Color, Phenolics and Antioxidant Activity of Blackberry (*Rubus glaucus* Benth.), Blueberry (*Vaccinium floribundum* Kunth.) and Apple Wines from Ecuador

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Publicado en *Journal of Food Science* (2013)

ABSTRACT:

Seventy wines were produced in Ecuador under different processing conditions with local fruits: Andean blackberries (*Rubus glaucus* Benth.) and blueberries (*Vaccinium floribundum* Kunth.) and Golden Reinette apples. Wines were evaluated for antioxidant activity (AA) using the radical scavenging capacity (DPPH) method, total phenolic content (TPC) using the Folin-Ciocalteu method, total monomeric anthocyanins (TMA) using the pH differential test, and color parameters using VIS-spectrophotometry. For blackberry wines, ellagitannins and anthocyanins were also analyzed using HPLC-DAD. Apples wines (n = 40) had the lowest TPC (608 ± 86 mg/L) and AA (2.1 ± 0.3 mM Trolox). Blueberry wines (n = 12) had high TPC (1086 ± 194 mg/L) and moderate AA (5.4 ± 0.8 mM) but very low TMA (8 ± 3 mg/L), with a color evolved toward yellow and blue shades. Blackberry wines (n = 10) had the highest TPC (1265 ± 91 mg/L) and AA (12 ± 1 mM). Ellagitannins were the major phenolics (1172 ± 115 mg/L) and correlated well with AA (r = 0.88). Within anthocyanins (TMA 73 ± 16 mg/L), cyanidin-3-rutinoside (62%) and cyanidin-3-glucoside (15%) were predominant. Wines obtained by co-fermentation of apples and blackberries (n = 8) showed intermediate characteristics (TPC 999 ± 83 mg/L, AA 6.2 ± 0.7 mM, TMA 35 ± 22 mg/L) between the blackberry and blueberry wines. The results suggest that the Andean berries, particularly *R. glaucus*, are suitable raw materials to produce wines with an *in vitro* antioxidant capacity that is comparable to red grape wines.

Keywords: polyphenols, antioxidant activity, wine, anthocyanin, fruit

Practical Application:

Red wine is known to be a health-promoting product when consumed moderately, due to the presence of antioxidants, mainly phenolic compounds. In Ecuador, the cultivation of grapes for winemaking is not possible. However, in the Sierra region, wines are produced from other fruits such as apples and Andean fruits including Mora de Castilla (blackberry, *Rubus glaucus* Benth.) and Mortiño (blueberry, *Vaccinium floribundum* Kunth.). This study shows how these wines, particularly blackberry wines, are characterized by high polyphenol contents and antioxidant activities as compared to red grape wines. Therefore, winemaking can be a suitable fruit processing alternative in the region.

Introduction

Globally, grape wine is the most-produced fruit-fermented alcoholic beverage, followed by apple cider. However, other fruits are processed through wine-making procedures, particularly red berries, which are rich in anthocyanins and other phenolic-type compounds. From a technological perspective, phenolic compounds are key determinants of the definition and evolution of several wine characteristics, such as color, astringency and bitterness. Red wine color depends on the absolute and relative concentrations of anthocyanins in the fruit, the wine production method, and the multiple chemical reactions that occur during fermentation and aging. These reactions are responsible for the generation of new pigments and the natural evolution of the red wine color from red to orange nuances. On the other hand, polyphenols are interesting as health promoting compounds. Most of the previous reports on red berry wines included the evaluation of their *in vitro* antioxidant activity (Pinghero and Paliyath 2001, Sánchez-Moreno and others 2003, Rupasinghe and Clegg 2007, Yildirim 2007, Jung and others 2009, Johnson and Gonzalez de Mejia 2012, Lim and others 2012, Mudnic and others 2012).

South America has a wide variety of native berries that are rich in antioxidants with high commercialization potential (Schreckinger and others 2010a). In Ecuador, blackberries (*Rubus glaucus* Benth.) are widely cultivated in the Andean regions and consumed fresh or processed into products such as frozen pulp, juice, jam and wine. Research has only recently addressed the phenolic composition, mainly ellagitannins and anthocyanins, and antioxidant activity of raw fruit (Garzón and others 2009, Mertz and others 2007, Vasco and others 2008, 2009b) or fruit-derived products, such as wines (Arozarena and others 2012) and isotonic beverages (Estupiñan and others 2009). The Andean blueberry (*Vaccinium floribundum* Kunth.) is a fruit that is found in Ecuador at very high altitudes and almost exclusively in wild form. Commercial use of Andean blueberries is still low. Vasco and others (2009a) characterized the phenolic composition of this berry for the first time and reported that it is primarily characterized by anthocyanins and proanthocyanidins, in a lesser amount (Schreckinger and others 2010b). In Ecuador, different apple varieties are also produced. "Emilia" is the local name of a Golden Reinette variety that has diminished in commercial value over the past decade in comparison to other apple varieties.

The aims of this study were to characterize the total phenolic content, total anthocyanin content, color and *in vitro* antioxidant activity of fruit wines of *R. glaucus*, *V. floribundum* and "Emilia" apples produced in Ecuador and to compare them with previous findings for grape and fruit wines. For blackberry wines, ellagitannins and individual anthocyanins were also analyzed. In addition, this study evaluated the influence on all of the aforementioned variables of several technological factors including fermentation with several yeast strains, in presence or absence of fruit solids, with different water-to-fruit ratios, the addition of pectinases, and the co-fermentation of blackberries and apples.

Materials and Methods

Chemicals. All chromatographic solvents were HPLC grade. Methanol, acetonitrile, formic acid, hydrochloric acid, gallic acid, Folin-Ciocalteu reagent, sodium carbonate and sodium metabisulfite were purchased from Panreac (Spain). 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich (Germany). Cyanidin-3-glucoside (≥ 96%), cyanidin-3-rutinoside (≥ 96%), and ellagic acid (≥ 90%), were from Extrasynthese (France).

Wines. Seventy fruit wines divided into six independent sets were produced in Ecuador. The experimental design and the features of the wine-making procedure for each set of wines are described in Table 1.

The basic raw materials for wine production were fruit, cane sugar, and water. Fifty-eight wines were made at the Technical University of Ambato (Tungurahua province) with Andean blackberries (*Rubus glaucus* Benth.) and Golden Reinette apples that were purchased at the municipal market of Ambato. Blackberries had a total acidity of 0.75 ± 0.01 g malic acid/100 g and a pH of 3.01 ± 0.01. Apples had a total acidity of 0.12 ± 0.04 g malic acid/100 g and a pH of 4.17 ± 0.12. In addition, twelve blueberry wines were produced in Salinas de Guaranda (Bolivar province) at the facilities of the Cooperative Consortium "Gruppo Salinas" using wild fruits (mortiño or Andean blueberry *Vaccinium floribundum* Kunth.) that were picked in the fields surrounding the village. The total acidity of the blueberries was 0.32 ± 0.04 g malic acid/100 g, and the pH was 3.51± 0.09.

Wines were produced on a small scale in plastic containers of 25 L. The general wine-making procedure was as follows: the fruit was mixed with water in the proportions indicated in Table 1, sulfited (100 mg $K_2S_2O_5$/L) and crushed. The practice of diluting fruit with water is common in the region in order to diminish the viscosity and acidity of the juices, and also due to economic reasons. Immediately after crushing, and only in the S0 trials of set 1, the fruit solids were removed manually through a sieve (850 μm).

All of the musts were enriched up to 21 °Brix to acquire a potential alcohol level of approximately 12 % vol. The next step was the inoculation of the yeasts (0.3 g dry yeast/L). The soluble solids content of each trial was monitored daily during fermentation until it reached a constant level (usually 6-7°Brix).

Subsequently, the solids were removed, and the wines were transferred into a new container and sulfited (75 mg $K_2S_2O_5$/L). The wines were maintained at 18 ± 1°C for 2 months. Samples of all of the wines were sent to the Public University of Navarre (Spain) and kept refrigerated (4 °C) and under nitrogen until analyses.

Basic characteristics. Alcoholic degree (% vol.), pH and total acidity (% malic acid) were measured according to usual methods (Commission Regulation (EEC) No 2676/90).

Turbidity. This analysis was applied to the wines of sets 2 and 4, in which the study of enzymatic treatments with pectinases was included. Turbidity of wines was measured in a HACH 2100N Turbidimeter (Hach Company USA) calibrated with formazine standards within the range 20-1000 NTU.

Color measures. All the spectrophotometric measures were made in a double-beam spectrophotometer (Zuzi TU 1901 Spain). In the apple wines only the absorbance at 420 nm was measured (A420 nm). In the red wines the absorbances at 420, 520 and 620 nm were used to calculate color intensity (CI = A_{420} + A_{520} + A_{620}), yellow (100·A_{420}/CI) red (100·A_{520}/CI) and blue (100·A_{620}/CI) components (Glories, 1984), and hue (A_{420}/A_{520}) of the wines.

The method of Somers and Evans was used to obtain wine absorbance at 520 nm or wine color (WC), the color due to pigments resistant to SO_2 blanching (CDR_{SO2}) or residual absorbance of the wine containing 0.3 % sodium metabisulfite, the anthocyanin color (AC = WC – CDR_{SO2}) or the color mainly due to monomeric anthocyanins, and chemical age (CAW = CDR_{SO2}·100/WC).

Antioxidant activity. DPPH assay (Rivero-Pérez and others 2008) was used to evaluate the radical scavenging activity of wines. Sixty microliters of wine (previously diluted 1:20 with methanol) was mixed with 2940 µl of a 60 µM DPPH methanolic solution. The difference between the absorbance at 515 nm at time zero and at 60 minutes was employed to quantify the antioxidant activity as millimoles of Trolox equivalents (TE) per liter (0.1-1 mM TE R^2 = 0.999).

Total phenolic content. Total polyphenol content (TPC) was determined by the Folin-Ciocalteu method (Commission Regulation (EEC) No 2676/90). TPC was expressed as milligrams per liter of gallic acid equivalents (100–600 mg GAE/L, R^2 = 0.999).

Total monomeric anthocyanins. Total monomeric anthocyanins (TMA) were determined by the pH-differential method described by Giusti and Wrolstad

(2005). TMA were expressed as cyanidin-3-glucoside equivalents in milligram per liter.

HPLC-DAD analysis of anthocyanins and ellagitannins. These analyses were applied to the wines elaborated with Andean blackberries (sets 4 and 5). Anthocyanins were separated and quantified with a modified version of the method described by Vasco and others (2009b), while ellagitannins were estimated through their acid hydrolysis and the subsequent analysis by HPLC-DAD of the hydrolytic products, according to the method reported by Vrhovsek and others (2006), with some modifications. All the methods were described in detail in a previous work (Arozarena and others 2012).

Statistical analysis

Means and standard deviation were obtained from at least three repetitions. One-way ANOVA and Tukey's Range Test were used to evaluate the differences among wine sets and the effects of technological factors within each set of wines. Pearson's correlation coefficients were used to establish the relationship among antioxidant activity and the rest of analytical parameters. Principal Component and Cluster Analyses were used to achieve a better description and discrimination of the red fruit wines. All the statistical analyses were made with the Statgraphics Centurion XVI software (StatPoint Technologies Inc., Warrenton, Virginia, USA).

Results and Discussion

Fermentation

First, the sugar attenuation during fermentation and the turbidity of wines will be discussed briefly to clarify subsequent sections. For each trial, fermentation was completed when the soluble solids reached a value below 7 ºBrix. As shown in Figure 1, the time of fermentation differed depending on the fruit that was used as the raw material. For the blueberry wines (Blue), fermentation finished 55-76 days after yeast inoculation. The extremely slow rate of sugar decrease was most likely caused by the low temperature of juices during fermentation (14-18ºC), due to the cold climate of Salinas de Guaranda (3550 m altitude). The other wines were produced in Ambato (2570 m altitude), with temperatures fluctuating between 18 and 24ºC. The time of fermentation of these wines could be considered consistent to the time reported for wines produced from dessert apples (Satora and others 2008) and black raspberries (Lim and others 2012). Blackberry wines (Blk) fermented the fastest (12-17 days) and most regularly, while apple wines (Ap) needed 30 to 45 days to finish the process, and wines produced through the co-fermentation of apples and blackberries (ApBlk) had an intermediate behavior. Furthermore, within the latter wines, it was observed that the larger the proportion of blackberry in the initial juice, the shorter the fermentation time. As all of the wines were prepared from juices with similar soluble solids levels (21 ºBrix), these results suggest that blackberries might provide nutrients other than carbohydrates in a higher concentration than apples. The richness of nutrients in the juices might also be the reason because of, within the Blue wines, those with the lowest water-to-fruit ratio (BlueW2) finished the fermentation first. In contrast, this finding could not be verified for the Ap3 wines. In the Ap1 and Ap2 sets, the fermentations with the bread yeast strain were significantly slower than those developed by the wine yeast strains. Finally, neither the presence/absence of fruit solids (Ap1) nor the pectinases (Blk, Ap2) affected the course of fermentation.

Turbidity

Approximately two months after the end of fermentation, turbidity was measured in the Blk and Ap2 wines. The use of pectinases is common in cider processing. As expected, the addition of pectinases, either before or after fermentation, significantly reduced ($p < 0.01$) the turbidity of the Ap2 wines from 150-280 NTU to 14-36 NTU. Neither the time at which pectinases were added nor the type of yeast strain affected the final turbidity value. For the Blk wines, no differences were observed among treatments, with low turbidities (15-33 NTU) in all of the trials. Therefore, the addition of pectinases in these wines would not be required, since the process occurred naturally in the untreated wines.

Color and phenolics

The characteristics of each type of wines are summarized in Table 2. The total phenolic content (TPC) of the Ap wines ranged from 471 to 801 mg GAE/L, which is consistent with previous findings in Spanish ciders (Picinelli and others 2009) and apple wines from Turkey (Yildirim 2006, Satora and others 2008). The Blk, ApBlk and Blue wines had TPC levels (854-1400 mg GAE/L) that were similar to those previously reported in wines produced from blackberries (Yildirim 2006, Amidzic-Klaric and others 2011, Johnson and Gonzalez de Mejia 2012, Mudnic and others 2012), blueberries (Sanchez-Moreno and others 2003, Rupasinghe and Clegg 2007, Su and Chien 2007) and other red berries (Yildirim 2006, Rupasinghe and Clegg 2007, Jung and others 2009, Schmitzer and others 2010, Lim and others 2012).

Blackberry (*R. glaucus*) wines also had the highest monomeric anthocyanin concentration (TMA), which was similar to previous findings in commercial blackberry wines from Croatia (Klaric and others 2011) and Illinois (Johnson and Gonzalez de Mejia 2012). The main anthocyanin compounds in the Blk wines (Figure 2a) were cyanidin-3-rutinoside (62%) and cyanidin-3-glucoside (15%), in agreement with the typical anthocyanin profile of *Rubus glaucus* (Mertz and others 2007, Vasco and others 2009b). In addition, a substantial proportion of other minor pigments (23%) was observed. Among these pigments, several pyroanthocyanins produced during the fermentation and maturation of wines were identified in a previous report (Arozarena and others 2012). TMA in the *V. floribundum* wines was very low in comparison with the levels found in the Blk and ApBlk wines and other blueberry wines (Su and Chien 2007, Johnson and Gonzalez de Mejia 2012). It is well-documented that the free anthocyanin content of any anthocyanin-rich juice declines during fermentation (Rommel and others 1992, Czyzowska and Pogorzelski 2004, Amidzic-Klaric and others 2011, Arozarena and others 2012). Considering the extremely long duration of fermentation in the Blue trials, the low TMA levels found in the wines are not surprising. Furthermore, these levels are consistent with the values observed for the color parameters. According to the variables Hue, Yellow, Red, and Blue, the blueberry wines showed a more evolved color than the Blk and ApBlk wines. In addition, the chemical age (CAW) of the Blue wines was high, indicating that almost half (48%) of the wine absorbance at 520 nm was attributable to pigments resistant to the SO_2 blanching, that is assumed to be generated from the native free anthocyanins during fermentation and aging.

Antioxidant activity

Higher antioxidant activities (AA) were shown for red wines; in particular, the AA of the Blk wines was almost twice that of the ApBlk and Blue wines (Table 2). The results agreed with the radical scavenging capacities that were previously detected in Andean blackberry wines (3.8 to 14.2 mM TE, Arozarena and others 2012), in elderberry wines (6.3-9.95 mM TE, Schmitzer and others 2010) and in red grape wines (4.7-17.4 mM TE, Fernández-Pachón and others 2004), through the DPPH method. Furthermore, the AA of the Ap wines was comparable to that reported by the latter authors in white grape wines (0.3-2.68 mM TE).

AA was highly correlated with TPC in the four types of wines (Figure 3), as is usually observed in most phenolic-rich products, including fruit wines (Sanchez-Moreno and others 2003, Rupasinghe and Clegg 2007, Satora and others 2008, Amidzic-Klaric and others 2011, Lim and others 2012, Johnson and Gonzalez de Mejia 2012, among others). The AA/TPC ratios for the Blk, ApBlk, Blue and Ap wines were, 9.2 ± 0.2, 6.2 ± 0.4, 5.0 ± 0.3 and 3.5 ± 0.2 mmoles TE/g GAE, respectively. On the other hand, values of 4.5 ± 0.6, 4.9 ± 1.5, and 3.4 ± 2.3 mmoles TE/g GAE were calculated from the data previously reported in wines from elderberries (Schmitzer and others 2010), red grapes and white grapes (Fernández-Pachón and others 2004), respectively. These data suggest that, in relative terms, the Blk wines were the most effective in vitro antioxidants among all of the aforementioned wines, which is in agreement with previous findings comparing the superoxide anion scavenging capacity (Pinghero and Paliyath 2001) and the ferric reducing antioxidant power (Mudnic and others 2012) of blackberry and red grape wines. This observation might be attributed to the different nature of the major phenolic compounds in each type of wine. In red grape wines, AA are particularly associated with flavanols and anthocyanins (Fernández-Pachón and others 2004). Both in elderberry wines (Schmitzer and others 2010), and in *V. floribundum* berries (Vasco and others 2009a), anthocyanins are predominant. Schreckinger and others (2010b) showed that the AA of *V. floribundum* was more highly correlated with anthocyanins than with proanthocyanidins. Flavanols and hydroxycinnamic acids are known to be the most abundant phenolic compounds found in apples (Khanizadeh and others 2008), apple wines (Satora and others 2008) and ciders (Rodriguez-Madrera and others 2006, Picinelli and others 2009). In the Blk wines, radical scavenging activity was related to the content of ellagitannins (r = 0.881, Figure 3), but no significant correlation with anthocyanins could be verified. Similar findings were previously reported in other *Rubus glaucus* wine samples (Arozarena and others 2012) and in ellagitannin-rich berries such as *Rubus adenotricus* (Acosta-Montoya and others 2010) and *Rubus idaeus* (Borges and others 2010). In contrast, in the ApBlk wines, AA was correlated with ellagitannins (r = 0.882, Figure 3), but also with TMA (r = 0.853), cyanidin-3-rutinoside (r = 0.874) and cyanidin-3-glucoside (r = 0.870). Finally, in the Blue wines, AA was correlated with CDR_{SO2} (r = 0.842), which appears to be consistent with the above observations regarding the low content in TMA and the advanced evolution of the color of these wines.

Influence of technological factors

The significance of the effects of the technological factors on each of the sets of apple wines is summarized in Table 3. The yeast strain factor (Y) was irrelevant in the Ap1 and Ap2 wines. In the Ap2 wines, the reduction of turbidity caused by the pectinases was accompanied by a significant decrease of the AA, and this effect was more important for the post-fermentative treatments (18% of reduction) than for the pre-fermentative treatments (5%). These results are consistent to those reported by Hubert and others (2007) showing that enzymatic depectination followed by sedimentation removed 14% of the flavanols in apple musts. However, other factors had a more profound effect than pectinases on apple wine characteristics. When fruit solids were not removed prior to fermentation, the TPC and AA of the Ap1 wines showed increases of 19% and 12%, respectively. Within Ap3 wines, those obtained from juices with the lowest water-to-fruit ratio (1 L/kg) had 29% higher TPC values and 24% higher AA values than those from musts prepared with 2 L and 3 L of water per kg of apple fruit.

The results for the influence of technological factors on the red fruit wines are shown in Table 4 and Figures 4 and 5. The Blk wines are shown as a homogeneous group of wines characterized by an appreciable richness in polyphenols, a very high AA, and an intense reddish color that is predominantly linked to TMA. The pectinolytic treatments produced neither positive nor negative effects on these characteristics. The method used to prepare the musts might partially explain these findings. In blackberries, anthocyanins are located in the flesh, while ellagitannins are distributed throughout the fruits, with the seeds being the main source (Siriwoharn and Wrolstad 2004, Hager and others 2008). Crushing the raw material in a highly diluted medium (two parts water to one part fruit) caused an intense disruption of the fruit drupelets that would produce a massive extraction of anthocyanins from the fruit flesh so that subsequent addition of enzymes would not have any additional effect on anthocyanin concentration. Pectinases cannot attack the hard tissues of seeds and therefore cannot influence the extraction of ellagitannins from them.

In Figure 4, the Blue wines are located on the opposite side of the blackberry wines. These wines were also rich in polyphenols but had a moderate antioxidant activity and a very low TMA, with a color approaching blue and yellow shades. The BlueW2 wines, with the highest fruit proportion, are clearly separated from the remaining blueberry wines (Table 4, Figures 4 and 5). The BlueW2 showed 37% and 29% higher values for TPC and AA, respectively, than the BlueW0 and BlueW1 wines. This result was also verified for the variables related to the color concentration: CI (55%), WC (45%), AC (27%), and CDR_{SO2} (65%). In contrast, no differences were observed for TMA and the variables linked to the color shade (Hue, Yellow, Red, or Blue).

Finally, the ApBlk wines are located between the Blue and Blk wines (Figure 4). The ApBlkF2 wines, with some characteristics resembling those of the Blk wines, are separated from the ApBlkF1 wines, which are closer to the Blue wines, particularly to the BlueW0 and BlueW1 trials. As expected, the ApBlkF2 wines were a greater source of antioxidants and colorants than the ApBlkF1 wines. On average, when the blackberry proportion was increased, the changes observed were as follows: 13% TPC, 22% AA, 46% CI, 63% WC, 128% AC, 113% ellagitannins 265% TMA, 580% cyanidin-3-glucoside, and 1085% cyanidin-3-rutinoside (Table 4). This explains why in ApBlk wines both ellagitannins as well as anthocyanins were correlated with AA, as it was mentioned above. On the other hand, the predominance of apples in the ApBlkF1 wines gave them a more evolved color, with less red and more yellow and blue, and a CAW two times higher than that of the ApBlkF2 wines. This result was consistent with the finding that in the ApBlkF1 the minor compounds detected using HPLC-DAD represented on average 68% of the sum of areas recorded at 520 nm, while in the ApBlkF2 wines this percentage was only 19%, being the native anthocyanins cyanidin-3-rutinoside (66%), and cyanidin-3-glucoside (15%) predominant (Figures 2b and 2c).

Conclusions

Wines produced from Andean red berries from Ecuador have high total polyphenol contents that are correlated with their *in vitro* antioxidant activity, which is comparable with that of red grape wines. *Rubus glaucus* blackberries are highly available in Ecuador and have outstanding levels of anthocyanins and ellagitannins, making these berries particularly interesting as raw materials for winemaking. The combination of Andean blackberries with other less-acidic fruits such as apples may also be a good end-use alternative for both fruits. In contrast, the lack of widespread crops of the *Vaccinium floribundum* blueberry in Ecuador hinders its exploitation. Further research is needed to evaluate the best processing practices for the production of wines or other products derived from these fruits.

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Acknowledgments

This work was possible by financial assistance from the AECID (Spanish Agency of International Cooperation for Development): Projects PCI-A/016087/08 and PCI-A/023823/09. Thanks to M.J. Andrade, I. Córdova, V. Criollo, P. Guano, I. Ocaña, G. Salazar, and K. Tapia, from the Universidad Técnica de Ambato (Ecuador), J. Sánchez from Gruppo Salinas (Salinas de Guaranda, Ecuador); and I. Urretavizcaya, H. Ortega and S. Salvatierra, from the Universidad Pública de Navarra (Spain), for their collaboration in the projects.

Table 1. Experimental designs

Set 1 – Apple wines (Ap1)	
Factor Y - yeast strain Y0 - LEVAPAN - *S. cerevisiae* var. *cerevisiae* Y1 - Lalvin ICV OPALE - *S. cerevisiae* var. *cerevisiae* Y2 - Lalvin EC1118 - *S. cerevisiae* var. *oviformis* Y3 - Lalvin QA 23 - *S. cerevisiae* var. *oviformis* Factor S - Fruit solids in fermentation S0 - Without solids S1 - With solids Number of trials: 4 x 2 x 2 repetitions = 16 wines	Description: sixteen apple wines were obtained in either presence or absence of fruit solids using four different yeast strains, three wine dry active yeast strains supplied by Lallemand (Canada), and one bread instantaneous dry active yeast supplied by LEVAPAN (Ecuador). The latter is the cheapest, most readily available and most used commercial yeast in the region. Year of production = 2009 Temperature of fermentation = 24.1-26.4°C
Set 2 – Apple wines (Ap2)	
Factor E - Pectinolytic enzymes E0 – No treatment E1 – Lallyzme EX: 0.03 g/kg fruit (prior-fermentation) E2 – Lallzyme EX: 0.0075 g/L wine (post-fermentation) Factor Y (yeast strain) Y0 - LEVAPAN - *S. cerevisiae* var. *cerevisiae* Y1 - Lalvin EC1118 - *S. cerevisiae* var. *oviformis* Number of trials: 3 x 2 x 2 repetitions = 12 wines	Description: twelve wines were obtained with a wine yeast strain or with the same bread yeast used in the set 1 and submitted to enzymatic clarification treatments with Lallzyme C-MAX (Lallemand Inc., Canada) prior or after the fermentation. Treatments were carried out at ambient temperature, as is customary in enological practices, and doses of the enzymes were established according to the supplier's recommendations. Year of production = 2009 Temperature of fermentation = 23.8-26.1°C
Set 3 – Apple wines (Ap3)	
Factor W - proportions of water and fruit in the musts W0 – 3 L water/kg fruit W1 – 2 L water/kg fruit W2 – 1 L water/kg fruit Number of trials: 3 x 4 repetitions = 12 wines	Description: twelve wines were obtained from musts prepared by mixing fruit and water in three different proportions. Year of production = 2010 Temperature of fermentation = 18.2-19.9°C
Set 4 – Blackberry wines (Blk)	
Factor E - Pectinolytic enzymes E0 – No treatment E1 – Lallyzme EX: 0.02 g/kg fruit (prior-fermentation) E2 – Lallzyme EX: 0.03 g /kg fruit (prior-fermentation) E3 – Lallzyme C-MAX: 0.0013 g/L (post-fermentation) E4 – Lallzyme C-MAX: 0.0025 g/L (post-fermentation) Number of trials: 5 x 2 repetitions = 10 wines	Description: two doses of two commercial pectinases (Lallemand Inc., Canada) were added at different moments. Lallzyme EX, is recommended for the red grape maceration process to increase the amount of juice and color extraction. It was added to the must just before the beginning of the fermentation process. Lallzyme C-MAX which is recommended to clarify must and wines was added once the alcoholic fermentation concluded. As in set 2, treatments were done at ambient temperature. Year of production = 2009 Temperature of fermentation = 23.1-26.9°C
Set 5 - Apple and Blackberry wines (ApBlk)	
Factor F – Proportions of each fruit in the musts F1 – 2 parts of apples and 1 part of blackberries F2 – 1 part of apples and 2 parts of blackberries Number of trials: 2 x 4 repetitions = 8 wines	Description: eight wines were produced from musts in which two different mixtures of apples and blackberries were used. Year of production = 2010 Temperature of fermentation = 17.5-19.5°C
Set 6 – Blueberry wines (Blue)	
Factor W – Proportions of water and fruit in the musts W0 – 4 L water/kg fruit W1 – 3 L water/kg fruit W2 – 2 L water/kg fruit Number of trials: 3 x 4 repetitions = 12 wines	Description: twelve wines were obtained from musts prepared by mixing fruit and water in three different proportions. Year of production = 2010 Temperature of fermentation = 14.2-18.3°C

Wines of sets 1 and 2, were obtained from juices prepared with 3 L water per kg of fruit; 2 L in the case of sets 4 and 5

Wines of sets 2, 3, 4, 5, and 6 were fermented in the presence of fruit solids.

Wines of sets 3, 4, 5, and 6 were fermented with the same yeast strain (Lalvin QA 23).

Wines of sets 1, 3 and 5 were clarified after fermentation with pectinases (Lallzyme C-MAX)

Table 2. Summary of basic characteristics, color parameters, phenolic composition and antioxidant activity of wines.

Variables \ Wines	Blackberry (n = 10)		Apple/Blackberry (n = 8)		Blueberry (n = 12)		Apple (n = 40)	
	Mean ± SD	Min-Max	Mean ± SD	Min-Max	Mean ± SD	Min-Max	Mean ± SD	Min-Max
AD (% vol.)	12.3 ± 0.4	11.9-12.8	12.1 ± 0.6	11.6-12.9	12.3 ± 0.3	12.0-12.9	12.1 ± 0.9	11.2-12.9
pH	3.0 ± 0.1	2.9-3.1	3.3 ± 0.1	3.1-3.5	2.9 ± 0.1	2.8-3.0	3.2 ± 0.1	3.1-3.4
TA (% malic acid)	0.7 ± 0.1	0.6-0.8	0.3 ± 0.5	0.1-1.0	0.5 ± 0.1	0.4-0.6	0.3 ± 0.1	0.2-0.4
TPC (mg GAE/L)	1265 ± 91	1122-1400	999 ± 83	862-1077	1090 ± 190	854-1386	608 ± 86	471-801
AA (mM TE)	11.6 ± 0.7	10.6-12.5	6.2 ± 0.7	5.2-7.2	5.4 ± 0.8	4-7	2.1 ± 0.3	1.6-2.8
CI	8.3 ± 0.6	6.9-9.0	2.9 ± 0.6	2.2-3.7	4.5 ± 1.2	2.9-6.4	0.21 ± 0.03	0.14-0.25
Hue	0.50 ± 0.02	0.46-0.55	0.60 ± 0.06	0.53-0.67	1.15 ± 0.10	1.00-1.32	--	--
Yellow (%)	31 ± 1	30-32	35 ± 1	34-36	44 ± 2	42-48	--	--
Red (%)	63 ± 2	60-66	58 ± 4	54-63	39 ± 2	36-42	--	--
Blue (%)	6 ± 1	4-8	6 ± 7	3-10	17 ± 1	13-18	--	--
WC	5.2 ± 0.5	4.4-5.9	1.7 ± 0.5	1.2-2.3	1.7 ± 0.4	1.0-2.4	--	--
AC	3.5 ± 0.5	2.5-4.5	1.2 ± 0.5	0.6-1.8	0.9 ± 0.2	0.5-1.2	--	--
CDR_{SO2}	1.7 ± 0.3	1-2	0.5 ± 0.1	0.3-0.7	0.8 ± 0.3	0.5-1.3	--	--
CAW (%)	33 ± 7	21-43	33 ± 13	18-52	48 ± 5	42-60	--	--
TMA (mg/L)	73 ± 16	52-105	35 ± 22	11-63	8 ± 3	5-13	--	--
CyGlu (mg/L)	11 ± 4	8-18	7 ± 6	1-15	--	--	--	--
CyRut (mg/L)	44 ± 15	25-69	30 ± 28	3-64	--	--	--	--
A_min (mg/L)	15 ± 3	9-19	14 ± 2	12-17	--	--	--	--
Ellagitannins (mg/L)	1172 ± 115	1010-1312	361 ± 144	206-538	--	--	--	--

* AD: alcoholic degree, TA: total acidity, TPC: total polyphenol content, AA: antioxidant activity, CI: color intensity, WC: wine color, AC: anthocyanin color, CDRSO2: color of pigments resistant to SO2 decoloration, CAW: chemical age, TMA: Total monomeric anthocyanins, CyGlu: cyanidin-3-glucoside, CyRut: cyanidin-3-rutinoside, A_min: sum of minor anthocyanin peaks.

Table 3. Effects of technological factors in apple wines.

Set 1 - Ap1 (factor S: fruit solids in fermentation)				
Variables	*p*-value	Ap1S0	Ap1S1	
TPC (mg GAE/L)	0.0012	574 ± 28a	682 ± 48b	
AA (mM TE)	0.0089	2.1 ± 0.1a	2.3 ± 0.1b	
A420	0.4424	0.21 ± 0.01a	0.21 ± 0.01a	
Set 2 - Ap2 (factor E: pectinolytic enzymes)				
Variables	*p*-value	Ap2E0	Ap2E1	Ap2E2
TPC (mg GAE/L)	0.0716	567 ± 30a	523 ± 31a	509 ± 34a
AA (mM TrE)	0.0105	2.1 ± 0.1b	2.0 ± 0.2b	1.7 ± 0.1a
A420	0.0906	0.23 ± 0.01a	0.21 ± 0.02a	0.21 ± 0.01a
Set 3 - Ap3 (factor W: proportions of water and fruit)				
Variables	*p*-value	Ap3W0	Ap3W1	Ap3W2
TPC (mg GAE/L)	0.0001	630 ± 13a	563 ± 56a	772 ± 22b
AA (mM TrE)	0.0001	2.3 ± 0.1b	1.9 ± 0.1a	2.6 ± 0.2c
A420	0.0000	0.16 ± 0.01a	0.15 ± 0.01a	0.22 ± 0.01b

* For each set of wines, values within a raw followed by different letters are significantly different (Tukey's test, $p < 0.05$). Data for each factor level are represented as the mean ± SD. Identification of factor levels in table 1. Results regarding the effect of factor Y (yeast strain) for Ap1 and Ap2 wines not included due to the lack of significance.

Table 4. Effects of technological factors in red wines

Variables \ Wines	Set 5 – Apple/Blackberry wines (Factor F: proportions of each fruit)			Set 6 – Blueberry wines (Factor W: proportions of water and fruit)			
	p-value	ApBlkF1	ApBlkF2	*p*-value	BlueW0	BlueW1	BlueW2
TPC (mg GAE/L)	0.0150	936 ± 72a	1061 ± 18b	0.0003	934 ± 60a	1001 ± 110a	1322 ± 89b
AA (mM TE)	0.0025	5.6 ± 0.4a	6.8 ± 0.3b	0.0000	4.7 ± 0.3a	5.0 ± 0.1a	6.3 ± 0.3b
CI	0.0009	2.4 ± 0.2a	3.4 ± 0.3b	0.0009	3.3 ± 0.3a	4.2 ± 0.9a	5.8 ± 0.4b
Hue	0.0008	0.65 ± 0.02b	0.56 ± 0.03a	0.1495	1.15 ± 0.14a	1.07 ± 0.03a	1.21 ± 0.07a
Yellow (%)	0.0039	36 ± 1b	34 ± 1a	0.4470	45 ± 2a	44 ± 1a	45 ± 1a
Red (%)	0.0008	55 ± 1a	62 ± 2b	0.0736	39 ± 3a	41 ± 1a	37 ± 1a
Blue (%)	0.0010	9 ± 1b	4 ± 1a	0.0701	16 ± 1a	16 ± 2a	18 ± 1a
WC	0.0001	1.3 ± 0.1a	2.1 ± 0.2b	0.0028	1.3 ± 0.2a	1.7 ± 0.3ab	2.2 ± 0.2b
AC	0.0000	0.7 ± 0.1a	1.6 ± 0.1b	0.0765	0.7 ± 0.1a	0.9 ± 0.2a	1.0 ± 0.2a
CDR_{SO2}	0.1713	0.6 ± 0.1a	0.5 ± 0.1a	0.0002	0.6 ± 0.1a	0.8 ± 0.1a	1.1 ± 0.1b
CAW (%)	0.0017	44 ± 8b	22 ± 4a	0.0961	47 ± 2a	46 ± 4a	53 ± 5a
TMA (mg/L)	0.0001	15 ± 3a	55 ± 9b	0.4119	7 ± 2a	9 ± 2a	8 ± 4a
CyGlu (mg/L)	0.0004	2 ± 1a	12 ± 3b	--	--	--	--
CyRut (mg/L)	0.0001	5 ± 2a	55 ± 10b	--	--	--	--
A_min (mg/L)	0.0506	13 ± 2a	15 ± 1a	--	--	--	--
Ellagitannins (mg/L)	0.0001	231 ± 25a	492 ± 51b	--	--	--	--

* For each set of wines, values within a raw followed by different letters are significantly different (Tukey's test, $p < 0.05$). Data for each factor level are represented as the mean ± SD. Identification of factor levels in table 1. TPC: total polyphenol content, AA: antioxidant activity, CI: color intensity, WC: wine color, AC: anthocyanin color, CDRSO2: color of pigments resistant to SO2 decoloration, CAW: chemical age, TMA: Total monomeric anthocyanins, CyGlu: cyanidin-3-glucoside, CyRut: cyanidin-3-rutinoside, A_min: sum of minor anthocyanin peaks. Results regarding the effect of factor E (pectinolytic enzymes) for blackberry wines (set 4) not included due to the lack of significance.

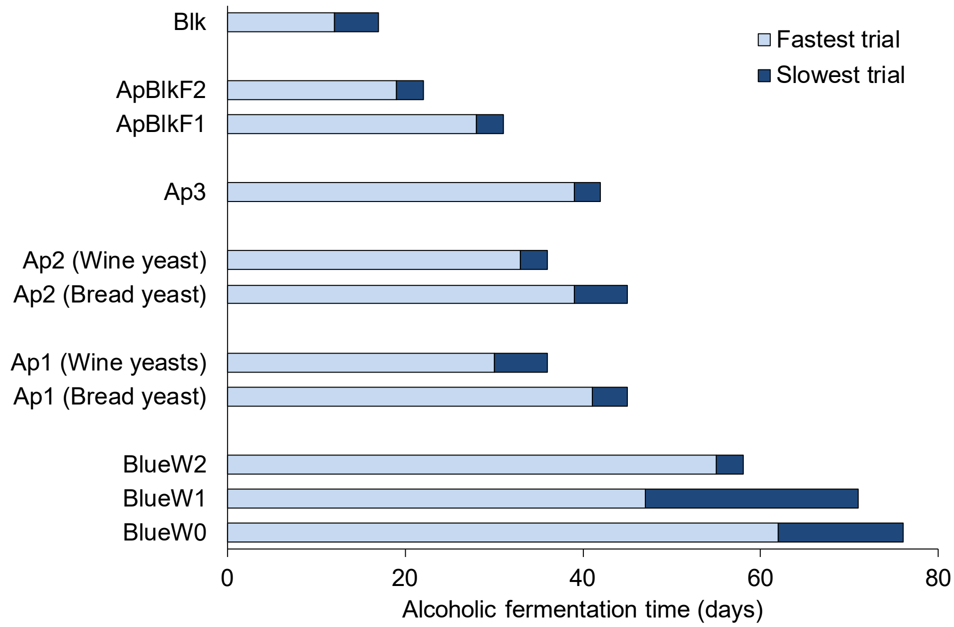

Figure 1. Alcoholic fermentations time.

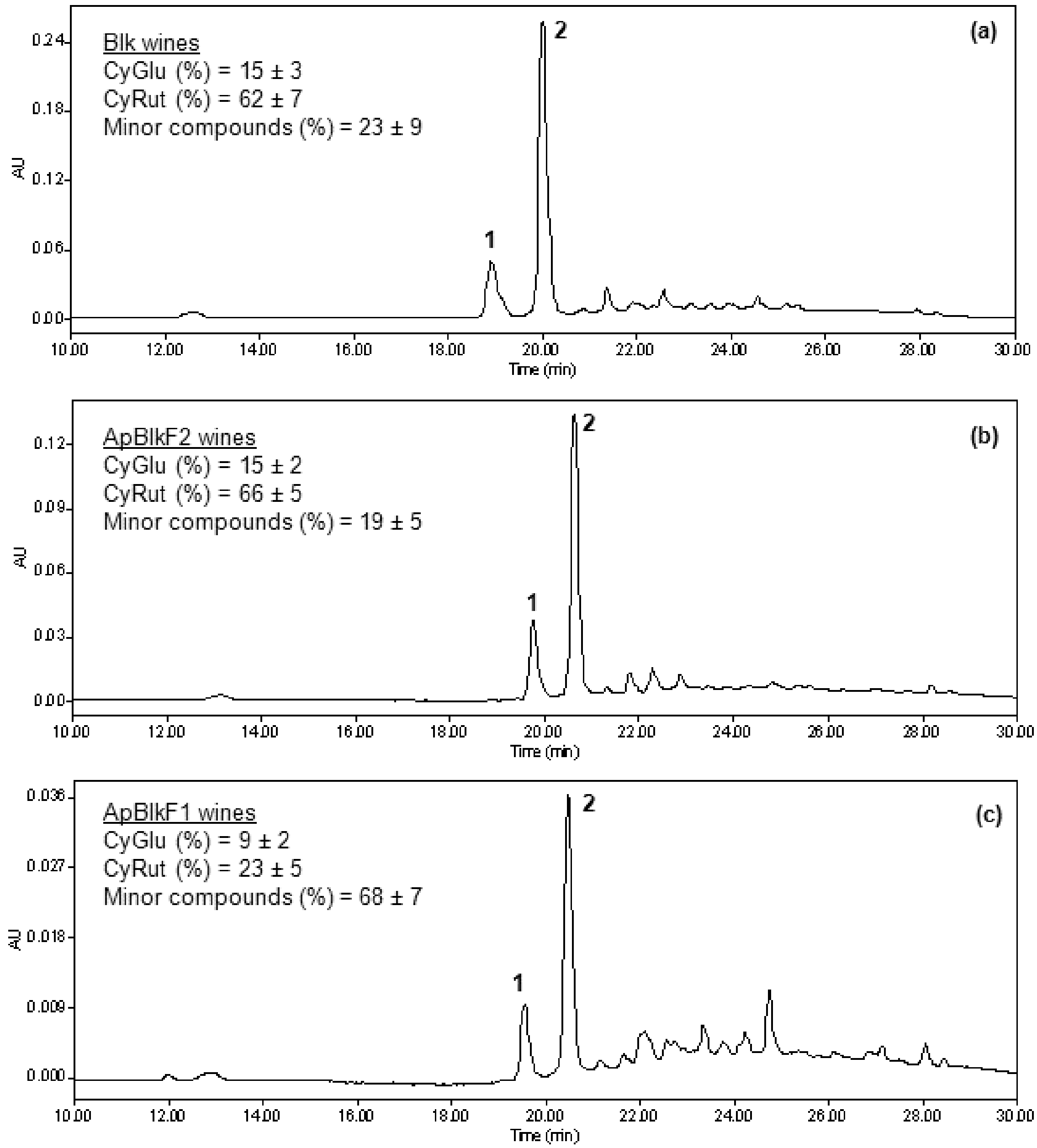

Figure 2. Chromatograms at 520 nm of samples of Blk (a), ApBlkF2 (b), and ApBlkF1 (c) wines (Peak 1: cyanidin 3-glucoside. Peak 2: cyanidin 3-rutinoside).

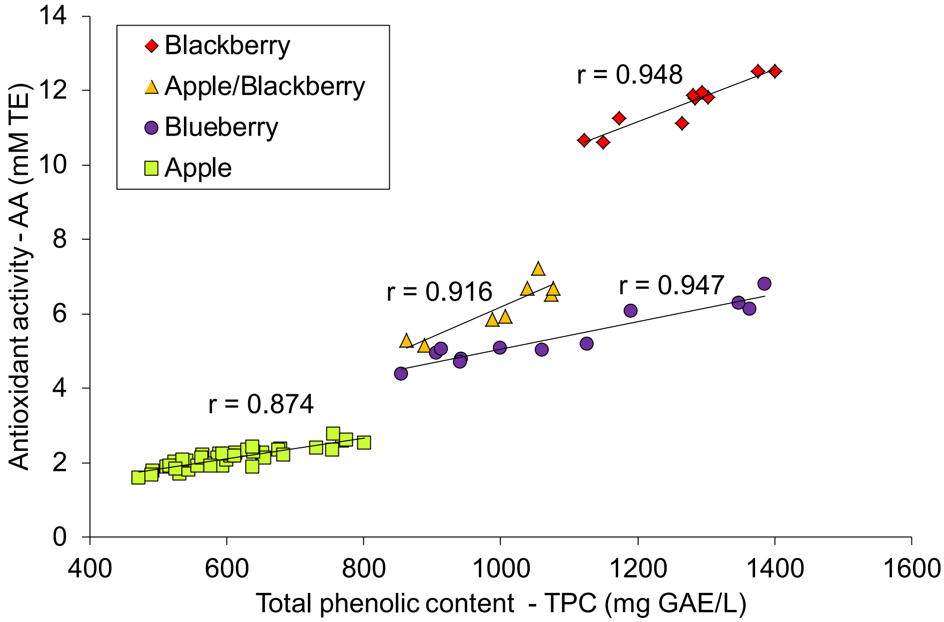

Figure 3. Relationship between the antioxidant activity and the total phenolic content of wines

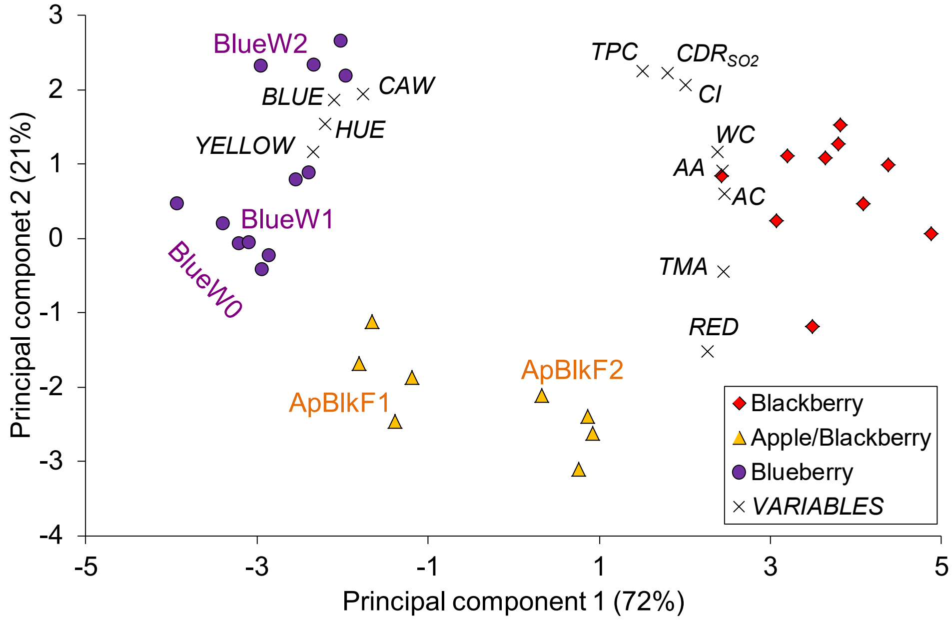

Figure 4. Principal component analysis for the red fruit wines: Biplot of variables and wines.

* AA: antioxidant activity, AC: anthocyanin color, CAW: chemical age, CDRSO_2: color of pigments resistant to SO_2 bleaching, CI: color intensity, WC: wine color, TMA: Total monomeric anthocyanins, TPC: total polyphenol content.

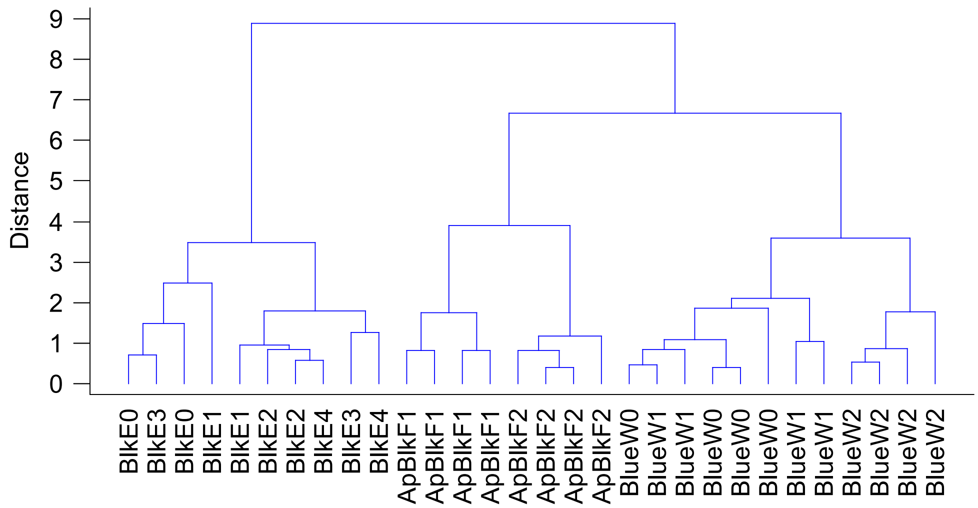

Figure 5. Dendogram from the complete linkage clustering (euclidean distance) of the red fruit wines.