used to detect mislabeling and counterfeiting in the sugar market and other sugar related markets, such as wine, honey, and among others.

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CONFLICT OF INTEREST STATEMENT

The authors confirm that they have no conflict of interest to declare for this publication.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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