Research Article

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Evaluation of foliar applications of urea at three concentrations on grape amino acids composition

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Abstract

BACKGROUND: Grape nitrogen composition directly affects the development of alcoholic fermentation and also influences the final wine aromatic composition. Moreover, different factors influence grape amino acids composition, such as rate and timing of nitrogen application. The aim 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 grapes during two consecutive seasons.

RESULTS: Urea treatments did not affect vineyard yield, oenological parameters of the grapes and yeast assimilable nitrogen. However, amino acids concentration in the musts increased at both moments of urea application (pre-veraison and veraison), but the lower urea concentrations and sprayed at pre-veraison improved most of the amino acids in the musts, during two vintages. Moreover, when the year was rainy, the higher dose treatment (9 kg N ha^{-1}) applied at pre-veraison and veraison improved the amino acid concentration in the must.

CONCLUSION: Foliar applications of urea could be an interesting viticulture practice in order to increase the amino acids concentration in Tempranillo musts.

Supporting information may be found in the online version of this article.

Keywords: foliar application; urea; YAN; amino acids; grapes; phenological stage

INTRODUCTION

Grapevine is a crop that are often cultivated on nutrient-poor soils, so it is necessary to provide nutrients. The soil is the main source of nutrients for vineyards, but foliar fertilization could satisfy more quickly and efficiently the requirements of nitrogen (N) needs during the plant vegetative development.[1] Plants can assimilate the nutrient solution applied to the leaves by contact. Leaf adsorbs the nutrient solution, translocate and utilize the absorbed nutrients.[2] Urea is characterized by having a small molecular size, non-ionic nature and has high solubility, so that it may be rapidly absorbed by the foliage.[3] Consequently, optimal N management is obtained, N losses to the environment are reduced and fertilization costs could be lower. For these reasons, urea can be an alternative to traditional fertilization.[2] Nitrogen is a very important element in the must because it is necessary for correct yeast growth, proper fermentation of the must and the wine quality,[4] as some amino acids are precursors of several volatile compounds formed during fermentation, such as higher alcohols and esters.[5] Therefore, N concentration less than 140 mg N L^{-1} can slow down yeast growth and fermentation or may even result in a stuck fermentation and formation of off-flavours during alcoholic fermentation, such as volatile thiols.[6]

Prior studies have shown that foliar application of urea increased yeast assimilable nitrogen (YAN),[7,8] the amino acid concentration,[9,10] and improve wine bouquet.[11] However, many factors can condition the grape N, such as N application timing,[12] N application rate[13] grapevine variety,[8,12] and cultural practices,[14] among other factors.[6]

Regarding application timing, previous studies assessed the effect of providing sprays at different times during the growing season at different frequencies of application. Most studies have evaluated the impact of foliar applications sprayed at one

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moment, and veraison was the most common moment for urea treatments, such as Tozzini *et al.*,[15] Garde-Cerdán *et al.*,[9] Pérez-Álvarez *et al.*[13] and Jiménez-Moreno *et al.*[10] However, there are other studies that started spraying treatments before veraison and finish at post-veraison such as Hannam *et al.*,[16] Havlin *et al.*[17] and Lasa *et al.*[3] which sprayed at three different plant developmental stages (pre-veraison, veraison and post-veraison). Moreover, previous urea foliar application investigations were developed with various urea doses. This range is between 0.9 kg N ha^{-1} [9] and 50 kg N ha^{-1} [3] and most of the previous research usually applied one dose[8,18] or two doses.[3,10,13] Lasa *et al.*[3] sprayed two doses of urea (10 and 50 kg N ha^{-1}) at three times (pre-veraison, veraison and post-veraison) and observed that treatments applied at veraison increased some amino acids. In addition, other studies that applied lower doses than Lasa *et al.*[3] reported the same effect. Pérez-Álvarez *et al.*[19] sprayed 3 and 6 kg N ha^{-1} at veraison, Jiménez-Moreno *et al.*[10] applied urea from veraison to harvest (2 and 4 kg N ha^{-1}), Garde-Cerdán *et al.*[9] sprayed 0.9 kg N ha^{-1} at veraison and Havlin *et al.*[17] sprayed different urea doses (between 11.2 and 44.8 kg N ha^{-1}) from pre-veraison to post-veraison. Moreover, Hannam *et al.*[8] described an improvement in YAN concentration in the grapes and that grape leaves were susceptible to burning when concentrations were increased above 4% urea (*w/v*). Regarding application timing, Porro *et al.*[12] determined that more N was taken up and translocated to the clusters in the phase between pre-bunch closure and veraison. However, in other studies, as in the case of Gutiérrez-Gamboa *et al.*,[18] Pérez-Álvarez *et al.*[13] and Tozzini *et al.*[15] described that the amino acids and YAN concentrations hardly improved after foliar application of urea at veraison.

In previous works carried out by our research group, the impact of foliar fertilization with two doses of urea on the concentration of amino acids and YAN in Tempranillo grapes was studied.[13,19] It was observed that YAN concentrations in grapes were low and, moreover, the highest concentration of urea (3 kg N ha^{-1}) tended to increase some amino acids concentration. As described earlier, there are many studies in reference to foliar application of urea but we have not found any work that applied three low doses of urea and studied the effect on grape N composition. In addition, there are few works that study the effects of urea applications sprayed at pre-veraison and veraison.

For these reasons, the aim of this work was to evaluate the effect and the efficiency of three concentrations of urea and to determine the optimal moment to spray this N compound (pre-veraison or veraison) in order to improve the N composition of Tempranillo grapes over two seasons.

MATERIALS AND METHODS

Vineyard plot description

This study was carried out in 2018 and 2019 vintages, in a vineyard located in Uruñuela, in La Rioja region, in the north of Spain (latitude: 42° 27′ 21.08″ N; longitude: 2° 40′ 59.63″ W; 552 m above mean sea level). Grapevines of *Vitis vinifera* L. cv. Tempranillo were grafted on 110 Ritcher rootstock. The vineyard was planted in 1995 at a density of 2900 vines ha^{-1}, with spacing of 1.20 m between the vines and 2.30 m between rows. Vine-training system was gobelet and left a maximum of 12 buds per vine. The soil characteristics of the experimental vineyard were described by Pérez-Álvarez *et al.*[13] This vineyard soil was classified as *Petrocalcic Palexerolls* and was characterized by a medium organic matter content, pH 8.5 (soil/water, 1:5 *w/v*), low N levels and limited water holding capacity.[13] Moreover, YAN concentrations were between 36.4 and 100.3 mg N L^{-1} in previous studies.[13,19] These low YAN concentrations could result in a stuck fermentation. Due to the soil characteristics and the low YAN levels, we decided to continue applying urea in the same vineyard.

The plot was neither fertilized nor irrigated, during the two study seasons. It is really important not to fertilize the soil with a N fertilizer because this is a study where a N source was applied. In addition, the vineyard was not irrigated because vineyard cultivation has traditionally been considered a non-irrigated crop. Climatic data were obtained from the Agroclimatic Information Service of La Rioja (SIAR). The weather station was located near to the plot (longitude: 2° 42′ 45.7218″ W; latitude: 42° 27′ 40.0379″ N; altitude 465 m above mean sea level). Annual precipitation was 600 and 534 L m^{-2} for 2018 and 2019 vintages, respectively, so 2018 vintage was a littler rainier than 2019 vintage. The accumulated precipitation from bud breaking to harvest (April–September) was 274 (46% of annual precipitation) and 263 L m^{-2} (50% of annual precipitation) for 2018 and 2019 vintages, respectively. Over the growing season (April–September), the average maximum temperature was similar in the 2 years (25.2 °C in 2018 vintage and 25.5 °C in 2019 vintage). The reference evapotranspiration (ET_0) from bud breaking to harvest date in 2019 vintage was slightly higher than in 2018 vintage (757 and 714 mm, respectively). The 2018 harvests were on 3 October and the 2019 harvests were on 24 September.

In addition, leaf samples were collected at flowering and veraison (before applying urea treatments), according to the methodology proposed by Romero *et al.*[20] Total N in leaf blades and petioles samples were determined by dry combustion analysis (Leco CNS, St Joseph, MI, USA) using the Dumas method.[21] For other nutrients, phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), and boron (B) were determined using the method described by Hoenig *et al.*[22] and the samples were analysed by inductively coupled plasma-optical emission spectrometry (Optima 3000DV; PerkinElmer, Norwalk, CT, USA). In this foliar analysis, no significant differences were found between the control and the treatments. Therefore, the nutritional status of the plot was homogeneous (no data shown).

Treatments and samples

In this research, plants were sprayed with water (control, C), and with urea at three doses: 3 kg N ha^{-1} (U3), 6 kg N ha^{-1} (U6), and 9 kg N ha^{-1} (U9). Tween 80 (Sigma-Aldrich, Madrid, Spain) was added to all solutions (1 mL L^{-1}) and used as a wetting agent. Treatments were performed in triplicate and the block design was random. In each treatment and repetition, there were eight vines.

Every year, the treatments were performed in two different phenological stages. In the first one, grapes were started ripening (pre-veraison, Pre) and in the second one, the grapes were approximately 60–70% ripe (veraison, Ver). In 2018 vintage, pre-veraison foliar treatments were carried out on 7 August, and veraison treatments were applied on 16 August. The maximum, average and minimum temperature were 30.8°C, 24.2°C and 18.5°C on 7 August, and 25.4°C, 20.2°C and 14.9°C on 6 August. However, there was no rainfall during these dates. In 2019 vintage, the foliar applications were carried out on 2 August for pre-veraison treatments, and 14 August for veraison treatments. The maximum, average and minimum temperature were 30.4°C, 21.9°C and 16.8°C on 2 August, and 31.9°C, 20.6°C and 10.0°C

on 14 August. During the foliar application dates it only rained on 9 August (4 mm). Also, each of the treatments was repeated 1 week later and the leaves were sprayed with 200 mL of solution per plant. The applications were dosed with knapsack sprayer and were carried out early in the morning to maximize the absorption of urea by the plant.

Oenological parameters and nitrogen fractions

Each year, grapevines were harvest at the optimal moment of technological maturation. One day before harvest, 100 grapes from each treatment and replicate were picked up, counted and weight to obtain their average weight of 100 berries. The following parameters were determined in the grapes: probable alcohol, pH, total acidity and K concentration according to the official methods.[23] Tartaric acid was determined according to Rebelein method.[24] 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 YAN was calculated by sum of NH_4^+ and NH_2^+. Colour intensity (CI) was calculated as the sum of optical density at 420, 520 and 620 nm, and tonality was the ratio between the absorbance at 420 and 520 nm. Total anthocyanins were determined using the methodology proposed by Ribéreau-Gayon *et al.*[25] and total polyphenol index (TPI) was determined by measuring absorbance at 280 nm. In addition, grapes from each treatment and replicate were weighed separately to determine the yield on the harvest day.

Then, the different treatment grapes were crushed and destemmed. Must aliquots were taken before addition of potassium metabisulphite and were frozen (−20 °C) until amino acids concentration was analysed. As the treatments were performed in triplicate, the results of oenological parameters are shown as the average of three analyses ($n = 3$).

Analysis of amino acids in the musts by HPLC

The analysis of amino acids was carried out by the method described by Garde-Cerdán *et al.*[26] and Gómez-Alonso *et al.*[27] Before analysing, all the must samples were centrifuged at 2500 × *g* for 15 min. The amino acids derivatization was carried out inside a screw cap test tube which was added to 1.75 mL of borate buffer 1 mol L^{-1} (pH 9) (Sigma-Aldrich), 750 µL of methanol (PanReacAppliChem, Barcelona, Spain), 1 mL of sample and 30 µL of diethyl ethoxymethylenemalonate (DEEMM) (Sigma-Aldrich). The tubes were introduced into a DU-100 ultrasonic bath (ArgoLab, Carpi, Italy) for 30 min and then, were heated in an oven up to 75 °C for 2 h, in order to degrade the excess of DEEMM. Finally, the samples were filtered using 0.22 µm polyvinylidene fluoride (PVDF) syringe filters (Proquinorte, Bilbao, Spain) and introduced into high-performance liquid chromatography (HPLC) vials. Chromatography analysis was made on a Shimadzu Nexera X2 ultra-high-performance liquid chromatography (UHPLC) machine (Shimadzu, Kyoto, Japan) equipped with an automatic liquid sampler (ALS) and a diode array detector (DAD). Chromatographic separation was made in an ACE C18-HL column (Aberdeen, UK), particle size 5 µm (250 mm × 4.6 mm), at 20 °C. The mobile phases used were always filtered through a 0.45 µm Durapore membrane pore filter (Merck, Dublin, Ireland). Phase A was composed of 25 mmol L^{-1} acetate buffer (pH 5.8) (Sigma-Aldrich) with 0.4 g L^{-1} of sodium azide (Sigma-Aldrich). Phase B was composed of 80:20 (% *v/v*) of acetonitrile (PanReacAppliChem) and methanol. DAD at 280 nm was used to detect and quantify the amino acids.

The amino acids quantified were: aspartic acid (Asp), glutamic acid (Glu), asparagine (Asn), 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), cysteine (Cys), isoleucine (Ile), tryptophan (Trp), leucine (Leu), phenylalanine (Phe), ornithine (Orn), and lysine (Lys). These amino acids were identified according to the retention times and ultraviolet-visible (UV-vis) spectral characteristics of the corresponding standards (Sigma-Aldrich) and were quantified using the external standard method. Quantification of amino acids was performed using the calibration graphs of the respective standards ($R^2 > 0.95$), which were derivatized in the same way as the samples.

Statistical analysis

Statistical analysis of oenological parameters, N fractions and amino acids data was performed by one-way analysis of variance (ANOVA). The differences between means were compared using Duncan's test ($P \leq 0.05$). Besides, a multivariate factorial analysis was carried out with the concentration of amino acids in the must samples. Lastly, a discriminant analysis was performed on data expressing amino acids concentration in grapes to classify the samples according to urea concentrations, application timing and vintage. All statistical analyses were performed using SPSS version 22.0 (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

Oenological parameters

The yield and must oenological parameters from foliar application of the three doses of urea (3, 6 and 9 kg N ha^{-1}) and control, which were applied at pre-veraison (Pre) and veraison (Ver), in 2018 and 2019 vintages, are shown in Tables 1 and 2, respectively.

In 2018 vintage, the foliar applications of urea did not affect yield, weight of 100 berries and probable alcohol (Table 1). However, the values of pH, tartaric acid, malic acid, K, CI, tonality and TPI were affected by some urea applications. Regarding the results from pre-veraison, U6-Pre treatment increased the values of tartaric acid, malic acid, K, and TPI in must samples. U9-Pre treatment increased the value of pH and K in must samples. In the case of veraison treatments, U3-Ver treatment improved CI. U6-Ver treatments only increased concentration of tartaric acid. Finally, U9-Ver treatment increased tonality in the must with respect to the control must (Table 1).

In 2019 vintage, the yield and oenological parameters analysed in Tempranillo grapes were not modified by any of three urea doses and any of two application moments (pre-veraison and veraison), with the exception of total anthocyanins and K, which concentrations were increased in the must samples by U3-Pre and U6-Ver treatments, respectively (Table 2).These results are in agreement with previous studies, where it was observed that foliar applications of urea did not increase production and berry weight.[13,16,28] Moreover, in previous works, most of the oenological parameters of musts were not effect by urea application.[8,9,13-15,28,29] However, other previous studies observed the oenological parameters were increased. Ancín-Azpilicueta *et al.*[11] found that foliar application increased pH and total acidity in Tempranillo must and Havlin *et al.*[17] found that malic acid concentration increased after applying urea. In other previous studies, some oenological parameters were decreased, such as pH[18] and total acidity.[8]

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Table 1. Yield and oenological parameters of the grapes in 2018 vintage for the different treatments: Control (C); foliar application with 3 kg N ha $^{-1}$ (U3), 6 kg N ha $^{-1}$ (U6), and 9 kg N ha $^{-1}$ (U9) of urea (U), at the different phenological stages: pre-veraison (Pre) and veraison (Ver)

	Pre-veraison (Pre)				Veraison (Ver)			
	C	U3	U6	U9	C	U3	U6	U9
Yield (kg grapes per vine)	1.30 ± 0.29a	2.20 ± 0.99a	2.35 ± 0.74a	2.46 ± 1.50a	3.16 ± 0.87AB	1.96 ± 0.62A	3.69 ± 0.49B	2.14 ± 0.73A
Weight of 100 berries (g)	181.13 ± 18.59a	198.07 ± 25.68a	226.63 ± 22.26a	213.33 ± 27.56a	265.10 ± 14.11A	243.63 ± 10.26A	240.93 ± 36.24A	261.03 ± 10.02A
Probable alcohol (% *v/v*)	14.27 ± 0.20a	14.58 ± 0.28a	14.79 ± 0.27a	14.29 ± 0.57a	14.39 ± 0.15AB	15.01 ± 0.14B	14.52 ± 0.56AB	14.08 ± 0.36A
pH	3.30 ± 0.02ab	3.26 ± 0.02a	3.38 ± 0.03bc	3.41 ± 0.10c	3.34 ± 0.08A	3.33 ± 0.06A	3.37 ± 0.08A	3.33 ± 0.03A
Total acidity (g L $^{-1}$)[†]	4.29 ± 0.13a	4.68 ± 0.19a	4.75 ± 0.19a	4.53 ± 0.58a	4.40 ± 0.36A	4.40 ± 0.33A	4.23 ± 0.20A	4.30 ± 0.15A
Tartaric acid (g L $^{-1}$)	5.80 ± 0.22a	6.31 ± 0.23ab	6.59 ± 0.25b	6.30 ± 0.39ab	5.96 ± 0.04A	5.81 ± 0.12A	6.33 ± 0.33B	5.79 ± 0.16A
Malic acid (g L $^{-1}$)	1.34 ± 0.02a	1.67 ± 0.27ab	1.77 ± 0.15b	1.62 ± 0.26ab	1.49 ± 0.25AB	1.23 ± 0.08A	1.81 ± 0.24B	1.54 ± 0.23AB
Potassium (mg L $^{-1}$)	1081.00 ± 35.00a	1139.67 ± 98.72ab	1454.67 ± 33.31c	1265.33 ± 96.15b	1091.33 ± 127.58A	1097.33 ± 67.53A	1196.67 ± 57.84A	1010.33 ± 121.56A
Colour intensity	7.46 ± 1.16a	8.50 ± 2.29a	8.81 ± 0.60a	8.55 ± 0.44a	6.82 ± 0.66A	9.09 ± 0.68B	7.84 ± 1.33AB	6.90 ± 0.42A
Tonality	0.42 ± 0.04a	0.41 ± 0.04a	0.42 ± 0.03a	0.48 ± 0.05a	0.39 ± 0.02A	0.40 ± 0.02AB	0.40 ± 0.02AB	0.43 ± 0.02B
Total anthocyanins (mg g $^{-1}$)	2.23 ± 0.35a	2.24 ± 0.54a	2.10 ± 0.17a	2.16 ± 0.42a	1.80 ± 0.01A	2.04 ± 0.10A	2.08 ± 0.46A	1.67 ± 0.20A
Total polyphenol index	88.15 ± 9.45a	103.05 ± 8.57ab	105.59 ± 8.36b	98.73 ± 6.29ab	92.93 ± 1.80A	100.09 ± 5.09A	97.01 ± 7.37A	98.33 ± 10.52A

Note: 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 $^{-1}$ tartaric acid.

Table 2. Yield and oenological parameters of the grapes in 2019 vintage for the different treatments: Control (C); foliar application with 3 kg N ha^{-1} (U3), 6 kg N ha^{-1} (U6), and 9 kg N ha^{-1} (U9) of urea (U), at the different phenological stages: pre-veraison (Pre) and veraison (Ver)

	Pre-veraison (Pre)				Veraison (Ver)			
	C	U3	U6	U9	C	U3	U6	U9
Yield (kg grapes per vine)	1.09 ± 0.99a	1.15 ± 0.59a	1.86 ± 0.43a	1.71 ± 1.12a	2.00 ± 1.33A	1.40 ± 0.32A	2.27 ± 1.19A	1.32 ± 0.60A
Weight of 100 berries (g)	171.73 ± 28.66a	167.05 ± 19.54a	202.12 ± 20.50a	190.70 ± 28.54a	196.38 ± 12.67AB	183.67 ± 11.32A	207.60 ± 6.77B	197.78 ± 10.12AB
Probable alcohol (% *v/v*)	13.62 ± 0.60a	13.91 ± 0.42a	14.13 ± 0.89a	13.41 ± 0.56a	13.61 ± 0.32A	13.64 ± 0.15A	13.94 ± 0.64A	13.89 ± 0.11A
pH	3.23 ± 0.05a	3.26 ± 0.05a	3.27 ± 0.09a	3.25 ± 0.13a	3.22 ± 0.02A	3.27 ± 0.04A	3.30 ± 0.06A	3.29 ± 0.05A
Total acidity (g L $^{-1}$)[†]	5.02 ± 0.30a	5.23 ± 0.17a	5.29 ± 0.14a	5.07 ± 0.73a	5.20 ± 0.22AB	4.82 ± 0.42A	5.38 ± 0.18B	5.00 ± 0.13AB
Tartaric acid (g L $^{-1}$)	6.21 ± 0.41a	6.35 ± 0.11a	6.38 ± 0.21a	6.25 ± 0.26a	6.15 ± 0.29AB	5.88 ± 0.11A	6.30 ± 0.06B	5.93 ± 0.19A
Malic acid (g L $^{-1}$)	1.79 ± 0.36a	1.96 ± 0.19a	2.11 ± 0.23a	1.83 ± 0.39a	1.81 ± 0.10AB	1.69 ± 0.35A	2.25 ± 0.22B	1.96 ± 0.13AB
Potassium (mg L $^{-1}$)	1010.33 ± 98.47a	1101.33 ± 85.98a	1187.00 ± 162.10a	997.33 ± 65.21a	1018.00 ± 30.51A	995.00 ± 14.00A	1143.00 ± 55.05B	1024.33 ± 38.00A
Colour intensity	8.00 ± 2.08a	10.22 ± 2.83a	8.95 ± 2.32a	8.01 ± 1.76a	7.96 ± 0.57A	8.32 ± 0.50A	9.43 ± 1.68A	8.93 ± 0.95A
Tonality	0.43 ± 0.01a	0.44 ± 0.03a	0.43 ± 0.03a	0.44 ± 0.04a	0.43 ± 0.03A	0.43 ± 0.03A	0.48 ± 0.03A	0.47 ± 0.01A
Total anthocyanins (mg g $^{-1}$)	1.97 ± 0.16a	2.54 ± 0.19b	2.20 ± 0.22ab	2.05 ± 0.44ab	2.06 ± 0.11AB	2.24 ± 0.09B	2.17 ± 0.23AB	1.91 ± 0.04A
Total polyphenol index	90.19 ± 9.83a	102.78 ± 2.23a	99.96 ± 3.55a	98.00 ± 11.97a	99.18 ± 7.36A	96.88 ± 5.59A	96.33 ± 10.7A	102.22 ± 5.21A

Note: 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 polyphenol index (TPI).
[†] Total acidity as g L $^{-1}$ tartaric acid.

Regarding the different K and malic acid concentrations found in this study, it could be that the K concentration in berries can be affected by K level in the soil, grape variety, viticultural practices,[30,31] climatic conditions, the relationship between root absorption and the distribution of this cation in the plant (sink-source competition).[32] Furthermore, K is absorbed by the roots and distributed to all parts of the vine and after veraison, a sharp increase in berry K is observed as a result of K redistribution from leaves to berries.[33]

The concentrations of malic acid fall during ripening because of berry growth. Moreover, temperature, affect malic acid concentration in ripe grapes[34] because temperature shifts the activities of enzymes involved in malic acid degradation.[35] However, high K levels in the berry may decrease the rate of malic acid degradation by impairing malate transport from the storage pools in the vacuole to the cytoplasm.[36] This could explain the high malic acid concentration found in grapes samples when the K concentration was high.

According to multifactorial analysis (Supporting Information Table SS1), treatment factor mainly affected tartaric acid, malic acid, K in Tempranillo grapes. And it has been observed that K, tartaric acid and malic acid concentration was higher in the samples of U6-Pre treatment from 2018 vintage. The foliar application moment (phenological stage factor), affected weight of 100 berries, acid tartaric, K and total anthocyanins in must samples. The year factor affected most of the oenological parameters. However, it did not affect tartaric acid, CI, total anthocyanins and TPI. The interaction between treatment and phenological stage factor only affected tartaric acid concentration. The interaction between phenological stage and year factors affected the weight of 100 berries, K concentration and tonality in grapes. The interaction between treatments, phenological stages and year factors only affected K concentration in Tempranillo grapes.

Amino acids concentration in grapes samples

In Tables 3 and 4 the results are shown for the 2018 and 2019 vintages, respectively, of the 22 amino acids, sum of the all amino acids (total amino acids), sum of amino acids without proline (total amino acids − Pro), NH_4^+, NH_2^+ and YAN in control must and in the must from the three urea treatments (3, 6 and 9 kg N ha^{-1}), which were applied at pre-veraison (Pre) and veraison (Ver).

In 2018 vintage, U3-Pre application had a significant influence on the concentration of 15 amino acids; therefore, this treatment increased the concentration of most of the amino acids analysed (Table 3). However, the other two treatments applied at pre-veraison only improved the concentration of six amino acids: U6-Pre treatment rose Asp, Glu, Cit + Thr, Ala, and GABA concentrations; and U9-Pre treatment enhanced Cit + Thr, Ala, GABA, Tyr, and Lys concentration. Nevertheless, a different behaviour had been observed in the samples from veraison treatments, because the concentration of several amino acids was lower than in the control musts (Table 3). Thus, U3-Ver treatment reduced Arg, Phe and Orn concentrations; U6-Ver application decreased the concentration of 10 amino acids compared to the control samples (Gln, His, Arg, Pro, Val, Met, Ile, Leu, Phe, and Orn); and U9-Ver treatment only reduced Phe concentration (Table 3). Whereas, U6-Ver treatment improved Cit + Thr concentration; and U9-Ver application enhanced Arg and Orn concentrations in the musts (Table 3). Therefore, total amino acids and total amino acids without Pro concentrations were higher in U3-Pre than in control samples (Table 3).

In 2019 vintage (Table 4), U3-Pre and U9-Pre application in the vineyard improved the concentration of 12 and 15 amino acids, respectively, together with the sum of amino acids and the sum of amino acids without Pro concentration. However, U6-Pre treatment only increased Asp, Glu, Gly and Tyr. Therefore, U9-Pre was the treatment that most increased the content in the musts of the different amino acids, and therefore, the sum of all amino acids and sum of amino acids without Pro concentration (Table 4). Similar behaviour was observed in foliar applications at veraison, since U3-Ver and U9-Ver treatments increased the concentration of 8 and 15 amino acids, respectively. Thus, the sum of all amino acids concentrations and the sum of all amino acids without Pro concentration were improved by these two foliar treatments (Table 4). In this case, U9-Ver was the treatment that enhanced more the amino acids content in the grape samples. By contrast, U6-Ver treatment increased the concentration of two amino acids (Asp and Pro) and reduced Gln, Val and Leu concentration.

In 2018 vintage, Gln, Arg and Glu (Table 3) were the most abundant amino acids and represented around 46% of total amino acids of the musts from pre-veraison urea applications, and 50% of amino acids of samples from veraison treatments. In 2019 vintage, the most abundant amino acids were Gln, Arg and GABA (Table 4) and represented about 46% of total amino acids in the musts of pre-veraison and veraison treatments. Whereas Orn, and Gly were among the least abundant amino acids, accounting about 0.5% (2018 vintage) and 0.3% (2019 vintage) of total amino acids in the musts. The obtained results show a different effect of supplying foliar urea spraying at pre-veraison compared to veraison. Previous studies observed that the increase of amino acid concentration was greater after foliar application at veraison than in other phenological stages.[3] However, in this study, an increase in the concentration of amino acids was observed when the foliar urea treatment was sprayed at pre-veraison. Porro *et al.*[12] observed that the quantity of isotope nirogen-15 (^{15}N) taken up and translocated to the clusters was very consistent between pre-bunch closure and veraison, while foliar application at veraison or pre-harvest, ^{15}N was reallocated to storage sinks (perennial or annual organs that will be used as N storage for growth initiation the next year parts). This is because fruit sink strength increases after veraison but still competes with shoot growth.[37] These could explain amino acids concentration decrease in must from treatments applied at veraison and N could have been translocated from berries to other parts of the vine. In a previous study that was carried out in the same vineyard, Pérez-Álvarez *et al.*[13] described that amino acids concentration was only improved in the second year of foliar application with the highest concentration of urea (3 kg N ha^{-1}). Furthermore, Gutiérrez-Gamboa-*et al.*[18] and Garde-Cerdán *et al.*[29] did not observe any improvement in amino acids concentration of Tempranillo must when urea doses were 0.9 kg N ha^{-1} in both studies. However, other studies observed that some amino acids concentrations in must were increased after applied urea to the foliage.[8,14] In other cases, the concentration of most of amino acids[9,10,19] and the total amino acids were increased.[9]

These results suggest that 3 kg N ha^{-1} and 9 kg N ha^{-1} doses were more easily absorbed by leaves or were more efficiently transported from the leaves to the berries compared to 6 kg N ha^{-1} doses. Jiménez-Moreno *et al.*[10] have reported that after applying two doses of urea from veraison to harvest (2 and 4 kg N ha^{-1}), some amino acids concentration did not increase after the application of a high dose of urea (4 kg N ha^{-1}).

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Table 3. Amino acids concentration (mg L^{-1}) and nitrogen fractions (mg N L^{-1}) of the grapes in 2018 vintage for the different treatments: Control (C); foliar application with 3 kg N ha^{-1} (U3), 6 kg N ha^{-1} (U6), and 9 kg N ha^{-1} (U9), at the different phenological stages: pre-veraison (Pre) and veraison (Ver)

	Pre-veraison (Pre)				Veraison (Ver)			
Amino acids	C	U3	U6	U9	C	U3	U6	U9
Asp	27.86 ± 1.84a	29.51 ± 2.91ab	35.21 ± 5.64b	27.11 ± 2.88a	18.39 ± 2.29A	17.50 ± 1.41A	20.94 ± 2.82A	20.01 ± 1.50A
Glu	39.07 ± 7.41a	47.75 ± 4.20ab	55.20 ± 3.39b	48.23 ± 5.16ab	53.80 ± 5.88A	53.39 ± 3.67A	59.48 ± 7.19A	55.38 ± 3.06A
Ser	21.77 ± 1.41a	22.33 ± 2.09a	20.67 ± 1.61a	22.16 ± 5.06a	21.59 ± 0.16A	22.47 ± 4.05A	22.40 ± 3.66A	23.53 ± 3.39A
Gln	63.65 ± 5.11a	98.96 ± 15.43b	62.85 ± 4.74a	67.46 ± 12.03a	87.86 ± 12.58 BC	77.76 ± 9.26AB	62.48 ± 0.31A	104.20 ± 8.99C
His	7.97 ± 1.01a	12.29 ± 1.79b	7.34 ± 1.08a	7.83 ± 0.78a	10.98 ± 1.82 BC	8.80 ± 1.15AB	7.50 ± 0.45A	13.33 ± 1.42C
Gly	1.13 ± 0.04a	1.16 ± 0.05a	0.97 ± 0.03a	1.15 ± 0.23a	1.11 ± 0.05A	1.09 ± 0.14A	0.96 ± 0.01A	1.08 ± 0.11A
Cit + Thr	17.90 ± 2.24a	24.27 ± 1.80b	21.75 ± 2.64b	22.40 ± 0.44b	23.82 ± 1.31A	25.23 ± 1.98AB	29.39 ± 1.04B	27.45 ± 3.89AB
Arg	43.29 ± 6.13ab	79.46 ± 13.03c	31.70 ± 0.83a	51.98 ± 1.74b	77.19 ± 3.43B	42.97 ± 11.90A	48.62 ± 15.11A	104.62 ± 14.25C
Ala	19.11 ± 1.92a	25.88 ± 0.69c	22.18 ± 1.65b	22.61 ± 1.21b	25.15 ± 1.87A	26.06 ± 2.08A	22.36 ± 2.67A	25.49 ± 3.68A
GABA	25.97 ± 3.52a	36.08 ± 1.54b	32.65 ± 1.65b	32.31 ± 0.81b	41.29 ± 2.10A	37.67 ± 7.05A	39.37 ± 4.77A	39.15 ± 5.82A
Pro	9.23 ± 0.23a	13.23 ± 1.51b	8.46 ± 1.11a	9.60 ± 1.85a	12.20 ± 0.40 BC	11.52 ± 0.49B	9.67 ± 1.46A	13.82 ± 0.92C
Tyr	3.03 ± 0.24a	4.35 ± 0.45c	3.22 ± 0.20ab	3.64 ± 0.14b	4.15 ± 0.63AB	3.26 ± 0.48A	3.22 ± 0.42A	4.53 ± 0.86B
Val	9.23 ± 0.23a	12.85 ± 2.40b	9.11 ± 1.06a	9.43 ± 2.00a	13.12 ± 1.82B	11.09 ± 1.13B	8.52 ± 0.33A	11.55 ± 1.42B
Met	1.26 ± 0.15ab	1.38 ± 0.15b	1.04 ± 0.14ab	0.99 ± 0.28a	1.15 ± 0.17B	1.26 ± 0.09B	0.82 ± 0.12A	1.33 ± 0.10B
Cys	1.28 ± 0.20a	1.36 ± 0.13a	1.19 ± 0.12a	1.26 ± 0.12a	1.55 ± 0.17AB	1.63 ± 0.20B	1.26 ± 0.14A	1.28 ± 0.08A
Ile	5.73 ± 0.002a	8.82 ± 0.49b	5.19 ± 0.76a	5.22 ± 1.34a	7.84 ± 1.19B	6.65 ± 0.54AB	5.32 ± 0.73A	6.28 ± 0.77AB
Trp	10.32 ± 0.51a	13.49 ± 1.99b	11.57 ± 0.64ab	10.83 ± 1.76ab	13.84 ± 1.82A	13.43 ± 2.43A	12.24 ± 2.09A	12.18 ± 0.92A
Leu	5.63 ± 0.24a	9.34 ± 0.75b	5.05 ± 0.51a	5.43 ± 1.19a	8.07 ± 1.34B	6.58 ± 0.65AB	4.94 ± 0.26A	6.78 ± 0.97B
Phe	4.66 ± 0.66a	7.47 ± 0.52b	5.26 ± 0.11a	5.34 ± 0.72a	7.54 ± 0.86B	5.69 ± 0.66A	5.25 ± 0.58A	5.78 ± 0.74A
Orn	0.79 ± 0.14ab	1.03 ± 0.16b	0.72 ± 0.12a	0.81 ± 0.05ab	0.81 ± 0.14B	0.60 ± 0.04A	0.62 ± 0.04A	1.00 ± 0.02C
Lys	1.60 ± 0.13a	2.18 ± 0.20c	1.61 ± 0.08a	1.87 ± 0.06b	2.16 ± 0.24A	1.85 ± 0.30A	1.82 ± 0.17A	2.08 ± 0.38A
Total amino acids	320.71 ± 32.44a	453.19 ± 26.68b	342.95 ± 18.44a	357.66 ± 30.58a	433.63 ± 21.34AB	376.48 ± 38.51A	367.16 ± 36.02A	480.86 ± 41.12B
Total amino acids − Pro	311.26 ± 32.80a	439.96 ± 27.39b	334.49 ± 18.68a	348.07 ± 28.73a	421.43 ± 21.32AB	364.96 ± 38.37A	357.5 ± 34.81A	467.04 ± 41.03B
Ammonium nitrogen	76.87 ± 15.47a	78.69 ± 12.81a	57.72 ± 6.47a	57.85 ± 5.05a	85.42 ± 4.85B	61.60 ± 7.05A	51.64 ± 6.60A	83.09 ± 8.96B
Amino nitrogen	62.36 ± 10.36ab	67.60 ± 6.62b	53.33 ± 6.30a	68.19 ± 2.62b	73.00 ± 2.19B	54.20 ± 3.50A	47.64 ± 1.31A	71.10 ± 7.07B
YAN	139.23 ± 25.83ab	146.29 ± 8.68b	111.05 ± 11.18a	126.04 ± 7.67ab	158.41 ± 5.78B	115.80 ± 8.99A	99.28 ± 7.91A	154.19 ± 15.63B

Note: 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 amino acids – Pro: total amino acids without proline. YAN: yeast assimilable nitrogen.

Table 4. Amino acids concentration (mg L^{-1}) and nitrogen fractions (mg N L^{-1}) of the grapes in 2019 vintage for the different treatments: Control (C); foliar application with 3 kg N ha^{-1} (U3), 6 kg N ha^{-1} (U6) and 9 kg N ha^{-1} (U9), at the different phenological stages: pre-veraison (Pre) and veraison (Ver)

	Pre-veraison (Pre)				Veraison (Ver)			
Amino acids	C	U3	U6	U9	C	U3	U6	U9
Asp	16.12 ± 0.60a	25.79 ± 2.43c	20.75 ± 2.11b	22.55 ± 1.45bc	19.82 ± 1.03AB	17.36 ± 1.80A	25.34 ± 1.98C	22.17 ± 0.89B
Glu	46.05 ± 2.55a	63.30 ± 2.31b	60.92 ± 7.83b	62.53 ± 3.38b	55.35 ± 0.65AB	52.01 ± 4.37A	57.49 ± 7.19AB	62.98 ± 3.12C
Ser	21.80 ± 2.52a	26.68 ± 0.79a	23.88 ± 2.80a	25.74 ± 4.47a	22.60 ± 0.17AB	23.89 ± 1.29B	21.84 ± 1.04A	27.82 ± 0.68C
Gln	65.46 ± 7.95a	93.46 ± 9.32b	65.51 ± 7.49a	106.07 ± 4.42b	73.16 ± 4.85B	87.70 ± 8.37C	60.72 ± 1.68A	114.79 ± 8.28 D
His	16.70 ± 1.78a	23.65 ± 2.43b	17.88 ± 3.35ab	30.21 ± 4.38c	17.09 ± 0.94A	23.00 ± 2.21B	18.74 ± 0.48A	26.47 ± 3.15B
Gly	0.50 ± 0.06a	0.75 ± 0.07b	0.72 ± 0.01b	0.85 ± 0.16b	0.59 ± 0.01A	0.91 ± 0.11B	0.68 ± 0.14A	0.79 ± 0.12AB
Cit + Thr	31.55 ± 4.53a	40.21 ± 1.25b	36.39 ± 5.89ab	55.46 ± 2.87c	35.92 ± 6.07A	41.50 ± 4.77AB	37.61 ± 2.97A	48.32 ± 3.85B
Arg	52.99 ± 10.07a	102.24 ± 16.48b	77.92 ± 2.86ab	160.88 ± 20.93c	52.28 ± 3.52A	110.09 ± 8.44B	66.02 ± 1.45A	127.65 ± 24.62B
Ala	25.23 ± 2.30a	32.79 ± 2.18b	27.58 ± 2.31a	39.68 ± 0.37c	28.37 ± 3.72AB	32.69 ± 2.76 BC	26.14 ± 0.14A	35.36 ± 2.78C
GABA	58.69 ± 6.07a	68.10 ± 3.04a	67.41 ± 8.53a	68.99 ± 12.44a	68.14 ± 10.90A	74.91 ± 3.51A	64.62 ± 2.76A	73.84 ± 7.82A
Pro	29.43 ± 0.56ab	37.99 ± 3.84c	27.93 ± 1.20a	32.46 ± 0.64b	31.67 ± 2.60A	36.54 ± 1.41B	36.21 ± 1.11B	38.81 ± 2.82B
Tyr	2.37 ± 0.40a	3.32 ± 0.37b	3.13 ± 0.16b	4.61 ± 0.27c	2.86 ± 0.52A	3.34 ± 0.44AB	2.67 ± 0.12A	4.09 ± 0.41B
Val	8.21 ± 1.06a	8.10 ± 0.50a	7.74 ± 1.16a	11.67 ± 1.37b	9.57 ± 0.96B	9.77 ± 0.69B	7.13 ± 0.52A	10.75 ± 0.26B
Met	1.17 ± 0.23a	1.04 ± 0.14a	0.91 ± 0.09a	1.72 ± 0.24b	1.04 ± 0.13AB	1.12 ± 0.10 BC	0.90 ± 0.02A	1.23 ± 0.05C
Cys	n.d.	n.d.	n.d.	n.d.	0.87 ± 0.12A	1.01 ± 0.16AB	1.11 ± 0.16AB	1.24 ± 0.15B
Ile	4.84 ± 0.98a	4.10 ± 0.06a	4.17 ± 0.61a	6.92 ± 0.74b	5.56 ± 0.10AB	5.65 ± 0.84AB	4.46 ± 0.89A	5.84 ± 0.45B
Trp	11.13 ± 1.69a	12.93 ± 0.87a	12.12 ± 1.61a	15.37 ± 0.71b	10.52 ± 0.00A	12.43 ± 0.76A	11.70 ± 1.84A	11.41 ± 0.64A
Leu	7.10 ± 0.77a	7.49 ± 1.34a	6.28 ± 0.65a	8.67 ± 3.12a	7.90 ± 0.44B	8.21 ± 0.89B	6.29 ± 0.43A	8.96 ± 0.71B
Phe	6.07 ± 1.00a	7.86 ± 1.11a	7.26 ± 1.39a	6.86 ± 0.59a	6.37 ± 0.13A	7.56 ± 0.30B	6.91 ± 0.51AB	7.93 ± 0.93B
Orn	0.29 ± 0.02a	0.71 ± 0.08b	0.52 ± 0.03ab	2.11 ± 0.34c	0.34 ± 0.02A	0.75 ± 0.07B	0.42 ± 0.06A	0.82 ± 0.13B
Lys	1.10 ± 0.17a	1.45 ± 0.15b	1.25 ± 0.21ab	1.92 ± 0.12c	1.19 ± 0.01A	1.62 ± 0.23B	1.26 ± 0.08A	1.70 ± 0.25B
Total amino acids	406.79 ± 25.52a	561.94 ± 33.98b	470.26 ± 43.55a	665.28 ± 47.21c	451.21 ± 18.91A	552.07 ± 28.80B	458.26 ± 4.91A	632.98 ± 45.51C
Total amino acids − Pro	377.36 ± 25.51a	523.95 ± 32.6b	442.33 ± 42.38a	632.82 ± 47.32c	419.53 ± 20.57A	515.53 ± 27.47B	422.05 ± 5.97A	594.17 ± 48.19C
Ammonium nitrogen	92.15 ± 18.25a	104.31 ± 16.61a	87.75 ± 14.75a	95.51 ± 2.80a	88.26 ± 11.0A	123.98 ± 7.34B	108.71 ± 14.25AB	102.24 ± 14.33AB
Amino nitrogen	46.92 ± 3.94a	57.11 ± 7.07a	46.04 ± 5.12a	56.82 ± 6.99a	48.08 ± 5.2A	58.86 ± 6.79B	54.20 ± 3.50AB	61.19 ± 5.46B
YAN	139.06 ± 20.73a	161.43 ± 23.40a	133.79 ± 18.30a	152.33 ± 9.73a	136.36 ± 16.22A	183 ± 12.07B	162.91 ± 15.13AB	163.43 ± 15.44AB

Note: 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 amino acids – Pro: total amino acids without proline. YAN: yeast assimilable nitrogen. n.d.: not detected.

However, Lasa *et al.*[3] have reported that higher concentrations of urea increased the value of total amino acid concentrations in grape must. The results of this study showed no clear behaviour. It could be a different factor such as berry maturity. Several studies have shown that the degree of berry maturity has a strong influence on its amino acid composition.[29,38] Amino acids accumulate during the first phase of berry growth (early fruit development).[36] Therefore, N must be made available to grapevines during fruit maturation to obtain well-developed berries with an amino acid concentration that will sustain vinification.[39] The main amino acids increased during maturation and before veraison, total amino acids increased slowly and after veraison, amino acid concentration increased rapidly to reach a plateau before maturity.[39]

Regarding amino acid concentrations, they were modified differently by the different doses of urea. GABA and Trp concentrations were not affected by urea applications. This agrees with Rodríguez-Lovelle and Gaudillère[39] and Jiménez-Moreno *et al.*[10] who classified GABA concentration as relatively independent of N supply rate. In both years, Gln and Arg were the most abundant amino acid in must samples. This result is consistent with that described by Stines *et al.*[38] and Rodríguez-Lovelle and Gaudillère[39] who described that Gln is the most predominant amino acid in early berry development and can act as a precursor of other amino acids as Pro and Arg via glutamate. Therefore, glutamine decreased throughout fruit development.[38] However, the role of Pro or Arg in the grape berry and the factors responsible for different patterns of Pro and Arg accumulation remain unknown.[38]

Proline concentration increases at the end of the grape ripening.[9,38] The concentration of this amino acid increases as the °Brix value increases in accordance with an exponential equation.[40] In addition, previous studies have linked Pro synthesis in plant tissues to osmotic stress,[41] and in the case of grapevines, with the high osmotic pressure caused by the accumulation of sugar during ripening.[42] In addition, Stines *et al.*[38] showed that the concentration of Pro changed only during the later stages of fruit ripening, when its accumulation paralleled the increasing sugar concentration. Comparing the result from 2018 and 2019 vintages, Pro concentration from 2019 vintage was higher than in 2018 vintage, because 2019 vintage was drier than 2018 vintage. This effect was described by Esteban *et al.*[40] who observed that the concentration of Pro was higher in the non-irrigated treatments than in irrigated treatments.

Arginine concentration increases during berry ripening. Moreover, the biosynthetic pathways of Arg and Pro are closely related.[38] In this study, Arg concentration was higher in berries samples sprayed with higher doses (9 kg N ha^{-1}). This could be that Arg is more related to the N nutrient status of the grapevine.[36] Nevertheless, Arg concentration was lower in 2018 vintage, this reduction could indicate the remobilization of N toward the storage organs (i.e., roots), in order to prepare for the following season. Alternatively, Arg could be converted to Pro or other compounds such as polyamines, guanidines and other amino acids.[43] This effect was observed by Bell and Henschke[6] and Garde-Cerdán *et al.*,[26] they described a reduction in Arg after maturation.

Ornithine concentration was a lower amino acid which was detected at low concentrations in berry in both vintages, this could be that Orn is an intermediate in the synthesis of Arg from glutamate and also a product of Arg degradation via arginase.[37]

The difference in the concentration of amino acids between the two vintages could be due to rainfall because it rained more in 2018 vintage than in 2019 vintage. Previous studies reported that the season had a strong significant effect on the average concentration of all amino acids[13,38] and the years with the mildest temperatures with little rainfall during the ripening process produced the grapes with the highest concentration of amino acids.[4]

Nitrogen fraction in grapes samples

The analysis of N fraction (NH_4^+, $NH_2^+$and YAN) of Tempranillo musts from 2018 and 2019 vintages are shown in Tables 3 and 4, respectively. In 2018 vintage, U3-Ver and U6-Ver reduced the NH_4^+, $NH_2^+$and YAN (Table 3). These decrease in the nitrogen fractions could be due to the fact that during ripening, NH_4^+ is transformed in amino acids, thus its concentration decreases.[44] However, no significant differences were observed in the other treatments in these three N fractions (Table 3). However, in 2019 vintage, the NH_4^+, $NH_2^+$and YAN concentrations rose in the U3-Ver must samples, and U9-Ver application improved the NH_2^+concentration in the musts. However, no significant differences were observed in the other treatments for these N fractions (Table 4). The results obtained during these two vintages showed no clear effect of foliar applications on the three N fractions. This agrees with Lasa *et al.*[3] who said that foliar application did not have a clear effect over YAN in grape juice. Moreover, in a previous study carried out on this vineyard, Pérez-Álvarez *et al.*[13] showed that urea application did not increase YAN concentration despite the fact that initial YAN concentration (control samples) was below 140 mg N L^{-1}. In other cases, YAN concentration did not increase when initial YAN concentration in control sample was higher than 140 mg N L^{-1}.[3,15,18] However, in other previous studies, YAN concentration increased when initial YAN concentration was lower[8,14,19] or higher[9-11,15-17,28] than 140 mg N L^{-1}.

Multifactorial analysis is shown in Table 5. This analysis was carried out by each amino acid concentration, total amino acids, total amino acids without Pro and N fractions (NH_4^+, $NH_2^+$and YAN) according to the main factors of variability (treatment, phenological stage and year) and their interactions. Moreover, in Table 5 are shown means and significant differences for each of the factors analysed.

The treatments factor had an intense significant effect on amino acid, total amino acids, total amino acids without Pro concentrations, $NH_2^+$and YAN concentration, except for GABA and Trp, which had no significant effect. Moreover, U9 treatment had a greater effect on amino acid concentration and YAN (Table 5).

The phenological stages factor had no effect on most of the amino acids concentrations. However, this factor had a strong significant effect on the average concentration of Asp, Pro, Orn and a weak significant effect on the average concentration of Glu, Gln, Cit + Thr, GABA, total sum of amino acids and NH_4^+ concentration. Consequently, the effect was higher at veraison samples (Table 5).

The year factor had a strong effect on most of the amino acids concentrations, total sum of amino acids, total amino acids without Pro and the N fraction (NH_4^+, $NH_2^+$and YAN) concentrations. On the contrary, this factor had no significant effect on Met, Trp and Orn concentrations in must samples. Thus, the highest amino acids concentration was found in musts from 2019 vintage (Table 5).

The interaction between treatment and phenological stage factors was dominant in Gln, Pro, Phe, Orn, total amino acids and total amino acids without Pro concentrations. However, the

Table 5. Multifactor analysis of variance of amino acids concentration (mg L^{-1}) and nitrogen fractions (mg N L^{-1}) in grapes from 2018 and 2019 vintages, for the different treatments: Control (C); foliar application with 3 kg N ha^{-1} (U3), 6 kg N ha^{-1} (U6) and 9 kg N ha^{-1} (U9), and different phenological stages: pre-veraison (Pre) and veraison (Ver)

	Treatments				Phenological stages		Years		Interactions						
Amino acids	C	U3	U6	U9	Pre	Ver	2018	2019	T	Ps	Y	T × Ps	T × Y	Ps × Y	T × Ps × Y
Asp	20.55a	22.54b	25.56c	22.96b	25.61b	20.19a	24.57b	21.24a	***	***	***	**	NS	***	**
Glu	48.57a	54.11b	58.27b	57.28b	52.88a	56.24b	51.54a	57.58b	***	*	***	**	NS	**	NS
Ser	21.94a	23.84ab	22.20a	24.81b	23.13a	23.27a	22.12a	24.28a	*	NS	**	NS	NS	NS	NS
Gln	72.53b	89.47c	62.89a	98.13d	77.93a	83.58b	78.15a	83.36b	***	*	*	***	***	NS	*
His	13.19a	16.94b	12.86a	19.46c	15.48a	15.74a	9.51a	21.72b	***	NS	***	NS	***	NS	**
Gly	0.83a	0.98b	0.83a	0.97b	0.90a	0.90a	1.08a	0.72a	***	NS	***	NS	**	NS	NS
Cit + Thr	27.30a	32.80b	31.28b	38.41c	31.24a	33.65b	24.02a	40.87a	***	*	***	NS	***	*	NS
Arg	56.44a	83.69b	56.06a	111.28c	75.06a	78.68a	59.98a	93.76b	***	NS	***	*	***	***	***
Ala	24.47a	29.35b	24.57a	30.78b	26.88a	27.70a	23.61a	30.98b	***	NS	***	*	***	*	NS
GABA	48.52a	54.19b	51.01ab	53.57ab	48.77a	54.87b	35.56a	68.09a	NS	**	***	NS	NS	NS	NS
Pro	20.69a	24.82b	20.57a	23.67b	21.07a	23.80b	10.99a	33.88b	***	***	***	***	**	*	*
Tyr	3.11a	3.57b	3.06a	4.22c	3.46a	3.52a	3.67b	3.30a	***	NS	**	**	*	NS	**
Val	10.03b	10.46b	8.13a	10.85b	9.54a	10.19a	10.6b	9.12a	***	NS	***	*	**	NS	*
Met	1.15b	1.20bc	0.92a	1.32c	1.19a	1.11a	1.16a	1.14a	***	NS	NS	NS	***	NS	***
Ile	5.99b	6.30b	4.78a	6.07b	5.62a	5.95a	6.38b	5.19a	***	NS	***	*	***	NS	***
Trp	11.46a	13.07b	11.91ab	12.45ab	12.22a	12.22a	12.24a	12.20a	NS	NS	NS	NS	NS	**	NS
Leu	7.17b	7.90b	5.64a	7.46b	6.87a	7.22a	6.48a	7.61b	***	NS	**	*	*	NS	*
Phe	6.16a	7.14b	6.17a	6.48a	6.35a	6.63a	5.87a	7.10b	**	NS	***	***	*	NS	*
Orn	0.56a	0.77b	0.57a	1.18c	0.87b	0.67a	0.80b	0.75a	***	***	NS	***	***	**	***
Lys	1.51a	1.77b	1.49a	1.89b	1.62a	1.71a	1.90a	1.44a	***	NS	***	NS	*	NS	*
Total amino acids	403.09a	485.92b	409.66a	534.20c	447.35a	469.08b	391.58a	524.85b	***	*	***	***	***	*	**
Total amino acids − Pro	382.40a	461.10b	389.09a	510.52c	426.28a	445.28a	380.59a	490.97b	***	NS	***	***	***	*	**
Ammonium nitrogen	85.68ab	92.15b	76.45a	84.67ab	81.36a	88.12b	69.11a	100.364b	*	*	***	NS	**	NS	**
Amino nitrogen	57.59b	59.45b	50.30a	64.33c	57.30a	58.53a	62.18b	53.654a	***	NS	***	NS	***	NS	*
YAN	143.27b	151.60b	126.75a	149.00b	138.66a	146.65a	131.29a	154.018b	***	NS	***	NS	***	NS	**

Note: Different letters indicate significant differences between treatments ($P \leq 0.05$). Not significant (NS), $P \geq 0.05$ (*), $P \leq 0.01$(**), $P \leq 0.001$(***). T: Treatment; Ps: Phenological stage; Y: year.

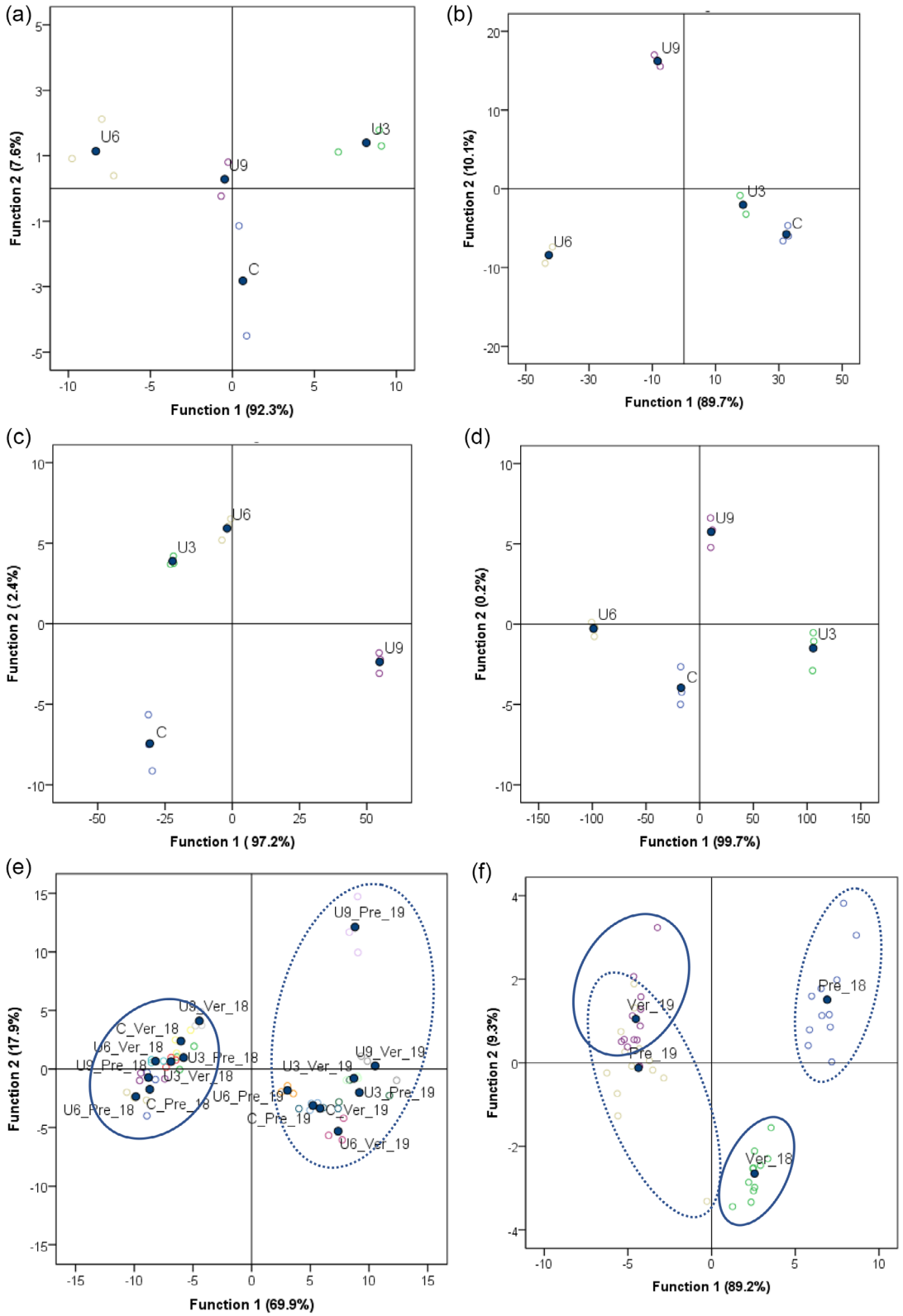

Figure 1. Discriminant analysis carried out with amino acid concentrations (mg L^{-1}) in Tempranillo grapes from 2018 and 2019 vintages, for the different treatments: Control (C); foliar application with 3 kg N ha^{-1} (U3), 6 kg N ha^{-1} (U6), and 9 kg N ha^{-1} (U9) and different phenological stages: pre-veraison and veraison. (a) Pre-veraison treatments in 2018 vintage, (b) veraison treatments in 2018 vintage, (c) pre-veraison treatments in 2019, (d) veraison treatments in 2019 vintage, (e) all treatments and control in 2018 and 2019 vintages and (f) pre-veraison and veraison in 2018 and 2019 vintages.

interaction between these factors did not affect Ser, His, Gly, Cit + Thr, Met, Trp, Lys and N fraction concentrations in must samples (Table 5).

The interaction between treatment and year factors were dominant in Gln, His, Cit + Thr, Arg, Ala, Met, Ile, Orn, total amino acids, total amino acids without Pro, $NH_2^+$and YAN concentrations.

However, this interaction had no significant effect on Asp, Glu, Ser, GABA and Trp concentrations in must samples (Table 5).

The interaction between phenological stage and year factors did not affect most of the amino acids concentrations in musts (Ser, Gln, His, Gly, GABA, Tyr, Val, Met, Ile, Leu, Phe and Lys). However, this interaction was dominant in Asp and Arg (Table 5).

The interaction between treatment, phenological stage and year factors did not affect Glu, Ser, Gly, Cit + Thr, Ala, GABA, Trp and N fraction concentrations in musts. However, the interaction between these three factors had a strong effect on Arg, Met, Ile and Orn concentrations (Table 5).

In summary, treatment, year and the interaction between treatment and year were the factors that most affected the amino acid concentration and N fraction of Tempranillo musts. While phenological stage, the interaction between treatment and phenological stage, the interaction between phenological stage and year, and the interaction between phenological stage, year and treatment explained only a minor fraction of the observed variation. Thus, the urea doses and climatological conditions are an important factor when urea is applied in vineyards. This disagrees with previous studies, such as Pérez-Álvarez *et al.*[13] who showed the effect of year on increase of must total amino acid concentrations is more important than effect of the doses applied.

Discriminant analysis of the must amino acid concentration

Figure 1 shows the discriminate analysis performed with amino acids concentration of control and treated samples (3, 6 and 9 kg N ha^{-1}) during 2018 and 2019 vintages, from pre-veraison treatments in 2018 vintage (Fig. 1(a)), veraison treatments in 2018 vintage (Fig. 1(b)), pre-veraison treatments in 2019 vintage (Fig. 1(c)), veraison treatments in 2019 vintage (Fig. 1(d)), all treatments applied in 2018 and 2019 vintage, including controls (Fig. 1(e)) and both vintages as phenological stage as discriminant factor (Fig. 1(f)).

Figure 1 exposed a clear separation of the musts from control and treatments. In 2018 vintage, the pre-veraison samples (Fig. 1(a)), Function 1 and Function 2 explained 92.3% and 7.6%, respectively (total of variance explained was 99.3%). Arg, Ala and GABA were the amino acids that most contributed to the constitution of both functions. The discriminant figure showed a good separation between samples. U3 treatment is located in the positive side, because the sample had more Arg and Ala content than control, U6 and U9 samples (Table 3). However, the veraison samples (Fig. 1(b)), Function 1 explained 89.7% and Function 2 explained 10.1% of this variance (99.8% of the total variance). The variables that contributed most to the discriminate model were Phe, Met, Leu and Tyr (Function 1) and Tyr, Leu, Met, Gln (Function 2). This discriminant figure showed a good separation among different treatments. U3 samples were located in the same sector and very close to the control samples, while U6 and U9 samples were located further away from the control samples. This distribution could be explained by the fact that the U9 samples have more Gln concentration and U6 samples had less Phe, Met and Gln concentration than control samples (Table 3).

However, in 2019 vintage, the pre-veraison samples (Fig. 1(c)), Function 1 explained 97.2% and Function 2 explained 2.4% of variance. The variables that contributed most to the discriminant model were Orn, Gly, Glu and Cit + Thr (Function 1) and Cit + Thr, Glu, Gly and Tyr (Function 2). This discriminant figure showed a clear separation between the treatment samples. U9 samples were located in the positive side because the samples had more Gly, Tyr and Orn concentration than control, U3 and U6 samples (Table 4). The veraison samples, (Fig. 1(d)) Function 1 explained 99.7% and Function 2 explained 0.2% of variance. The variables that contributed most to the discriminant model were Asp, Trp, Ala and Pro (Function 1), and Asp, Arg, Trp and Gln (Function 2). In this case, the separation between veraison samples was different (Fig. 1(d)), U9 and U3 treatments were positioned on the positive side, and control and U6 treatments were positioned on the negative side, this result could be owing to the fact that U9 samples had more Gln concentration than control, U3 and U6 samples. Figure 1(e) exposed a separation of the samples from treatment, phenological stages and year. Function 1 explained 69.9% and Function 2 explained 17.9% of this variance (86.8% of the total variance). The variables that contributed most to the discriminant model were Pro, Gly, Orn and Asp (Function 1), and Orn, Asp, Gly and Glu (Function 2). Function 1 separated musts from 2019 vintage, located on the positive side, from musts from 2018 vintage, located on the negative side. This coincides with a higher Pro concentration in the musts from 2019 vintage (Table 5).

Finally, Fig. 1(f) shows the results classify the samples from phenological stage and year. Function 1 explained 89.2% of the variance and Function 2 explained 9.3%. Function 1 separated must samples from 2018 vintage between 2019 vintage. The samples from 2018 vintage were located on the positive side, whereas samples from 2019 vintage were located on the negative side. The amino acids that contributed positively to the constitution of Function 1 were Asp, Trp, Gly and Val. Furthermore, Asp, Glu, GABA and Trp contributed to construct Function 2. This coincides with a higher concentration of Asp in the pre-veraison from 2018 vintage (Table 5).

CONCLUSIONS

Foliar urea application did not clearly modify must oenology parameters. During 2018 vintage, amino acids concentrations were modified by foliar application, mostly with lower urea dose (3 kg N ha^{-1}) and applied at pre-veraison. Moreover, 2019 vintage, U3 and U9 treatments, applied at pre-veraison and veraison, increased the amino acids concentrations. The year and the treatment were the most important factors influencing amino acid concentration in Tempranillo musts. In addition, the application at veraison was the best phenological stage to apply urea in order to increase amino acids concentrations in Tempranillo musts. Thus, the foliar application at veraison could be considered as a good strategy to increased N composition in Tempranillo grape.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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