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**ASSESSMENT OF METAL BIOAVAILABILITY IN THE VINEYARD SOIL-
GRAPEVINE SYSTEM USING DIFFERENT EXTRACTION METHODS**

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Abbreviated running title: Metal availability in soil-grapevine system using different extractions

Abstract

This study was focused on the assessment of single and sequential extraction methods to predict the bioavailability of metals in the vineyard soil-grapevine system. The modified BCR sequential extraction method and two single-step extraction methods based on the use of EDTA and acetic acid were applied to differently amended vineyard soils. The variety effect was studied on the uptake of metals by leaves and grapes. Most of the elements studied (Ca, Mg, Cu, Fe, Mn, Zn and Pb) were weakly mobilized from vineyard soils, with the exception of Cu and Mn. The determination of total metal content in leaves and grapes showed a different accumulation pattern in the two parts of the vine. A significant relationship was observed, for all the elements studied except for Fe, between the content bioavailable in the soil and the accumulated in both leaves and grapes ($R= 0.602-0.775$, $p < 0.01$).

Keywords: bioavailability, metals, vineyard soil, leaves, grape berries, sequential extraction methods, single extractions

1. Introduction

Wine is one of the most traditional and widely-consumed alcoholic beverages worldwide. The presence of trace metals in the wine is important from a toxicological and organoleptic point of view (Pyrzyńska, 2004). The contents of trace metals depend on environmental and anthropogenic factors including the type of vineyard soil, the metal intake from the soil into the grapes, grape variety and maturity, climatic conditions, viticulture management practices, environmental pollution, winemaking additives, winery equipment, wine preservation and bottling (Pyrzyńska, 2004; Pohl, 2007; Grindlay, Mora, Gras, & de Loos-Vollebregt, 2011). Despite the variability associated with viticultural and winemaking practices, recent studies have reported a successful discrimination of wines produced in different growing areas based on the content of trace metals (González, Llorens, Cervera, Armenta, & de la Guardia, 2009; Rodrigues et al., 2011; Martin, Watling, & Lee, 2012). Therefore, soil and climatic conditions are primary factors determining the elemental composition of grapes and wines. Furthermore, the accumulation patterns of trace metals depend on the grape cultivar (Ko, Vogeler, Bolan, Clothier, Green, & Kennedy, 2007; Vystavna, Rushenko, Diadin, Klymenko, & Klymenko, 2014).

It is well known that mineral nutrients and other trace metals are absorbed by plants, and the mobility and extractability from soils influence their bioavailability (Kabata-Pendias, 2004). The total metal content is a poor indicator of metal bioavailability because it basically depends on the chemical association with the different soil components. Therefore, the distribution of metals among soil components is important to assess the soil potential for supplying enough mineral nutrients and to evaluate the toxicity to plants. Various single and sequential extraction procedures have been proposed to assess the mobility and bioavailability of metals in soils.

Many multistep extraction procedures have been widely used for sediment and soil analysis (Gleyzes, Tellier, & Astruc, 2002), but the establishment of standard and unified sequential extraction criteria for non-polluted agricultural soils is required. So, the three-step sequential extraction procedure proposed by the Community Bureau of Reference (BCR, now superseded by the Standards, Measurement and Testing Programme) was initially developed for sediment analysis, but it has been also applied to soils (Rauret et al., 2000; Sahuquillo, Rauret, Rehenert, & Muntau, 2003). The BCR sequential extraction procedure was modified in order to improve the reproducibility of the results obtained (Sahuquillo et al., 1999). Both BCR sequential extraction methods (original and modified) allow the differentiation of extractable metals (available for plants) according to the following fractions: acid soluble, reducible or associated with Fe-Mn oxides, and oxidizable or associated with organic matter.

To considerably simplify the experimental task associated with the use of multistep extraction procedures for estimating the labile fraction of metals, leaching tests based on single extractions have been developed. In this sense, the use of ethylenediaminetetraacetic acid (EDTA) and acetic acid single extraction procedures is widely extended, and even certified reference materials with EDTA and acetic acid extractable contents of metals are available for quality control purposes (Quevauviller et al., 1997; Pueyo et al., 2001).

Although numerous attempts have been made to establish correlations between metal fractionation in soils and their uptake by plants (Álvarez, López-Valdivia, Novillo, Obrador, & Rico, 2006), the problem still remains unsolved because a general behavior has not been found. In fact, the absorption of metals by plants depends on several factors including the type of plant and the soil properties and, therefore, each soil-plant system can be different. There are a few published studies on the bioavailability of trace metals in the vineyard soil-vine-grape system (Orescanin, Katunar, Kutle, & Valkovic, 2003; Tokalioglu, Kartal, & Güneş, 2004; Vystavna et al., 2014) in which the acid soluble and exchangeable fractions were

determined using dilute hydrochloric or nitric acids and ammonium acetate, respectively, as single extractants. Particularly, the application of organic wastes to improve the physical properties of soil affects the total content and extractable fractions of metals in amended vineyard soils (Ramos, 2006).

Therefore, the present work was focused on: i) the determination of the extractable contents of some macronutrients (Ca and Mg), micronutrients (Cu, Zn, Mn and Fe) and heavy metals (Pb) in vineyard soils amended with different organic fertilizers (cattle and sheep manures) using the modified BCR sequential extraction method as well as EDTA and acetic acid single extractions; ii) the determination of total metal contents in leaves and grapes of two Galician white grapevine varieties (Treixadura and Loureira) grown in the above mentioned soils; iii) the establishment of correlations between extractable metal contents from soils (using single and sequential procedures) and those accumulated in leaves and grapes. This study could strongly contribute to the development of empirical models to predict the bioavailability of Ca, Mg, Cu, Fe, Mn, Zn and Pb in the vineyard soil-vine-grape system because the different responses of the grapevine varieties to the intake of essential and non-essential elements from soils amended with different animal manures are considered.

2. Materials and methods

2.1. Study area

The study area is located in the Ribeiro region (Ourense province), in North-West Spain (42° 21' 41" N; 8° 7' 2.5" W). This region has an Atlantic climate characterized by abundant rainfalls, particularly in winter and spring seasons. The annual precipitations are about 600 mm. Daily average temperature varies between 7 and 13 °C in the cold season and ranges from 15 to 24 °C in the hot season. The high intensity rainfall causes significant

erosion, which leads to nutrient losses and alterations of the soil surface. Therefore, the application of organic amendments is a common practice to improve the physical properties of the soil and to enrich it in mineral nutrients.

Two Galician white Treixadura and Loureira varieties (*Vitis vinifera* L.), grown in the same vineyard, were studied. The vineyard soil was amended with different organic fertilizers (cattle and sheep manures) at a dose of 50 Mg/ha. The cattle manure was applied four years before sampling to some Treixadura vines of twelve years old (sample 1). The sheep manure was applied one and four years before sampling to other Treixadura vines of six years old (samples 2 and 3, respectively) and one year before sampling to all Loureira vines of six years old (sample 4). In February, a mineral amendment (600 kg/ha of Lithothamne 400, Timac Agro, Navarra, Spain) to provide Ca and Mg, and an organo-mineral fertilizer composed of N, P, K and humic/fulvic acids (1000 kg/ha of VITALOR ATB K, Timac Agro, Navarra, Spain) were applied to the soil. Grapevine treatments were also used to control vine fungal diseases. When the vines began to sprout, non-phytotoxic organic fungicides were applied as treatment against mildew at intervals of 12-15 days. At the end of spring, organo-cupric fungicides were used, whereas in summer, when the vegetation growth slowed, copper-based fungicides (oxychlorides and sulfates) were preferred against mildew. Anti-oidium treatments were also used: powdered sulfur was applied at the beginning of May, and organic fungicides were then applied in May-July at intervals of 25-30 days. Finally, a foliar fertilizer was applied in August (400 mL/hL of Fertileader, Timac Agro, Navarra, Spain).

2.2. Sample collection and pretreatment

The vineyard topsoil was sampled at the depth interval 0–20 cm using a handle steel soil sampler. At the four sampling locations (samples 1-4), soil samples (samples S) were collected in two consecutive seasons (winter, samples S1A-S4A; spring, samples S1B-S4B)

to evaluate the influence of the season on the mobility of nutrients and heavy metals. The soil samples were diagonally collected from three rows representing the one-fourth, middle and three-fourths rows into each sampling location of the vineyard. The three subsamples were combined to obtain a composite sample (approximately 3 kg). In the same sampling zones (samples 1-4), leaf samples (samples H) were also collected in spring (samples H1B-H4B) and at harvest (samples H1C-H4C), and grapes (samples G) were sampled at harvest (samples G1C-G4C). Thirty leaves and ten bunches per sample were randomly picked up from ten plants.

Once in the laboratory, soil samples were initially air-dried and then oven dried at 40 °C. The dry soil samples were sieved using a 2 mm stainless-steel sieve. Leaf samples were oven dried at 40 °C and cut in small pieces (1-3 cm). Afterwards, the soil and leaf samples were separately ground using a S-100 ball-mill (Retsch, Haan, Germany) and sieved with a 70 µm nylon-sieve. All the berries of each cluster were manually separated from the stalk, and the skins and seeds were manually removed from the pulp using a plastic spatula. Skins, seeds and pulps were separately oven dried at 40 °C, powdered using an agate mortar and a ball-mill, and sieved with a 500 µm stainless steel-sieve. All samples were stored in polyethylene bottles in a desiccator until analysis.

2.3. Reagents and standards

All chemicals were of analytical-reagent grade and purchased from Panreac (Barcelona, Spain) and Merck (Madrid, Spain). The solutions were prepared in deionized water produced by a Purelab Classic system (Elga Labwater, Marlow, United Kingdom). The stock standard solutions of metals (1000 mg/L) were prepared by dissolving the appropriate amount of the pure metal or a high purity salt in dilute acid and stored in a refrigerator at 4 °C. The stock standard solution of mercury (1000 mg/L) was supplied by Merck. The working

standard solutions for each individual element were prepared daily by appropriately diluting the stock standard solutions.

The stannous chloride (100 g/L) reducing agent for mercury determination was prepared daily by dissolving the appropriate amount of stannous chloride dihydrate (Panreac) in a minimum volume of 37% m/m hydrochloric acid. The carrier was an aqueous solution of sulfuric acid (50% v/v).

Eight reference materials were used for analytical quality control purposes: CRM BCR-701 (lake sediment), CRM BCR-483 (sewage sludge amended soil), ERM-CC580 (estuarine sediment), CRM BCR-060 (aquatic plant) and CRM BCR-596 (aquatic plant) were obtained from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium), ERM-CC-135a (contaminated soil) from LGC (Teddington, Middlesex, UK), IAEA-359 (cabbage) from the International Atomic Energy Agency (Vienna, Austria), and CRM DORM-2 (Dogfish muscle) from the National Research Council of Canada (Ottawa, Ontario, Canada).

2.4. Extraction procedures and determination

To evaluate the extractable metal contents in soils, two single extraction procedures were applied. Representative aliquots of each sample were treated with EDTA and acetic acid solutions as briefly commented (Quevauviller et al., 1997). The soil sample (2.5 g) was treated with 25 mL of 0.05 mol L⁻¹ EDTA for 1 h at room temperature with continuous agitation. Instead, 1 g of soil and 40 mL of 0.43 mol L⁻¹ acetic acid were shaken for 16 h at room temperature. After centrifugation at 2500 rpm for 10 min using a 5100 Kubota centrifuge (Tokyo, Japan), the resulting supernatants were analyzed.

Metal fractionation in soils was performed according to the modified BCR three-step sequential extraction procedure (Quevauviller, 2002). Briefly, the soil sample (2 g) was first

treated with 20 mL of 0.11 mol/L acetic acid for 16 h at room temperature with continuous agitation and then centrifuged at 2500 rpm for 5 min. The aqueous phase was carefully separated and used to determine the acid soluble fraction (the most easily leachable fraction). For the extraction of the reducible fraction, a second step was required where the remaining solid phase was shaken with 20 mL of a 0.5 mol/L hydroxylamine hydrochloride solution of pH 1.5 for 16 h at room temperature. In the third sequential step, the solid residue of the second step was treated twice with 5 mL of 300 g/L hydrogen peroxide for 1 h at room temperature with continuous agitation (only the first time) and for 1 h at 85 °C in a water bath. After evaporation, the solid residue was treated with 25 mL of a 1 mol/L ammonium acetate solution of pH 2 for 16 h at room temperature with continuous agitation. The supernatant was carefully separated by centrifugation and used to determine the oxidizable fraction.

Total metal contents were determined in soils, leaves and grapes after microwave-assisted acid digestion of the samples using a 900 W microwave oven (Moulinex, Barcelona, Spain) according to the method proposed by Lavilla, Pérez-Cid and Bendicho (1998) but slightly modified for leaves and grapes. The soil sample (0.1 g) was digested with 4 mL of 70% m/m nitric acid, 1 mL of 37% m/m hydrochloric acid and 2 mL of 48% m/m hydrofluoric acid in a 4782 Parr medium-pressure reactor (Moline, IL, USA) heated in the microwave oven at 540 W for 2 min. The solution obtained was evaporated to dryness in a sand bath, and the residue was dissolved in 37% m/m hydrochloric acid. The leaf and grape samples (0.2 g) were mineralized with 4 mL of 70% m/m nitric acid and 1 mL of 37% m/m hydrochloric acid in the Parr reactor by heating in a microwave oven at 510 W for 1 min. After cooling to room temperature, 2 mL of 300 g/L hydrogen peroxide were added, and the Parr reactor was again microwave-irradiated at 510 W for 2 min. In the case of ICP-MS determinations, only 0.12 g of leaf and grape samples were digested with 5 mL of 70% m/m nitric acid and 1 mL of 37% m/m hydrochloric acid at 510 W for 1 min.

Absorbance was measured with a 2380 Perkin-Elmer atomic absorption spectrophotometer (Norwalk, CT, USA) equipped with an air–acetylene flame. Hollow cathode lamps were used as the radiation source. The resonance line, lamp intensity and slit width were selected for each element according to the manufacturer's recommendations. In the case of Hg, a RA-3000 Nippon mercury analyzer (Texas, USA) was used. Pb, Cd and Cr were determined in the leaf and grape samples by Inductively coupled plasma mass spectrometry (ICP-MS) using a Thermo Elemental-X7 Quadrupole spectrometer (Thermo Scientific, Waltham, MA, USA) because of their low contents.

For each procedure, reagent blanks were prepared and analyzed. The quality assurance was assessed by the analysis of blanks and reference materials. Three replicates were used for each sample and procedure. The analyses were done by duplicate, and the average value was reported. All material was cleaned by soaking in 10% v/v nitric acid and then rinsed with deionized water.

2.5. Statistical analysis

Statistical analyses were performed using the SPSS Statistics software package version 19.0 (IBM Corporation, Armonk, NY, USA). One-way analysis of variance (ANOVA) was used to establish significant differences. Pearson's correlation coefficients were calculated to determine significant relationships between the metal contents obtained in the same samples using different extraction methods or between those found in the different types of samples studied in the soil-plant system.

3. Results and discussion

3.1. Soils

The digestion method used for the determination of total metal contents was validated by analyzing three reference materials (CRM DORM-2, ERM-CC-135a and ERM-CC580). The results obtained were not significantly different from the certified values (t -test, $p = 0.05$). The total metal contents in the vineyard soil samples collected in winter (samples S1A-S4A) and spring (samples S1B-S4B) are shown in Table 1. Ca, K and Fe were the most abundant elements in all samples with total contents ranging from 2079 to 26860 mg/kg, from 17955 to 27568 mg/kg and from 21114 to 26434 mg/kg, respectively. Other major elements were Mg and Mn, whose total contents varied from 786 to 2610 mg/kg and from 493 to 795 mg/kg, respectively. Cu and Zn showed total contents in the range of 133-306 mg/kg and 98.9-156 mg/kg, respectively. Among heavy metals, Pb was the most abundant element with total contents between 72.6 and 104 mg/kg, followed by Cr (3.13-5.09 mg/kg), Cd (43.9-101 $\mu\text{g/kg}$) and Hg (21.2-29.9 $\mu\text{g/kg}$). This relative distribution agreed with other previously published for vineyard soils (Almeida & Vasconcelos, 2003; Orescanin et al., 2003; Ribero de Lima et al., 2004), although the metal contents vary among growing areas and even among vineyards of the same growing area (Almeida & Vasconcelos, 2003; Ribero de Lima et al., 2004; Aceto et al., 2013).

The greatest differences among soil samples corresponded to the total content of Ca. The sample S1 (cattle manure applied to Treixadura vines) showed higher total contents of K, Ca, Fe and Mg in the two sampling dates, although the differences with respect to the other samples were only significant ($p < 0.001$) for Ca in the two dates and for K and Mg in the first date (samples SA). Cd and Pb were also more abundant in the sample S1. Instead, the sample S4 (sheep manure applied to Loureira vines) had lower total contents of Fe and higher ones of Hg, but they did not differ significantly from those corresponding to the sample S2 ($p > 0.05$). The sample S4 was also characterized by relatively low total contents of Pb. In general, the soil amended with cattle manure was richer in mineral nutrients.

Metal fractionation in vineyard soils was assessed according to the modified BCR three-step sequential extraction method, and the metal contents found in the different extractable fractions are reported in Table 2. Cd, Cr and Hg were not determined in the extractable fractions because of the low total contents found in all the vineyard soils studied (Table 1). The analytical quality of the results was assured by the analysis of a reference material CRM BCR-701 with certified extractable metal contents in the three fractions of the modified BCR method. The experimental results were not significantly different from the certified values (*t*-test, $p = 0.05$).

The fractionation of macronutrients (Ca and Mg) was similar in all samples. They were mainly associated with the residual fraction (66.8-95.2% for Ca and 61.0-92.7% for Mg, except for the sample S4A), and therefore they are poorly mobilized (Reid, Spencer, & Shotbolt, 2011). In most cases, the extraction percentage for each of the three extractable fractions was lower than 10%. Nevertheless, the acid soluble fraction achieved extraction percentages ranging from 21.7 to 46.7% for Ca, and from 17.3 to 39.4% for Mg in the samples S3A, S4A and S4B. A decrease in the extraction efficiency of Ca and Mg associated with the acid soluble fraction was observed from samples A to samples B, which was more significant for the samples S3 and S4. This effect was also observed in the reducible fraction, although it was smaller than in the acid soluble fraction. This decrease could be attributed to the partial absorption of Ca and Mg by the plant (Pérez-de-los-Reyes, Amorós Ortíz-Villajos, García Navarro, Bravo Martín-Consuegra, & Jiménez Ballesta, 2013).

Cu was primarily associated with extractable fractions, particularly the oxidizable fraction with extraction percentages ranging from 29.2 to 51.3% for most of the samples. Only in the sample S3B, the extractable content of Cu was equally distributed in the three fractions. Therefore, Cu has a high affinity for organic ligands. These results agreed with those previously published (Sahuquillo, Rigol, & Rauret, 2002; Kubová, Matus, Bujdos,

Hagarova, & Medved, 2008; Reid et al., 2011). In this case, the extraction efficiency of Cu in the samples B was always higher than in the samples A, which could be attributed to treatments with organo-cupric fungicides.

Fe was mainly associated with the residual fraction, with extraction percentages between 92.4 and 95.6% for all the samples studied. Despite Fe is one of the most abundant elements in the vineyard soils analyzed, it is little mobilized. Similar results have been found in previous studies (Herencia, Ruiz, Morillo, Melero, Villaverde, & Maqueda, 2008; Reid et al., 2011). Instead, Mn was predominantly associated with the extractable fractions with extraction efficiencies ranging from 43.4 to 79.8%. Particularly, Mn was largely associated with the reducible fraction (21.4-43.4%) and to a lesser extent with the acid soluble fraction (13.1-30.9%). These results agreed with those observed in agricultural soils (Kubová et al., 2008; Bakircioglu, Bakircioglu Kurtulus, & Ibar, 2011). A decreased extraction percentage of Mn in the reducible and oxidizable fractions in the samples B with respect to the samples A could be attributed to Mn uptake by the plant due to its functions as micronutrient, whereas the increased relative amount of Mn in the acid soluble fraction could be related to the use of plaguicides and fertilizers (López-Rayó, Lucena, & Lucena, 2014).

Zn was also primarily associated with the residual fraction with extraction percentages between 57.6 and 93.0%. Regarding the extractable fractions, Zn was more abundant in the reducible fraction for some samples (11.1 and 23.1%), but the extractable content of this element was equally distributed in the three extractable fractions in the samples S1B, S2A, S2B and S3B. This distribution is consistent with that found in other previous studies on agricultural soils (Kubová et al., 2008; Rozansky, 2013). In all soil samples where the Treixadura variety was grown (S1, S2 and S3), a decrease was observed in the relative amount of Zn in the three extractable fractions of the samples B when compared with the

samples A. This decrease was more pronounced in the reducible fraction, suggesting that the plant absorbs primarily Zn associated with this fraction.

Pb was mainly associated with the residual fraction with extraction percentages ranging from 74.5 to 83.1%, and therefore this element is little mobilized. These results agreed with those found in previous studies (García-Delgado, Sánchez-Martín, Lorenzo, & Sánchezcamanazo, 2002; Kubová et al., 2008). The extractable content of Pb was associated with the reducible and oxidizable fractions as occurred in other studies on agricultural soils (Kubová et al., 2008; Rozansky, 2013). The sum of these two extractable fractions was very similar in all samples, although in the samples B the extractable content of Pb increased in the reducible fraction in detriment of the oxidizable fraction.

The extraction efficiency of the two single-step extraction methods (EDTA and acetic acid) was compared with that obtained using the modified BCR three-step sequential extraction method. The two single-step extraction methods were validated by the analysis of a reference material CRM BCR-483 with certified extractable metal contents in the EDTA and acetic acid fractions. The results obtained were not significantly different from the certified values (*t*-test, $p = 0.05$). Afterwards, the extractable content of each metal (expressed as extraction percentage relative to the total metal content) in the first two fractions of the modified BCR sequential extraction method (modified BCR Σ_{1-2} , the most easily mobilized fractions, Table 2) was compared with that obtained using EDTA and acetic acid single-step extractions (Table 3) in Figure 1.

For Ca, Mg and Zn, very similar extraction percentages were obtained using the three methods (Figure 1_[a,b,g]). The differences found for the three elements were not significant (*t*-test, $p = 0.05$) and always lower than 9%. Other authors reported good correlations between the results obtained for Zn in agricultural soils using the modified BCR and EDTA extraction methods (Alvarez et al., 2006; Bakircioglu et al., 2011) and even in soils affected by mining

activities (Anju & Banerjee, 2011). In the case of Cu, the extractable content obtained using the modified BCR sequential method was comparable to that obtained using the acetic acid single extraction method (t -test, $p = 0.05$) with differences less than 10%. Instead, the extractable content of Cu with EDTA was significantly higher than that found using the other two extraction methods (Figure 1_[c]). This confirms the affinity of this metal for chelating ligands (Rao, Sahuquillo, & Sánchez, 2008). Regarding Mn, the extraction percentage using the modified BCR sequential method (42-61%) was higher than that obtained using acetic acid (28-36%) and EDTA (10-21%) single extractions (Figure 1_[e]), except for the samples S4A and S4B with extraction efficiencies of about 62 and 55% for acetic acid extractions and 32 and 40% for EDTA extractions, respectively. Other researchers did not find significant differences in the extractable contents of Mn in agricultural soils when the BCR and EDTA extraction methods were used (Alvarez et al., 2006; Bakircioglu et al., 2011). In the present work, the extraction efficiency of the two single extraction methods was not significantly different for Mn (t -test, $p = 0.01$). The extraction percentages of Pb and Fe were slightly higher for the modified BCR sequential extraction method than those obtained for the EDTA single extraction method, although these differences were always less than 11% (Figure 1_[d,f]). In fact, the differences found in the extraction efficiency of Pb using these two methods were not significantly different (t -test, $p = 0.01$). The extraction of Pb and Fe with acetic acid was negligible (Rao et al., 2008; Abedin, Beckett, & Spiers, 2012). Therefore, EDTA single-step extraction can be considered a good alternative to estimate the available content of Ca, Mg, Zn and Pb in vineyard soils, as well as acetic acid single-step extraction for Ca, Mg, Cu and Zn. This represents a great simplification of the operating procedure and a considerable reduction of total time of analysis.

Considering these results, correlations were established between the extractable metal content in the first two fractions of the modified BCR sequential extraction method (modified

BCR Σ_{1-2}) and in EDTA or acetic acid for all elements together. The most satisfactory Pearson correlation coefficient (R) corresponded to the modified BCR and acetic acid (HAc) extraction methods ($R = 0.890$, $p < 0.001$). The equation of linear regression was: modified BCR $\Sigma_{1-2} = 4.214 + 0.976 \times \text{HAc}$. The Pearson coefficient for the correlation between the modified BCR and EDTA extraction methods was lower ($R = 0.514$, $p < 0.001$).

3.2. Leaves and grapes

The performance of the digestion method used for the determination of total metal contents was assessed by analyzing four reference materials (CRM DORM-2, IAEA-359, CRM BCR-060 and CRM BCR-596). The analytical quality of the method used was assured because the results obtained were not significantly different from the certified values (t -test, $p = 0.05$). The total metal contents in the leaf samples collected in spring (samples H1B-H4B, prior to fruit development) and at harvest (samples H1C-H4C), and in the grape samples collected at the harvest date (samples G1C-G4C) are shown in Table 4. All of them were sampled in the same locations that the soils.

Regarding leaf samples, the major elements were K (5187-17974 mg/kg), Ca (6010-33682 mg/kg) and Mg (1767-3991 mg/kg). Mn was the most abundant micronutrient with total contents ranging from 377 to 875 mg/kg. The total contents of Cu, Fe and Zn were considerably lower (15.9-263 mg/kg), except for Cu in the samples H1C-H4C with total contents ranging from 882 to 1882 mg/kg. The results obtained for most of the elements studied agreed with those published in previous studies on vine leaves (Angelova, Ivanov, & Braikov, 1999; Bora, Bunea, Rusu, & Pop, 2015). Among heavy metals, the major element was Cr with total contents ranging from 3.03 to 3.96 mg/kg, followed by Pb with total contents between 0.221 and 0.703 mg/kg. These contents of Pb were lower than those found in other studies (Angelova et al., 1999; Bora et al., 2015). Cd and Hg were present in amounts

lower than 0.06 mg/kg. Although it is difficult to establish a general trend, the higher contents of most elements were found in the samples H4, they being significantly different for Mn in the sample H4B and for Ca, Mg, Cu, Mn, Zn and Pb in the sample H4C ($p < 0.001$) from those found in the other samples HB and HC, respectively.

The total contents of Ca, Mg, Cu, Fe and Mn experienced a significant increase from the leaf samples B to the samples C ($p < 0.001$), regardless of the variety studied and manure applied to the soil. This could be related to the absorption of these metals by the plant during fruit development. In the case of Cu, the increase (46-97 times) could be attributed to fungicide treatments, including the use of copper sulfate or copper oxychloride in the summer season (July-August). However, the total contents of K and Zn were lower in the leaf samples C ($p < 0.001$). According to the transfer coefficients must-canes and leaves-canes reported by other authors (Bora et al., 2015), the transference of Zn towards berries is higher and therefore its migration towards leaves decreased in relation to that of other elements. The same trend could occur for K. Heavy metals (Cd, Cr, Pb and Hg) did not show an important variation in the total contents between the two leaf samples (B and C), although a significant increase in the total contents of Pb and Hg was observed with the development of the fruit ($p < 0.01$), probably due to vine treatments. The increasing trend was confirmed by the establishment of a relationship between the two data sets for all elements together (except for K). A significant linear regression ($p < 0.001$) was obtained with a satisfactory correlation coefficient ($R = 0.965$). The equation of linear regression was: $HC = -294.73 + 3.431 \times HB$. The variety and manure effects were not significant for the total contents of the elements studied in vine leaves ($p > 0.05$).

In grape berries, the total content of each element was assessed as the sum of the contents in the skin, pulp and seeds (Σ skin + pulp + seeds). K was the most abundant element with total contents ranging from 34959 to 46306 mg/kg, followed by Ca (from 3655 to 5244

mg/kg) and Mg (from 2077 to 2458 mg/kg). Among micronutrients, Fe was the major element with total contents between 137 and 249 mg/kg, followed by Mn (from 93.5 to 114 mg/kg) and Cu (from 55.0 to 78.5 mg/kg). Zn had lower contents (close to 30 mg/kg). The contents of macronutrients were slightly higher than other previously published (Zhu, Du, Li, Zhang, & Li, 2012; Grembecka & Szefer, 2013), whereas the relative distribution of micronutrients (Cu, Fe, Mn and Zn) was similar to that found in different grape cultivars (Zhu et al., 2012). The total contents of heavy metals showed a similar profile to that reported for the leaf samples. Therefore, Cr was the predominant heavy metal with total contents between 2.77 and 3.51 mg/kg. The total content of Pb varied between 92.0 and 251 µg/g, and Hg had very low contents ranging from 15.2 to 18.9 µg/g. Cd was not quantifiable in these samples (Zhu et al., 2012). Metal contents significantly change depending on the cultivar (Zhu et al., 2012). Ca, Mg, Fe, Mn and Zn were mainly associated with the seeds (47.9-80.5% of the total content of the berry), whereas K, Cu and Hg were mainly related to the skins (41.5-67.1%). Pb and Cr had a very similar distribution in the three parts of the berry (26.2-31.9% for seeds, and 32.6-44.7% for pulp and skins).

With the aim of evaluating the relationship between the amounts of metals in leaves and grapes, the total metal contents in the two parts of the vine were compared when they were sampled simultaneously (samples C, H1C-H4C and G1C-G4C). The total contents of Ca, Mg, Mn and Cu in leaves were significantly higher than in berries regardless of the variety studied. Similar behavior was observed for Fe, Pb and Zn, although the differences were less pronounced. This trend agreed with that found for Cu, Zn, Pb and Cd (Angelova et al., 1999). Contrarily, K was significantly more abundant in berries than in leaves, and this finding is in agreement with other studies (Amorós Ortiz-Villajos et al., 2012). For Cr and Hg, the differences between leaves and grapes were not significant, but the total contents were slightly higher in leaves. Taking into account that, for most of the elements studied, the total

content found in the leaves sampled at the harvest date was higher than in the berries, the two sets of data were statistically related for all elements together, excluding K. A significant linear regression ($p < 0.001$) was obtained with a satisfactory correlation coefficient ($R = 0.933$). The equation of linear regression was: $HC = -387.11 + 4.689 \times GC$.

3.3. Soil-plant system

To establish relationships for the different elements studied in the soil-plant system, the metal content mobilized from vineyard soils (samples B, S1B-S4B) according to the modified BCR sequential extraction method (modified BCR Σ_{1-2} , Table 2) was compared with the total metal content found in vine leaves (samples B and C, H1B-H4B and H1C-H4C, Table 4) and berries (samples C, G1C-G4C, Table 4). The total contents of Ca, Mg, Mn and Zn in the leaf samples were always greater than those leached from the soil, regardless of the sampling location. These differences were more significant for Ca and Mg (macronutrients) than for Mn and Zn (micronutrients), possibly because the latter two elements are present in lower contents in the soil-plant system. In any case, there was a mismatch between the content mobilized from the soil and the total content found in leaves, which could be due to an external supply of mineral fertilizers. In fact, sodium nitrate and calcium nitrate fertilizers are highly soluble and of fast action when they are applied directly on the plant. There are foliar fertilizers and pesticides containing Mn and Zn (Webb, Bartos, Boles, Hasty, Thuotte, & Thiex, 2014), as those used in the present work. With the exception of Mn, the same behavior was observed for these elements when the content mobilized from the vineyard soil was compared with the total metal content found in the berries. Instead, the total content of Mn in berries was lower than the content mobilized from the soil for all sampling locations.

Regarding Fe and Pb, their content released from the soil was much higher than the total content found in both leaves and berries, indicating poor absorption of these elements by

the vine (Pérez-de-los-Reyes et al., 2013). In the case of Cu, there was a great similarity between the content mobilized from the soil and the total content found in the leaf samples B. The application of phytosanitary treatments based on copper during the summer season (July-August) caused a strong increase of Cu in the leaf samples C. Despite the treatments applied to the vineyard during the development and maturation of the fruit, the Cu content released from the soil was also similar to the total content found in berries, showing a limited uptake of this element by the berries.

The next step was the establishment of more robust relationships between the metal content mobilized from the soil (samples S1B-S4B) and the total content found in both vine leaves (samples H1B-H4B and H1C-H4C, Table 4) and berries (samples G1C-G4C, Table 4) considering all elements together, with the exception of Fe. In this case, the extractable metal content was assessed according to the modified BCR sequential extraction method (modified BCR Σ_{1-2} , Table 2) and the EDTA and acetic acid single-step extraction methods (Table 3). Fe was the only element with significant differences in the mobilized content according to the three extraction methods. The significant Pearson correlation coefficients were reported in Table 5. For both leaves and berries, the most significant and strongest correlations corresponded to the assessment of the metal content mobilized from the soil using the EDTA single-step extraction method with coefficients (R) ranging from 0.649 to 0.775 ($p < 0.001$), followed by the modified BCR sequential extraction method (modified BCR Σ_{1-2}) with coefficients between 0.607 ($p < 0.01$) and 0.740 ($p < 0.001$). Taking into account the sample, the metal content bioavailable in the soil was significantly correlated with the total metal content found in the leaf samples B and C as well as in the berries (from $R = 0.602$, $p < 0.01$ to $R = 0.775$, $p < 0.001$). The total metal content in the leaf samples C was better correlated with the metal content released from the soil ($R = 0.709$ - 0.775 , $p < 0.001$), particularly when the latter was assessed using the EDTA single-step extraction method ($R = 0.775$, $p < 0.001$).

These results show a good balance in the soil-plant system studied between the metal content bioavailable in the soil and the absorbed by the vine during the development and maturation of the fruit. Other researchers have also found a good correlation between the metal content mobilized from the soil (using single extractions) and that found in wheat grains (Bakircioglu et al., 2011) or in some grass species (Abedin et al., 2012).

4. Conclusions

According to the modified BCR sequential extraction method, Cu and Mn are the more easily mobilized and available metals to crops. Although Ca and Mg are major elements, their mobility is relatively low. Fe and Pb are the more weakly mobilized minor elements in the vineyard soils studied. Using the single-step extraction methods based on the use of EDTA or acetic acid, it is possible to predict the extractable content corresponding to the first two fractions of the modified BCR sequential extraction method for all metals, with the exception of Mn and Fe. Diverse patterns of micronutrient accumulation in leaves and grapes were observed, being Mn more intensively accumulated in leaves while Fe in grapes. In the vineyard soil-grapevine system, although differences were found between the content bioavailable in the soil (assessed by either single or sequential extraction methods) of the elements studied and the total content accumulated in both leaves and grapes, significant relationships were found when all elements were considered together with the exception of Fe, regardless of the vinegrape variety and manure applied to the soil. Single-step extraction methods were satisfactory strategies to evaluate the metal uptake by the plant, simplifying the operating procedure and reducing considerably the total time of analysis.

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FIGURE CAPTIONS

Fig. 1. Extractable content using modified BCR sequential extraction procedure (Σ_{1-2}), and EDTA and acetic acid single-step extraction procedures for Ca (a), Mg (b), Cu (c), Fe (d), Mn (e), Pb (f) and Zn (g) in soils.

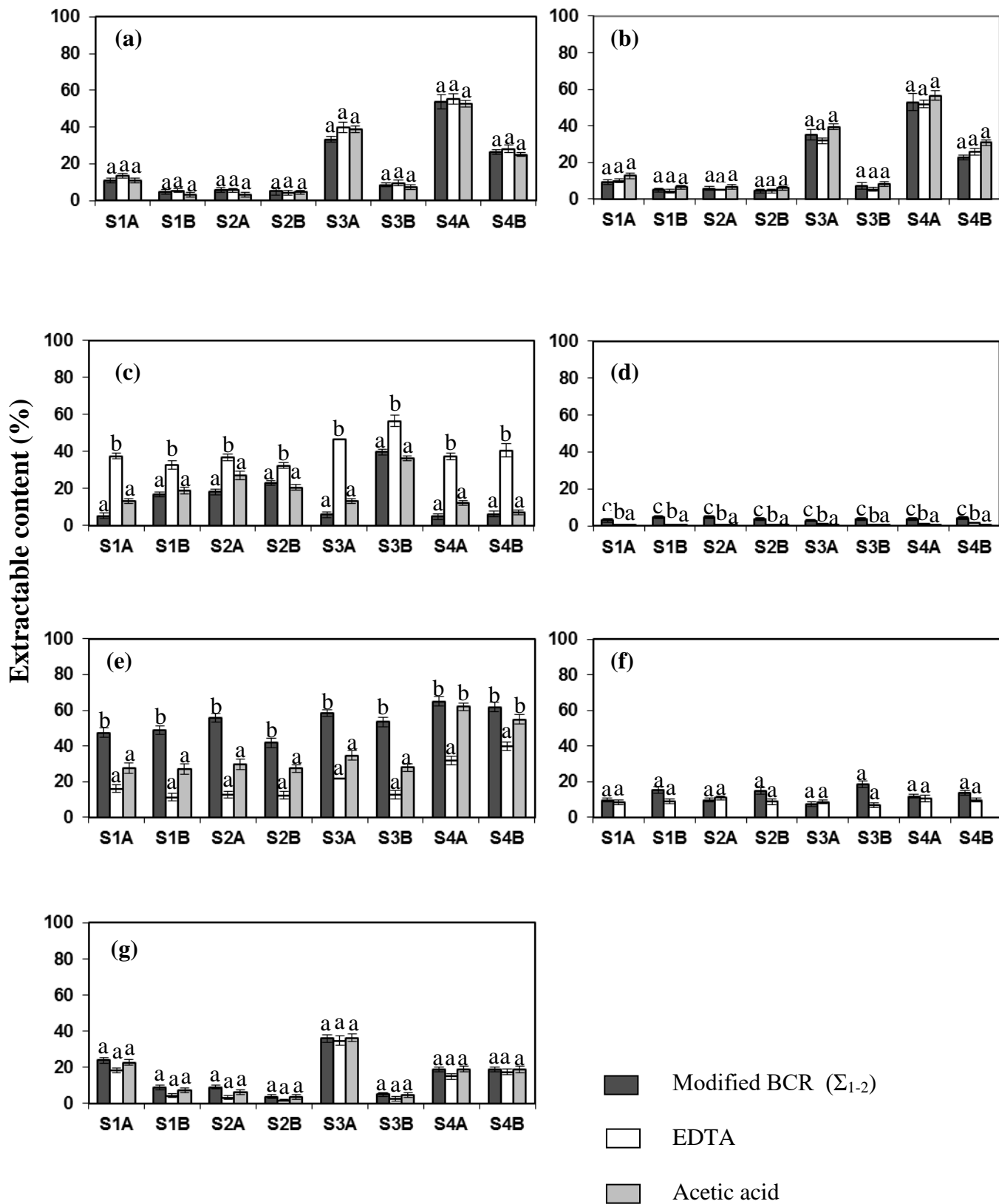


Figure 1

Table 1
Metal composition of vineyard soil samples (mg/kg).

Sample	K	Ca	Mg	Cu	Fe	Mn	Zn	Cd	Cr	Pb	Hg
S1A	27568±1957c	26860±747d	2487±130d	193±6a	25335±725c	674±8a	129±5c	0.0761±0.0071b	4.92±0.08b	104±8	0.0213±0.0015a
S2A	22479±541b	2336±175a	1947±37c	302±3c	23243±731ab	795±72b	101±2a	0.0669±0.0065ab	5.09±0.15b	98.7±1.7	0.0256±0.0015ab
S3A	21382±1323b	13910±90c	1656±108b	306±3c	23872±1308bc	643±34a	156±2d	0.0491±0.0047a	4.06±0.32a	91.4±6.5	0.0224±0.0020a
S4A	17955±1285a	3458±67b	786±62a	216±7b	21546±768a	604±24a	114±2b	0.0660±0.0053ab	5.00±0.30b	84.9±7.5	0.0297±0.0023b
Sign ^a	***	***	***	***	**	**	***	*	*	ns	*
S1B	24675±580b	15921±521c	2610±244b	258±2d	26434±552c	691±9b	126±4c	0.101±0.003c	3.37±0.06b	96.3±3.2b	0.0239±0.0023ab
S2B	24085±932ab	2560±204a	2327±220b	133±1a	22069±1772ab	663±21b	98.9±2.5a	0.0801±0.0060b	4.61±0.09c	99.3±1.3b	0.0278±0.0014bc
S3B	22221±569a	2079±182a	1733±151a	145±1b	24281±1692bc	493±36a	108±4b	0.0439±0.0043a	3.13±0.05a	72.6±5.1a	0.0212±0.0018a
S4B	23083±296ab	8547±118b	2590±148b	158±4c	21114±1269a	682±59b	123±5c	0.0940±0.0081bc	4.94±0.05d	78.4±5.4a	0.0299±0.0027c
Sign ^a	*	***	**	***	***	***	***	***	***	**	*
Sign ^b	ns,*,ns,**	***,ns,***,***	ns,*,ns,***	***,***,***,***	ns,ns,ns,ns	ns,*,**,ns	ns,ns,***,*	*,ns,ns,ns	** ,ns,ns,ns	ns,ns,ns,ns	ns,ns,ns,ns

All data are expressed as average value ± standard deviation (n = 4). ^{a,b}Sign: *, **, *** and ns indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant, respectively, for the differences (a) among sampling zones for each date and (b) among sampling dates for each zone. ^aDifferent Latin letters within the same column indicate significant differences (Tukey-b test; $p < 0.05$).

Table 2

Extractable metal content in vineyard soil samples (mg/kg) according to the modified BCR sequential extraction method.

Fraction	Sample	Ca	Mg	Cu	Fe	Mn	Zn	Pb
Acid soluble	S1A	2522 ±74	169±7	1.86±0.03	10.5±0.4	88.4±3.9	12.8±0.5	<LOD
	S2A	100±6	87.6±1.7	25.1±0.7	11.2±1.2	114±2	4.15±0.06	<LOD
	S3A	3740 ±46	459±1	4.35±0.22	9.88±0.79	106±1	19.9±0.1	<LOD
	S4A	1616 ±95	310±36	3.18±0.07	23.7±8.6	129±1	7.78±0.13	<LOD
	S1B	663 ±18	90.9±0.7	21.3±0.1	14.4±0.8	123±2	5.90±0.22	0.696±0.003
	S2B	108±3	72.1±0.4	17.1±0.1	15.7±0.2	134±3	1.76±0.01	0.846±0.006
	S3B	144 ±1	82.5±0.7	31.8±0.3	6.81±0.53	97.9±0.6	2.79±0.02	<LOD
	S4B	1855±32	449±10	5.05±0.10	12.9±0.1	211±3	9.13±0.10	0.848±0.030
Reducible	S1A	348±9	62.9±0.8	7.38±0.57	768±23	321±3	18.1±0.6	10.1±0.1
	S2A	37.3 ±1.4	22.2±0.3	29.9±1.8	1126±27	328±5	9.48±0.04	9.46±0.14
	S3A	883 ±18	123±3	11.6±0.2	682±2	268±1	36.1±1.7	6.87±0.18
	S4A	248±10	105±2	6.12±0.60	771±31	262±5	13.6±0.2	9.99±0.31
	S1B	93.5 ±4.1	37.6±2.3	21.9±0.1	1263±4	213±5	5.20±0.03	15.1±0.8
	S2B	18.8±0.3	32.2±1.1	13.7±0.1	841±5	142±3	1.88±0.01	13.8±0.7
	S3B	30.1 ±1.4	38.4±1.9	25.8±0.1	892±7	165±4	2.57±0.11	13.3±0.4
	S4B	398±16	139±6	4.88±0.84	872±6	210±5	13.6±0.1	9.84±0.11
Oxidizable	S1A	6.07±0.55	41.6±0.8	97.4±3.1	521±19	67.2±2.3	7.08±0.20	10.2±0.3
	S2A	3.49 ±0.55	20.6±0.4	88.1±0.3	285±9	43.1±0.1	4.17±0.29	9.47±0.45
	S3A	6.07 ±0.66	63.0±4.3	157±1	643±25	90.6±0.1	10.1±0.1	9.44±0.68
	S4A	2.62±0.14	38.1±0.9	104±2	499±14	91.0±9.0	7.07±0.47	11.7±0.5
	S1B	3.30±0.80	60.3±1.5	116±2	229±9	19.0±0.1	5.10±0.01	3.92±0.24
	S2B	0.135±0.098	56.5±1.5	45.4±0.4	150±9	12.0±0.3	3.26±0.09	2.10±0.20
	S3B	0.326±0.031	60.6±3.4	33.9±0.3	175±10	16.9±1.1	3.72±0.02	0.900±0.053
	S4B	0.981±0.131	116±0	77.4±1.7	715±1	27.3±0.0	9.90±0.01	4.47±0.44

All data are expressed as average value ± standard deviation (n = 4). LOD= limit of detection.

LOD for Pb in the acid soluble fraction= 0.563 mg/kg.

Table 3

Extractable metal content in vineyard soil samples (mg/kg) according to EDTA and acetic acid single-step extraction methods.

Fraction	Sample	Ca	Mg	Cu	Fe	Mn	Zn	Pb
EDTA	S1A	3612±114	245±1	72.7±0.7	178±7	101±2	23.1±0.5	8.68±0.32
	S2A	131±2	101±0	111±2	158±3	94.1±1.6	3.05±0.07	10.8±0.3
	S3A	5536±238	529±5	142±0	298±5	132±0	54.3±3.0	7.85±0.13
	S4A	1918±62	408±10	80.9±1.5	240±8	191±3	17.0±0.8	9.07±0.60
	S1B	875±32	106±1	84.9±3.1	183±10	71.1±2.8	4.90±0.01	8.55±0.18
	S2B	109±8	101±5	43.1±0.7	152±9	75.8±2.0	1.55±0.03	8.89±0.55
	S3B	198±11	91.7±1.4	81.9±3.0	94.4±4.7	57.3±3.2	2.65±0.14	5.09±0.12
	S4B	2398±93	666±25	64.2±4.7	323±14	272±5	21.3±0.6	7.67±0.02
Acetic acid	S1A	2964±77	313±16	27.5±0.7	18.5±0.2	192±7	29.5±0.6	<LOD
	S2A	76.9±5.1	129±3	85.1±4.1	34.6±2.4	245±10	6.38±0.38	<LOD
	S3A	5393±90	671±9	43.3±1.2	14.1±1.1	229±5	56.7±1.8	<LOD
	S4A	1824±25	445±14	28.3±0.3	23.1±1.1	374±7	21.5±0.6	<LOD
	S1B	517±13	168±1	48.8±1.5	30.9±2.8	194±7	9.22±0.26	<LOD
	S2B	114±4	138±2	27.6±0.6	50.0±2.2	188±1	3.39±0.26	<LOD
	S3B	147±5	140±6	52.7±0.3	50.5±1.5	142±1	4.83±0.22	<LOD
	S4B	2142±6	799±14	11.3±0.5	81.4±3.4	374±2	22.9±1.1	<LOD

All data are expressed as average value ± standard deviation (n = 4). LOD= limit of detection.
LOD for Pb in the acetic acid fraction= 2.25 mg/kg.

Table 4
Metal composition of leaf and grape samples (mg/kg).

Sample type	Sample	K	Ca	Mg	Cu	Fe	Mn	Zn	Cd	Cr	Pb	Hg
Leaf	H1B	13567±54a	7270±71b	1767±16a	15.9±1.6a	135±5c	483±7b	45.1±1.5a	0.0328±0.0031b	3.03±0.27	0.333±0.004b	0.0205±0.0009b
	H2B	17974±207b	6010±243a	1827±28a	26.2±0.5c	139±0c	377±5a	57.1±0.4b	0.0254±0.0023a	3.14±0.07	0.378±0.016c	0.0220±0.0015b
	H3B	14084±490a	7146±254b	2221±46b	24.3±1.8c	118±6b	473±5b	60.1±1.4c	0.0336±0.0013b	3.30±0.19	0.344±0.023bc	0.0275±0.0011c
	H4B	13448±175a	7470±522b	2141±185b	19.5±0.2b	105±4a	697±34c	60.3±1.0c	0.0353±0.0028b	3.26±0.18	0.221±0.013a	0.0109±0.0009a
	Sign ^a	***	***	***	***	***	***	***	*	ns	***	***
Leaf	H1C	9521±270bc	22478±1358b	3128±92b	882±17a	246±17	643±13b	31.1±1.5a	0.0241±0.0020a	3.15±0.20a	0.421±0.025a	0.0531±0.0018c
	H2C	10173±419c	22508±729b	2939±24a	1249±14c	230±16	606±10a	39.5±0.6b	0.0228±0.0022a	3.55±0.19ab	0.511±0.025b	0.0428±0.0034b
	H3C	9188±542b	19582±605a	3626±80c	1128±15b	257±1	649±17b	39.4±1.0b	0.0251±0.0015ab	3.96±0.28b	0.454±0.029ab	0.0439±0.0016b
	H4C	5187±314a	33682±767c	3991±59d	1882±20d	263±17	875±14c	44.0±1.7c	0.0291±0.0011b	3.55±0.28ab	0.703±0.040c	0.0251±0.0012a
	Sign ^a	***	***	***	***	ns	***	***	*	*	***	***
	Sign ^b	***,***, ***,***	***,***, ***,***	***,***, ***,***	***,***, ***,***	***,***, ***,***	***,***, ***,***	***,***, ***,***	*,ns, **,*	ns,ns, ns,ns	***,***, ***,***	***,***, ***,***
Grape	G1C	38055±482b	4688±270b	2087±30a	58.9±1.8b	185±9b	93.5±1.8a	30.6±1.6ab	<LOD	3.48±0.13b	0.185±0.009c	0.0158±0.0009a
	G2C	34959±348a	5156±204c	2200±54b	76.3±2.3c	152±9a	114±3b	32.7±2.2b	<LOD	3.51±0.19b	0.0920±0.0045a	0.0154±0.0005a
	G3C	46229±1033c	3655±163a	2458±49c	55.0±0.6a	137±5a	110±4b	27.8±1.0a	<LOD	3.08±0.12a	0.151±0.007b	0.0189±0.0009b
	G4C	46306±950c	5244±153c	2077±12a	78.5±0.6c	249±10c	99.8±2.1a	31.4±1.6ab	<LOD	2.77±0.11a	0.251±0.012d	0.0152±0.0009a
	Sign ^a	***	***	***	***	***	***	*		***	***	**

All data are expressed as average value ± standard deviation (n = 4). ^{a,b}Sign: *, **, *** and ns indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant, respectively, for the differences (a) among sampling zones for each date and (b) among sampling dates for each zone.

^aDifferent Latin letters within the same column indicate significant differences (Tukey-b test; $p < 0.05$).

LOD= limit of detection. LOD for Cd in grape samples= 0.0010 mg/kg.

Table 5

Pearson correlation coefficients for the soil-grapevine system.

Sample		modified BCR \sum_{1-2}	EDTA	Acetic acid
H1B-H4B	R	0.635**	0.668***	0.607**
	<i>p</i>	0.001	0.000	0.002
H1C-H4C	R	0.740***	0.775***	0.709***
	<i>p</i>	0.000	0.000	0.000
G1C-G4C	R	0.607**	0.649***	0.602**
	<i>p</i>	0.002	0.001	0.002

** $p < 0.01$, *** $p < 0.001$. ($n = 24$). Fe has been excluded. R = Pearson correlation coefficient.