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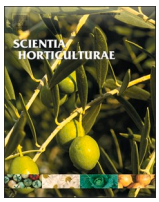

Influence of three doses of urea applied at two different phenological stages on the nitrogen composition of Tempranillo Blanco must over two seasons

Rebeca Murillo-Peña, José María Martínez-Vidaurre *, Teresa Garde-Cerdán *

Instituto de Ciencias de la Vid y del Vino (Gobierno de La Rioja, CSIC, Universidad de La Rioja). Ctra. de Burgos, Km. 6. 26007 Logroño, La Rioja, Spain

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ABSTRACT

Urea is frequently used as a fertiliser in agriculture. In addition, foliar applications of urea in the vineyard have improved the nitrogen composition of the grapes and could improve their quality. There are many studies about the effect of foliar application of urea on red grapevines, but there are no works about the effect of timing application and three doses of urea on nitrogen composition in Tempranillo Blanco grapes. Consequently, the objective of this study was to determine the influence of three doses of urea, applied at two different phenological stages (pre-veraison and veraison), on the nitrogen composition of Tempranillo Blanco grapes during two consecutive years. The results showed that, in general, the foliar application of urea, at both phenological stages, increased the amino acids concentration in Tempranillo Blanco grapes, without modified oenology parameters and yield. A different behaviour was observed between the three doses applied at pre-veraison and veraison, because 6 kg N ha^{-1} treatment, applied at pre-veraison, and 9 kg N ha^{-1} treatment, applied at veraison, improved the concentration of amino acids during the two years. In addition, amino acids concentrations were mainly affected by year and treatment factors. Consequently, foliar application could be considered a good strategy to increase amino acids concentration in Tempranillo Blanco grapes that are poor in these compounds.

1. Introduction

In 1988, cv. Tempranillo Blanco (*Vitis vinifera* L.) was found in a vineyard located in La Rioja (North of Spain). This cultivar is the result of a natural genetic mutation from a single cane of one Tempranillo grapevine. This somatic variant of Tempranillo has been selected and subsequently authorized by Qualified Designation of Origin Rioja (D.O. Ca. Rioja) in 2008 year (Martínez and García-Escudero Domínguez, 2017). Nowadays, Tempranillo Blanco is the second most planted white cultivar in D.O.Ca. Rioja, covers an area of 763 ha (≈ 13 % of white grape cultivars) (Consejo Regulador de la D.O.Ca. Rioja, 2021). Regarding its agronomic behaviour, Tempranillo Blanco is a white cultivar of a short cycle, because it presents a late budburst and early ripening. Moreover, this variety presents a good vegetative-productive balance (Martínez and García-Escudero Domínguez, 2017). Tempranillo Blanco wines have a higher average concentration of acetate esters, ethyl esters, and volatile fatty acids than Tempranillo wines. Therefore, Tempranillo Blanco wines are characterised by their fruity and floral aromas (Garde-Cerdán et al., 2021b)

Nitrogen is one of the most abundant element in vines. The nitrogen status influence vine growth and yield, as well as grape composition (Bell and Henschke, 2005). Moreover, nitrogen is important in the must because it is necessary for correct yeast growth and proper fermentation (Hernández-Orte et al., 1999); moreover, some amino acids are precursors of volatile compounds formed during fermentation, such as volatile thiols, esters, higher alcohols, and fatty acids (Bell and Henschke, 2005), so it could affect wine quality (Garde-Cerdán and Ancín-Azpilicueta, 2008).

Fertilisers are inorganic materials that can supply nutrients and trace elements, usually applied to the soil to promote crop growth, and that have a high nutritional value and a defined composition (Koli et al., 2019). Urea, $CO(NH_2)_2$, is a commonly used fertiliser, characterised by its small molecular size, non-ionic and high solubility, and it is generally absorbed rapidly through the leaf cuticle (Lasa et al., 2012). Consequently, optimal nitrogen management is obtained, nitrogen losses to the environment are reduced and therefore fertilisation costs could be lower. For these reasons, urea can be an alternative to traditional fertilisation (Fernández et al., 2013).

The majority of earlier studies investigating the effects of urea foliar applications and its impact on nitrogen composition focused on red

* Corresponding author.
E-mail addresses: jmvidaurre@larioja.org (J.M. Martínez-Vidaurre), teresa.garde.cerdan@csic.es (T. Garde-Cerdán).

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grape varieties, such as Cabernet Sauvignon (Gutiérrez-Gamboa et al., 2017a, 2017b; Hannam et al., 2014), Pinot Gris (Hannam et al., 2016, 2014), Pinot Noir (Hannam et al., 2014), Merlot (Hannam et al., 2016, 2014; Lasa et al., 2012), Tempranillo (Garde-Cerdán et al., 2017, Garde-Cerdán et al., 2014; Gutiérrez-Gamboa et al., 2018; Jiménez-Moreno et al., 2020; Murillo-Peña et al., 2023; Pérez-Álvarez et al., 2021), and Monastrell (Garde-Cerdán et al., 2017). But, there are few studies about white grapes, such as Sauvignon Blanc (Lasa et al., 2012), Chardonnay (Tozzini et al., 2013), Viognier (Hannam et al., 2014), Chasselas (Verdenal et al., 2015), Italian Riesling (Janjanin et al., 2016), Trebbiano Romagnolo (Baldi et al., 2017) and Greco (Mataffo et al., 2020). These studies were conducted differently. Lasa et al. (2012) examined the effects of two doses of urea (10 and 50 Kg N ha^{-1}) applied at three phenological stages (pre-veraison, veraison and post-veraison). Tozzini et al. (2013) and Verdenal et al. (2015) applied one dose of urea (1 % w v^{-1} and 5 kg N ha^{-1}, respectively) at veraison. Baldi et al. (2017) applied one dose of urea 32.5 kg N ha^{-1} at post-bloom. K.D. Hannam et al. (2014) investigated the effect of two doses of urea (4.4 and 10.5 kg N ha^{-1}) around veraison. Janjanin et al. (2016) applied NPK to the soil (250 Kg ha^{-1}) and urea to the leaves (1 % w v^{-1}) and the foliar application were repeated at four growth stage from young shoot with eight leaves. Finally, Mataffo et al. (2020) studied the effect of urea enriched with amino acids (0.28 and 0.64 Kg N ha^{-1}) at veraison onset and full veraison. On the other hand, Tempranillo Blanco is a white grape variety that has been little studied. This grape variety has been mainly evaluated agronomically and oenologically (Martínez et al., 2017, 2011). Moreover, previous studies have evaluated the effect of some cultural practices, such as irrigation (Baroja et al., 2014) or resident cover crop on D.O.Ca. Rioja (García-Escudero et al., 2014), and the adaptation of this variety to climate change (Kizildeniz et al., 2021).

However, few woks study the amino acids content in Tempranillo Blanco grapes. Only Gutiérrez-Gamboa et al. (2020) studied the effect of seaweed application on the amino acid content of Tempranillo Blanco grapes, Garde-Cerdán et al. (2021a) compared the content of the amino acids between Tempranillo and Tempranillo Blanco grapes and wines, and Sáenz de Urturi et al. (2023) studied the effect of methyl jasmonate application on the aromatic, phenolic and nitrogen composition of Tempranillo Blanco grapes. But no paper describes the effect of foliar application of urea on Tempranillo Blanco grapes. For these reasons, the aims of this work were (1) to study the amino acid composition of Tempranillo Blanco variety; (2) to evaluate the effect of three doses of urea applied to Tempranillo Blanco grapevines on must amino acids content; and (3) to determine the optimal timing of urea application (pre-veraison or veraison) during two consecutive seasons.

2. Material and methods

2.1. Vineyard and climatic characteristics

This research was carried out in a vineyard located in Logroño, North of Spain (42°26′26″ North Latitude; 2°30′52″ West Longitude, at 447 m above sea level) during two consecutive seasons, 2019 and 2020. Moreover, the vineyard belongs to Qualified Designation of Origin Rioja (D.O.Ca. Rioja). The foliar applications were performed in a Tempranillo Blanco (*Vitis vinifera* L.) vineyard, that was grafted on Richter-110 (R-110) and was planted in 2001. The space between vines was 1.10 m and between rows was 2.90 m (3134 vines·ha^{-1}). Vine-training system was double Royat cordon and throughout this time, the vineyard was neither fertilised nor irrigated.

The climatic data were obtained from Agroclimatic Information Service of La Rioja (SIAR). The agroclimatic station is installed at an altitude of 465 m above sea level. Annual precipitation was 520 L m^{-2} (2019) and 498 L m^{-2} (2020). The rainfall from bud to harvest (growing season) was 183 L m^{-2} (2019) and 190 L m^{-2} (2020), which correspond to 35.2 % and 38.1 % of annual precipitation, respectively. In 2019 year, the rainfall was not very intense during the first and second pre-veraison applications (1.8 mm accumulated in seven days). However, there was no rainfall during the veraison applications. In 2020 year, the precipitation was 3.6 mm in seven days (pre-veraison treatments). Nevertheless, the precipitation was higher during the week of veraison applications (28.4 mm were accumulated in seven days). Therefore, 2019 year was slightly dried than 2020 year, during the weeks of foliar applications of the urea treatments. The reference evapotranspiration (ET_0) throughout the growing season (April-August) was 767 and 708 mm in 2019 and 2020 year, respectively. Over the growing season, the average maximum temperature was slightly higher in 2019 year (33.7 °C) than in 2020 year (31.8 °C). However, the average maximum temperature was slightly lower in 2019 year (32.9 °C, at pre-veraison, and 28.7 °C, at veraison) than in 2020 year (30.2 °C, at pre-veraison, and 33.1 °C, at veraison), during the week of urea applications.

2.2. Treatments and foliar applications

The study design was a randomised block with each treatment in triplicate, using 10 vines for each replication. The treatments used in the earlier Tempranillo vineyard study were used again (Murillo-Peña et al., 2023). The treatments applied to the vineyard were a control (C), and three different urea doses (3, 6 and 9 kg N ha^{-1}, called as U3, U6 and U9, respectively). In addition, 1 mL L^{-1} of the wetting agent Tween® 80 (Sigma-Aldrich, Madrid, Spain) was added to each solution. Each of the plants was dosed with 200 mL of the solution. In both years, urea applications were sprayed at pre-veraison (Pre), which corresponds to BBCH-scale code 81, and veraison (Ver), which corresponds to BBCH-scale code 83 (Lorenz et al., 1995), and each treatment was repeated one week later.

In 2019 year, pre-veraison treatments were carried out on 2nd August, and veraison treatments were applied on 13th August. In 2020 year, pre-veraison treatments were applied on 29th July, and veraison treatments were carried out on 5th August.

2.3. Oenological parameters of the samples and nitrogen fractions

All grape samples were harvested manually at their optimum technological maturation, when the total soluble solids reached close to 22.7°Brix . The 2019 harvest was on 2nd September, and the 2020 harvest was on 7th September. One day before harvest, 500 berries from each treatment and replicate were picked up. Immediately, 100 berries were counted and weight to obtain their average weight. The remaining grapes were used to determine the oenological parameters: probable alcohol, pH, total acidity and tartaric acid, according to the official methods of International Organisation of Vine and Wine (OIV) (OIV, 2019). Malic acid, ammonium nitrogen (NH_4^+) and amino nitrogen (NH_2^+) were measured using the enzymatic equipment Miura One (Tecnología Difusión Ibérica, Barcelona, Spain). The yeast assimilable nitrogen content (YAN) was calculated as sum of NH_4^+ and NH_2^+. In order to calculate the yield per vine on the day of harvest, samples of grape bunches were weighed separately. Following that, grapes from each treatment were crushed and destemmed separately. Must aliquots were taken before the addition of potassium metabisulfite ($K_2S_2O_5$), and were frozen (−20 °C) to subsequent determine of the amino acids concentration in Tempranillo Blanco must. In winter, the shoots of each replicate were pruned and weighed independently, on 29th January 2020 and 19th January 2021, respectively. In addition, Ravaz index was calculated as the ratio between the yield and pruning weight. As the treatments were performed in triplicate, the results of oenological parameters and nitrogen fractions are shown as the average of three analyses ($n = 3$).

2.4. Analysis of amino acids in the musts by HPLC

The determination of the amino acids content in the musts was carried out by liquid chromatography on a Shimadzu Nexera X2 Ultra-

High-Performance Liquid Chromatograph (UHPLC) machine (Shimadzu, Kyoto, Japan) equipped with an Automatic Liquid Sampler (ALS) and a Diode Array Detector (DAD), and this analysis was carried out by the method described by Murillo-Peña et al. (2023). The sample preparation was as follows: first, the must samples were centrifuged at 2500 x g for 15 min. After that, in a screw cap test tube was added 1.75 mL of borate buffer 1 M (pH = 9) (Sigma-Aldrich), 750 μL of methanol (PanReacAppliChem, Barcelona, Spain), 1 mL of sample, and 30 μL of diethyl ethoxymethylenemalonate (DEEMM) (Sigma-Aldrich). Then, the tubes were introduced into DU-100 Digital ultrasonic (ArgoLab, Carpi, Italy) for 30 min (derivation reaction), and after this time, the tubes were heated at 75 °C for 2 h. During the heating process, degradation of excess DEEMM and reagent by-products occurred. Lastly, each of the samples was filtered using 0.22 μm polyvinylidene fluoride (PVDF) syringe filters (Proquinorte, Bilbao, Spain) and introduced into UHPLC autosampler vials. The mobile phases were: Phase A was composed of 25 mM acetate buffer (pH 5.8) with 0.4 g L^{-1} of sodium azide. Phase B was composed of 80:20 (% v/v) of acetonitrile (PanReacAppliChem) and methanol (PanReacAppliChem). The mobile phases were always filtered through a filter a 0.45 μm Durapore® membrane pore filter (Merck, Dublin, Ireland). The injected volume of derivatized samples was 50 μL. All separations were performed on the ACE C18-HL column (Aberdeen, Scotland), particle size 5 μm (250 mm × 4.6 mm) and heated up to 20 °C. Nitrogen compounds in grapes were detected at 280 nm by a diode array detector (DAD).

The 21 amino acids measured were as follows: aspartic acid (Asp), glutamic acid (Glu), serine (Ser), glutamine (Gln), histidine (His), glycine (Gly), citrulline + threonine (Cit+ Thr), arginine (Arg), alanine (Ala), γ-aminobutyric acid (GABA), proline (Pro), tyrosine (Tyr), valine (Val), methionine (Met), isoleucine + tryptophan (Ile+Trp), leucine (Leu), phenylalanine (Phe), ornithine (Orn), and lysine (Lys). The ultraviolet-visible (UV–Vis) spectral properties and retention times of the corresponding standards (Sigma-Aldrich) were used to identify these amino acids. They were measured with the aid of the external standard method and calibration graphs of the pertinent standards ($R^2 > 0.96$), which underwent the same derivatization process as the samples. As the treatments were performed in triplicate, the results of amino acids concentration are shown as the average of three analyses ($n = 3$).

2.5. Statistical analysis

Analysis of variance (ANOVA) was used in the statistical analysis of oenological parameters, nitrogen fractions, and amino acids data. The variations between the different must samples were compared using Duncan's test ($p \leq 0.05$). Discriminant analysis was also carried out on the concentration of amino acids in must samples in order to separate the must according to urea doses, application time, and year. SPSS version 22.0 (SPSS, Chicago, IL) was used for all statistical analysis.

3. Results

3.1. Yield and oenological parameters

Tables 1 and 2 show the yield and the oenological parameters of the Tempranillo Blanco grapes from 2019 to 2020 years, respectively. These results show that foliar applications of urea affected neither the yield nor oenological parameters, except in 2019 year, when malic acid concentration was increased slightly by the U9-Pre treatment (Table 1).

The results of the two years showed that, while the yield and weight of 100 berries in 2020 were marginally higher than in 2019, the oenological parameters in both years were comparable (Tables 1 and 2).

3.2. Nitrogen composition

The amino acids composition and nitrogen fractions of the Tempranillo Blanco grapes were represented in Table 3 (2019 year) and Table 4 (2020 year). In both years, the most abundant amino acids detected in Tempranillo Blanco must were Arg, Gln, Cit+Thr, and Glu, accounting for 62.1 % of the total amino acids, while the amino acids least abundant were Gly, Orn, Lys, and Met, representing 0.9 % of the total.

Regarding the effects of the foliar treatments applied to the Tempranillo Blanco vineyard in 2019 year, the U3-Pre treatment increased the concentration of 6 amino acids in the musts, i.e. Gln, Gly, Leu, Phe, Orn, and Lys; U6-Pre treatment enhanced the content of 7 amino acids in the samples, i.e. Gln, His, Gly, Arg, Tyr, Orn, and Lys; and U9-Pre treatment increased the concentration in the musts of 7 amino acids, i.e. Gln, Gly, Arg, Ala, GABA, Orn, and Lys (Table 3). Therefore, the total amino acids and total amino acids without Pro were increased by the three urea foliar treatments, independent of the doses (Table 3). However, the treatments applied at veraison had a moderate effect, except U9-Ver treatment, which increased the concentration of most amino acids (Table 3). U3-Ver treatment increased the concentration of 3 amino acids, i.e. Gly, Pro, and Orn; U6-Ver treatment also enhanced the content of 3 amino acids, i.e. Ser, Gly, and Orn; whereas U9-Ver treatment increased the concentration of 11 amino acids, i.e. Ser, Gln, His, Gly, Arg, Ala, Tyr, Val, Met, Orn, and Lys (Table 3). Consequently, only the U9-Ver treatment led to an increase in total amino acids and total amino acids without Pro (Table 3).

The effect of the urea foliar treatments on the amino acids content in the samples was different in 2020 than in 2019. U6-Pre treatment was the only one that improved the concentration of 9 amino acids, i.e. His, Arg, Ala, GABA, Tyr, Val, Leu, Orn, and Lys, the total amino acids and the total amino acids without Pro in Tempranillo Blanco grapes (Table 4). On the other hand, U3-Pre and U9-Pre treatments only enhanced the content in the samples of 1 amino acid, i.e. Gly, and 3 amino acids, i.e. Gly, Tyr, and Orn, respectively (Table 4). Nevertheless, the effect of the treatments applied at veraison were different than those

Table 1
Yield and oenological parameters of the grapes in 2019 for the different treatments: Control (C) and foliar application with 3 kg N ha^{-1} (U3), 6 kg N ha^{-1} (U6), and 9 kg N ha^{-1} (U9) at different phenological stages: pre-veraison and veraison.

	Pre-veraison (Pre)				Veraison (Ver)			
	C	U3	U6	U9	C	U3	U6	U9
Yield (kg $vine^{-1}$)	1.53 ± 0.09a	1.74 ± 0.09a	1.50 ± 0.24a	1.70 ± 0.19a	1.63 ± 0.07A	1.40 ± 0.38A	1.55 ± 0.19A	1.77 ± 0.16A
Weight of 100 berries (g)	147.00 ± 5.17a	155.17 ± 4.90a	143.2 ± 14.67a	148.77 ± 7.1a	146.33 ± 2.85A	139.33 ± 14.11A	145.2 ± 18.56A	144.33 ± 10.43A
Pruning weight (kg $vine^{-1}$)	0.49 ± 0.05 a	0.50 ± 0.03 a	0.50 ± 0.09 a	0.51 ± 0.08 a	0.49 ± 0.05 AB	0.42 ± 0.03 A	0.52 ± 0.07 B	0.52 ± 0.03B
Ravaz index	3.13 ± 0.14 a	3.47 ± 0.15 a	3.00 ± 0.31 a	3.36 ± 0.59 a	3.36 ± 0.46 A	3.28 ± 0.66 A	2.99 ± 0.18 A	3.40 ± 0.12A
Probable alcohol (% v v^{-1})	12.99 ± 0.15a	13.18 ± 0.24a	13.11 ± 0.42a	13.27 ± 0.88a	12.74 ± 0.17A	13.38 ± 0.66A	13.15 ± 0.42A	12.9 ± 0.3A
pH	3.31 ± 0.02a	3.35 ± 0.05a	3.37 ± 0.03a	3.38 ± 0.03a	3.38 ± 0.01A	3.43 ± 0.02A	3.39 ± 0.09A	3.42 ± 0.08A
Total acidity (g L^{-1})*	6.39 ± 0.11a	6.22 ± 0.33a	6.31 ± 0.26a	6.33 ± 0.28a	5.63 ± 0.16A	5.46 ± 0.36A	5.76 ± 0.4A	5.84 ± 0.41A
Malic acid (g L^{-1})	2.45 ± 0.14a	2.63 ± 0.07ab	2.74 ± 0.14ab	2.77 ± 0.22b	2.51 ± 0.25A	2.34 ± 0.26A	2.71 ± 0.2A	2.69 ± 0.18A

For each parameter, different lowercase and capital letters indicate significant differences ($p \leq 0.05$) between treatments applied at pre-veraison and veraison, respectively. All parameters are shown with the standard deviation ($n = 3$). * Total acidity as g L^{-1} of tartaric acid.

Table 2
Yield and oenological parameters of the grapes in 2020 for the different treatments: Control (C) and foliar application with 3 kg N ha $^{-1}$ (U3), 6 kg N ha $^{-1}$ (U6), and 9 kg N ha $^{-1}$ (U9) at different phenological stages: pre-veraison and veraison.

	Pre-veraison (Pre)				Veraison (Ver)			
	C	U3	U6	U9	C	U3	U6	U9
Yield (kg vine^{-1})	2.89 ± 0.25a	3.17 ± 0.17a	3.05 ± 0.55a	3.00 ± 0.32a	3.01 ± 0.26A	2.40 ± 0.56A	3.25 ± 0.47A	3.09 ± 0.34A
Weight of 100 berries (g)	204.27 ± 10.6a	211.97 ± 21.57a	203.80 ± 9.27a	203.50 ± 6.08a	203.10 ± 8.43A	199.37 ± 8.22A	196.57 ± 15.88A	205.70 ± 11.44A
Pruning weight (kg vine^{-1})	0.71 ± 0.08a	0.71 ± 0.06 a	0.75 ± 0.08a	0.74 ± 0.04a	0.79 ± 0.13A	0.62 ± 0.09A	1.01 ± 0.45A	0.77 ± 0.09A
Ravaz index	4.10 ± 0.30a	4.46 ± 0.310 a	4.12 ± 0.92a	4.04 ± 0.28a	3.90 ± 0.94A	4.00 ± 1.39A	3.70 ± 1.69A	4.05 ± 0.76A
Probable alcohol (% v v^{-1})	12.38 ± 0.44a	12.77 ± 0.79a	12.74 ± 0.28a	12.85 ± 0.74a	12.24 ± 0.17A	13.04 ± 0.59A	12.67 ± 0.61A	12.55 ± 0.78A
pH	3.42 ± 0.05a	3.39 ± 0.07a	3.41 ± 0.03a	3.53 ± 0.26a	3.42 ± 0.07A	3.46 ± 0.04A	3.48 ± 0.06A	3.47 ± 0.04A
Total acidity (g L^{-1})*	5.56 ± 0.21a	5.69 ± 0.47a	5.64 ± 0.06a	5.62 ± 0.39a	5.20 ± 0.07A	5.06 ± 0.24A	5.03 ± 0.21A	5.26 ± 0.19A
Malic acid (g L^{-1})	2.55 ± 0.06a	2.53 ± 0.23a	2.60 ± 0.17a	2.46 ± 0.35a	2.45 ± 0.20A	2.46 ± 0.28A	2.48 ± 0.13A	2.62 ± 0.06A

For each parameter, different lowercase and capital letters indicate significant differences ($p \leq 0.05$) between treatments applied at pre-veraison and veraison, respectively. All parameters are shown with the standard deviation ($n = 3$). * Total acidity as g L^{-1} of tartaric acid.

Table 3
Amino acids concentration (mg L^{-1}) and nitrogen fractions content (mg N L^{-1}) of the grapes in 2019 for different phenological stages: pre-veraison and veraison, and different treatments: Control (C) and foliar application with 3 kg N ha $^{-1}$ (U3), 6 kg N ha $^{-1}$ (U6), and 9 kg N ha $^{-1}$ (U9).

	Pre-veraison (Pre)				Veraison (Ver)			
	C	U3	U6	U9	C	U3	U6	U9
Amino acids								
Asp	63.11 ± 1.71a	66.63 ± 0.67a	64.12 ± 4.41a	70.26 ± 10.81a	83.12 ± 7.89B	62.44 ± 6.26A	68.11 ± 0.89A	65.79 ± 1.64A
Glu	83.82 ± 6.63a	91.42 ± 6.12a	91.03 ± 5.06a	93.10 ± 8.42a	76.89 ± 5.06AB	66.81 ± 6.15A	76.85 ± 6.05AB	86.54 ± 6.53B
Ser	39.29 ± 4.52a	43.78 ± 4.79a	43.57 ± 6.04a	46.96 ± 4.21a	4.40 ± 0.50A	3.31 ± 0.13A	10.20 ± 1.24B	39.05 ± 4.40C
Gln	122.54 ± 9.49a	170.39 ± 2.43b	154.06 ± 22.85b	171.23 ± 9.21b	109.34 ± 10.13AB	102.93 ± 6.84A	134.07 ± 23.92B	207.36 ± 8.26C
His	29.19 ± 3.19a	32.96 ± 4.69ab	40.18 ± 5.70b	35.78 ± 1.98ab	30.00 ± 1.47B	26.50 ± 3.26AB	23.40 ± 0.39A	49.02 ± 5.09C
Gly	0.32 ± 0.03a	0.45 ± 0.03b	0.44 ± 0.07b	0.43 ± 0.06b	0.20 ± 0.01A	0.33 ± 0.03B	0.27 ± 0.02B	0.49 ± 0.05C
Cit +Thr	88.37 ± 8.87a	106.39 ± 13.55a	101.06 ± 18.29a	111.68 ± 4.73a	80.20 ± 7.82AB	67.41 ± 7.31A	82.35 ± 6.81AB	86.55 ± 8.47B
Arg	159.14 ± 15.00a	207.49 ± 33.73ab	218.37 ± 43.24b	243.49 ± 8.79b	139.76 ± 18.32A	157.15 ± 3.96AB	152.28 ± 17.92AB	180.56 ± 21.96B
Ala	42.98 ± 5.33a	51.13 ± 6.45ab	50.98 ± 5.73ab	53.84 ± 3.12b	31.13 ± 3.26A	32.76 ± 2.36A	37.91 ± 4.09A	59.33 ± 5.33B
GABA	28.42 ± 3.06a	28.83 ± 1.23a	30.02 ± 3.55ab	33.83 ± 1.26b	25.94 ± 1.86AB	23.65 ± 0.67A	28.37 ± 1.25B	27.85 ± 0.97B
Pro	38.10 ± 3.16a	42.22 ± 3.98a	40.16 ± 2.44a	38.78 ± 3.49a	34.22 ± 3.49B	54.28 ± 8.89C	33.61 ± 1.23B	20.99 ± 1.53A
Tyr	9.78 ± 1.12a	12.06 ± 0.95ab	13.50 ± 2.68b	12.55 ± 1.53ab	8.64 ± 1.39A	8.37 ± 0.58A	8.22 ± 0.26A	13.85 ± 0.92B
Val	12.21 ± 0.35a	13.17 ± 0.95a	13.28 ± 1.33a	14.02 ± 1.64a	10.45 ± 1.41A	10.33 ± 1.46A	8.72 ± 0.29A	13.15 ± 1.23B
Met	1.90 ± 0.20a	2.10 ± 0.33a	2.37 ± 0.25a	2.07 ± 0.35a	1.78 ± 0.09B	1.70 ± 0.28A	1.46 ± 0.10AB	2.84 ± 0.08C
Ile +Trp	22.35 ± 0.62a	25.66 ± 4.62a	23.30 ± 2.23a	24.93 ± 2.55a	21.23 ± 1.64A	18.92 ± 1.17A	20.29 ± 1.79A	21.04 ± 2.45A
Leu	9.20 ± 0.17a	11.65 ± 1.12b	10.38 ± 0.94ab	11.08 ± 1.39ab	8.50 ± 0.88A	8.30 ± 0.93A	9.12 ± 0.65A	9.66 ± 0.57A
Phe	9.25 ± 0.86a	14.68 ± 1.90b	11.10 ± 1.57a	11.32 ± 1.85a	8.70 ± 1.25AB	9.25 ± 1.69AB	7.07 ± 0.37A	10.62 ± 1.22B
Orn	1.09 ± 0.14a	1.95 ± 0.37b	2.98 ± 0.39c	2.61 ± 0.28c	0.55 ± 0.04A	1.51 ± 0.29C	1.07 ± 0.06B	2.45 ± 0.30D
Lys	1.53 ± 0.09a	2.03 ± 0.01b	2.17 ± 0.20b	2.17 ± 0.12b	1.50 ± 0.19A	1.37 ± 0.17A	1.50 ± 0.28A	2.16 ± 0.17B
Total Aa	762.62 ± 48.52a	924.98 ± 75.97b	913.09 ± 108.95b	980.14 ± 44.24b	676.55 ± 61.21A	657.33 ± 20.01A	704.89 ± 45.65A	899.29 ± 61.09B
Total Aa-Pro	724.52 ± 51.67a	882.76 ± 73.43b	872.93 ± 111.29b	941.36 ± 41.26b	642.33 ± 58.12A	603.05 ± 12.51A	671.28 ± 46.87A	878.30 ± 60.03B
Nitrogen fractions								
NH_2^+	95.87 ± 8.49a	109.57 ± 19.04ab	124.14 ± 9.93bc	133.75 ± 4.01c	97.33 ± 5.12A	112.19 ± 14.13AB	117.73 ± 3.53B	142.79 ± 14.21C
NH_4^+	100.43 ± 6.23a	104.57 ± 8.93ab	113.11 ± 2.5b	115.18 ± 5.05b	86.71 ± 10.13A	104.57 ± 13.98AB	107.68 ± 7AB	118.81 ± 11.73B
YAN	196.30 ± 13.68a	214.14 ± 26.27ab	237.25 ± 12.42bc	248.93 ± 3.71c	184.04 ± 5.03A	216.76 ± 27.86AB	225.4 ± 10.13B	261.59 ± 22.08C

For each parameter, different lowercase and capital letters indicate significant differences ($p \leq 0.05$) between treatments applied at pre-veraison and veraison, respectively. All parameters are shown with the standard deviation ($n = 3$). Total Aa: total amino acids, total Aa – Pro: total amino acids without proline, NH_2^+: amino nitrogen, NH_4^+: ammonium nitrogen, YAN: yeast assimilable nitrogen.

carried out at pre-veraison. U3-Ver and U6-Ver applications only improved the concentration in the musts of 3 and 2 amino acids, respectively (Table 4). On the contrary, U9-Ver treatment enhanced most of the amino acids content, and therefore, the total amino acids and the total amino acids without Pro in Tempranillo Blanco grapes (Table 4).

Regarding the nitrogen fractions, NH_2^+, NH_4^+, and YAN were increased in the musts by U6-Pre and U9-Pre treatments, applied in 2019 season (Table 3). In addition, $NH_2^+$and YAN were enhanced by U6-Ver and U9-Ver treatments, whereas NH_4^+ was only increased by U9-Ver treatment (Table 3). However, the effect of the urea treatments was different in 2020 year. U6-Pre treatment was the only one that improved NH_4^+ and YAN in Tempranillo Blanco grapes, and U9-Ver treatment enhanced NH_2^+ and YAN in these grapes (Table 4).

Table 5 shows the multifactorial analysis performed with amino acids content, total amino acids, total amino acids without Pro, and nitrogen fractions (NH_2^+, NH_4^+ and YAN) according to the three factors studied: treatment (T), phenological stage (Ps), year (Y) and their

Table 4
Amino acids concentration (mg L^{-1}) and nitrogen fractions content (mg N L^{-1}) of the grapes in 2020 for different phenological stages: pre-veraison and veraison, and different treatments: Control (C) and foliar application with 3 kg N ha^{-1} (U3), 6 kg N ha^{-1} (U6), and 9 kg N ha^{-1} (U9).

	Pre-veraison (Pre)				Veraison (Ver)			
	C	U3	U6	U9	C	U3	U6	U9
Amino acids								
Asp	62.44 ± 4.08a	55.53 ± 6.69a	60.81 ± 6.13a	53.80 ± 7.39a	48.96 ± 7.21A	41.10 ± 2.86A	39.96 ± 2.78A	43.82 ± 4.81A
Glu	67.81 ± 7.51ab	56.09 ± 5.00a	78.13 ± 9.81b	67.44 ± 9.64ab	64.55 ± 2.38A	59.17 ± 0.52A	61.97 ± 0.90A	83.59 ± 7.66B
Ser	35.40 ± 5.98ab	28.76 ± 3.05a	43.43 ± 4.54b	37.19 ± 5.02ab	34.22 ± 1.50A	31.16 ± 2.66A	34.01 ± 0.64A	46.24 ± 1.43B
Gln	152.69 ± 28.19a	153.00 ± 19.75a	190.07 ± 9.36a	161.22 ± 30.42a	138.42 ± 3.96A	169.55 ± 23.23B	173.68 ± 19.37B	239.82 ± 3.43C
His	36.78 ± 7.01a	39.02 ± 6.37ab	49.50 ± 2.99b	41.9 ± 4.54ab	39.95 ± 2.46A	45.22 ± 2.76A	43.64 ± 6.85A	55.83 ± 0.69B
Gly	1.33 ± 0.01b	1.81 ± 0.08c	1.06 ± 0.10a	1.98 ± 0.22c	1.42 ± 0.12B	1.17 ± 0.09A	1.16 ± 0.21A	2.13 ± 0.00C
Cit +Thr	89.70 ± 16.14a	96.94 ± 13.24a	110.75 ± 9.22a	93.02 ± 16.22a	90.32 ± 4.02A	76.87 ± 9.61B	88.64 ± 4.30B	127.73 ± 3.98C
Arg	230.77 ± 26.81ab	207.23 ± 24.07a	349.09 ± 28.46c	280.59 ± 36.24b	265.79 ± 23.05A	310.43 ± 36.83AB	268.67 ± 38.90A	369.48 ± 25.00B
Ala	41.86 ± 2.71a	51.76 ± 5.86ab	62.49 ± 6.68b	52.29 ± 5.83ab	48.68 ± 2.96AB	44.94 ± 3.21A	55.94 ± 6.89B	68.73 ± 1.31C
GABA	35.58 ± 3.61a	31.73 ± 4.40a	45.42 ± 1.38b	40.54 ± 7.02ab	34.04 ± 4.25A	34.23 ± 0.98A	38.74 ± 4.71A	49.03 ± 2.86B
Pro	51.79 ± 2.85a	52.49 ± 2.23a	52.88 ± 2.41a	53.85 ± 7.30a	50.74 ± 4.53B	59.75 ± 0.84C	53.31 ± 1.69B	44.41 ± 2.26A
Tyr	12.78 ± 1.18a	11.93 ± 0.69a	19.73 ± 2.08c	16.03 ± 2.21b	14.37 ± 1.63A	13.02 ± 2.42A	13.56 ± 0.41A	20.20 ± 0.03B
Val	15.00 ± 2.58a	17.84 ± 1.95ab	19.85 ± 1.23b	17.36 ± 2.05ab	15.47 ± 0.42B	13.8 ± 0.95A	15.80 ± 0.87B	22.23 ± 0.40C
Met	4.12 ± 0.81a	4.60 ± 0.27a	4.44 ± 0.46a	4.41 ± 0.60a	4.24 ± 0.27BC	3.17 ± 0.12A	3.90 ± 0.18B	4.35 ± 0.17C
Ile +Trp	23.01 ± 2.72a	21.83 ± 4.53a	26.13 ± 1.65a	22.85 ± 1.91a	22.58 ± 1.31B	20.23 ± 0.79A	21.48 ± 1.36AB	27.14 ± 0.02C
Leu	9.18 ± 1.51a	10.37 ± 1.31ab	12.07 ± 1.01b	10.33 ± 1.19ab	9.40 ± 0.72AB	8.22 ± 0.99A	9.78 ± 0.65B	13.44 ± 0.10C
Phe	14.28 ± 1.41a	16.25 ± 1.44a	17.05 ± 1.51a	15.43 ± 1.97a	13.54 ± 0.63AB	13.17 ± 0.50A	16.4 ± 3.06BC	19.22 ± 0.58C
Orn	2.31 ± 0.31a	2.28 ± 0.29a	3.71 ± 0.28c	2.83 ± 0.10b	2.55 ± 0.35AB	2.89 ± 0.28B	2.39 ± 0.21A	3.97 ± 0.10C
Lys	1.91 ± 0.25a	2.36 ± 0.32a	2.93 ± 0.26b	2.25 ± 0.22a	2.18 ± 0.14A	2.60 ± 0.38AB	2.36 ± 0.21A	3.01 ± 0.16B
Total Aa	888.74 ± 91.21a	861.82 ± 54.12a	1149.54 ± 75.90b	975.32 ± 113.00a	901.41 ± 43.26A	950.67 ± 55.64A	945.38 ± 82.54A	1244.39 ± 44.27B
Total Aa – Pro	836.95 ± 93.71a	809.33 ± 55.67a	1096.66 ± 78.17b	921.47 ± 120.05a	850.67 ± 39.76A	890.92 ± 55.53A	892.07 ± 80.99A	1199.98 ± 42.17B
Nitrogen fractions								
NH_2^+	128.51 ± 22.06a	142.06 ± 8.30a	150.65 ± 14.43a	150.36 ± 17.06a	131.13 ± 16.19A	139.29 ± 18.01A	148.03 ± 4.31A	173.53 ± 3.06B
NH_4^+	115.18 ± 5.17a	135.50 ± 14.37a	161.51 ± 18.09b	124.50 ± 2.37a	125.28 ± 18.08A	110.52 ± 9.83A	113.89 ± 11.32A	118.03 ± 13.25A
YAN	243.69 ± 27.15a	277.56 ± 6.06ab	312.17 ± 16.78b	274.86 ± 18.77a	256.41 ± 5.76A	249.81 ± 27.27A	261.92 ± 13.19AB	291.56 ± 12.78B

For each parameter, different lowercase and capital letters indicate significant differences ($p \leq 0.05$) between treatments applied at pre-veraison and veraison, respectively. All parameters are shown with the standard deviation ($n = 3$). Total Aa: total amino acids, total Aa – Pro: total amino acids without proline, NH_2^+: amino nitrogen, NH_4^+: ammonium nitrogen, YAN: yeast assimilable nitrogen.

interactions in Tempranillo Blanco musts.

Regarding the treatment factor, significant differences were found in all amino acids content, except Ile+Trp (Table 5). Most of the amino acids, total amino acids, total amino acids without Pro, and nitrogen fractions were affected by U6 and U9 treatments (Table 5). Furthermore, the treatment was the main factor affecting Gln, Ala and total amino acids without Pro, with a weight of 40.77 %, 38.37 % and 29.04 %, respectively. In addition, the weight of the treatment factor was the most important factor explaining variations of NH_2^+ concentration with a rate of 35.66 % (Table 5). Furthermore, the treatment factor was the second most important factor after the year factor. The weight of the treatment factor in this case was 18.50 % for Ser, 22.63 % for His, 9.62 % for Gly, 20.94 % for GABA, 19.08 % for Pro, 24.86 % for Tyr, 13.03 % for Val, 23.12 % for Leu, 26.51 % for total amino acids and 27.22 % for YAN (Table 5).

The phenological stage factor had significant differences in amino acids concentration, except Gln, His, Arg, GABA, Lys, NH_2^+, and YAN (Table 5). As a result, the average amino acids concentration was higher in pre-veraison samples than in veraison samples (Table 5). However, this factor did not have a great impact on the amino acids content, except for Ser, which the Ps factor explained 26.24 % of the variation in its content.

The year factor was the factor that had more influence on the changes in amino acids and YAN, i.e. Asp (50.19 %), Glu (38.40 %), His (32.49 %), Gly (79.46 %), Arg (52.07 %), GABA (49.92 %), Pro (48.81 %), Tyr (35.96 %), Val (52.94 %), Met (81.96 %), Phe (56.93 %), Orn (35.08 %), Lys (41.31 %), total amino acids (29.38 %) and YAN (44.02 %). Moreover, the year was the second most important factor influencing Ile+Trp (17.14 %), total amino acids without Pro (33.39 %) and NH_4^+ (23.38 %) (Table 5).

The interaction between the treatment and the phenological stage affected most of the amino acids concentrations, except GABA, Ile+Trp, NH_2^+, NH_4^+, and YAN (Table 5). In the case of Ala, this one was the second most important factor, accounting for 18.21 % of the total.

The interaction between the treatment and the year factors only affected Ser, Gly, GABA, Pro, Tyr, Val, Leu, Phe and Orn content in musts (Table 5).

The interaction between the phenological stage and the year factors affected most of the amino acids concentration, except Gly, Pro, Met, NH_2^+, NH_4^+ and YAN. This interaction had the greatest effect on Ser (27.02 %) and it was the second most important factor conditioning the Asp concentration in the must with 14.63 % (Table 5).

The interaction between the treatment, the phenological stage and the year factors affected some of the concentrations of the amino acids: Asp, Glu, Ser, Gln, Gly, Cit + Thr, Arg, Ala, GABA, Val, Met, Leu and NH_4^+ (Table 5).

Table 5
Multifactor analysis of variance of amino acids (mg L^{-1}) and nitrogen fractions (mg N L^{-1}) in grapes for different treatments: Control (C) and foliar application with 3 kg N ha^{-1} (U3), 6 kg N ha^{-1} (U6), and 9 kg N ha^{-1} (U9), at different phenological stages: pre-veraison (Pre), veraison (Ver), and grapes from 2019, and 2020 year.

	Treatment				Phenological stage		Year		Percentages (%)							
	C	U3	U6	U9	Pre	Ver	2019	2020	T	Ps	Y	T x Ps	T x Y	Ps x Y	T x Ps x Y	Residual
Amino acids																
Asp	64.41b	56.42a	58.30a	58.42a	62.09b	56.66a	67.95b	50.80a	6.18 **	5.03 **	50.19 ***	4.40 *	0.31 ^{NS}	14.63 ***	5.35 *	13.91
Glu	73.27ab	68.37a	76.99b	82.67c	78.61b	72.05a	83.31b	67.34a	16.46 ***	6.48 **	38.40 ***	8.40 **	1.54 ^{NS}	6.38 **	5.85 *	16.49
Ser	28.33a	26.75a	32.81b	42.36c	39.80b	25.32a	28.82a	36.30b	18.50 ***	26.24 ***	7.01 ***	9.63 ***	3.98 ***	27.02 ***	3.01 **	4.62
Gln	130.75a	148.97b	162.97b	194.91c	159.40a	159.40a	146.49a	172.31b	40.77 ***	0.00 ^{NS}	12.33 ***	20.62 ***	1.72 ^{NS}	4.81 **	5.45 *	14.28
His	33.98a	35.93ab	39.18b	45.63c	38.16a	39.20a	33.38a	43.98b	22.63 ***	0.31 ^{NS}	32.49 ***	22.58 ***	2.97 ^{NS}	3.20 *	1.95 ^{NS}	13.87
Gly	0.82b	0.94c	0.74a	1.26d	0.98b	0.90a	0.37a	1.51b	9.62 ***	0.41 **	79.46 ***	1.96 ***	5.56 ***	0.00 ^{NS}	1.52 ***	1.47
Cit +Thr	87.15a	86.9a	95.70a	104.75b	99.74b	87.51a	90.50a	96.75b	18.70 **	13.01 ***	3.39 *	15.80 **	1.47 ^{NS}	9.61 **	12.26 **	25.77
Arg	198.86a	220.57a	247.11b	268.53b	237.02a	230.52a	182.28a	285.26b	13.65 ***	0.21 ^{NS}	52.07 ***	7.52 ***	1.52 ^{NS}	9.16 ***	6.13 **	9.75
Ala	41.16a	45.15b	51.83c	58.55d	50.92b	47.43a	45.01a	53.33b	38.37 ***	2.67 *	15.18 ***	18.21 ***	3.54 ^{NS}	7.78 ***	1.04 ^{NS}	13.22
GABA	30.99a	29.61a	35.64b	37.81b	34.30a	32.73a	28.36a	38.66b	20.94 ***	1.15 ^{NS}	49.92 ***	1.76 ^{NS}	4.68 *	2.40 *	6.26 **	12.89
Pro	43.72b	52.18c	44.99b	39.51a	46.29b	43.91a	37.80a	52.40b	19.08 ***	1.29 *	48.81 ***	15.53 ***	3.99 **	0.64 ^{NS}	1.50 ^{NS}	9.16
Tyr	11.39a	11.34a	13.75b	15.66c	13.55b	12.53a	10.87a	15.20b	24.86 ***	1.99 *	35.96 ***	18.16 ***	3.23 *	2.71 **	2.02 ^{NS}	11.07
Val	13.28a	13.79a	14.41a	16.29b	15.34b	13.74a	11.92a	17.17b	13.03 ***	4.90 ***	52.94 ***	11.82 ***	3.16 *	1.59 *	3.17 *	9.39
Met	3.01a	2.89a	3.04a	3.42b	3.25b	2.93a	2.03a	4.15b	2.81 **	1.87 **	81.96 ***	4.90 ***	0.55 ^{NS}	0.44 ^{NS}	1.79 *	5.68
Ile +Trp	23.13ab	22.50a	23.64ab	24.83b	24.60b	22.45a	22.22a	24.83b	7.33 ^{NS}	11.52 **	17.14 ***	8.97 ^{NS}	4.45 ^{NS}	5.99 *	9.12 ^{NS}	35.46
Leu	9.07a	9.64ab	10.34bc	11.13c	10.53b	9.55a	9.74a	10.35b	23.12 ***	9.32 **	3.65 *	18.53 ***	6.86 *	4.79 *	9.73 *	24.00
Phe	11.44a	13.33b	12.91b	14.15b	13.67b	12.25a	10.25a	15.67b	7.50 **	3.92 **	56.93 ***	8.85 ***	6.39 **	3.05 **	1.46 ^{NS}	11.89
Orn	1.63a	2.16b	2.54c	2.97d	2.47b	2.17a	1.78a	2.87b	28.57 ***	2.66 ***	35.08 ***	18.64 ***	2.78 **	6.40 ***	0.55 ^{NS}	5.33
Lys	1.78a	2.09b	2.24bc	2.40c	2.17a	2.08a	1.80a	2.45b	20.54 ***	0.72 ^{NS}	41.31 ***	13.61 ***	2.29 ^{NS}	6.56 ***	2.59 ^{NS}	12.37
Total Aa	807.33a	848.70a	928.23b	1024.78c	932.03b	872.49a	814.86a	989.66b	26.51 ***	3.41 **	29.38 ***	11.19 ***	1.84 ^{NS}	9.84 ***	5.75 **	12.09
Total Aa - Pro	763.62a	796.51a	883.24b	985.28c	885.75b	825.58a	777.07a	937.26b	29.04 ***	3.21 **	25.24 ***	12.48 ***	1.66 ^{NS}	9.73 ***	6.03 **	12.60
Nitrogen fractions																
NH_2^+	113.21a	122.10ab	135.14bc	145.34c	127.52a	130.36a	116.67a	141.22b	35.66 ***	1.26 ^{NS}	33.39 ***	2.62 ^{NS}	0.83 ^{NS}	0.58 ^{NS}	0.29 ^{NS}	25.37
NH_4^+	106.90a	110.78ab	124.05c	119.13bc	129.74b	110.69a	106.38a	124.05b	13.65 **	6.15 *	23.38 ***	7.92 ^{NS}	7.13 ^{NS}	2.01 ^{NS}	10.25 *	29.51
YAN	220.11a	233.88a	259.18b	264.46b	247.27a	241.05a	223.05a	265.27b	27.22 ***	0.99 ^{NS}	44.02 ***	5.42 ^{NS}	2.89 ^{NS}	0.47 ^{NS}	3.16 ^{NS}	15.83

For each factor, different letters indicate significant differences between samples ($p \leq 0.05$). For the percentages, NS: not significant, $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). Total Aa – Pro: total amino acids without proline, NH_2^+: amino nitrogen, NH_4^+: ammonium nitrogen, YAN: yeast assimilable nitrogen. T: Treatment; Ps: Phenological stage; Y: year.

In general, the three studied factors explained the changes in amino acids content and nitrogen fractions, except for the content of Cit+Thr, Ile+Trp, Leu and NH_4^+, which had a high weight of the residual factor (25.77 %, 35.46 %, 24.00 % and 29.5 %, respectively) (Table 5).

3.3. Discriminant analysis of the must amino acids concentration

Fig. 1 shows the discriminant analysis performed with amino acids concentration in control and treated samples from a) 2019 year, b) 2020 year, and c) both years together. Regarding samples from 2019 year (Fig. 1a), Function 1 explained 56.8 % and Function 2 explained 37.5 %, so 94.3 % of the variance was explained. Arg, Gly, Orn, and Met were the amino acids that more contributed to the Function 1; and Ser, Gly, and Arg contributed to most to the Function 2. The discriminant model shows a good separation between pre-veraison and veraison samples from 2019 year (Fig. 1a). The pre-veraison and veraison samples formed two different groups in the discriminant, except U9-Ver samples. U9-Ver samples were furthest away from the other veraison samples because it had a higher concentration of Ser, Gly, Arg, Met, and Orn (Table 3).

Regarding samples from 2020 year (Fig. 1b), Function 1 explained 79.5 % and Function 2 explained 14.1 %, so 93.6 % of the variance was explained. The variables that contributed most to the discriminant model were Leu, Cit+Thr, Asp, and Gly (Function 1), and Cit+Thr, Gly, Orn, and Asp (Function 2). The discriminant model revealed no distinction between pre-veraison and veraison samples from 2020 year (Fig. 1b). However, the veraison samples were situated centrally with the pre-veraison samples surrounding them. The veraison samples were more closely grouped together, except for the U9-Ver samples. This

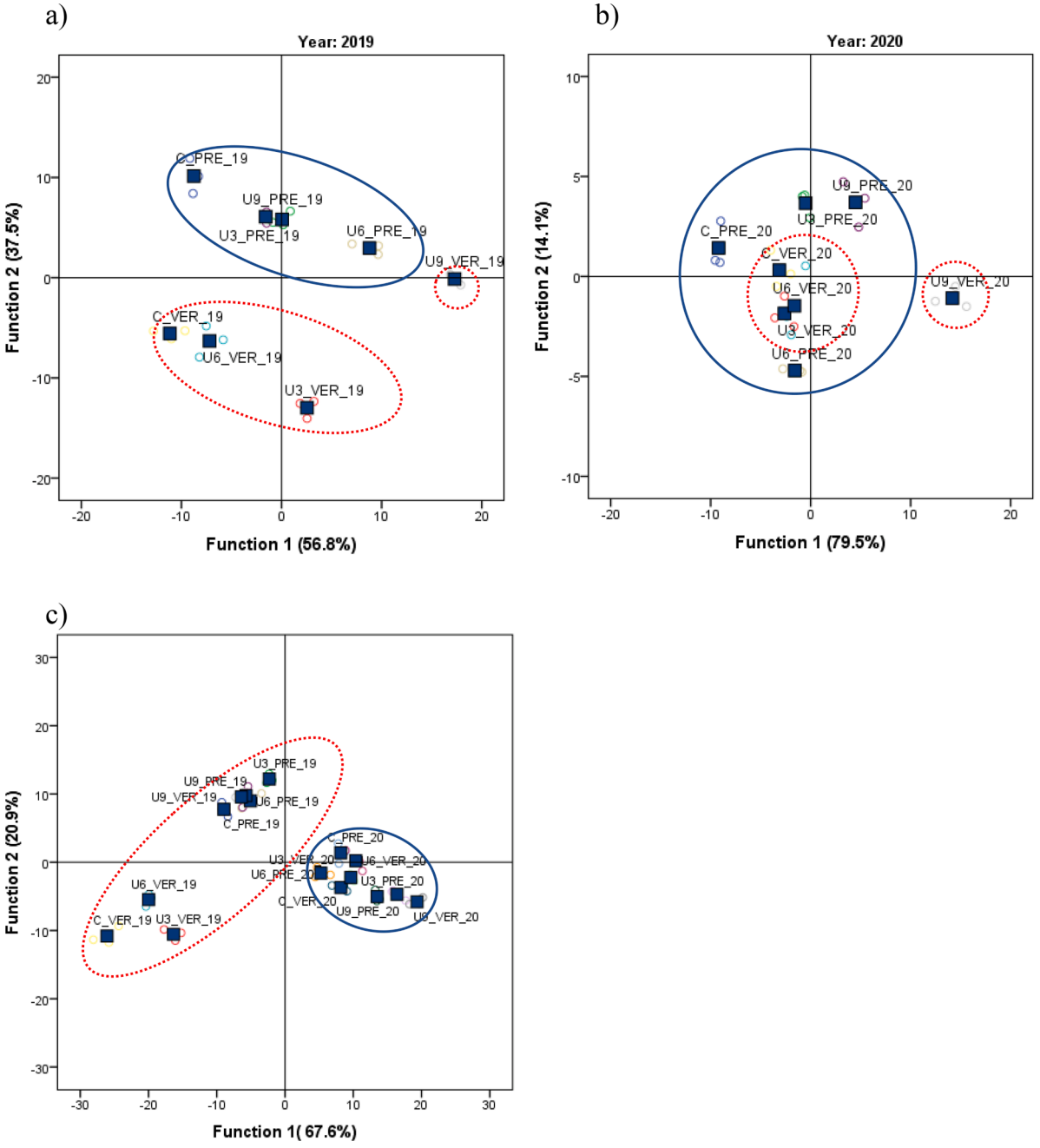

Fig. 1. Discriminant analysis carried out with amino acids concentration (mg L^{-1}) in Tempranillo Blanco grapes for different treatments: Control (C) and foliar application with 3 kg N ha^{-1} (U3), 6 kg N ha^{-1} (U6) and 9 kg N ha^{-1} (U9), at two different phenological stages: pre-veraison and veraison. a) 2019 year, b) 2020 year, c) both years (2019 and 2020).

exception could be due to the elevated Leu, Cit+Thr and Gly concentrations (Table 4).

For samples from 2019 to 2020 years (Fig. 1c), Function 1 and 2 explained 67.6 % and 20.9 %, respectively (total of variance explained was 88.5 %). Ser, Gly, Glu, and Phe were the variables that most contributed to the Function 1. In addition, Ser, Val, Arg, and Glu contributed the most to the Function 2. This discriminant model shows a clear separation between samples from 2019 to 2020 years. However, it did not show a good separation between urea treatments and the timing of application (Fig. 1c). The veraison samples (2019 year) were clustered together and, except U9-Ver samples that were closer to the pre-veraison samples (Fig. 1c). This could be due to the content of Ser, Val, Arg, and Glu was more like veraison samples than to pre-veraison samples (Table 3).

4. Discussion

The results of yield and oenological parameters were similar to the results described in previous studies of the Tempranillo Blanco variety (Baroja et al., 2014; Gutiérrez-Gamboa et al., 2020; Martínez et al., 2014, 2011; Sáenz de Urturi et al., 2023). Moreover, these oenological parameters were not affected by the treatments in both years. These results are in the line with previous studies, which observed that the foliar applications of urea did not affect the oenological parameters of white grapes, such as Chasselas (Verdenal et al., 2015), Sauvignon Blanc (Lasa et al., 2012), Chardonnay (Tozzini et al., 2013), Viognier (Hannam et al., 2014), and Greco (Mataffo et al., 2020). However, the U9-Pre treatment in 2019 year presented higher concentration of malic acid. This response may be attributed to the fact that the U9-Pre bunches could be probably more protected by the leaves. Consequently, the brunches may have been receive less light and the enzymatic process may have been slower. Malic acid is accumulated during grapes berry development and is loss during subsequent ripening, especially from veraison to harvest. The decrease in malic acid content is mainly due to enzymatic processes (Ford, 2012) that are affected by environmental factors, such as temperature and light received by the bunches (Debolt et al., 2008; Kliewer, 1971).

The amino acids content in the must is influenced by grape variety, density of plantation, fertilisation and composition of soil, grape ripening and degree of *Botrytis cinerea* infection, harvesting procedure and climatic characteristics (Rapp and Versini, 1995). In general, the most abundant amino acids are Arg, Gln, Pro, Ala, Glu, Thr, Ser and GABA (Rapp and Versini, 1995). Regarding nitrogen composition, Arg, Gln, Cit+Thr, Glu, Asp and Pro were the most abundant amino acids in Tempranillo Blanco grapes. These findings are agree with Gutiérrez--Gamboa et al. (2020) and Martínez et al. (2015), who identified Arg, Pro, Glu and Thr as the most abundant amino acids in this white grape variety. Tempranillo Blanco grapes were shown to have the lowest concentrations of Gly, Orn, Lys, and Met. These same amino acids were found in the smallest amounts by Gutiérrez-Gamboa et al., al.(2020) and Martínez et al. (2015) in Tempranillo Blanco grapes.

Nitrogen metabolism plays an important role in the growth of *Saccharomyces cerevisiae*. Moreover, amino acids contribute to the formation of several compounds, such as esters, higher alcohols, hydrogen sulphide (H_2S), monoterpenes, and volatile thiols during the wine-making process (Bell and Henschke, 2005; Henschke and Jiranek, 1993). In both years, the U6-Pre treatment increased the concentration of Arg and the U9-Ver treatment increased the concentration of Arg, Ala and Ser in Tempranillo Blanco must. These amino acids are some of the most important, because the yeast metabolite them first during the alcoholic fermentation (Waterhouse et al., 2016). However, U9-Ver treatment reduced Pro concentration. Arg and Pro are frequently the most abundant amino acids in grapes, but Pro is an amino acid that yeast needs oxygen to metabolise (Waterhouse et al., 2016). At the end of alcoholic fermentation, a few hundred milligrams of amino acids per litre remain, half of which is usually proline (Ribereau-Gayon et al., 2006).

Catabolism of amino acids leads to the formation of α-keto acids and their corresponding aldehydes, which may be further reduced in higher alcohols. At moderate content of higher alcohols, desirable aromatic compounds, are found that contribute to the complexity of the wine fermentation bouquet (Verdenal et al., 2021). Higher alcohols are formed during the Ehrlich pathway from Val, Lue, Phe, Met and Ile; and sometimes its olfactory descriptions are described as solvent, fusel, boiled potato, rose, and honey. Although the treatments increased these amino acids, the production of higher alcohols depend by other factors during the fermentation, such as low YAN concentration, higher suspended solids, high temperature and yeasts strain (Waterhouse et al., 2016). Phe is not present in significant amounts in grapes, but it plays a crucial role as a precursor to 2-phenylethanol, which is a compound that contributes to the floral aroma in wine (Waterhouse et al., 2016). Met is also sulphur-containing amino acid, which is involved in yeast metabolism under certain conditions and can result in H_2S production (Bell and Henschke, 2005).

Esters are important compounds that can be classified into two groups: acetate esters and ethyl esters. These compounds are formed by the enzymatic or non-enzymatic esterification of carboxylic acids during alcoholic fermentation and storage (Waterhouse et al., 2016). Esters contribute to wine aroma such as flowers, ripe fruits aroma (Waterhouse et al., 2016). Garde-Cerdán and Ancín-Azpilicueta (2008) described that esters formation was directly proportional to the amount of amino acids in the must. The U6-Ver and U9-Pre treatments increased the total concentration of amino acids in the musts, thus, these results could produce more floral wines.

The YAN concentration of the treated grapes ranged from 188 to 304 mg L^{-1} (Tables 3 and 4). Thus, all samples exceeded the minimum for a satisfactory fermentation (140 mg L^{-1}) (Bell and Henschke, 2005). Moreover, when the YAN content in musts is low, undesirable volatile compounds, such as sulphur hydrogen sulphide (H_2S), some higher alcohols and thiols/mercaptans can be produced (Waterhouse et al., 2016). On the other hand, higher YAN concentration in must can foment the formation of biogenic amines, which can be dangerous to human health and produce undesirable odours (Bell and Henschke, 2005). The main biogenic amines in wine are histamine, tyramine and putrescine, which are derived from the decarboxylation of the amino acids His, Tyr and Orn, respectively (Moreno-Arribas and Polo, 2009).

The year factor, which includes climatic characteristic such as temperature and precipitation, had significant differences in all amino acids concentrations. The amino acids concentration was higher in 2020 than in 2019 (Table 5). In 2020 year, rainfall was slightly lower than in 2019 (498 and 520 mm, respectively). These results coincide with the previous study about white grapes, such as Bouzas-Cid et al. (2015) who analysed cv. Godello and Treixadura during two consecutive years and observed greater amino acids concentrations in the warmer and drier year than in wet year.

In summary, the year was the main factor that affected the amino acids and YAN content. These results are in according with results presented for another grapevine cultivar, such as Tempranillo (Pérez-Álvarez et al., 2017).

5. Conclusions

In this work, the foliar applications of urea were carried out at two phenological stages (pre-veraison and veraison) and with three doses of urea (3, 6 and 9 Kg N ha^{-1}) to Tempranillo Blanco vineyard. Moreover, this is the first study that analysed the effect of the urea application on the nitrogen composition of Tempranillo Blanco grapes.

In this study, neither oenology parameters nor yield were modified by the urea applications. Regarding the results from 2019 year, these showed that amino acids concentrations were improved by the three urea doses applied at pre-veraison and only with the highest applied at veraison. However, in 2020 year, the dose of 6 kg N ha^{-1}, applied at pre-veraison, and the one of 9 kg N ha^{-1}, applied at veraison, increased the

concentration of the amino acids in the white grape samples. Moreover, the year was the most important factor influencing amino acids and YAN content in the Tempranillo Blanco musts; while only two amino acids, Gln and Ala, were significantly affected by the treatment factor. However, the phenological stage factor had the smallest effect on the amino acids and nitrogen fractions concentrations in the Tempranillo Blanco must. Therefore, foliar application of urea with 6 kg N ha^{-1} at pre-veraison and with 9 kg N ha^{-1} at veraison increased amino acids concentration, so more aromatic wines could be elaborated from these grapes. Consequently, the foliar application of urea may be considered as a good viticulture practice in order to improve nitrogen composition in Tempranillo Blanco grapes.

CRediT authorship contribution statement

Rebeca Murillo-Peña: Writing – original draft, Methodology, Investigation, Formal analysis. **José María Martínez-Vidaurre:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization. **Teresa Garde-Cerdán:** .

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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