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Master thesis

Unveiling the Role of Technological Processes on the Strontium Isotopic Ratio, Fingerprint of Wines' Geographical Origin

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Abstract

Based on the close relationship of strontium isotopic ratio $^{87}\text{Sr}/^{86}\text{Sr}$ between soil and plants, this isotopic ratio has been reported as fingerprint tool to verify wine geographical origin and its authenticity. In the last decade, some studies suggested that winemaking applications do not alter the $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratios from vineyard to the wine despite the variations of mineral concentration. However, information about wood impact on $^{87}\text{Sr}/^{86}\text{Sr}$ is lacking in the literature. In this study, we investigated the wood ageing effect on $^{87}\text{Sr}/^{86}\text{Sr}$, and also on the multi-elemental composition of wine, which to our best knowledge are novelties, thus representing important advances to this field of knowledge.

A red wine from Castelão grape variety was aged in stainless steel vats (34,000 L) with oak wood staves, in triplicate. The wines were sampled after 30, 60 and 90 days of ageing and evaluated in terms of: $^{87}\text{Sr}/^{86}\text{Sr}$, by Q-ICP-MS after Sr and Rb separation by ion exchange chromatography; multi-elemental analysis (Be, Mg, Al, Sc, Ti, V, Mn, Co, Ni, Cu, Zn, Ga, Ge, As, Rb, Sr, Y, Zr, Mo, Sb, Cs, Ba, Pr, Nd, Sm, Eu, Dy, Ho, Er, Yb, Lu, Tl, Pb) by Q-ICP-MS; Na, K, Ca, Fe by FAAS. Wood ageing effect on total polysaccharides concentration, chromatic characteristics and phenolic composition was also evaluated. Statistical analysis showed that there was no significant difference of the $^{87}\text{Sr}/^{86}\text{Sr}$ between the control wine and wine aged with wood. The results show that strontium isotopic ratio of wines was not altered even after ageing with wood (0.710 at initial time and 0.709 after 90 days) despite having a significant increase in Sr concentration by wood stage. This suggests that $^{87}\text{Sr}/^{86}\text{Sr}$ might be used as a reliable geographical indicator. Time had significant impact on Al, V, Zn, Ni, Cs, Pb and also on Na, K, Ca and Fe while wood stage only altered concentrations of Mg, V, Co, Ni and Sr. Our study suggests that wood ageing does not impact the $^{87}\text{Sr}/^{86}\text{Sr}$, accordingly it will shed some light for further studies.

Key words: wine, authenticity, wood stage, strontium isotopic ratio $^{87}\text{Sr}/^{86}\text{Sr}$, elemental composition

Resumo

A razão isotópica de estrôncio $^{87}\text{Sr}/^{86}\text{Sr}$ tem sido apontada como impressão digital do vinho para verificação da origem geográfica e autenticidade. Alguns estudos, realizados na última década, sugerem que a razão $^{87}\text{Sr}/^{86}\text{Sr}$ não é significativamente alterada pelas práticas enológicas, não obstante as alterações na composição mineral. Contudo, falta informação na literatura sobre o impacto do estágio em madeira na $^{87}\text{Sr}/^{86}\text{Sr}$ do vinho. No presente estudo, foi investigado o efeito do envelhecimento em contacto com aduelas de madeira de carvalho na razão isotópica do estrôncio do vinho. Complementarmente foi avaliado o efeito deste tratamento na composição multi-elementar do vinho, o que representa igualmente um importante avanço nesta área de conhecimento. Para tal, um vinho tinto foi estagiado, à escala industrial, em depósitos de aço inoxidável com aduelas de madeira de carvalho, em triplicado. Foram colhidas amostras ao fim de 30, 60 e 90 dias de estágio e avaliados os seguintes parâmetros: $^{87}\text{Sr}/^{86}\text{Sr}$, por Q-ICP-MS após separação do Sr e Rb por cromatografia de troca iónica; composição multi-elementar (Li, Be, Mg, Al, Sc, Ti, V, Mn, Co, Ni, Cu, Zn, Ga, Ge, As, Rb, Sr, Y, Zr, Mo, Sb, Cs, Ba, Pr, Nd, Sm, Eu, Dy, Ho, Er, Yb, Lu, Tl, Pb) por Q-ICP-MS; elementos Na, K, Ca, Fe por FAAS. Foi ainda avaliado o efeito do estágio com madeira nas características cromáticas, composição fenólica e polissacáridos totais. Nas condições experimentais descritas não foi observado efeito significativo do estágio com madeira na razão isotópica $^{87}\text{Sr}/^{86}\text{Sr}$ do vinho, não obstante o aumento significativo da concentração de Sr do vinho decorrente do contacto com a madeira. O valor de $^{87}\text{Sr}/^{86}\text{Sr}$ no início (0.710) e no final do estágio (0.709) não variou significativamente, indicando a robustez desta razão isotópica como marcador de origem geográfica. O estágio com madeira afectou significativamente as concentrações de Mg, V, Co, Ni e Sr. Ao longo do tempo de estágio verificaram-se alterações significativas nas concentrações dos metais Al, V, Zn, Ni, Cs e Pb.

Palavras-chave: vinho, autenticidade, estágio em madeira, razão isotópica de estrôncio $^{87}\text{Sr}/^{86}\text{Sr}$, composição multi-elementar

Resumo Alargado

As Denominações de Origem e Indicações Geográficas, assim como o desenvolvimento de ferramentas que permitam verificar a autenticidade dos produtos vitivinícolas no que respeita à sua origem geográfica, são aspectos importantes para os intervenientes do sector vitivinícola e consumidores de vinho.

A avaliação da composição multi-elementar de alimentos, relacionando-a com o solo de origem, tem sido relatada frequentemente na literatura como uma ferramenta útil para avaliação da proveniência geográfica. As razões isotópicas $^2\text{H}/^1\text{H}$, $^{13}\text{C}/^{12}\text{C}$, $^{18}\text{O}/^{16}\text{O}$ apresentam utilidade na avaliação da autenticidade do vinho principalmente no que respeita à origem botânica e detecção de práticas fraudulentas clássicas, tais como adição de água, ou etanol com outra origem que não no açúcar da uva.

Mais recentemente, a razão de isótopos $^{87}\text{Sr}/^{86}\text{Sr}$ de estrôncio, cuja composição isotópica natural varia com a idade geológica e conseqüentemente com a região geográfica, entrou neste campo de aplicação. A aplicabilidade da razão $^{87}\text{Sr}/^{86}\text{Sr}$ para rastreabilidade de Denominações de Origem Portuguesas foi recentemente demonstrada. De facto, a proporção do isótopo radiogénico ^{87}Sr , enriquecido ao longo do tempo por decaimento radioativo do ^{87}Rb , relativamente ao ^{86}Sr , varia de acordo com a composição e idade geológica. A razão $^{87}\text{Sr}/^{86}\text{Sr}$ do vinho reflete a razão $^{87}\text{Sr}/^{86}\text{Sr}$ da formação geológica/solo, uma vez que a videira não promove o fracionamento isotópico do Sr pela e a influência de aditivos enológicos é negligenciável.

Alguns estudos indicam que esta razão isotópica não sofre alteração significativa durante o processo de vinificação, apesar das variações da concentração mineral do vinho. Contudo, a literatura carece de informação sobre o impacto do envelhecimento em/com madeira na razão isotópica $^{87}\text{Sr}/^{86}\text{Sr}$ do vinho. Assim, o objectivo principal deste estudo consistiu na avaliação do impacto do estágio do vinho com madeira de carvalho nesta razão isotópica e na evolução da composição multi-elementar do vinho ao longo do envelhecimento.

A utilização de barricas de madeira de carvalho no envelhecimento do vinho é um processo tradicional na produção de vinho. Durante o período de envelhecimento, ocorrem várias reações, como interações entre compostos no vinho e na madeira. Actualmente, como alternativa às barricas de carvalho, devido ao seu elevado preço e espaço necessário, é autorizada a utilização de fragmentos de madeira de carvalho e de castanho.

No presente estudo, foi investigado o efeito do envelhecimento em contacto com aduelas de madeira de carvalho na razão isotópica do estrôncio do vinho. Complementarmente foi avaliado o efeito deste tratamento na composição multi-elementar do vinho, o que representa igualmente um importante avanço nesta área de conhecimento. Para tal, um

vinho tinto foi estagiado, à escala industrial, em depósitos de inox com aduelas de madeira de carvalho, em triplicado. Foram colhidas amostras ao fim de 30, 60 e 90 dias de estágio e avaliados os seguintes parâmetros: $^{87}\text{Sr}/^{86}\text{Sr}$, por Q-ICP-MS após separação do Sr e Rb por cromatografia de troca iónica; composição multi-elementar (Li, Be, Mg, Al, Sc, Ti, V, Mn, Co, Ni, Cu, Zn, Ga, Ge, As, Rb, Sr, Y, Zr, Mo, Sb, Cs, Ba, Pr, Nd, Sm, Eu, Dy, Ho, Er, Yb, Lu, Tl, Pb) por Q-ICP-MS; elementos Na, K, Ca, Fe por FAAS. Foi ainda avaliado o efeito do estágio com madeira nas características cromáticas, composição fenólica e polissacáridos totais. Nas condições experimentais descritas não foi observado efeito significativo do estágio com madeira na razão isotópica $^{87}\text{Sr}/^{86}\text{Sr}$ do vinho, não obstante o aumento significativo da concentração de Sr do vinho decorrente do contacto com a madeira. O valor de $^{87}\text{Sr}/^{86}\text{Sr}$ no início (0.710) e no final do estágio (0.709) não variou significativamente, indicando a robustez desta razão isotópica como marcador de origem geográfica. O estágio com madeira afectou significativamente as concentrações de Mg, V, Co, Ni and Sr. Ao longo do tempo de estágio verificaram-se alterações significativas nas concentrações dos metais Al, V, Zn, Ni, Cs e Pb.

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1. General Introduction

Wine is one of the oldest fermented drinks in the world and consumed by number of people with a growing interest day by day. 274, 4 millions of hectoliters of wine are produced in the world with Europe's half share of the global production (OIV - International Vine and Wine Organization, 2015). These numbers provide a clear insight how the wine sector is important for consumers, producers and all the actors in this sector. As commonly known common attributes like aroma, color, mouth feeling, are the main quality indicators of wine. As a result of containing many organic and inorganic substances, it is a complex beverage. Therefore, it has several quality criteria besides its physical and chemical composition, such as grape variety, origin, soil and climate, winemaking practices, traditional applications and *terroir*. *Terroir* is a concept that is attributed to build a holistic approach regarding interactions between physical, chemical, biological environment and applied viticultural practices that represent the characteristics of products from a particular territory. The term includes specific soil, topography, climate, landscape and biodiversity and grape variety. The biggest contribution factor to classify the wines is *terroir* (OIV, 2010).

With regards to especially *terroir* aspect, the determination of wines' authenticity has been an important issue for wine producers and consumers for decades. As a consequence of being one of the most consumed alcoholic drinks, and having easily adulteration potential, it is subjected to some falsification applications in the industry, hence posing risks for producers and actors as well as for consumers. While some of these falsifications are of quality risks and result in financial losses due to reputation damage such as incorrect grape variety, addition of water, sugar or alcohol, while some can lead to serious health risks due to high heavy metal concentration if they are superior to safe limits. One research study that was done with wide variety of wines from different countries showed that most of the samples exhibited high level risks regarding target hazard quotients (THQ) (Naughton and Petróczi, 2008). Therefore, this situation makes researchers develop reliable methods to reveal the illegal applications and adulterations. Due to certain protected and certificated wine regions, classification of wines are strictly regulated in EU by label regulations which producers need to declare the qualities of the wine and to give certain and correct information to the consumers. In Europe, label legislation is maintained for protecting the designation of the region and encourages traditional and good practices under the name of protected geographical indication (PGI) that wine's characteristics are related exclusively to the geographic origin, including natural and human factors and also protected designation of origin (PDO) and traditional specialities guaranteed (TSG). PDO covers agricultural products and foodstuffs which are produced, processed and prepared in a given geographical area

using recognized traditional practices while PGI covers agricultural products and foodstuffs closely linked to the geographical area. At least one of the stages of production, processing or preparation takes place in the area. TSG highlights traditional character, either in the composition or means of production (OIV, 2015).

From the consumer's perspective, it is difficult to rely on every bottle in the market as some may not have fair declarations. Fraudulent practices in the industry are not only on premium wines but also on fine/medium quality wines. There are several types that producers may apply at different stages of the chain. Easily applied adulterations, that eventually provide high profit margins, pose challenging risk for counterfeiting. Sweetening (chaptalization), substitutions by cheaper products which can be addition of different source of sugars, coloring agents, water, preservatives, flavor note and CO₂ and acidity regulators are commonly known fraud uses. Traceability of the whole chain by the authorities is not always easy and requires specific tools and methods for authentication purposes. In Europe, the quality of wine is controlled by taking several criteria into account about the sensory characteristics, label declarations such as alcohol content, vintage, blending, geographical origin and also chemical adulterations as mentioned above. The objective of these detection tools is to verify the accuracy of the information that is declared on the labels and uncover the counterfeiting applications so that buyers and consumers are protected and not misled.

In addition to revealing the falsifications, authenticity of wine's origin on the labels provides dignity to the product in the eyes of customers. Moreover, designated origins, that are also called appellation of origin, add high values to the wines. As there are several regions (*terroir*) in the world that are known by their fine quality wines, people are willing to pay more for the wines from these regions as long as they are reliably identified.

In the literature, recently, one of the most commonly used analytical techniques for fingerprinting is the multi-element composition of the product. However, it is still in research that there is no officially established method by OIV for authentication purpose by using multi-elemental analyses. Multi-elemental analysis is applied to determine the mineral pattern and concentration of wine. Statistical techniques make it possible the discrimination of wines according to the products' geographical area where they are grown. However, mineral composition of wine (macro and micro elements) changes with different agricultural applications, such as fungicide, pesticide and fertilization applications, winemaking practices and environmental conditions (Kment *et al.*, 2005; Pohl, 2007; Catarino *et al.*, 2008a, Catarino *et al.*, 2008b). Therefore, it makes it difficult to depend on only multivariate methods as a provenance tool due to uncertainties and insufficiencies. It is necessary to find new alternatives with more precise and accurate results related to the geographical origin. Thus,

analytical methods are developed by using trace elements whose concentrations are lower in the nature than other elements that had slight but significant changes according to the geological regions that seem to give more trustworthy results.

With provenance verification purpose, isotope abundance ratios have been used for several decades for determination of archeological sources and also in geo-sciences. Growing interest in food provenance enabled new applications and methods also in agriculture. As an alternative to the above mentioned methods regarding authenticity and wine fingerprinting, Horn *et al.* (1993) published a study by using isotopic ratio of strontium for wine traceability with promising results. The study emphasizes that different geological bedrocks have different mineral composition in the earth stratum. The mineral composition of rocks and soils differs from one region to another by representing different strontium isotopic ratios. Even though, the ratios vary slightly from one area to another, they might be significantly different from each other. Strontium isotopic ratios (Sr IR) can lead to recognition of adulterations or frauds as soils from different wine regions carry different $^{87}\text{Sr}/^{86}\text{Sr}$ values (Horn *et al.*, 1993). Hence, strontium isotopic ratio can be used as a provenance assignment tool in regard to its close relation between $^{87}\text{Sr}/^{86}\text{Sr}$ of soil origin and the plant. The study by Horn's team encouraged researchers to focus on strontium element for traceability purpose in order to prevent false declarations of wines with denominations of origins. In the last two decades, there have been many studies proving the reliability on $^{87}\text{Sr}/^{86}\text{Sr}$ for wine fingerprinting. Nevertheless, there are also number of studies reporting the analytical uncertainties and discrepancies of Sr isotopes analyses in wines and difficulties to match them with their substrata data (Martins *et al.*, 2014; Durante *et al.*, 2015; Marchionni *et al.*, 2016).

One study that analyzed the soil composition and grape juices, seeds, skins, and wine samples in each step of the vinification process, suggests that strontium isotopic ratio may be carried inalterably by the different stages of wine making processes (Almeida and Vasconcelos, 2004). Even though, several vinification steps do not seem to alter the strontium ratio of soil that is transferred to the vines, according to the recent studies in the literature, to our knowledge, there is no study that reveals the oak ageing impacts on the strontium isotopic ratio. The evolution of wine's mineral composition with ageing is also lacking in the literature as some of these elements are with special interest such as heavy metals due to their food safety issues in addition to the evolution of physical – chemical characteristics of wine. Hence, it is important to unveil their development by wood contact for future studies.

2. Objective of the Thesis

Our objective in this study was to observe the evolution of $^{87}\text{Sr}/^{86}\text{Sr}$ in wine by oak ageing and to reveal if there is any significant change in strontium isotopic ratio over time as well as evaluating the alterations in physical, chemical and chromatic characteristics of wine during ageing. The results will contribute to the state of art if strontium isotopic ratio can be a reliable tool for justifying wine provenance, moreover, whether they can be used even for aged wines. Also, the developments of the mineral composition, during ageing, were studied, which to the best of our knowledge is a novelty.

3. State of Art

It is stated that authenticity and origin of wines might be identified relying on the specific mineral and trace element patterns by means of chemometric methods (Baxter *et al.*, 1997; Thiel *et al.*, 2004).

With the increasing awareness of consumers about the origin of the food, authorities intensely focused on discovering new practices for food fingerprinting. Recently reliable analytical methods are being emerged. The use of stable isotopes of water and alcohol of wine has been a reliable tool for authentication as they vary depending on the climatic factors. Analyses recognized by OIV for authenticity purpose are shortly presented hereinafter for detecting the frauds and tracing the authentication in wines concerning the falsification and authenticity issues.

In terms of authenticity in wine, stable isotopic ratio analyses of $^{13}\text{C}/^{12}\text{C}$, $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ were the first methods that were recognized by OIV for detecting the sugar origin in wine. Nevertheless, these tools still stay to be limited in terms of interpreting the data and relating them to the wines' provenance. The most commonly used frauds are shortly explained as follows:

Chaptalization is identified by ethanol analysis in order to identify the sugar source in wine. Because sugar cane and maize are from C4 group plants while grapes are of C3 plants. Grapes showing lower isotopic ratio of $^{13}\text{C}/^{12}\text{C}$ in ethanol than other sugar sources makes easy to detect the foreign sugar addition into the wine (OIV-MA-AS312-06 Type II method; OIV, 2015). Another method based on the distribution of the deuterium (heavy H) in the grape must reveals the addition of exogenous sugar while also giving the climatology of the production area (Commission Regulation No 2348/91). This technique is also known as Site-specific Isotope Fractionation measured by Nuclear Magnetic Resonance (SNIF NMR™).

Water addition is identified by isotopic ratio of $^{18}\text{O}/^{16}\text{O}$ water in musts and wines by means of stable isotopes ratio mass spectrometry method (IRMS). The ratio of $^{18}\text{O}/^{16}\text{O}$ reflects the geographic origins and the climatic conditions of the products (Commission Regulation No 1932/97).

Carbon dioxide in wine must come from the botanic origin of *Vitis Vinifera*. In order to justify its origin exogenous CO_2 can be detected by the isotopic ratio of $^{13}\text{C}/^{12}\text{C}$ by IRMS method especially for sparkling wines (OIV Method OIV-MA-AS314 Type method: II; OIV, 2015).

Glycerol; The determination of $^{13}\text{C}/^{12}\text{C}$ in wines by Gas Chromatography Combustion or High performance Liquid Chromatography coupled to Isotopic Ratio Mass Spectrometry (GC-C-IRMS or HPLC-IRMS, respectively) enables the detection glycerol addition (OIV Method OIVMA-AS312-07 - Type method: II).

In the international methods of analysis by OIV, wine authenticity for fingerprinting is assessed by analyses with targeted compounds that are, as some are already indicated above, nuclear magnetic resonance techniques (NMR), stable isotopes ratio mass spectrometry (IRMS), high performance liquid chromatography (HPLC) and gas chromatography for determination of light isotopes (Roullier-Gall *et al.*, 2015).

In the case that the results are inconsistent, additional information to the isotope analysis of $^{18}\text{O}/^{16}\text{O}$ of wine water can be used. For instance, in a study, the minerals in wine (Mg and Rb) in addition to $^{18}\text{O}/^{16}\text{O}$ isotopic information made it possible to classify the different regions in Brazil by means of discrimination statistical analysis (Dutra *et al.*, 2011).

A very interesting study was done in 1999 in Bordeaux, with combination of methods of isotope ratios (C, H and O) and trace elements. The production year and different denominations were efficiently differentiated by IRMS and SNIF-NMR (Martin *et al.*, 1999). Very similar study by using multivariate analysis, this time with combination of IRMS and H-NMR made it possible to discriminate three different regions in Spain by their ^2H and ^{13}C content variation of fermentative ethanol based on environmental and eco-physiological factors within three years (Giménez-Miralles *et al.*, 1999).

Furthermore, frauds are not only about exogenous addition of sugars or waters but also on the labels that can be related to the vintage year, ageing time and grape variety. There are several studies that established methods to reveal these characteristics' of wine. One research team published a study with different wine samples from three different vintage years (1996, 1997 and 1998) with the aim of provenance tool and they were successfully separated according to their geographical origin that compares the coastal region and continental regions with combination of site-specific natural isotopic fractionation nuclear

magnetic resonance (SNIF-NMR) method and IRMS method that used $\delta^{13}\text{C}$. However, separation of two continental regions was only possible when $^{18}\text{O}/^{16}\text{O}$ was included in the statistical evaluations (Ogrinc *et al.*, 2001). It promotes to note again that the integration of different methods and tools are essential for additional information in the scope of authenticity.

It is difficult to determine the vintage years of wines. Analytical measurements are established by the determination of ^{14}C content with successful findings. However, ^{14}C content of a wine can differ from one year to another due to nuclear testing that was done in 1950s and 1960s. Isotopic ratio $^2\text{H}/^1\text{H}$ ethanol is also correlated with the environmental conditions of the vintage year. Yet again, any supportive environmental data to the $^2\text{H}/^1\text{H}$ or to any fingerprinting method about soils are necessary for authentic results (Arvanitoyannis *et al.*, 1999). Alternatively, DNA-based detection systems are alternative methods for wine varietal identification as long as they are not modified through the chain (Fernandes *et al.*, 2015). DNA based techniques (PCR, capillary electrophoresis, capillary gel electrophoresis with laser-induced fluorescence) by extracting DNA fragments from must or wine for authenticity purpose are also used when the spectrometric techniques need additional information (Pereira *et al.*, 2016). Baleiras-Couto and Eiras-Dias (2006) reported that molecular methods on the basis of residual DNA successfully detected the grape cultivar DNA fragments in the wine. However, a contradictory study stated that, since the DNA can be degraded during winemaking processes and can be found in low concentration after fermentation, molecular markers based on DNA analysis cannot be a reliable tool for commercial wines (García-Beneytez *et al.*, 2002).

In addition to analytical tools, sensory analyses are carried out by experts who are able to distinguish which geographical origin the wine is made in (Elortondo *et al.*, 2007). However as Palade and Popa (2014) stated this method might be significantly uncertain which makes it controversial despite having well-trained panelists who are thoroughly qualified to perform the sensory evaluation. Because, determination of wines' origins depends on the qualification of the panel which means it is difficult to have reliable results. Uncertainty of this method may cause insufficient results and requires combination of additional techniques such as analysis of volatile compounds, amino acidic profile, phenolic composition, mineral profile and stable isotope ratios as the principal methods (Danezis *et al.*, 2016).

In order to assess the must varietal origin, molecular markers can also be used. Microsatellite-based system (microsatellite SSR) is found to have a strong correlation with leaf and must samples. The method is based on DNA extraction. In spite of the fact that DNA

extraction from vine and wine is relatively more difficult due to reasons such as maceration process, use of microorganisms during the process, nevertheless, for grape vine variety characterization, it is considered to be a very efficient method once they are independent of environmental factors (Pereira *et al.*, 2012).

Composition of wine phenolic compounds varies according to the grape variety and is affected by maceration, fermentation and ageing conditions that consequently determine the wine color and mouth feel due to especially anthocyanin compounds (Mazza, 1995; Gao *et al.*, 1997; Vázquez *et al.*, 2010). Anthocyanins form polymeric compounds that create more stable wines by the time (Ribéreau-Gayon *et al.*, 2006). They have been also found as the most indicative phenolic profile of geological origin and phenolic compounds are the biggest contributors for organoleptic characteristics of wine. The determination is carried out by high performance liquid chromatography (HPLC). Several studies showed the use of anthocyanin profiles for distinguishing wines with the ratio of acetylated and coumaroylated anthocyanins ($R_{ac/coum}$) (Von Baer *et al.*, 2008). In the course of maturation of wines in barrels or tanks, due to the presence of oxygen, red color gets degraded and yellow/brown color increases. This phenomenon is because of the shift reactions of anthocyanins by polymeric pigments and it highly contributes to the taste and flavor qualities of wine. The formation of the polymeric pigments is caused by acetaldehyde condensation and co-pigmentation as the stability of the compounds in wine are dependent upon several aspects, such as oxygen, temperature (storage conditions), pH, the density of molecules, SO₂, and acetaldehyde (Mazza, 1995).

The effect of different regions on phenolic characteristics of specific botanical origins can be also representative tool of classification of wines. In a research study, it is reported that environmental conditions from different vineyards exhibit significantly different anthocyanin profile that can be a useful tool for young red wines' authenticity (González *et al.*, 2004). Another study with the scope of categorization of wines based on the grapes variety and origin by using their phenol content emphasizes that the polyphenolic profile of a particular variety considerably reflects its genetic character. Therefore, study reports that many wine samples from several regions were differentiated by eliminating the oak ageing and technology impact on phenol content (Makris *et al.*, 2006). One case study on vinification impact, investigated six different winemaking variables and techniques, revealed that phenolic concentration increased by vinification processes such as thermo-vinification, pectolytic enzyme treatment and extended maceration. Moreover, yeast selection, carbonic maceration, skin and juice mixing practices caused inconstant results depending on the grape varieties (Sacchi *et al.*, 2005).

It is also important to highlight the impacts of vinification processes and ageing on Castelhão wines as it is our case study's variety. The study by Spranger *et al.* (2004) made it possible

to differentiate wines from the same cultivar on the basis of the winemaking technologies. The carbonic maceration wine had less color intensity than skin fermented wines, lower concentrations of phenolic compounds and increasing maceration time decreased both total and some individual anthocyanins. The volatile composition was not significantly affected by the presence of stems. It is once again confirmed that phenolic and volatile composition seemed to be a potent tool to classify the wines.

On the other hand, one opponent study about using the phenolic composition for geographical indication purpose was published. The study on Italian red wines showed that it was not possible to differentiate the wines by their phenolic composition (Gambelli and Santaroni, 2004).

3.1. Multi-elemental Markers

As illustrated on figure 1, the metal content of the wines can be influenced by factors such as the levels of these elements in soil, fertilizing practices and processing conditions (Álvarez *et al.*, 2007; Pohl, 2007). Effect of winemaking techniques on the mineral composition of wines was investigated by ICP-MS if the mineral content changes by the addition of yeast, fining products, etc. from must to wine. The study was in micro-vinification scale. Only few elements (Li, B, Mg, Ca, Rb, Cs and Pb) out of 63 were found to stay in constant concentration during the winemaking processes while the rest of the elements' concentrations varied due to fermentation and winemaking processes (Castiñeira-Gomez *et al.*, 2004).

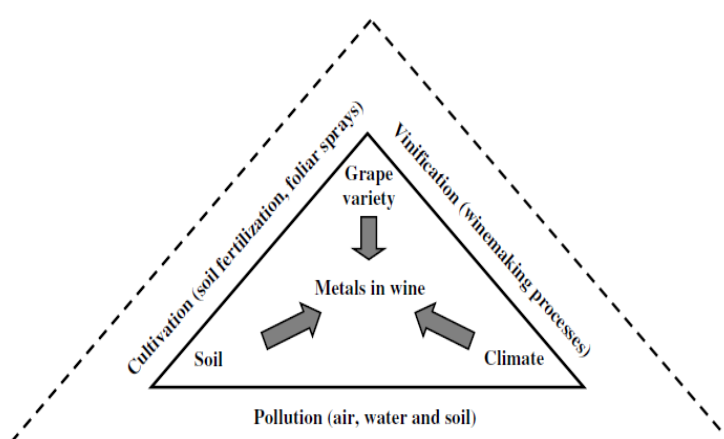


Figure 1. Metal sources in wine - endogenous (solid line) and exogenous (dotted line) (Pohl, 2007)

In another study, the effects of different winemaking techniques (addition of pectolytic enzymes, Ganimede autowinemaker, prefermentative maceration, addition of oak chips, delestage, and conventional procedure) on the metal content (Ca, Cu, Fe, Mn, Mg, K, and Zn) and phenolic composition of red wines were investigated. Fe, Mn, Ca were selected as descriptor elements that discriminated different winemaking practices. Other descriptor elements were also found significantly correlated with the phenolic composition and anthocyanin compounds (Soto Vázquez *et al.*, 2013). The team also highlighted that, depending on the winemaking process, metal concentration can be influenced due to metal-tannins combinations if there is addition of tannins to the wine. Additionally, the effect of vine variety on the metal absorption from soil was reported by another study with the purpose of assessing the origin of wines by their mineral composition. Some elements (Mg, Mn, Ca, Na, K) out of 11 elements were found to have almost identical concentration in the soil, juice, and wine. In the study Mg is suggested to be used as a marker for wine provenance (Fabani *et al.*, 2009).

According to another study by Marengo and Aceto (2003), a successful classification by using 56 metal elements in wine made it possible to identify the wines related to their geographical origin. The study suggests that elemental composition of wines could be a good tool to characterize and identify wine samples (Marengo and Aceto, 2003).

Despite the fact that above methods seem to give us information about the wine's origin, variety and authenticity, they are not strongly reliable and appropriate methods particularly for wine fingerprinting. In order to establish a reliable tool for geographical origin determination, the selected abundance ratio of the element must not be altered by the agricultural and winemaking practices through the chain and the wines must reflect the same ratios as in the soil and the rocks where the vines grow (Almeida and Vasconcelos, 2004).

As is known, the elements naturally occur in Nature as heavy and light elements with number of their isotopes. The types of mass fractionations of light elements' isotopic ratios represent information about only their history. Besides that, elements with atomic weights greater than 50, heavy elements, have large relative mass differences, have very small $\delta m/m$ ratio. Accordingly, mass fractionation and technical processes do not alter the abundance ratios. Therefore, heavy elements isotopic ratios can give information about the plant's origin. (Horn *et al.*, 1993) Heavy isotopes group, most commonly used ratio in food authentication in the literature are $^{87}\text{Sr}/^{86}\text{Sr}$ and more rarely $^{206}\text{Pb}/^{204}\text{Pb}$, $^{207}\text{Pb}/^{204}\text{Pb}$, $^{208}\text{Pb}/^{204}\text{Pb}$, $^{143}\text{Nd}/^{144}\text{Nd}$ (Danezis *et al.*, 2016).

Mineral composition of wines is the most valuable tool for identifying the wine provenance. While some mineral elements' content change during winemaking process, some elements so-called trace elements (Co, Cr, Li, etc.) undergo a change in small amount. Although they are found in very small quantities, these slightly changed elements are considered to be good tools for tracing the wines' origin and could give statistically significant results (Palade and Popa, 2014). Marengo and Aceto (2003) state in their study that Al, B, Ba, Li, Mn, Mo, Rb, Si, Sr and Ti are elements not expected to be influenced by the production process. However, they may be altered by the winemaking processes (Marengo and Aceto, 2003). The effect of vinification process on composition of Cr, Cd and Pb was studied by Kristl *et al.* (2002) on the grapes, pressed pomace, deposit of must, lees deposit, must and wine which were sampled from three different wineries. Cr, Cd and Pb were maintained in higher amounts in wine than in the must. This confirms the metal contamination from the equipment. This result also shows that these elements cannot be used as origin tracers as they may undergo a change during winemaking and change the concentration of elements in the course of contact with equipment. For instance, a study was performed in order to reveal the evolution of lead isotopic ratio, which is a trace element, along the several vintages whose grapes were harvested from the same vineyard. The concentration has been measured with high accuracy by isotope dilution mass spectrometry. Over the years, lead concentration was found to be significantly lower than it was in the past (Rosman *et al.*, 1998).

Multi-element composition of heavy and light elements is used for fingerprinting of wine. Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) and Thermal Ionization Mass Spectrometry (TIMS) methods can quantify heavy isotopes by spectroscopic methods. A large number of studies have been published with especially ICP-MS technique for characterizing the mineral composition of wines and correlating them with their geographical origin with the close soil – vine relation (Jakubowski *et al.*, 1999; Barbaste *et al.*, 2002; Almeida and Vasconcelos, 2003; Fortunato *et al.*, 2004; Kment *et al.*, 2005; Mihaljevič *et al.*, 2006; Di Paola-Naranjo *et al.*, 2011; Martin *et al.*, 2012; Zou *et al.*, 2012; Martins *et al.*, 2014; Catarino *et al.*, 2014).

Thiel *et al.* (2004) analyzed the multi-elemental composition of several wine samples and 32 elements were insignificantly influenced by climate and winemaking technology. Several methods have been developed in the wine and food sector for tracing the agricultural chain. An early study by Baxter *et al.* (1997) used multi-elemental analysis for discrimination of 112 wines by using ICP-MS method. It is stated that 48 trace elements helped successfully discriminate of English and Spanish wines by their geographical origin. ICP-MS is known to be one of the best techniques for multi-element determinations (Baxter *et al.*, 1997).

Recently, spectroscopic methods are very commonly used to identify the wine to find out adulterations of targeted and non-targeted ingredients. Methods with targeted approach are applied by specified compounds of interest. These techniques give results of different parameters in very short time by characterizing the wine in terms of quantification. On the other hand, non-targeted approach, that can be also called as food fingerprinting, permits having information about authenticity parameters such as vintage, grape variety, provenance and help determine the falsifications. However, this method requires a thorough statistical analysis such as, principal component analysis (PCA), linear discriminant analysis (LDA), and a large database with the aim of classifying the wines according to the place of origin.

Being a spectroscopic method, ICP-MS by liquid/gas chromatographic identify minimal analytical differences between compounds by means of analytical methods and again, statistical evaluation. One challenge about using non-targeted path is that the regulations are not strictly established by the authorities yet; therefore the data may differ from one end to another because of specific applications of each laboratory. To give an example study, 6 different Slovakian wines were analyzed with Atomic Absorption Spectrometry (AAS) and the data were evaluated by the statistical method Principal Component Factoring (PCF) and PCA. B, Ba, Ca, Co, Cr, Li, Mg Rb, Sr, Sn and V elements out of 36 different elements in Slovakia soils were discriminated as markers. PCF method was found to be more effective in this study than PCA for differentiation of Slovakian wines from Portuguese, Spanish, French and Italian wines (Koreňovská and Suhaj, 2005). It was even possible to discriminate red and white wines successfully from 23 different wineries in a relatively small region (1000 km²) by using multi-elemental analysis under favor of statistical methods (Coetzee *et al.*, 2014).

The statistical approach on multi-elemental analysis was assessed in terms of its power to differentiate the wines according to their relation with the soil in some other study. The data comprised of 23 elements in 103 wines. ANOVA and PCA were used to choose the most indicative elements. The study confirmed the reliability of the multi-elemental method as wine classification tool even with a large number of data emphasizing that the selection of the elements is essential and the use of lower measured concentrations of elements will give more reliable results (Serapinas *et al.*, 2008).

In two different Canadian regions (Okanagan Valley and Niagara Peninsula), multivariate analyses were carried out in order to identify the regions by their mineral compositions with large number of wine samples confirming the ICP-MS as a useful tool with good precision (Taylor *et al.*, 2003). Another similar study found 4 discriminant elements (Rb, Na, Mn and Sr) of wines from three denomination regions of Canary Islands (Frías *et al.*, 2003).

The metal content of sparkling wines also discriminated cava and champagne wines by means of multi-elemental analysis (by ICP-MS) and discriminant analysis (Jos *et al.*, 2004).

In the literature, large number of case studies, using the multivariate analysis allied to the statistical data analysis, confirms the method as a useful discrimination method of wines from different denomination of regions. To give examples, one case study with multivariate statistical analysis in Southern Italian regions (Basilicata, Calabria and Campania) enabled to discriminate 120 wines according to their provenance that were produced in two different vintages. Results showed that elemental composition was independent from the production year (Galgano *et al.*, 2008). Fino wines from two Andalusian Denominations of Origin (DO) were perfectly separated as well (Álvarez *et al.*, 2007). Same success was achieved in Valencian wines from three protected designation of origins. Discrimination was done by 38 elements including Sr as our element of interest (González *et al.*, 2009). Similar study by ICP-MS methodology was validated for correct determination the concentration of 17 elements (Al, As, B, Ba, Ca, Co, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, Sr, Zn) in Portuguese wines from regions of Alentejo, Bairrada, Dão and Vinho Verde (Rodrigues *et al.*, 2011). Catarino *et al.* (2011) analyzed the composition of fourteen rare earth elements multi-elemental characteristics of soils, grape musts and wines in three different regions (Dão, Óbidos and Palmela) of Portugal. Discriminant analysis made it possible to relate the wines to their vineyard origin by means of Li, Mn, Sr and RE elements (Catarino *et al.*, 2011).

3.2. Strontium Isotopic Ratio as Provenance Indicator

As given in IUPAC technical report, strontium element has four naturally occurring isotopes. Three of them are stable (^{84}Sr , ^{86}Sr and ^{88}Sr) with constant values which means they are non-radiogenic while ^{87}Sr is derived from radioactive β -decay of ^{87}Rb (half-life of 48.8 billion years). Oldest $^{87}\text{Sr}/^{86}\text{Sr}$ isotope abundance ratios (0.699) come from meteorites and mass fractionation is minute. Hence, $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of a rock depends on its age and content of Rb. Absolute amounts of the stable ^{86}Sr remains constant. Basaltic rocks (young rocks) have the lowest IR (0.702–0.706), granites (silicate rocks) show the highest abundance ratios (0.710–0.750). Lime stones contain high Sr content with a medium level of IR values (0.706–0.709) (Garcia *et al.*, 2007; Horn, 1993).

The $^{87}\text{Sr}/^{86}\text{Sr}$ isotope abundance variations given by IUPAC fall in a range of 82.29–82.75 % for ^{88}Sr , 6.94–7.14 % for ^{87}Sr , 9.75–9.99 % for ^{86}Sr and 0.55–0.58 % for ^{84}Sr , respectively (Rosman and Taylor, 1998; Fortunato *et al.*, 2004).

Observed range in the nature for ^{87}Sr is 0.0694–0.0714 mole fractions while ^{86}Sr is 0.0975–0.0999 which gives the ratio range from 0.695 to 0.732 as also indicated in IUPAC technical report (Berglund and Wieser, 2011).

Geological age of a rock determines how many nuclides are formed. Nevertheless, despite having constant decay rates of radiogenic elements, overlapping of $^{87}\text{Sr}/^{86}\text{Sr}$ in different regions is possible due to human factors such as fertilizer use. In this case, in spite of being a reliable fingerprint of abundance ratios of heavy and light elements, taking another element's isotope ratio into consideration would give more efficiency for provenance tracing purpose, such as use of $^{144}\text{Nd}/^{143}\text{Nd}$ (Horn *et al.*, 1998).

Bearing in mind the above qualities of naturally occurring isotopes of elements, such as strontium (Sr), are considered to be good tools for characterizing soil-vegetation relationship. Multiple-collector inductively coupled plasma mass spectrometry (MC-ICP-MS) has been used for the precise determination of variations in the isotopic composition of Sr (Capo *et al.*, 1998; Fortunato *et al.*, 2004).

Considering that the wines contain several minerals, only a few of them are affected by the soil structure and composition that allow soil - vine close relation to be used as geographical indicator (Thiel *et al.*, 2004). In the literature, most recently, trace elements and stable isotopes are used to classify wines to the geographical origins. The trace elements in grape juice are up taken by the plant from the soil. Lately, strontium is one of the most focused elements as wine fingerprinting tool. Sr element is absorbed by the roots of the plant. Strontium isotopic ratio is independent of the vine species that, reflects the value of the geological parent rock (Horn *et al.*, 1993; Gosz and Moore, 1998; Tescione *et al.*, 2015). Therefore, it is important to choose specific elements that are particularly carried from rock to the soil and pass from soil to the grape in the same isotopic ratios as in the soil (García-Ruiz *et al.*, 2007).

Recently, there have been a number of studies revealing the relation between the soil and food products representing their traceability of each *terroir* with analytical parameters Almeida and Vasconcelos (2003) urged the close relationship between soil and the wines by using multi-elemental composition of soils, grape juices, and wine samples. The method used was ICP-MS. In the study, it is indicated that vinification processes altered the elemental composition of wine because of precipitation and co-precipitation phenomenon. In addition, during the maturation of wine, there was Cd, Cr and Pb contamination from the equipment. Despite this contamination fact, strong correlations were still found in terms of their strontium isotopic ratio between wine and respective grape juices. Hence, using an

element's isotopic ratio would be more efficient for fingerprinting particularly on fine wines produced by specific grape varieties as the contamination sources might be better controlled (Almeida and Vasconcelos, 2003). It is also important to take into consideration anthropogenic human factors such as irrigation water, pollution and fertilizers that can contribute as mineral sources (Rosman *et al.*, 1998; Avram *et al.*, 2014; Petrini *et al.*, 2015). To give an example, a study focusing on atmospheric inputs of forested ecosystems in New Mexico reported that $^{87}\text{Sr}/^{86}\text{Sr}$ isotope ratio varied from 0.7090 to 0.7131 significantly between years due to the precipitation (Gosz and Moore, 1998). What is to point out here is that the use of mineral composition is only reliable for determining the wine's origin as long as they are not altered during production processes (Almeida and Vasconcelos, 2003; Suhaj and Korenovska, 2005). Winemaking involves several practices that can modify the element content significantly. Therefore, elements to classify with high discrimination power must be carefully selected (Thiel *et al.*, 2004).

Strontium isotope ratios for provenance tracing have also been used on different food and agricultural products in several studies, such as wheat, butter, cheese and ginseng, mineral water, beer and olive oil (Branch *et al.*, 2003; Rossmann *et al.*, 2003; Fortunato *et al.*, 2004; Montgomery *et al.*, 2006; Rosner, 2010; Medini *et al.*, 2015; Bong *et al.*, 2016). Another study compared the changes in $^{87}\text{Sr}/^{86}\text{Sr}$ ratio in different levels of a forest ecosystem by analyzing the soil, caterpillars, leaves, snails and eggshells. The team affirmed that strontium isotopic ratio was not modified within the food chain (Blum *et al.*, 2000).

As specified earlier, it is always necessary to have complementary information for authentication of wine by means of relation between its chemical compositions and the territory where the grapes grow (Thiel *et al.*, 2004).

In a study with cider, $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratio with together with Na, Mg, Al, K, Ca, Ti, V, Mn, Zn, As, Rb, Sr, Mo, Ba elements allowed distinguishing wines from different countries by linear discriminant analysis (García-Ruiz *et al.*, 2007).

For example, according to a research, $^{87}\text{Sr}/^{86}\text{Sr}$ values along with Mg concentrations were best discriminator minerals of wine origin. Additionally, inserting phenolic characteristics of wine provided even a more efficient discrimination between the varieties from the same region (Di Paola-Naranjo *et al.*, 2011).

Such studies about isotopic ratios of radiogenic elements of geological region interest have drawn attention for wine traceability (Horn *et al.*, 1993; Almeida and Vasconcelos, 2004). Once again, $^{87}\text{Sr}/^{86}\text{Sr}$ can be used as a fingerprint of wine origin, if a considerable correlation between the value of this parameter in the soil and in a wine is observed and if significantly

different values are observed in wines from different geological bedrocks (Almeida and Vasconcelos, 2004).

Horn's team published a study two decades ago, stating that soils of respective vineyards regions have different $^{87}\text{Sr}/^{86}\text{Sr}$ ratios. The elements, which are absorbed by the plants, maintain the same isotopic ratios as in the soil and rocks. The team used thermal ionisation mass spectrometry (TIMS) method for Sr isotopic ratio measurement. This technique gives both accurate and precise results (Horn *et al.*, 1993). However, being an inconvenient method with long sample preparation and an expensive method, this technique is less favored and unfeasible. Thanks to its fast and simpler sample preparation, low sample analysis cost, ICP-MS method has recently been used in isotope ratio determination. It has 0.01 % relative standard deviation (RSD) that is considered to be not enough for accurate results while TIMS have 0.002 % RSD. 0.01 % is the minimum precision that is needed. However, Barbaste *et al.* (2002) achieved a ratio between 0.002 – 0.003 % with ICP- sector field multi collector MS that is based on relative differences on the precision which was better than the R.S.D of 0.3 % achieved by Almeida and Vasconcelos (2001). The technique can play an important role due to its fast sample throughput, low sample analysis cost, instrument robustness and simplified sample preparation comparing to TIMS (Barbaste *et al.*, 2002; Marguí *et al.*, 2006). The lower precision obtained by ICP-MS compared to TIMS can be explained considering that the isotopes are usually measured sequentially by using strontium isotopic ratio as tracer element which relates the grapes with the substratum (García-Ruiz *et al.*, 2007). However, it is necessary to highlight that, in our work, precision of the results is more fundamental than the accuracy.

Another publication achieved correct classification of soils by means of multi elemental data. Also 100 % of the wines analyzed from the three regions in Argentina using seven elements (K, Fe, Ca, Cr, Mg, Zn and Mn) were successfully distinguished, 85 % of correlation variability was observed between the wines and respective soils in which the vines were grown (Fabani *et al.*, 2010).

Almeida and Vasconcelos (2004) state that although winemaking processes, and chemical applications in the vineyard change the element composition of must and wine, a strong correlation in terms of Sr isotopic ratio between wine and grape juice is still found. A similar study also reports that the analytical results of red and white wines did not show significant differences between oenological food chains. Sr isotopic values of red and white wines were not affected by addition of fining agents (yeast and bentonite) during winemaking process proving the close relation with the vineyard (Tescione *et al.*, 2015). One recent study by Durante *et al.* (2016) shed light on the use of $^{87}\text{Sr}/^{86}\text{Sr}$ isotope ratio as geographical tracer,

investigated the cellar practices that used different additives, such as clarification or deacidification agents as well as concentrated musts. The hypothesis was that strontium concentration could be modified; hence the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of wine may be affected by these practices. Considering constancy of Sr IR from the soil to the plant and eventually into the wine, samples from grape juice, intermediate products and wine were taken at each step. Once again, the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio was found to be a powerful tool discriminate the wines based on their region (Durante *et al.*, 2016).

In another study targeting the IR measurements for B and Sr element by ICP-MS, in four different South African regions, achieved the precision value of 0.1 RSD. While significant results to differentiate the wine regions were obtained with $^{11}\text{B}/^{10}\text{B}$, it is reported that $^{87}\text{Sr}/^{86}\text{Sr}$ values did not give a useful correlation between the wines' respective origins that might be explained due to isobaric overlapping of ^{87}Rb with ^{87}Sr (Vorster *et al.*, 2015).

A Romanian team has investigated the mineral content and isotopic ratios of $^{206}\text{Pb}/^{207}\text{Pb}$, $^{207}\text{Pb}/^{208}\text{Pb}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ of 27 white wines from four regions from three vintages. ICP-MS method was used. Some agricultural practices such as fertilization and pesticide uses can make difficult the tracing of wine origin. They quantified the concentration of three mineral groups: macro-elements (superior to 10 mg/L), micro-elements (between 0.1 – 10 mg/L) and trace elements, such as Pb, Cd, Cs, Sr, whose concentrations are below 0.1 mg/L. The results show that using winemaking and agricultural practices cause adulterations on the concentration of micro and macro elements while trace elements are quite stable from year to year (Avram *et al.*, 2014). As long as anthropogenic contamination does not arise during winemaking, strontium isotopic ratio (Sr IR) stays constant. Yet, in order to justify this, Sr IR of the vineyard soil must be determined (Almeida and Vasconcelos, 2004).

Mercurio and his team studied on volcanic vineyards to find a correlation between rare earth elements, micro-nutrients and Sr isotopic ratio for a reliable fingerprinting method. However, no element was found to be traced as an indicator of the wine provenance except for $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic composition (Mercurio *et al.*, 2014).

A correlative study by Martins *et al.* investigated the soil strontium isotopic ratio values from Portuguese appellation regions of Dão, Óbidos and Palmela. It was reported that the regions were successfully discriminated by their geographical indications with the precision ratio between 0.04 and 0.23 (Martins *et al.*, 2014).

Furthermore, $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratios of 45 bottles of wine from four different regions of Italy were analyzed by TIMS which is a reliable method with high precision and accuracy. It is demonstrated that Sr isotopic ratios in wines were in the same range as the vineyards soil. Large range variations were observed between different geographical areas even from

different vintage years, which make Sr IR a reliable mechanism for traceability (Marchionni *et al.*, 2013).

Very recent study on Cesanese wines from different vintage years focused on the only strontium composition change during winemaking process. It was found that $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratios that were determined in soil, grape juice and in wines have been found statistically identical. It confirms that Sr isotopic ratio in substrata of the vineyards are highly correlated with the wine Sr IR of the Cesanese region and are independent from winemaking process and vintage year unless contamination occurs (Marchionni *et al.*, 2016).

Two recent studies from Vinciguerra *et al.* (2016) and Geană *et al.* (2016) announced very promising results by attributing a strong relationship between $^{87}\text{Sr}/^{86}\text{Sr}$ on grape and wine and soils from different vineyards in Quebec and in Romania, respectively.

Being a reliable tool of Sr IR as a geographical indicator, study reports that musts from the different vineyards are also characterized by variable $^{87}\text{Sr}/^{86}\text{Sr}$ ratio, which remains reproducible in three different harvests. However, the variation of the $^{87}\text{Sr}/^{86}\text{Sr}$ in must, seeds and stem overlaps is explained by experimental uncertainties (Petrini *et al.*, 2015).

3.3. Wood Ageing and Oak Wood Alternatives in Enology

Traditional winemaking involves ageing in oak barrels for maturation of the wine in order to obtain favorable aroma and flavors by means of extraction of volatile compounds (and phenolic compounds, namely hydrolyzable tannins) from the wood and also for the wine's color improvement. Oak ageing has a big influence on wine's chemical and organoleptic characteristics. Oak wood composition depends on its geographical origin, oak species and the cooperage processes. Oak comes from *Quercus* genus. The species that are used for ageing of alcoholic beverages are *Q. alba* L. (American oak), *Q. sessiliflora* Salisb and *Quercus robur* (French oaks) and *Quercus pyrenaica* Willd. The first two species are mainly used for the ageing of wine while *Q. robur* is used for the ageing of Cognac wine-brandy (Garde-Cerdán and Ancín-Azpilicueta, 2006).

The most important quality of oak barrels for wine ageing is being porous to let the oxygen contact with wine and develop the wine's aroma and taste and color, affect the phenolic content and improve its stability (Mazza, 1995; Rubio-Bretón *et al.*, 2012). Porous characteristics lead to aeration that increases the color intensity of wines (Ribéreau-Gayon *et al.*, 1983).

Physical and chemical changes that occur during oak ageing can be described as degradation of anthocyanins, tannins polymerizations, copolymerization of tannins and anthocyanins, formation of new pigments, extraction of pigments from the oak (Rubio-Bretón *et al.*, 2012). The anthocyanin composition changes during wood ageing by means of formation of pyranoanthocyanins and anthocyanin derived pigments. Although grapes do not contain pyranoanthocyanins, during ageing they are produced with a reaction of anthocyanins and several compounds (hydroxycinnamic acids, pyruvic acid, and the corresponding vinylphenols). That is why it is important to take into consideration of the formation of pyranoanthocyanins while determining the anthocyanins in wine (Von Baer *et al.*, 2008).

During the ageing in oak barrels, wine composition changes also due to the extracted compounds, such as lignins, tannins, gallic acid, ellagic acid, aldehydes, aromatic carboxylic acids (Chira and Teissedre, 2013). However, ellagic tannins and volatile substances decrease by the time as barrels are continuously used.

The wine's development depends on various parameters during ageing such as oxygen, SO₂, temperature, time, its phenolic composition, total polysaccharides concentration and interactions between tannins and anthocyanins (Ribéreau-Gayon *et al.*, 2006).

The oak barrels are subjected to heating with different temperatures. The components (tannins, lignin derivatives, volatile compounds) that are transferred into the wine with thermal degradation alter wines' organoleptic characteristics. Maturation of the oak depends on the species, geographical origin, forest origin, stave variation, barrel's age and volume (Koussissi, 2009). In addition to these factors, toasting process, (toasting time and temperature) contact period play important roles on the wine's final composition. Lignins exhibit a large amount of aromatic compounds in the course of toasting treatment. Frequency of using the barrels also influences the oak wood composition (Garde-Cerdán and Ancín-Azpilicueta, 2006). Old barrels have less contribution to the wines organoleptic development than the new barrels since they release fewer compounds. Toasting is considered to be the process that has the greatest impact on chemical and polyphenolic composition of the oaks (De Simon *et al.*, 2010). Medium level of toasting is known to give the optimal combination of several compounds due to the newly formed polymers or polymers degradation (Chatonnet *et al.*, 1989).

However, being space occupant, costly, a long-lasting practice of production of oak barrels and also having the risk of microbial growth of undesired organisms (*Brettanomyces*) of reused barrels, cost-effective alternatives to oak-ageing are developed in the industry, such as, using pieces of woods so-called oak chips and oak staves (larger fragments of oak chips) that provide the desired quality in a shorter time (Campbell *et al.*, 2006). It has been found

that use of chips in wine provides better extracted wood aromas than oak barrels in shorter time (Gutiérrez Afonso, 2002). The use of oak chips and staves are regulated by International Oenological Codex (OIV, 2015)

Dimensions of oak staves play an important role on the wine flavor. For example, guaiacol was extracted in higher amounts when larger sized chips were used (Arapitsas *et al.*, 2004)

A study that was carried out to see the impacts of use of oak chips, oak staves and oak barrels on wine's composition reported that vanillin was extracted in greater quantity by oak chips than oak staves and oak barrels while barrels and staves provided similar extracted amounts in wine (Del Alamo Sanza *et al.*, 2004). Another study by De Simón *et al.* (2010) stated that although different type of oak species exhibit specific extraction behavior, still, the size of wood pieces has a greater impact on the volatile composition of the wines than the oak species.

Nevertheless, it is important to state that these alternative accelerated uses of oak woods may not provide the same results as oak barrels (Rubio-Bretón *et al.*, 2012). In addition, micro-oxygenation in stainless steel vats is used in order to mimic the oxidation that occurs in oak barrels.

3.3.1. Chemical Composition of Oak Wood

Chemical composition of oak can be categorized in two groups: extractable compounds and macromolecules. 90% of the dry oak wood consists of macromolecules which are polymers of cellulose, hemicelluloses and lignin (Rubio-Bretón *et al.*, 2012). Oak wood lignins have significant effect on barrel characteristics, regarding their contribution to the hydrophobicity and impermeability (Le Floch *et al.*, 2015). Wood composition with percentages is in table 1. In the table, cells with more than one value represents values from different references in the literature.

Table 1. Wood chemical compositions from different species (% of dry weight) (Canas and Caldeira, 2015)

Species	Cellulose	Hemicellulose	Lignin	Extractables
<i>Castanea sativa</i>	47.3	-	31.8; 45	14; 16.1; 18.4
<i>Quercus robur</i>	38.1; 41.1		26.6; 52; 29.6	12.6; 14.5
<i>Quercus alba</i>	50; 49-52		32; 31-33	6; 13.3
<i>Alnus glutinosa</i>	43,4		23,9	3,8
<i>Fagus sylvatica</i>	49,1	22	23,8	0,8
<i>Robinia pseudoacacia</i>	50,1		20,6	3 - 4
<i>Fraxinus excelsior</i>	37,9		25,6	

Cis-oak lactone and *trans*-oak lactone are the main compounds of oak wood. Guaiacol and 4-methylguaiacol are the compounds that form by the degradation of lignins while furfuryl compounds (furfural and 5- methylfurfural) also form from hemicellulose with toasting the oak. The quantity of guaiacol and 4-methylguaiacol are in very small quantities when the toasting temperature is under 230°C (Bautista-Ortín *et al.*, 2008). These compounds are transferred to the wine in the course of ageing. Oak lactones in untreated oaks are in small amounts while vanillin, smoky aromas come from coopered barrels. There are several studies confirming that American white oak species release higher quantity of *cis*-oak lactones to the wine than European oak species (Gómez-Plaza *et al.*, 2004). Cooperage by heat treatment with the aim of likening the original flavors can also be applied to oak pieces as well as to the barrels. Toasting that is also called heat treatment can be light, medium and heavy (Chatonnet *et al.*, 1989). It significantly alters the volatile concentration that consequently influences the wine flavor and its balance. Hence, it is an important decision to make to what extent the barrels, or the staves or chips must be heated. Chemical and sensory analyses assist in this respect (Campbell *et al.*, 2006). Another factor that affects the volatile extraction from the oak is the alcohol content of the wine. Higher amounts of volatile compounds are extracted in higher alcohol content (Rubio-Bretón *et al.*, 2012).

Chip size is also an important factor regarding their impact on extraction of volatiles from the wood. A study with comparison of two different sized oak chips, as 15 x 15 x 5 mm and 10 x 2 x 2 mm states that larger amounts of *cis*-oak lactones were extracted from finer chips than smaller chips during 30 days (Campbell *et al.*, 2006). Another study indicates that heating larger staves at 235°C releases two to four times greater quantity of guaiacol and 4-methylguaiacol than smaller staves for French and American oak. However, it is stated that oak chips that are significantly small in diameter (0. 1 – 0. 5 mm) lose their volatility.

3.3.2. Wood Ageing Impact on Wine Characteristics

Ageing in wood highly contributes to improve the sensory characteristics of wine by means of extracted compounds from the oak into the wine as mentioned earlier. These extracted compounds are dependent upon wine composition, oak wood composition and the time that wine is in contact with wood (Garde-Cerdán and Ancín-Azpilicueta, 2006).

Oxidation, copigmentation, polymerization and condensation phenomena occur during wine and oak contact time that ensure the stability and improve the wine's taste by reducing its astringency (Boulton, 2001).

Another study investigated the micro oxygenation and maturing with oak impacts on the chromatic characteristics of wine. The micro-oxygenated and the oak matured wines showed higher color intensity than control wine. Nevertheless, after six months in bottle, the oak matured wines showed a more stable color than micro-oxygenated wines that can be because of extracted compounds from wood (e.g. ellagitannins or wood aldehydes) (Cano-López *et al.*, 2010).

Using new or old barrels is also an important factor in wine's characteristics. A case study, which compares the contact time, size of chips and impact of new and old barrels on aroma compounds states that the highest concentration of furfural was found by the end of 3 months ageing in the wines that are aged in new barrels and those in used barrels with oak cubes (Bautista-Ortín *et al.*, 2008).

It is emphasized by many authors that the technological processes and contact of wine with vineyard equipment increase metal concentrations during maturation and ageing (Hernández *et al.*, 1996; Al Nasir *et al.*, 2001; Sauvage *et al.*, 2002; Galani-Nikolakaki *et al.*, 2002; Pohl, 2007; Catarino *et al.*, 2008a). In spite of several studies indicating that $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratio remains stable after winemaking processes, the literature lacks the information on the wood ageing influence on $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratio. The aim of this study is to uncover the impact of wood aging by oak staves on strontium composition whether they change the Sr composition or $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratio during ageing in red wines and shed some light to the metal extraction from wood to wine and suggest that $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratio could be a good fingerprinting method for wines' authenticity.

4. References

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5. Effect of Wood Ageing on Wine Mineral Composition and $^{87}\text{Sr}/^{86}\text{Sr}$ Isotopic Ratio*

5.1. Abstract

Recently, the close relationship of strontium isotopic ratios between soil and plants has been reported to be used as fingerprinting tool in wine. Researchers are focused on the impacts of anthropogenic factors and technological processes of isotopic ratios of authenticity indicator elements. In the last decade, some studies suggested that winemaking technologies do not alter the $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratios from vineyard to the wine. In this study, we investigated the evolution of strontium isotopic ratio (Sr IR) during wood ageing. The experiment was carried out with French oak staves (*Quercus sessiliflora* Salisb.) in stainless steel tanks with three industrial scale replicates. The wines samples were obtained in Palmela DO. The variance analysis was applied to samples at time-zero and to ones at the end of ageing (90 days). Multi-elemental composition of wines was also analyzed by quadrupole inductively coupled plasma mass spectrometry Q-ICP-MS and macro elements (Na, K, Ca and Fe) by flame atomic absorption spectrometry (AAS). In the end of ageing period, wines exhibited statistically identical Sr isotopic ratios compared to control wine. To our knowledge, effect of wood stage on multi-elemental composition is also a novelty. Following elements were analyzed: Li, Be, Mg, Al, Sc, Ti, V, Mn, Co, Ni, Cu, Zn, Ga, Ge, As, Rb, Sr, Y, Zr, Mo, Sb, Cs, Ba, Pr, Nd, Sm, Eu, Dy, Ho, Er, Yb, Lu, Tl, Pb by using Q-ICP-MS and Na, K, Ca, Fe concentrations were determined by flame AAS technique. Our study suggests that wood ageing does not impact the $^{87}\text{Sr}/^{86}\text{Sr}$, accordingly it will shed some light for further studies.

Key words: wine, authenticity, woodstage, strontium isotopic ratio $^{87}\text{Sr}/^{86}\text{Sr}$, elemental composition

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5.2. Introduction

Determination of wines' authenticity has been an important issue for wine producers and consumers for decades. Due to certain protected and certificated wine regions, classifications of wines are strictly regulated in EU by label regulations which producers need to declare the qualities of the wine and to give certain and correct information to the consumers. Provenance declaration also provides added value to the products as well as verifying the products' origins. Widely accepted tool for authentication determination has been the use of stable isotope ratios of C, H, N, O (Rossmann *et al.*, 2000). Besides, heavy elements such as lead and strontium gained a critical importance recently (Almeida and Vasconcelos, 2001; Mihaljevič *et al.*, 2006; Martins *et al.*, 2014).

Large number of studies indicates that mineral composition and concentration of wine can give information about their geological origin and multi-elemental analyses can determine the wine fingerprint (Taylor *et al.*, 2003). Furthermore, by means of statistical techniques, the discrimination of elements according to the products' geographical area where they are grown is obtained. However, mineral composition of wine changes with different agricultural applications, such as fungicide, pesticide and fertilization applications, winemaking practices and environmental conditions (Kment *et al.*, 2005; Pohl, 2007; Catarino *et al.*, 2008a; Catarino *et al.*, 2008b).

Therefore, in order to establish a reliable tool for geographical origin determination of wines, the selected abundance ratio of the element must not be altered by the agricultural and winemaking practices through the chain from vineyard to bottle, in other words the wines must reflect the same ratios as in the soil or in the rocks where the vines are grown (Almeida and Vasconcelos, 2003). It is also important to take into consideration anthropogenic human factors such as irrigation water, pollution and fertilizers that can contribute as mineral sources to the plants (Rosman *et al.*, 1998).

Horn *et al.* (1993) brought to our attention that different geological bedrocks have different mineral composition in the earth stratum. The mineral composition of rocks and soils differs from one region to another by representing different strontium isotopic ratios. Even though, the ratios vary slightly from one area to another, they might be significantly different from each other to be used as a discriminant factor. To achieve that, elements with high discrimination power must be selected (Barbaste *et al.*, 2002; Thiel *et al.*, 2004). Multi-element composition of heavy and light elements is used for wine fingerprint. Techniques of ICP-MS and Thermal Ionization Mass Spectrometry (TIMS) can quantify heavy isotopes by spectroscopic methods. Recently, a large number of studies has been published with especially ICP-MS technique for characterizing the mineral composition of wines and correlating them with their geographical origin (Jakubowski *et al.*, 1999; Barbaste *et al.*,

2002; Almeida and Vasconcelos, 2003; Kment *et al.*, 2005; Mihaljevič *et al.*, 2006; Di Paola-Naranjo *et al.*, 2011; Zou *et al.*, 2012; Catarino *et al.*, 2014; Martins *et al.*, 2014). There is several authenticity methods published, such as analysis of volatile compounds, amino acidic profile, phenolic composition, mineral profile and stable isotope ratios as principal methods. Among all, the most commonly used tool is multi-elemental analysis (rare earth and trace elements) for geographic origin authentication while most commonly used heavy isotopes in food authentication is $^{87}\text{Sr}/^{86}\text{Sr}$ and more rarely $^{206}\text{Pb}/^{204}\text{Pb}$, $^{207}\text{Pb}/^{204}\text{Pb}$, $^{208}\text{Pb}/^{204}\text{Pb}$, $^{143}\text{Nd}/^{144}\text{Nd}$ (Danezis *et al.*, 2016).

Being of our element of interest, strontium occurs in the Nature as four isotopes. Three of them are stable (^{84}Sr , ^{86}Sr and ^{88}Sr) with constant values which means they are non-radiogenic while ^{87}Sr is radiogenic and is derived from radioactive β -decay of ^{87}Rb and absolute amounts of the stable ^{86}Sr remains constant (Horn *et al.*, 1993). Hence, Sr IR of a rock, depending on its age and content of Rb gives particular data on different geological regions. Horn's team stated that soils of respective vineyards regions have different $^{87}\text{Sr}/^{86}\text{Sr}$ ratios. The elements, which are absorbed by the plants, maintain the same isotopic ratios as in the soil and rocks. The team used thermal ionisation mass spectrometry (TIMS) method for Sr isotopic ratio measurement. This technique gives both accurate and precise results (Horn *et al.*, 1993). However, being an inconvenient method with long sample preparation and expensive, the technique is less favored and unfeasible. Thanks to its fast and simpler sample preparation, low sample analysis cost, ICP-MS method has recently been used in isotope ratio determination.

Lately, there is number of researches who focus on the wine authenticity tools, especially on $^{87}\text{Sr}/^{86}\text{Sr}$ ratios, associating the wines with their vineyards. Many studies suggest Sr IR as one of the most reliable fingerprint tools when it comes to relating the plants to the soil (Almeida and Vasconcelos, 2004; Mercurio *et al.*, 2014; Martins *et al.*, 2014; Tescione *et al.*, 2015; Vorster *et al.*, 2015; Durante *et al.*, 2016; Geană *et al.*, 2016; Marchionni *et al.*, 2016; Vinciguerra *et al.*, 2016).

Almeida and Vasconcelos (2004) state that although winemaking processes, chemical applications in the vineyard change the elemental composition of must and wine, a strong correlation in terms of Sr isotopic ratio between wine and grape juice is still found. A similar study also reports that the analytical results of red and white wines did not show significant differences between oenological food chains (Tescione *et al.*, 2015). Sr isotopic values of red and white wines were not affected by addition of fining agents (yeast and bentonite) during winemaking process proving the close relation with the vineyard. One recent study by Durante *et al.* (2016) shed light on the use of $^{87}\text{Sr}/^{86}\text{Sr}$ isotope ratio as geographical tracer, investigated the cellar practices that used different additives, such as clarification or

deacidification agents as well as concentrated musts. The hypothesis was that strontium concentration could be modified; hence, the objective in the study was to see whether the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of wine is affected by these practices. Considering constancy of Sr IR from the soil to the plant and eventually into the wine, samples from grape juice, intermediate products and wine were taken at each step. Once again, the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio was not altered and found to be a powerful tool to discriminate the wines based on their region (Durante *et al.*, 2016).

A very recent study from Moreira *et al.* (2016) studied also the effect of nanofiltration (NF) on the isotopic ratio $^{87}\text{Sr}/^{86}\text{Sr}$ and it was confirmed that there is no significance on $^{87}\text{Sr}/^{86}\text{Sr}$ of wines.

In addition to Sr IR data, it is always necessary to have complementary information for authentication of wine by means of relation between its chemical compositions and the territory where the grapes grow (Thiel *et al.*, 2004). For instance, according to a research, $^{87}\text{Sr}/^{86}\text{Sr}$ values along with Mg concentrations were best discriminator minerals of wine origin. Additionally, another study even suggests that inserting phenolic characteristics of wine provided a more efficient discrimination between the varieties from the same region (Di Paola-Naranjo *et al.*, 2011).

A study in Australia also justifies the robustness of the strontium isotopic ratio method in order to differentiate between Australian wines' and Non-Australian wines. Nevertheless, overlap of strontium isotopic ratio from different countries was observed. This suggests that the use of only one fingerprinting method may not be sufficient (Wilkes *et al.*, 2016).

As commonly known in the literature, ageing in wood highly contributes to improve the sensory characteristics of wine by means of extracted compounds from the oak into the wine as mentioned earlier. These extracted compounds are dependent upon wine composition, oak wood composition and the time that wine is in contact with wood (Garde-Cerdán and Ancín-Azpilicueta, 2006). Oxidation, copigmentation, polymerization and condensation phenomena occur during wine and oak contact time that ensure the stability and improve the wine's taste by reducing its astringency (Boulton, 2001).

It is emphasized by many authors that the technological processes and contact of wine with vineyard equipment increase metal concentrations during maturation and ageing (Hernández *et al.*, 1996; Al Nasir *et al.*, 1999; Sauvage *et al.*, 2002; Galani-Nikolakaki *et al.*, 2002; Pohl, 2007; Catarino *et al.*, 2008). In spite of several studies indicating that $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratio

remains stable after winemaking processes, the literature lacks the information on the wood ageing influence on $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratio. The aim of this study is to uncover the impact of wood aging by oak staves on strontium composition whether they change the Sr composition or $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratio during ageing of red wines and shed some light to the metal extraction from wood to wine.

5.3. Materials and Methods

5.3.1. Wines and Wood Material

A red wine of Castelão grape variety from 2014 vintage from a winery in Península de Setúbal region of Palmela DO was aged with French oak (*Quercus sessiliflora* Salisb.) staves in steel vats with the volume of 34.000 L during 90 days. The experiment was carried out in industrial scale in the cellars of the winery. The wines did not suffer any stabilization treatment before ageing in wood.

The physical-chemical characteristics of wine before starting ageing were: alcoholic strength 13.6 %vol.; total acidity 5.40 g/L (expressed in tartaric acid); volatile acidity 0.7 g/L (expressed in acetic acid); pH 3.62; free sulphur dioxide 14 mg/L; total sulphur dioxide 45 mg/L with completed malolactic fermentation.

5.3.2. Experimental Conditions / Experimental Design

Three stainless steel vats have been added medium plus toasted (< 200 °C) oak staves in dimensions of 91 cm x 6.4 cm x 0.95 cm made from French oak (*Quercus sessiliflora* Salisb.) in February, 2016. Typical drying time of oak woods was 18-24 months. One oak piece was added per 40 L of wine with the surface to volume ratio of 33.75 cm²/L. Micro-oxygenation was applied with the amount of 3 mg/L per month. Storing temperature of tanks was close to 18°C. Sampling of the wines was performed each month. It is worth to note that stave dimensions and micro-oxygenation amount were decided by the winery according to their experiences. The experiment was carried out as one control vat without oak staves which is designated as Control and three identical vats (triplicates) with oak staves that are designated as Wood.

5.3.2.1. General Physical/Chemical Analysis

Physical and chemical analysis and determinations are carried out by using OIV analytical methods (OIV, 2015). Alcoholic strength by distillation and determination by hydrometry, free and total SO₂ by titration, volatile acidity by distillation followed by acid-base titrimetry, density by aerometry, total dry extract by calculation, pH value by potentiometry, total acidity by acid-base titrimetry, malolactic fermentation verification by paper ascending chromatography, reducing substances, chromatic characteristics; total anthocyanins and total phenolics index by spectrophotometry and ash determination. Total polysaccharides content in wine is determined by an adopted method which was established by Segarra *et al.* (1995). In this method, settling the wine samples that are diluted with ethanol (96% vol.) about 12 - 14 hours in order to eliminate the phenolic and other compounds, and then washing the precipitation were important steps for the determination. All the analyses except for total polysaccharides are followed in duplicates. As the total polysaccharide determination protocol is relatively low precise and robust, it is performed in triplicate.

5.3.2.2. Phenolic Composition and Chromatic Characteristics

The chromatic characteristics of wines (tonality and color intensity) and total phenolic index were measured in the framework of OIV standards (OIV, 2015). Total anthocyanins concentrations were analyzed by the method that was established by Somers and Evans (1977), based on their responses to pH change and to sulphur dioxide addition. The determination of color intensity, tonality and total phenolic index is carried out by measuring absorbances in different wavelengths with spectrophotometer as follows.

$$\text{Color intensity} = A_{420} + A_{520} + A_{620}$$

$$\text{Tonality} = A_{420} / A_{520}$$

$$\text{Total Phenolic Index (TPI)} = A_{280} \times 100$$

5.3.2.3. Mineral Composition

- ***Major mineral elements***

The wine samples were analyzed in terms of their Na, K, Ca and Fe content by flame atomic absorption spectrophotometry methods described by OIV (OIV, 2015). The equipment Perkin Elmer, Analyst 100, equipped with an air-acetylene burner and appropriate hollow cathode lamps, was used.

- ***Multi-elemental Analysis***

Multi-elemental analysis by ICP-MS, the following elements were determined by quadrupole inductively coupled plasma mass spectrometry (Q-ICP-MS) semi-quantitative methodology that was described in the study of Catarino *et al.* (2006) : Li, Be, Mg, Al, Sc, Ti, V, Mn, Co, Ni, Cu, Zn, Ga, As, Rb, Sr, Y, Sb, Cs, Ba, Pr, Nd, Eu, Dy, Ho, Er, Yb, Lu, Tl, Pb as given in table 9. Some trace elements are neglected due to high relative standard deviation values. Perkin-Elmer SCIEX Elan 9000 utilized with Gilson pump and a Scott-type spray chamber, a crossflow nebulizer and nickel cones.

In order to optimize operational conditions, monoelement standard solutions of Be, Co and in 1000 mg/L (Merck) and a multi-element solution with Mg, Cu, Rh, Cd, In, Ba, Ce, Pb and U 10 µg/L (Perkin-Elmer) were used. Wash, blank and standard solutions were prepared with ultrapure concentrated HNO₃ Ultrex II 70% (v/v) (J.T. Baker). Analytical calibration was established with a standard solution with 30 elements, 10 mg/L (Perkin-Elmer). Standard solutions of Rh and Re 1000 mg/L (Merck) were used for internal standardization (Catarino *et al.*, 2006).

5.3.2.4. Strontium Isotopic Ratio Analysis

- ***Sample Preparation***

Prior to ⁸⁷Sr/⁸⁶Sr determination by Q-ICP-MS, following three main analytical steps in sequence are performed as described by Martins *et al.* (2014): Sample digestion by High Pressure Microwave Digestion (HPMW) (1), chromatographic separation of ⁸⁷Sr and ⁸⁷Rb (2) and determination of Sr and Rb content by Q-ICP-MS (3).

(1) For elimination of organic substances in order to prevent any interference during chromatographic separation the wine was digested by High Pressure Microwave Digestion (HPMW, Milestone ETHOS Plus Microwave Labstation) (Catarino *et al.*, 2010).

(2) Separation of Sr from Rb is an essential step for correct determination due to isobaric overlapping ^{87}Rb and ^{87}Sr . An HIPEX Duran column was filled up by using Dowex 50W-X8/400 (Sigma-Aldrich) mesh resin and EDTA as eluent. Separation consists of four phases which are resin activation/pre-treatment; resin conditioning; sample preparation/dilution and elution (Martins *et al.*, 2014). Determination is done in duplicates.

(3) Sr and Rb total content and $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratio were measured by a Perkin Elmer Sciex Elan 9000 ICP-MS. After first two steps, Sr containing fraction was used for Q-ICP-MS analysis. The device is calibrated with Sr isotopic standard to have to most precise measurement. Determination of Sr and Rb total contents in Sr-containing fractions previously to the isotopic measurement is important in order to keep Rb concentration less than 1% of the Sr content in Sr-fraction. The SRM 987 (SrCO_3) from National Institute of Standards and Technology (NIST) are used as an isotopic reference material for correction of mass bias phenomenon (Martins *et al.*, 2014). Analytical steps for Sr and Rb isotopic ratio determination is summarized in figure 2.

- ***Isotopic Ratio Determination***

After separation of Rb and Sr by ion-exchange chromatography, analytical determinations of Rb and Sr concentration and $^{87}\text{Sr}/^{86}\text{Sr}$ were carried out by ICP-MS using Perkin-Elmer SCIEX 9000 ICP-MS (Norwalk, CT, USA) equipment. In figure 2, it is illustrated. Detailed analytical protocol is in the study of Martins *et al.* (2014).

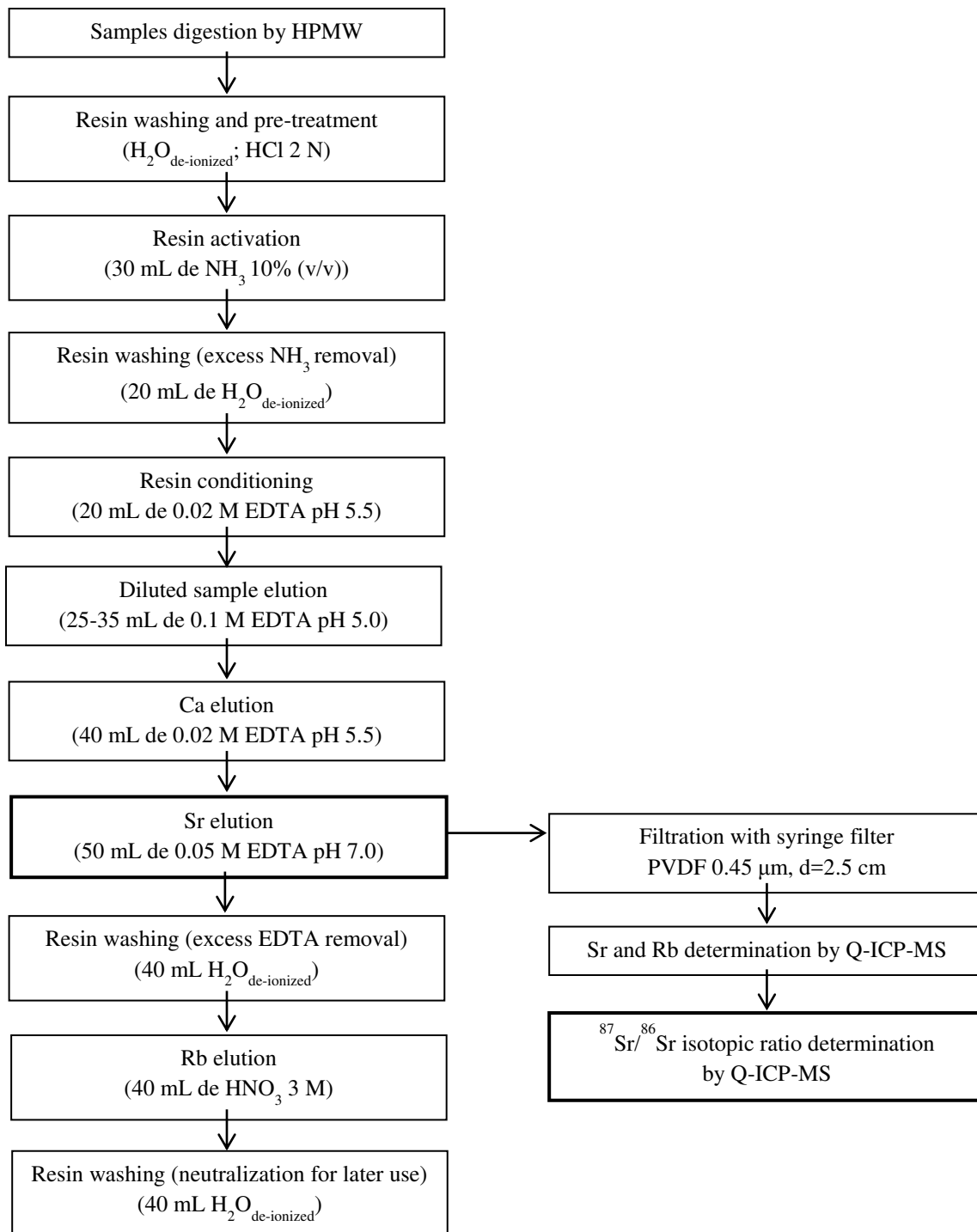


Figure 2. Analytical procedure of strontium isotopic ratio determination (Moreira *et al.*, 2016)

5.4. Statistical Analysis

The two-way analysis of variance (ANOVA) was performed to evaluate the effects of wood ageing and ageing time on the studied wines. Significant differences are compared with posthoc LSD (Tukey's test) by using Statistica 7 software (StatSoft, Inc. 2004). *p*-values that are lower than 0.05 were considered to be significant ($p < 0.05$).

5.5. Results and Discussion

5.5.1. General Physical and Chemical Analysis

Results of the physical-chemical analyses of the wines are listed in table 2. Control value is a mean based on analytical duplicates while Wood represents three experimental replicates and respective analytical duplicates. The values from each month sampling are presented and they are in the range of usual range of variation for each parameter. Castelão wines exhibit the following physical and chemical characteristics of the wine at time –zero: 13.6 % vol. of alcoholic strength, 0.9921 g/mL of density, 3.62 of pH, 3.2 g/L of ash, 45 mg/L SO₂, 14 mg/L of free SO₂, 0.7 g/L volatile acidity, 5.4 g/L total acidity, 30 g/L of total dry matter and 2.4 g/L of reducing substances. Results are expressed as average values. As seen in table 2, there is no significant effect of ageing with wood and time on major part of parameters. Exceptionally, total SO₂ and free SO₂ concentrations have significantly changed that might be explained by SO₂ corrections. Statistical significance was found also on reducing substances. However, it is considered to be a slight increase in terms of wine parameters.

Table 2. Effect of wood stage and time on the physical chemical characteristics of wine.

	Wood Stage			Time			Time*Wood Interaction
	Wood Effect	Control	Wood	Time Effect	T-0	T-90	
Density (20°C) (g/mL)	<i>n.s.</i>	0.9919 (0.0002)	0.9918 (0.0001)	<i>n.s.</i>	0.9919 (0.0002)	0.9919 (0.0002)	<i>n.s.</i>
Alcohol (% vol.)	<i>n.s.</i>	13.5 (0.1)	13.5 (0.1)	<i>n.s.</i>	13.5 (0.1)	13.5 (0.0)	<i>n.s.</i>
pH	<i>n.s.</i>	3.61 (0.01)	3.61 (0.01)	<i>n.s.</i>	3.62 (0.00)	3.60 (0.01)	<i>n.s.</i>
Ash (g/L)	<i>n.s.</i>	3.24 (0.01)	3.11 (0.25)	<i>n.s.</i>	3.31 (0.13)	2.98 (0.16)	<i>n.s.</i>
Total SO₂ (mg/L)	<i>n.s.</i>	55.8 (12.5)	56 (7)	***	48 (5) a	63 (3) b	*
Free SO₂ (mg/L)	<i>n.s.</i>	17 (3)	17 (2)	***	15 (2) a	18 (2) b	<i>n.s.</i>
Volatile Acidity (g/L)	<i>n.s.</i>	0.7 (0.0)	0.7 (0.0)	<i>n.s.</i>	0.7 (0.0)	0.7 (0.0)	<i>n.s.</i>
Total Acidity (g/L)	<i>n.s.</i>	5.1 (0.2)	5.3 (0.2)	<i>n.s.</i>	5.3 (0.2)	5.1 (0.2)	<i>n.s.</i>
Total Dry Matter (g/L)	<i>n.s.</i>	28.6 (0.2)	28.8 (0.6)	<i>n.s.</i>	29.1 (0.6)	28.4 (0.0)	<i>n.s.</i>
Reducing Substances (g/L)	<i>n.s.</i>	2.6 (0.3)	2.6 (0.3)	*	2.3 (0.0)	2.9 (0.1)	<i>n.s.</i>

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-90: 90 days.

Regarding the wood effect, Control and Wood results correspond to mean values of the corresponding levels of time (T-0 and T-90).

Concerning time effect, for each level (T-0 and T-90), the results are based on average values of Control and Wood.

Relative standard deviations are presented in parenthesis. Mean values followed by the same letter are not significantly different at 0.05* or 0.001*** level of significance; *n.s.* without significant difference

5.5.2. Total Polysaccharides

Total polysaccharides concentrations at the initial state and after 90 days are given in table 3. Wood ageing did not have a significant impact on total polysaccharides. The wood is known to have high amount of cellulose, hemicellulose. Hence, it might be possible to have an increase due to polysaccharides release from the wood. However, as seen in table 3, wood does not play a significant impact. It is also important to note that the method that was applied for total polysaccharides determination is relatively low precise and robust.

On the other hand, there is a significant increase of total polysaccharides over the time. This increase might be also due to the addition of any enological product that has polysaccharides content even though to our knowledge there was no addition of any additive. Evolution of total polysaccharides during ageing is given in table 11 in annex.

Table 3. Effect of wood stage and time on Total polysaccharides concentrations of wine (mg/L)

	Wood Stage			Time Effect	Time		Time*Wood Interaction
	Wood Effect	Control	Wood		T-0	T-90	
Total polysaccharides	<i>n.s.</i>	437 (83)	416 (77)	***	350 (26) a	493 (28) b	<i>n.s.</i>

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-90: 90 days.

Regarding the wood effect, Control and Wood results correspond to mean values of the corresponding levels of time (T-0 and T-90).

Concerning time effect, for each level (T-0 and T-90), the results are based on average values of Control and Wood.

Relative standard deviations are presented in parenthesis. Mean values followed by the same letter are not significantly different at 0.05* or 0.001*** level of significance; *n.s.* without significant difference

5.5.3. Phenolic Composition and Chromatic Characteristics

5.5.3.1. Total Phenolics Index (TPI)

In terms of total phenolics index, the wines show relatively higher values (54.3 at initial time, table 12, annex) than the wines studied by Jordão *et al.* (2015) from same region Palmela on Castelão wines (TPI of 19). On the other hand Spranger *et al.* (2004) reported a study about the effect of with four different winemaking techniques on the same grape variety. TPI values of the samples of our experiment are in accordance with study of Spranger *et al.* (2004). Yet, the slight difference is probably due to different vintage year and winemaking

techniques. They still fall within the medium range as usual TPI values for red wines (Ribéreau-Gayon, 2006). As it is seen in table 4, there is a significant increase by the wood effect, from 52.9 to 54.9. This might be most probably explained by the phenolic compounds that are released from wood to wine that are ellagic tannins as the major phenolic compounds in oak. Our results also justify the findings of Cruz *et al.* (2012) as they had found out that total phenolic index significantly increased after wood ageing of wine brandies. The evolution that occurred during three months can be seen in table 12 in annex.

Table 4. Effect of wood stage and time on the -Total Phenolic Index (TPI)

	Wood Stage			Time			Time*Wood Interaction
	Wood Effect	Control	Wood	Time Effect	T-0	T-90	
TPI	*	52.9 (2.0) a	54.9 (1.9) b	<i>n.s.</i>	53.7 (1.4)	55.1 (2.4)	***

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-90: 90 days.

Regarding the wood effect, Control and Wood results correspond to mean values of the corresponding levels of time (T-0 and T-90).

Concerning time effect, for each level (T-0 and T-90), the results are based on average values of Control and Wood.

Relative standard deviations are presented in parenthesis. Mean values followed by the same letter are not significantly different at 0.05* or 0.001*** level of significance; *n.s.* without significant difference

5.5.3.2. Total Anthocyanins

The average anthocyanins concentration values are given in table 5. The values are similar to those observed by Spranger *et al.* (2004) in wines from the same grape variety. When compared to other grape varieties, according to the literature, Castelão wines seem to have relatively lower anthocyanin levels as they can go from 200 to 2000 mg/L. As it is seen, there is a significant increase on the concentrations in the end of three months. However, use of wood did not have a significant impact on anthocyanin levels.

Table 5. Effect of wood stage and time on Total anthocyanins concentrations (mg/L) of wines

	Wood ageing			Time			
	Wood Effect	Control	Wood	Time Effect	T-0	T-90	Time*Wood Interaction
Total Anthocyanins	<i>n.s.</i>	320 (32)	303 (36)	***	278 (23) a	337 (12) b	<i>n.s.</i>

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-90: 90 days.

Regarding the wood effect, Control and Wood results correspond to mean values of the corresponding levels of time (T-0 and T-90).

Concerning time effect, for each level (T-0 and T-90), the results are based on average values of Control and Wood.

Relative standard deviations are presented in parenthesis. Mean values followed by the same letter are not significantly different at 0.05* or 0.001*** level of significance; *n.s.* without significant difference

Generally, total anthocyanins concentration is expected to decrease by ageing due to degradation reactions. However, in our study total anthocyanins seem to increase in concentration from 278 to 337 mg/L in the wine without oak staves after three months. There is a slight decrease in the end of 30 days and then they reach to a higher concentration after two months as seen in table 12 in annex. In our study total anthocyanin concentrations exhibit an increasing trend as in the study of Tavares (2015) that was carried out with acacia and cherry wood chips. This might be due to the complex composition of wine and some reactions occurred by precursors even without wood contact. Figure 3 shows the evolution of our wine during 90 days in terms of anthocyanins content.

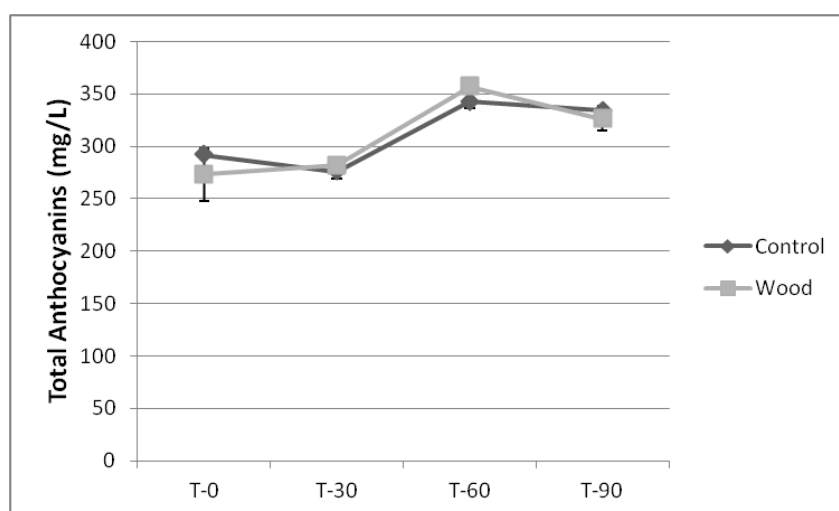


Figure 3. Evolution of total anthocyanins concentration during wood ageing

Jarauta *et al.* (2005) stated that there are several phenomena regarding the concentration of compounds changes in during wine ageing. For example compounds such as aliphatic lactones are wood-extractable lactones that are released into the wine and also compounds highly extracted from wood surface (such as fatty acids) in the end of three month ageing. In our study, most probably, we have extracted the compounds only from the wood surface considering short ageing time. Yet, keeping in mind the extraction process with oak staves is faster than oak barrels will help to interpretate these results. Nevertheless, another study where six different winemaking techniques were evaluated, including wood chips, revealed that anthocyanins concentration was not significantly altered with wood contact (Vázquez *et al.*, 2010).

Color Intensity

Mean values of color intensity with corresponding standard deviation values are given in table 6.

Table 6. Effect of wood stage and time on color intensity of wine

	Wood Stage			Time			Time*Wood Interaction
	Wood Effect	Control	Wood	Time Effect	T-0	T-90	
Color Intensity	<i>n.s.</i>	10.5 (0.6)	10.7 (0.5)	***	11.1 (0.1) b	10.2 (0.3) a	<i>n.s.</i>

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-90: 90 days.

Regarding the wood effect, Control and Wood results correspond to mean values of the corresponding levels of time (T-0 and T-90).

Concerning time effect, for each level (T-0 and T-90), the results are based on average values of Control and Wood.

Relative standard deviations are presented in parenthesis. Mean values followed by the same letter are not significantly different at 0.05* or 0.001*** level of significance; *n.s.* without significant difference

It is known that phenolic content has the highest contribution to color intensity. Interactions that occur during wine ageing such as condensation and polymerization of tannins, promoted by aeration play an important role on the color. In the course of wood ageing, despite the decrease in total anthocyanins concentration, due to breakdown phenomena and precipitation of free anthocyanins, polymeric pigments are formed resulting in the increase in color intensity and stabilizing it. Thanks to presence of oxygen, oxidation of ethanol forms

traces of ethanal during ageing and stimulates an increase in color (Ribéreau-Gayon *et al.*, 2006)

In our experiment, there is a significant decrease in color intensity. This might be due to precipitation of colloidal colorant matter. Therefore, loss of those molecules leads to decrease in color intensity. Also, it might be due to short ageing time that was not sufficient to create condensation reactions with tannins that eventually induce an increase of color intensity. The statistical data show that oak staves addition did not affect the color. It is important to remember that condensation reactions are slow and the experiment ended after 90 days. It is possible that also anthocyanins breakdown reactions occurred and led to color loss.

Another reason could be because of insufficient micro-oxygenation that was not enough for condensation formation as copolymerization of anthocyanins and tannins occur in the presence of ethanol and oxygen (Ribéreau-Gayon *et al.*, 1983).

Color intensity values of Castelão variety in Évora region from another study by Cabrita *et al.* (2003) present relatively lower values than ours in the years of 1997, 1998, 1999 that are 5.54, 5.01, and 6.05, respectively. This difference might be due to *terroir* and vintage year difference and also different winemaking practices.

Tonality

The absorbencies at different wavelengths represent different colors. Absorbance at 520 nm represents the red color that mainly represents free anthocyanins while 420 nm is responsible for yellow.

Table 7. Effect of wood ageing and time on tonality

	Wood Stage			Time			Time*Wood Interaction
	Wood Effect	Control	Wood	Time Effect	T-0	T-90	
Tonality	<i>n.s.</i>	0.771 (0.012)	0.779 (0.022)	***	0.760 (0.002) a	0.795 (0.013) b	*

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-90: 90 days.

Regarding the wood effect, Control and Wood results correspond to mean values of the corresponding levels of time (T-0 and T-90). Concerning time effect, for each level (T-0 and T-90), the results are based on average values of Control and Wood.

Relative standard deviations are presented in parenthesis. Mean values followed by the same letter are not significantly different at 0.05* or 0.001*** level of significance; *n.s.* without significant difference

As seen in table 7, time had significant impact on tonality causing a little bit degradation in red color as expected. This increase in tonality might be due to the anthocyanin and tannin breakdown reactions and wine's tendency to yellowish color. For a desired color in wine, the molar ratio between tannins and anthocyanins (T/A) is crucial and necessary to be superior to 2. Therefore, as the wine may be considered not very rich in tannins and also relatively low anthocyanins (varying from 273 to 347 mg/L), the red color did not improve as desired. These reactions depend on the phenolic content and structure, tannin origin.

5.5.4. Mineral Composition

Mineral composition of wines is of great interest due to its potential effect on physical chemical stability, sensory characteristics, wine food safety and legal limits as well as help discriminating the wines as their composition change from rock to soil to the vines. There are several factors that can alter the multi-elemental composition of grapes and wines such as winemaking practices, agricultural practices and climatic conditions (Catarino *et al.*, 2008a). The concentrations with two-way ANOVA results are given below in table 8 and 9.

5.5.4.1. Major and Micro Elements

Concentrations of Na, K, Ca and Fe were measured by flame atomic absorption spectrometry. As the concentrations of these elements are relatively higher in wine, the measurement is easily carried out by atomic absorption. The results can be seen in table 8 in mg/L. Control values (designated as control) represent the mean values of duplicates of analysis. Wines of analysis with wood staves (designated as wood) represent three experimental replicates and two analysis replicates. As seen in table 8, Na, K, Ca, Fe showed a significant decrease in concentration by the time. Having 56 mg/L of Ca in our control wine (table 15, annex), which is under the empiric limit of 80 mg/L regarding the susceptibility to calcium tartrate precipitation. However, significant decrease effect by wood ageing in Na, K, Ca and Fe might be explained by precipitation of these metal ions. Although the study is carried out with red wine, Fe concentration is important for the stability of the wines. Especially during wine ageing, it plays role in oxidative reactions. Iron forms soluble compounds with organic acids in wine. However, excessive amounts of iron can cause turbidity that is called ferric casse. Concentration above 5 - 10 mg/L can cause undesired sensory characteristics in wine (Ribéreau-Gayon, 2006). However, our wine is under this limit, by having 2.42 mg/L (table 15 in annex) in control wine after three months that has

decreased 16 %, has no risk of iron casse or toxic effect, as at usual concentrations there is no toxic risk in wines.

Moreover, another enological interest of major elements in wine is content of K and Ca as they form tartaric salts and lead to precipitation. It is seen in table 8 that K concentration has slightly but statistically decreased in the end of ageing period. Nevertheless, wood stage did not play a significant role even though it shows a slight increase. Values can be found in annex from each sampling month in table 15 in annex. It is known that potassium mainly comes from soils, fertilizers and anthropogenic resources. And $K_2S_2O_5$ (potassium metabisulphite) can be also used to inhibit bacterial growth and for its antioxidant characteristic (Avram *et al.*, 2014).

Table 8. Effect of wood stage and time on macro elements composition (mg/L)

	Wood ageing			Time			Time*Wood Interaction
	Wood Effect	Control	Wood	Time Effect	T-0	T-90	
Na	<i>n.s.</i>	29.5 (1)	30.4 (0.2)	***	30.4 (0.3) b	29.9 (1) a	***
K	<i>n.s.</i>	1064 (97)	1102 (37)	***	1135 (11) b	1050 (50) a	*
Ca	<i>n.s.</i>	56 (1)	54 (1)	***	56 (2) b	53 (1) a	<i>n.s.</i>
Fe	<i>n.s.</i>	2.7 (0.4)	2.9 (0.3)	***	3.1 (0.1) b	2.6 (0.2) a	<i>n.s.</i>

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-90: 90 days.

Regarding the wood effect, Control and Wood results correspond to mean values of the corresponding levels of time (T-0 and T-90). Concerning time effect, for each level (T-0 and T-90), the results are based on average values of Control and Wood.

Relative standard deviations are presented in parenthesis. Mean values followed by the same letter are not significantly different at 0.05* or 0.001*** level of significance; *n.s.* without significant difference

Figures 4, 5, 6 and 7 are given below concerning the evolution through the time of Na, K, Ca and Fe elements, respectively.

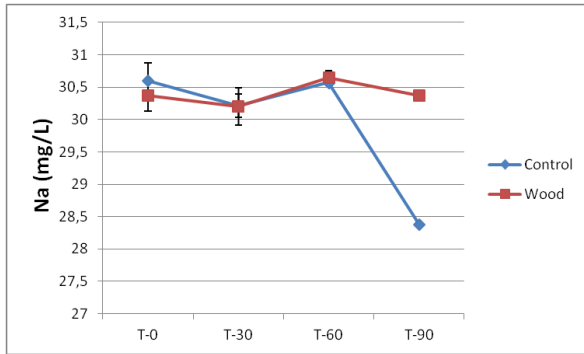


Figure 4. Evolution of Na (mg/L) in wines over time

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-30: 30 days, T-60: 60 days, T-90: 90 days. Results are expressed in mg/L. Regarding Control wines, results correspond to mean values of the corresponding analytical duplicates (n=2); Concerning Wood wines results mean values (and corresponding standard deviation) of three experimental replicates and corresponding analytical duplicates (n=6).

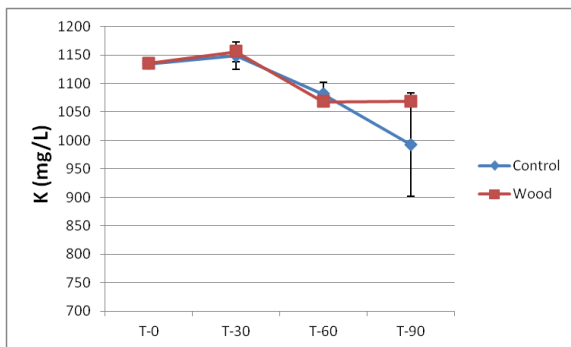


Figure 5. Evolution of K (mg/L) in wines over time

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-30: 30 days, T-60: 60 days, T-90: 90 days. Results are expressed in mg/L. Regarding Control wines, results correspond to mean values of the corresponding analytical duplicates (n=2); Concerning Wood wines results mean values (and corresponding standard deviation) of three experimental replicates and corresponding analytical duplicates (n=6).

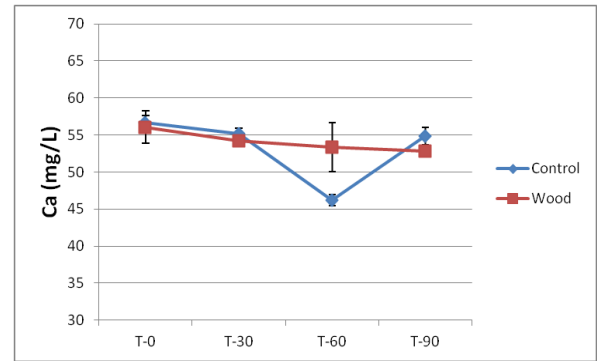


Figure 6. Evolution of Ca (mg/L) in wines over time

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-30: 30 days, T-60: 60 days, T-90: 90 days. Results are expressed in mg/L. Regarding Control wines, results correspond to mean values of the corresponding analytical duplicates (n=2); Concerning Wood wines results mean values (and corresponding standard deviation) of three experimental replicates and corresponding analytical duplicates (n=6).

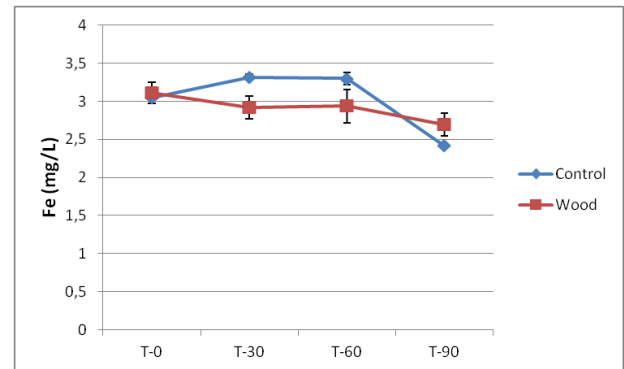


Figure 7. Evolution of Fe (mg/L) in wines over time

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-30: 30 days, T-60: 60 days, T-90: 90 days. Results are expressed in mg/L. Regarding Control wines, results correspond to mean values of the corresponding analytical duplicates (n=2); Concerning Wood wines results mean values (and corresponding standard deviation in parenthesis) of three experimental replicates and corresponding analytical duplicates (n=6).

5.5.4.2. Multi-elements

As can be seen in table 9, studied wines did not show significant differences for the following elements after wood ageing period for neither time nor wood factor: Li, Be, Sc, Ti, Mn, Cu, Ga, As, Rb, Y, Sb, Ba, Pr, Nd, Eu, Dy, Ho, Er, Yb, Lu and Tl. Time had a significant impact on the concentrations of Al, V, Co, Ni, Zn, Cs and Pb, also can be seen in figure 8, 9, 10, 11, 12, 13. All the elements that time has significant impact show a decrease in their content except for Zn. This incidence is accordance with precipitation phenomena of metals over the time. Regarding ageing impact on the mineral content, we can see from table 9 that Mg, V, Co, Ni and Sr are the only elements that wood ageing significantly affected and suffered an increase. Sr concentration is altered by wood from 249 to 258 µg/L. As Sr is an important element regarding wine fingerprinting concerning its $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratio, increase in its concentrations might cause also a change in this ratio. It is substantial to observe if alterations on the concentration cause changes in Sr abundance ratios in order to contribute to authenticity verification applications.

Table 9. Effect of wood stage and time on multi-elemental composition

	Wood Ageing			Time			Time*Wood Interaction
	Wood Effect	Control	Wood	Time Effect	T-0	T-90	
Li	<i>n.s.</i>	11300 (162)	11756 (640)	<i>n.s.</i>	11451 (318)	11790 (720)	<i>n.s.</i>
Be	<i>n.s.</i>	102 (47)	110 (36)	<i>n.s.</i>	91 (26)	126 (39)	<i>n.s.</i>
Mg	*	55943 (1432) a	58186 (2339) b	<i>n.s.</i>	56448 (1151)	58810 (2664)	<i>n.s.</i>
Al	<i>n.s.</i>	180 (12)	184 (7)	***	188 (4) b	178 (8) a	<i>n.s.</i>
Sc	<i>n.s.</i>	671 (169)	915 (121)	<i>n.s.</i>	858 (212)	860 (53)	<i>n.s.</i>
Ti	<i>n.s.</i>	1253 (84)	1346 (104)	<i>n.s.</i>	1313 (122)	1333 (93)	<i>n.s.</i>
V	***	672 (277) a	961 (56) b	***	938 (90) b	839 (245) a	***
Mn	<i>n.s.</i>	770 (22)	806 (47)	<i>n.s.</i>	777 (24)	812 (52)	<i>n.s.</i>
Co	***	2392 (534) a	2833 (110) b	***	2866 (92) b	2580 (412) a	***
Ni	***	33691 (24380) a	53440 (2440) b	***	55094 (708) b	41911 (18205) a	***
Cu	<i>n.s.</i>	21673 (639)	24365 (6241)	<i>n.s.</i>	22018 (7083)	25367 (2814)	<i>n.s.</i>
Zn	<i>n.s.</i>	323 (37)	330 (21)	***	344 (20) b	313 (19) a	<i>n.s.</i>
Ga	<i>n.s.</i>	2770 (171)	2865 (137)	<i>n.s.</i>	2903 (88)	2779 (172)	<i>n.s.</i>
As	<i>n.s.</i>	976 (98)	1058 (71)	<i>n.s.</i>	1060 (48)	1016 (107)	<i>n.s.</i>
Rb	<i>n.s.</i>	1174 (41)	1220 (47)	<i>n.s.</i>	1187 (32)	1231 (54)	<i>n.s.</i>
Sr	*	240 (7) a	258 (12) b	<i>n.s.</i>	249 (6)	258 (17)	<i>n.s.</i>
Y	<i>n.s.</i>	401 (48)	418 (41)	<i>n.s.</i>	406 (33)	423 (51)	<i>n.s.</i>
Sb	<i>n.s.</i>	76 (7)	67 (6)	<i>n.s.</i>	67 (9)	72 (13)	<i>n.s.</i>
Cs	<i>n.s.</i>	4941 (443)	5202 (208)	***	5338 (117) b	4935 (273) a	*
Ba	<i>n.s.</i>	976 (98)	1058 (71)	<i>n.s.</i>	1060 (48)	1016 (107)	<i>n.s.</i>
Pr	<i>n.s.</i>	50 (12)	45 (8)	<i>n.s.</i>	43 (11)	49 (6)	<i>n.s.</i>
Nd	<i>n.s.</i>	123 (11)	181 (78)	<i>n.s.</i>	189 (89)	135 (18)	<i>n.s.</i>
Eu	<i>n.s.</i>	109 (20)	111 (18)	<i>n.s.</i>	106 (19)	115 (16)	<i>n.s.</i>
Dy	<i>n.s.</i>	32 (17)	43 (8)	<i>n.s.</i>	36 (11)	47 (9)	<i>n.s.</i>
Ho	<i>n.s.</i>	13 (5)	13 (3)	<i>n.s.</i>	15 (3)	11 (4)	<i>n.s.</i>
Er	<i>n.s.</i>	30 (13)	44 (10)	<i>n.s.</i>	42 (6)	38 (16)	<i>n.s.</i>
Yb	<i>n.s.</i>	37 (13)	44 (12)	<i>n.s.</i>	47 (12)	38 (12)	<i>n.s.</i>
Lu	<i>n.s.</i>	15 (5)	13 (3)	<i>n.s.</i>	13 (3)	13 (3)	<i>n.s.</i>
Tl	<i>n.s.</i>	310 (21)	324 (15)	<i>n.s.</i>	322 (13)	320 (21)	<i>n.s.</i>
Pb	<i>n.s.</i>	4912 (1292)	6567 (2678)	***	7956 (2353) b	4351 (449) a	<i>n.s.</i>

Results are expressed in µg/L for Mg, Al, Mn, Zn, Rb, Sr, and Ba. The rest of the elements are in ng/L.

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-90: 90 days.

Regarding the wood effect, Control and Wood results correspond to mean values of the corresponding levels of time (T-0 and T-90).

Concerning time effect, for each level (T-0 and T-90), the results are based on average values of Control and Wood.

Relative standard deviations are presented in parenthesis. Mean values followed by the same letter are not significantly different at 0.05* or 0.001*** level of significance; *n.s.* without significant difference

Mean values followed by the same letter are not significantly different at 0.05* or 0.001*** level of significance; *n.s.* without significant difference

Two-way ANOVA is applied only to T-0 and T-90 values.

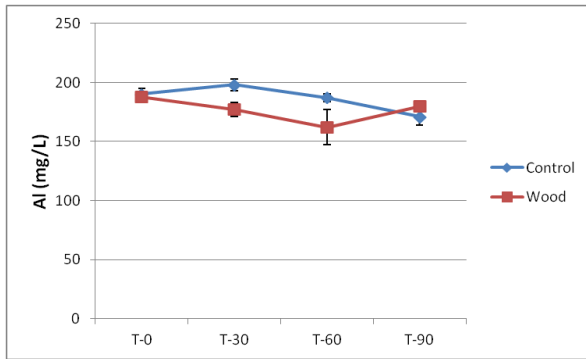


Figure 8. Evolution of Al (mg/L) in Control and Wood over time

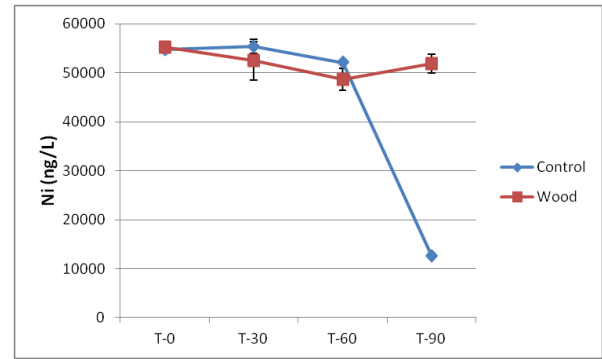


Figure 11. Evolution of Ni (ng/L) in Control and Wood over time

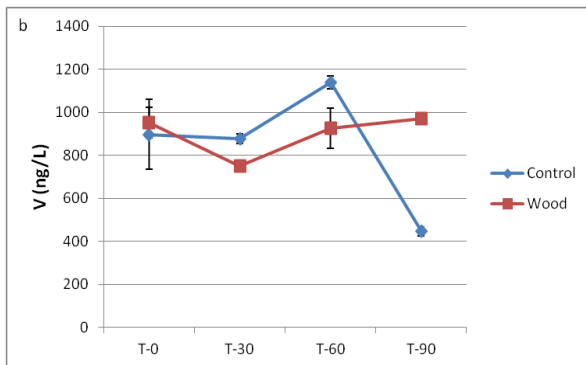


Figure 9. Evolution of V (ng/L) in Control and Wood over time

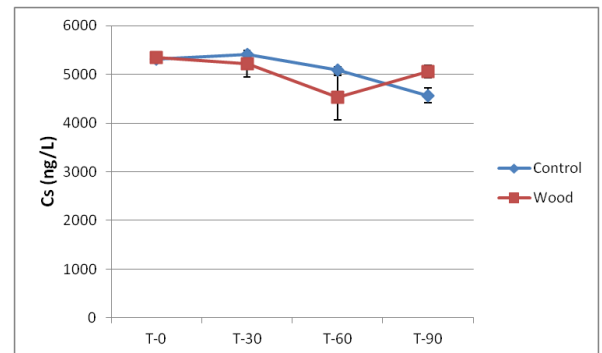


Figure 12. Evolution of Cs (ng/L) in Control and Wood over time

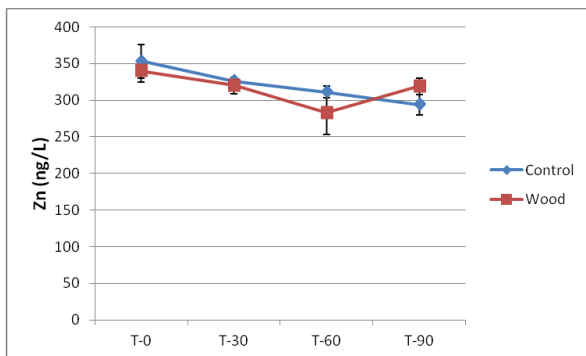


Figure 10. Evolution of Zn (ng/L) in Control and Wood over time

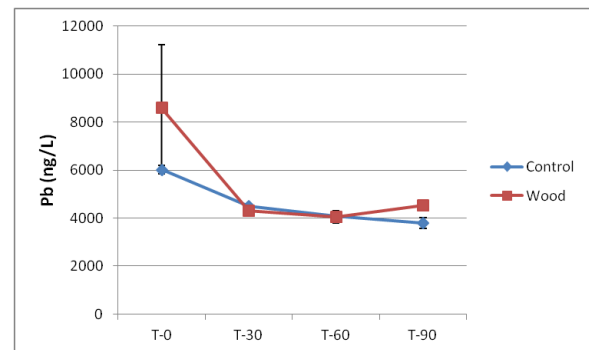


Figure 13. Evolution of Pb (ng/L) in Control and Wood over time

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-30: 30 days, T-60: 60 days, T-90: 90 days. Results are expressed in mg/L. Regarding Control wines, results correspond to mean values of the corresponding analytical duplicates (n=2); Concerning Wood wines results mean values (and corresponding standard deviation) of three experimental replicates and corresponding analytical duplicates (n=6).

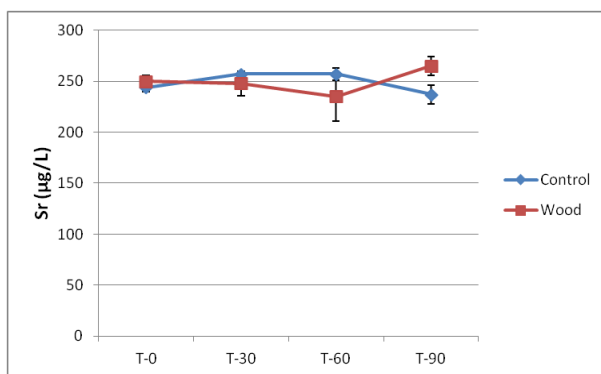


Figure 14. Evolution of Sr (µg/L) in Control and Wood over time

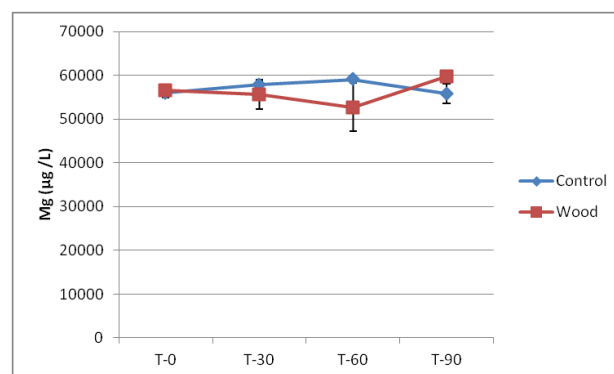


Figure 15. Evolution of Mg (µg/L) in Control and Wood over time

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-30: 30 days, T-60: 60 days, T-90: 90 days. Results are expressed in mg/L. Regarding Control wines, results correspond to mean values of the corresponding analytical duplicates (n=2); Concerning Wood wines results mean values (and corresponding standard deviation) of three experimental replicates and corresponding analytical duplicates (n=6).

Maximum acceptable limits by OIV of critical elements are defined as 1, 5, 0.2 and 0.15 mg/L, for Cu, Zn, As, Pb respectively (OIV, 2015). Our wines show low amount of these elements which are 0.02, 0.0004, 0.001, 0.01 mg/L, respectively.

Cu and Fe play role in the oxidation of ellagic tannins (Vivas and Glories, 1996) that makes them important for ageing phenomena. However, there was no impact of wood ageing on Cu and Fe while time significantly caused a decrease in Fe concentration.

Wines are in the range of 60 – 150 mg/L in terms of Mg content (Ribéreau-Gayon *et al.*, 2006). Wine samples have medium concentration with average of 58 mg/L. As it can be seen in figure 15, Mg concentration has increased after wood ageing. One of magnesium's important roles in wine is to have ion exchange character. Mg resins can be used to inhibit tartrate precipitation by elimination K and Ca cations (Ribéreau- Gayon *et al.*, 2006). They also contribute to the buffer capacity and metallic structure of wine.

5.5.5. Strontium Isotopic Ratios

Separation of rubidium and strontium

As previously stated, the separation of Sr and Rb is a mandatory step before isotopic analysis by ICP-MS. In this study, successful separation of Rb and Sr was achieved. The concentration ratios of Rb/Sr after ion-exchange chromatography are given in table 13 in annex. It can be confidently said that in the samples that Sr isotopic ratios were measured

were not interfered by ^{87}Rb isotope, because in all the chromatographic fractions containing Sr, the ratio Rb/Sr was always lower than 0.93%. Relative standard deviation was obtained as optimal as possible for ICP-MS method.

$^{87}\text{Sr}/^{86}\text{Sr}$ Isotopic Ratios

$^{87}\text{Sr}/^{86}\text{Sr}$ isotope abundance ratio values determined are given in table 10. In total, 32 wine samples were analyzed from initial time until the end of 90 days. Yet, as previously indicated, it is important to consider anthropogenic sources of strontium while using it as geographical indicator.

Strontium isotopic ratio that is taken by the roots of the vines shows identical values as in the soil as well as in the wine after wood ageing. As previously stated, regarding Sr concentration, the wine samples in the end of three months exhibit significantly higher concentration values. However, as it is illustrated on figure 16, strontium isotopic ratios were not modified after wood ageing and they are significantly identical before and after which are independent from the Sr concentrations even though, as given in table 9, the concentration has slightly increased by wood ageing factor. These results justify our hypothesis about using Sr isotopic ratio as wine provenance tool. Our findings are also in accordance with the results from Martins *et al.* (2014). The team analyzed isotopic ratio of the soil from Palmela region that was 0.711 ± 0.0003 . In this study the ratios vary from 0.709 to 0.710. This suggests strong relationship of $^{87}\text{Sr}/^{86}\text{Sr}$ between soil and wines.

Nevertheless more studies are necessary for the reliability of this method and the database must have enough sources of data to be used as a reliable method.

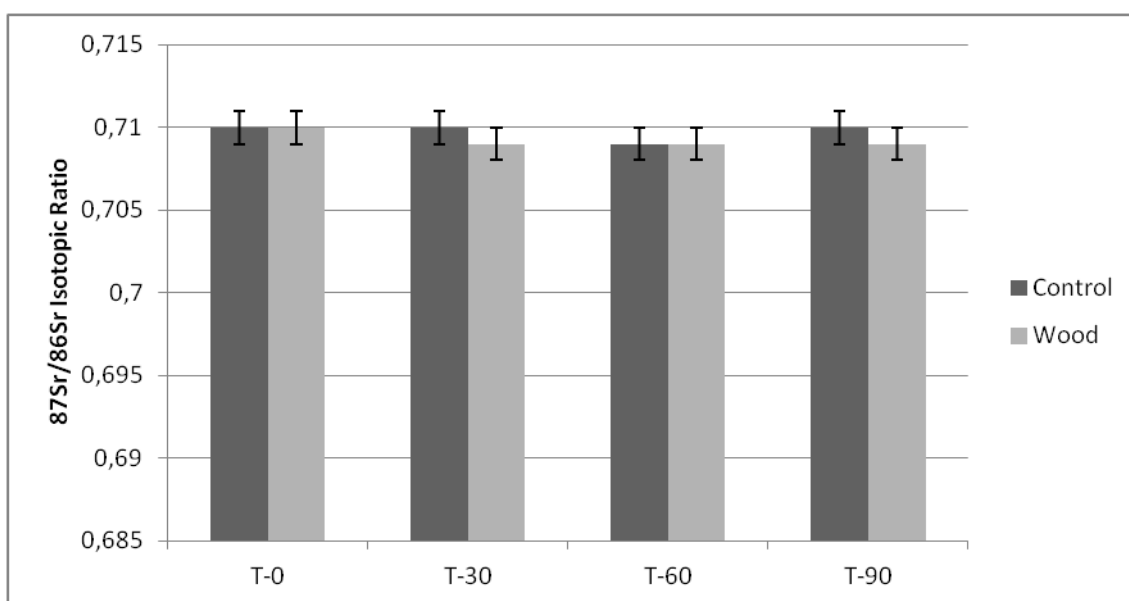


Figure 16. $^{87}\text{Sr}/^{86}\text{Sr}$ Isotopic Ratios in wines over time

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-30: 30 days, T-60: 60 days, T-90: 90 days. Regarding Control wine results correspond to mean values of the corresponding analytical duplicates ($n=2$); Wood wines results correspond to mean values (and corresponding standard deviation with error bars) of three experimental replicates and corresponding analytical duplicates ($n=6$).

Table 10. Effect of wood stage and time on strontium isotopic ratio

	Mean (S.D.)	Time	Wood Effect	Time Effect	Time* Wood Interaction
Control	0.710 (0.001)	T-0	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>
Wood	0.710 (0.001)	T-0	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>
Control	0.710 (0.001)	T-30	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>
Wood	0.709 (0.001)	T-30	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>
Control	0.709 (0.001)	T-60	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>
Wood	0.709 (0.001)	T-60	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>
Control	0.710 (0.001)	T-90	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>
Wood	0.709 (0.001)	T-90	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-30: 30 days, T-60: 60 days, T-90: 90 days.

Regarding Control wine results correspond to mean values of the corresponding analytical duplicates ($n=2$); Concerning Wood wines results mean values (and corresponding standard deviation in parenthesis) of three experimental replicates and corresponding analytical duplicates ($n=6$). Mean values followed by the same letter are not significantly different at 0.05* or 0.001*** level of significance; *n.s.* without significant difference

5.6. Final Considerations and Conclusion

After three months of ageing time with oak staves in stainless steel tanks with three experimental replicates, it is found that wood ageing and time did not alter $^{87}\text{Sr}/^{86}\text{Sr}$ ratios. However, some significant changes on elements' compositions occurred that might be explained by oenological treatments, precipitations and interactions between several compounds in wine such as anthocyanins, proteins and tannins. Effect of wood ageing on the chromatic characteristics and total polysaccharides content of wine was also studied.

This study allowed us to reveal the impacts of wood ageing on wines mineral composition and on strontium isotopic ratio as a novelty to the best of our knowledge. Findings of this work can shed some light on the authenticity methods and contribute to future similar studies and can be developed in different regions, especially the ones that have appellations, by analyzing the soil and the wines. In this way establishing a database will help new studies that can relate soils' Sr isotopic ratios with the wines. It is also worth to mention that in order to add value to the high quality fine wines, and prevent false declarations; this method can be very useful.

Considering that this study was carried out with one type of wine treating with oak staves from a single wood species, it can be developed by assaying ageing wines with different oak species, oaks with different cooperage processes, with oak barrels and diverse winemaking techniques in order to reveal impacts of such practices and alterations on the strontium isotopic ratios of wine. Different grape varieties from different regions would also contribute to a better understanding of the reliability of the method. After several supportive studies, studied method can be recognized by OIV for fingerprinting of wines.

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Scientific Disclosure

Part of the results of this work has been included in the communication “*Effect of new and conventional technological processes on the terroir marker $^{87}\text{Sr}/^{86}\text{Sr}$* ” and was presented orally in OIV Congress which has taken place in October, 2016 in Bento Gonçalves, Brazil.

The corresponding proceeding is published at Bio Web of Conferences: CATARINO S., Moreira C., Bruno de Sousa R., Curvelo-Garcia A.S., de Pinho M., Ricardo-da-Silva J.M., 2016. Effect of new and conventional technological processes on the *terroir* marker $^{87}\text{Sr}/^{86}\text{Sr}$. *Bio Web of Conferences*, 7, 02003. 39th World Congress of Vine and Wine, Bento Gonçalves, Brasil. (DOI: 10.1051/bioconf/20160702003)

ANNEX

Table 11. Total polysaccharides concentrations values (mg/L)

	Mean (S.D.) (mg/L)	Time
Control	362 (13)	T-0
Wood	346 (27)	T-0
Control	321 (33)	T-30
Wood	391 (50)	T-30
Control	306 (65)	T-60
Wood	313 (36)	T-60
Control	512 (12)	T-90
Wood	486 (28)	T-90

Wood: Mean values with standard deviation values (in parenthesis) of wines with wood staves; averages present 6 replicates, which three replicates are in industrial scale and two in analytical scale ($n=9$); Control: Mean values of analytical duplicates of control wines. ($n=3$) T-0: Time-zero, T-30: After 30 days, T-60: After 60 days, T-90: After 90 days.

Table 12. Mean values of chromatic characteristics

	Color Intensity (a.u)	Tonality	Total Phenolic Index	Total Anthocyanins	Time
	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)	Mean (S.D.) (mg/L)	
Control	11.0 (0.0)	0.761 (0.000)	54.3 (1.9)	292 (1)	T-0
Wood	11.1 (0.1)	0.762 (0.001)	53.5 (0.5)	273 (25)	T-0
Control	10.3 (0.1)	0.783 (0.005)	55.2 (0.6)	276 (7)	T-30
Wood	10.4 (0.4)	0.780 (0.000)	55.3 (0.6)	282 (3)	T-30
Control	10.0 (0.2)	0.833 (0.040)	56.6 (0.3)	343 (7)	T-60
Wood	9.8 (0.1)	0.773 (0.007)	56.4 (0.3)	357 (2)	T-60
Control	10.0 (0.0)	0.781 (0.002)	51.5 (0.2)	334 (11)	T-90
Wood	10.3 (0.3)	0.798 (0.005)	56.4 (0.8)	326 (5)	T-90

Wood: Mean values with standard deviation values (in parenthesis) of wines with wood staves; averages present 6 replicates, which three replicates are in industrial scale and two in analytical scale ($n=6$); Control: Mean values of analytical duplicates of control wines. ($n=2$) T-0: Time-zero, T-30: After 30 days, T-60: After 60 days, T-90: After 90 days.

Table 13. Sr and Rb concentrations (ng/L) and effectiveness of chromatic separation values.

Sample	[Sr]	[Rb]	Rb/Sr (%)	Time
Control	476.374,81	186,556	0,04	T-0
Control	403.257,88	261,732	0,06	T-0
Wood	390.171,28	309,037	0,08	T-0
Wood	462.771,22	1.212,22	0,26	T-0
Wood	396.319,58	99,308	0,03	T-0
Wood	374.400,32	215,502	0,06	T-0
Wood	386.536,90	131,51	0,03	T-0
Wood	431.072,10	2.276,19	0,53	T-0
Control	369.026,16	143,239	0,04	T-30
Control	397.660,52	138,274	0,03	T-30
Wood	413.214,57	129,178	0,03	T-30
Wood	450.056,92	128,156	0,03	T-30
Wood	489.660,08	73,92	0,02	T-30
Wood	488.694,54	137,926	0,03	T-30
Wood	347.574,32	114,198	0,03	T-30
Wood	443.194,73	235,825	0,05	T-30
Control	434.147,23	159,085	0,04	T-60
Control	366.838,91	145,876	0,04	T-60
Wood	257.964,94	241,658	0,09	T-60
Wood	449.726,50	153,345	0,03	T-60
Wood	371.054,54	275,886	0,07	T-60
Wood	296.450,04	224,284	0,08	T-60
Wood	348.958,12	116,651	0,03	T-60
Wood	369.569,72	284,345	0,08	T-60
Control	340.269,04	178,737	0,05	T-90
Control	344.313,93	128,707	0,04	T-90
Wood	452.184,52	4.220,29	0,93	T-90
Wood	488.444,01	302,552	0,06	T-90
Wood	122.849,63	253,738	0,21	T-90
Wood	117.479,24	218,478	0,19	T-90
Wood	395.178,72	115,722	0,03	T-90
Wood	342.629,93	133,283	0,04	T-90

Wood: Wines with wood staves which three replicates are in industrial scale and two in analytical scale ($n=6$); Control: analytical duplicates of control wines ($n=2$). T-0: Time-zero, T-30: After 30 days, T-60: After 60 days, T-90: After 90 days.

Table 14. Physical – chemical characteristics of wines

	Density (20°C) (g/mL)	Alcohol (% vol.)	pH	Ash (g/L)	Total SO ₂ (mg/L)	Free SO ₂ (mg/L)	Volatile Acidity (g/L)	Total Acidity (g/L)	Total Dry Matter (g/L)	Reducing Substances (g/L)	Time
Control	0.9921 (0.0000)	13.6 (0.0)	3.62 (0.00)	3.2 (0.0)	45 (0)	14 (0)	0.7 (0.0)	5.4 (0.2)	30.0 (0.0)	2.4 (0.0)	T-0
Wood	0.9918 (0.0002)	13.5 (0.1)	3.61 (0.00)	3.4 (0.1)	51 (6)	16 (2)	0.7 (0.4)	5.2(0.1)	28.8 (0.1)	2.2 (0.1)	T-0
Control	0.9915 (0.000)	13.4 (0.0)	3.61 (0.00)	2.3 (0.0)	40 (0)	14 (0)	0.7 (0.0)	5.3 (0.0)	28.9 (0.0)	2.0 (0.0)	T-30
Wood	0.9916 (0.002)	13.4 (0.0)	3.61 (0.0)	2.2 (0.0)	39 (4)	9 (1)	0.7 (0.0)	5.4 (0.1)	28.9 (0.0)	2.1 (0.0)	T-30
Control	0.9919 (0.000)	13.4 (0.0)	3.62 (0.0)	2.7 (0.0)	53 (4)	14 (0)	0.6 (0.0)	5.4 (0.1)	28.4 (0.4)	2.3 (0.1)	T-60
Wood	0.9918 (0.007)	13.4 (0.1)	3.61 (0.0)	2.6 (0.0)	63 (3)	16 (1)	0.6 (0.1)	5.4 (0.1)	28.0 (1.1)	2.3 (0.1)	T-60
Control	0.9918 (0.0001)	13.5 (0.0)	3.60 (0.0)	3.2(0.0)	66 (2)	19 (1)	0.7 (0.0)	5.3 (0.0)	28.4 (0.4)	2.8 (0.0)	T-90
Wood	0.9919 (0.0002)	13.5 (0.0)	3.60 (0.0)	2.9 (0.0)	61 (1)	18 (2)	0.7 (0.0)	5.1 (0.2)	28.4 (0.0)	2.9 (0.2)	T-90

Mean values with standard deviation values for each modality (T-0, T-30, T-60 and T-90)

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-30: 30 days, T-60: 60 days, T-90: 90 days.

Regarding Control wine results correspond to mean values of the corresponding analytical duplicates (n=2); Concerning Wood wines results mean values (and corresponding standard deviation in parenthesis) of three experimental replicates and corresponding analytical duplicates (n=6).

Table 15. Major and micro elements compositions of wines

	T-0		T-30		T-60		T-90	
	Control	Wood	Control	Wood	Control	Wood	Control	Wood
Na	30.6 (0.3)	30.4 (0.2)	30.2 (0.2)	30.2 (0.3)	30.6 (0.0)	30.7 (0.1)	28.4 (0.0)	30.4 (0.1)
K	1134.6 (1.4)	1135.1 (3.1)	1148.2 (24.0)	1155.7 (17.0)	1081.2 (20.4)	1067.9 (8.3)	992.6 (90.8)	1068.7 (3.6)
Ca	56.64 (1.02)	56.06 (2.20)	55.15 (0.75)	54.18 (0.15)	46.18 (0.74)	53.38 (3.29)	54.84 (1.22)	52.80 (0.58)
Fe	3.05 (0.08)	3.11 (0.14)	3.32 (0.04)	2.92 (0.15)	3.30 (0.08)	2.94 (0.22)	2.42 (0.03)	2.70 (0.15)

Mean values with standard deviation values for each modality (T-0, T-30, T-60 and T-90)

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-30: 30 days, T-60: 60 days, T-90: 90 days.

Regarding Control wine results correspond to mean values of the corresponding analytical duplicates (n=2); Concerning Wood wines results mean values (and corresponding standard deviation in parenthesis) of three experimental replicates and corresponding analytical duplicates (n=6).

Table 16. Multi-elemental compositions of wines

	T-0		T-30		T-60		T-90	
	Control	Wood	Control	Wood	Control	Wood	Control	Wood
Li	11277 (128)	11459 (101)	11621 (483)	10507 (717)	12308 (334)	10566 (1474)	11307 (268)	12058 (400)
Be	75 (2)	94 (15)	104 (21)	79 (11)	81 (13)	79 (89)	156 (0)#	142 (21)
Mg	56020 (1012)	56581 (1106)	57842 (986)	55651 (3432)	59113 (533)	52717 (5541)	55867 (2259)	59792 (643)
Al	190 (5)	188 (3)	198 (5)	177 (6)	187 (3)	162 (15)	171 (7)	180 (1)
Sc	575 (42)	952 (70)	615 (144)	752 (183)	413 (78)	523 (194)	863 (0) #	844 (53)
Ti	1201 (8)	1350 (122)	1554 (66)	1362 (56)	1355 (32)	1228 (22)	1305 (102)	1342 (59)
V	897 (162)	952 (71)	877 (23)	751 (20)	1139 (30)	926 (93)	446 (20)	970 (4)
Mn	759 (32)	784 (18)	793 (8)	777 (46)	794 (3)	736 (76)	755 (37)	833 (37)
Co	2850 (127)	2871 (91)	2932 (27)	2808 (146)	2818 (102)	2530 (216)	1934 (12)	2795 (37)
Ni	54804 (265)	55191 (542)	55380 (1409)	52451 (3913)	52097 (1042)	48596 (2240)	12578 (470)	51869 (1858)
Cu	21409 (146)	22221 (9353)	17517 (331)	13477 (778)	17331 (719)	20497 (4245)	21936 (962)	26510 (2236)
Zn	353 (23)	340 (15)	326 (3)	320 (11)	311 (8)	283 (30)	294 (14)	319 (11)
Ga	2893 (1)	2906 (84)	2867 (149)	2702 (150)	3026 (92)	2378 (378)	2648 (165)	2824 (69)
As	1022 (80)	1073 (14)	1015 (33)	968 (79)	1058 (7)	892 (143)	931 (118)	1044 (47)
Rb	1173 (38)	1191 (33)	1219 (1)	1184 (38)	1241 (33)	1117 (117)	1175 (59)	1249 (21)
Sr	244 (4)	250 (6)	257 (0)	248 (12)	257 (6)	235 (24)	237 (9)	265 (9)
Y	441 (20)	394 (13)	385 (56)	388 (16)	419 (50)	377 (61)	361 (4)	443 (18)
Sb	77 (9)	63 (3)	94 (2)	80 (8)	123 (24)	59 (7)	73 (0) #	72 (2)
Cs	5315 (76)	5346 (116)	5403 (83)	5218 (266)	5086 (63)	4524 (460)	4567 (153)	5057 (129)
Ba	214 (1)	221 (6)	219 (1)	215 (12)	205 (2)	192 (19)	198 (7)	214 (10)
Pr	53 (16)	40 (3)	34 (4)	34 (2)	40 (0) #	38 (4)	40 (0) #	50 (2)
Nd	120 (0) #	201 (34)	273 (0)	235 (19)	180 (0)	189 (10)	125 (19)	141 (18)
Eu	111 (34)	105 (7)	111 (8)	88 (8)	93 (9)	91 (10)	107 (5)	118 (15)
Dy	22 (2)	41 (7)	37 (5)	35 (7)	51 (0) #	43 (12)	41 (15)	50 (10)

Ho	17 (1)	14 (3)	10 (0)	13 (4)	13 (1)	10 (2)	9 (0)	12 (2)
Er	41 (7)	44 (6)	38 (4)	40 (1)	37 (7)	35 (5)	19 (1)	44 (14)
Yb	44 (18)	48 (9)	35 (3)	36 (15)	41 (0) #	37 (11)	31 (2)	35 (5)
Lu	18 (1)	12 (2)	14 (2)	9 (2)	12 (0) #	11 (2)	9 (3)	13 (2)
Tl	328 (8)	320 (15)	318 (16)	302 (25)	342 (5)	282 (34)	293 (9)	329 (16)
Pb	6020 (188)	8601 (2638)	4504 (35)	4316 (22)	4066 (3)	4048 (257)	3803 (226)	4534 (221)

Mean values with standard deviation values for each modality (T-0, T-30, T-60 and T-90)

Control: Wine without wood staves; Wood: Wine with wood staves; T-0: Time-zero, T-30: 30 days, T-60: 60 days, T-90: 90 days.

Regarding Control wine results correspond to mean values of the corresponding analytical duplicates (n=2); Concerning Wood wines results mean values (and corresponding standard deviation in parenthesis) of three experimental replicates and corresponding analytical duplicates (n=6).

Values with '#' represent one value without replicates due to high standard deviation values.

Effect of new and conventional technological processes on the terroir marker $^{87}\text{Sr}/^{86}\text{Sr}$

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Abstract. Aiming to use the strontium isotopic ratio $^{87}\text{Sr}/^{86}\text{Sr}$ for traceability and authentication of wine, it is crucial to understand the impacts of anthropogenic factor and technological processes on this parameter. In this study the effect of two technologies: nanofiltration (NF), applied for wine dealcoholisation (1), and ageing with oak wood staves (2), on $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of wines from Portuguese Designations of Origin (DO), was assessed. Control wines, NF processed wines and wooden aged wines were evaluated for chemical composition, multi-elemental composition and $^{87}\text{Sr}/^{86}\text{Sr}$ ratio by Q-ICP-MS. Regarding NF experiments, high rejections to mineral elements, including heavy metals, were observed. Bearing in mind the several roles played by these elements in wine, this knowledge is of strategic importance in order to guarantee that NF process does not adversely affect product quality. Concerning wood ageing experiment, results indicate the influence of this technological process on metal physical-chemical stability. In the experimental conditions of this study, no significant effect of NF process and wood ageing on $^{87}\text{Sr}/^{86}\text{Sr}$ ratio was observed, suggesting that the application of these technologies in wine production is not a limitative factor for the use of $^{87}\text{Sr}/^{86}\text{Sr}$ ratio as a geographical origin marker.

1. Introduction

Closely related to the idea that *terroir* determines the quality and character of wines, many efforts have been made to identify potential markers and develop reliable analytical methods to determine the wine's geographical origin.

The isotopic ratio $^{87}\text{Sr}/^{86}\text{Sr}$ is a well-established tool for dating and tracing the origin of rocks and minerals [1] with special interest for wine traceability. Several studies on its use for wine geographical origin assessment can be found in literature [2–8]. Recently, within a research program regarding strategies for wine fingerprinting, the authors confirmed $^{87}\text{Sr}/^{86}\text{Sr}$ as a viable tool for traceability of Portuguese DO, where soils were developed on different geological formations [9, 10].

The use of this parameter as a marker of wine geographical origin is based on the assumption that a relation between soil, plants and wine exists. Therefore, $^{87}\text{Sr}/^{86}\text{Sr}$ ratio should not be significantly modified during wine processing. Aiming to use $^{87}\text{Sr}/^{86}\text{Sr}$ for traceability and authentication of wine, it is mandatory to clarify the impacts of anthropogenic factors and technological processes on this isotopic ratio. This is a current subject of major importance addressed in this study.

NF is a versatile membrane process, with several applications in wine production where its use is increasing, e.g. for ethanol content modulation but also for reduction of the sugar content in musts, volatile acidity correction

and to remove excess of ethyl-phenols [11]. NF effects on wine composition have been assessed, namely on alcoholic strength, total acidity and phenolic composition [12, 13]. Nevertheless, to the best of our knowledge, its influence on mineral composition and wine $^{87}\text{Sr}/^{86}\text{Sr}$ is not known.

Wood aging is a traditional practice widely used in wine production. Currently, as alternative to wood barrels, in order to improve the traditional process in conditions that sustain the economic viability of this practice, the use of wood pieces in stainless-steel tanks is widespread. In spite of substantial understanding of the organic extractable compounds, little has been published on metal extraction from wood to wine, and no data about its influence on $^{87}\text{Sr}/^{86}\text{Sr}$.

In spite of a few studies suggesting that $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratio remains stable after enological processes [3, 5, 14, 15], the literature lacks the information on the NF process and wood ageing influence on $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratio. The main aim of this study was to uncover the effect of NF and wood ageing by oak staves on strontium $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratio. Furthermore, information on the effect of these technologies on mineral composition of wines was obtained.

2. Materials and methods

2.1. Nanofiltration experiments

For NF experiments, three white wines (WW) and three red wines (RW) from the Portuguese DO Dão, Óbidos and Palmela were used. White wines, from 2014 vintage,

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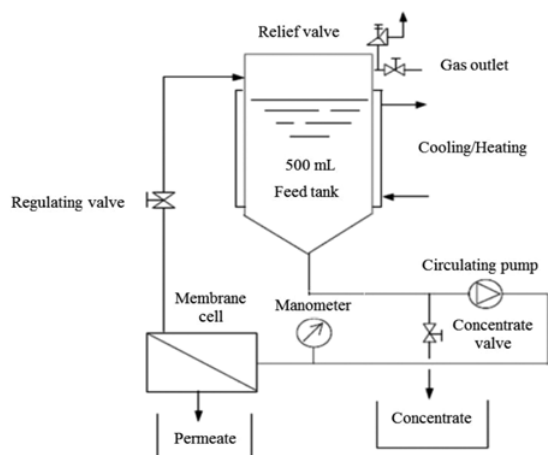


Figure 1. Set up of the nanofiltration unit.

were produced using the following grape varieties (*Vitis vinifera* L.): Fernão Pires (Óbidos); Verdelho and Viosinho (Palmela); Verdelho (Dão). Red wines, from 2013 vintage, resulted from the following grape varieties (*Vitis vinifera* L.): Calladoc, Syrah and Alicante Bouchet (Óbidos); Castelão (Palmela); Touriga Nacional (Dão).

NF experiments were carried out at laboratory scale in the unit Celfa P-28 for dealcoholisation of white and red wines (Fig. 1).

Regarding the NF membrane used, a cellulose acetate membrane was laboratory made and characterized, presenting hydraulic permeability of 0.313 kg/(h.m².bar) and rejection coefficients of 90.3% to NaCl, 95.3% to Na₂SO₄, 92.8% to CaSO₄ and 90.3% to glucose. Rejection coefficients (f) to solutes were determined using equation 1, where C_f and C_p are the concentrations in feed and permeate, respectively:

$$f = (C_f - C_p)/C_f \quad (1)$$

NF experiments were carried out at a transmembrane pressure of 17 bar and a feed recirculation flow rate of 2.1 L/min. Each NF fraction (initial feed, final feed and permeate) was evaluated through summary analysis and total polysaccharides, total anthocyanins, total phenolic index, multi-elemental composition and ⁸⁷Sr/⁸⁶Sr isotopic ratio.

2.2. Ageing experiments

A red wine from Castelão grape variety (Palmela DO), 2014 vintage, was aged in stainless steel vats (34,000 L) with wood staves, in triplicate. French oak (*Quercus sessiliflora* Salisb.) staves, with medium plus toasting level (<200 °C), in dimensions of 91 cm (length) × 6.4 cm (width) × 0.95 cm (thickness) were used, one oak stave per 40 L of wine. Micro-oxygenation was applied with the amount of 3 mg/L per month. Storing temperature of the tanks was 18 °C. The experiment was carried out with one control vat without oak staves.

The wines were sampled after 30, 60 and 90 days of ageing (a total of 16 samples) and evaluated in terms of general analysis, total polysaccharides, total anthocyanins, total phenols index, multi-elemental composition and ⁸⁷Sr/⁸⁶Sr isotopic ratio.

2.3. Analytical determinations

2.3.1. Physical-chemical characterization

Alcoholic strength, total acidity, volatile acidity, tartaric acid, malic acid, lactic acid, total dry matter, ash, density, pH, total sulphur dioxide, reducing substances, colour intensity and tonality, were determined according to OIV analysis methods [16], in the samples from ageing experiments.

Due to the low sample volume available for analysis, wine NF streams (initial feed, final feed and permeate) were analysed for the before referred analytical parameters by Fourier Transform Infrared (FTIR) technique using a Foss WineScan FT 120 equipment.

Total polysaccharides were assessed through phenol-sulfuric method (colorimetric method), after the separation of polysaccharides by ethanol. Total anthocyanins and total phenols index determinations were carried out as described by Somers and Evans [17].

2.3.2. Multi-elemental analysis

Thirty-six minor and trace elements were measured by quadrupole inductively coupled plasma mass spectrometry (Q-ICP-MS) semi-quantitative methodology according to the protocol described by Catarino et al. [18].

A Perkin-Elmer SCIEX Elan 9000 Q-ICP-MS apparatus, equipped with a peristaltic sample delivery pump with four channels model Gilson, a Scott-type spray chamber made of Rytan, a crossflow nebulizer, nickel cones and an autosampler Perkin-Elmer AS-93 Plus, protected by a laminar-flow-chamber clean room class 100 was used. The system was controlled by software Elan – 6100Windows NT (Version 2.4).

Major mineral elements (Na, K, Ca, Fe) were determined in wines from ageing experiments by Flame Atomic Absorption Spectrophotometry (FAAS) according to the methods described by OIV [16].

2.3.3. ⁸⁷Sr/⁸⁶Sr isotopic ratio determination

Due to isobaric overlap of ⁸⁷Rb and ⁸⁷Sr, an effective Rb and Sr separation is required before ICP-MS analysis, in order to accurate determination of ⁸⁷Sr/⁸⁶Sr isotopic ratios. Wine samples treatment is composed by three steps: digestion by High Pressure Microwave (HPMW), chromatographic separation of Sr and Rb and filtration of Sr chromatographic fraction.

⁸⁷Sr/⁸⁶Sr isotopic ratio determination by Q-ICP-MS was carried out according to protocol described by the authors [9], using the Q-ICP-MS equipment previously described. Analytical calibration and stability control in ⁸⁷Sr/⁸⁶Sr isotopic ratio analysis were made with SrCO₃ (50 µg/L), prepared from certified reference material NIST SRM 987 (SrCO₃).

2.4. Statistical analysis

Analysis of variance (ANOVA) was carried out to assess the effect of nanofiltration and wooden ageing on the ⁸⁷Sr/⁸⁶Sr isotopic ratio of wines. Fisher's least significant difference (LSD, significance level of 0.05) test was applied to compare the different averages. All calculations were carried out using Statistica version 7 (StatSoft Inc., Tulsa, USA).

Table 1. Physical-chemical characteristics of wines nanofiltration streams (I.F. – initial feed; F.F. – final feed; P. – permeate).

	WW Dão DO			RW Óbidos DO		
	I.F.	F.F.	P.	I.F.	F.F.	P.
Alcoholic strength at 20 °C (% v/v)	12.6	12.5	11.7	11.9	11.9	11.3
Total acidity (g/L tartaric acid)	5.60	4.86	0.74	4.71	4.98	1.31
Volatile acidity (g/L acetic acid)	0.29	0.28	0.18	0.56	0.56	0.44
Tartaric acid (g/L)	1.51	1.17	nd	0.87	0.83	nd
Malic acid (g/L)	1.90	1.56	nd	–	–	–
Lactic acid (g/L)	0.08	0.16	0.16	1.72	1.89	0.44
Total dry matter (g/L)	20.7	17.9	1.5	27.5	30.7	2.6
Ash (g/L)	2.17	1.92	0.43	3.32	3.60	0.43
Density at 20 °C (g/mL)	0.9897	0.9887	0.9832	0.9931	0.9943	0.9841
pH	3.22	3.22	3.13	3.59	3.63	3.14
Total sulfur dioxide (mg/L)	95	89	56	53	57	67
Reducing substances (g/L)	3.24	2.57	0.62	3.12	3.60	0.26
Total polysaccharides (mg/L)	416	583	nq	717	977	nq
Color intensity (u.a)	–	–	–	13.810	14.945	0.012
Tonality	–	–	–	0.697	0.760	21.000
Total anthocyanins (mg/L)	–	–	–	229	296	13
Total phenols index (u.a.)	6	7	1	53	62	1

nd – not detected; nq – not quantified.

3. Results and discussion

3.1. Nanofiltration of wines

3.1.1. Physical-chemical characteristics of NF streams

Physical-chemical characteristics of NF streams (initial feed, final feed and permeate) from a white wine and a red wine (WW Dão DO and RW Óbidos DO, as examples) are displayed in Table 1.

As expected, ethanol was preferentially permeated by membrane. Rejections to tartaric, malic, lactic and acetic wines were in general high. Regarding polysaccharides, a complete retention was verified. Results relatively to total phenols index and anthocyanins indicate a high rejection to phenolic compounds. It was observed the increase of colour intensity and tonality. For the majority of chemical compounds concentrations in final feed increased with NF treatment due to water and ethanol removal.

In general, rejections higher than 90% to mineral elements, including heavy metals, were observed. Concentrations in final feed were slightly higher than concentrations in initial feed, due to water and ethanol removal. Bearing in mind the several roles played by these elements in wine, this knowledge is of strategic importance in order to guarantee that NF process does not adversely affect product quality.

Rejection to Sr was mostly higher than 95%, suggesting that NF does not change the ⁸⁷Sr/⁸⁶Sr isotopic ratio. Table 2 shows the ⁸⁷Sr/⁸⁶Sr isotopic ratios of wine NF fractions. Strontium isotopic ratio in final feed was not significantly different from ⁸⁷Sr/⁸⁶Sr initial feed, revealing that no preferential permeation of any strontium isotopes occurred.

As expected, ⁸⁷Sr/⁸⁶Sr values were higher for DO Dão, characterized by granitic soils (older). Palmela and Óbidos DO, mainly characterized by sandy and clay soils, showed lower ⁸⁷Sr/⁸⁶Sr ratios. These results are in accordance with previous research of the authors involving soils from these DO [9]. The highest ⁸⁷Sr/⁸⁶Sr values were found in soils from Dão, pointing out a relation between soils and wines.

Table 2. ⁸⁷Sr/⁸⁶Sr isotopic ratio in wine NF streams (I.F. – Initial feed; F.F. – final feed; P. – Permeate).

Wine NF stream	⁸⁷ Sr/ ⁸⁶ Sr
Palmela WW I.F.	0.7075 ± 0.0007 a,b,c
Palmela WW F.F.	0.7075 ± 0.0007 a,b,c
Palmela RW I.F.	0.709 ± 0.000 c,d
Palmela RW F.F.	0.7075 ± 0.0007 a,b,c
Óbidos WW I.F.	0.7080 ± 0.0008 a,b,c,d
Óbidos WW F.F.	0.709 ± 0.001 b,c,d
Óbidos RW I.F.	0.709 ± 0.000 c,d
Óbidos RW F.F.	0.7095 ± 0.0007 d
Dão WW I.F.	0.713 ± 0.000 e
Dão WW F.F.	0.714 ± 0.000 e,f
Dão RW I.F.	0.715 ± 0.001 f
Dão RW F.F.	0.714 ± 0.001 e,f

Results are based on average values of two independent replicates and respective standard deviation. Means followed by the same letter are not significantly different at the 0.05 level of significance.

3.2. Wood ageing

3.2.1. Mineral composition and ⁸⁷Sr/⁸⁶Sr isotopic ratio

Table 3 displays the concentrations of some elements in Control and Wood wines at the end of the experiment (90 days). Wine treated with oak wood staves showed significantly different concentrations for the elements Na, V, Co, Ni, Sr, Cs and Pb, compared to the Control. Particular attention must be paid to the differences in V, Co and Ni concentrations between Control and Wood wines. Higher concentrations of these elements in Wood in comparison with Control are most probably explained by its more intense precipitation through time in the wine without wood staves. This hypothesis is supported by the concentrations of V, Co and Ni in Control wine at the beginning of the ageing experiment (results not shown). For the rest of the elements, despite no significant effect of wood treatment was observed, metals concentrations tend to be higher in the wine treated with oak wood staves.

Table 3. Mineral composition of the wine treated with oak wood staves and of the control wine at the end of the experiment, 90 days (Results are given in $\mu\text{g/L}$, except for Na, Mg, Al, K, Ca, Mn, Fe and Zn, which are expressed as mg/L)^a.

	Treatment effect ^b	Control	Wood
Li	n.s.	11.3 ± 0.3	12.2 ± 0.2
Na	***	28.38 ± 0.03 a	30.37 ± 0.04 b
Mg	n.s.	56 ± 2	59.7 ± 0.5
Al	n.s.	171 ± 7	180.3 ± 0.5
K	n.s.	993 ± 91	1069 ± 3
Ca	n.s.	55 ± 1	53.3 ± 0.6
V	***	0.45 ± 0.02 a	0.970 ± 0.004 b
Mn	n.s.	0.76 ± 0.04	0.83 ± 0.03
Fe	n.s.	2.42 ± 0.03	2.7 ± 0.1
Co	***	1.9 ± 0.1 a	2.80 ± 0.03 b
Ni	***	12.6 ± 0.5 a	52 ± 2 b
Cu	n.s.	21.9 ± 0.9	26 ± 2
Zn	n.s.	0.3 ± 0.1	0.319 ± 0.009
Ga	n.s.	2.6 ± 0.2	2.8 ± 0.6
As	n.s.	0.9 ± 0.1	1.04 ± 0.04
Rb	n.s.	1175 ± 59	1249 ± 17
Sr	*	237 ± 9 a	265 ± 7 b
Cs	*	4.6 ± 0.2 a	5.1 ± 0.1 b
Tl	n.s.	0.293 ± 0.009	0.33 ± 0.01
Pb	*	3.8 ± 0.2 a	4.5 ± 0.2 b

^a Control: the results are based on average values of analysis duplicates. Wood: the results are based on average values of experimental triplicates and analyses duplicates. ^b Means followed by the same letter in a line are not significantly different at the 0.05* or 0.001*** level of significance; n.s. = without significant difference. ^b Control: the results are based on average values of analysis duplicates. Wood: the results are based on average values of experimental triplicates and analyses duplicates.

Concerning ⁸⁷Sr/⁸⁶Sr isotopic ratio, the values were of 0.710 ± 0.001 and 0.709 ± 0.001 in Control and Wood, respectively. Under these experimental conditions, the ⁸⁷Sr/⁸⁶Sr isotopic ratio of wine was not significantly affected by the wood treatment suggesting that wood ageing does not preclude the use of this parameter as a marker of geographical origin.

Additional results and comprehensive discussion concerning this research are being prepared for publication.

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