Technical Note

Comparing Methods for the Analysis of δ^{13}C in *Falanghina* Grape Must from Different Pedoclimatic Conditions

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Abstract: Agroforestry applications in viticulture are considered a promising strategy to improve vine water status by mitigating the threats of increasing drought due to climate change. The analysis of δ^{13}C is often used in viticulture to understand vine water use. In this study, the analysis of δ^{13}C was performed on the must of Falanghina grapevines growing in different pedoclimatic conditions. The aim was to compare the results obtained by the application of two different methodologies, using the whole must or extracted sugars as the matrix. The results showed that the δ^{13}C values obtained by applying the two methodologies were comparable in all analyzed vineyards independently from the pedoclimatic conditions. Indeed, the proposed method of extraction of the δ^{13}C on the must as a whole can be both cost- and time-saving for the analysis. This is valuable, considering that the δ^{13}C of must is becoming more and more used as indicator of vines' water use. Therefore, the possibility to utilize a simplified method of extraction would enhance the application of the δ^{13}C at a larger scale to evaluate vine adaptation in the context of climate-change-driven increases in drought.

Keywords: agroforestry; carbon isotopic discrimination (δ^{13}C); must analysis; sustainable viticulture; *Vitis vinifera*; drought-stress; water-use efficiency (WUE)

1. Introduction

Ongoing climate change in the Mediterranean region is exposing plants to more and more extreme environmental conditions, such as frequent, prolonged, and severe periods of water scarcity, high temperatures, and strong winds [1]. Grapevine (*Vitis vinifera* L. subsp. *vinifera*) is a crop cultivated in the rainfed regime in many Mediterranean cultivation areas, as requested by the production disciplinary of quality and origin labels. Therefore, this crop is facing increasing problems due to drought-stress during summer, when high evapotranspiration is accompanied by very low precipitation. In the near future, this phenomenon might endanger viticultural suitability of the traditional wine-producing areas, with the risk of a reconfiguration of vineyard locations, especially in Mediterranean cultivation areas [2]. It has been recently underlined by Favor and Udawatta et al. [3] that agroforestry applications in viticulture are instead promising, although severely overlooked. Indeed, the incorporation of trees in vineyards may ameliorate grapevine water status and mitigate stress conditions due to many mechanisms that are mainly linked to the alteration of microclimate and belowground interactions [3]. The main objective of agronomic techniques is to maintain environmental conditions to guarantee a balanced vegetative growth directed towards quality production [4]. Indeed, it has been recently shown that the control of vine water status can help achieve a rebalance of the gap between technological and aromatic maturity [4]. Therefore, in order to adopt suitable cultivation strategies to mitigate vines drought-stress, the monitoring of vine water status is needed over the course of years to understand the vines' responses to year-to-year environmental variability, including possible agroforestry applications [3,5]. An important

current challenge in the biological research applied to viticulture systems is to identify an analytical method that is quick to perform and effective for evaluating the plant water status and specifically water-use efficiency (WUE). In the water-stressed vines, the decrease in leaf area and photosynthesis leads to physiological and biochemical disorders, which can have a negative impact on plant growth, structure and chemistry of the leaves, (soil-)nutrient uptake, and berry ripening [6,7]. All of these aspects affect the yield and berry composition (e.g., content of organic acids, sugars, and polyphenols also responsible for aroma) and are ultimately associated with lowering wine quality. Therefore, the improvement of WUE is among the main aims of viticulture to achieve an environmentally friendly and sustainable viticulture.

The concept of WUE always reflects a balance between gains (carbon acquisition or crop yield, AN) and costs (water consumed by transpiration and water applied, E). This balance can be measured at different levels from instantaneous fluxes of CO_2 and water vapor at the leaf, plant, and crop levels; however, in a wider context, this concept is also applied to whole agricultural systems [8]. At the leaf level, WUE can be assessed with an infra-red gas-analyzer, allowing us to determine the "instantaneous water use efficiency (AN/E, WUE_{inst})" and the "intrinsic water use efficiency (AN/gs, WUE_{i}-gs stomatal conductance)". These are in-vivo measurements which, although repeated during the growth cycle at specific intervals (often in correspondence of specific phenological stages), are not representative of the annual water status of the vines [9]. The carbon isotope ratio of dry matter ($\delta^{13}C$) is instead used as an integrated measurement of intrinsic water-use efficiency and can provide important information on the water status of the different plant- organs or of the whole plants [10]. The carbon isotope composition of plant material depends on the discrimination against $^{13}CO_2$ during photosynthetic process, due to fractionation events happening first during CO_2 stomata assimilation and then by Rubisco (Ribulose 1,5-bisphosphate carboxylase/oxygenase) activity [10]. More stomatal closure, i.e., in limited-water-availability conditions, determines increasing $\delta^{13}C$ values in plant tissues [11]. Therefore, the analysis of carbon isotopes composition in different plant tissues allows us to determine the integrated value of WUE, and, thus, the increase of $\delta^{13}C$ corresponds to an increase of WUE. In the case of grapevine, significant relations between plant water status and the carbon isotope ratio of grapevine organs have been observed under both glasshouse [12,13] and field conditions [14–20].

Several approaches to analyze the $\delta^{13}C$ isotope in viticulture have been performed by using different matrices with different procedures. Various plant tissues can be taken as samples for $\delta^{13}C$ detection, with leaves [16] and berries [17] being the most common ones. Concerning representativeness, under field conditions, leaves have been reported to be the least representative organ, as their carbon isotope ratio is less related to water-use efficiency [16], or to water potential [15,16] (R^2 = 0.17), compared to the data from pulp (R^2 = 0.74) and from whole grape (R^2 = 0.62). This is probably due to the fact that leaves are formed early in the season, before any significant water deficit is experienced [15,16]. In Santesteban et al. [21], it was showed that the vine water status of the Tempranillo cultivar was related to $\delta^{13}C$ more strictly during the ripening period (from veraison to harvest) than during the berry herbaceous development (from fruit-set to veraison). Seeds were included in the $\delta^{13}C$ whole-berry analysis that could indeed have caused a slight decrease in the $\delta^{13}C$ values as an average, considering that values are 1–2‰ lower in seeds than in berry flesh [16,22] and seeds represent just a 10–15% of berry dry weight [23,24]. The range of $\delta^{13}C$ observed in the study was also very broad, ranging from −23‰ to −29‰. In Coulouma et al. [25], the samples of whole berries were ground, centrifuged, and oven-dried, and the resulting powder was analyzed by a continuous-flow isotope-ratio mass spectrometer. As expected, the driest year (2016) presented a significantly higher $\delta^{13}C$ mean and the highest maximum value. In conclusion, berry pulp appears to be the most sensitive tissue [16], although whole berries follow a similar trend [16]. Indeed, it is not clear yet if the whole berry's pulp can be considered the most representative matrix of plant water status during the growing season. A translocation of sucrose from leaves

to fruits occurs during berries' maturation, with a subsequent conversion in glucose and fructose. This might indicate that sugar berries' carbon isotope signature better reflects the leaf carbon photosynthetic discrimination [14]. If so, the $\delta^{13}C$ of must sugars should be considered the most representative physiological indicator of grape water status [26]. However, sugar extraction is a time-consuming procedure, and, to our knowledge, only a few reports, limited to a single study case, have compared the different methodologies to find the more convenient method to assess grapevine water status over the growing season and in a large number of plots [14].

In this study, the attention was focused on methods for $\delta^{13}C$ determination in must in order to evaluate the possibility to perform the analysis of isotopes on must as a whole and not on the extracted sugars. Our hypothesis is that no fractionation occurs during the formation of sugars and that, since the must contains more that 20% sugar, the $\delta^{13}C$ signal, if present at the photosynthesis level, it will be present at the level of must, too.

The method was evaluated in vineyards growing in different pedoclimatic conditions in order to extend the methodology in a larger number of conditions.

The possibility to perform the analysis directly on must would allow us to save time and resources to perform the analysis and to expand the isotope analysis on vineyards at a larger scale.

2. Materials and Methods

2.1. Sampling Vineyards

The study area is located in a hilly environment in Southern Italy (Guardia Sanfra-mondi, Benevento, Campania region) (Figure 1). The experimental sites were selected within the vineyards of the La Guardiense farm, as follows: (1) SL-Santa Lucia (41° 14′ 45″ N; 14° 34′ 16″ E, 194 m a.s.l.); (2) CA-Calvese (41° 14′ 19″ N; 14° 35′ 11″ E, 163 m a.s.l.); (3) GR-Grottole (41° 14′ 21″ N; 14° 34′ 56″ E, 158 m a.s.l.); and (4) AC-Acquafredde (41°13′44″ N; 14°35′33″ E, 84 m a.s.l.).
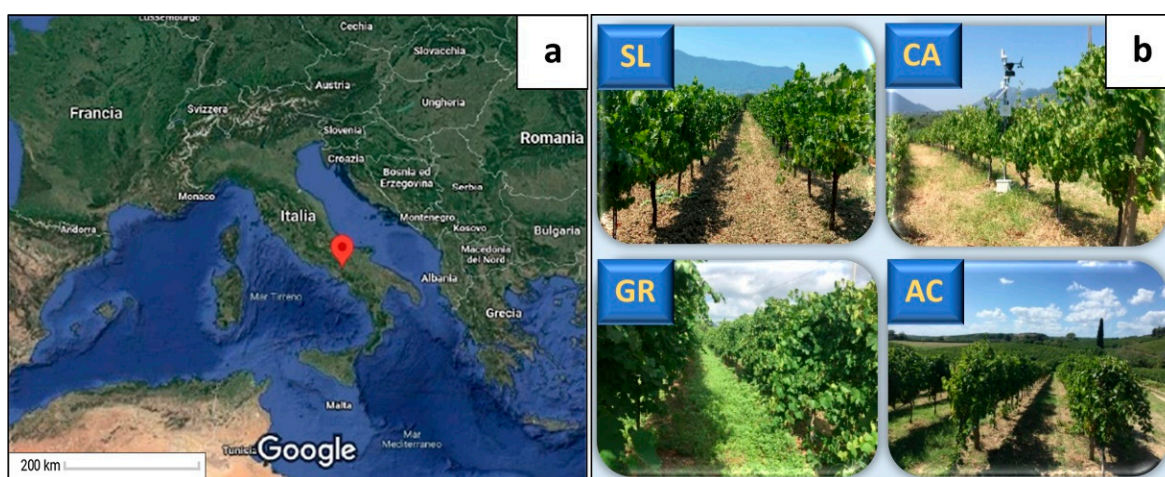

Figure 1. Location of the study area in Southern Italy (**a**) and images of the four study sites: SL, Santa Lucia; CA, Calvese; GR, Grottole; and AC, Acquefredde (**b**). Source: Google Maps ©2021.

The vineyards were selected, as much as possible, based on the use of similar plant material and cultivation techniques, apart from the water availability [27]. Therefore, in the four vineyards, the same cultivar, *Vitis vinifera* L. subsp. *vinifera* 'Falanghina' (Controlled designation of origin—DOC/AOC), is grafted on the same rootstock (157-11 Couderc), and vines are characterized by similar age, training system (double Guyot), pruning manage-ment, and spacing ($\approx$4545 vines/ha). Some information on environmental characteristics, soil type and management, vineyard agronomic management, and vine productivity of the four sites are reported in Table 1. The pedoclimatic conditions (data reported from Bonfante et al. [28,29] and Terribile et al. [30]) and agronomic management determine different plant

vigor and productivity measured as yield (bunch weight per plant, kg) divided by TCSA (trunk cross-sectional area, cm^2) in 10 vines per site (Table 1).

Table 1. Information on environmental and productivity at the four sites (SL—Santa Lucia, CA—Calvese, GR—Grottole, and AC—Acquefredde). For yield efficiency, mean values and standard errors are shown; different letters correspond to significant differences.

Site and Vineyards Information	SL	CA	GR	AC
Row orientation	N-S	E-W	E-W	E-W
Landscape Systems *	Hills	Hills	Hills	Ancient alluvial terraces
Soil type **	Typic calciustolls	Typic calciustolls	Typic calciustolls	Typic calciustolls
Soil series	Consociazione dei suoli Pennine	Consociazione dei suoli Pennine	Consociazione dei suoli Pennine	Consociazione dei suoli Taverna Starze
Soil management	Tillage	Natural coverage	Natural coverage	Tillage
Average Amerine & Winkler index (DDA) ***	1697	1697	1697	1827
Average Potential CWSIcum (%)—Total stress ***	6	6	6	20
Irrigation management	Rainfed	Rainfed	Rainfed	Supplemental irrigation
Yield efficiency (Kg/cm^2)	0.951 ± 0.115 a	0.288 ± 0.024 c	0.591 ± 0.038 b	0.924 ± 0.126 a

* Data from Bonfante et al., 2018 [29]. ** Data from Terribile et al., 1996 [30]. *** Data from Bonfante et al. 2011 [28]. DDA, degree-day average; CWSIcum, Crop Water Stress Index.

For sampling, in the year 2019, all the bunches from 3 vines per each vineyard were collected, and the berries were pressed to achieve the must (3 samples × 4 vineyards). Selected vines did not show any sign of disease and/or mechanical stress.

2.2. Freeze-Drying of Samples

Starting from frozen samples, 10 mL of defrosted must was taken and placed in a 50 mL plastic tube and stored in freezer at −20 °C for one day. The samples were freeze-dried in a VirTis wizard 2.0 SP Scientific for 2 days and stored away from light, in cool and dry place.

2.3. Soluble Sugars Extraction and Carbon Isotope Analysis

A modified version of the method proposed by Devaux et al. [31] and Perini et al. [32] was used. Starting from samples of freeze-dried must, 45–55 mg of sample was taken with the help of a spatula, and 15 mL of deionized water was added. The samples were stirred for 60 min at temperature, i.e., 4 °C, and then placed in a thermostatic bath at a temperature of 95 °C for 10 min (protein denaturation and precipitation phase). After the thermostatic bath, the samples were centrifuged at 1200 rpm for 10 min, twice. With the help of a pipettor, the final supernatant (the extracted sugars) was removed and placed in the freezer at a temperature of −20 °C and freeze-dried again for subsequent isotopic analysis.

Carbon isotope composition was measured on must's extracted sugars (ES) in mature grapes and on must (M) before the sugar extraction, weighing about 0.1 mg of both sample types in tin capsules. The δ ^{13}C isotopic analyses were performed at the iCONa lab of the University of Campania Luigi Vanvitelli, using an isotope-ratio mass spectrometer Delta V Advantage (Thermo Scientific, Waltham, MA, USA) (for details see Ricci et al. [33]) connected, in continuous flow mode (CONFLO III), with an elemental analyzer Flash EA 1112 series (Thermo Scientific, Waltham, MA, USA). IAEA C3 (δ^{13}C VPDB = −24.91 ± 0.49‰), IAEA CH6 (δ^{13}C VPDB = −10.45 ± 0.03‰), and IAEA CH3

(δ^{13}C VPDB = -24.72 ± 0.04‰) international standards were used to calibrate samples data. All results are expressed in delta notation, as computed in Equation (1):

$$\delta^{13}\text{C} = [(R\text{sample}/R\text{std}) - 1] \times 1000 \tag{1}$$

where Rsample and Rstd are the absolute δ^{13}C/δ^{12}C ratios for sample and standard, respectively; values of δ^{13}C are reported in parts per thousand (‰), relative to the Vienna Pee Dee Belemnite (VPDB) international reference.

2.4. Data Analysis

Data were analyzed with the SPSS 13 statistical software (SPSS Inc., Chicago, IL, USA). A two-way ANOVA was performed by using the site and matrix as main factors and using the Duncan's coefficient for multiple comparison tests. Pearson's rank correlation coefficient was calculated between data series from must (M) and extracted sugars (ES).

3. Results

The data analysis showed that the site had a significant effect as main factor ($p < 0.001$), while interaction with the matrix (Site $\times$ Matrix) did not have significant effect (Table 2). There were no significant differences between the δ^{13}C values obtained with the two methods (must as a whole or extracted sugars) for all the analyzed vineyards (Figure 2). The most negative values were found in the AC vineyard, i.e., -28.04‰ ± 0.25 on must (M) and -27.82‰ ± 0.24 on extracted sugars (ES), which were significantly lower ($p < 0.001$) than those for SL vineyard, i.e., -26.87‰ ± 0.40 on M and -26.87‰ ± 0.36 on ES. The SL values were, in turn, significantly lower ($p < 0.001$) than CA -25.03‰ ± 0.08 on M and -25.02‰ ± 0.11 on ES and GR -24.93‰ ± 0.12 on M and -24.94‰ ± 0.12 on ES, respectively, with no differences among them. The graph in Figure 3 shows a linear correlation between the δ^{13}C ‰ values calculated on must and sugars extract. The correlation between data from the two matrixes was significant ($R^2 = 0.9935$, $p < 0.001$).

Table 2. Results of the two-way-ANOVA of δ^{13}C values (‰ vs. PDB), using the matrix (must and sugars) and vineyards (SL, Santa Lucia; CA, Calvese; GR, Grottole; AC, Acquefredde) as factors. Mean values and standard errors are shown. Different letters correspond to significant differences.

	d^{13} C
	‰ vs. PDB
Site	
SL	-26.87 ± 0.24 [b]
CA	-25.03 ± 0.06 [a]
GR	-24.94 ± 0.08 [a]
AC	-27.93 ± 0.16 [c]
Matrix	
Must (M)	-26.22 ± 0.41 [a]
Extracted sugars (ES)	-26.16 ± 0.38 [a]
Significance	
Site	***
Matrix	NS
Site $\times$ Matrix	NS

NS, *, **, and ***, Not significant or significant at $p < 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan multiple comparison tests ($p \leq 0.05$).
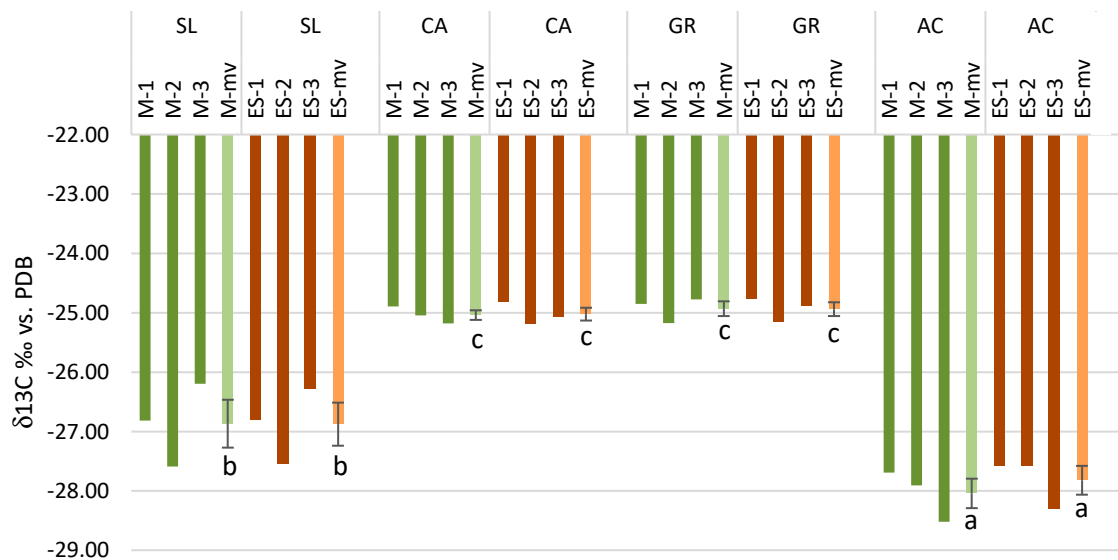
Figure 2. Comparison of $\delta^{13}C$ values (‰ vs. PDB) in must (M, 1-2-3 replicates) and extracted sugars (ES, 1-2-3 replicates) in the four vineyards (SL, Santa Lucia; CA, Calvese; GR, Grottole; AC, Acquefredde). Raw data, mean values (mv), and standard errors are shown. Different letters correspond to significant differences among vineyards according to Duncan's multiple comparison tests of one-way ANOVA.
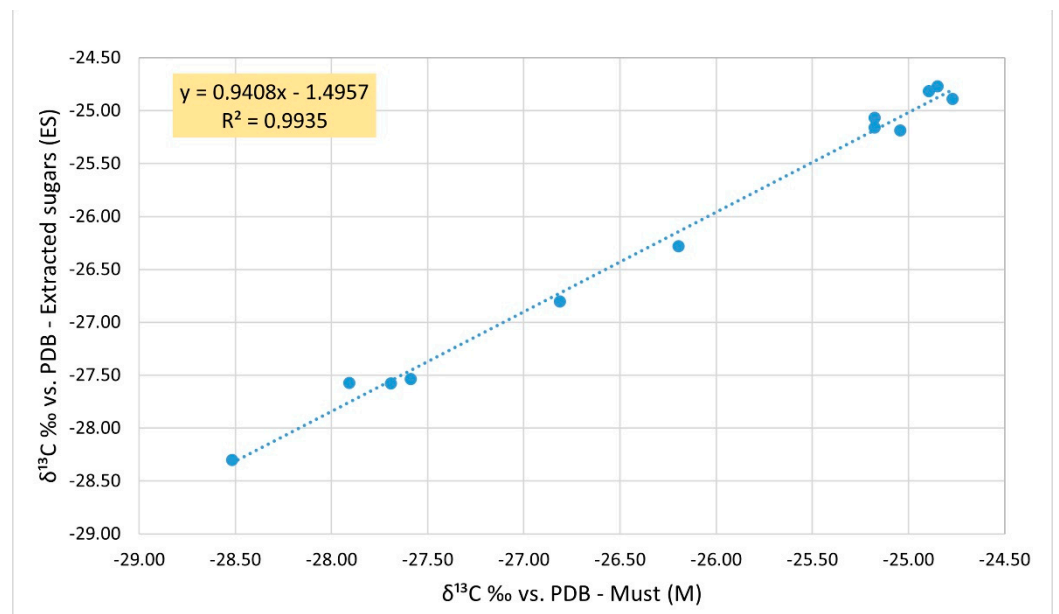

Figure 3. Relation between $\delta^{13}C$ values obtained from the analysis of must (M) samples with the corresponding extracted sugars (ES) samples, considering all the samples collected in the four study sites.

4. Discussion

In plants, such as *V. vinifera*, sugar fraction (mainly glucose and fructose) is the largest carbon pool in berries, since it is the primary photosynthetic product that is produced during the current growing season. Thus, it is important to understand how much it can influence the $\delta^{13}C$ signal of the whole must, where other components (such as organic acids) are present and where a carbon remobilization from reserves could occur [34].

In this study, the δ^{13}C values on the two matrices, M and ES, collected in Falanghina vineyards did not show significant differences. A linear correlation between δ^{13}C calculated on M and ES was found ($R^2 = 0.9935$). The comparison between data obtained by the two matrixes showed that not only the same trends of δ^{13}C values among the samples from the different pedoclimatic conditions were found, but also no statistical differences in the absolute values, thus reinforcing the idea to utilize directly must as a whole for δ^{13}C analyses. The result is in agreement with a previous study performed by Gaudillere et al., [14] who proposed to use must without sugar extraction in Merlot, Carbernet Souvignon, and Cabernet Franc but recommend to test the procedure in a broader range of grapevine plots. Perini et al. [32] arrived at the same results when comparing different procedures, but they underlined the necessity to extract sugar when aiming to improve the detection of authenticity of grape must.

In the challenge to develop a powerful analytical tool for quantifying how climate changes and water scarcity affect or may affect the plant water status, the application of the carbon isotopes is a tool with great potential demonstrated by numerous researches on this subject. Santesteban et al. [35] proposed a correspondence between δ^{13}C and the water deficit via the vine water status measured in a set of studies. The water deficit is considered as weak or null with a δ^{13}C lower than $-26‰$; conversely, the water deficit is considered severe with a δ^{13}C higher than $-24‰$ [35]. Concerning water-use efficiency, it is a parameter that is often used to summarize the water state of the plant, and its correlations with δ^{13}C were studied by several scientists. In Amani Bchir et al. [36], in Spanish cultivars Tempranillo and Grenache, a correlation was found between berries δ^{13}C and the WUE achieving values of 0.98. In this study, dried berry powder was used and analyzed in an isotope ratio mass spectrometer, as in Farquar et al. [10]. In De Souza et al. [16], the researchers evaluated the effect of deficit irrigation on intrinsic WUE and carbon isotope composition (δ^{13}C) of Moscatel and Castelao grapevine cultivars growing in a commercial Portugal vineyard. The results show for δ^{13}C of the dried pulp and dried whole berry the best determination coefficient (respectively $R^2 = 0.74$ and $R^2 = 0.62$) with WUE as compared to the δ^{13}C of leaves ($R^2 = 0.17$) in both cultivars and years considered, showing the less representativeness of this last tissue. In Gómez-Alonso et al. [37], the determination of δ^{13}C was performed on the must of four Spanish autochthonous grapevines Airén, Macabeo (white grapes), Tempranillo, and Garnacha (red grapes), and four foreign ones, Chardonnay, Sauvignon Blanc (white grapes), Cabernet Sauvignon, and Merlot (red grapes), comparing irrigated and non-irrigated vines. The results showed significantly ($p < 0.001$) lower ^{13}C/^{12}C ratios of irrigated grapes, confirming the δ^{13}C enrichment berries of drought-stressed vines. All of these studies show the importance of the δ^{13}C use as an indicator of the crop water status when researchers tried to analyze different matrices with different results during the years.

In accordance with Santesteban et al. [35], who proposed thresholds of δ^{13}C to evaluate weak/null and severe water deficit, in the present study case, the vineyards SL and AC seem to be not in drought-stressed conditions, while CA and GR would be classified as drought-stressed. Indeed, in water-limited conditions, there is a strong stomatal regulation, which leads to partial or total stomatal closure determining a decrease in ^{13}CO$_2$ discrimination and an increase of δ^{13}C values [10]. In our study, the lower values of δ^{13}C found in AC indicate that the supplemental irrigation at this site has likely reduced the stress experienced by the vineyard (also in line with higher yield efficiency), notwithstanding the higher potential stressful conditions, as suggested by the higher Average Amerine and Winkler index and CWSI, compared to the other sites. Indeed, supplemental irrigation is a practice applied to essentially rainfed crops to improve and stabilize yields under periods that are particularly dry [38]. In CA and GR, the lower yield efficiency, accompanied by higher δ^{13}C compared to SL, with the same Amerine and Winkler index and CWSI, may be ascribed to the effect of different soil-management methods. Indeed, the natural coverage in CA and GR, in contrast to the tillage in SL, could have induced resources competition between the vines and cover crops during the period of water-stress conditions.

In fact, the use of cover crops is considered a strategy that could possibly have a positive influence on water-use efficiency in vineyards, but it can induce a negative effect on vineyard productivity in the case of limiting environmental conditions [39]. Our data confirmed our working hypothesis of there being a linear relationship between sugar and whole berries, and demonstrated that the two types of matrixes gave the same $\delta^{13}C$ values in all analyzed vineyards, characterized by different pedoclimatic conditions, reinforcing the idea to utilize directly must as whole for $\delta^{13}C$ analyses. This study aimed to evaluate the presence of correlation between must- and extracted-sugars-derived data in Falanghina vineyards, irrespective of different pedoclimatic conditions likely responsible for different levels of $\delta^{13}C$. Gained data suggested a different water use in the four vineyards that should be supported by further analyses considering also other growth and productivity traits. However, the methodological findings were valuable, given that the $\delta^{13}C$ of must is becoming more and more used as an indicator of vines' water use: the introduction of a simplified method of extraction would enhance the application of the $\delta^{13}C$ at a larger scale, allowing us to save both time and costs for the analysis.

5. Conclusions

Nowadays the negative effects of climate changes are increasing the need to implement strategies to monitor the water status of main crops, such as grapevine, in order to be able to implement corrective actions to improve the health status of the crops also in agroforestry systems. Therefore, seeing the representativeness of the carbon isotope ratio of grapevine organs on plant water status increasingly used to obtain such information, it is important to compare the protocols faster and more cost-effective to calculate the $\delta^{13}C$. There is a great diversity in the organs that have been chosen for carbon isotope analysis in the last decade. The proposed method of measurement of the $\delta^{13}C$ on the must as a whole—that is, without sugar extraction—allows us to save on both costs and time for the analysis; this result is promising, given that the application of the $\delta^{13}C$ analysis on grapevine must is being used more and more as an indicator of vines' water status, especially to evaluate vine adaptation ability in the context of climate-change-driven increases in drought.

As a result of this, in the future, we can evaluate the possibility to perform the analysis directly on must to spread the study of the $\delta^{13}C$ rate, a tool that is easy and quick in relation to determining the vine's water status.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the results of this study are accessible from the corresponding author, upon reasonable request.

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