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ORIGINAL ARTICLE

Determination of exogenous water in grape juice through the isotopic analysis of $^{18}O/^{16}O$

Determinação de água exógena em suco de uva através da razão isotópica do $^{18}O/^{16}O$

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Abstract

The consumption of grape juice has been growing significantly, so its quality is becoming an issue of great importance, both for the consumer and for the industry. However, identifying adulteration in juice is a great challenge and requires a reliable analytical process. The isotope ratio ($^{18}O/^{16}O$) is an important tool to determine the addition of exogenous water in beverages, however, there is no official method for juice in Brazil. This study aimed to develop and validate a method for detecting exogenous water in grape juice through isotopic analysis of $^{18}O/^{16}O$. The development and validation of the analytical method were performed using Isotope Ratio Mass Spectrometry (IRMS). The effect of temperature and evaporation of $\delta^{18}O$ in experimental juices was evaluated, and reference values were found for juices based on the $\delta\ ^{18}O$ of musts. The influence of the juice industrial production process on ^{18}O values was verified, and commercial juices were evaluated in relation to the values of reference regarding the addition of water. The temperature and evaporation parameters did not influence the results of the ^{18}O of the juice, as they presented differences lower than the method uncertainty. The heat exchanger system did not influence the proposed method. The reference values for juice can come from the musts, without affecting the interpretation of the final results. Of the thirty real juices analyzed, nine had exogenous water, three proved to be reconstituted juices and eighteen were considered to have no exogenous water. The method proposed and validated in this study presented values for the limit of detection (LOD) of 0.24‰, the limit of quantification (LOQ) of 0.97‰ and measurement uncertainty of 0.71‰, proving to be effective for the detection of exogenous water in grape juice, through of the analysis of the isotopic ratio of $^{18}O/^{16}O$ by IRMS.

Keywords: Isotopes; IRMS; Adulteration; Method validation; Musts; Biotechnology.

Resumo

O consumo de suco de uva vem crescendo significativamente, tornando sua qualidade uma questão de grande importância, tanto para o consumidor quanto para a indústria. Assim, identificar a adulteração em suco é um grande desafio e requer um processo analítico confiável. A razão isotópica ($^{18}O/^{16}O$) é uma importante ferramenta para determinar a adição de água exógena em bebidas, porém não existe um método oficial para suco, no Brasil. O objetivo deste trabalho foi desenvolver e validar um método para detecção de água exógena em suco de uva através da razão isotópica do $^{18}O/^{16}O$. A validação e o desenvolvimento do método analítico foram realizados através de espectrometria de massas de razão isotópica (IRMS). Foi avaliado o efeito da temperatura e da evaporação de $\delta^{18}O$ em sucos experimentais, sendo encontrados valores de referência para sucos baseado no $\delta^{18}O$ de mostos. Também foi verificada a influência do processo industrial de fabricação de sucos nos valores de $\delta^{18}O$ e avaliados sucos comerciais em relação aos valores de referência quanto à adição de água. Os parâmetros de temperatura e evaporação não influenciaram os resultados do $\delta^{18}O$ do suco, pois apresentaram diferenças inferiores à incerteza do método. O sistema trocador de calor não exerceu influência sobre o método proposto. Os valores de referência para suco podem ser provenientes dos mostos, sem prejudicar a interpretação dos resultados finais. Dos trinta sucos comerciais analisados, nove apresentaram água exógena, três demonstraram ser sucos reconstituídos e 18 foram considerados com ausência de água exógena. O método proposto e validado neste estudo apresentou valores para o limite de detecção (LD) de 0,24‰, limite de quantificação (LQ) de 0,97‰ e incerteza de medição de 0,71‰, mostrando-se eficaz para a detecção de água exógena em suco de uva, por meio da análise da razão isotópica do $^{18}O/^{16}O$ por IRMS.

Palavras-chave: Isótopos; IRMS; Adulteração; Validação de método; Mostos; Biotecnologia.

Highlights

- This proposed and validated method can be used to detect exogenous water in grape juice.
- The isotopic reference values for juice may come from the musts.
- Of the thirty Brazilian commercial juices analyzed, nine had exogenous water in their composition.

1 Introduction

Fruit juices contain many nutrients such as minerals, vitamins (especially vitamin C), antioxidants, carotenoids, phytochemicals and dietary fiber, which are essential for human health (Abdel-Rahman et al., 2019). Grape juice is an unfermented drink, obtained from grape must which has undergone authorised practices and treatments (International Organisation of Vine and Wine, 2021).

The growing search for healthy habits leads to an increase in the intake of foods and beverages that contain beneficial compounds to health. World consumption of fruit juice reached a volume of 44.12 billion liters, in 2020, and by 2024, it is expected to reach a volume of 50.6 billion liters (Research and Markets, 2021). Brazil is the third largest non-alcoholic beverage market in the world and the state of Rio Grande do Sul (RS) is responsible for 90% of the production of grape juices in the country, where more than 15 thousand families work in planting and harvesting the grape, used in the production of juices (Associação Brasileira das Indústrias de Refrigerantes e de Bebidas não Alcoólicas, 2020). The consumption of grape juice in Brazil increased approximately 10% between 2018 and 2019, from 1.33 L/inhabitant to 1.46 L/inhabitant in 2019 (Mello & Machado, 2020).

In addition to RS, other Brazilian regions stand out in the production of quality grape juice. The sub-region of São Francisco Valley (SFV) presents a favorable climate for the adaptation of the vines, with high exposure to the sun, absence of low temperatures and irrigation of the São Francisco River, which allows for the harvesting of grapes throughout the entire year as well as the production of juices recognized for their high antioxidant activity (Santos, 2019; Padilha et al., 2017). The state of São Paulo (SP) has also been standing out, with the production of 10.4% of the grape in Brazil, behind Rio Grande do Sul (45.3%) and Pernambuco (29.6%). However, there are projects under development for the production of organic juices and wines in this region, that is, in the state of São Paulo (Tivelli, 2021).

The popularity of fruit juices has been increasing at the same rate as attempts at adulteration, especially to natural juice. Due to their highly appreciated sensory characteristics, whole juices have more value in the market and are more susceptible to fraud (Palade & Popa, 2018; Włodarska et al., 2018). The most common adulteration methods include tap water addition, sugars, extraneous matter like acids, prohibited dyes and preservatives or even using cheaper fruit juices, or yet, adding juices from other fruits and diluting with water. In many cases, the addition of water has the objective of increasing the juice yield, thus aiming at a profit (Calle et al., 2022; Banti, 2020). The increase in cases of fruit juice fraud hinders the development of this industry. Juice authenticity represents an important quality and food safety parameter and, furthermore, adulteration in juices certainly has negative effects on juice quality (Xu et al., 2021).

The control of the origin of the raw materials used in the production of food and beverages is recommended in order to guarantee their quality and authenticity. Many techniques have been applied in fruit juices authenticity assessment, such as Isotope Ratio Mass Spectrometry (IRMS), Nuclear Magnetic Resonance (NMR) (International Organisation of Vine and Wine, 2021), Ethanol Isotope Measurement - Isotope Ratio Mass Spectrometry (EIM-IRMS) (Smajlovic et al., 2019).

Isotopic analyzes have been proposed by the International Organisation of Vine and Wine to detect the addition of water (International Organisation of Vine and Wine, 1996) and sugar (International Organisation of Vine and Wine, 2001) to wines and derivatives. In Brazil, isotopic analysis in grape juices has been used since 2007 to detect adulteration with sugarcane added to the juice (Brasil, 2007). However, there is no official methodology for detecting exogenous water in grape juice in Brazil, which represents a demand from the sector in order to improve the quality of juices and avoid possible adulterations. The presence of water in the juice does not constitute health problems, however, if it is not declared, it is characterized as a fraud to the consumer. Therefore, this study aimed to validate and apply a methodology for detecting exogenous water in grape juice, through the analysis of isotopic ratio $^{18}O/^{16}O$, aiming to detect the tap water addition in grape juice.

2 Material and methods

2.1 Standards and reagents

The VSMOW (Vienna Standard Mean Ocean Water) (NIST, IAEA - 8535a) and GISP (Greenland Ice Sheet Project) (NIST, IAEA – 8536) standards were used to validate the method. Sorbic acid (Merck®) was used as a preservative. The gases used in the IRMS were a mixture of 0.3% CO_2 in Helium 6.0 (Air Liquide), CO_2 4.8 (Air Liquide) as a reference gas and Helium 6.0 (Air Liquide) as carrier gas.

2.2 Samples

2.2.1 Samples for experimental juices

Grapes from the cultivars Ives, Isabella and White Niagara were selected, in the region of Serra Gaúcha (RS), of the 2017 harvest. Each variety was worked in triplicate, totaling twelve samples in the experiment.

2.2.2 Samples to assess the influence of the process of elaboration of industrial juices on isotopic values

Five musts and five juices of the Isabella variety were compared. For this purpose, 1.200 kg of Isabella variety grapes were processed in a juice industry in São Marcos/RS (Brazil). The must that entered the heat exchanger resulted in the final juice.

2.2.3 Samples for isotopic reference values

The main grapes used in the production of grape juices in the industry were selected. Grapes from many regions of the state of RS (Brazil) concerning the varieties Ives, Concord, Isabella, White Niagara, Rose Niagara, hybrid varieties like Isabel Precoce, BRS Cora, BRS Violeta and BRS Lorena, from the 2017 harvest were collected, totaling 177 grape samples (159 red grapes and 18 white grapes).

2.2.4 Samples of market juices

Thirty whole grape juices were collected in supermarkets in Caxias do Sul/RS (Brazil), 27 whole red grape juices and three whole white grape juices.

2.3 Method validation

The method validation was performed according to the requirements established by the ABNT NBR ISO/IEC 17025:2017 standard of Instituto Nacional de Metrologia Qualidade e Tecnologia (2020) (INMETRO).

2.3.1 Selectivity

The selectivity was assessed by determining $\delta^{18}O$ in whole red grape juice and water (VSMOW standard), performing nine repetitions for each sample. The analysis of variance was performed by Analysis of Variance (ANOVA) at a 5% significance level, followed by F-test as a decision criterion.

2.3.2 Linearity

The linearity was established by adding water to whole red grape juice at increasing concentrations of 0; 0.5; 1; 5; 10; 20; 30; 50; 75; 90; 99.5 and 100%. The triplicate of each concentration was analyzed nine times, obtaining 27 results for each point. Afterward, the analytical curve was constructed, and the evaluation criterion of the decision used was the coefficient of determination (R^2). The calculation of the residuals of each curve was performed through the difference between the observed average value and the real value of each concentration.

2.3.3 Limit of detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were calculated using the results of $\delta^{18}O$ analysis of the juice referring to the lowest concentration of added water (0.5%) in the linearity test. To calculate LOD, 27 replicates were analyzed, considering 26 degrees of freedom and a Student's t-distribution of 2,479. The LOQ was calculated using the sample blank with the lowest concentration of the analyte and 27 replicates were analyzed.

2.3.4 Tendency

The tendency was evaluated by calculating six repetitions of the GISP standard, which has a known value of -24.78‰, instead VSMOW standard, whose value is zero. The observed value was divided by the expected value and multiplied by 100%. The tendency was evaluated using the Association of Official Analytical Chemists (AOAC) criteria (Association of Official Analytical Chemists, 2016).

2.3.5 Recovery

The recovery was calculated by analyzing the $\delta^{18}O$ of different concentrations of water added to the grape juice. The analyzed concentrations were 20; 30; 50; 75; 90 and 99.5%, in triplicate, and the acceptance criteria for the recovery of the Association of Official Analytical Chemists (2016) were used.

2.3.6 Accuracy

The accuracy was assessed by determining the $\delta^{18}O$ of 27 repetitions of the GISP standard. The mean values and standard deviation of the tests were compared with the real value of the standard (-24.78‰) and the z-score was calculated.

2.3.7 Repeatability

To determine repeatability, 27 repetitions were performed in the same juice, on the same day, with the same analyst and equipment, and under the same conditions. The result was expressed by the coefficient of variation (relative standard deviation), and the acceptance criteria was based on the Association of Official Analytical Chemists (2016).

2.3.8 Reproducibility

The reproducibility was performed by two analysts considering 14 repetitions of the same juice. The result was expressed by the limit of reproducibility (Albano & Raya-Rodriguez, 2015).

2.3.9 Intermediate precision

The intermediate precision was calculated by variation of the $\delta^{18}O$ reading of two different matrices, water (VSMOW standard) and grape juice. The result was calculated according to INMETRO (Instituto Nacional de Metrologia Qualidade e Tecnologia, 2020).

2.3.10 Robustness

For the robustness evaluation, a temperature variation test was performed on the Delta V automatic sampler block, at temperatures of 24°C, 25°C, 26°C and 27°C, to verify whether this parameter interferes with the analysis results.

2.3.11 Measurement uncertainty

The measurement uncertainty of the method was calculated according to document DOQ-CGCRE-008 of INMETRO (Instituto Nacional de Metrologia Qualidade e Tecnologia, 2020), considering the data distribution according to the guide for measurement uncertainty RM 68 of the Rede Metrológica do Rio Grande do Sul (2013).

2.4 Experiment

2.4.1 Evaluation of the effect of temperature and evaporation on the juice elaboration process

To evaluate the influence of evaporation, five samples of grape must were selected of the varieties Isabella and White Niagara. These grapes represent the largest production in the state of RS and are highly representative in the production of grape juice.

The grapes were destemmed and crushed manually and then passed through a pneumatic press to collect 3.2 L of must from each grape sample, in a 5 L Erlenmeyer. Then, 3.2 g of sorbic acid were added to the must to avoid fermentation and collected in 12 mL flasks, triplicate aliquots of each sample for analysis of $\delta^{18}O$ without heating (“zero point”). 200 mL of the must was measured in a graduated cylinder and transferred to 1000 mL amber flasks with a lid. In another 1000 mL amber flask, containing 200 mL of must, a thermometer was attached. Then, the samples were heated in a water bath, at temperatures of 50°C, 80°C, 90°C and 95°C, for 15 and 30 min.

The effect of evaporation on the value of $\delta^{18}O$ was evaluated by leaving the lid of one of the test flasks open. After cooling at room temperature, all samples of the experiment were transferred to 300 mL amber flasks previously identified and, subsequently, the analysis of the isotopic oxygen ratio ($^{18}O/^{16}O$) in triplicate was performed.

2.4.2 Evaluation of the influence of the process of elaboration of industrial juices in the isotopic values

Samples of initial and final musts of Isabella grape juices were collected in industry. The Isabella variety was chosen because it is the grape with the highest production in the state of RS and, consequently, with the greatest representation in commercial juices. For this purpose, 12 mL of must and 12 mL of juice were collected from the same grape must Isabella. Collections were carried out in triplicate, at the beginning (must) and at the end (juice) of the process, on different days, during the harvest of this variety, and in addition, on days when the industry was processing only Isabella grape.

The first collection (must) was carried out after the grape went through the destemmer. Then, the must was passed through the heat exchanger, where it was heated to a temperature of 85°C for 10 to 12 s and then cooled to 45°C. Then, it went through a pneumatic press, where it was centrifuged at 3000 rpm and, later, pasteurized at 85°C, finishing the elaboration process and collecting the second sample (juice) for the comparison of $\delta^{18}O$.

2.5 Determination of the isotopic oxygen ratio by IRMS

The oxygen isotope ratio of the juices was performed using a Delta V Advantage isotope ratio mass spectrometer coupled to the GasBench II gas chromatograph (ThermoFinnigan®, Bremen - Germany). In addition, 500 µl of the musts and juices samples were pipetted in a 12 mL flask, hermetically sealed with a plastic cap and a new teflon-silicone septum. The vials were stored in the GasBench II automatic sampler. A 0.3% of CO_2 mixture in He 6.0 was automatically inserted into the flasks through a syringe in the *flush* and *fill* system. The sample remained in equilibrium at a controlled temperature (25ºC ± 0.5ºC) for 24 h. After the complete gas exchange of the CO_2 of gas with the CO_2 of the water molecules, the $\delta^{18}O$ of the top equilibrium gas (*headspace*) was analyzed, which reflected the $\delta^{18}O$ of the sample. The values of $^{18}O/^{16}O$ were expressed on the $\delta^{18}O$‰ scale and were obtained comparatively in relation to the international VSMOW2 standard (International Organisation of Vine and Wine, 2009; International Union of Pure and Applied Chemistry, 2002).

2.6. Interpretation of the results for market juices

For the interpretation of exogenous water, the $\delta^{18}O$ results for market juices were evaluated against to the reference values obtained from musts. If the $\delta^{18}O$ value found for market juice was more negative when compared to the reference value, the results were considered juice with added tap water. For juices that are in the measurement uncertainty range the result was considered authentic.

2.7 Statistical analyses

The results were analyzed using means and standard deviations, ANOVA and Tukey's multiple comparison test at the 5% significance level. For the tests with the open caps of item *2.3.1*, the paired t-test was performed. The influence of juice elaboration process was evaluated by the coefficient of determination (R^2) and linear regression. All statistical analyses were conducted using the statistical software SPSS 20.0 for Windows.

To calculate the isotopic reference values, the weighted average is used. To obtain the weighted average, the isotopic value of each grape sample is multiplied by the total grape production (kg), declared by each producer, in the Sistema de Declarações Vinícolas (SISDEVIN) (Sistema de Declarações Vinícolas, 2019) related to the previous year's harvest. Then, the value found is divided by the sum of the total kg and the weighted value is obtained. Thus, the weighting effect is to balance the impact of the volume of production according to the grape samples. The final reference value was corrected according to the equation obtained from the industrial test that evaluated the influence of juice elaboration process.

3 Results and discussion

3.1 Method validation

The method was selective for both whole red grape juice and water (VSMOW standard). The selectivity was proven because the Fcalculate (0.35) was lower than the Ftabulated (3.44) ($p = 0.57$). Therefore, the matrix does not interfere in the determination method. The linearity data exhibited linear results in the working range of the studied concentrations, with a coefficient of determination (R^2) of 0.99, showing adjustment of the correlations in the linear regression (Chein, 2019). The residuals profile demonstrated randomly, which means that there are no systematic errors and tendency in its variability over the different points.

The LOD and LOQ limits found were 0.24‰ and 0.97‰, respectively. The recovery of the method proved to be adequate, obtaining an average recovery of 100% (satisfactory range between 98% and 102%) (Association of Official Analytical Chemists, 2016), as well as a 99.32% of tendency, being in accordance with the parameters proposed by Association of Official Analytical Chemists (2016). Repeatability was satisfactory with coefficient of variation (CV) of 0.48%, being lower than that proposed by the Association of Official Analytical Chemists (2016) which, for the 100% concentration of the analyte, is 1.30%. Likewise, the reproducibility was assessed with the limit of reproducibility, where the result obtained was 0.29‰, also considered satisfactory (Albano & Raya-Rodriguez, 2015).

The accuracy results were considered satisfactory and were within the accepted limits for the validation of methods. The results obtained for the robustness test showed that the temperature used in the automatic sampler that least varied was 25°C, which is the temperature used by the $\delta^{18}O$ determination method by IRMS, demonstrating to be the ideal temperature for analysis. The result found for the measurement uncertainty was 0.71‰, being characterized as adequate when compared to other methods, such as, for example, the official method for determining exogenous water in wines in Brazil, which is 0.55‰ (Brasil, 2009).

3.2 Effect of temperature on isotopic fractionation in experimental juices

There is an increase in temperature in the process of making grape juice. Therefore, in this test, it was evaluated whether this temperature influences the isotopic values of $\delta^{18}O$. This aspect is important because the isotopic reference values were made in musts that did not undergo the heating process and to certify the validity of these data, it must be ensured that the values of $\delta^{18}O$ are not influenced by the juice elaboration process.

The average values of $\delta^{18}O$ of the musts of white and red grapes, heated for 15 and 30 min at different temperatures are shown in Table 1. Some results presented statistically significant differences; however, it cannot be inferred that there was an increase or decrease in isotopic values as a function of temperature, regardless of the exposure time, demonstrating that the variation in $\delta^{18}O$ results was random. Thus, it can be inferred that the variations that occurred may be a consequence of the determination method itself since there is less variation than the calculated measurement uncertainty.

Table 1. Averages of $\delta^{18}O$ of samples exposed to different temperatures for 15 and 30 min, expressed in %.

Varieties	Samples	Exposure temperatures (°C) for 15 min				
		0	50	80	90	95
Ives	1	$3.25^{a} \pm 0.10$	$3.04^{b} \pm 0.10$	$3.39^{c} \pm 0.10$	$3.21^{a} \pm 0.10$	$3.35^{c} \pm 0.16$
	2	$1.50^{a} \pm 0.11$	$1.57^{b} \pm 0.09$	$1.58^{b} \pm 0.06$	$1.57^{b} \pm 0.07$	$1.56^{ab} \pm 0.06$
	3	$0.19^{a} \pm 0.12$	$0.43^{b} \pm 0.10$	$0.32^{c} \pm 0.11$	$0.59^{d} \pm 0.09$	$0.43^{b} \pm 0.12$
Concord	4	$-0.08^{a} \pm 0.13$	$-0.03^{a} \pm 0.11$	$-0.54^{c} \pm 0.07$	$-0.19^{ab} \pm 0.06$	$0.05^{d} \pm 0.06$
	5	$-0.08^{a} \pm 0.13$	$-0.11^{a} \pm 0.09$	$0.10^{b} \pm 0.07$	$0.38^{c} \pm 0.08$	$0.62^{d} \pm 0.07$
	6	$1.32^{ac} \pm 0.09$	$0.88^{b} \pm 0.05$	$1.17^{d} \pm 0.07$	$1.26^{c} \pm 0.10$	$1.38^{a} \pm 0.09$
Isabella	7	$-1.13^{a} \pm 0.11$	$-0.93^{b} \pm 0.17$	$-1.16^{a} \pm 0.07$	$-1.15^{a} \pm 0.08$	$-1.01^{b} \pm 0.07$
	8	$-1.63^{a} \pm 0.08$	$-1.08^{b} \pm 0.07$	$-1.43^{c} \pm 0.15$	$-1.49^{c} \pm 0.08$	$-1.07^{b} \pm 0.10$
	9	$-1.62^{a} \pm 0.07$	$-1.14^{b} \pm 0.09$	$-1.10^{b} \pm 0.11$	$-1.38^{c} \pm 0.11$	$-1.13^{b} \pm 0.08$
White Niagara	10	$-0.39^{a} \pm 0.05$	$-0.41^{a} \pm 0.05$	$-0.10^{b} \pm 0.07$	$-0.15^{bc} \pm 0.07$	$-0.21^{c} \pm 0.07$
	11	$-0.45^{a} \pm 0.13$	$-0.49^{ac} \pm 0.06$	$-0.47^{ac} \pm 0.09$	$-0.65^{b} \pm 0.09$	$-0.56^{bc} \pm 0.09$
	12	$-1.22^{a} \pm 0.09$	$-1.20^{a} \pm 0.08$	$-0.97^{b} \pm 0.06$	$-0.95^{b} \pm 0.07$	$-1.17^{a} \pm 0.05$
		Exposure temperatures (°C) for 30 min				
Ives	1	$3.25^{ac} \pm 0.10$	$3.18^{a} \pm 0.07$	$3.07^{b} \pm 0.10$	$3.29^{ac} \pm 0.09$	$3.36^{ac} \pm 0.08$
	2	$1.50^{ac} \pm 0.11$	$1.68^{b} \pm 0.08$	$1.58^{ac} \pm 0.06$	$1.57^{ac} \pm 0.08$	$1.56^{ac} \pm 0.09$
	3	$0.19^{a} \pm 0.12$	$0.05^{b} \pm 0.14$	$0.12^{ac} \pm 0.12$	$0.12^{ac} \pm 0.08$	$0.08^{abc} \pm 0.09$
Concord	4	$-0.08^{a} \pm 0.13$	$-0.60^{c} \pm 0.09$	$-0.18^{b} \pm 0.05$	$-0.18^{b} \pm 0.09$	$0.05^{d} \pm 0.06$
	5	$-0.08^{a} \pm 0.13$	$-0.07^{a} \pm 0.08$	$0.00^{a} \pm 0.09$	$0.81^{b} \pm 0.07$	$0.75^{b} \pm 0.09$
	6	$1.32^{a} \pm 0.09$	$0.99^{b} \pm 0.05$	$1.33^{a} \pm 0.05$	$1.30^{a} \pm 0.07$	$1.06^{b} \pm 0.12$
Isabella	7	$-1.13^{a} \pm 0.11$	$-1.25^{b} \pm 0.13$	$-1.12^{a} \pm 0.10$	$-1.07^{ac} \pm 0.06$	$-1.02^{ac} \pm 0.06$
	8	$-1.63^{a} \pm 0.08$	$-1.29^{b} \pm 0.10$	$-1.30^{b} \pm 0.12$	$-1.89^{d} \pm 0.26$	$-0.99^{c} \pm 0.12$
	9	$-1.62^{a} \pm 0.07$	$-1.22^{b} \pm 0.10$	$-1.03^{c} \pm 0.10$	$-1.05^{c} \pm 0.11$	$-1.16^{b} \pm 0.10$
White Niagara	10	$-0.39^{a} \pm 0.05$	$-0.39^{a} \pm 0.06$	$-0.34^{ab} \pm 0.13$	$-0.30^{b} \pm 0.06$	$-0.09^{c} \pm 0.07$
	11	$-0.45^{a} \pm 0.13$	$-0.29^{b} \pm 0.10$	$-0.32^{b} \pm 0.08$	$-0.42^{a} \pm 0.09$	$-0.45^{a} \pm 0.09$
	12	$-1.22^{a} \pm 0.09$	$-1.23^{a} \pm 0.07$	$-1.32^{b} \pm 0.03$	$-1.00^{c} \pm 0.06$	$-0.97^{c} \pm 0.04$

Means followed by equal letters in the lines do not differ significantly by ANOVA complemented by Tukey's multiple comparison test; significance level of 5% ($p < 0.05$).

3.3 Effect of evaporation on the value of $\delta^{18}O$ in experimental juices

The results of the effect of evaporation on the values of $\delta^{18}O$ in experimental juices are presented in Table 1. A statistically significant difference was observed between the initial point and the final point of heating of samples exposed to different temperatures for 15 and 30 min. The average difference calculated between the initial point and the final point of heating at the time of 15 min was 0.18‰ and at the time of 30 min was -0.19‰. Although it presents these differences, this is lower than the uncertainty of the method (0.71‰).

Isotopic fractionation processes occur naturally during the cycle of vapor formation, vapor condensation and moisture transport. According to some authors, an isotopic fractionation can occur due to heating, and the extent of this fractionation depends on factors such as relative humidity, wind speed, pressure, precipitation and temperature (Balagizi & Liotta, 2019; Rahul et al., 2018). In this test it was possible to observe that the isotopic results, after heating the juices with both the open lids and the closed lids, did not present statistically significant differences, demonstrating that the heating that occurs during the process of making juices did not interfere with isotopic results and, consequently, did not influence the method of analysis.

Isotopic enrichment occurs naturally during the development of the vine so the levels of ^{18}O in grape water are significantly higher than in groundwater or irrigation, which are absorbed by the plant's root system. This enrichment is generally linked to the loss of ^{16}O (lighter) isotopes during leaf evapotranspiration, with consequent enrichment of grape water in ^{18}O (heavier) isotopes (Cueni et al., 2021). Other authors claim that fruit grown in hot and dry climates has greater isotopic enrichment than fruit grown in colder and more humid areas (Simpkins et al., 1999).

The present study demonstrated, however, that the effect of evaporation is insignificant in the temperatures applied in the process of making juices. In this sense, in the method of preparing the juice by the heat exchanger, there is no contact with atmospheric air, the fluids in the system are in close contact with each other, but are prevented from being mixed by a physical barrier, being the heat exchanger a closed system (Rašković et al., 2010).

Furthermore, it was observed that both in the evaluation of the influence of temperature (item 3.2) and of evaporation, the isotopic values showed differences lower than the method's uncertainty. In this way, it can be stated that the reference values for juice may come from the musts, without prejudice to the interpretation of the results in the final juice. Several studies (Dordevic et al., 2013; Magdas et al., 2012a; Perini et al., 2015; Thomas et al., 2013; Preti, 2019; Dasenaki & Thomaidis, 2019; Lei et al., 2022) have shown that the analysis of the isotopic ratio of $^{18}O/^{16}O$ is a good tool to characterize the origin and detect adulterations as well as illegal water addition in beverages. Therefore, the methodology proposed in the present study can be applied to detect the addition of exogenous water in grape juices elaborated by the heat exchanger system.

3.4 $\delta^{18}O$ of musts and industrial juices

The analysis of the isotopic ratio of samples collected in the industry, at the beginning and at the end of the juice elaboration process, showed values that ranged from -2.92‰ to -1.60‰ for initial musts and -2.49‰ to -1.41‰ for final juices, these values are negative because represents the influence of the climatic conditions in the harvest. From these data, the linear regression was calculated and the linear equation ($y = ax + b$) and the coefficient of determination (R^2) were found. The linear equation established the relation between the initial must and the final juice and was $\delta^{18}O_{(juice)} = 0.90\delta^{18}O_{(must)}+0.13$ with a coefficient of determination (R^2) of 0.96 (Figure 1), showing a strong correlation between the results (Chein, 2019). This equation was used to correct the reference values.

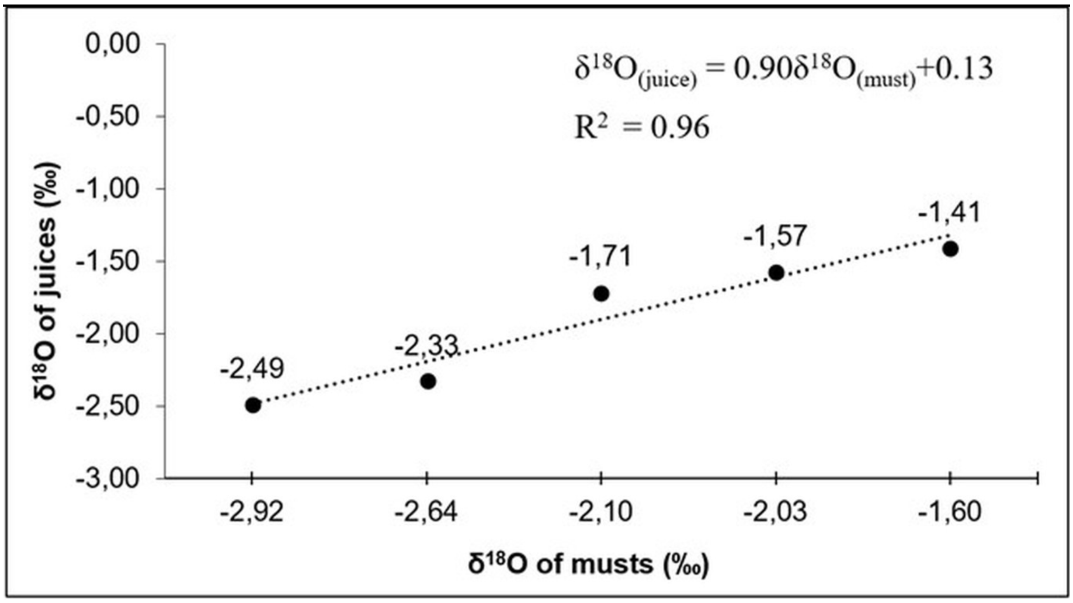

Figure 1. Linear regression of musts and juices from Isabella grape collected in the industry.

The slight isotopic differences found between the values of the corresponding musts and juices, were lower than the measurement uncertainty, which is 0.71‰, showing that the process of the heat exchanger system did not significantly interfere with the final results. In addition, the more positive values of the juices in relation to the musts showed that there was no incorporation of exogenous water since the water has lower $\delta^{18}O$ values. In rainfall water studies with rain samples collected in Indiana in the United States of America (USA), the $\delta^{18}O$ values ranged from -28.10‰ to -3.23‰, with an average of -6.54‰. Swiss waters showed values of -9.05‰ (Tian et al., 2018). In Brazilian lagoons waters, the values found ranged from -7.30‰ to -5.24‰, with higher values in areas of high evaporation (Linhares et al., 2017). These results proved that the addition of exogenous water would make the results more negative, a fact that did not occur in this study.

Considering the strong correlation between the $\delta^{18}O$ results of the initial must and the final juice, the linear regression found was used to construct the reference values for authentic juices. In this way, the reference values calculated for the juice from the musts were used to verify the authenticity of market juices.

3.5 Isotopic reference values for authentic juices

The reference values are shown in Table 2 separated by varieties, which are classified as red and white grapes. These values allow comparison with market juices that, for the most part, do not have variety information on the label. Therefore, white juices were compared with white grapes and red juices were compared with red grapes. When there is information about the variety of the grape on the juice label, the evaluation is much more accurate, since it works with a more specific contingent, minimizing the influence of the variety on the reference value, giving more security to the final result when affirming whether or not there is water in the juice.

Table 2. Reference values for musts and juices separated by red and white grapes and grapes varieties.

Grapes and varieties	$\delta^{18}O$ for Musts Mean ± SD (‰)	$\delta^{18}O$ for Juices (‰) $\delta^{18}O_{(juice)} = 0.90\delta^{18}O_{(must)}+0.13$
Red grapes (n=159)	-1,01 ± 0,82	-0,78
White grapes (n=18)	-1,69 ± 0,86	-1,39
Ives (n=57)	-0.99 ± 1.00	-0.76
Isabella (n=62)	-0.89 ± 0.61	-0.67
Concord (n=22)	-0.49 ± 0.52	-0.31
White Niagara (n=11)	-0.55 ± 0.63	-0.36

SD: standard deviation. n: samples of grapes.

The results of this study are very similar to Dutra et al. (2013). These authors observed that grapes and fine wines from the same region (Serra Gaúcha), but from the 2007 harvest, obtained values of 0.38‰ for the Merlot variety and 0.28‰ for Cabernet Sauvignon variety. The difference found can be attributed to the phenotype of the variety or, also, by the different climatic conditions of the harvest, since negative values of $\delta^{18}O$ can indicate particular climatic conditions and rainfall during the grape harvest (Warmling et al., 2018). Factors that greatly influence $\delta^{18}O$ values are modified during ripening and the harvest period. The influence of the climate and the harvest justify the need to find the reference values for each harvest, to consider the isotopic variation in the interpretation of the results (Camin et al., 2017).

3.6 $\delta^{18}O$ values from market juices

The $\delta^{18}O$ values of market juices and musts are shown in Table 3. It was observed a statistically significant difference between market juices authentic must.

Table 3. $\delta^{18}O$ results from musts and fruit juices and the final result based on the reference values expressed as ‰ relative to V.SMOW2.

Samples	Red grape juices	
	$\delta^{18}O$ (‰)	**Final results**
Red must (n=159)	-0.78 ± 0.82	Authentic
1	-3.64 ± 0.11*	Added tap water
2	-0.80 ± 0.15	Authentic
3	-0.67 ± 0.15	Authentic
4	-1.54 ± 0.11*	Added tap water
5	-4.44 ± 0.07*	Added tap water
6	-0.75 ± 0.13	Authentic
7	-1.07 ± 0.14	Authentic
8	-2.46 ± 0.11*	Added tap water
9	-0.57 ± 0.13	Authentic
10	-0.42 ± 0.15	Authentic
11	-4.40 ± 0.16*	Added tap water
12	-1.05 ± 0.15	Authentic
13	-4.90 ± 0.21*	Added tap water
14	-0.79 ± 0.13	Authentic
15	-0.77 ± 0.08	Authentic
16	-1.02 ± 0.10	Authentic
17	-2.06 ± 0.12*	Added tap water
18	-2.08 ± 0.07*	Added tap water
19	-2.30 ± 0.09*	Added tap water
20	0.19 ± 0.12	Authentic
21	-1.04 ± 0.15	Authentic
22	-4.69 ± 0.10*	Added tap water
23	0.26 ± 0.14	Authentic
24	0.02 ± 0.12	Authentic
25	0.62 ± 0.13	Authentic
26	-0.95 ± 0.12	Authentic
27	0.13 ± 0.06	Authentic
	White grape juices	
White must (n=18)	-1.89 ± 0.86	Authentic
28	-0.72 ± 0.06	Authentic
29	-4.50 ± 0.06*	Added tap water
30	-7.15 ± 0.05*	Added tap water

*Means differ significantly in the column by Tukey's test; significance level of 5%.

The results of the red juices declared "100% whole grape juice" ranged from -4.90‰ (sample 22) to 0.62‰ (sample 25), being -0.78‰ the reference value for must from red grapes. These values were lower than a study by Magdas et al. (2012b) that investigated the isotopic characterization in fruit juices, whose values for grape juices ranged from –2.7‰ to 6.7‰. Samples 1, 4, 8, 17, 18, 19 and 22 showed a statistically significant difference in relation to the authentic must, which indicated the addition of tap water, even declared on the label as "100% red grape juices". The value defined for tap water in Brazil is -5.55‰ (Brasil, 2009), when tap water is added to the juice the values become more negative, closer to -5.55‰. The addition of 41% water changed the isotopic value of oxygen by around 2‰, in a study realized by Magdas et al. (2012b). Therefore, when exogenous tap water is added, the $\delta^{18}O$ value of the juice decreases linearly (Bontempo et al., 2014). In addition, more negative values $\delta^{18}O$ values of the juice may indicate the addition of water, characterizing a type of adulteration.

Samples 5, 11 and 13 are labeled with "100% grape juice", at the same time, the first ingredient on the label is water. This fact was confirmed by the statistically significant difference when compared to the reference value for red must (-0.78‰). In addition, the negative $\delta^{18}O$ values of the juices (-4.44‰, -4.40‰, and -4.90‰ respectively) are closer to the value of tap water. However, none of these juices used the word “whole” on the label, which can be characterized as “reconstituted grape juice”, in accordance with Brazilian legislation. The term “whole” is exclusive to juice in its natural concentration and without the addition of sugars, water, dyes or flavorings (Brasil, 2018).

The samples 20, 23, 24 and 25 juices did not contain tap water in their composition, showing more positive $\delta^{18}O$ values than the reference value for red must. With regard to the numbers 2, 3, 6, 9, 10, 12, 14, 15, 16, 21, 26 and 27 juices when compared to the reference value of -0.78‰ and, taking into account, the measurement uncertainty of 0.71‰, we could not have the final result for these samples. In the present study, these juices were interpreted as authentic. The same occurred for juice number 7, which declared being “100% Isabella grapes” on the label, where the reference value for this grape variety was -0.67‰ (Table 2) and the isotopic value found was -1.07‰, considered as authentic.

The white juices labeled as “100% white grape juice,” (samples 29 and 30) showed a statistically significant difference in relation to the white must, with much more negative values when compared to the reference value of -1.39‰ for white must, characterizing tap water addition in its composition. The number 28 juice did not contain tap water in its composition, since the result found was more positive than the reference value.

4 Conclusion

The method using the isotopic ratio of $^{18}O/^{16}O$ was effective in detecting the addition of tap water to grape juice. The reference values used for interpretation of the result may come from musts, indicating when juice is adulterated with tap water. It is important to update the musts reference values every year. Related to market juice, 30% of the samples showed tap water addition in their composition, indicating an adulteration according to Brazilian legislation. As a perspective, we can mention the extension of this work to grapes and musts from other regions of great importance for viticulture, such as the tropical climate region of the San Francisco Valley, as well as subtropical regions such as São Paulo, in order to obtain greater robustness in the methodology.

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