



## RESEARCH ARTICLE

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# Leaf blade and petiole nutritional evolution and variability throughout the crop season for *Vitis vinifera* L. cv. Graciano

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## Abstract

An adequate nutritional state of a crop can be kept by means of a well-designed fertilization plan based on the assessment of the nutrient availability throughout the growing season. The objective of this study was to determine the reliability of leaf blade and petiole diagnosis and the period of validity of their references at both flowering and veraison by means of systematic monitoring throughout the complete growing season. The study was carried out in two plots planted with *Vitis vinifera* L. cv. Graciano within the AOC Rioja (Spain). Blades and petioles were collected throughout a growing season (2006) and total N, P, K, Ca, Mg, Fe, Mn, Zn, Cu and B concentrations were analyzed in both tissues. Results suggest, in general, that petioles have higher variability and lower analysis reproducibility than blades. Therefore, blade could be a more appropriate tissue to evaluate N, P, K, Ca, and Mg at both flowering and veraison in this variety. Micronutrients in blade and petiole showed different variability behaviour in each of the vineyards studied, therefore, based on our results, it was difficult to determine which one could be the best tissue for the nutritional diagnosis of the 'Graciano' variety. Seasonal changes of nutrient concentration in both tissues also confirmed the need for reference values for each tissue and each phenological stage.

**Additional key words:** nutrition; grapevine; leaf analysis; nutritional references.

**Abbreviations used:** AOC (appellation d'origine contrôlée or appellation of controlled origin); CV (coefficient of variation percentage, %); DOP (deviation from optimum percentage method); DRIS (diagnosis and recommendation integrated system); DW (dry weight); PC (principal component); PCA (principal components analysis); RA (Rioja Alta vineyard); RM (Rioja Media vineyard).

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## Introduction

Fertilization is one of the farming techniques which the wine-grower uses to modify the nutritional status of the plant in search of the correct balance between yield and crop quality. Nevertheless, an inadequate application of fertilizers can unbalance the availability of nutrients, negatively affecting the nutritional status of plants, the final harvest quality and even the environment. The current nutritional status of plants is key in designing an adequate fertilization plan.

The knowledge of the desirable level for each nutrient in representative tissues allows to define its excess or deficiency in the plant and the design of a correct

fertilization program. Therefore, nutrition can be monitored by the analysis of the chosen tissues, applying the different available methodologies of plant diagnosis such as diagnosis and recommendation integrated system (DRIS), deviation from optimum percentage (DOP), sap analysis from conductive tissues, flower analysis, analyses of active metabolites (e.g. N-NO<sub>3</sub><sup>-</sup> in petiole), or even studies of specific enzymatic activity (Cook & Kishaba, 1956; Bonilla *et al.*, 1980; Lucena, 1997; Robinson, 2005).

Many of the diagnosis methodologies of leaf tissue use the individual total nutrient concentrations in dry tissue, comparing those values to a reference. However, the physical and chemical characteristics of the

soil, rootstock, variety, climatic conditions, cultural practices, possible diseases, training system, sampling time and the analyzed organ could modify the mineral composition of the plant (Delas, 1990; Benito *et al.*, 2013; Romero *et al.*, 2013) reducing the accuracy of these methods. Thus, it is essential to standardize the sampling method for nutritional diagnosis (Delas, 2000) and a wide range of criteria are used with respect to the sampling time, the analyzed tissue, or its position on the plant (Romero *et al.*, 2010, 2013; Benito *et al.*, 2013).

Current bibliography shows a disparity of opinions with regard to the tissue and the ideal moment for sampling with diagnostic purposes. Concerning the organ to be analyzed, initially the whole leaf (blade and petiole together) was used, before analyzing the leaf blades and petioles separately. With respect to sampling time, different papers propose two phenological stages: end of flowering or fruit-set, and/or veraison. For instance, Failla *et al.* (1995) used the leaf blade at flowering and veraison; Navarro *et al.* (2008) analyzed the leaf blade at veraison; García-Escudero *et al.* (2002) and Wolpert & Anderson (2007) chose flowering and veraison phenological stages analyzing both leaf blades and petioles separately; Christensen (1984) and Robinson (2005) analyzed petioles at flowering, while Delas (2000) used petioles at veraison.

Previous studies with cv. ‘Tempranillo’ and cv. ‘Red Grenache’ showed the usefulness of carrying out monitoring studies throughout the complete crop season in order to determine the reliability of leaf blade and petiole diagnosis and the period of validity of their references (Benito *et al.*, 2013; Romero *et al.*, 2013). According to these and other studies (Fraguas *et al.*, 2003; Wolpert & Anderson, 2007; Benito *et al.*, 2013; Romero *et al.*, 2013), the higher variation for nutrient concentrations in petiole implied, in general, a lower reproducibility for this tissue. Comparing the analysis of blade and petiole, Christensen (1984) and Champagnol (1990) indicated that the variation intervals of phosphorus (P), potassium (K) and magnesium (Mg) in petiole were higher than in leaf blade. Nevertheless, Champagnol (1990) suggested that the significance of a result obtained from both tissues would be identical; also other studies showed that the individual variability for each nutrient was sometimes different between different vine varieties (Benito *et al.*, 2013; Romero *et al.*, 2013). On the other hand, the nutrition level for each element can change widely among different vineyards, especially with respect to K and Mg nutritional status in the vines (Champagnol, 1990; Loué, 1990).

‘Graciano’ is a grapevine variety with a Spanish origin which is currently grown in other winemaking regions, *e.g. for example* Australia, California and

Argentina. ‘Graciano’ is also grown in the French-Midi and South Africa (cv. Morrastel among other synonyms), and Portugal (cv. Tinta Miuda).

The objective of this study was to determine the reliability of leaf blade and petiole diagnosis of ‘Graciano’ variety by means of systematic monitoring studies throughout a complete growing season. This procedure also allows us to establish the period of validity of a reference suggested for both flowering and veraison phenological stages, the periods for which fertilization references are usually set.

## Material and methods

A year-long (2006) monitoring study was carried out in two AOC Rioja vineyards. Both were representative of an average vineyard within the Rioja region and their yield and wine quality were within the usual values for the AOC Rioja. Vineyards were located in Haro, sited at the Rioja Alta subzone (RA) within the AOC, and Logroño, sited at the Rioja Media subzone (RM) (La Rioja, Spain). They were more than five years old and had similar agronomic characteristics.

The vineyards were planted with cv. Graciano (*Vitis vinifera* L.) grafted on 110 Richter rootstock. Plants were trained to a single cordon on a vertical-shoot-positioned (VSP) trellis system and pruned leaving 10 to 12 buds per vine. Planting density was 3,100 vines/ha for RA and 3,350 vines/ha for RM. The experimental design divided each vineyard in three homogeneous subplots, with 108 vines per subplot distributed in three consecutive rows, to obtain the three necessary replicates to perform statistical analyses.

The recorded annual rainfall was 468 mm in RA and 405 mm in RM. The vineyards were not irrigated and both were managed according to the ordinary practices used by growers in the region.

The grape juice quality parameters were optimal at harvest with values within those common for the AOC Rioja. The RA vineyard showed an average bunch weight of 400 g, while grape juice showed a sugar concentration of 17.8 °Brix (refractometer), titratable acidity was 7.28 g/L tartaric acid, and pH was 3.09 (pH meter). On the other hand, for the RM vineyard the average bunch weight was 344 g, and the grape juice showed a sugar concentration of 25.0 °Brix, titratable acidity was 6.78 g/L tartaric acid and pH was 3.32.

The RA soil was classified as typic *Calcixerepts* while the RM soil was classified as typic *Xerofluvents* (Soil Survey Staff, 2010). Both soil textures were loamy in the topsoil ( $A_p$ ) and in the subsurface horizon (B). Physical and chemical properties are shown in Table 1.

**Table 1.** Physical and chemical properties of the soils of two vineyards, RA (Haro) and RM (Logroño), used in the study at the two surface horizons at different depths (0-30 cm, 30-60 cm, 0-39 cm, 39-78 cm)

Properties	RA		RM	
	AP	B	AP	BW
	0-30	30-60	0-39	39-78
Organic matter, % (DW)	1.71	1.53	1.18	0.94
Total carbonate <sup>1</sup> , % (DW)	16.2	20.9	13.4	13.2
Active limestone <sup>2</sup> , % (DW)	8.8	11.5	4.4	4.8
pH <sup>3</sup>	8.5	8.2	7.6	7.5
EC <sup>3</sup> (mmhos/cm)	0.15	0.18	0.38	0.15
P <sup>4</sup> (mg/kg DW)	64.1	32.4	27.4	6.9
CEC <sup>5</sup> (mmolc/kg DW)	162	170	94	96
Ca <sup>6</sup> (mmolc/kg DW)	163	178	102	100
Mg <sup>6</sup> (mmolc/kg DW)	9.0	8.8	12.6	16.0
K <sup>7</sup> (mmolc/kg DW)	18.2	12.6	5.0	2.0
Na <sup>7</sup> (mmolc/kg DW)	3.1	3.0	6.0	3.0

DW: dry weight. <sup>1</sup>Bernard calcimeter. <sup>2</sup>Drouineau method. <sup>3</sup>pH and electrical conductivity, 1:5 (v:v), 25°C. <sup>4</sup>Olsen method. <sup>5</sup>Cation exchangeable capacity, NaAc (1 M) and Na determination by atomic absorption. <sup>6</sup>Cobalt hexamine extraction. <sup>7</sup>NH<sub>4</sub>Ac (1 M) extraction.

## Sampling

An exhaustive leaf sampling, according to the sampling intervals shown in Table 2, was carried out from pre-flowering to post-harvest. Thirty complete, disease free and non-senescent leaves were sampled in each replicate. Sampling was carried out between 10 a.m. and 12 a.m., taking one leaf per plant, proceeding from fruit-bearing shoots of average vigour, out of 30 vines chosen at random within each replicate. The leaves collected were opposite to the first bunch from pre-flowering until pre-veraison, and opposite to the second bunch from veraison to ripening (Romero *et al.*, 2010). Sunlight exposure over the canopy was also considered at sampling time and, therefore, leaves were sampled alternatively on both sides of the trellis. Samples were codified using the percentage of time that had elapsed in each phenological stage (Table 2).

## Leaves chemical analysis

Leaf blades and petioles were separated, washed three times with tap water, and then rinsed with distilled water and oven-dried (Dry-big; J.P. Selecta,

Barcelona, Spain) at 70°C for 48 hours. Finally, dried samples were ground and homogenized with an ultra-centrifugal mill (ZM1; Retsch, Haan, Germany), in order to pass through a 0.5 mm mesh.

For total N determination (N-organic+N-NH<sub>4</sub><sup>+</sup>) in leaf blades and petioles, 0.20 g of the ground sample was used to carry out a wet digestion using the Kjeldahl method (Jones *et al.*, 1991). For the remaining nutrients (P, K, Ca, Mg, Fe, Mn, Zn, Cu and B), 0.20 g was used to carry out a wet digestion with 95% H<sub>2</sub>SO<sub>4</sub> and 30% H<sub>2</sub>O<sub>2</sub> (Hoenig *et al.*, 1998) and afterwards were analyzed by inductively coupled plasma-optical emission spectrometry (Optima 3000DV, Perkin Elmer, Norwalk, CT, USA). Double deionised water (Milli-Q; Milli-pore, Bedford, MA, USA) was used for all dilutions. Concentrations were expressed on a dry weight (DW) basis.

## Statistical analysis

A principal components analysis (PCA) was performed to detect the vectors that differ from the ten dependent variables (N, P, K, Ca, Mg, Fe, Mn, Zn, Cu, B) data. A non-rotated component matrix was used as factor analysis and the extraction method was performed by a scree plot with eigenvalue >1. The variables were adequately represented within the model. Furthermore, comparisons for equality of means, between blade and petiole tissues at each sampling time, were performed using the parametric t-Student test.

Nutrient trends were fitted with Tablecurve 2D (vers. 5.1; SYSTAT Software Inc., San Jose, CA, USA). The coefficient of variation (CV) percentage between replicates was analyzed for each nutrient, tissue, and sampling date as a measurement of the reproducibility of the analysis within each vineyard.

Finally, the differences between consecutive sampling dates were determined by means of a monofactorial ANOVA followed by a post-hoc test (Tukey,  $p \leq 0.05$ ) in order to define the periods of validity for references at different phenological stages. The ANOVA procedure was performed by comparing groups of five consecutive samplings, as a *moving window* throughout the growing season, displacing the ANOVA procedure throughout the period to the next sampling and discarding the first sampling of the previous ANOVA to assemble the new group of five samplings to perform the next ANOVA procedure. Furthermore, when a long period with no significance differences was detected, the first sampling of the first *window* was additionally compared with the last four samplings of the last *window* to confirm the absence of differences over the whole period. This procedure prevents error accumulation as it increases the number of comparisons within the ANOVA.

**Table 2.** Sampling calendar: days after bud break (DAB), phenological stage, sampling code, and E-L development stage in two vineyards: RA (Haro) and RM (Logroño)

Sampling	DAB			Phenological stage		
	RM	RA	Code <sup>1</sup>	Description	E-L number <sup>2</sup>	
1	35	38	H25	1 month	16	
2	41	47	H75	20 days	before flowering	18
3	47	–	H100	2 weeks		19
4	55	–	I40	40%		
5	–	59	I50	50%	caps fallen (flowering)	23
6	62	–	I100	100%		26
7	70	69	J		fruit set	27
8	–	77	K25	25%		29
9	77	–	K30	30%		
10	–	89	K50	50%		31
11	83	–	K60	60%	berry growth	
12	91	–	K70	70%		
13	98	101	K75	75%		
14	103	–	K80	80%		
15	111	–	K90	90%		32
16	119	111	L		closed cluster	33
17	126	–	M5	5%		
18	–	123	M15	15%		
19	134	–	M25	25%		
20	140	–	M50	50%	berries coloured (veraison)	35
21	–	133	M60	60%		
22	147	–	M75	75%		
23	155	143	M100	100%		
24	–	161	N50	50%	ripening	37
25	–	188	N100	100%		38
26	–	209	O		Post-harvest	43
Total samples	18	14				

<sup>1</sup>Code: Numerical value is the percentage of time that had passed in each phenological stage. <sup>2</sup>E-L number: Eichhorn and Lorenz phenological stage according to Coombe (1995)

Statistical procedures were performed with SPSS (vers. 15.0; SPSS Inc., Chicago, IL, USA) and Statgraphics Plus (vers. 5.1; Manugistics, Inc., Rockville, MD, USA).

## Results

### Principal components analysis

PCAs are employed to reduce a large set of inter-correlated variables to a smaller number of uncorrelated variables. When applying this method, if the variables are highly correlated, some of the first components are able to describe most of the data variability. In our study three principal components (PC) contained approxi-

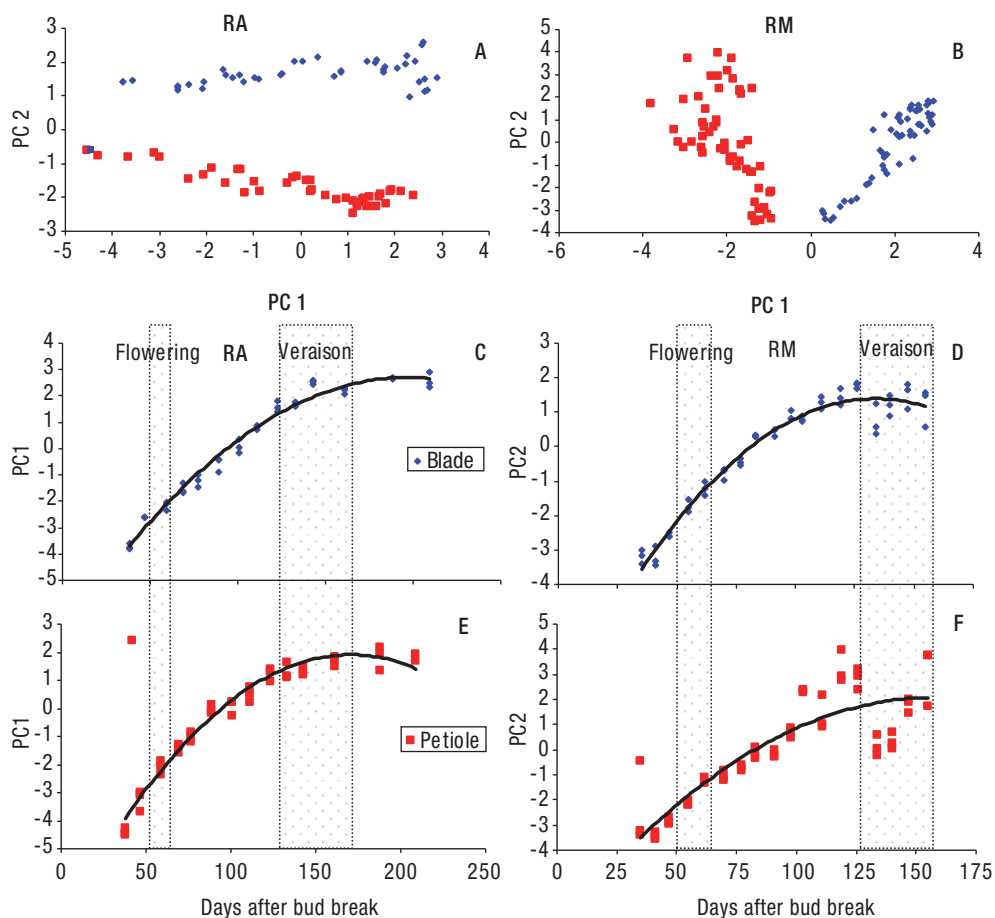
mately 83% of the total variance of the ten original variables (Table 3). The first PC (PC1) explained 41% of the total variance for both vineyards. The second component (PC2) explained 30% of the variance for the RA and 32% for the RM dataset. The third component (PC3) explained 12.6% of the variance for RA and 10% for RM.

The highest component loadings for the RA vineyard were: P, K, Ca, Mn and B for PC1, N, Mg, and Fe for PC2, and Zn for PC3. On the other hand, the highest component loadings for the RM vineyard were: K, Mg, Fe, Zn, and Cu for PC1, N, P, Ca, and Mn for PC2, and B for PC3. The PCA for the RA dataset showed the existence of a strong relationship between PC1 and the phenological stage as well as between PC2 and the analyzed tissue (Fig. 1A). Inversely, for the RM dataset, PC1 showed a strong relationship with the tissue and

**Table 3.** Principal components analysis matrix. Component loadings for the original variables (nutrient concentrations) and the first three standardized variables (PCs); relative variance contribution of each PC to the total variance (%) of the data set; and cumulative percentage of total variance due to PCs in two vineyards, RA (Haro) and RM (Logroño)

Variables	RA			RM		
	PC1	PC2	PC3	PC1	PC2	PC3
	Phenological stage	Tissue	–	Tissue	Phenological stage	–
N	-0.506	<b>0.794</b>	0.115	0.631	<b>-0.648</b>	-0.207
P	<b>-0.887</b>	0.206	0.343	-0.135	<b>-0.875</b>	-0.268
K	<b>-0.653</b>	-0.474	0.214	<b>-0.775</b>	-0.244	0.337
Ca	<b>0.935</b>	0.240	-0.070	0.295	<b>0.923</b>	0.073
Mg	0.487	<b>-0.827</b>	-0.089	<b>-0.752</b>	0.591	-0.096
Fe	0.327	<b>0.919</b>	-0.002	<b>0.907</b>	0.264	0.150
Mn	<b>0.878</b>	-0.007	0.116	-0.319	<b>0.790</b>	-0.311
Zn	0.097	-0.148	<b>0.867</b>	<b>-0.901</b>	-0.078	-0.111
Cu	0.568	<b>0.622</b>	0.264	<b>0.808</b>	0.419	0.191
B	<b>-0.571</b>	0.313	-0.483	0.094	-0.351	<b>0.695</b>
Variance (%)	41.17	29.90	12.56	40.69	31.51	10.05
Cumulative percentage	41.17	71.07	83.64	40.69	72.20	82.25

<sup>1</sup> Loadings in bold: higher loading values for each original variable and vineyard.



**Figure 1.** Principal components analysis (PCA): PC1 vs PC2 for RA (A) and RM (B) vineyards. Seasonal evolution of PC1 (RA) and PC2 (RM) vs time for blade (◆) (C and D) and petiole (■) (E and F).



PC2 with the phenological stage (Fig. 1B). These two components together explained more than 71% of the total variance in both cases.

The positive correlations between the original nutrient concentrations and PC2 for RA, and between those concentrations and PC1 for RM, indicated that nutrient concentration in blade was higher than in petiole (Table 3). Therefore, blades showed higher concentrations than petioles for N, Fe and Cu, and slightly higher for Ca and B (Figs. 2 & 3; Table 4). Consequently, petioles had higher concentrations of K, Mg, and, to a lesser extent, Mn, and Zn. Phosphorus did not show clear behaviour as it had higher concentration in blades from RA but lower in blades from RM (Table 4; Figs. 2C,D).

Concerning the phenological stage, component loadings of the original nutrient concentrations for PC1 for RA, and for PC2 for RM (Table 3) indicated that the nutrients which positively correlated with the PC showed an accumulating trend in the analyzed tissue throughout the growing cycle (Table 3; Figs. 1C,D). That was the case for Ca, Mg, Fe, Mn, and Cu; while N, P, K, and B showed a negative correlation with the PC and, therefore, a reduction in the concentration in both tissues throughout the growing season. Finally, Zn showed a low correlation with respect to the PC, which indicates that the Zn requirements were relatively unsteady throughout the crop season in both tissues, mainly for the RA vineyard (Table 3; Figs. 3E,F).

The third principal component (PC3) explained 12% and 10% of the global variance, showing a low influence in the total variability compared to the other PCs, phenological stage and tissue (Table 3).

## Trends in nutrient concentrations

Nitrogen showed maximum concentration at pre-bloom sampling. Its concentration decreased over the course of the growth cycle until a stabilization trend for blade was noted around the veraison phenological stage (Figs. 2A,B). Phosphorus also showed a declining pattern of concentration from flowering to harvest (Figs. 2C,D).

Potassium showed a declining pattern for blades (Figs. 2E,F). However, petioles only showed this pattern for RA vineyard (Fig. 2E), while petioles for RM showed an irregular trend throughout the growth cycle (Figs. 2F). On the other hand, Ca and Mg showed an increasing concentration in both tissues (Figs. 2G-J). In general, macronutrients showed a nutrient evolution adjusted to a logarithmic curve in blade and petiole, with a decreasing slope until values near to zero when the veraison phenological stage was reached (Figs. 2A-J).

With respect to micronutrients, trends were not as easily recognizable as for macronutrients (Fig. 3). Some

micronutrients showed a logarithmic trend (B, Fe and Zn in petiole for RM); others showed a polynomial fitting (Fe and Cu in petiole and Zn in blade for RA, as well as Cu for RM); and the others fitted to a sigmoidal curve (Fe, Mn, and B in blade and Zn in petiole for RA). Those nutrients with a sigmoidal curve showed two periods where the slope tends to zero, the first around flowering and the second at veraison (Fig. 3).

Iron concentration increased throughout the cycle except at the beginning of bloom and at veraison for both tissues (Figs. 3A,B). Manganese concentration increased in both tissues until veraison, and then its concentration remained stable until the end of the growth cycle (Figs. 3C,D).

Zinc concentration in RA decreased until half of the berry growth phenological stage, increasing thereafter until the end of veraison and then maintaining concentration levels up to harvest in leaf blade and petiole (Fig. 3E). However, Zn concentration in blades from the RM vineyard maintained its levels after fruit set until the end of veraison, while the petiole showed a constant Zn concentration increasing from half of the berry growth until the end of veraison which denoted a different behaviour (Fig. 3F).

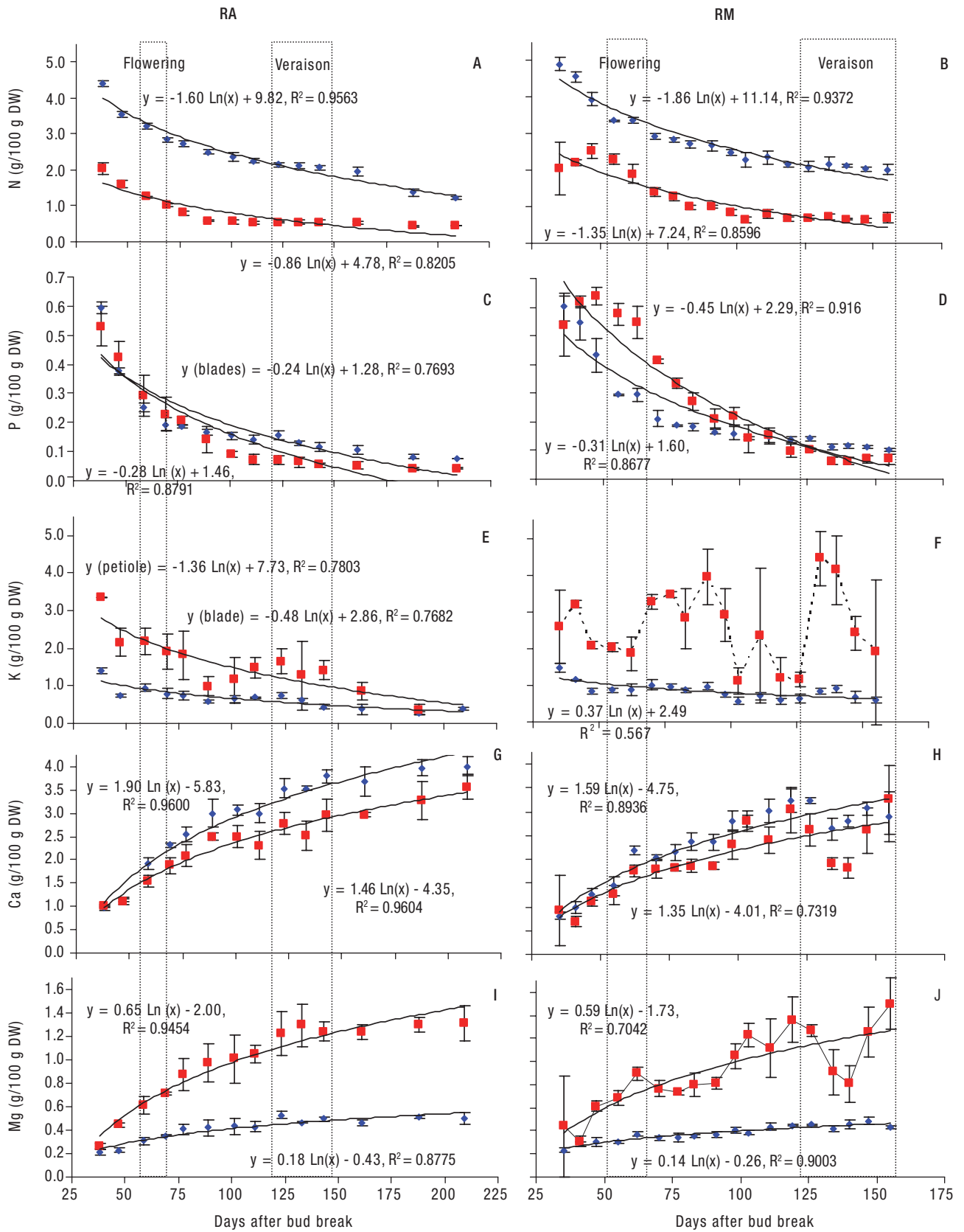
Copper maintained relatively constant levels of concentration in both leaf blades and petioles until flowering and even to early pea-sized berries. However it was noted that the application of phytosanitary sprays with a copper-based formulation prompted an increase in blade Cu concentration in the early maturation stage (Figs. 3G,H).

Finally, B concentration increased at the beginning of the cycle, afterwards showing a sharp decrease in blades (Figs. 3I,J).

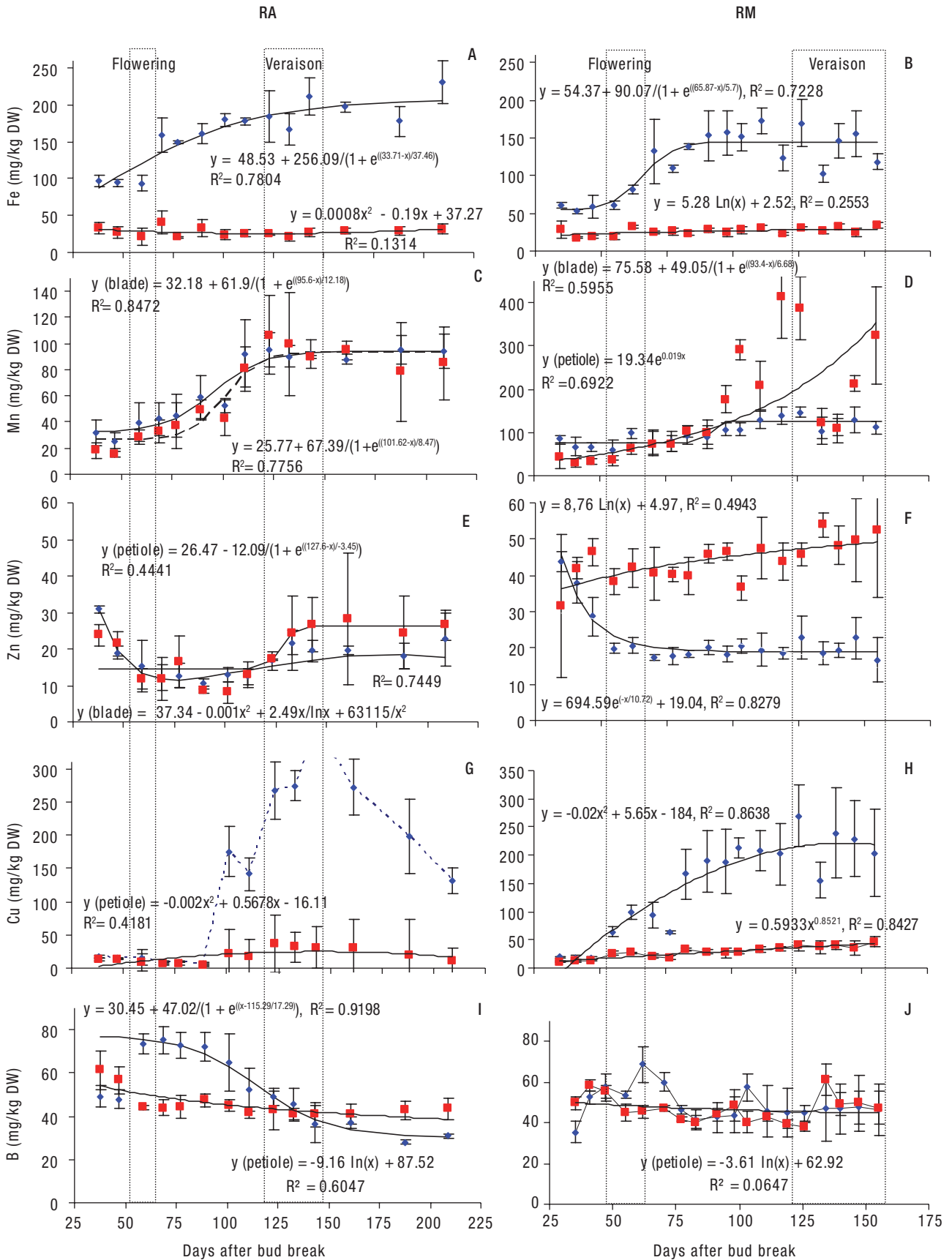
## Stability periods

A stability period is defined as the period where the nutrient concentration remains stable, allowing its comparison with a reference which, therefore, would be reliable for that complete period. However, the length of each stability period is difficult to be delimited only since the slope. Due to it, a stability period could also be defined by the absence of statistical differences between consecutive samplings.

Different stability periods of various lengths in time, were obtained for each nutrient and tissue because of the different behaviour of the nutrient concentration throughout the season. From these different stability periods, the period of validity for references proposed for flowering or veraison stages was determined. Furthermore, a period of sampling for blade or petiole could be also delimited for a common diagnosis of all the analyzed nutrients.



**Figure 2.** Seasonal trend for blade (◆) and petiole (■) macronutrients concentration (g/100 g DW) throughout the growing cycle in two vineyards, RA (Haro) and RM (Logroño).



**Figure 3.** Seasonal trend for blade (♦) and petiole (■) micronutrients concentration (mg/kg DW) throughout the growing cycle in RA (Haro) and RM (Logroño).



**Table 4.** Significance level for the difference between blade and petiole nutrient concentrations throughout a growing season in two vineyards, RA (Haro) and RM (Logroño)

Phenological stage	DAB <sup>1</sup>	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	B
<b>RM</b>											
Pre-flowering	35	***	NS	NS	NS	NS	**	*	NS	**	*
	41	***	NS	***	*	NS	***	*	NS	*	*
	47	***	**	***	NS	***	**	**	**	*	NS
Flowering	55	***	***	***	NS	***	***	NS	***	***	*
	62	***	***	**	**	***	***	*	***	***	**
Fruit setting	70	***	***	***	NS	***	**	NS	**	**	***
Berry growth	77	***	***	***	*	***	***	NS	***	***	*
	83	***	**	**	**	***	***	NS	***	**	NS
	91	***	*	***	**	***	***	NS	***	**	NS
	98	***	*	**	*	***	***	*	***	**	NS
	103	***	NS	*	NS	***	***	***	***	***	**
	111	***	NS	NS	*	**	***	*	**	***	NS
	Closed cluster	119	***	*	NS	NS	***	***	**	***	**
Veraison	126	***	***	**	*	***	***	**	**	***	*
	134	***	**	***	**	**	***	NS	***	***	NS
	140	***	**	***	***	**	***	NS	***	**	NS
	147	***	**	***	NS	***	***	**	**	**	NS
	155	***	*	NS	NS	***	***	*	*	*	NS
<b>RA</b>											
Pre-flowering	38	***	*	***	NS	*	***	*	***	*	*
	47	***	NS	***	NS	***	***	*	NS	*	NS
Flowering	59	***	NS	**	**	***	***	NS	NS	NS	***
Fruit setting	69	***	NS	**	**	***	***	NS	NS	NS	***
Berry growth	77	***	*	*	*	**	***	NS	NS	**	***
	89	***	NS	*	*	**	***	NS	NS	**	**
	101	***	***	NS	**	**	***	NS	*	***	*
Closed cluster	111	***	**	**	*	***	***	NS	NS	***	NS
Veraison	123	***	***	**	**	***	***	NS	NS	***	NS
	133	***	**	NS	**	***	***	NS	NS	***	NS
	143	***	***	***	**	***	***	NS	NS	***	NS
Ripening	161	***	**	*	**	***	***	NS	NS	***	NS
	188	***	***	NS	*	***	***	NS	NS	**	**
Post-harvest	209	***	***	*	*	***	***	NS	NS	***	**

<sup>1</sup> DAB: days after bud break. <sup>2</sup> Significant differences at \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$  (t-Student test). <sup>3</sup> NS: non-significant differences.

## Leaf blades

Fig. 4 shows the stability periods for macronutrients and micronutrients concentration in blades for both vineyards. Only those periods which included the flowering or veraison phenological stages are shown.

Blade results showed that there was no period which included the complete flowering phenological stage for any nutrient in the RA vineyard (Fig. 4) because of the absence of sufficient samplings within this phenological stage for this vineyard (Table 2). Nevertheless, for N, P, K, Mn, Zn and B a period which included the

Blade Code	Pre-flowering			Flowering			Berry development										Veraison					Ripening		Harvest			
	H25	H75	H100	I40	I50	I100	J	K25	K30	K50	K60	K70	K75	K80	K90	L	M5	M15	M25	M50	M60	M75	M100	N50	N100	O	
RA				3.21a			2.84b	2.74b								2.26c		2.14c		2.12cd				1.96cd			
RM				3.35a		3.36d	2.93b	2.84b								2.15c	2.09c	2.18c	2.14c		2.06d		2.02c				
RA				0.25a			0.16b	0.19b	0.17b			0.15c				0.14c	0.16c			0.13d				0.11d			
RM				0.29a		0.29a	0.21b	0.19b	0.18b			0.16bc	0.14c	0.15c	0.14c	0.11c	0.14c			0.11c		0.11c		0.11c			
RA				0.94a			0.80a					0.65b				0.69b	0.73b			0.62bc				0.44c		0.38c	0.28c
RM			0.83b	0.87b		0.87b	1.01a	0.97a	0.86a	0.96a						0.64b	0.85b	0.90b		0.68b	0.6b						
RA				1.92c			2.31b	2.56b								2.82a	2.87a	3.01a	3.23a		3.53a			3.79a		3.68a	
RM			1.26d	1.43d		2.18c	2.03c	2.14c								2.64b	2.80b			3.07b	2.89b						
RA				0.31c			0.35c	0.41c				0.43b				0.43b	0.53ab			0.47ab				0.46a	0.51a	0.50a	
RM				0.28d	0.31d	0.37c	0.34c	0.34c	0.35c	0.37c						0.43b	0.45b	0.42b	0.45ab	0.48a	0.43a						
RA	97.0c	94.4c		93.8c			159.1b	149.3b	161.1b	180.1ab	177.5ab	180.1ab	177.5ab			183.5a	167.4a			210.9a				197.25a	171.31a		
RM	60.5d	52.71d	59.24d	60.18d		81.4c	132.5a	109.2a	138.6a	153.3a	157.1a	123a	157.1a			145.4ab	145.4ab	103b	155.8a	118.5a							
RA				39.7b			42.5b	45b	59b	52.4b						92.1a	95.4a			92.1a				87.7a	94.9a	93.9a	
RM	85.0b	66.8b	66.8b	58.4b	97.9b		77.1b	79.6b	93.1b	89.4b	106.7b	111.9b	128.4ab	139.9a		146.9a	104.1a	112.4a	129.9a	111.5a							
RA				15.3b			12.1b	12.6b	10.5b	12.9b						16.8a	21.7a			19.6a				19.5a	18.1a	22.6a	
RM				19.9a	20.6a		17.4a	17.8a	18.3a	20.3a	18.3a	20.4a	19.4a	18.6a		22.9a	18.6a	19.3a	22.7a	16.6a							
RA				73.5a			75.3a	72.6a	72.2a	64.6a						48.8b	45.3bc			36.5c				37.16c			
RM	52.6b	57.3b	68.9a	55.8b			60.0a	60.0a	41.6b	42.7b	43.8b	57.4b	45.7b	45.3b		44.8b	46.9b	47.5b	46.4b								

**Figure 4.** Stability periods for leaf blade nutrients concentration in RA (Haro) and RM (Logroño). Different letters among phenological stages express significant differences at  $p \leq 0.05$  (Tukey test). Shaded periods show common periods for a general diagnosis of all nutrients for ‘Graciano’.

complete flowering stage was found for the RM vineyard. This period was limited to flowering for N and P, and was slightly broader for K and B. For Mn, this period was extended to K75, and even until complete veraison for Zn due to the high variability of the Zn concentration in blade at some moments during the growing cycle (Fig. 4). This latter fact was the origin of the absence of significant differences between samplings for Zn at RM.

The RM vineyard showed a period of stability which included the whole of veraison (the last sampling time of this vineyard) for N, P, K, Mn, Zn and B. However, the stability period for Ca ranged from 25% to 100% of veraison, and for Mg and Fe it comprised 50% to 100% of veraison (Fig. 4).

On the other hand, in the RA vineyard only Fe and Mn had a stability period that lasted all veraison while the other nutrients showed stability periods which ranged, in general, between M50 (50% of veraison) and N50, or 50% of ripening (Fig. 4).

## Leaf petioles

The stability periods for petioles at both vineyards are shown in Fig. 5. For petioles at flowering, the nutrient concentration and thus the stability periods behaved similarly to blades. Petiole results showed that there was not a period which included the complete flowering phenological stage for N, P, Ca, Fe and B in the RA vineyard (Fig. 5). However, the absence of sampling for H100 and I40 from this vineyard (Table 2) rules out an adequate evaluation of the flowering stability period length for the RA vineyard. On the other hand, Ca, Mg and Fe had stability periods that did not include the complete flowering stage in the RM vineyard (Fig. 5). Therefore, Ca, Mg and Fe would need one reference at the beginning of flowering and another at the end due to the rapid change in the concentration of these elements in petioles (Fig. 5).

With respect to veraison, the RA vineyard showed a stability period which included this phenological stage completely for all nutrients except Ca, which ranged from M15 to 50% of ripening (N50). Nevertheless, the RM vineyard only showed a stability period for the complete veraison phenological stage for N, P and Fe. Furthermore in the RM vineyard, Zn and B showed a stability period from M15 until the end of veraison (the last sampling time of this vineyard), K from M50 to the end of veraison, and finally Ca, Mg and Mn only showed a stability period within M15 to M75 phenological stages (Fig. 5). In these cases, a reference obtained at M50 for Ca, Mg and Mn would not be valid for a sampling at the beginning (M5) or at

the end (M100) of this phenological stage due to the change in the concentration of these elements in petioles (Fig. 5).

## Coefficient of variation and analysis of reliability

The CV is used to determine the most appropriate tissue for nutritional diagnosis. The maximum CV values for macronutrients were, in general, greater in petioles than blades. The greatest differences between both tissues were found mainly at veraison especially for N, K and Ca in the RM (Figs. 6B,F,H). At flowering, differences between both tissues were often lower than at veraison (Fig. 6).

Micronutrients did not show clear differences between blades and petioles. The maximum CV values were usually lower than 50% and, in general, higher in petioles than blades (Fig. 7). Furthermore, differences were found between vineyards. Iron, Mn and Zn showed greater CV in petioles from RA vineyard throughout the crop season (Figs. 7A,C,E) whereas results from RM did not show such marked differences.

## Discussion

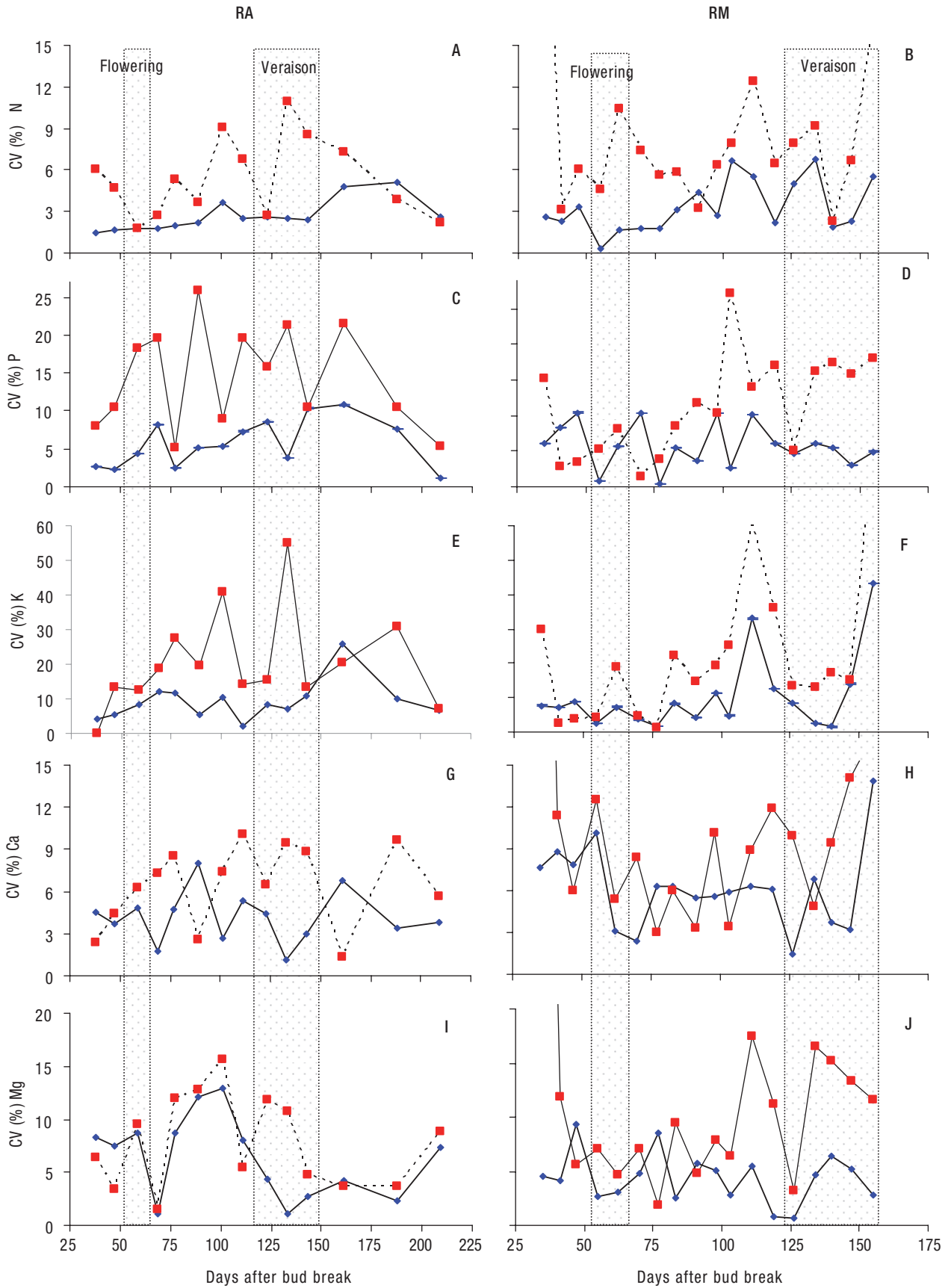
### Principal components analysis

The differences between both tissues for N has been described for varieties such as 'Tempranillo' (Romero *et al.*, 2013), 'red Grenache' (Benito *et al.*, 2013) and others (Christensen, 1984; Champagnol, 1990; Kliewer, 1991). Besides this, the higher concentrations in blades for Ca, Fe, B and Cu have also been observed in previous reports (Christensen, 1984; Benito *et al.*, 2013; Romero *et al.*, 2013). This is also the case for the higher concentration in petioles described in the bibliography for K (Christensen, 1984; Schreiner & Scagel, 2006), Mg (Schreiner & Scagel, 2006), Mn (Romero *et al.*, 2010) and Zn (Christensen, 1984; Romero *et al.*, 2013). However, the differences between both tissues were not so marked for K, Mn and Zn in the RA vineyard or for B in the RM plot. Finally, Atalay (1978) found a higher P concentration in petiole from vineyards with high P concentration in the soil, while Benito *et al.* (2013) observed that P concentration was higher in blades than in petioles. These results show the importance of proposing specific reference levels for blade and petiole tissues, separately.

The variation of the nutrient content in blade tissues throughout the crop season has been widely discussed

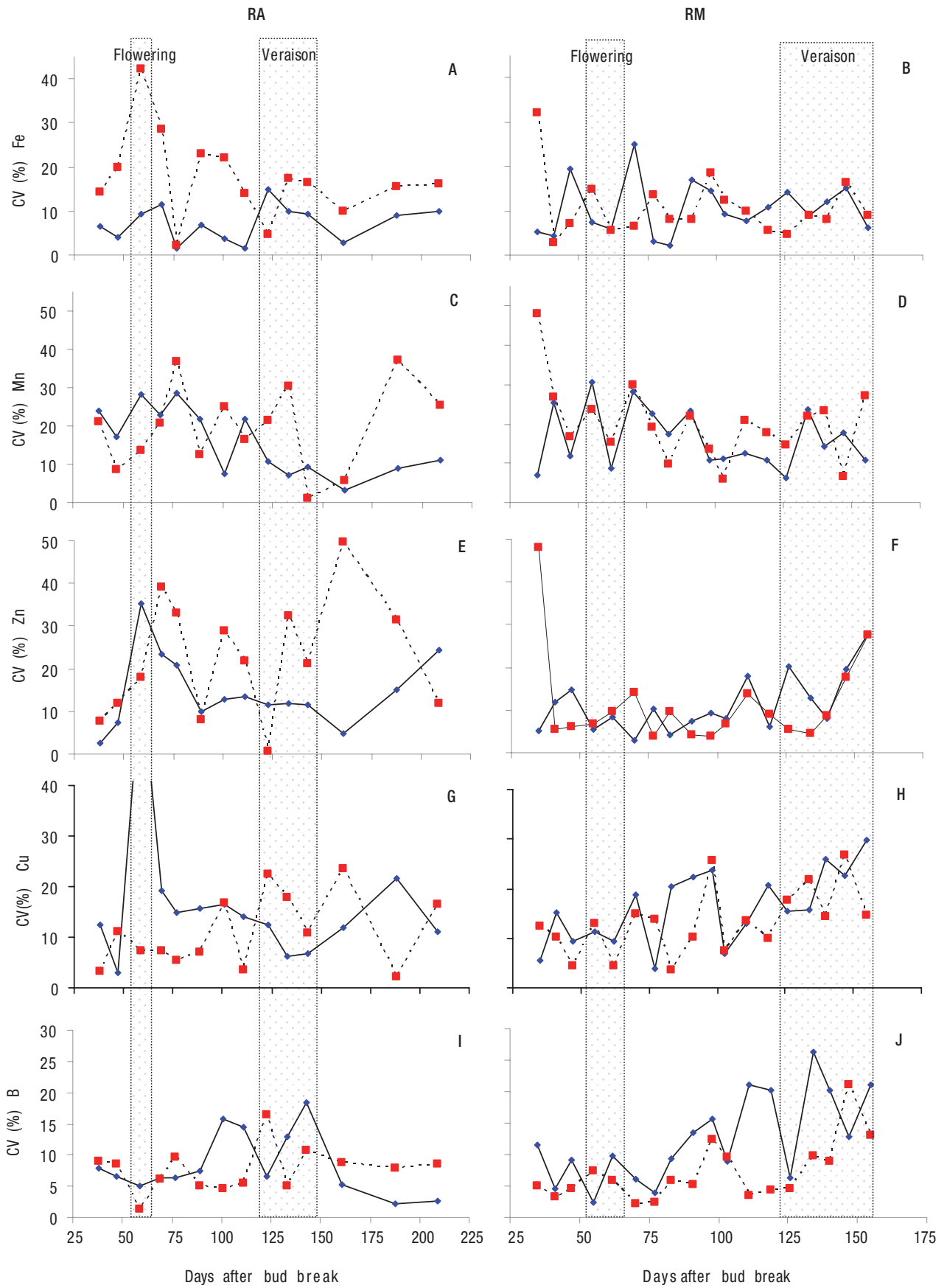
Petiole Code	Pre-flowering			Flowering			Berry development										Veraison			Ripening		Harvest						
	H25	H75	H100	I40	I50	I100	J	K25	K30	K50	K60	K70	K75	K80	K90	L	M5	M15	M25	M50	M60		M75	M100	N50	N100	O	
RA				1.24a			1.00b			0.59c		0.56c			0.54c						0.55c							
RM	2.05a	2.20a	2.52a	2.29a	1.90a		1.40b	1.27b					0.65c	0.81c	0.67c		0.70c		0.73c	0.66c	0.63c	0.69c						
RA				0.29a			0.23a	0.21a							0.73b					0.65b		0.54b						
RM	0.53a	0.62a	0.64a	0.57a	0.54a		0.41b								0.10c		0.10c		0.06cd									
RA				2.18a			1.92a	1.81a					1.16b		1.49b		1.65b			1.27b		1.27b						
RM			2.09c	2.03c	1.86c		3.27b	3.47b	2.82b	3.94b	2.91b	1.10d	2.35d	1.19d		1.15d			4.45a	4.21a	2.42b	1.90b						
RA				1.53b			1.88b													2.52a		2.95a						
RM			1.10c	1.25c	1.77b		1.80b	1.82b	1.86b	1.86b		2.79a	2.41a	3.04a	2.62a						2.61ab	3.25a						
RA				0.61b			0.71b					1.01a			1.04a		1.22a			1.30a		1.24a		1.24a	1.30a	1.31a		
RM				0.69d			0.77c								1.23a	1.12a	1.36a	1.27a			1.25a	1.36a						
RA	32.9b	27.2b		21.3b			41.2a					23.9b			25.5b		24.5b			21.1b		27.1b		29.1b	29.4b	31.5b		
RM			17.5d	18.6d	32.3a		24.0c	25.6c	23.5c	27.6c	24.1c	27.9c	30.7c	22.4c		29.7b	26.0b	31.9b			24.8b	34.1a						
RA				28.5b			32.5b	36.8b							80.5a		106.7a			100.0a		90.3a		94.9a	78.4a	85.0a		
RM			33.5c	35.6c	64.0cd		73.8c	73.8c	101.8c	99.4c					413.5a		388.7a			212.3b		323.1a						
RA				11.8b			11.8b	16.5b	8.7b	8.0b					12.9ab		17.2ab			24.2ab		26.6a		28.2a	24.5a	26.5a		
RM				38.3c	42.2c		40.5c	40.2c	40.0c				47.4a	43.9a		45.8a	53.9a	48.3a			49.7a	53.3a						
RA				44.0a			43.8a	44.5a	47.4a	44.7a					41.7a		42.9a			41.0a		40.8a		41.1a	42.8a	43.4a		
RM				45.0b	45.4b		47.1b	41.8b				40.4c	42.5c	3967c	37.9c					61.2a	49.2a	49.6a	47.0a					

**Figure 5.** Stability periods for leaf petiole nutrients concentration in RA (Haro) and RM (Logroño). Different letters among phenological stages express significant differences at  $p \leq 0.05$  (Tukey test). Shaded periods show common periods for a general diagnosis of all nutrients for ‘Graciano’.



**Figure 6.** Coefficient of variation (%) for macronutrient concentrations in blade (♦) and petiole (■) throughout the growing season in RA (Haro) and RM (Logroño).





**Figure 7.** Coefficient of variation (%) for micronutrient concentrations in blade (♦) and petiole (■) throughout the growing season in RA (Haro) and RM (Logroño).

by different authors (Colugnati *et al.*, 1992; Parejo *et al.*, 1992; Benito *et al.*, 2013; Romero *et al.*, 2013). In a PCA when one of the PC is highly related to the phenological stage, the original variables (nutrients) with sharp changes in concentration over the growing season are related to that PC.

Therefore, in our study, those elements showing slight concentration changes over the growing season present a weak relation with PC1 for RA and with PC2 for RM (Table 3). This result shows the importance of proposing specific reference levels for different phenological stages, mainly for those nutrients with strong evolutionary behaviour over the crop season.

In addition, our results showed that the vineyard origin and/or the year effect could modify the nutrient concentration. Other studies showed that the year effect was low on the total variance of datasets obtained for single vineyards throughout different years (Benito *et al.*, 2013; Romero *et al.*, 2013).

### Trends in nutrient concentrations

Most of the trends, mainly for macronutrients, showed curves with slopes near to zero at veraison (Fig. 2). Furthermore, some nutrients with sigmoidal behaviour curves presented two periods with slopes near to zero, one at the flowering phenological stage, which was shorter than that found at veraison (Fig. 3).

In general, seasonal trends for macronutrients in blades and petioles (Fig. 2) concurred with results found for other cultivars, such as 'Chardonnay' (Parejo *et al.*, 1992), 'Pinot noir' (Schreiner, 2005), 'red Grenache' (Benito *et al.*, 2013) or 'Tempranillo' (Romero *et al.*, 2013).

The N and P behaviour is in agreement with results found by other authors (Christensen, 1984; García-Escudero *et al.*, 2002; Schreiner & Scagel, 2006; Benito *et al.*, 2013; Romero *et al.*, 2013). The decreasing pattern for K was also found for Colugnati *et al.* (1992) and Porro *et al.* (1995). Furthermore, the increasing concentration of Ca and Mg in both tissues has also been shown by different authors (Stevens, 2005; Schreiner & Scagel, 2006; Benito *et al.*, 2013; Romero *et al.*, 2013).

Regarding micronutrients, the increases in Fe and Mn, agree with previous reports for other cultivars (Schreiner, 2005; Benito *et al.*, 2013; Romero *et al.*, 2013). The different trends observed by the Zn were probably due to either different climatic conditions and/or the soil effect (Figs. 3F,G). The trend observed by the Zn in RA was observed by Christensen (1984). Finally, the B pattern was also reported by Failla *et al.* (1995).

With respect to Cu, Schreiner (2005) observed a decreasing trend for Cu concentration in blade throughout the season for 'Pinot noir' cultivar while, in this study, blade increased Cu concentration in the early maturation stage. Therefore, the application of fungicides impeded the study of Cu behaviour (Benito *et al.*, 2013; Romero *et al.*, 2013).

Finally, the differences between nutrient concentration for leaf blades and petioles, as well as the seasonal changes throughout the growing season, confirm that it is necessary to design specific reference levels for each tissue and phenological stage.

### Evaluation of two sampling periods for nutrient diagnosis purposes

The most adequate moment for nutritional diagnosis would correspond to the period in which nutrient concentrations remain highly stable for some time in the tissue analyzed allowing a reliable comparison with their references. Therefore, the best sampling times are those where the concentration trend shows a slope as near as possible to zero. Most of the trends, mainly for macronutrients, showed curves with slopes near to zero at veraison (Fig. 2), making that phenological stage an adequate moment to propose useful references. Furthermore, those nutrients with sigmoidal behaviour trends presented a shorter stability period at flowering (Figs. 2 & 3).

Flowering and veraison are the preferred phenological stages to evaluate the grapevine nutritional status (Robinson, 2005). Therefore, the available bibliography offers different reference values for nutritional diagnosis in grapevine tissues at these two phenological stages. However, the length of each stability period is not fully defined and it is difficult to delimit it considering only the slope, especially at flowering. Therefore, a statistical determination of the differences between sampling times becomes necessary to enclose the validity period for each nutrient reference at flowering and veraison.

Considering all constraints, the best time to perform a multi-element analysis of leaf blades and petioles during flowering for the 'Graciano' variety is at 100% flowering as it is an easy recognizable phenological stage, and references provided for this moment remain valid until fruit set. This recommendation agrees with results obtained for other varieties (Benito *et al.*, 2013; Romero *et al.*, 2013).

Regarding veraison, the recommendable period to carry out a general nutritional diagnosis of the ten nutrients studied for blades extends from 50% veraison to beginning of ripening, except for K. This situation is not similar to recommendations obtained for other

varieties in longer monitoring studies, where the stability period covers all the veraison period or even allows an early diagnosis, up to a month before the start of this phenological stage (Benito *et al.*, 2013; Romero *et al.*, 2013). Therefore, the results suggest the need for more years of study to avoid the year and/or the soil effect on the nutrient concentrations in blades.

With respect to petioles, the optimum period for a common diagnosis at veraison ranged from two weeks after closed bunch until one week before the end of the veraison (75% of the berries coloured), except for K. Benito *et al.* (2013) and Romero *et al.* (2013), for ‘red Grenache’ and ‘Tempranillo’ varieties respectively, showed that the diagnosis in veraison with respect to a reference for 50% of coloured berries (M50), ranged from two weeks before the veraison to the beginning of ripening. This difference might be due to variety behaviour. However it is also necessary to extend the years of study to adjust these results for petiole. Finally, phytosanitary treatments did not permit a correct copper diagnosis throughout the growing season.

### Coefficient of variation and analysis of reliability

To evaluate the general nutritional status of a vineyard, the vine grower usually carries out a single analysis that compares the results to the standards. Therefore, an adequate tissue for nutritional diagnosis should provide acceptable analysis reproducibility (Wolpert & Anderson, 2007). A high variability between replicates from a uniform vineyard implies that obtaining representative nutrient concentrations is difficult. Due to that, the CV is used for the evaluation of the analysis reproducibility in both tissues at each phenological stage throughout the growing season.

Considering the diagnosis of individual nutrients, results suggest that blade was a more appropriate tissue than petiole to compare N, P and K concentrations with respect to their references (Figs. 6A-F). Calcium and Mg did not show clear differences between blades and petioles throughout the growing season, and CV in both cases was lower than 20%, but results suggest that blade would be preferable at flowering and veraison phenological stages (Figs. 6G-J). Some of these results agreed with those found for ‘Tempranillo’ (Romero *et al.*, 2013) as for N and K at both phenological stages and for P and Mg at veraison. Furthermore, results are also in agreement with those found for N, P and K for ‘red Grenache’ cultivar (Benito *et al.*, 2013).

The greater CV values in petioles implied a lower reproducibility of the analysis that may lead to diagnosis errors when the vinegrower compares a routine

single analysis with the macronutrient reference values (Benito *et al.*, 2013). Furthermore, CV result also suggest that the analysis of blade and petiole separately would be more appropriate than whole leaf since one tissue will always show a lower CV.

With regard to the micronutrients, results found for both vineyards are contradictory and therefore it is not possible to establish a blade *vs.* petiole preference sampling policy (Fig. 7).

In summary, the higher variability within petiole nutrient concentrations for the cv. Graciano suggests that, for routine single analysis, leaf blades would be preferable to petioles for N, P, K, Ca and Mg diagnosis at both flowering and veraison. With regard to micronutrients, the differences found between both vineyards do not allow to define the best tissue to analyze micronutrients. In general, references for blade and petiole at full flowering could be valid for diagnosis of all nutrients even in late samplings at fruit set. On the other hand, references obtained at 50% veraison, would allow the simultaneous diagnosis for leaf blades of all the studied nutrients between the middle of veraison and ripening. For petioles, the simultaneous diagnosis period ranges from the beginning of veraison until one week before the end of this phenological stage.

The methodology employed is appropriate in establishing the most adequate tissue and moment for the diagnosis of ten different nutrients; however, it is necessary to extend this study for a longer period to include data from different pedoclimatic subzones, in order to obtain conclusive results for ‘Graciano’ and to evaluate the local effects on nutrient behaviour.

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