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Francesco Salvo

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Statistical study of the influence of fungicides treatments (mancozeb, zoxamide and copper oxychloride) on heavy metals concentration in Sicilian red wines.

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1 **Statistical study of the influence of fungicide treatments (mancozeb, zoxamide**
2 **and copper oxychloride) on heavy metals concentrations in Sicilian red wine**

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Abstract

The purpose of this work was to assess the influence of mancozeb, zoxamide and copper oxychloride fungicide treatments on Mn, Zn, Cu, Cd and Pb concentrations in Sicilian red wines, grapes, marcs and grape stalks; the experimentation was carried out over two crop years, 2003 and 2004. Trace metals analysis was performed by derivative stripping chronopotentiometry which allowed detection of concentrations lower than 1 ng g^{-1} . The data which was obtained gave evidence that the levels of Mn and Zn in wines from plots treated with zoxamide-mancozeb were about 3-fold higher than those observed in the control. Wines treated with Cu oxychloride had a significant increase in Cu (II) concentrations with respect to the control; in particular samples from 2004 showed a 50% increase in Cu levels. Furthermore, as shown in a previous paper, the fungicides treatments which were studied led to a moderate increase in Pb (II) and Cd (II) levels in treated samples with respect to the control. Wines from 2004 had higher Cu and Pb amounts than wines from 2003; but the concentrations of all the other metals were similar. The statistical analysis of the data Linear Discriminant Analysis (LDA) and Kruskal-Wallis test confirmed that both zoxamide-mancozeb treatments and copper oxychloride treatments exerted a significant influence on Mn (II), Zn (II) Cu (II), Pb (II) and Cd (II) concentrations in wines, grapes, marcs and grape stalks samples from both the studied vintages.

Running head: Fungicide treatments influence on metals content of wine

Keywords: fungicide treatment, grape, grape-stalk, heavy metals, marc, statistical analysis, wine.

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3 41 **INTRODUCTION**

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7 43 In recent years, great importance has been directed towards the monitoring of food products for
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9 44 heavy metals contamination, and the assessment of the source of food contamination. The presence
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11 45 of trace elements in wines, depends on many factors such as the type of soil, wine processing
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13 46 equipment, vinification methods (Hsia et al., 1975; Golimowsky et al, 1979 b; Karadjova et al.,
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15 47 2002). Nowadays, a number of different metallic compounds are used in agricultural chemicals and
16
17 48 can, as a result, contaminate crops. Fungicides are considered to be indispensable aids in agriculture
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19 49 and are extensively used in vine-growing; many studies report the toxic effect of these chemicals on
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21 50 human health but there are few data about the influence of the use of pesticides on wine
22
23 51 composition and particularly on metal concentrations. Trace metals in wine, including Cu, Mn and
24
25 52 Zn are essential for human, animals, and plants and particularly Zn at low concentrations plays an
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27 53 important role in wine fermentation. On the other hand, Cu and Mn at high concentrations
28
29 54 participate significantly in the destabilization of wines and in their oxidative evolution (Benítez et
30
31 55 al., 2002). At the same time the analysis for certain elements, such as Pb and Cd, in wines is of
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33 56 special interest due to their toxicity in case of excessive intake (Ribéreau-Gayon and Peynaud, 1976;
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35 57 Gennaro et al, 1986; Mc Kinnon and Scollary, 1997; Fernandez Pereira, 1998). A recent study has
36
37 58 provided evidence that the use of some organic and inorganic fungicides - quinoxifen, dinocap,
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39 59 penconazole, azoxystrobin and powder of sulphur-in wine-growing, may moderately influence the
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41 60 concentration of trace elements (Cd, Cu, Pb and Zn) in red and white wines (Salvo et al., 2003).

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51 62 The present study has been focussed on the possible correlation between the presence of Mn (II), Zn
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53 63 (II), Cu (II), Cd, (II), and Pb (II), in samples of Sicilian red wines and mancozeb-zoxamide and
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55 64 copper oxychloride treatments that occurred during viticulture; in order to better investigate the
56
57 65 effects of these fungicide treatments, heavy metals analysis were also performed on grapes, marcs,
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59 66 and grape stalks; furthermore data concerning heavy metal levels in soil near the plots were

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3 67 available as background data. The experimentation was carried out over two crop years, 2003 and
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5 68 2004. Ethylenebisdithiocarbamate of manganese and zinc (mancozeb)- $[C_4H_6N_2S_4Mn]_x(Zn)_y$ -
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8 69 belongs to a wider group of ethylenebisdithiocarbamate fungicides and is widely used in
9
10 70 agriculture, mainly on fruits, ornamental plants and vegetables (Maroni et al., 2000). It is
11
12 71 characterized by low acute toxicity and short environmental persistence, for this reason the total
13
14 72 amount of mancozeb used is increasing worldwide. Zoxamide [3, 5-dichloro-N-(3-chloro-1-ethyl-1-
15
16 73 methyl-2-oxopropyl)-4-methylbenzamide] is a new fungicide for foliar use on potatoes, grapevines
17
18 74 and vegetables to control Oomycete fungi (Egan et al., 1998). Despite the production of a wide
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20 75 variety of synthetic organic fungicides, copper oxychloride- $CuCl_2 \cdot 3Cu(OH)_2$ -still predominates
21
22 76 the field of fungicidal plants disease control and its wide use is also due to its general low toxicity
23
24 77 for humans (Helling et al., 2000).
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31 78
32 79 The determination of Mn, Zn, Cu, Cd and Pb in grapes, grape stalks, marcs and wines was carried
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34 80 out by using derivative stripping chronopotentiometry (dSCP), an electro-analytical technique
35
36 81 widely employed for trace and ultra-trace metals analysis in food and environmental matrices
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38 82 (Salvo et al, 2003; La Pera et al., 2003; Dugo et al., 2004; Dugo et al., 2005a; Dugo et al., 2005b;
39
40 83 Dugo et al., 2005c; Matarese Palmieri et al., 2005). The analytical techniques conventionally used
41
42 84 for metal determinations are both emission and absorption spectrophotometry, as well as
43
44 85 voltammetry (Balduini et al., 1999). These methods require sample pre-treatment which enable the
45
46 86 complete destruction of the organic matrix such as wet digestion, dry ashing and microwave oven
47
48 87 dissolution (Reid et al., 1995). Derivative stripping chronopotentiometry allows the direct
49
50 88 determination of heavy metals in wines without the need of a laborious sample preparation
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52 89 procedure but acid adjustment, as the glassy (GCMFE) is inert towards organic molecules
53
54 90 interferences during the signal collection phase (Town and van Leewen, 2001 and 2002).
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92 **Materials and methods**

93 *Apparatus*

94 Metals analysis were carried out by using a PSA ION 3 potentiometric stripping analyzer
95 (Steroglass, S. Martino in Campo, Perugia, Italy). The analyzer was equipped with a conventional
96 three-electrodes cell. The plating process with mercury of the glassy carbon working electrode, the
97 reference and the auxiliary electrodes, have been described in previous papers (Salvo et al., 2003;
98 La Pera, et al. 2003; Dugo et al, 2005 a, b, c; Matarese Palmieri et. al 2005; Dugo et al., 2004 b)

99 *Reagents*

100 All glassware was rinsed with 10% (v/v) nitric acid. Ultra-pure water ($18.2 \text{ Mohm cm}^{-1}$) was
101 obtained from a Pure Lab RO and a Pure Lab UV system (USF, Ransbach-Baumbach, Germany).
102 Ultra pure hydrochloric acid (34-37%), Hg (II) (1000 mgL^{-1} , 1M in hydrochloric acid) and Cd (II),
103 Cu (II), Pb (II), Zn (II), and Mn (II) (1000 mgL^{-1} , 0.5 N in nitric acid) standard solutions were
104 purchased from Panreac Quimica (Barcelona, Spain). Glucose, fructose, gallic acid, tartaric acid
105 and potassium tartrate used to prepare the model wine solution (14%v/v aqueous ethanol, 2 gL^{-1}
106 fructose, 2 gL^{-1} glucose, 1 gL^{-1} gallic acid, saturated with potassium hydrogen tartrate and adjusted
107 to pH 3.5 with tartaric acid), were purchased from Sigma Aldrich (Milwaukee, WI, USA). Supel-
108 ENVI carbon columns were obtained from Supelco (Bellefonte, PA, USA)

109 *Wines characteristics and pesticides treatments*

110 Trials were conducted in five plots located in the Sicilian wine-producing zone of Etna; grapevines
111 were from the Nerello Mascalese variety. Plots were investigated over two crop years 2003 and
112 2004. Plots B-C were characterized by the use of mancozeb and zoxamide and they differed for the
113 time elapsed from the last fungicide treatment and the grape harvesting (Δt) ; plots D-E by the use
114 of copper oxychloride and they also differed for the Δt , and plot A was characterized by the use of
115 water and represented the control plot (Table 1). All the studied plots measured between 0.25-0.5

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3 116 ha. Pesticides were used at the doses recommended by the manufacturer and the treatments were
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6 117 applied by the wine farmers with a manual sprayer. In all the considered crop years (2003 and
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8 118 2004), the grapes were harvested in the period from Aug. 25 to Sept.10. Newly cropped *Vitis*
9
10 119 *vinifera* were crushed and de-stemmed, then spiked with SO₂ (5 g/hL), selected yeasts (30 g/hL)
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13 120 and let to ferment for 10 days at 28°C, making three fullings a day. The wine was drawn from the
14
15 121 vat and the marcs were crushed by a hydraulic press. After 4 weeks the lees were removed and the
16
17 122 wine was spiked with SO₂ (2 g/hL). Subsequently the wine was spiked again with SO₂ (2 g/hL) and
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20 123 bottled in dark bottles at 4°C for the duration of the experimentation. Each sample was separately
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22 124 fermented and bottled, following the protocol mentioned above. From each investigated plot, both
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25 125 in 2003 and 2004, representative samples of grapes (about 10 kg), grape-stalks (about 1.4 kg),
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27 126 marcs (about 0.5 Kg), and wines (7.5 L) were delivered to the laboratory and stored at -10°C.
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29 127 *Pedoclimatic conditions*

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32 128 The vineyards lay on calcareous soil (300 m asl) that contains on average 37% of sand, 36 % of
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34 129 silt, 27% of clay and 4.5 % of organic matter; water contents at 10–20 cm depths were 30.1% by
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37 130 volume. Heavy metal concentrations of soil and groundwater samples collected in different points
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39 131 of each plot before the first pesticides application both in 2003 and 2004, determined in an external
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42 132 laboratory by ICP-AES, were available as background data (Table 2). In the studied area the climate
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44 133 is arid and the low rainfall results in a lack of water except in the winter months. The environmental
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46 134 conditions were recorded by an AD-2 automatic weather station from April to September; during
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49 135 the experiments, the minimum and maximum average temperatures were 12-35 °C in 2003 and
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51 136 12.6-35.5 °C in 2004. The average precipitation rate over the studied area was 665 mm/year in 2003
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54 137 and 600 mm/year in 2004; in particular the rainfall was 0.7 mm in spring and 0.1 mm in summer on
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56 138 1 rainy day in 2003 and 0.55 mm in spring and 0.0 mm in summer on 1 rainy day in 2004.
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58 139 *Sample preparation*

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3 140 Wine samples were directly analyzed after acidification to pH 2 with 5 M HCl and filtration through
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6 141 a Carbon column as described in previous studies (Salvo et al, 2003; Dugo et al, 2005 b). Grapes
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8 142 from each plot were beaten by a blender equipped with titanium blades to avoid metal
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10 143 contamination, until a homogeneous juice was obtained. An aliquot of 2 mL of the juice was placed
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13 144 into a teflon beaker with 4.0 mL of 37% HCl and 6.0 mL of ultrapure water and extracted as
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15 145 described in a previous paper (Dugo et al., 2005 a; Dugo et al., 2004 b). Marcs and grape-stalks
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17 146 from each plot were separately dried at 110 C° to constant weight in order to measure their moisture
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20 147 contents. An aliquot of 1 g of dried samples was dissolved in 10.0 mL of 35% HCl and extracted at
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22 148 80 °C as discussed in previous studies concerning metal analysis in vegetable matrices (Dugo et al,
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25 149 2005 c, Matarese Palmieri et. al 2005).

27 150 *Derivative chronopotentiometric stripping analysis*

30 151 Cd (II), Cu (II), Pb (II) and Zn (II) were determined simultaneously in wine, grapes, grape-stalks
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33 152 and marcs as described in a previous papers (Salvo et al, 2003, Dugo et al, 2005 b, c; Matarese
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35 153 Palmieri et. al 2005); Mn (II) was determined separately by placing 500 µL of acidified wine (or
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38 154 acid extract of grapes, marc, grape-stalks) in the electrochemical cell together with 1 mL of 0.5 M
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40 155 ammonia-ammonium chloride buffer (pH 9.5), 10.0 mL of ultrapure water and 1.0 mL of 1000 µg
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42 156 mL⁻¹ Hg (II) standard solution as oxidant agent, and de-oxygenating for 5 min by N₂ purging. The
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45 157 best electrochemical conditions were optimized by fixing the electrolysis potential at -1700 mV, the
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47 158 final acquisition potential and the cleaning potential respectively at -500 mV and -1500 mV, and
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50 159 varying the electrolysis time as afterwards described; the solution was stirred at 1000 rpm; the
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52 160 manganese peak was registered around -1460 mV (Dugo et al., 2005 c). The quantitative analysis
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54 161 of metals was done by the multiple-point standard additions method. Optimum precision and
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57 162 accuracy were obtained with two additions of appropriate aliquots of standard solution of Mn (II) in
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59 163 order to double the sample peak area, and performing the measurements five times for each addition
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164 (La Pera, et al. 2003; Dugo et al, 2005 a, b, c; Matarese Palmieri et. al 2005; Renman and Jagner,

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3 165 1997). Calibration curve with correlation coefficients $> 99.5\%$ were obtained. The linear
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6 166 concentration range was $0-10000 \mu\text{gL}^{-1}$. Under optimized electrochemical conditions, the analysis
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8 167 lasted 25 minutes.
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10 11 168 ***Method performance: sensitivity, accuracy and repeatability***

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13 169 Method performances for Cu, Cd, Mn, Pb and Zn chronopotentiometric analysis in wines and
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16 170 vegetables have been reported in previous papers (Salvo et al, 2003; La Pera, et al. 2003; Dugo et
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18 171 al, 2005 a, b, c; Matarese Palmieri et. al 2005; Dugo, et al.2004).
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20 21 172 ***Statistical methods***

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23 173 The statistical elaboration of the data was performed by two softwares: SPSS for Windows (version
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25 174 12.0; 2003) and STATISTICA package for Windows (version 6.0, 2000). The non parametric test
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28 175 of Kruskal-Wallis and Linear Discriminant Analysis (LDA) were performed grouping wines,
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30 176 grapes, marcs and grape stalks according to the pesticide treatments applied; data expressing
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32 177 concentrations of Mn, Zn, Cu, Pb and Cd in wines, marcs, grapes and grape-stalks samples from
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35 178 2003 and 2004 vintages were processed. In order to define statistically the difference between
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37 179 samples submitted to a specific fungicide treatment (A, B, C, D, E), the data were elaborated using
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39 180 a multivariate non-parametric Analysis of Variance and subsequently a Linear Discriminant
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41
42 181 Analysis in order to distinguish samples coming from one of the five treatment groups according to
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44 182 a simple statistical model (Dugo et al., 2005 b). The choice to perform a non-parametric analysis
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46
47 183 was related to the fact that the distribution of Mn, Zn, Pb, Cd and Cu levels did not seem to meet
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49 184 the necessary conditions required to apply a simple Analysis of Variance (ANOVA), because there
50
51 185 was not a homogeneity of variance between samples coming from different groups. Moreover
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54 186 samples belonging to different treatments did not show a normal distribution over the same group.
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56 187 Therefore the five variables (Mn, Zn, Pb, Cd and Cu concentrations expressed in mg/L), were
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58 188 submitted to the non-parametric test of Kruskal-Wallis to verify the significances of differences
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60 189 between the control and treated samples. This procedure tested the hypothesis that n independent
190 samples came from populations having the same distribution.

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RESULTS AND DISCUSSION

Derivative stripping chronopotentiometry for the analysis of metals in enological samples

Growing concern about heavy metals food contamination has led to increasing needs to develop sensitive analytical techniques capable to detect trace and ultra-trace concentrations of metals in a variety of alimentary matrices. Derivative stripping chronopotentiometry has been recognised as a powerful tool for measuring trace metals in food samples. Its remarkable sensitivity is attributed to the concentration step during which the metals are accumulated onto the working electrode. Under optimized analytical conditions, detection limits lower than 1 ng g^{-1} have been obtained for the studied metals in different food matrices; the analytical performances of SCP tested on certified reference materials are reported in the cited literature (La Pera, et al. 2003; Dugo et al, 2005 a, b, c; Matarese Palmieri et. al 2005; Renman and Jagner, 1997). Sample preparation played an important role in ensuring the accuracy of determination, because metals were present in very low concentrations in complex matrices such as wine, grape, marcs and stalks. Interferences from organic molecules are minimized in SCP, hence a simple and rapid sample pre-treatment based on concentrated hydrochloric acid extraction at 80°C was required which enabled the extraction of the metals in less than 1 h. Wine may be analysed after acidification to pH 2, followed by filtration through a carbon column, without the need for sample pre-treatment. A speciation study performed on a certified reference wine has shown that at $\text{pH} < 3$ heavy metals are present in the soluble form (Azenha and Vasconcelos, 2000; Karadjova et al., 2002). A recovery test carried out on a model wine spiked at different levels with Cu (II), Mn (II), Zn (II), Cd (II) and Pb (II) standard solutions, acidified to pH 2 with HCl and filtered trough a carbon column. The obtained recovery factors (95-99%) provided evidence that metal loss did not occur during sample pre-treatment. Comparative analysis between filtered and unfiltered red wine discussed in a previous paper, demonstrated that metal quantification was not affected by the filtration procedure and a higher analytical response was achieved by passing the wine sample trough the carbon column prior to the

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3 217 chronopotentiometric measures. (Dugo et al, 2005b); the repeatability of the chronopotentiometric
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6 218 method, expressed as RSD%, was $1.1\pm 0.1\%$ for Cd, $2.0\pm 0.3\%$ for Cu, $1.5\pm 0.2\%$ for Pb, $3.0\pm 0.1\%$
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8 219 for Mn and $1.0\pm 0.1\%$ for Zn (La Pera and Dugo, 2005). A previous study reports parallel analysis
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10 220 of red and white wine samples by graphite furnace atomic absorption spectroscopic and the
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13 221 obtained results agreed within 5% (Salvo et al., 2003). SCP coupled high sensitivity and resolution,
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15 222 rapid sample preparation with low costs of the equipment; moreover the small dimensions of the
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18 223 analyzer allowed its transport for in situ analysis. For all these features, SCP is well suited for
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20 224 routine analysis.

225 ***Influence of fungicide treatments on Cu, Cd, Mn, Pb and Zn levels***

226 *Results from untreated plots*

227 In control wines samples from 2003 (plot A), metal levels decreased in the order
228
229 Zn>Mn>Cu>Pb>Cd, whereas in samples from the following crop year in the order Zn \cong Mn
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231 \cong Cu>Pb>Cd (Table 3). Mn, Cu and Zn were the most abundant metals in grape-stalks from 2003
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233 and 2004, followed by Cd and Pb (Table 4). As it was observed for wine samples, in grapes from
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235 2003 metals concentrations decreased in the order Zn>Cu>Mn>Pb>Cd, whereas in samples from
236
237 the following crop year in the order Cu>Zn>Mn > Cd > Pb (Table 5). Furthermore, at **vintaging**
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239 both in 2003 and 2004 grapes from untreated plots did not show any fungal infection since weather
240
241 conditions, characterized by high temperatures and reduced rainfall were unfavourable for fungal
242
243 development. In marcs samples from both vintages, metal concentrations decreased in the order
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245 Cu>Mn>Zn, Cd and Pb levels in the controls were lower than the instrumental detection limits
246
247 (Table 6). Cadmium levels in soil from plot A sampled in 2003 were higher than those found in the
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249 other plots; this might have resulted in an increased cadmium concentration in grape-stalks and
250
251 grapes grown in the control vineyard.

241 *Results from plots treated with mancozeb-zoxamide*

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3 242 As Table 3 reports, Mn and Zn levels in 2003 wines from zoxamide-mancozeb treatments (plot B
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6 243 and C) were about 3 fold and 2 fold higher than those observed in the control samples, respectively;
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8 244 the Δt parameter did not greatly influence the concentration of these elements. Mn levels in 2004
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11 245 wines from zoxamide-mancozeb treatments (plot B and C) were about 3-fold higher than those
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13 246 observed in the control samples; the Δt parameter seemed to exert a significant influence on Zn
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16 247 level as the metal concentration was 3.5 fold higher than that observed in the control in samples
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18 248 harvested 14 days after the last treatment (plot B) and 6.5 fold higher in samples harvested 28 days
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20 249 after the last treatment (plot C). Moreover the studied treatments lead to a significant increase of Pb
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23 250 and Cd concentrations (an average of 150% over the control) in wines from 2003, meanwhile only a
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25 251 slight enhancement in Pb levels in samples from 2004 was observed. As given in Table 4, Mn levels
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27 252 in 2003 grape-stalks from plots B and C were from 2.4 to 6.5-fold higher than those observed in the
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30 253 control and from 5.6 to 6.8-fold higher in 2004 grape-stalks; in particular, samples harvested 28
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32 254 days after the last treatment showed the highest values. Zn levels were from 1.5 to 2.6-fold higher
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34 255 with respect to the control in grape-stalks from 2003 and from 4 to 6 higher in 2004 samples; in
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37 256 particular, samples harvested 14 days after the last treatment showed the highest values. Pb and Cd
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39 257 concentrations in 2003 grape-stalks from zoxamide-mancozeb treatments were from 5 to 7 fold
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42 258 higher than those observed in the blanc, whereas samples from 2004 showed a slight increase of Pb
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44 259 concentration as compared to the control and a great enhancement of Cd levels (7 to 11 fold higher
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46 260 with respect to the blanc). As Table 5 reports, the Δt parameter seemed to exert a significant
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49 261 influence on Mn level in grapes from both the crop years: samples from 2003 harvested 14 days
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51 262 after the last treatment (plot B) presented Mn concentrations 11 fold higher with respect to the
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53
54 263 control, whereas samples harvested 28 days after the last treatment (plot C) 4-fold higher than the
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56 264 control; on the contrary samples from 2004 harvested in the plot B showed Mn levels 2-fold higher
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58 265 with respect to the blanc, whereas samples harvested in the plot C presented Mn levels 4-fold higher
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60 266 than the blanc. A similar profile was observed for Zn levels, particularly for grapes from 2004.

267 Furthermore a remarkable influence of the treatments over Pb and Cd levels, particularly in grapes

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3 268 from 2003 was noticed. Mn and Zn levels in 2003 and 2004 marcs from plots B and C were from 2
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6 269 to 4-fold higher than those observed in the controls; treated samples from 2004 showed an increase
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8 270 of Pb levels with respect to the control, whereas no significant influence of the treatments on Cd
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10 271 levels in marcs was noticed (Table 6).

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15 273 *Results from plots treated with copper-oxychloride*

16
17 274 Cu levels in wines from 2003 were very much lower with respect to those observed in the following
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20 275 crop year; moreover Cu concentrations in 2003 wines from all the plots were very similar, whereas
21
22 276 a significant influence of copper-oxychloride treatments was noticed in wines from 2004 (plots D
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25 277 and E) that showed a Cu amount increased by 50% with respect to the control; moreover copper-
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27 278 oxychloride applications lead to a moderate increase of Pb and Cd concentrations in wines from
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29 279 both the crop years (Table 3). Copper-oxychloride treatments greatly influenced Cu levels in grape-
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32 280 stalks from 2003 and 2004 that presented concentrations from 4 to 50-fold higher than those
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34 281 detected in the control; samples harvested 28 days (Δt) after the last treatment (plot E) showed the
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37 282 highest Cu amounts. In particular, copper-oxychloride applications seemed to exert a remarkable
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39 283 effect on Pb levels in grape-stalks which are 24-fold higher than that of the control in samples from
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41 284 2003, nevertheless this effect was not observed in samples from the following crop year (Table 4).
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44 285 As was already observed for wines, grapes samples from 2003 presented lower Cu concentrations
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46 286 than those detected in samples from 2004; the influence of copper-oxychloride treatments over Cu
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49 287 concentrations in grapes from both the vintages was particularly evident since treated samples
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51 288 presented values 3-4 fold higher than those of the controls (Table 5). A significant influence of Cu
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53 289 oxychloride treatments over Cu levels in marcs from both the crop years was noticed since samples
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56 290 from plots D and E had concentrations 3-5 fold higher than the control; moreover treated samples
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58 291 from 2004 showed an increase of Pb levels with respect to the control, whereas no significant
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60 292 influence of the treatments on Cd levels in marcs was noticed (Table 6).

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3 294 ***Statistical analysis***
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6 295 As shown in Tables 3-6, all the four types of enological matrices (wines, marcs, grapes and grape-
7 stalks) presented a significant difference through the five treatments. This is explained by the
8 296 calculated significance levels (p) from Kruskal-Wallis test, that resulted $\leq .001$ for all of the five
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10 297 variables considered independently over the two years. This result suggests that the studied
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12 298 fungicide treatments greatly influenced the concentrations of the Mn, Zn, Pb, Cd and Cu in the
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14 299 wines, marcs, grapes and grape-stalks samples from both the vintages. The concentrations of Mn,
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16 300 Zn, Pb, Cd and Cu have been further studied using *Linear Discriminant Analysis (LDA)* in order to
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18 301 create a model that, through some classification functions, could separate samples in relation to the
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20 302 particular treatment used. LDA was performed separately on wines, marcs, grapes and grape-stalks
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22 303 samples from the two crop years. The results have shown a very good degree of classification
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24 304 (100%) of the samples according to the functions created in every analysis. Scatterplots (Figures 1-
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26 305 4) of discriminant functions, that explained on average the 93% of the total variance over the
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28 306 samples, have shown a good separation of the five groups representing various fungicide treatments
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30 307 applied to grapevines. Generally it can be observed (overall for the wine samples) a little proximity
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32 308 between samples belonging to treatments “B” with those from the treatment “C”; and between
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34 309 samples from treatments “D” and “E”. This means that these groups of treatments influence in a
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36 310 common way the concentrations of metals in the samples. Moreover any classification function can
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38 311 be represented as a linear combination of the five variables, providing a good statistical model to
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40 312 characterize the treatments. For example, the two classification functions regarding wines from
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42 313 2003 can be described by the following equations:
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55 316 $\text{Root1} = -2,420\text{Mn} -5,477\text{Zn} +53,807\text{Cu} -76,533\text{Pb} -230,871\text{Cd} +10,013;$

56 317 $\text{Root2} = 2,995\text{Mn} -0,379\text{Zn} +472,674\text{Cu} -117,649\text{Pb} +190,004\text{Cd} -22,525;$

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319 where Root1 is strongly negatively correlated with the Zn level and Root2 is positively correlated
320 with the Cu level.

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322 On the whole the statistical analysis of the data has evidenced that wines, grapes, grape-stalks and
323 marcs from both the crop years treated with zoxamide-mancozeb and with copper oxychloride
324 showed a significant increase of Mn (II), Zn (II) and Cu (II) concentrations with respect to the
325 control; the effect of time elapsed between the last application of fungicides and the harvesting (Δt)
326 on the concentration of Mn, Zn, Pb, Cd and Cu in wines, grapes, grape-stalks and marcs has not
327 been yet elucidated as heavy metal levels in treated samples irregularly increased in a Δt -dependent
328 manner. Furthermore Mancozeb-zoxamide and copper-oxychloride treatments lead to a moderate
329 increase of Pb (II) levels in treated wines, grape-stalks, grapes and marcs with respect to the control.
330 As shown in a previous paper (Salvo et al, 2003), the increase of Cd and Pb levels in treated
331 samples could be the result of the presence of trace concentrations of these contaminants in the
332 chemicals used for the treatments.

334 *Inter-seasonal variations*

335 Pesticide treatments were the variables that mostly influenced metal levels in the enological
336 matrices from 2003 and 2004 vintages . Therefore, in order to estimate inter-seasonal variations due
337 to the environmental conditions, only the control plots have been considered. Tables 3-6 give
338 evidence that Cu and Pb levels in 2004 wines, grapes, and marcs were much higher than those
339 found in 2003, whereas Mn and particularly Zn levels in 2003 wines, grapes, grape-stalks and marcs
340 were higher than those found in 2004. As Table 2 reports, these results are related to metal levels in
341 the soil/groundwater sampled in the control plots in both the crop years. The relationship between
342 metal levels in the enological matrices and in soil/groundwater system may explain the observed
343 inter-seasonal variations. It must be added that it is very difficult to asses all the factors including,

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3 344 pollution, volcanic emissions, harvesting and storage that that may influence metal levels in food
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6 345 plants grown in the same zone in two different crop years.
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10 347 ***Nutritional evaluation***
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13 348 Zinc, copper and manganese are essential micronutrients since they are co-factors of many
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15 349 enzymes, a low dietary intake of these microelements may cause severe deficiency syndromes; on
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17 350 the other hand at high concentrations these trace elements may have toxic effects. For this reason
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20 351 Recommended Daily Allowances (RDAs) have been established by the Food and Drug
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22 352 Administration; for a 70 kg adult they were 18 mg Zn/day, 5 mg Mn/day, 3 mg Cu/day (La Pera and
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25 353 Dugo, 2005). Considering the concentration ranges of Zn ($0.4\text{-}3\text{ mgL}^{-1}$), Mn ($0.5\text{-}1.6\text{ mgL}^{-1}$) and Cu
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27 354 ($0.05\text{-}1\text{ mgL}^{-1}$) found in this study and a 0.25 L daily wine consumption, the RDA % were
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29 355 respectively of 0.5-4 %, 2-8 % and 0.3-8 %. Moreover Cu and Zn levels were always below the
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32 356 legal limits established by the Italian Republic, 1.0 and 5.0 mgL^{-1} respectively (Italian Republic,
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34 357 1986). Toxic elements such as Cd and Pb, present in foodstuff mainly as a result of a contamination
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36 358 process, were found in the studied treated and untreated wines in concentrations well below the
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39 359 legal limits established by the European Community (0.2 mg Pb L^{-1} and 0.1 mg Cd L^{-1}) (European
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41 360 Community, 2001). The nutritional evaluation of the obtained data has shown that a moderate daily
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44 361 consumption of treated wines from both the crop years, provides a Cu, Mn and Zn intake below the
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46 362 RDA and that Pb and Cd levels are within the legal limits; therefore a correct use of these
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48 363 fungicides in wine-growing does not represent a risk for human health.
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52 365 **References**
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3 **Table 1.** Fungicide treatments applied for each plot.
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PLOT	Fungicide	N. applications	Frequency of applications (days)	Δt (days)*
A	H ₂ O	4	7	28
B	200g/hL of Zoxamide 8.3% and Mancozeb 66.7%	4	7	14
C	200g/hL of Zoxamide 8.3% and Mancozeb 66.7%	4	7	28
D	350g/hL of Cu oxychloride 50%WP**	4	7	14
E	350g/hL of Cu oxychloride 50%WP	4	7	28

2 *Δt (days): time elapsed from the last fungicides treatment and the grape harvesting

3 **WP= wet powder

4
5 **Table 2.** Heavy metal concentrations (as well as pH) in soil (mgkg⁻¹ d.w.) and groundwater (μgL⁻¹)
6 samples collected in different points of the studied plots. Each result is the mean of three replicates.

Plot	pH	Mn	Zn	Cu	Pb	Cd
2003						
A s*	7.9	100.2±11.3	85.4±7.2	11.1±1.3	1.0±0.2	1.5±0.1
A g*	7.3	198.2±15.6	67.1±8.8	5.9±0.5	0.1±0.0	0.2±0.0
B s	8.0	95.5±19.1	73.2±8.4	10.5±1.1	1.5±0.5	1.0±0.3
B g	7.4	177.5±25.9	58.3±5.5	5.0±0.5	0.2±0.0	<0.1
C s	7.9	103.4±16.3	78.8±5.1	8.9±0.9	1.6±0.4	1.3±0.3
C g	7.2	212.2±25.3	66.3±7.1	3.8±0.3	0.2±0.0	0.1±0.0
D s	7.9	89.0±9.6	81.4±9.4	9.1±0.9	1.3±0.7	1.6±0.2
D g	7.3	161.1±14.3	70.0±5.9	4.1±0.3	0.1±0.0	0.1±0.0
E s	7.8	106.7±13.5	75.6±8.3	11.7±1.9	1.3±0.6	1.0±0.2
E g	7.5	224.0±21.5	62.5±5.8	6.0±0.6	0.1±0.0	<0.1
2004						
A s	8.0	80.2±10.0	74.3±7.2	13.2±1.0	1.7±0.3	1.1±0.1
A g	7.5	68.5±6.1	49.9±5.1	7.3±0.6	0.2±0.0	<0.1
B s	8.1	85.5±9.4	83.0±8.5	15.2±2.0	1.9±0.7	1.0±0.3
B g	7.5	62.3±5.5	54.2±5.9	8.5±0.6	0.2±0.0	<0.1
C s	7.8	103.0±11.2	94.2±7.3	10.5±1.5	2.0±0.2	1.9±0.3
C g	7.3	70.0±6.9	58.6±4.8	7.0±0.5	0.2±0.0	0.1±0.0
D s	7.9	101.7±8.5	82.8±8.8	8.5±0.9	2.1±0.5	1.4±0.2
D g	7.4	69.8±6.3	55.6±6.1	7.0±0.6	0.2±0.0	<0.1
E s	7.9	78.9±7.5	67.4±6.7	8.2±1.1	1.8±0.6	1.0±0.2
E g	7.3	75.2±7.0	41.5±3.9	6.3±0.4	0.1±0.0	0.1

7 *s=soil, g=groundwater

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3 **Table 3** Metal concentrations (mean±st.dev) in wines subjected to different pesticide treatments, as
4 indicated in Table 1; five samples from each plot were analysed in triplicate (mean±st.dev).
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7 Significances of differences between blank and treated samples are also reported as chi-square (χ^2)
8 and p values from Kruskal- Wallis test*
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10 *the treatments number was considered as the grouping variable; the freedom degrees were 4
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year	2003				
PLOT	Mn (mgL ⁻¹)	Zn (mgL ⁻¹)	Cu (µgL ⁻¹)	Pb (µgL ⁻¹)	Cd (µgL ⁻¹)
A	0.5±0.1	1.4±0.2	43.5±2.0	10.4±10.1	5.5±1.0
B	1.7±0.1	3.0±0.1	50.2±1.3	31.3±5.2	9.3±4.2
C	1.2±0.2	2.6±0.2	47.3±3.4	18.4±1.5	13.5±3.9
D	0.5±0.1	0.5±0.1	57.2±2.7	13.0±1.5	2.2±1.0
E	0.6±0.1	0.5±0.0	44.0±2.1	19.3±1.4	6.3±1.0
χ^2	19.579	21.840	19.818	20.427	18.298
p <	0.000	0.000	0.000	0.000	0.000
	2004				
A	0.5±0.0	0.4±0.0	552.2±92.1	117.1±25.5	<0.5
B	1.5±0.1	1.4±0.1	335.4±16.4	190.4±21.9	<0.5
C	1.7±0.2	2.6±0.3	760.4±14.4	<0.1	<0.5
D	0.3±0.1	0.7±0.0	1112.1±84.5	32.6±7.3	<0.5
E	0.6±0.0	1.1±0.1	1100.0±73.3	152.2±6.3	<0.5
χ^2	21.948	22.995	21.940	22.564	0.0
p <	0.000	0.000	0.000	0.000	1

Table 4. Metal concentrations in grape-stalks subjected to different pesticide treatments; five samples for each plot were analysed in triplicate (mean±st.dev). Significances of differences between blank and treated samples are also reported as chi-square (χ^2) and p values from Kruskal-Wallis test

year	2003				
PLOT	Mn (mgL ⁻¹)	Zn (mgL ⁻¹)	Cu (mgL ⁻¹)	Pb (µgL ⁻¹)	Cd (µgL ⁻¹)
A	7.4±1.2	17.3±1.4	6.44±0.75	8.3±1.2	17.2±1.2
B	18.8±1.0	47.2±2.0	8.84±1.47	55.1±1.4	86.1±2.2
C	48.4±1.6	26.2±1.0	11.2±1.2	58.4±2.9	73.9±2.5
D	10.4±1.5	15.6±1.4	196±12	191.1±11.0	62.1±1.3
E	4.5±0.6	16.2±1.2	314±12	192.8±2.5	17.4±2.1
χ^2	22.900	19.483	22.390	21.986	23.529
p <	0.000	0.000	0.000	0.000	0.000
year	2004				
A	7.0±0.2	5.0±0.4	28.08±0.92	87.0±9.1	11.1±1.7
B	39.8±5.9	29.0±4.2	24.5±2.3	92.3±8.6	82.2±11.0
C	47.7±3.2	19.2±1.3	54.7±4.5	103.0±12.1	126.8±80.3
D	7.6±0.8	4.4±0.4	124.0±13.4	164.8±13.4	67.2±7.4
E	15.6±1.7	6.4±0.7	177.0±15.1	<0.1	<0.1
χ^2	21.629	22.250	22.900	19.672	21.718
p <	0.000	0.000	0.000	0.000	0.000

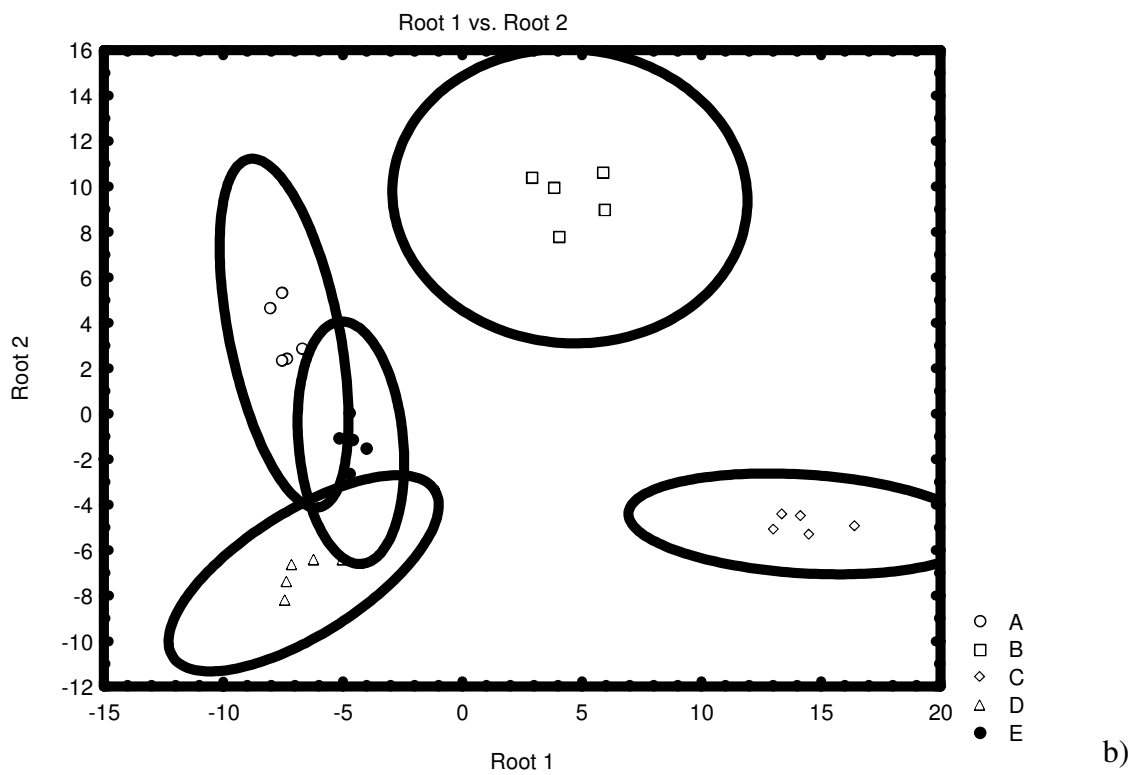
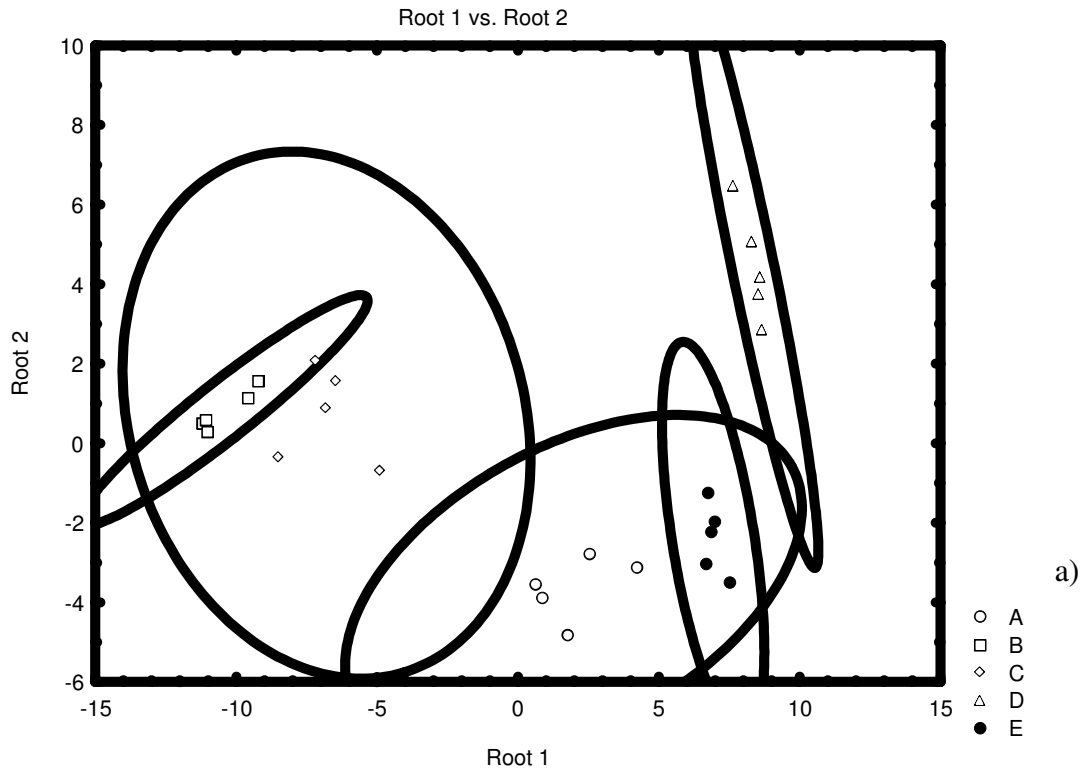
Table 5. Metal concentrations in grapes subjected to different pesticide treatments; five samples for each plot were analysed in triplicate (mean±st.dev). Significances of differences between blank and treated samples are also reported as chi-square (χ^2) and p values from Kruskal- Wallis test

year		2003				
PLOT	Mn (mgL ⁻¹)	Zn (mgL ⁻¹)	Cu (mgL ⁻¹)	Pb (µgL ⁻¹)	Cd (µgL ⁻¹)	
A	2.2±0.7	24.2±3.3	3.60±0.77	<0.1	<0.1	
B	23.3±4.1	61.8±10.0	5.40±1.29	196.1±46.4	143.2±20	
C	8.0±1.8	27.0±6.0	12.9±1.8	22.3±9.5	76.1±18	
D	4.3±1.4	24.2±6.7	12.9±2.1	265.4±28.1	40.2±9.9	
E	3.3±0.5	15.1±1.7	9.78±1.92	288.0±22.3	52.9±10.4	
χ^2	21.494	19.080	19.947	21.492	22.825	
p <	0.000	0.001	0.001	0.000	0.000	
		2004				
A	5.0±0.7	11.5±0.7	20.0±0.9	<0.1	54.2±3.2	
B	8.9±0.9	19.8±0.9	18.6±1.0	41.1±1.3	66.1±4.5	
C	21.5±2.3	54.9±2.6	10.6±1.4	<0.1	156.3±10.2	
D	4.7±0.7	14.0±0.8	83.2±2.7	<0.1	161.2±7.8	
E	2.0±0.4	3.9±0.50	120±4	<0.1	183.4±8.2	
χ^2	21.998	23.077	22.530	23.641	22.030	
p <	0.000	0.000	0.000	0.000	0.000	

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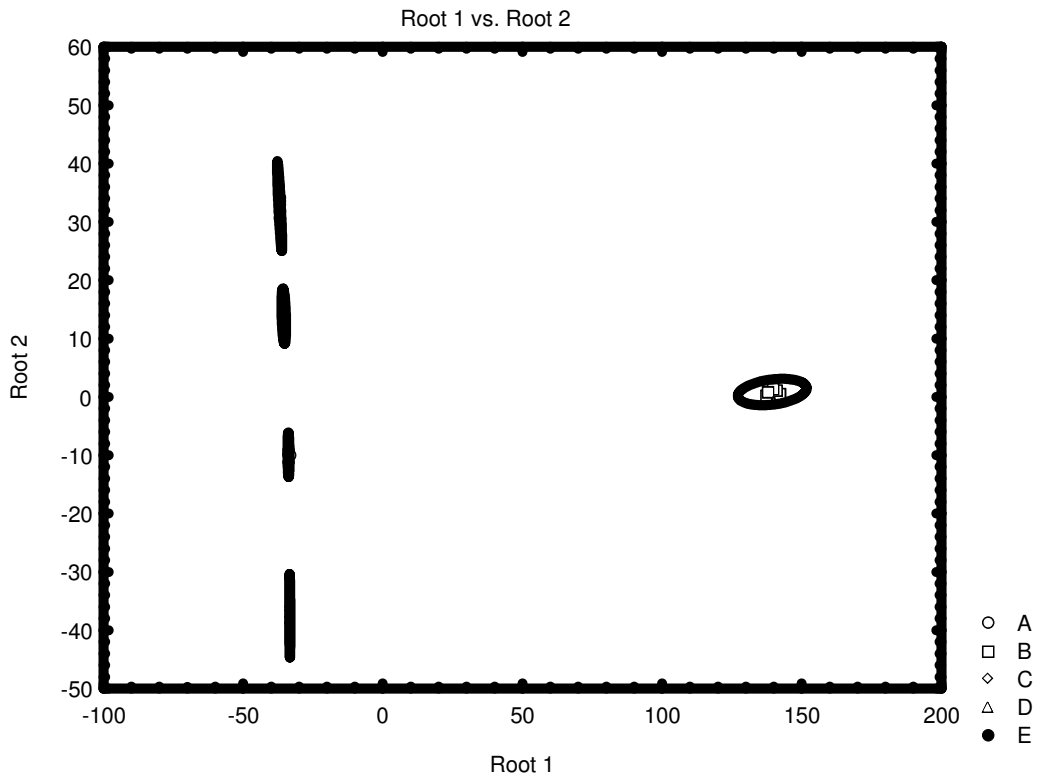
Table 6. Metal concentrations in marcs subjected to different pesticide treatments; five samples for each plot were analysed in triplicate (mean±st.dev). Significances of differences between blank and treated samples are also reported as chi-square (χ^2) and p values from Kruskal- Wallis test

year		2003				
PLOT	Mn (mgL ⁻¹)	Zn (mgL ⁻¹)	Cu (mgL ⁻¹)	Pb (µgL ⁻¹)	Cd (µgL ⁻¹)	
A	7.4±1.6	2.5±0.3	31.2±3.7	<0.1	<0.5	
B	19.3±2.8	7.2±0.7	56.1±6.9	<0.1	31.3±7.2	
C	33.2±4.0	9.1±1.5	51.4±2.9	<0.1	80.0±6.1	
D	3.9±0.7	8.7±0.5	161.7±10.1	<0.1	<0.5	
E	12.2±1.7	4.6±0.4	150.3±12.6	<0.1	<0.5	
χ^2	23.077	21.150	21.267	23.622	23.529	
p <	0.000	0.000	0.000	0.000	0.000	
		2004				
A	5.6±0.92	3.0±0.5	32.2±4.8	<0.1	<0.5	
B	10.7±2.0	4.7±0.7	33.2±3.6	71.1±2.2	<0.5	
C	12.4±1.2	10.9±1.4	31.7±2.9	42.3±6.4	63.4±8.3	
D	5.5±0.9	0.6±0.1	86.1±7.4	46.8±7.1	<0.5	
E	2.8±0.8	9.2±0.9	93.5±3.7	<0.1	<0.5	
χ^2	21.186	22.457	17.935	22.401	23.622	
p <	0.000	0.000	0.001	0.000	0.000	

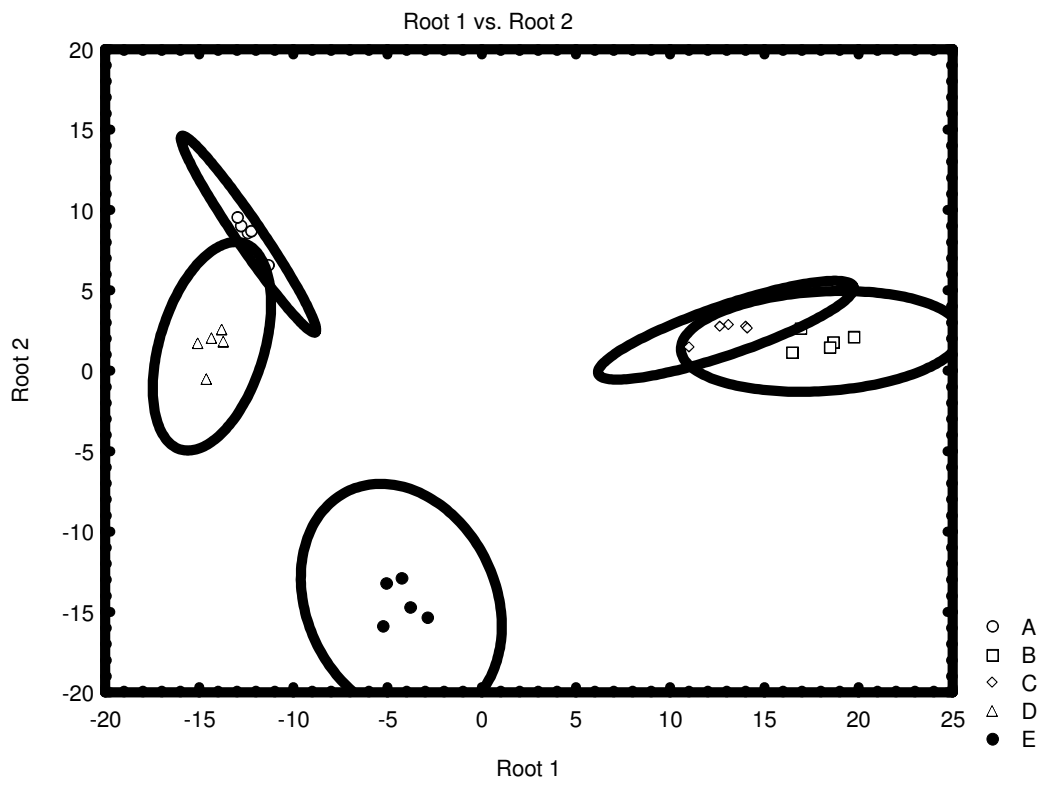


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8 **Figure 1:** Linear Discriminant Analysis for wines from 2003 (a) and 2004 (b)

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Figure 2: Linear Discriminant Analysis for grape stalks from 2003 (a) and 2004 (b)

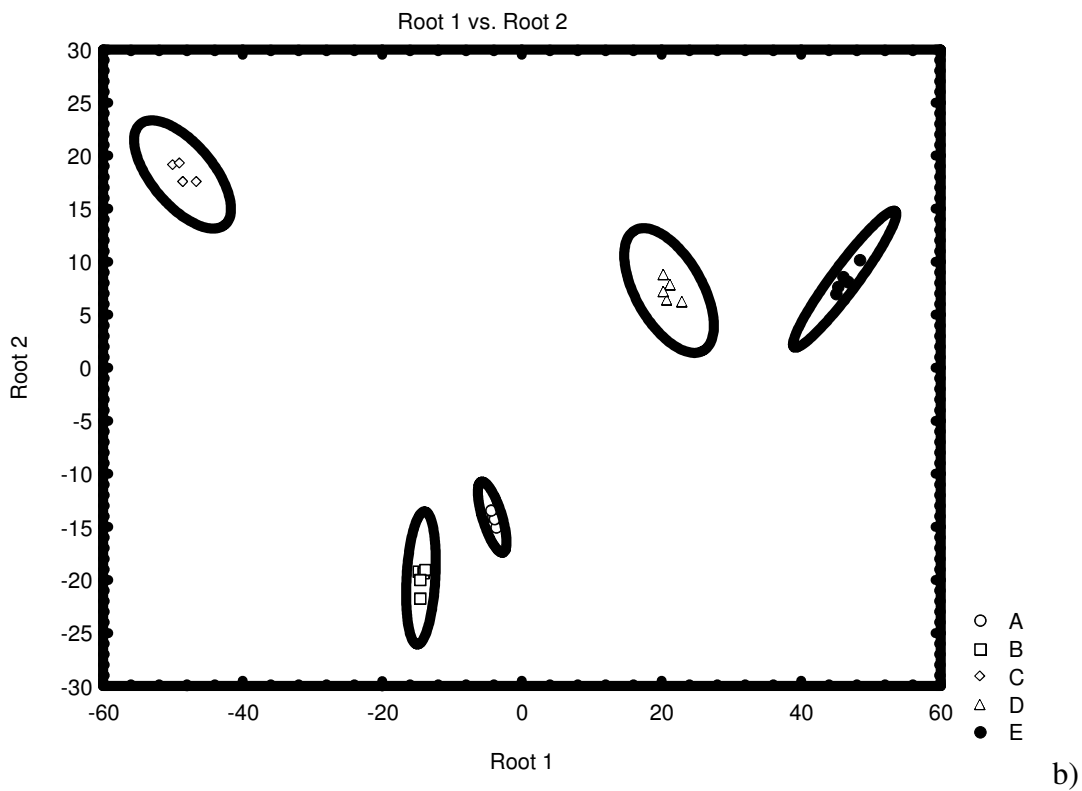
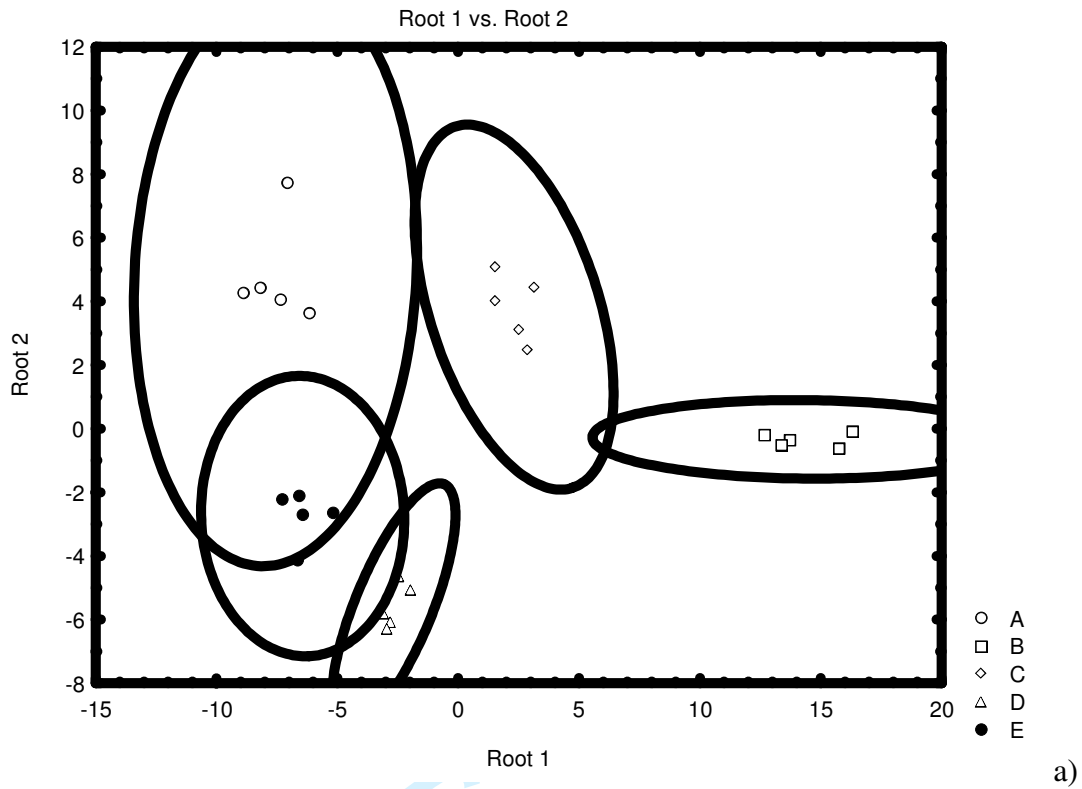


Figure 3: Linear Discriminant Analysis for grapes from 2003 (a) and 2004 (b)

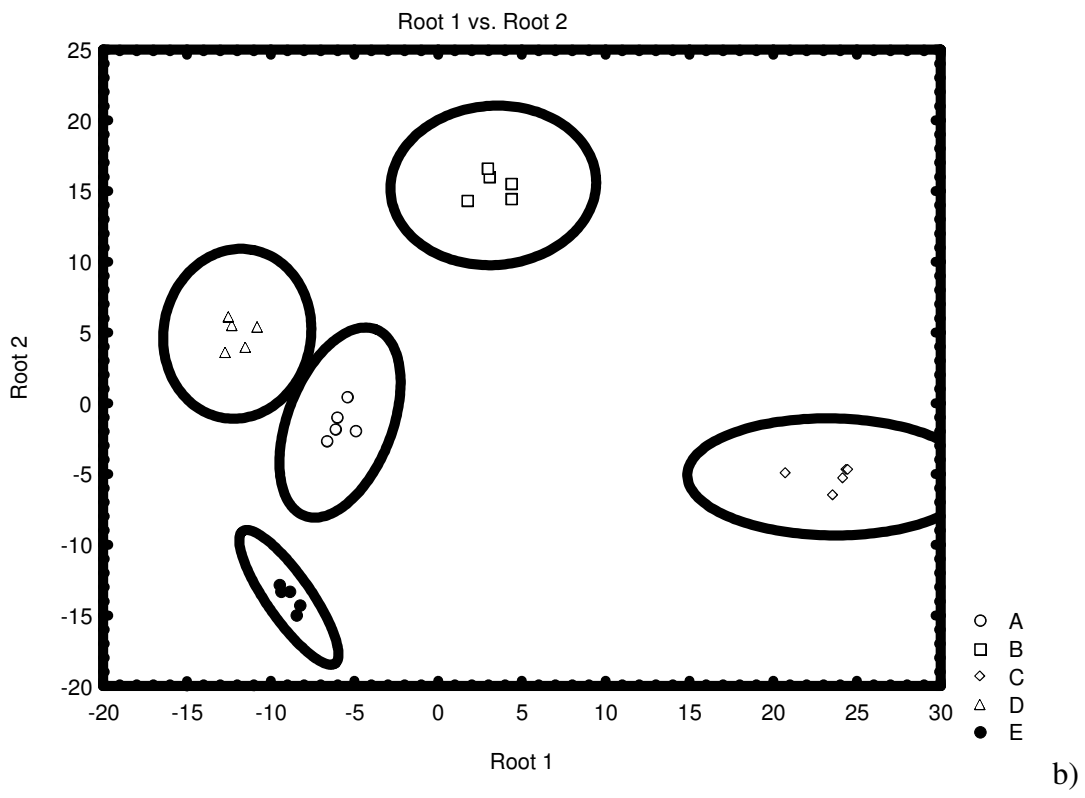
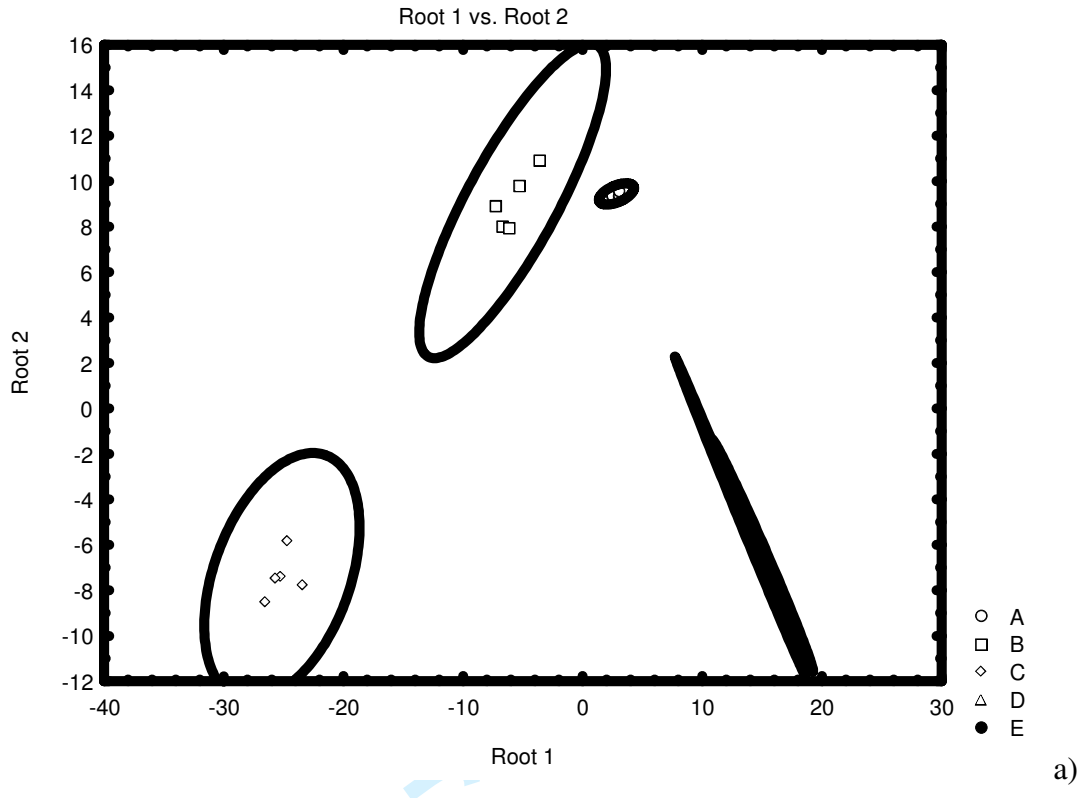


Figure 4: Linear Discriminant Analysis for marcs from 2003 (a) and 2004 (b)