“New thoughts about red winemaking using the Portuguese emblematic red grape variety Touriga Nacional growing in Lisbon”

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Abstract

Touriga Nacional is one of the most important grapes variety in Portugal. It is used to create varied type of wine due to the high polyphenols content, the characteristic aroma and the good sugar accumulation. By one hand, consumer ask for sustainable wine, with a low content of alcohol; by the other hand, a slice of consumer ask for deeply coloured and astringent wines. The work lead to better understand chemical phenomena in wines obtained by *Vitis Vinifera Sativa* "Touriga Nacional". The work differentiates a "standard protocol" by an "AEB Protocol" and study difference between two vinification methods with the support of laboratory analysis that are focused in the study of phenols compounds, colour and aroma. A study of two different wine obtained by Touriga Nacional grapes was carried out. The work lead to better understand efficiency of some oenological products used as pectic enzymes, yeast and their supplies, exogenous tannins (both hydrolysable and condensed). The wines were subjected to a maceration post fermentation of fifteen days.

In order to evaluate differences, in a part of must oenological product named above wasn't added (Control), while another part was processed with oenological products addition (Treatment). Both wines were subjected to maceration of 15 days. The finished wines were bottled and analysed in laboratory, with a particular focus on chromatic and phenolic characteristics (CIE-Lab analysis, phenolic concentration, pigments and their polymerization). The data obtained suggested that the use of pectolytic enzymes, exogenous tannins, yeast and their supplies can effectively modify important oenological parameters as colour intensity, tonality, anthocyanins concentration (and their polymerization), pigments and phenols. CIE-Lab data suggested that differences can't be found with naked eyes, by the way several study must be done, especially regarding aging.

The results show that the use of specific oenological products, applied in different vinification phases, can effectively modify wines chemical composition, by increasing phenolic composition, colour intensity and polymerized pigments but with a reduction in anthocyanins and tonality.

Each analysis was performed in triplicate and a comparison T-test (independent trial), after a variance study was carried out. More study should be done in the next years, especially using independent replicates of vinification and the same vinification procedure.

Keywords

Wine –Oenological Products – Maceration post fermentation - Winemaking - Touriga Nacional

Resumo

Foi realizado um estudo de dois vinhos diferentes obtidos pelas uvas Touriga Nacional. O trabalho levou a entender melhor a eficiência de alguns produtos enológicos utilizados como enzimas pécticas, leveduras e seus suprimentos, taninos exógenos (hidrolisáveis e condensados). Os vinhos foram submetidos a uma maceração após fermentação de quinze dias.

Para avaliar as diferenças, uma parte do produto enológico obrigatório mencionado acima não foi adicionada (Controle), enquanto outra parte foi processada com adição de produtos enológicos (Tratamento). Ambos os vinhos foram submetidos a maceração quinzes dias. Os vinhos acabados foram engarrafados e analisados em laboratório, com foco particular nas características cromáticas e fenólicas (análise CIE-Lab, concentração fenólica, pigmentos e polimerização).

Os dados obtidos sugeriram que o uso de enzimas pécticas, taninos exógenos, leveduras e seus suprimentos pode efetivamente modificar parâmetros enológicos importantes como intensidade de cor, tonalidade, concentração de antocianinas (e sua polimerização), pigmentos e fenóis. Os dados do CIE-Lab sugeriram que não podem ser encontradas diferenças a olho nu, pela maneira como vários estudos devem ser feitos, principalmente com relação ao envelhecimento.

Os resultados mostram que o uso de produtos enológicos específicos, aplicados em diferentes fases da vinificação, pode efetivamente modificar a composição química dos vinhos, aumentando a composição fenólica, a intensidade da cor e os pigmentos polimerizados, mas com uma redução de antocianinas e tonalidade.

Cada análise foi repetida 3 vezes e um teste T de comparação (ensaio independente), após a realização de um estudo de variação. Mais estudos serão feitos nos próximos anos.

Palavras-chave

Vinho - produtos enológicos- Maceração após fermentação - Vinificacao - Touriga Nacional

Resumo ampliado

Foi realizado um estudo de dois vinhos diferentes obtidos pelas uvas Touriga Nacional. O trabalho levou a entender melhor a reação química e avaliar a eficiência de alguns produtos enológicos utilizados como enzimas pécticas, leveduras e seus suprimentos, taninos exógenos (hidrolisáveis e condensados). Os vinhos foram submetidos a uma maceração após fermentação de quinze dias.

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O ensaio AEB mostrou uma intensidade de cor mais alta e uma tonalidade mais baixa, com um Abs520 mais alto (comprimento de onda vermelho) e um Abs420 mais baixo (comprimento de onda amarelo). Ambos os vinhos têm uma cor elevada devido à copigmentação:

normalmente o valor, para um vinho jovem, oscila entre 30% e 50% ((Ribéreau-Gayon, et al. 2015), enquanto CTRL tem um valor de 55,1% e AEB um valor de 56,6%. A cor devido à copigmentação tende a diminuir com o envelhecimento devido à ação do álcool. A menor concentração de antocianinas totais e o maior índice de polimerização (21% para amostra AEB é um valor muito alto, considerando que o vinho é bastante jovem; Tavares et al. 2017, por exemplo, encontraram um índice de polimerização em torno de 7% em um vinho lotado de 80% de Tinta Roriz e 20% de Touriga Nacional) na amostra AEB sugerem que compostos fenólicos evoluíram rapidamente e surgiram pigmentos poliméricos também. O maior grau de ionização de antocianinas sugere que a amostra AEB evolui mais rápido do que CTRL, na verdade, Ribéreau-Gayon, et al. 2015) valores em torno de 10% e 30% são típicos de vinho jovem, enquanto um vinho envelhecido pode atingir também valores em torno 80-90%.

AEB tem um ângulo de matiz H * de 1,54 °, isso significa que o componente amarelo já aparece, aliás as diferenças entre AEB e CTRL são imperceptíveis de se ver, de fato, como é possível ver com ΔL <3, eles estão além do visível.

Em relação ao poder de bronzeamento, a amostra de CTRL apresenta um valor inferior, provavelmente devido ao maior teor de flavanóis monoméricos também se o BSA for mais reativo contra as proantocianidinas polimerizadas (que nas duas amostras não são estatisticamente diferentes). O valor total de fenóis é maior (embora visivelmente) na amostra AEB (76,33 a.u): é um valor frequentemente encontrado (García-Marino et al. 2013, Ribéreau-Gayon, et al. 2015, Tavares et al. 2017). O CTRL apresentou maior conteúdo em flavonóides monoméricos (33,29 mg / L) do que AEB (19,48 mg / L); em relação aos flavonóis oligoméricos, o CTRL apresentou uma concentração mais elevada enquanto que a concentração dos flavonóis poliméricos não é estatisticamente diferente. Esses resultados sugerem que a amostra AEB apresenta menor concentração em flavonóides.

Palavras-chave

Vinho - AEB produtos enológicos- Maceração após fermentação - Vinificacao - Touriga Nacional

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List of abbreviation

TN (pg.10) = Touriga Nacional
LAI (pg.11) = Leaf area index
RDI (pg. 11) = Regular deficit irrigation
MP (pg.13) = Mannoproteins
MW (pg.13) = Molecular weight
N-SY (pg.13) = Non-Saccharomyces yeast
PVPP (pg.15) = Poly-vinyl-poly-pyrrolidone
MPF (pg.15) = Maceration post fermentation
TPI (pg. 16) = Total phenols index
mDP (pg. 17,41) = Medium degree of polymerization
GSH (pg. 19) = Glutathione
PG (pg. 20) = Polygalacturonase
MLF (pg. 21) = Malolactic fermentation
GC-MS (pg. 21) = Gas chromatography-mass spectrometry
PL (pg. 23) = Pectin Lyase
HPLC (pg. 23) = High Performance Liquid Chromatography
TA (pg. 25,37,39) = Total acidity
PPO (pg. 26) = Polyphenol-oxidase
TP (pg. 34) = Tanning power
Tot. Ant. (pg. 37) = Total anthocyanins
VA (pg. 39) = Volatile acidity
CTRL (pg. 39-43) = Control

1) Introduction

1.1) Portugal's features

Portugal is a country based on an industrial production and is driven by manufacturing sector and tourism as well. Portugal is based also in agriculture products as beef (pork especially), fish, cheese, fruit (as Rocha, pear) and cork production depending by zone and climate too.

"Portugal is well known for its Mediterranean climate, with warm and sunny summers, and a fresh breeze from the Atlantic coast." (Consultoria Agro-Industrial, Lda. 2020). The north of the state is quite cooler than the centre/south and the topography is mainly consisting of steep slope. The wooded area occupies most of the ha. The Alentejo region, in the south, is dryer, dominated by permanent pastures and produce fruits and vegetables, which have increased substantially in area and value in the last decade. It is famous also for the cork production. The average annual temperature varies dependently by longitude: it is around 7°C in the north and 18°C in the south, nearly to the west coast. As the temperature, also the precipitation depends by longitude: 3000 mm can be reach in the mountain of north-west, but in the south the precipitation can be below 500 mm (in this case, irrigation is mandatory in agriculture).

The agriculture, in Portugal, occupies around 3.641.691 ha (PorData 2016) divided in wooded land (around 18% of total ha) and production of tomato, corn, olive oil, horticulture products and grapes too.
Viticulture represents the 5,28 % of the total agriculture area (192.322 ha, (OIV 2019)). It is distributed around all the state, in particular nearly to the coast, but also in the inland.

Portugal has a long history concern grapes and wines production: it counts 14 wine regions and produce 24 Denominations of Origin and Geographical Indications. The wine grapes are the majority and the 5 variety most used are (OIV 2017):

- Aragonez (9% of total ha);
- Touriga Franca (7,5%)
- Castelao (6,5%)
- Fernao Pires (6,5%)
- Touriga Nacional (6,5%).

In the 2017, the Portugal produced around 6,1 millions of hL of wine with a revenue, through export, nearly to 804 millions of € (OIV 2019).

2) Aims of work

Due to high demand for high quality wines obtained from Portugal regarding Touriga Nacional, several studies are suggested to improve knowledge about factors that could influence chemical composition and biological effects in this variety.
The work lead to better understand chemical phenomena in wines obtained by *Vitis Vinifera Sativa* "Touriga Nacional". The work differentiates a "standard protocol" by an "AEB Protocol" and study difference between two vinification methods with the support of laboratory analysis that are focused in the study of phenols compounds, colour and aroma.
The main objectives of this study are the evaluation of distinct oenological products used (Yeast, Enzymes, oenological tannins, nitrogen sources) in a wine (obtained by Touriga Nacional grapes, cultivated in Lisbon) subjected to a red winemaking process with prolonged maceration post-fermentative of 15 day.

3) Literature Review

3.1) Touriga Nacional

One of the most common variety in Portugal is the Touriga Nacional, a *Vitis Vinifera sub. Sativa* red variety. Touriga Nacional is an historical variety: since 1703 England start a wine commerce with Portugal, after Metheun treaty which saw the relations between England and France intensify and an increase of duty for French wine. The history of Touriga Nacional is very particular because, after the Second World War the surface cultivated with this variety increased while in 1990, the cultivated area drastically decreased and reached 2.760 hectares. The cultivated area of Touriga Nacional are in 2010 7268 ha (VIVC 2010): it has an increase because it can increase the quality of the wine.
Some important characteristics are the high vigour, low yields, and the small dark blue berries (particularly rich in phenols compounds). It cannot develop in insufficient sunlight or in region with high drought stress (Santos *et al.* 2017).
It's well know that Touriga Nacional is cultivated in other state, as Brazil, South Africa, California (especially to do some fortified wines): the tropical climate in some zones (as São Francisco Valley) allow to do multiple harvests in one year, by the way some differences can be found in grapes composition between first harvest and second harvest. Grapes and wines

obtained by first harvest have a better quality in sensory analysis, especially in acidity, body, floral aromas and limpidity: this sensorial characterization was found also in analytical parameters. Sugar, tonality and pH was higher in second harvest, while total acidity, polymeric tannins, polymerization index was higher in first harvest (de Oliveira *et al.* 2018).

In region with altitude >300 meters as Douro region, the yield is lower because of lower cluster weights, and lower berry volumes. This effect might be the result of lower soil fertility and lower water availability at higher elevations. Also, the exposition plays an important role in yield: best results was obtained with a S-E and N-E exposition (Oliveira and Magalhães 2004).

Touriga Nacional is used mainly in Douro, but also in the other Portugal wine regions, where is blended with up to 80 variety as Touriga Franca, Aragonez etc. (Wine-Searcher 2018).

Dão winemaking region has an high viticulture potential but, due to the high variability climate in years, Touriga Nacional's health and composition (total acidity, total soluble solid, anthocyanins content etc.) may be compromised (Pedroso *et al.* 2012).

Douro winemaking region is a particular Portugal region famous all over the world thanks to wines quality. The 70% of vineyards are planted with a steep slope, higher than 30% (it's for this that Douro Valley is part of the "World heritage list UNESCO"). Viticulture, in this region, due to the pedological soil characteristics, is allowed through the construction of terraces that drastically decrease the number of vines per hectare. In order to ensure a good yield, expanded training system could be used: though the musts obtained by this grapes have a lower sugar concentration, a satisfactory quality is achieved (Queiroz et al. 2013).

The variety is used to create common table dry wine and in the blends of Porto wines, but it isn't famous only in Portugal: it has been spread world-wide. The high tannins concentration gives structure and body to the wine, allowing a long aging to wine, especially in wood (Santos *et al.* 2017). Touriga Nacional is characterized by small, thick-skinned grapes, with a high skin/ volume ratio and high anthocyanin pigment content that results in a higher release of anthocyanins into the must during the maceration process (Jordão *et al.* 2012) as know, anthocyanins are released especially during first fermentation phase. Anthocyanins, as other pigments as carotenoids, protects photosynthetic apparatus damage (due to excessive radiation, drought, heatwave) by removing the triplets states of chlorophyll molecules but also by scavenging free radical (Koyama 1991, Edge *et al.* 1997, Carvalho *et al.* 2016).
The black fruits flavours and fruity-citric aroma (due to the presence of linalool, linalyl acetate (Silva Ferreira *et al.* 2006) and C_{13}-Norisoprenoids give a particular bouquet to the wine. Oliveira *et al.* 2006 showed that Touriga Nacional has a low-medium concentration in

carotenoids (927 μg/kg. of grapes as average of total carotenoids of 2001-2002-2003), a low concentration in bounded C_{13}-Norisoprenoids and high concentration in free C_{13}-Norisoprenoids. In contrast, a good concentration of β-ionone (3.1 μg/L as average of 2001-2002-2003), 1,1,6,-trimethyl-1,2-dihydronapthalene (TDN) (10 μg/L as average of 2001-2002-2003), and vitispirane (9 μg/L as average of 2001-2002-2003) was founded in the wine. These results can explain, in part, the floral and violet aroma characteristics of Touriga Nacional wines. A trial regarding Touriga Nacional's aroma descriptor (Silva Ferreira *et al.* 2006) showed that the cultivar is particularly rich in a-pinene (described as pine-like aroma), γ-terpinene, (E)-β-ocimene and β-phellandrene (which were important for the bergamot essential oil aroma). Petronilho *et al.* (2020) described Touriga Nacional's aroma also like tree, tropical, and berry fruit; In the same study, the cultivar expressed also a sweet and oxidized note, with a toasted aroma, probably due to 2-methyl-3-furanthiol. The study showed also a good composition in lactones (γ-nonalactone,γ- and δ-decalactone), phenols (guaiacol, eugenol, 3-propylguaiacol, and 4-vinylguaiacol) but in a small quantity. Falqué *et al.* (2004) formed a panel test and analysed Touriga Nacional wines aroma describing it with plum brandy, dry raisin, wild fruit aroma-like.

About pathological status of TN, it is proved that the variety is mildly/severely susceptible to Plasmopara viticola infection (downy mildew) with an important sporulation rate, necrosis rate and oil stains(yellow area visible on the upper leaf surface) (Boso Alonso *et al.* 2008). The variety is highly susceptible to *Botrytis Cinerea* attack (in France, Grande Ferrade site): more than 98% incidence were diagnosed, together with cultivar as Aragonez and Cabernet Franc (Paňitrur *et al.* 2018)

The variety can be used also for a biomedical and alimentary applications: a study done by Mendes *et al.* (2013) proposed a pre-extraction with apolar solvent in order to extract some valuable extractives as cellulase, xylan and triterpenes acids(especially oleanolic acid); pectins and glucomannan can be extract by using hot water.

3.2) New strategies to obtain quality wines

Nowadays, consumers ask for characteristic wines deeply coloured and with a full body: several methods are purposed as use of regulated deficit irrigation (R.D.I.) (Casassa *et al.* 2013, Wenter *et al.* 2018), use of pectolytic enzymes, thermovinification, pre-fermentative maceration, high frequency of pumping over, punch down and délestage (with a bigger volume), but seems that the contact length time of wine, skin and seed is the main factor (Gil *et al.* 2012). Thanks to new applied strategies both in winery and vineyards, the wines overall

quality can increase. Starting from pruning (both winter and green pruning), in fact, it's possible to handle yield and (indirectly) berries metabolites concentration. With the study of water potential, it's possible to induce different water stress levels that could increase berries quality and maximize yields at the same time (it's possible to modulate stomal conductance, peculiarity that is principle of photosynthesis ratio (Malheiro *et al.* 2020)) . Fertilizations can be advantageous in poor soil, whereas deficiencies occur in order to reach a mineral and organic equilibrium already in grapes. The soil particularly rich in mineral substances, in fact, give higher dry extract, a peculiarity of full wines during taste.

3.3) Distinct vineyard technique

According with Fregoni 2015, wines quality can be modulated starting from vine. Partial rootzone drying (P.R.D.) and regulated deficit irrigation can modulate berry's diameter, increase pulp/skin ratio and modulate oenological parameter, by improving phenolic and aromatic potential.

3.3.1) Beneficial water stress

According with Barreales *et al.* 2019, Touriga Nacional (cultivated in Douro region, PT) has a good fits to water stress by closing stomas and reducing photosynthesis activity (well known as isohydric response): in the study, a Non-irrigated portion of vineyards was compared with one portion subjected to regulated water deficit (25% of evapotranspiration). The RDI treatment increase kg of grapes/vine of 24,5% (from 0.77 ± 0.44 to 1.02 ± 0.62): parameters as total acidity and total polyphenols index wasn't affected.

3.3.2) Kaolin/potassium silicate spray

Due to climate change scenario (drought, high sun exposure), several methods to retard sugar accumulation (promoting phenolic evolution) are purposed as use of kaolin or potassium silicate: in Touriga Nacional cultivated in Douro, foliar application can influence sugar accumulation without interfering with pH, tannins and anthocyanins content and average weight of the grapes berry (Singh *et al.* 2020).

3.3.3) Canopy management

Downey *et al.* (2006) observed that light and temperature can modulate anthocyanins synthesis and accumulation (temperature has greater effect than light).

The basal defoliation seems to influence anthocyanins biosynthesis and its accumulation in grapes (Gouveia *et al.* 2015; Fregoni, 2015). Castro *et al.* (2004) proved that basal defoliation doesn't influence yield and average cluster weight but can increase wine colour intensity (this technique can be useful in wet zone, in order to promote cluster aeration and better control

diseases). The technique practically seems to work, but some precautions are needed: dependently by variety and phenological stage, it can give different results (Harrison 2018).
Sternad Lemut *et al.* 2011 studied the defoliation effect in Pinot Noir discovering that anthocyanins increase in grapes that came from vine early (berry set) defoliated, while the same treatment done in veraison leaded to a lower anthocyanins content
In a trial conducted by Hunter *et al.* (1991), a defoliation performed in C. Sauvignon during veraison conduced to an higher anthocyanins content, while the same treatment applied after fruit-set and pea-sized berries didn't showed the same result. Climate, variety, intervention period are just some variables that need to be taken in account.

3.4) Distinct winemaking strategies

Touriga Nacional is an important variety in wine scene: it can be proven by numerous tests and trials done with goal to obtain a better product, qualitatively speaking. Touriga Nacional is a ductile variety that can be vinificated in different way dependently by oenological goals and by grape's quality. For this reason, several vinification methods were purposed during last years.

3.4.1) Lees Addiction/Mannoprotein

In a study done by Rodrigues *et al.* 2013, after a cold pre-maceration, the use of yeast lees (ghost) was studied. The lees, as known, can be implicated in adsorption phenomena with phenols compounds, in particular with polymeric pigments: this can explain that the "control" wine (without lees addition) has a higher colour intensity and polymeric pigments than "treated-1" (produced with winery by-product older lees) and "treated-2" (with newly fermented lees). The lower parameters founded in the two treatment could be due to the higher release in mannoproteins. The study showed that proanthocyanidins decrease with contact with lees, in particular that one with more -OH group (galloylated more than non-galloylated)
A taste panel was created and judged **"**treated-1" as less equilibrated in mouthfeel that could be due to the greater amount of external white wine yeast lees that was added to the wine, but **"**treated-2" had a better mouthfeel than "control". The study shows that yeast ghost can be added in Touriga Nacional and, depending by the type, the wine can reach a better quality in less time.
As showed by Rodrigues *et al.* 2012, depending by mannoproteins molecular weight, it's possible to stop tannins polymerization increasing. In a trial done with Touriga Nacional, Mannoproteins with a Molecular Weight of 19-44 kDa (two peaks almost similar), due to the small dimension, can stabilize tannins growth around an MDP of 8-14, also after 60 days at 35°C. This result can be explained by a steric stabilization: due to the small-size particle, mannoproteins can interact with tannins more efficiently.

3.4.2) Non-Saccharomyces yeast (N-SY)

The use of Non-saccharomyces yeast in the last year increased due to their contribution to diversify the final product. The study Teixeira *et al.*, 2015 talks about autochthonous yeasts that could be found up the skin of Touriga Nacional in some Portugal wine regions (Douro, Lisbon, Alentejo). By one hand, N-SY (Especially *Starmerella Bacillaris*, produce more fructophilic character, glycerol, lower Volatile Acidity; by other hand, N-SY have a low fermentative capacity, for this reason it can be done in co-fermentation or in a sequential fermentation with saccharomyces, to complete fermentation and improve aroma and general quality too. The Table 1 shows some wine's parameters depending by yeast used.
It doesn't include Total phenols index and colour Intensity because no significative differences were founded. Alcoholic strength is higher in reference (*Saccharomyces cerevisiae*), as expected. Many times, fructose is not converted at all during fermentation: some Non-Saccharomyces yeast (as *S. bacillaris*) can consume it in order to avoid residual sugar in the final wine. Volatile acidity is lower by using *Pichia terricola*, but its fermented a little part of sugar and, at the same time, it consumed an important part of malic acid by
producing a half g/L of lactic acid.

A panel taste composed by 13 tasters evaluated wine by quantitative sensory descriptive analysis. The panel analysed wines by orthonasal aroma descriptor, retronasal aroma descriptor and mouthfeel perception. Wine obtained by fermentation of *Starmerella bacillaris* and *Candida diversa* have an overall quality higher than the other yeasts used, maybe due to complexity and softness.

Table 1: Some oenological parameters that used yeasts can influence(adapted by Teixeira *et al.*, 2015)

Parameter	% (v/v) Alcohol	F (g/L)	VA (g/L)	TA (g/L)	pH	G (g/L)	L-Ma (g/L)	CO2 (g/L*day)
Effect	***	***	***	***	***	***	***	***
Saccharomyces cerevisiae	12,79 g	0,81 a	0,78 bc	5,95 bcd	3,47 de	4,17 a	2,1 b	22,2 f
Candida diversa	7,8 cd	70,98 e	1,08 d	6,5 d	3,41 cd	13,11 e	2,8 g	9,4 bc
Hanseniaspora guilliermondii	8,17 cd	22,10 b	0,82 bc	5,9 bc	3,41 c	10,9 c	2,6 ef	15,6 de
Hanseniaspora opuntiae	7,3 c	35,60 c	1,33 e	6,04 bcd	3,42 cd	10,17 c	2,45 cde	12 c
Hanseniaspora uvarum	6,2 b	51,28 d	0,88 c	5,7 b	3,43 cd	11,93 d	2,45 de	14,5 d
Pichia kudriavzevii	10,89 ef	33,41 c	1,09 de	6,21 cd	3,44 cde	7,68 b	2,75 fg	7 b
Pichia terricola	4,96 a	90,40 f	0,42 a	4,97 a	3,48 e	13,4 e	1,57 a	3,3 a
Starmerella bacillaris	10,41 e	1,56 a	0,71 b	6,21 d	3,37 b	13,34 e	2,3 bc	16,7 e
Zygoascus hellenicus	4,18 a	106,42 g	0,27 a	5,21 a	3,4 bc	13,9 e	2,35 bcd	3,8 a
Zygosaccharomyces bailii	11,35 f	2,16 a	0,68 b	7,2 e	3,43 cd	5,27 a	1,7 a	15,3 de
Zygosaccharomyces bisporus	8,65 d	3,13 a	1,05 cd	7,79 f	3,31 a	10,96 c	2,43 cde	14,9 de

F=Fructose; G=Glicerol; L-Ma= L-Malic acid; TA=Total acidity

The worst inconvenience founded in these two trials was the low-sugar consumption: in order to finish all sugars, co-fermentation or sequential fermentation must be done.

3.4.3) Use of alternative wood chips from new botanical species

A trial conducted by Santos *et al.* 2017 allow to better understand molecules extraction from wood chips by evaluating oenological parameters as color, total phenols and with a global evaluation by sensorial analysis. Acacia and cherry chips increase total phenols, flavonoid phenols in Touriga Nacional's rose wine. The contact with acacia's chips, persisted for 20 days,

had a higher tannin power than other chips: the result suggested that contact with acacia wood, increase astringency perception. The study of CIE/Lab (L*a*b*) parameters allow to understand color differences in wines: ΔE was > 2 in cherry, American oak and acacia samples subjected to fining agents (fining treatments has a lower ΔE, except for cherry sample that was higher). Acacia's sample had also a higher anthocyanins concentration, probably due to the higher release in non-flavonoid phenols, that preserve monomeric anthocyanins by oxidation. The degree of ionized anthocyanins did not show statistically differences by samples after the fining treatment, probably due to the reduced contact time. Polymerization index was higher for French oak, American oak and cherry before fining treatment; after the fining treatment, the index deeply decreased. After a sensorial evaluation, cherry chips seem to have higher values for the majority of the parameters (non-fined). In addition, PVPP, Isinglass and bentonite can deeply modify Touriga Nacional wines characteristics.

3.4.4) Maceration post-fermentation

Maceration post-fermentation (MPF) is a technique that complete the release of constituents pre-formed during pre-fermentative and fermentative maceration. It allows an increase (positively or negatively dependently by grapes and wine) of phenolic compounds and their subsequent polymerization of tannins with anthocyanins (it means that this technique could increase colour stability too: wines result richer and more complex, with a higher aging potential). It can be conducted at hot temperature (40-45°C) or cold (room temperature or less) dependently by grapes status and wine sought (OIV 2013), (Ioannidou and Ricardo 2012).

Many study purpose a prolonged maceration to increase phenolic composition in red wine as proven by (Gambuti *et al.* 2004, Francesca *et al.* 2014) with Aglianico, Nerello Mascalese and Piedirosso.

Gambuti *et al.* (2004) focused their study in the bioavailability of polyphenols due to the potential benefits in favour of human health and They tried to find a relationship between maceration time and marc pressing. In the study was proved that phenolic compounds concentration depends, first of all, by:

- **Variety**, as proved by Fuleki and Ricardo-Da-Silva 2003, that discovered that Seyval and Niagara were highest in procyanidins content while Chardonnay and Elvira were highest in catechins content (for white wines) while, for red wines, Vincent, Foch, and Baco were highest in catechins.

- **Maceration time**, (according with Karumanchiri and Ng 1995 in a trial with Merlot grapes) depending by compounds, in fact, trans-resveratrol and (+)-catechin doesn't follow a linear trend (Gambuti *et al.* 2004). Once they increase, after a certain amount of time, tend to decrease probably due to precipitation, adsorption on yeast lees or marc and isomerization to cis-resveratrol.

- **Press marc**, compounds as (+)-catechin and (-)-epicatechin was statistically different compared to free run (Gambuti *et al.* 2004), but variety influence release kinetics and final concentration.

Francesca *et al.* (2014) investigated the microbiological (yeast and lactic acid bacteria), chemical (release of polyphenol compounds from skin and seeds, antioxidant activity) and sensory characteristics (Volatile organic compounds) of Aglianico di Taurasi (cultivated in Avellino, Campania, Italy) subjected to post-fermentation maceration (the maceration length varies from 13 days to 90 days). Seven different maceration length was studied (PMF of 13,20,40,50,60,70 and 90 days). Regarding:

Microbiological aspect: it's possible to say that yeasts stay alive also after alcoholic fermentation keeping a concentration≈7 in Log (CFU/mL) until fortieth day, and then decrease with various swings. The study of 5.8S and 26S rRNA gene with Polymerase chain reaction (PCR) allow to identify, in MPF: Saccharomyces Cerevisiae, debaryomyces Carsonii (after 60 days; is important that some strain can degree biogenic amines (Bäumlisberger *et al.* 2015)) and Zygosaccharomyces bisporus (after 60 days). The study shows that Brettanomyces wasn't detected (note that the presence of yeasts in wine is also dictated by their presence in the cellar environment).

Chemical analysis: an increase in maceration length allow to convert all sugar, increase polyphenol concentration, increase flavan-3-ol as (+)-catechin and (-)-epicatechin (with a non-linear trend), increase the sum of stilbenes and hydroxybenzoic acids, increase esters concentration.
Concentration of other odorous compounds as alcohols, carbonyl compounds, fatty acids, lactones, phenols didn't followed linear trend but, a sensory analysis showed that, in terms of aroma, intensity, complexity was higher after a MPF of 90 days than others, with an higher perception of dried fruits, aromatic herbs and spices; in terms of taste, complexity and sweetness was higher than other trials after a MPF of 90 day. Bitterness and astringency were higher in trials with MPF of 13 and 20 days. The study showed that MPF of 40-50 can deeply modulate chemical and sensorial evaluation of Aglianico wines.

Gil *et al.* 2012 studied influence of Maceration length on colour, phenolic composition and polysaccharide content in Tempranillo and Cabernet Sauvignon wines in Tarragona, Spain. The study showed that total anthocyanins diminished as the maceration time increase in both cultivar) probably due to adsorption by yeast/tank surface or their transformation in new pigments with different wavelength. TPI increased in both cultivars. Mean degree of polymerization (mDP) increased between first and second weeks in both cultivars, but, after, it decreased significatively: probably the solubilization kinetics of skin and seeds proanthocyanidins are different (proanthocyanidins are released quickly from skins than seeds). Polysaccharides increased as maceration length became higher: this can be explained by the longer contact time and the simultaneous release of mannoproteins and polysaccharides by yeast.

3.4.5) Bloodletting (Cold Saignée in French, Salasso in Italian)

Wine can be enriched in phenols compounds (and others) with an increase in ratio grape marc/juice by removing a part of juice by rank. This technique have some lack as concentration in off-flavours, defects (sanitary status, excessive phenolic concentration) and economical disadvantages (volume losses that can be avoided by creating different products as white or rosé wines, Ribéreau-Gayon, *et al.* 2015).

3.4.6) Remountage techniques (Pump-Over, Punch-Down, Délestage)

The regulation of pump-over and punch-down allow the berries constituent release, especially when alcohol start to accumulate in must. Punch-down are suggested especially during last fermentation phase, in order to avoid aerobically bacteria growth and continue extraction also after fermentation. In order to avoid excessive extraction that could increase bitterness sensation, these techniques need continuous taste and periodical analysis (gelatine index, HCL index etc. Ribéreau-Gayon, *et al.* 2015, Ribéreau-Gayon, *et al.* 2015) especially in that wines particularly rich in alcohol.

3.4.7) Cold maceration

According with Langbecker *et al.* 2018, seems that a pre-fermentative cold maceration of three days could give positive results regarding Touriga Nacional wines overall quality. It allows to preserve a higher acidity and a lower p; the tone ($\frac{Abs420}{Abs\ 520}$) was higher in control (wine subjected to a standard maceration during fermentation). Other parameters as total polyphenols index, total anthocyanins, HCl Index didn't shows statistically differences.

3.5) General information on enological products used in vinification used in the trial

3.5.1) Yeast

Yeast, as known, is one of the most important micro-organisms responsible of wines quality. It allows conversion of sugar in alcohol, the production of odorous compounds (as example, (Winter *et al.* 2011) prove that 3-mercaptohexanol can be formed by yeast in cytoplasm, starting from cysteine/glutathione bounded with Trans-2-Esenale) etc.
The French Institute of wine and viticulture of Nantes selected yeast strains coming from Moldavia, (LEVULIA® T.P.C.O commercialized by AEB France). The yeast is a *Saccharomyces cerevisiae var. bayanus*, a subspecies adopted especially in the production of wine with high alcohol content (it can ferment until 16% v/v, (Istitut Francais de la Vigne et du Vin 2018)) with fast fermentative kinetic and low production of volatile acidity (0,07 g/L in $CH_3CO\text{-}OH$). The yeast can allow thiols production due to expression of IRC7 gene pathway (Uniprot 2020), with a ß-Lyase activity: once cysteine bound with a substituent (-ethyl, -3MH and -4MMP), IRC7 can explicate its liasi activity, following the reaction:

S-substituted-L-Cysteine + H_2O → A thiol + NH_4^+ + Pyruvate

IRC7 gene has a preference to use 4-mercapto-4-methylpentan-2-one (4MMP)-Cysteine (Roncoroni *et al.* 2011), that can smells as cat urine at high concentration. It works also with glutathione precursor, but lower. Yeasts that have this gene expressed, can create, starting from precursor, an higher thiols quantitative.

3.5.2) Nutrient, vitamins and yeast derived product

Yeast nutrients are essential during alcoholic fermentation in order to allow yeast growth and propagation. There are many substances that are useful for yeast growth and their fermentation:

- Vitamins: as thiamine, biotin, or pantothenic acid (etc.) play a key-role in yeast's survival. Pantothenic acid (Vitamin B_5), as example, is the cofactor in methylation of homoserine in methionine; is proved that it strongly influences yeast growth. Their concentrations can be modified in grapes and by adding yeast ghost to wine that

promote its release (Ough *et al.* 1989): its addition as pure substances are considered adulteration, therefore strongly prohibited.

- Fatty acids: as stearic, oleic, linoleic and palmitic acid keep integrity of yeast's membrane and viability during alcoholic fermentation, promoting sugar-alcohol and amino acids exchange by cellular membrane, by making it more permeable (Gobert *et al.* 2019), (Ribéreau-Gayon, *et al.* 2015). Their synthesis is favoured by O_2: oxygenation, especially during first fermentation phase, allow yeast to produce its.
- Nitrogen: is used by yeast to create amino acids, peptides, polypeptides, protein (and enzymes too) useful for: formation of cellular walls and membranes, reproduction (formation of sexual pheromone, a peptides with 12 amino acids), etc. (Ribéreau-Gayon, *et al.* 2015). Nitrogen forms as amino acids or ammonium ion (NH_4^+) speeds up fermentation because it increases cells count. It is consumed by *Saccharomyces cerevisiae* in 24-48 after addition: its addition should be done during first fermentation phase, because alcohol can inhibit yeast's permease enzymes and slow down its absorption (Mendes-Ferreira *et al.* 2004).

Commercial preparation done by yeast ghost rich in amino acids and glutathione (as FermoPlus® Energy Glu 3.0) are useful as fermentation starter because it influences yeasts multiplication velocity (AEB 2018)[a]. Nutrient (as FermoPlus® Integrateur) composed by **ammonium phosphate** (used by yeast as nitrogen source), **yeast ghost** (useful in difficult fermentation, as one with high alcohol content, due their actions of heavy metal chelating, and the release of some particular compounds as sterols and unsaturated fatty acids that facilitates trans-membrane exchange), **yeast autolysates** and **perlite** (as filtration aid) (AEB 2019)[b]. FermoPlus® Alfa is a nutrient composed by yeast ghost and yeast autolysates characterized by a slow release. It allows yeast to create proteins and enzymes (AEB 2019)[c]. Preparation which contain yeast ghost and *Saccharomyces Cerevisiae's* autolysates rich in glutathione (as Elevage Glu®) can: regular redox equilibrium (AEB 2018)[d]; partially react with quinone produced by phenols oxidation (the neo-formed compound, can be oxidized by laccases but not by grapes tyrosinase (Ribéreau-Gayon, *et al.* 2015)), thanks to -SH group; protect thiols, delay sotolone formation and reducing yellowing in white wines; improve aroma (a chemical/enzymatical pathway suggested by Clark and Deed, (2018) shows that glutathione can reduce trans-2-exenal molecule and can form an odorous molecule of 3-mercapto-esanol (3-MH). Glutathione is a tripeptide formed by L-Glutamate, L-Cysteine and glycine and derived by grapes and yeasts too. It can be found in two forms: reduced (GSH) and oxidized (GSSG) with a reversible reaction. According with Dragojlović *et al.*, (2019), glutathione can react with oxidized phenols compounds and quinones (oxidized by PPO in must or during chemical oxidation in wine). Few milligrams per litre of GSH can effectively protect aroma (thiols, terpenes, esters etc.), by acting as a competitor for quinone reduction with -SH group.

Glutathione content in wine is not imputable to grapes varieties, while yeasts strain has an important influence in wine aged for one year and half.

3.5.3) Oenological enzymes

Enzymes are globular proteins that catalyse reaction. They are extremely specific for a substrate(Nepgen *et al.* 2016). Grapes contain many enzymes as pectin-methyl-esterase, polygalatturonase, glucosidase, lipoxygenase, oxidoreductase, peroxidase etc. with a variable activity dependently by pH, grapes maturity, substrate concentration etc. Enzymes as Pectine Lyase, Polygalatturonase and Cellulase, used before Alcoholic fermentation (as Endozym® Rouge), allow a better extraction of colour and aroma, especially with light skin grapes (AEB 2018)[e] and a viscosity wine reduction, favouring a better clarify (Ribéreau-Gayon, *et al.* 2015). Larsen *et al.* (2019) showed that anthocyanins can interact with pectin in model solution, especially anthocyanins aglycone with hydrophobic and hydrophilic interaction. Zhu (2018) proved that phenolic compounds can bond by non-covalent interaction, with cell wall polysaccharides: as consequence, the quality result deeply changed.

The pectins are localized inside the berry, mainly in the skin cell wall. Pectins, together with polygalacturonic acids, can intensify wine turbidity and make it less filterable (both filtering times and filter clogging increase, Ribéreau-Gayon, *et al.* 2015).

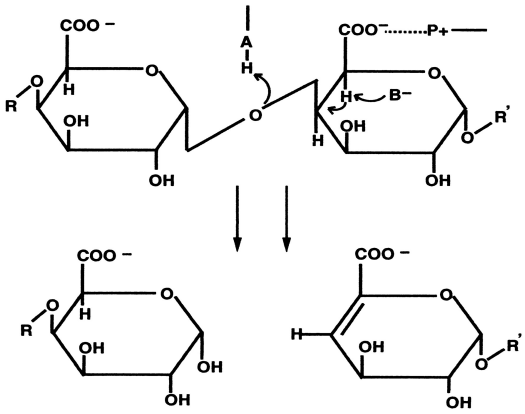

Figure 1: Pectine Lyase mechanism

- Pectin Lyase broke bound between two galacturonic acids (in a 1,4 – Polygalacturonic Acids, a pectin → Figure 1) by creating a single molecule of galacturonic acids and a compound with a double bond in C_4-C_5.

- Polygalacturonase (PG) are a class of enzymes that can separate units of polygalacturonic acids (bounded in a pectin): it's divided in eso-PG (follow a sequential hydrolysis from external pectin part to the internal one) and endo-PG (random act in the pectin chain).

- Pectin-esterase are enzymes that attack ester bond situated in galacturonic acid (pectin single unit) and release methanol: methanol release should be as less as

possible due to its toxicity for human; grapes have this enzymes, while enzymatic preparation used in oenology must have a low content in this enzymatic activity.

- Cellulase and hemicellulase are an enzymes hydrolysis family that catalyse the break of cellulose in glucose monomeric units, can also depolymerize glucan (in this case glucanasi) produced by Botrytis Cinerea (Ribéreau-Gayon, *et al.* 2015). Nogales-Bueno *et al.* (2020) showed that cellulase enzymes had a positive effect in extraction of phenolic compounds (especially flavanols and anthocyanins) in Tempranillo and Syrah.

A trial carried out by Symington *et al.* (2011) showed how enzymes preparation, rich in ß-glucosidase activity, can modulate key odorants responsible for Touriga Nacional's bergamot-aroma. The reaction is called enzymatic hydrolysis and allow to separate aroma compounds by glucose. In the study two controls were compared with 2 treatments:

- One control was compared with one treatment in a rosé wine (the addition was done after alcoholic fermentation, thus avoiding the inhibition carried out by glucose towards ß-glucosidase enzymes);

- One control was compared with one treatment in post-MLF red wine.

Aroma compounds as α-pinene, linalool, linalyl acetate, α-terpineol and citronellol were detected with GC-MS and sensorial analysis and, as expected, rosé wine showed lower results than red wine, due to the shorter contact with skin which led to a minor extraction of aromatic precursors (placed more in the skin than in the pulp (Ribéreau-Gayon, *et al.* 2015). The treatment with enzymes influenced terpenols tenor in both trials as showed in table 2 (consider that linalool and geraniol have a perception threshold, respectively of 15 and 30 µg/L)

Table 2: total terpenols founded in Touriga Nacional 2010 wines compared by control and treated wine. Adapted by (Symington *et al.*, 2011)

WINE	**Total terpenols (µg/L)**	
	Control	**Enzymes**
TN2010 Rosè	58.1 ± 17.7	71.0 ± 18.2
TN2010 Post-MLF	134.9 ± 22.9	248.3 ± 9.9

TN = Touriga Nacional

In the wine, α-terpineol was founded in higher concentration than other terpenols, probably act by acid catalysis and rearrangement/cyclisation reaction. Study showed that use of enzymes

led to increase [α-terpineol] concentration, also if after 3 years, its concentration decreases ½. After 3 years, all of trials lost an important part of terpenols, maybe due to formation of terpene-diols, compound catalysed by acid hydrolysis and formed by inserting of one hydroxyl group (-OH) in the molecule, that led to increase it hydrophilicity, decrease it volatility and make it less noticeable during tasting.

In a trial conducted by Rogerson *et al.* 2000 with TN processed in order to create a Porto wine, the use of pectolytic enzymes (pectintranseliminase, poly-galacturonase, pectin-esterase and arabinanase) can:

- Increase juice yields, but not in Touriga Nacional;
- Induce an increase nearly to 30% of wine colour (5,96 without enzymes addition and 7,46 with enzymes addition), total pigment (16,87 without enzymes addition and 20,81 with enzymes addition) and total phenols (48,5 without enzymes addition and 61,8 with enzymes addition);
- Reduce time taken from crossflow micro-filtration nearly to 8%.

3.5.4) Sulphur Dioxide (SO_2)

Sulphur Dioxide is a compound very useful in winemaking process due to its actions antiseptic, antioxidant and antioxidasic. It is highly soluble in water (112 g/L at 293 K) and, depending by pH stabilizes the following balances:

$$SO_2 + H_2O \rightarrow HSO_3^- + H^+;$$
$$HSO_3^- \rightarrow SO_3^{--} + H^+.$$

BOTH REACTIONS ARE REVERSIBLE.

SO_2 has two acids functions: the pK_1= 1,81 and the pK_2= 6,91: it means that in wine's pH, we have the form HSO_3^- dissociated (Ribéreau-Gayon, *et al.* 2015).
Its action and its availability depend by temperature, alcohol strength and some compounds that can bound it in a more or less stable way (aldehydes, anthocyanins, ketone acids etc.).
It is a ductile molecule, used in all winemaking phases, with different goals:

- Pre alcoholic fermentation phases: inhibition of bacteria growth, retard of fermentation (useful in white winemaking process, in order to clarify), avoid uncontrolled oxidation, inhibit deleterious enzymes (as Laccase from Botrytis Cinerea), etc.

- Post alcoholic fermentation phases: avoid oxidation, inhibit malolactic fermentation (useful in white wine or in red wines with low acidity and that will be consumed soon);

- During rank/filtration/addition: to avoid oxidation;

- Pre-bottling: to have a stable wine from a microbiological point of view and against oxidation.

3.5.5) Oenological tannins. Brief overview about grape and wine phenolic compounds

Phenolic compounds are a class of molecule contained in plants (tea, cinnamon, thyme etc.), wood (chestnut, oak, quebracho, fir), fruits (peach, blueberries, blackberries, Hachiya etc.), grapes and in wines too. As well know, they have oenological, medical, aesthetics application and they are used in clothes and alimentary industries.
They are classified in simple phenols and polyphenols that can be divided in flavonoids and non-flavonoids (Vardhan and Shukla 2017), dependently by the number of carbons atoms in the middle ring of the molecule.

Tannins, as definition, are a class of compounds that can react with proteins (proline, salivary protein, interact with tannins and give astringency perception during swallow) and other vegetals polymer as polysaccharides (Ribéreau-Gayon, *et al.* 2015).
They are present in grapes (in particular in the skin and in the seeds) and can be sold to wine with maceration and particular type of vinification (by using hot temperature, as example). There are many phenolic compounds classes that are present in the grapes, as Phenolic Acids, flavones, anthocyanins and flavanol.

Some of that may react among them in order to create compounds weightier, with a molecular weight higher as proanthocyanidins. These reactions occur during wine aging and create different compounds depending by many factors (oxygen availability, available reagents, tanks used, SO_2 quantity, temperature, pH etc.) and are responsible of many wine characteristics (bitterness, astringency, colour, aroma etc.). Oenological tannins can be divided in two important groups:

- Hydrolysable tannins: esters of phenolic acids, gallotannins, ellagitannins etc.

- Condensed tannins: flavan-3-ol polymers bonded with C_4-C_8 and C_4-C_6 (direct polymerization) or mediated by aldehydes (as ethanal).

A study carried on by Silva and Queiroz 2016 shows phenolic compounds that can be found in Touriga Nacional (and other grapes cultivated in Portugal) by using HPLC (high-performance liquid chromatography) to identify and quantify them. The results show that Touriga Nacional has higher concentration in anthocyanins and non-coloured phenols than other grapes used in the study (cultivated in Portugal) as Table 3 shows.

Table 3: Content in Non-Colored Phenolic compounds and Anthocyanins obtained by lyophilized grapes of Dão region (adapted by Silva and Queiroz, 2016)

	Jaen	**Touriga Nacional**	**Alfrocheiro**	**Aragonez**	**Syrah**
[Anthocyanins] mg/kg	1359,6	5336,3	1827,6	2603	2297,6
[Phenols N-C] mg/kg	343,8	1328,3	437,4	405,4	867,8

[Anthocyanins] mg/kg of Malvidin-3-O-glucoside

Anthocyanins are a class of coloured phenolic compounds that increase in grapes from the veraison stage. Grapes mainly accumulate it in the skin but, dependently by variety, it can be accumulated in pulp too. The final anthocyanins content depends by numerous factors first of all their:

- accumulation in grapes (dependently by variety, clones and climatic condition during vintage; as proved by Costa *et al.* 2015, anthocyanins accumulation depends also by region, light, exposure, temperature, altitude etc. in fact, in Dão region, the wines has an higher concentration vis-à-vis to Douro region Jordao 2020);
- extraction: it's necessary to know that anthocyanins are mainly localized in vacuole of hypodema's cells, a little layer of skin cells that are nearly in contact with pectin and protein that immobilize anthocyanins. Their release happens with extracting power of alcohol, pH, warm temperature (anthocyanins are thermolabile and hot temperature can denature them, Danişman *et al.* 2015), skin contact, mechanical broke of cells with high pressure as example or enzymes catalysis by broke bound with pectins and proteins, etc. As showed by Rolle *et al.* 2012, anthocyanins can't be extract totally from grapes (as tannins) with a maceration process because an exiguous part of them is detained by cell walls;
- storage: anthocyanins are a chemical class compound very reactive toward numerous chemical compounds and chemical reaction. They are photolabile and thermolabile and can bound with molecule as SO_2 (the -S desinence can catch

electron by aromatic rings), phenols compound (as catechin and tannins), aldehydes as acetaldehydes formed by partial oxidation of ethanol), O_2 (it oxidise by forming quinone as reaction product, responsible of colour decay), proteins etc. They can precipitate once they bound in a anthocyanins-tannins complex with a molecular weight too high and they can be oxidised by oxidase enzymes. In many of those cases the total anthocyanins content in wine decrease.

The analysis of red grapes from Dão region (Portugal) by HPLC allowed the identification of eight anthocyanins: delphinidin-3-O-glucoside, cyanidin-3-O-glucoside, petunidin-3- O-glucoside, peonidin-3-O-glucoside, malvidin-3-O-glucoside, petunidin-3-O-p-coumaroyl-glucoside, peonidin-3-O-p-coumaroyl-glucoside, malvidin-3-O-p-coumaroyl-glucoside, all present in Syrah and Touriga Nacional. Table 4 shows quantity of anthocyanins found in the study.

Table 4: Differentiation and quantification of anthocyanins in Touriga Nacional lyophilized grapes (adapted by Silva and Queiroz, 2016)

	[Anthocyanins] mg/kg	**% of the total**
Delphinidin-3-O-glucoside	609,1 ± 4,0	11,41
Cyanidin-3-O-glucoside	75,9 ± 0,1	1,4
Petunidin-3-O-glucoside	644,8 ± 2,5	12,08
Peonidin-3-O–glucoside	427,1 ± 7,9	8
Malvidin-3-O-glucoside	2800,2 ± 11,6	52,47
Petunidin-3-O-p-coumaroyl-glucoside	91,1 ± 0,9	1,7
Peonidin-3-O-p-coumaroyl-glucoside	114,5 ± 0,3	2,1
Malvidin-3-O-p-coumaroyl-glucoside	573,6 ± 0,7	10,7

[Anthocyanins] expressed in mg/kg of Malvidin-3-O-glucoside

As mentioned by Ribéreau-Gayon, *et al.* 2015, concentration of Malvidin is higher than others anthocyanins due to the two substituents -OCH3 present in the side ring, that make molecule more stable against oxidation. After vinification, anthocyanins content rapidly decrease (they bound with tannins or other molecules to create compounds more stable; they degrade by temperature, oxygen, light effect; etc.). Glycosylation and acylation move colour towards hypochromic shift: it means that colour goes towards orange. Touriga Nacional, together with Tinta Roriz, has an higher total anthocyanins content than French variety (Merlot, Syrah, Alicante Bouschet etc.); acetylated and p-coumaryl derivates result very high in Touriga Nacional, especially in cooler climates as Douro Valley (Jordao 2020).

The analysis by HPLC allowed the identification and quantification of 15 non-coloured phenolics in Touriga Nacional: one hydroxy-benzoic acid, four hydroxy-cinnamic acids, one

stilbene, three flavan-3-ols and six flavonols. The table 5 shows the concentration for each class.

Table 5: Differentiation and quantification of non-colored phenols compounds in Touriga Nacional lyophilized grapes (adapted by Silva and Queiroz, 2016)

	[N-C Phenols compounds] mg/kg	**% of the total**
Gallic acid	13,4 ± 0,3	1
t-Caftaric-TA	23,5	1,8
t-Coutaric-TA	107,6 ± 2,1	8,1
Epigallocatechin	128,7 ± 6,7	9,7
Catechin	267,9 ± 40,9	20,2
Caffeic acid	29,2 ± 0,9	2,2
Ferulic acid	8,8 ± 0,1	0,7
Epicatechin gallate	16,1 ± 0,2	1,2
Polydatin	93 ± 9,6	7
Quercetin-3-O-galactoside	97,6 ± 9,2	7,3
Quercetin-3-O-rutinoside	100,5 ± 4,2	7,6
Quercetin-3-O-glucoside	325,1 ± 5,8	24,4
Laricitrin-3-O-glucoside	59,8 ± 2,4	4,5
Isorhamnetin-3-O-glucoside	1,9 ± 0,5	0,1
Syringetin-3-O-glucoside	55,2 ± 1,5	4,1

Caftaric-TA mg/kg of caffeic acid; Coutaric-TA mg/kg of p-coumaric acid; Syringetin-3-O-glucoside and laricitrin-3-O-glucoside mg/kg of myricetin-3-O-rhamnoside.

Differentiate and quantify the non-coloured phenols compounds can be useful to better understand co-pigmentation phenomena: phenolic acids, flavonoids, coumarins etc. can tie loosely anthocyanins (with hydrogen bonds and hydrophobic interactions, Mateus *et al.* 2002). The reaction happens during first fermentation phase (the O_2 dissolved in wine can oxidize anthocyanins) when the low content of alcohol doesn't allow an extraction of tannins situated in seeds. Copigmentation phenomena can happen also with metal cations that create, with anthocyanins with two -OH, complex responsible of bathochromic effect that allow to move wines colour towards blue shade. In practice, phenomena as copigmentation, by adopting a sandwich configuration, protects flavylium ions (chromophore) from water (it can attack anthocyanins due to its nucleophilicity) and reduce the formation of hemiketal and chalcone

forms (García-Marino *et al.* 2013): this phenomena (when the medium is particularly rich in phenols) give a bright red colour to young wine.

Once alcohol concentration increase, the characteristic bonds of co-pigmentation are broken, and anthocyanins can bound with tannins (create more stable compounds with direct and mediated-by-aldehydes bounds, dependently by wine's condition, Ribéreau-Gayon, *et al.* 2015, Gombau *et al.* 2019) and takes a red colour: The younger the wine, the higher ionization index.

Caffeic and p-coumaric acids in free form are precursors of volatile phenols: as showed by Silva and Queiroz 2016 and Lima *et al.* 2018 Touriga Nacional has an amount of this precursor lower than other varieties as Syrah, Aragonez, Vinhão, Trincadeira etc. These molecules (and many others as caftaric acid) can be substrate for PPO activity that give "grille", "gouache", "animals" aroma to wine. Contrarily, Touriga Nacional has highest concentration in bond form as esters, especially regarding caftaric acid esters (that could be break bound and became free through an acid hydrolysis or thanks to some yeasts aerobic metabolism): in order to avoid unwanted yeasts spoilage (Dekkera/Brettanomyces) all winery practice (as perfectly cleaning of environment, tank, barrel etc., SO_2 check etc.) are highly recommended.

Catechin and epigallocatechin can polymerize by themselves and reduce wines bitterness, promoting a sense of astringency during tasting.

It's also important that phenols compounds are considered bioactive grape/wine compounds with possible importance for human health and nutrition.

Phenols compounds can derive from exogenous sources, therefore added to wine, with the goal to protect wine against:

- Enzymatic oxidation (laccases, PPO, etc,): this addiction allows an oxidation of the added tannins (enzymes oxidase exogenous tannins → quinone formed react with other quinone → polymerization → precipitation → elimination by racking) and preserve the grapes tannins;
- Chemical oxidation: hydrolysable tannins have capacity, in presence of heavy metals as catalyser, to react with O_2 due to its chemical structure, rich of -OH (used especially in white wine);

Condensed tannins can be used also to improve wine's organoleptic characteristics, because they can reduce % sulphured compounds, that give reduction problems to wine.

A trial done by Vignault *et al.* 2019 shows that model solution with ellagitannins have an oxygen consumption rate higher than model solution with gallotannins and procyanidins, probably due to higher number of -OH; exogenous tannins can also inhibit laccase activity (best results was obtained with 40 g/hL of ellagitannins and seeds tannins, but, with a lower quantity of 20 g/hL, ellagitannins lost a bit of activity against laccase). All exogenous tannins used in the trial have a key role in copigmentation: quebracho/seed/skin tannins, ellagitannins, gallotannins and (-)-epicatechin help to increase Copigment/pigment ratio and lead to increase absorbance at 520_{nm}.

Blend of proanthocyanidins arising by skin and seed grapes and quebracho too (Fermotan SH®) can be used in grapes with an anthocyanins profile characterized by numerous acylated form, as Syrah, Touriga Nacional and Tinta Caiada (AEB 2018)[f], in order to fix its colour.
Mixture of ellagic tannins extracted by oak tree (as AEB Goud-Ron®) can reacts, inside wines, with oxygen and oxidizes ethanol, forming ethanal, that promote the polymerization of anthocyanins and flavan-3-ol in proanthocyanidins. Ellagic tannins, due to high substituents -OH can effectively catch free radicals, one of the responsible of aroma oxidation (AEB 2018)[g].

4) Materials and Methods

4.1) Materials

The study is based in a comparation between two wines obtained by Touriga Nacional, a red pigmented variety, which is cultivated in Lisbon (PT), Tapada da Ajuda (Instituto Superior de Agronomia). 100 kg of grapes were harvested 26-August-2019 and subjected at two type of vinification: one, as control, and one (Treatment) with some products added during alcoholic fermentation and maceration.

- **CONTROL**: Once crushed and destemmed, the must was sulphited with 50 mg/L of SO_2 and 20 g/hL of Fermol mediterrane (yeast) and Fermoplus Integrateur;

- **Treatment**: Once crushed and destemmed, the crushed grapes was sulphited with 50 mg/L of SO_2 and Endozym® Rouge Light Skin was added in a concentration of 3 g/hL. Fermotan SH® has been added in twice: first in a concentration of 20 g/hL during the enzyme's addition, and the second after two days with the same concentration of the first addition. The must was now inoculated with 20 g/hL of Levulia TPCO®. As nutrient, Fermal Energy Glu® 3.0, Fermoplus Integrateur® and Fermoplus Alfa® were added

respectively in 25% weight rehydrated yeast, 20 g/hL and 40 g/hL. After 7 days, EB Goud-Ron® in a concentration of 20 g/hL was added, together with Elevage Glu®.

Both wines were racked and pressed after a maceration post-fermentation of 15 days (8-September-2019). Until the end of the year the malolactic fermentation has ended and, after that, the wines were again racked and sulphur dioxide adjusted.

4.2) Analytical methods

The wine analysis was studied with different methods, which will be described in this chapter.

4.2.1) Total soluble solids (Method OIV-MA-AS2-02)

A refractometry technique is used: allow a quantification of sugar dissolved in grapes. It's done with the refractometer (a tool that study therefractive index of the light;the higher the propagation velocity, the lower the particles content dissolved in solution): it's necessary to put some grape juice drops on the glass and then read the number between light and shadow that indicate the °BRIX. With the table available on (OIV 2012) it's possible to find the corresponding sugar content. Values (mass of sucrose) must be declared with the first decimal, or until the fourth with refractive index.

4.2.2) Alcoholic strength (Method OIV-MA-AS312-01B)

Determined by volume, describe the number of litres of ethanol contained in 100 L of wine (% or mL/100 mL) measured at 20°C. The determination was done by putting the sample in a water bath of 20°C: once the temperature is reach, 250 mL are pipetted in Kjeldahl (Figure 2) digestion tube. In order to eliminate all residues from the pipette, distillation water was used to rinsed down. The receiver flask was filled up with distilled water (20°C) and shacked due to the high inhomogeneity (very important because the differences in density of the solution can cause error of 5% in vol.). 20 mL of distillate was placed in the autosampler and the total alcoholic volume was automatically performed following OIV method of densimeter.

Figure 2: Distillation systems from kjeldahl analysis (Model: Gerhardt VAPODEST, image took at ISA University)

4.2.3) pH (Method OIV-MA-AS313-15)

It is measured with the immersion of two electrodes in wine: one electrode has a potential that is a function of pH (measure $[H^+]$) and one is used as reference. Analysis can be done only after a calibration with buffer solution. It's expressed as negative logarithm of H^+ ions concentration. pH has a scale between 0 and 14: wines, usually, have $3 < pH < 4$. pH Values must be expressed until second decimal.

4.2.4) Total acidity (Method OIV-MA-AS313-01)

It is the sum of its titratable acidities when it is titrated to pH 7 against a standard alkaline solution. 50 ml of wine is stirred with 25 ml of distilled water and 1 ml of bromothymol blue: now titration with Na-OH 0,1M start until pH reach 7 (bromothymol blue reaches a green-apple colour). A.T. = 0,75 * ml of Na-OH (g/L expressed in Tartaric acid). If expressed in meq/L, values must include one decimal point, while if It's expressed in g/L of tartaric/sulfuric acid, it must include two decimal point.

4.2.5) Volatile acidity (Method OIV-MA-AS313-02)

It is one of the most important parameters in wines because can badly influence organoleptic parameter; it's subjected to legal limit ($\leq$ 20 meq/L in red wines). Once CO_2 is removed from wine (H_2CO_3 can acidify the medium), volatile acids are removed by a steam distillation and a titration (with sodium hydroxide) to estimate volatile acids (after the titration Total SO_2 must be removed from calculation). If expressed in meq/L it must include one decimal point; another one is added if it is expressed in sulfuric/acetic acid.

4.2.6) SO_2 (Method OIV-MA-AS323-04A)

The wines are subjected to a steam of nitrogen in order to carry sulphur dioxide that is oxidized and fixed by bobbling through hydrogen peroxide (in a neutral solution). Sodium hydroxide is used now to do a titrate neo-formed sulfuric acid. With a low temperature ($\cong$10°C) free SO_2 is ripped off from wine; with high temperature ($\cong$100°C) total SO_2 is ripped off from wine. It is expressed as mg/L of SO_2 to the nearest whole number.

4.2.7) Reducing substances (Method OIV-MA-AS311-01A)

The wine is treated with neutral lead acetate (for red wines) and Zinc ferrocyanide (for white wines) in order to eliminate other reducing substances as phenols. The content in reducing substances is founded by an indirect titration: once copper salts are added, the residual Cu^{2+} that don't oxide reducing substances is titrated with iodine. Starch is used as colour change indicator (from blue to yellow). It is expressed as g/L of inverted sugar, including the first decimal point.

4.2.8) CIE/Lab (L*a*b*) (Method OIV-MA-AS2-11(OIV 2006)

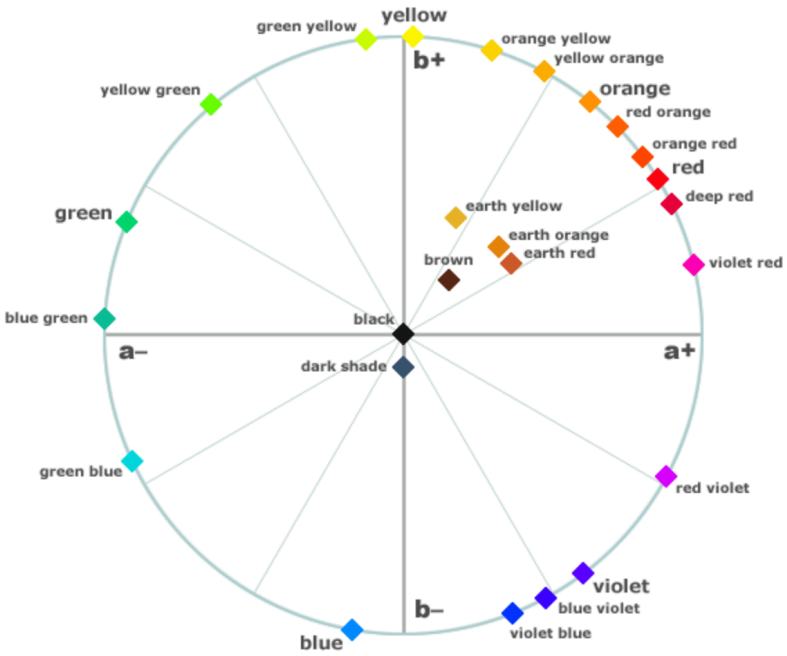

Figure 1: CIELAB diagram color space plane (it uses cylindrical coordinates, adapted by MacEvoy, 2015)

These systems allow users to evaluate colour attributes, identify inconsistencies, and accurately express their findings to others in numerical terms, in order to evaluate color in a graphic (Figure 3). The study was carried out with a spectrophotometer (Model Cary 100 UV-VIS): once the wine was centrifugated, the supernatant was placed in the spectrophotometer with different cuvettes depending by specific analysis. A cuvette of 1 mm was used to read the absorbance from 380 to 780 nanometres, using distilled water as reference, in order to establish the base line. The equipment setting must be with a data interval of 5 nm, with illuminants settled D65 and observers of 10* degrees. Interval (obtainable value) are cited in table 6.

The parameter found are:

- **Total colorimetric difference**: $\Delta \boldsymbol{E}^* = \sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]}$ → differences between two wines;
- **Clarity**: related to luminosity, is represented by **L*** value;
- **Chroma**: $\boldsymbol{C}^* = \sqrt{(a^{*2} + b^{*2})}$ → Chromaticness;
- **Angle of hue**: $\boldsymbol{H}^* = tg^{-1}(\frac{b^*}{a^*})$ → expressed in degree;
- **Difference in HUE**: $\Delta \boldsymbol{H}^* = \sqrt{[(\Delta E^*)^2 - (\Delta L^*)^2 - (\Delta C^*)^2}$.

Table 6: CIELAB parameter description

Obs. Tone is expressed as degree (°).

Colorimetric coordinates	Symbol	Interval	Decimal
Clarity	L*	0-100 0 black 100 white	1
Red/Green colour component	a*	>0 red <0 green	2
Yellow/Blue colour component	b*	>0 yellow <0 blue	2
Chroma	C*		2
Tone	H*	0-360°	2

4.2.9) Chromatic characterization

Anthocyanins and pigments (Somers and Evans 1977): after a centrifugation (5000 rpm for 10 minutes) it's necessary to use 1 mm cuvettes and then:

- read absorbance at 420-520-620 nanometres, using distilled water as reference;
- add 5 mL of $K_2S_2O_5$ (20%) and then read absorbance at 520 nm;
- add 100 mL of wine in 10 ml of HCL (1M) and then put the test tube in a thermal bath (25°C for 3-4 hours) and then read absorbance at 520 nm in a cuvette of 1 cm, using as references a solution with HCL (1M).

All Abs referee to corrected absorbance with 10 mm pathlength, except for Abs_{520}^{HCL} that must be multiplied for 101 dues to the dilution.

With this analysis it's possible to evaluate:

- **Colour intensity** = $Abs_{420} + Abs_{520} + Abs_{620}$;
- **Tonality** = $\frac{Abs_{420}}{Abs_{520}}$;
- **Total anthocyanins** (mg/L in malvidin 3-O-glucoside) = $20 * (Abs_{520}^{HCL} - \frac{5}{3} Abs_{520}^{SO2})$;
- **Degree of ionization of anthocyanins** (**a**) % = $\frac{(Abs_{520}) - (Abs_{520}^{SO2})}{(Abs_{520}^{HCL} - \frac{5}{3} Abs_{520}^{SO2})}$;
- **Ionized anthocyanins** (mg/L in malvidin-3-O-glucoside) = $\frac{\alpha}{100} * Total\ anthocyanins$;
- **Total pigments** = Abs_{520}^{HCL};

- **Polymerization index** (%) = $\frac{Abs_{520}^{SO2}}{Abs_{520}^{HCL}} * 100$;
- **Polymerized pigments** = Abs_{520}^{SO2}.

N.B. $\frac{5}{3}$ used in the total anthocyanin's formula explain that a part of polymeric anthocyanins is less sensible to pH change and need to be considered in order to allow the estimation of monomeric anthocyanins with a pH < 1. The choose of the factor ($\frac{5}{3}$) was based upon observation of the properties of polymeric pigment fractions.

4.2.10) Total phenols

After a centrifugation, 1 mL of sample is settled in 100 mL of distilled water: read the absorbance at 280 nm (UV) (Ribéreau-Gayon, et al. 2015). Once data are available, it's possible calculate:

$$\boldsymbol{Total\ phenol\ index} = Abs_{280} * 100;$$

Results must be expressed with two decimal point.

4.2.11) Tanning power

The tanning power simulates what happens in our mouth because procyanidins bind with the saliva protein (Ptyalin) that denatures, precipitates and give us a felt of astringency. The wine is added with some protein present is BSA (bovine serum albumin) that can bind with procyanidins. The reaction forms a precipitate that is directly imputable to association procyanidins-protein and can be measured by nephelometer (De Freitas and Mateus 2001).

Divided in two steps:

1) The wine is centrifugated and diluted 1/50 with a wine model solution (12% v/vm with 5 g/L of tartaric acid) previously filtrated with cardboard filter of 0,45 μm. The mix is subjected to a nephelometric analysis (1stL.).
2) 25 mL of diluted wine is added with 7,5 mL of BSA solution (BSA 0,8 g/L) and shacked. The wine is stored in a dark ambience for 45 minutes and then is putted in nephelometer in order to obtain 2ndL.

$$\boldsymbol{T.P.(NTU/mL)} = \frac{(2^{nd}L. - 1^{st}L.)}{0{,}08}$$

4.2.12) Colour due to copigmentation

The colour due to copigmentation is find by reading two absorbance at 520 nm (Boulton 2001):

1) Acetaldehyde is added in wine in order to untie the bonds between anthocyanins and SO_2 (10 mL of wine and 0,1 mL of acetaldehyde) and the first absorbance "520^a" is read;
2) Wine is diluted with and hydroalcoholic solution (composed by 12% v/v and with a pH of 3,2 adjusted with tartaric acid) in order to dissociate anthocyanins-Copigment complexes. After 45 minutes (in order to have a completely reaction) 2,5 mL of wine is diluted in 50 mL of hydroalcoholic solution and the "520^b" absorbance can be read.

$$\boldsymbol{CDTC}\ (a.u.)\ =\ 520^a\ -\ 520^b$$

$$\boldsymbol{CDTC}\ (mg/L\ of\ Malvidin-3-O-Glucoside)\ =\ CDTC\ (a.u.)\ *\ 20{,}3.$$

$$\boldsymbol{CDTC}\ (\%) = \frac{(520^a - 520^b)}{520^a}$$

4.2.13) Flavonoids and Non-Flavonoids

In order to determinate its, Kramling and Singleton 1969, purpose the following method:

1. Non-Flavonoids (N-F): in a centrifuge tube, it's necessary to add 10 mL of sample with 10 mL of HCL (previously diluted 1:4), 5 mL of Formaldehyde (previously diluted at 36% → 2,08 mL in a flask of 100 mL brought to volume with water) and nitrogen. After it's necessary to store samples in a dark place for 72 hours (red wines). After a centrifugation for 10 minutes at 3500 rpm, in a volumetric flask, 5 mL of samples are diluted with 45 mL of distilled water: the mix can be putted in the spectrophotometer in order to read the absorbance at 280 nm.

$$\mathbf{Non-Flavonoids}\ (mg/L\ of\ gallic\ acid)\ =\ \frac{[(ABS * 10) + 0{,}0344]}{0{,}038}$$

Flavonoids = Total Phenols - N-F

4.2.14) Proanthocyanidins in wines

Quantitative and qualitive analysis of proanthocyanidins conte wine are important to understand what is happening (in chemical terms) in wines. As example, a high catechins concentrations and a low polymeric proanthocyanidins content explain that wine is not evolving (or the evolution is very slow). The study of these compounds can be useful to direct the wine in a specific product category. They are studied by an optical analysis through

spectroscopy. The preparation of the trials in this case follow Sun *et al.* 1998 rules, with 5 passages:

1. two neutral Sep-Pak (tC18Sep-Pak and C18 Sep-Pak) cartridges are connected in series. The resin is activated with 10 mL of Methanol, 10 mL of distilled water and 15 mL of buffer solution (pH=7 → it can be done with 9,84 g of Na_2HPO_4 + 2,73 g of KH_2PO_4 in 1 L of distilled water).

2. Using a rotatory evaporator (that keep T° around 25-28°C), 5 mL of wine are dealcoholized → the volume obtained (less than 5 mL) is defined **V1**.

3. In order to elute catechins(**F_1**), oligomeric proanthocyanidins(**F_2**), 20 mL of pH=7 buffer is added to the dried wine sample: now it passes through the two Sep-Pak cartridges (in order to eliminate phenolic acids). The cartridges are now dry in N_2 (for 1 hour) and the eluent is thrown away. In order to elute catechins and oligomeric catechins (and other small molecules), 25 mL of ethyl-acetate is used: the collected fraction is dried out through evaporation (low pressure and T° of 25-30°C). 3 mL of pH=7 buffer is used to dissolve the dry residue. A second elution with 15 mL of methanol allow to sunder polymeric proanthocyanidins and anthocyanins (**F_3**). The cartridges are re-preconditioned in order to elute F_1 + F_2 (the cartridges are now dried with N_2 for 1 hour). Catechin (F_1) are eluted with 25 mL of ethyl-ether, while oligomeric proanthocyanidins (F_2) are eluted with 15 mL of methanol. In order to obtain dried fractions (F_1, F_2, F_3), each one is evaporated at low P and low T°. The dried residues are dissolved in 5 mL of methanol**.**

4. Fractions are detected with vanillin:

   - Monomeric flavanols (F_1) → 2 mL of obtained solution are mixed in two test tubes with 5 mL of sulfuric acid (dilution: 1:3): in the first tube it's necessary add 5 mL of methanol (obs. It is the blank, or "zero") while in the second one 5 mL of vanillin (1% of methanol). The reaction will take around 15 minutes: the absorbance can be read at 500 nm.
   - Oligomeric proanthocyanidins (F_2) → 2 mL of obtained solution are mixed in two test tubes with 5 mL of sulfuric acid (dilution: 1:3). In the first tube it's necessary add 5 mL of methanol (obs. It is the blank, or "zero") while in the second one 5 mL of vanillin (1% of methanol). The reaction will take around 1 hour: the absorbance

can be read at 500 nm. (Keep the cells closed with a stopper while reading → scan mode used in spectrophotometer).

- Polymeric proanthocyanidins (F_3) → as F_2.

5. Calculations:

$$\left[\frac{\text{mg}}{\text{L}}\text{of catechin}\right] = \frac{(\mathbf{vr\_s} * \mathbf{Abs})}{(\boldsymbol{b} * \boldsymbol{V1})}$$

Observation:

Abs = the absorbance read at 500 nm for each fraction;
B = inclination of the curve F_1 (0,0081), F_2 (0,0046), F_3 (0,0037)
V1 = initial volume of the sample (normally it's used 5 mL but, in the trial done with Touriga Nacional, o.d. 280 was > 1,2 so 3 mL was used).

4.3) Statistical analysis

In order to snatch differences between control and treated wines, a study of variance is performed with Fisher Test with a significance of ρ>0,05. Once variance is analysed a comparison test (T-test, independent samples) is done using a ρ<0,05 as significatively. The software used is Excel (version 16.37 for IOS devices). Results derive from mean of 3 simultaneous analysis.

5) Results and discussion

5.1) Grape's analysis

As showed in table 7, total soluble solids, pH, total acidity, total anthocyanins and total phenols was studied. The good content in anthocyanins and phenols allowed to harvest grapes in order to avoid higher loss acidity and an excessive pH raising. Total acidity, as well known, is strongly related with climate and temperature. Temperature, in fact, as severe water-stress (especially during June, July and August), negatively impact total acidity (strictly related with pH) also in the cultivar Touriga Nacional (Costa *et al.* 2020). Temperature, in fact, lead to malic acid respiration, which cause a drop of total acidity and an increase of pH. Temperature, however,

allow a good total soluble solids accumulation and promote a higher alcoholic content. Anthocyanins, due to their thermolability, are degraded with excessive temperature (during heatwaves, as example, particularly frequent in some zone of Douro, Lisbon and Alentejo region). Moderate correlations between minimum temperatures and anthocyanins at maturity were found in Douro, Dão and Alentejo with the cultivar Touriga Nacional. High temperature negatively influence also Total phenols index. Costa *et al.* 2020 define Touriga Nacional an anisohydric (genotypes with less stomal control) cultivar due to it elevate heat exchange capacity, especially in regions with an important water stock (or in irrigated vines). So far as the cultivar is subjected to a high temperature with a severe water stress, it could lose basal leaf, with a direct impact on berries quality.

Table 7: Basic grapes analysis

	Sampling date	**Total soluble solids (°BRIX)**	**Potential alcoholic strenght (%)**	**pH**	**TA (**g/L tartaric ac.)	**Tot. Ant.** (mg/L)	**TPI** (au)
Touriga Nacional grape' s analysis	6/ago	19,0	11,9	2,96	8,7	-	-
	12/ago	20,2	12,6	3,01	8,0	-	-
	19/ago	22,8	14,3	3,23	6,8	1735,38	69,70
	26/ago	22,7	14,2	3,37	6,0	1755,38	85,00

TPI = Total phenols index

5.2) Fermentation

The must obtained by grapes was separated in two vat in order to follow two different protocols: Treatment and CONTROL. In the table 8 it's possible to see fermentation course, with density and Temperature: both trials complete fermentation satisfactorily.

Table 8: Fermentation course in CTRL and Treatment trials

CTRL			Treatment		
Data	**Density**	**T°**	**Data**	**Density**	**T°**

26-aug	1105	27	**26-aug**	1105	27
28-aug	1060	29	**28-aug**	1070	29
30-aug	1025	28	**30-aug**	1034	29
31-aug	1006	26	**31-aug**	1009	27
01-sep	1004	25	**01-sep**	1002	25
02-sep	999	24	**02-sep**	997	24
03-sep	996	24	**03-sep**	996	24
04-sep	995	23	**04-sep**	995	23

5.3) Wine chemical characteristics

5.3.1) Basic analysis

After the alcoholic fermentation, the wines were subjected to a maceration post fermentation and then the pomaces were pressed, and the wines were racked and sulphited. The wines, then, were subjected to basic analysis (table 9).

Volatile acidity is quite high in both samples, probably due to a secondary fermentation as pyruvic glycerol that bring to the formation of secondary metabolities. The differences can be founded in the volatile acidity, lower in Treatment, probably due to the presence of exogenous tannins and glutathione; reducing substances are lower than 2, so the wines can be considered stable but CTRL has an higher risk in terms of wine aroma: due to the high volatile acidity (nearly to the perception threshold → 0,7 g/L), reducing substances can be degraded by lactic bacteria and, starting from it, they can create unwanted molecule as acetic acid. By the way, the high SO_2 content and the high alcohol content, should inhibit bacteria development in both trials. Considering that alcoholic concentration in both wines is very high and alcohol is one of the unhealthiest components in wine, a “Double Harvest” could be a solution inasmuch it don’t interfere, by modulating racking time, with quality parameters, as total anthocyanins or total polyphenol index of the final (blended) wine. By the way, depending by vintage, grapes could have a sensory character define as “green” too strong: in order to don’t badly modify wine sensory characteristics, a little delay of harvest is suggested (Martinez de Toda and Balda 2011).

Table 9: Basic analysis of wines

Treatments	**pH**	**VA** (g/L acetic acid)	**TA** (g/L tartaric acid)	**Free SO2** (mg/L)	**Total SO2** (mg/L)	**Alcohol content 20ºC** (%vol)	**Reducing substances** (g/L)
CTRL	3,88	0,64	6,8	49	62	16,1	1,8
Treatment	3,74	0,46	7,2	70	88	15,6	0,7

5.3.2) Colour evaluation

In the table 10 colour parameter are expressed. Colour intensity is a value which varies dependently by wines and varieties. It both trial it is medium/high. This could be explained by the high colour given by variety, Touriga Nacional. As example, other authors as Tavares et al. 2017 found a lower Colour intensity, nearly to 10 (a.u.).

Tonality, as expected, has values which reflect the young age of wines (according with Ribéreau-Gayon, *et al.* 2015). It is higher in CTRL and this could be explained by a higher yellow colour component (D.O. 420). According with (Somers and Evans 1974), ionised anthocyanins represent the percentage of total anthocyanins which are in the coloured form (flavylium ion form, free and combined too) and range between 6 and 25 % in young wines (can reach 80-90% in aged wines). The difference in the total anthocyanins between trials could be explained by a higher self-association in Treatment trials and a higher polymerization with condensed tannin and wood tannins released by oenological tannins used in the trial. Anthocyanins ionisation are related with copigmentation/self-association phenomena, polymerization with tannins, low SO_2 content etc. For the CTRL, degree of ionisation is a value founded also by Tavares *et al.* (2017), while the AEB samples is very high, nearly to the double. The differences between trials (Treatment is higher) are similar if expressed in mg/L but the percentage with total anthocyanins are higher in Treatment due to the lower total anthocyanins content. The use of exogenous tannins is still fundamental, because, as said above, they increase copigmentation phenomena (a normal range for a young wine is between 30 and 50 50% (Ribéreau-Gayon, *et al.* 2015). The Treatment trial, in fact, showed a higher Colour due to copigmentation.

Table 10: Colour wines analysis

	CTRL	**Treatment**
Colour Intensity *(u.a.)*	15,81 a	16,49 **b**
Tonality *(u.a.)*	0,686 **b**	0,677 a
Total anthocyanins *(mg/L of Malv-3-O-glu)*	530,3 **b**	303,9 a
Degree of ionisation of Ant. *(α %)*	15	27
Ionised anthocyanins *(mg/L of Malv-3-O-glu)*	79,5 **b**	74,4 a
Total Pigments *(u.a.)*	33,24 **b**	23,03 a
Polymerization index *(%)*	12	21
Polymerized pigments *(u.a.)*	4,0 a	4,7 **b**
Colour due to copigmentation *(mg/L of Malv-3-O-glu)*	10,85 a	12,47 **b**
Colour due to copigmentation (%)	55,1	56,6

In order to catch differences between trials, a study of colour with **CIE/Lab (L*a*b*)** was done (Table 11). Parameter as chroma, hue, angle of hue, chroma and colorimetric differences are useful to discriminate differences in colour, brightness, clarity etc. in wine and other substrates. Substantially, no differences were founded between samples. E* can help to understand differences between colour that can be catch through a visual analysis: the value is, in fact, < 3. Probably this value will change with aging, because AEB seems to have a good stability in terms of colour due to the higher polymerization. L* represent the lightness of wine, higher in CTRL, probably due to the higher presence of phenolic compounds in AEB, as mentioned by Jordão *et al.* 2006. A* represent the red/green colours: as expected, both wines have red colour but not so high for a very young wine (García-Marino *et al.* 2013 in fact, obtained higher value, with Tempranillo and Graciano varieties). B* represent the yellow/blue colours: AEB trials have a value > 0 (the opposite result was hoped because the AEB test showed higher yellow shade than CTRL; it means that maybe some molecules were oxidized by causing a hypochromic shift towards yellow wavelength).

Table 11: CIELab wines analysis

Average CIELAB	**CTRL**	Treatment	**Δ**
L* = Clarity	57,4	56,6	0,8
a*	37,8	39,1	-1,1
b*	-0,08	1,05	-1,1
E*			1,8
C*	37,8	39,1	1,2
H*	-0,13°	1,54°	

Depending by aging and storage condition: a* tend to decrease (it means that wine lose, in part, the classic red colour typical of young wines); it is desirable that b* tend to decrease in order to promote blue shade in wines, synonymous of a correct storage and micro-oxygenation; L* should increase in order to have an higher clarity. H* should be between 270° and 360° in order to have a blue-red colour (a negative b* and a positive a*)

5.3.3) Total phenols, non flavonoids phenols, Flavonoid phenols, Tanning power

Phenols are one of the main important class of compounds present in wines. Touriga Nacional is a variety particularly rich in phenols both coloured both non-coloured (Silva and Queiroz 2016). The study of these compounds, through analytical and sensorial analysis, allow to understand the perception of astringency and bitterness, in order to modulate it during winemaking process. Analysis done on Touriga Nacional shows that treatment AEB has statistically differences in tanning power, total phenols and flavonoids too.

Tanning power, as said above, simulate what happens during tasting through the interaction of phenols with BSA. In both trials, it is very high, but AEB showed a higher result. The differences can be explained by the higher flavonoids and total phenols. As showed by Bartolomé *et al.* 2000, the reactivity of BSA increase in compounds with two hydroxyl group (-OH) and with dimers. In the same way, Ricardo-da-Silva *et al.* 1991 demonstrated that protein shows a higher interaction with phenols that have a higher degree of polymerization, by the way the proanthocyanidins in this trial showed opposite results (CTRL has higher polymeric proanthocyanidins concentration than AEB, despite the lower tanning power). Ellagitannins are highly reactive with proteins too (McCallum *et al.* 2019) and it could means that ellagitannins interfere with tanning power analysis. Wojdyło *et al.* 2020 obtained an increase in total phenols with the use of pectolytic enzymes because cell wals are rich in polysaccharides, protein and glycogen too, formed by an high number of hydroxyl group (-OH) that can tie with hydrophobic force and hydrogen bond (Le Bourvellec *et al.* 2004) , by the way the increase wasn’t very high probably due to the interaction between protein portion of enzymes with phenols. Non-Flavonoids are similar in both samples, while flavonoids are higher, as expected, in AEB samples, probably due to the use of pectolytic enzymes that allow to split pectin and the consequent release of these compounds. The flavonoids (flavonols, anthocyanins, flavanols), in fact, as mentioned by Kramling and Singleton 1969, are mainly localized in grape skin and pomace solids, while non-flavonoids (stilbene, ellagitannins) compounds mainly derive from juice and don’t require a pushed mechanical/enzymatic extraction (as high pressure or hot temperature). As showed in table 12, total phenols founded in both trials are normally values (Tavares *et al*. 2017), by the way the content in flavonoids is really high, while the content in non-flavonoids compounds is very low. The tanning power is very high probably due to the prolongation of maceration for 15 days.

Table 12: Analysis of few phenols parameters in wines

	CTRL	Treatment
Tanning Power *(NTU/mL)*	441,4 a	480,7 **b**
Total Phenols Index *(u.a.)*	73 a	76 **b**
Total Phenols *(mg/L Gallic Acid)*	1932,4 a	2009,7 **b**
Non-Flavonoids *(mg/L Gallic Acid)*	186,9	189,6
Flavonoids *(mg/L Gallic Acid)*	1745,4 a	1820,1 **b**

5.3.4) Proanthocyanidins (flavanols)

As well know, catechins are monomeric units of flavanols and derive mainly from grape seeds. Oligomeric proanthocyanidins are formed by 3-10 flavanols units (both catechins and epicatechins). The bibliographic research is not clear about the polymeric proanthocyanidins provenance. Someone argue that they are localized mainly in grape's seed (Cheynier *et al.* 1997), in contraposition with the result obtained by Sun *et al.* 1999 that didn't found polymeric proanthocyanidins in seeds. (Bautista-Ortín *et al.* 2014) tried to remove seed during winemaking process observing that at least 40% of total proanthocyanidins are extracted by seeds, while anthocyanins concentration was not affected (as mentioned above, because anthocyanins are mainly localized in skin, and in some variety, in the pulp). Cosme *et al.* 2009 saw a higher concentration of oligomeric and polymeric proanthocyanidins in seeds instead of skin in all analysed varieties (Touriga Nacional, Trincadeira, Cabernet Sauvignon, Castelão and Syrah). The same study proves that oligomeric proanthocyanidins are present in larger quantities than monomeric and polymeric fractions. In particular, the mDP (medium degree of polymerisation) is centred between 2-7 units.

Furthermore, in wine, they can increase their content by a polymerization during aging. The polymeric proanthocyanidins are defined as such when the molecule reaches at least 10 flavanols units, bond through a covalent reaction exclusively between flavan-3-ols unit (Jordão *et al.* 2001). In wine, they give a sense of astringency during tasting: the higher the polymerization degree, the higher the sense of astringency but, when the molecule reaches a molecular weight too heavy, they precipitate and cause lost in astringency: the wine became much roundness in mouthfeel. The wine lost a part of its proanthocyanidins during aging. According with Cheynier *et al.* 1997, the mDP may be related to easier degradation of higher molecular weight proanthocyanidins. Proanthocyanidins are also useful for a good fermentation: a study done by Li *et al.* 2020 showed an higher intracellular levels in vitamin B_6, thiamine and cell availability with a decrease of saturated fatty acids and an increase of unsaturated fatty acids (useful for intermembrane exchange): the RNA-seq analysis allow to assert a greater expression of the corresponding metabolic pathway. The study showed that

catechins and oligomeric and polymeric proanthocyanidins are not statistically different between the two samples (Table 13).

Table 13: Content study of flavanols in CTRL and AEB trials

	CTRL	**Treatment**
F1 → Monomeric Flavanols *(mg/L)*	8,9	15,2
F2 → Olig. Proant. *(mg/L)*	71	57,5
F3 → Poly. Proant. *(mg/L)*	632,9	638,1

6) CONCLUSION

The work allows to better understand the exogenous tannins and enzymes role. By reacting with oxygen, exogenous tannins catch oxygen and allow to keep low volatile acidity. The lower total anthocyanins content in Treatment trial suggest that they could be polymerized, in fact the polymerization index wanders approximately around the 20% in Treatment sample and more or less the half in CTRL sample.

Treatment trial showed a higher color intensity and a lower tonality, with a higher Abs_{520} (red wavelength) and a lower Abs_{420} (yellow wavelength). Both wines have an high color due to copigmentation: normally the value, for a young wine, wanders between 30 % and 50 % (Ribéreau-Gayon, *et al.* 2015), while CTRL have a value of 55,1 % and Treatment a value of 56,6 %. The color due to copigmentation tend to decrease during aging due to the alcohol action. The lower total anthocyanins concentration and the higher polymerization index (21 % for Treatment sample is a really high value, considering that the wine is quite young; Tavares *et al.* (2017), as example, found a polymerization index around 7% in a blended wine of 80% of Tinta Roriz and 20% of Touriga Nacional) in Treatment sample suggest that phenolic compounds evolved fast and polymeric pigments appeared too. The higher degree of ionization of anthocyanins suggest that Treatment sample evolve faster than CTRL, in fact Ribéreau-Gayon, *et al.* 2015) values around 10% and 30 % are typical of young wine while an aged wine can reach also values around 80-90%.

Treatment have an angle of hue H* of 1,54°, it means that the yellow component already appears, by the way the differences between Treatment and CTRL are imperceptible to see, in fact, as it's possible to see with $\Delta L < 3$, they lie beyond the visible.

Regarding tanning power, CTRL sample show a lower value, probably due to the higher monomeric flavanols content also if BSA is higher reactive against polymerized proanthocyanidins (that in the two sample are not statistically different). Total phenols value is higher (albeit sightly) in Treatment sample (76,33 a.u): it's a value often found (García-Marino *et al.* 2013, Ribéreau-Gayon, *et al.* 2015, Tavares *et al.* 2017). The CTRL showed a higher

content in monomeric flavanols (33,29 mg/L) than Treatment (19,48 mg/L); regarding oligomeric flavanols CTRL showed an higher concentration while the concentration of polymeric flavanols are not statistically different. These results suggest that Treatment sample has a lower concentration in flavanols.

The high catechins content give a sense of bitterness to wine: in this case an aging in wood barrel/HPV vat/micro-oxygenation are suggested for Treatment trial. In this case, bond mediated-by-ethanal happens, together with direct reaction between anthocyanins and tannins, allow a complex enlargement, with an improvement of astringency (lost in bitterness) and a colour stabilization.

For the CTRL, the situation is more delicate because an absence in exogenous tannin didn't allow a copigmentation phenomenon. The anthocyanins, in fact, are free in the solution and can be oxidized by O_2 or, if presents, heavy metals dissolved in wine subjected to a partial oxidative aging in wood. In this sense, it's better to allow a condensation between tannins and anthocyanins in reducing atmosphere, as a stain less vat, with the formation of carbocation as intermediate step with a temperature < 18°C in order to prevent orange form formation and a subsequent lost in astringency. In this study, it's possible to see the positive effectiveness of prolonged maceration, use of enzymes and oenological tannins.

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