Evolution of metal and polyphenol content over one year period of vinification: Sample fractionation and correlation metals-anthocyanins

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ABSTRACT

Tempranillo grape variety (*Vitis vinifera*) samples obtained from 0 to 345-day period of vinification were analysed. Fe, Cu, Zn and Mn were quantified as well as polyphenols and anthocyanins. A good correlation has been found for both Fe and Cu with total polyphenols and anthocyanins. A selected sample was fractionated through an open column, allowing the individual correlation of both Zn and Cu with cyanidin-3-glucoside.

Keywords: anthocyanins, polyphenols, heavy metals, vinification, fractionation, wine colour.

1.- INTRODUCTION

The wine is a very complex matrix due to the high number of inorganic and organic compounds. The presence of metals like Fe, Cu, Mn, and Zn in grape has been studied elsewhere [1]. It is known that they act like catalysers of biological systems or as promoters of some enzymes. The metals act also in some redox processes that are required for the metabolism of the cells. They also play an important role in the stability, colour and clarity of wines and the role of some of these metals has been studied as they affect the organoleptic characteristics of the wine. It has been pointed out that Zn causes the

persistence of the sour taste and Fe changes the flavour of the wine. Other metals have been studied for its toxicity, as is the case of Pb [2].

The determination of metals in wine has been considered of great interest since it allows the definition of a "finger print" for each of them that permits to verify the certified brand of origin (CBO). This is related to the fact that the composition of wine is strongly influenced by many factors related to the specific area of production (grape varieties, soil and climate, culture, yeast and wine making practices). In this sense, metals were studied in wines from Galicia (Ribeira Sacra) [3] and from Greece [4].
Most of the papers published is this area make use of both Atomic Absorption Spectrometry (AAS) [3] and Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) [4-5] as analytical techniques and the performance of the two techniques has been compared in wine analysis [6].

The evolution of metals throughout the ripening of grapes and vinification processes has been studied to a lesser extent. Studies of the content of metals in the different parts of the grape (seeds, skin and pulp) have been carried out [7-8]. Another important parameter for red wines is the colour. The colour of the red wines depends, overall, on its phenolic composition and particularly on the presence of anthocyanin [9]. The polyphenolic composition of wine depends on the quality of the grape [10-11]. It is known that polyphenols increase their concentration during the first stages of fermentation reaching a maximum within the first 10 days [12].

Due to the great importance of polyphenolic compounds in wine, the structure of those organic molecules has been previously studied. Vivar *et al*. [13] carried out a fractionation of wine and employed HPLC to identify the malvidin-3-glucoside as the most abundant anthocyanin in red wines. Hakansson *et al.* [14] studied the organic reactions involved in the formation of these compounds. The evolution of anthocyanins during the ripening of grapes of Tempranillo (*Vitis Vinifera*) has been studied [15]. Those studies were taken along 33 days and it was concluded than anthocyanin content increased with the ripening time.

It turns out to be interesting to observe the polyphenol to metal content relation in wine. Natural polyphenols have a great complexing capacity, especially with Fe and Cu [16] Karadjova *et al*. [17] studied the fractionation of wine samples by using ionic exchange

resins and the speciation of Cu, Zn and Fe was analysed. They concluded that the majority of polyphenols form salts and chelates with metals, mainly Fe and Cu.

In the present work, the evolution of Fe, Cu, Mn and Zn as well as polyphenols was studied along 345 days of vinification. Metal content in grapes and their distribution in seeds, pulp and skin were also measured.

The aim of the present work is to correlate the evolution of metal and anthocyanin and polyphenol content, throughout the fermentation of wine. In order to get a deeper view of such relationship, a chosen sample was fractionated. The optimised separation procedure allowed us to identify and measure the individual concentrations of the main anthocyanins present, as well as the concentration of the heavy metals, in each fraction. As a result, we obtained a comprehensive data set from which a specific anthocyanin-metal matches were established. The experimental conditions for the fractionation procedure were selected in such a way that natural pH and metal complexation state in wine were not altered.

2.-EXPERIMENTAL

2.1.- Samples

The study was carried out with red wine, Tempranillo variety from Navarre. Must samples were taken from the first day of fermentation up to ca. 1 year (345 days). Initially, 800 kg of grapes were processed in the Station of Viticulture and Enology of Navarre (EVENA), following the usual steps in wine elaboration. For the first 15-day period samples were taken daily. Sampling was carried out weekly for the following 4-week period, and from then on monthly. After a 160-day period of vinification, in which alcoholic and malolactic fermentation were completed, must was removed from the fermentation tank and was transferred to two different American oak barrels. Just before the transfer half of the must was clarified and filtered while the other half was introduced in the barrel keeping the must in contact with the grape refuse. Therefore, from that day on, two sets of samples taken on the same day of vinification were available: a clarified and a non-clarified sample. Moreover, grapes from which must was produced were also analysed for metals in its three differentiated parts: skin, pulp and seeds.

2.2.- Analytical procedures

2.2.1.-Sample digestion

Ethos Plus microwave labstation (Milestone) with computer – controlled easywave software was used to digest all samples. Between 0.65 and 0.75 g of the dried sample (skin, pulp and seed of grape) and 1.5 mL aliquots of the liquid samples (wine) were digested. Unless otherwise stated replicated digestions have been trebled. The conditions for the digestion are:

- Sample: 0.65-0.75 g (dried samples); 1.5 mL (liquid samples)
- Nitric Acid sub boiling (s.b.): 8 mL
- Temperature:

Step 1: 0 – 90ºC for 9 min at 1000 W
Step 2: 90ºC for 5 min at 1000 W
Step 3: 90 – 120ºC for 8.5 min at 1000 W
Step 4: 120 – 170ºC for 6 min at 1000 W
Step 5: 170 ºC for 5 min at 1000 W
And cool to room temperature

Once digested, all samples were taken to a final volume of 10.00 mL with ultra-pure water (Waserlab G.R.Type I-Reagent grade-Water system; Millipore).

2.2.2.- Grape sample preparation for digestion

Studied grapes, of the Tempranillo variety, came from two close areas in Navarre: one from Leache and the other one from Olite (collected by Evena). Before the total metal content was determined in the different parts of the grape (skin, pulp and seeds) samples were dried and digested. 100 grapes were chosen randomly from different bunches; then, skin, pulp and seeds were carefully separated and dried at 105 ºC until constant weight was reached, which took approximately one week.

2.2.3.- AAS and ICP – AES measurements

The studied metals were measured by:

- Atomic absorption spectofotometry using an air/acetylene flame by means of an Atomic Absorption Spectrometer A Analyst 800 (Perkin Elmer). The instrumental conditions applied in the determination of each one of the three metals are:

Metal:	Zn	Fe	Cu
λ (nm):	213.9	248.3	324.8
Slit Width:	0.7	0.2	0.7
Lamp Current (mA):	15	30	15
Calibration Interval:	0 – 0.6 ppm	0 – 3 ppm	0 – 0.6 ppm
Calibration line	$y = 0.327x + 2.38 \cdot 10^{-5}$	$y = 0.055x + 1.2 \cdot 10^{-4}$	$y = 0.0619 x + 2.38 \cdot 10^{-5}$
Correlation coefficient:	0.9994	0.9989	0.9997
n:	4	4	4
Detection Limit* :	0.023 ppm	0.026 ppm	0.017 ppm
Quantification limit*:	0.077 ppm	0.085 ppm	0.056 ppm

*Detection and Quantification limits have been calculated according to the classical procedures [18].

- ICP-AES using an ISA – Jobin Yvon JY 38 S. The instrumental conditions for the determination of each one of the four elements, are:

Metal:	Zn	Fe	Cu	Mn
λ (nm):	213.9	248.3	324.8	257.6
Calibration Interval:	0 – 1.2 ppm	0 – 3 ppm	0 – 1.2 ppm	0 – 1.2 ppm
Calibration line	y = 737.9 x+58.81	y = 246.1 x + 46.1	y = 712.6 x + 119.1	y = 690.9 x + 18.8
Correlation coefficient:	0.9999	0.9986	0.9999	0.9999
n	4	4	4	4
Detection Limit* :	0.0063 ppm	0.034 ppm	0.0002 ppm	0.0021 ppm
Quantification limit*:	0.021 ppm	0.115 ppm	0.0007 ppm	0.007 ppm

*Detection and Quantification limits have been calculated using the background equivalent concentration (BEC) and following the procedure establish by M.Thompson *et al*.[19].

2.2.4.- Quantification of total content of phenolic compounds

Quantification of total polyphenols was done following a modified version of the "Prussian Blue" procedure described elsewhere [20-21].

Aliquots of the must sample of 1 mL were filtered through a sea sand bed and then dried by evaporation at room temperature and reduced pressure in a rotary evaporator. The solid residue obtained was dissolved in a 5 mL pH 4 buffered methanol-water (1:1) mixture. Next, 1.5 mL of 0.08 M potassium ferricyanide and 1.5 mL of 0.1 M ferric chloride were added to 0.5 mL of this solution, and taken to a volume of 100,00 mL with water. After 15 minutes, the absorbance of this final solution was read at 720 nm. The measurement conditions were:

Standard:	Gallic acid
λ (nm):	720
Calibration interval:	0.12 – 0.36 mg/mL
Calibration line	y = 2.040 x + 0.134
Correlation coefficient:	0.9949
n:	5
Detection limit* :	0.023 mg/mL
Quantification limit*:	0.215 mg/mL

2.2.5.- Quantification of anthocyanins

Total anthocyanins

The methodology used for quantification of anthocyanins is based on spectrophotometic measurements of absorbance to a specific wave length of 520 nm, which corresponds to the maximum of absorption of these compounds. Must samples were prepared following the procedure described before for the quantification of total polyphenols. All samples were filtered through 0.45 μm filters before measuring its absorbance. A calibration line was prepared for malvidin-3-glucoside. The experimental conditions were:

Standard:	Malvidin-3-glucoside
λ (nm):	520
Calibration Interval	0.0377 – 0.092 mg/mL
Calibration line:	y = 0.423 x – 0.056
Correlation coefficient:	0.9984
n:	5
Detection Limit*	0.005 mg/mL
Quantification limit*	0.110 mg/mL

Individual anthocyanins

Musts samples of 4.5 mL were filtered by sea sand and subsequently freeze-dried. The freeze-dried sample was dissolved in 3 mL of water. Samples were conditioned before being injected in the HPLC. The conditioning process starts when the aqueous solution obtained from the freeze-dried sample was passed through a C-18 Sep-Pack (Millipore, Waters) cartridge. The extract retained in the cartridge is washed with 3 mL of 10 % (w/w) formic acid and then dried with air. The purified extract was eluted with a 1 % methanol-

hydrochloric acid mixture. The C-18 Sep-Pack cartridge was previously activated by elution of 5 mL methanol and 5 mL water.

After the conditioning process, 40 μL of each sample were injected in a HPLC chromatograph Waters 2695. A Waters Novapack C-18 column (3.9 x 150 mm) was used. Solvents used were 1% formic acid (A) and acetonitrile (B), establishing the following procedure: 15% (A) over 10 min, 20% (A) over 15 min, 25% (A) over 10 min, 35% (A) over 10 min and 5% (A) over 10 min, at a flow rate of 1 mL min^{-1}. The detector was set at 520 and 280 nm, respectively .

The analytical method used for quantification of the individual anthocyanins was validated following the usual procedure [22], and commercial standards for malvidin-3-glucoside (Extrasynthese) and cyanidin-3-glucoside (Extrasynthese) were used.

2.2.6.- Sample fractionation

A procedure was optimised in order to be able to chromatographically fractionate samples with no alteration of the natural pH and metal-ligand complexes of must. Sephadex® LH 20 (Pharmacia Biotech) and a methanol-water (50:50) pH 4 mixture were used as stationary and mobile phases, respectively. The obtained fractions were studied by Thin Layer Chromatography (TLC) and finally analysed by High Performance Liquid Chromatography (HPLC).

The optimised methodology was used to fraction the must sample fermented for just six days. The fractionation started when 3 mL of the sample were placed in the Sephadex® column (50 x 1.5 cm) and eluted with a pH 4 buffer-methanol (50:50) solution which produced a total of 19 fractions. Once the different fractions were collected they were spotted on a TLC plate and subsequently developed in a n-butanol/glacial acetic acid/water (50:10:20) mixture.

The obtained fractions were freeze-dried, dissolved in 1 mL methanol and a portion of 40 μL was analysed by HPLC following the procedure described above. The remaining volume was digested with nitric acid following the procedure described before in order to quantify the metal content in each fraction. Digested samples were analysed by ICP-AES because of the low quantification limits reached by this technique.

2.3.- Ancillary data

An Orion 920A pH meter was used to measure the pH values of all samples. Evena has provided density data for all the studied samples.

3.-RESULTS AND DISCUSSION

3.1.- Optimisation of digestion conditions

An attempt was made to proceed to the digestion of the samples with the addition of the minimum possible amount of nitric acid. A volume of 1.5 mL of sample (that was previously estimated to be adequate to quantify the metals) was treated with 5.0 mL of HNO_3, made up to a 10.0 mL volume with water and was kept at room temperature and pressure for 24 h. For samples well within the fermentation process, that is to say, for those with a certain quantity of alcohol and with a diminished sugar concentration, the method proved to be satisfactory yielding clear solutions. However, samples with higher concentrations of sugar, i.e. those belonging to the first steps of the vinification process, were not completely digested giving rise to obstruction of the ICP nebulizer and the congestion of the capillary in the AAS. Accordingly, it was decided to use the microwave assisted digestion that, although shorter in time, means a higher ratio of added acid (see 2.2.1.).

3.2.- Total metal content evolution along one year of vinification

A total of 32 samples covering a period of vinification of up to 345 days were analysed following the above described optimised conditions of digestion. Results are summarised in Table 1. As it can be seen, different behaviours were observed for each individual metal. On one side, Cu and Fe concentrations showed a marked decrease along the first 5 days, the rate of that drop diminished afterwards up to c.a. 10 days, and –later on- a plateau was reached. On the other side Mn and Zn concentrations showed only slight changes with time, remaining practically constant at values of around 0.5 and 1 ppm, respectively.

One of the targets of this work is to compare the behaviour of must once it has been transferred to oak barrels depending on whether it is previously filtered and clarified or it is accompanied by the grape refuse into the barrel. Negligible differences were observed between these two sets of samples. These findings show that no further extraction from

either seeds or skin takes place into solution up to 345-day. However, those samples will be monitored for at least another two years in order to check any significant difference that might appear along that period of time.

It was our wish to check the performance of the analytical methodology by recourse to a reference material; unfortunately, there seems to be no available reference material of this kind. As an alternative, flame-AAS was employed for the quantification of those metals that appeared more relevant (Fe, Cu and Zn). In all instances it was seen that values calculated from AAS measurements were consistently lower (c.a.18%) than those obtained from ICP-AES. A fair correlation of data was nevertheless achieved for the analyses of all samples throughout the whole studied period when done by both techniques. This is specially true for the case of Cu and Fe, while Zn values offered a more scattered picture. Correlation data for the analyses of the three elements with the two techniques are:

Analysed element:	Fe	Cu	Zn
Correlation equation*:	y = 0.828x + 0.324	y = 0.821x + 0.236	y = 0.816x
R^2:	0.990	0.941	0.192

y: AAS values; x: ICP-AES values

Taking into account the dilution to which the samples are subjected, and the low concentration of the metals in the samples (except for those belonging to the first 5 days or so), and in spite of the inherent sensitivity of the spectroscopic techniques used, measurements have been done in ranges close to the theoretical quantification limits. Moreover, in the case of Cu, values belonging to vinification day 100 and over, are below its quantification limit.

The difference found between both techniques could be a consequence of the high acidic content of the digested samples (80%). It is well known that acidic samples produce a depletion in the signals obtained by AAS and ICP as repeatedly observed in other type of food samples [19, 23-24]. Nevertheless, all samples (including those coming from the fractionation, *vide infra*) could be measured by ICP, which suggest an easier way to handle these data and to compare with results obtained with those of organic compounds.

3.3.- Heavy metal distribution in the grape.

Grapes of equal variety coming from two different vineyards, one from Olite (the vineyard from where all our samples come) and one from a non far away vicinity (Leache)

were taken to study the metal distribution in the three different parts of the grain: skin, pulp and seeds. As it can be seen in Fig.1, the pattern is very close for both. Copper and Iron tend to be equally distributed in all three parts of the grape (although there is a higher presence of iron in the skin of the Olite grapes), whilst Mn and Zn are located mainly in the seeds. Tannins from skin and seeds are extracted from the start of maceration, although at a slower rate than anthocyanins. For the few first days, their structure is known to be more complex and polymerised and they come mainly from the skin; after a week, tannins come from the outer tissues of seeds, with a simpler structure and lesser degree of polymerisation, and their extraction is favoured for the increasing concentration of alcohol. Metals as Zn and Mn that are located mainly in the seeds, do not show an increase in their concentration along the maceration process, indicating that they are not directly associated with this type of tannins; this fact suggests that these metals are not easily extracted from seeds even in the presence of alcohol, as was latter confirmed by results for samples belonging to day 160 to 345, in which no significant difference was observed between filtered and not-filtered samples. The fact that metals distribute in a similar way in grapes of same variety but different origin, indicate that such a distribution is not a random one, but could be rather related with the polyphenols profile for this variety of grape.

On the other hand, these determinations were also replicated by both spectroscopic techniques. Obtained results correlate fairly well, what comes to confirm that either method is suitable for the quantification of these elements in this kind of matrix, the ICP-AES being more sensitive and thus more adequate for low concentration samples.

3.4.- Quantification of total content of phenolic compounds

Table 2 shows the total polyphenols concentrations calculated from day 1 to day 225 of vinification. Polyphenols steadily increase after the first day and highest values are found for days 6 to 9, that is consistent with alcoholic fermentation. Anthocyanins evolve in a similar way (Table 2); they are water soluble and extract very easily from grape skin into solution. After day 9 their concentration diminishes and get very stable. Some factors such as fixation to solid particles, colloidal precipitation and hydrolysis have been reported to occur to some extent during vinification [26-29]. These results are within the range of concentrations reported for a large variety of greek wines [4].

All samples were analysed by HPLC in order to identify individual anthocyanins. Their quantification required a previous validation of the chromatographic method [22]

(Table 3). The most abundant ones have been found to be petunidin-3-glucoside, cyanidin-3-glucoside, malvidin-3-glucoside and malvidin-3-glucoside acylated with p-cumaric acid. It can be observed that concentration profiles are very close to one another, and while total anthocyanins remain practically constant, the concentrations of these individual anthocyanins decrease after day 15. This behaviour can be due to the fact that once the extraction is complete, polymerisation starts to happen forming new aggregated molecules that are still quantified under identical conditions as total anthocyanins.

3.5.- Correlation between metals and organic compounds

When total concentrations of metals and those of polyphenols and anthocyanins were compared, a correlation was found for Fe and Cu, so that as polyphenols or anthocyanins concentrations increase, the metals concentrations decrease, giving rise to an inverse relationship as shown in Fig.2. This similarity for the behaviour of Fe and Cu can be understood if the possibility to participate in condensation reactions of tannins and Anthocyanins is considered for both metallic species [30]. Anthocyanins with two orto-hydroxyl groups, may form complexes with given metals such as Fe, Cu, Al, Mg, etc., this reaction being well known for the case of Fe, originating what is called "blue or black breakdown".

3.6.- Fractionation

Once the correlation of metal concentration (Fe, Cu) with either total polyphenols or anthocyanins was seen, a further step was taken in order to achieve fractionation of a given sample into its individual anthocyanins. The challenge was to find out the experimental settings that would allow fractionation of the must samples in an open column, without disturbing the natural equilibria and the existing complexes in must and without modification of the pH. Moreover, we tried to see whether quantification of metals was feasible in each of the individual fractions collected from the separation column. Since anthocyanins are mainly located in the grape skin, it was preferred to work with skin extracts when studying the experimental parameters. Six extracts were treated with either ethanol (12%, 20%, 50%, 96%) or methanol (50%) as solvent, all of them buffered at pH 4.

When the extracts were analysed by TLC and HPLC, it was concluded that best extraction of major anthocyanins was achieved by using the methanol (50%) buffered solvent, that was used henceforth.

A compromise was reached between analysis time, sample volume and effectiveness of separation by using a packing of Sephadex on an open column. By applying this experimental setting, individual identification and quantification of anthocyanins was accomplished. Furthermore, it was possible to isolate pure malvidin-3-glucoside (as confirmed by ^{1}H-RMN and ^{13}C-RMN).

After the procedure was established, a must corresponding to day 6 of vinification was chosen for this fractionation study. The selection was done having in mind that volume sample had to be relatively small and, accordingly, metals and anthocyanins would be quite diluted. In day 6 anthocyanins concentrations are close to its maximum, while at the same time metals concentrations still did not plummeted. The aim was to check whether any metal could be found associated to any particular anthocyanin.

A total of 19 fractions were collected at the bottom of the Sephadex column. When analysed by TLC, anthocyanins were seen to be present in fractions 7 to 12. Consequently, those fractions were afterwards injected in the HPLC chromatograph. The resulting pictures are shown in Fig 3. Despite the different scales used, and the different dry extract weight in every fraction, it is clear that each fraction shows a characteristic pattern. The main peaks were ascribed to correspond to the same anthocyanins identified in the whole samples *(vide supra, 3.4*). It was possible to quantify those species in every fraction and results are summarised in Table 4.

As for the metals, their concentration was, as expected, difficult to measure and only Cu and Zn were properly quantified. Mn concentrations were well below its quantification limit, and Fe concentrations were close to it, so that no definitive conclusions can be drawn for these last two metals. However a qualitative picture of results obtained for Fe show that it is detectable in all single fractions. These findings will induce further studies with greater volume samples in order to ascertain whether it is specifically associated with any anthocyanin or not.

On the contrary, results obtained for Cu and Zn show a very good correlation with the cyanidin-3-glucoside concentration profile in fractions 7 to 12 (R^2 = 0,897 for Cu; R^2 = 0,840 for Zn, as calculated from statistical analysis of variance, ANOVA) as shown in Fig. 4. The chemical structure of cyanidin-3-glucoside presents two hydroxyl groups in position *orto* (4' and 5') what enhances the formation of complexes with metals as sketched in Fig. 5

[31], while the other two major anthocyanins lack this functionality. These findings prove unequivocally the complexing capacity of the cyanidin-3-glucoside for metals and the occurrence of such complexes in musts from Tempranillo (*Vitis vinifera*) variety. It is foreseeable that Fe (either divalent or trivalent) might behave in a similar way. As a matter of fact, an inverse relationship with total anthocyanins (and -of course- with total polyphenols) has been found. That is the reason why further work will be carried out to try and find whether that expectation will be fulfilled or not.

To the best of our knowledge this is the first report in which a comprehensive 1-year following-up of metals and polyphenols has been done along the vinification of a Tempranillo variety. Besides it has been proved the close association of both Cu and Zn with one particular anthocyanin, i.e. cyanidin-3-glucoside. Work is on progress in our lab to study the electrochemical behaviour of these complexes.

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Table 1:

Vinification day	Zn (µg/g) ± SD[c]	Fe (µg/g) ± SD[c]	Mn (µg/g) ± SD[c]	Cu (µg/g) ± SD[c]
1	0.91 ± 0.19	13.21 ± 0.23	0.50 ± 0.05	3.67 ± 0.17
2	1.19 ± 0.08	8.87 ± 0.25	0.68 ± 0.04	2.53 ± 0.10
3	0.80 ± 0.03	3.17 ± 0.03	0.65 ± 0.02	1.03 ± 0.04
4	0.91 ± 0.02	2.35 ± 0.04	0.68 ± 0.02	0.84 ± 0.06
5	0.97 ± 0.01	1.98 ± 0.14	0.65 ± 0.02	0.74 ± 0.07
6	0.88 ± 0.03	1.79 ± 0.03	0.61 ± 0.01	0.59 ± 0.03
7	0.86 ± 0.02	1.75 ± 0.09	0.65 ± 0.02	0.57 ± 0.05
8	1.13 ± 0.07	1.56 ± 0.03	0.68 ± 0.02	0.27 ± 0.02
9	0.92 ± 0.07	1.60 ± 0.05	0.70 ± 0.03	0.09 ± 0.02
10	0.82 ± 0.03	1.48 ± 0.03	0.66 ± 0.02	0.20 ± 0.01
11	0.85 ± 0.11	1.17 ± 0.03	0.57 ± 0.02	0.08 ± 0.02
12	0.97 ± 0.10	0.98 ± 0.05	0.59 ± 0.02	0.15 ± 0.02
13	0.78 ± 0.11	0.94 ± 0.01	0.60 ± 0.02	0.11 ± 0.02
14	0.85 ± 0.03	0.96 ± 0.02	0.59 ± 0.03	< LQ
15	0.84 ± 0.05	0.99 ± 0.03	0.57 ± 0.02	< LQ
16	0.89 ± 0.05	0.86 ± 0.01	0.58 ± 0.06	0.31 ± 0.01
21	0.83 ± 0.03	1.79 ± 0.04	0.70 ± 0.02	0.36 ± 0.02
34	0.82 ± 0.05	1.70 ± 0.05	0.65 ± 0.04	0.13 ± 0.01
41	0.98 ± 0.04	1.52 ± 0.05	0.65 ± 0.01	0.22 ± 0.05
48	0.89 ± 0.10	1.45 ± 0.05	0.63 ± 0.01	0.46 ± 0.15
56	0.85 ± 0.03	1.36 ± 0.07	0.59 ± 0.02	0.34 ± 0.02
70	0.94 ± 0.01	1.52 ± 0.04	0.60 ± 0.03	0.31 ± 0.02
101	0.91 ± 0.05	1.79 ± 0.03	0.68 ± 0.02	0.06 ± 0.02
104	0.94 ± 0.01	1.28 ± 0.02	0.61 ± 0.01	< LQ
160 F[a]	1.00 ± 0.05	1.11 ± 0.04	0.65 ± 0.03	0.00 ± 0.02
160 R[b]	0.85 ± 0.01	0.63 ± 0.00	0.61 ± 0.02	0.03 ± 0.01
225 F	0.90 ± 0.04	0.96 ± 0.00	0.67 ± 0.01	< LQ
225 R	0.84 ± 0.05	1.15 ± 0.00	0.64 ± 0.02	<LQ
304 F	0.68 ± 0.02	1.03 ± 0.02	0.60 ± 0.01	<LQ
304 R	0.66 ± 0.02	1.01 ± 0.01	0.56 ± 0.00	<LQ
345 F	0.89 ± 0.03	1.12 ± 0.09	0.61 ± 0.01	<LQ
345 R	0.95 ± 0.01	0.86 ± 0.06	0.42 ± 0.01	0.06 ± 0.00

a: F = Filtered and clarified barrel
b: R = Grape refuse containing barrel
c: n = 6

Table 2:

Vinification day	Total polyphenols (mg/g) ± SD[a]	Total anthocyanin (mg/g) ± SD[a]
1	0.360 ± 0.006	0.204 ± 0.004
2	0.488 ± 0.082	0.435 ± 0.024
3	0.561 ± 0.107	0.476 ± 0.025
4	1.223 ± 0.010	1.096 ± 0.018
5	1.208 ± 0.008	1.611 ± 0.078
6	1.873 ± 0.010	1.547 ± 0.020
7	1.779 ± 0.009	1.695 ± 0.021
8	1.789 ± 0.014	1.631 ± 0.007
9	1.848 ± 0.062	1.813 ± 0.008
10	1.591 ± 0.012	1.787 ± 0.006
11	1.613 ± 0.071	1.544 ± 0.045
12	1.678 ± 0.273	1.496 ± 0.002
13	1.564 ± 0.257	1.485 ± 0.008
14	1.581 ± 0.111	1.506 ± 0.021
15	1.397 ± 0.179	1.470 ± 0.304
41	1.410 ± 0.386	1.403 ± 0.002
56	1.331 ± 0.068	1.326 ± 0.021
104	0.960 ± 0.009	1.469 ± 0.031
225	0.989 ± 0.052	1.494 ± 0.002

a: n = 4

Table 3 :

Vinification day	Pt-3-gluc (µg/g) ± SD[a]	Cy-3-gluc (µg/g) ± SD[a]	Mv-3-gluc (µg/g) ± SD[a]	Mv-3-p-cumaroil gluc (µg/g) ± SD[a]
1	8,36 ± 0,23	17,24 ± 0,42	74,44 ± 1,52	57,52 ± 0,01
2	19,72 ± 1,76	23,84 ± 1,01	59,81 ± 1,88	3,15 ± 0,04
3	19,14 ± 1,28	21,49 ± 0,57	71,14 ± 1,70	
4	133,29 ± 3,67	123,16 ± 2,85	328,29 ± 13,16	48,99 ± 0,14
6	153,73 ± 5,60	147,92 ± 5,18	408,93 ± 6,87	48,29 ± 1,45
7	177,85 ± 2,57	161,91 ± 4,10	407,95 ± 0,44	58,40 ± 1,15
8	148,97 ± 9,58	147,54 ± 3,46	396,07 ± 9,75	50,67 ± 1,42
9	164,15 ± 10,19	153,67 ± 2,01	386,55 ± 3,91	53,06 ± 0,03
10	168,91 ± 1,26	156,88 ± 0,66	422,19 ± 6,41	44,15 ± 1,00
11	127,39 ± 6,60	124,79 ± 2,45	348,28 ± 8,15	39,48 ± 2,06
12	150,23 ± 0,56	138,59 ± 2,20	380,87 ± 8,57	39,51 ± 2,35
13	155,93 ± 0,92	142,15 ± 1,78	382,65 ± 3,14	38,87 ± 0,02
14	151,56 ± 12,08	144,47 ± 2,69	391,48 ± 8,03	45,59 ± 0,36
15	123,19 ± 1,87	117,54 ± 1,37	333,19 ± 9,00	38,35 ± 1,80
16	112,73 ± 6,44	109,08 ± 1,90	287,86 ± 3,55	31,44 ± 1,06
21	44,86 ± 3,74	42,22 ± 1,45	121,51 ± 0,88	12,11 ± 0,04
34	74,38 ± 5,53	80,00 ± 1,57	248,88 ± 4,83	23,79 ± 0,55
41	63,57 ± 0,05	65,26 ± 0,21	188,83 ± 5,59	16,29 ± 1,44
48	91,92 ± 10,17	90,94 ± 0,47	260,51 ± 2,97	19,88 ± 5,49
56	82,82 ± 3,61	80,70 ± 3,04	232,10 ± 5,67	21,30 ± 1,21
70	85,31 ± 9,19	82,16 ± 2,49	239,63 ± 5,46	18,56 ± 5,56
101	81,34 ± 7,04	77,74 ± 1,47	227,57 ± 0,71	15,77 ± 3,30
104	84,37 ± 4,07	84,70 ± 3,31	255,43 ± 12,38	22,02 ± 0,94
160	66,91 ± 2,27	65,55 ± 0,91	207,10 ± 3,55	15,61 ± 0,25
225	64,40 ± 2,04	67,80 ± 3,00	196,29 ± 3,10	14,56 ± 0,57

a: n = 2

Pt-3-gluc: petudnidin-3-glucoside**; Cy-3-gluc:** cyanidin-3-glucoside**; Mv-3-gl:** malvidin-3-glucoside**; Mv-3-p-cumaroil gluc:** malvidin-3- glucoside acylated with p-cumaric

Table 4:

Fractions	Pt-3-gluc (mg/g) ± SD[a]	Cy-3-gluc (mg/g) ± SD[a]	Mv-3-gluc (mg/g) ± SD[a]	Mv-3-p-cumaroil gluc (mg/g) ± SD[a]	Cu ppm (µg/g) ±SD[b]	Zn ppm (µg/g) ±SD[b]
7	0.1864 ± 0.0065	0.6661 ± 0.0157	4.1560 ± 0.1034		0.0414 ± 0.0004	0.0485 ± 0.0003
8	0.2796 ± 0.0164	1.6142 ± 0.0176	3.6673 ± 0.0548		0.0892 ± 0.0014	0.4880 ± 0.0047
9	1.1506 ± 0.0852	1.1908 ± 0.0248	0.8961 ± 0.0002		0.0597 ± 0.0001	0.2097 ± 0.0006
10	0.8834 ± 0.3463	0.0414 ± 0.0380	0.1352 ± 0.0259		0.0331 ± 4.63E-05	0.0277 ± 1.18E-05
11	3.6371 ± 0.3887	0.0469 ± 0.0498	0.0704 ± 0.0202	0.0102 ± 0.0237	0.0100 ± 7.93E-06	0.0210 ± 1.43E-05
12	0.0321 ± 0.0103	0.0355 ± 0.0093	0.1239 ± 0.0030	0.2138 ± 0.0080	0.0095 ± 5.17E-05	0.0007 ± 6.62E-07

a: n = 2
b: n = 3

Pt-3-gluc: petudnidin-3-glucoside**; Cy-3-gluc:** cyanidin-3-glucoside**; Mv-3-gl:** malvidin-3-glucoside**; Mv-3-p-cumaroil gluc:** malvidin-3- glucoside acylated with p-cumaric

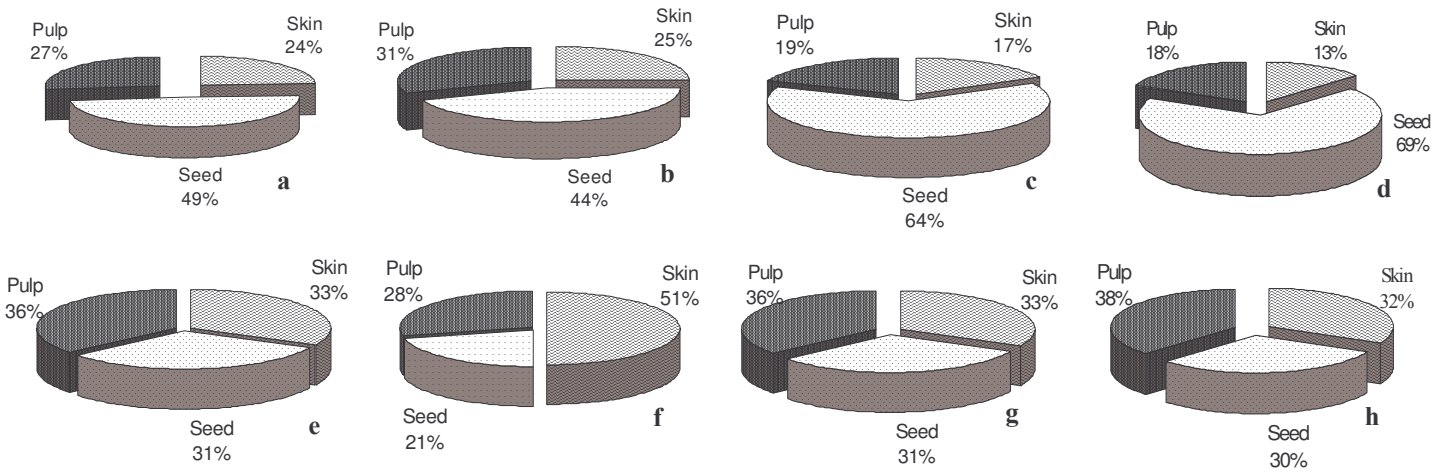

Pulp
27%
Skin
24%
Seed
49%
a
Pulp
31%
Skin
25%
Seed
44%
b
Pulp
19%
Skin
17%
Seed
64%
c
Pulp
18%
Skin
13%
Seed
69%
d
Pulp
36%
Skin
33%
Seed
31%
e
Pulp
28%
Skin
51%
Seed
21%
f
Pulp
36%
Skin
33%
Seed
31%
g
Pulp
38%
Skin
32%
Seed
30%
h

Figure 1

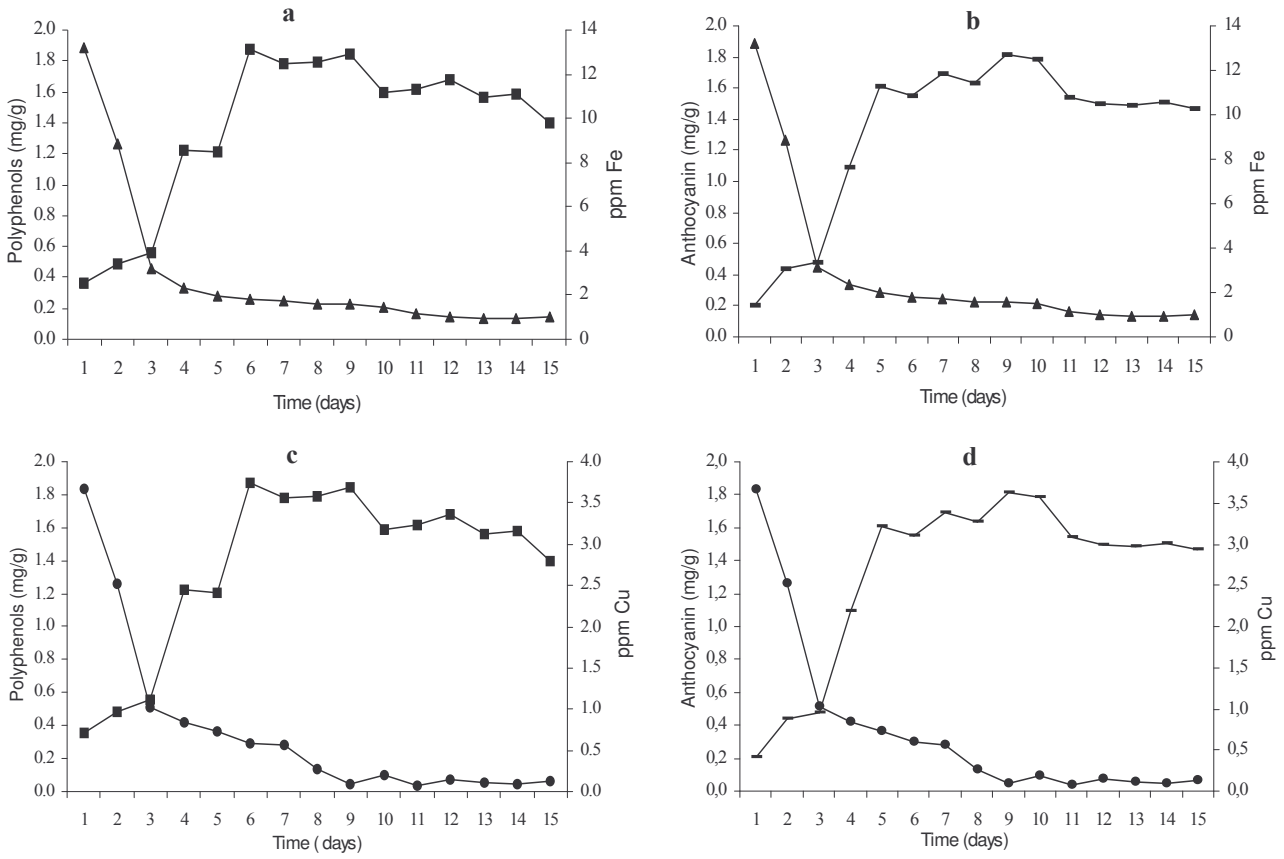
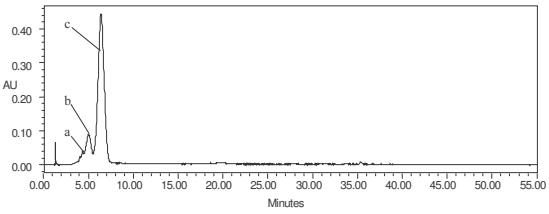
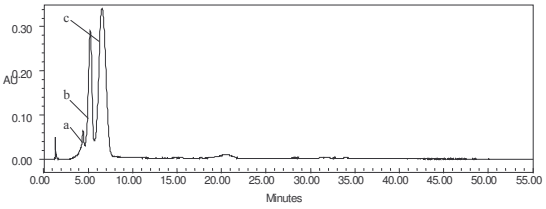

a
Polyphenols (mg/g)
ppm Fe
Time (days)
b
Anthocyanin (mg/g)
ppm Fe
Time (days)
c
Polyphenols (mg/g)
ppm Cu
Time (days)
d
Anthocyanin (mg/g)
ppm Cu
Time (days)

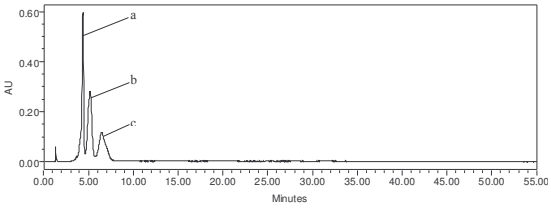
Figure 2



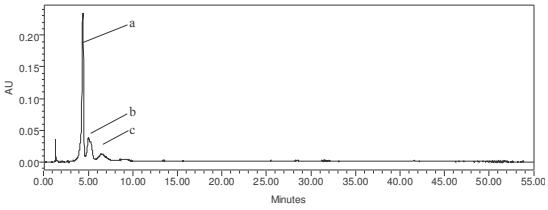
F 7



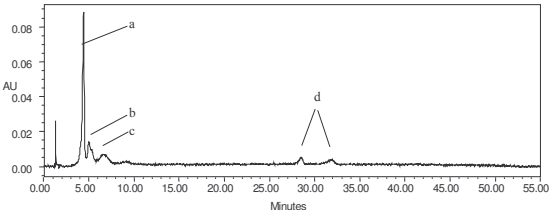
F 8



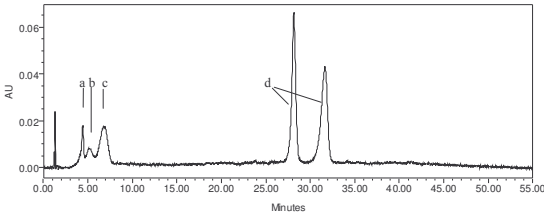
F9



F10



F 11



F 12

Figure 3

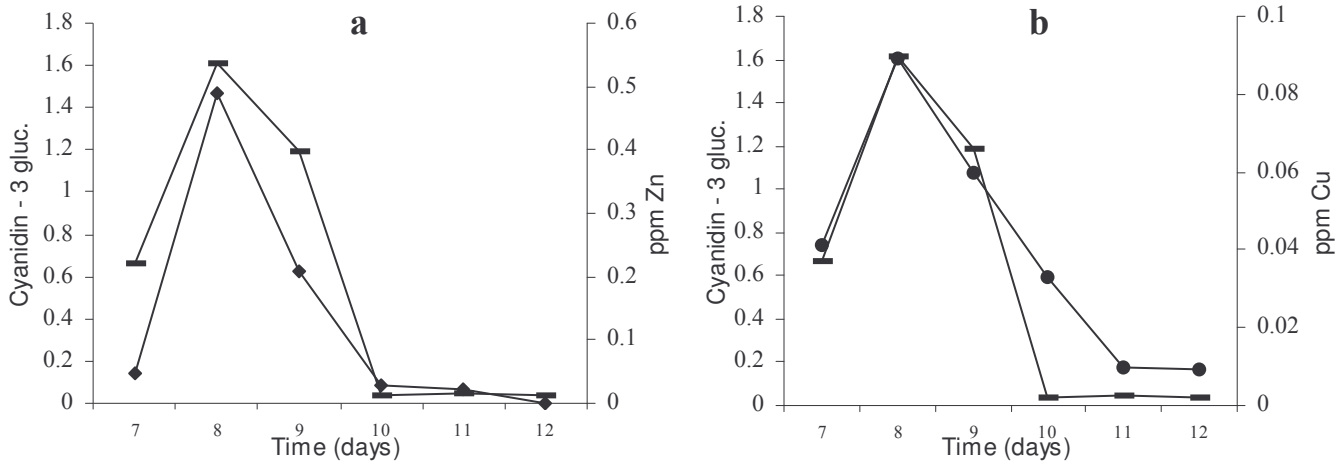

a
b
Cyanidin - 3 gluc.
ppm Zn
ppm Cu
Time (days)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
0.08
0.06
0.04
0.02
7
8
9
10
11
12

Figure 4

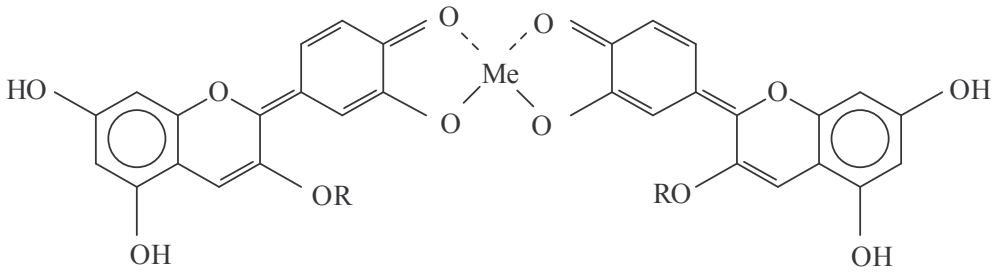


Figure 5

Table 1. Total metal concentration along 1-year vinification (mean ± Standard Deviation). Measurements have been done by ICP-AES.

Table 2: Polyphenols content along the days of vinification in mg/g of must (mean ± Standard Deviation).

Table 3: Evolution of individual anthocyanin during the vinification process (mean ± Standard Deviation).

Table 4: Concentrations of individual anthocyanins and metals present in the dried residue of each fraction (mean ± Standard Deviation) corresponding to day 6 vinification.

Figure 1: Percentages of metals in the different parts of grape from two origins: **a:** Zn of Leache; **b:** Zn of Olite**;** **c:** Mn of Leache; **d:** Mn of Olite; **e:** Fe of Leache; **f:** Fe of Olite; **g:** Cu of Leache; **h:** Cu of Olite.

Figure 2: Correlation of heavy metals (Fe and Cu) and the total polyphenols and anthocyanin during the first days of vinification
a: Fe–Polyphenols (▲= Fe; ■ = Total polyphenols); **b**: Fe–Anthocyanin (▲= Fe; ▁ = Total Anthocyanin); **c:** Cu–Polyphenols (● = Cu; ■ = Total polyphenols); **d:** Fe–Polyphenols (● = Fe; ▁ = Total Anthocyanin)

Figure 3: Chromatograms of the fractions containing individual anthocyanins (F7 to F12). Peak of: **a:** petudnidin-3-glucoside; **b:** cyanidin 3-glucoside; **c:** malvidin-3-glucoside; **d:** Mv-3-p-cumaroil gluc: malvidin-3-glucoside acylated with p-cumaric

Figure 4: Correlation between metals (Zn and Cu) and cyanidin-3-glucoside existing in the different fractions (F7 to F12)
a : Zn – Cy-3g (◆ = Zn; ▁ = Cy-3g); **b:** Cu – Cy-3g (● = Cu; ▁ = Cy–3g)

Figure 5: Molecular structure of the metal-cyanidin-3glucoside complex. (R = Glucoside)