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CICLO: XXXI

Characterization of anthocyanins and condensed tannins from grapes and their qualitative incidence on astringency and bitterness sensory properties

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In red wine, phenolic compounds are generally associated with the quality of products. Among them, anthocyanins extracted from skins are responsible for wine colour. The grapevine genomes determine the anthocyanins profiles, but several factors in the vineyard can influence their accumulation, as well as post-harvest techniques can modify their extraction during winemaking. Monomeric, oligomeric and polymeric flavanols from skins and seeds contribute to astringency and bitterness of wine and during winemaking and ageing complexes formation with anthocyanins modifies wine characteristics. Several publications are available to understand flavanols sensory characteristics, whereas anthocyanins role has not consensus in scientific literature.

This PhD thesis is composed by two parts. The first part deal with the evaluation of the use of gaseous ozone as post-harvest technique in red wine grapes Nebbiolo and Barbera used on both fresh grape and during withering. Ozone treatment is an innovative technology proved to avoid mycobiota spoilage and preserving from the use of sulphur dioxide. Its influence on flavanol and anthocyanin contents and extractabilities during maceration was evaluated, considering skin cell wall modification. In fresh grape, ozone influenced skin maceration for both the varieties, leading to a higher anthocyanin extraction in Nebbiolo grapes and lower in Barbera. Ozone did not influence the final individual anthocyanin extractability, respecting the varietal anthocyanin fingerprint. During dehydration, opposite trend was found: in Nebbiolo reported no change in the

content of total anthocyanins just after ozone-assisted dehydration, but their extraction yield was lower. On the contrary, although lower contents of anthocyanins were found in Barbera grapes no differences in final extractability was found. Regarding oligomeric and polymeric flavanols, their extractability was less affected by the ozone treatment. Only in Nebbiolo, both oligomeric and polymeric flavanol extraction was increased in fresh grape, whereas it is slightly decrease during dehydration. The ozone-induced modification of skin cell wall composition together with skin hardness parameters fitted well in multivariate models to predict anthocyanins, oligomeric flavanols and polymeric flavanols extraction. Therefore, the ozone treatment should be adapted depending on the variety and on the target wine.

In the second part, grape anthocyanins were isolated depending on the acylation patterns, i.e. glucoside, acetyl-glucoside, and cinnamoyl-glucoside by a combination of centrifugal partition chromatography (CPC) and preparative-HPLC. Protein precipitation analyses to assess astringency and sensorial analysis were carried out. Anthocyanins reacted with both bovine serum albumine and salivary proteins, in different extent, since higher interaction between anthocyanins and salivary proteins was found with a significative reduction of total extract and fractions glucoside, acetyl-glucoside, and cinnamoyl-glucoside. The latter in particular is the more reactive to salivary proteins. Sensorial analysis was carried out as detection threshold test. Best estimated threshold (BET) of anthocyanins were resulted in wine-range scale, in particular acetyl-glucoside and cinnamoyl-glucoside BET are lower of glucoside threshold, and descriptors reported were astringency and bitterness. These results show that anthocyanins can be detected as in-mouth properties contributors, and the magnitude of their involvement is related to anthocyanins acylation.

Keywords: Anthocyanins, Flavanols, Grapes, Ozone, Sensory properties, Wine

Caratterizzazione di antociani e tannini condensati di uva e loro incidenza sulle caratteristiche sensoriali di astringenza e amaro nei vini

I composti polifenolici dell'uva svolgono un importante ruolo nel determinare la qualità dei vini. Tra questi, gli antociani sono responsabili del colore dei vini rossi e sono presenti nelle bucce. Sebbene il profilo antocianico sia determinato dalla varietà, pratiche in campo e post-raccolta possono modificare il loro accumulo e la loro estraibilità. I flavan-3-oli monomeri, oligomeri e polimeri si trovano nelle bucce e nei semi d'uva influendo sulla percezione di astringenza e sul gusto amaro dei vini. Inoltre, durante la macerazione e l'invecchiamento possono formare complessi con gli antociani modificando le caratteristiche del vino. Allo stato dell'arte, numerose pubblicazioni sono presenti sull'influenza dei flavanoli sulle caratteristiche sensoriali dei vini, mentre per gli antociani non sono presenti risultati condivisi.

Questa tesi di dottorato si divide in due parti. Nella prima parte, l'utilizzo dell'ozono gassoso come trattamento post-raccolta ed il suo utilizzo durante l'appassimento è stato valutato sui vitigni a bacca rossa Nebbiolo e Barbera. Infatti, l'ozono gassoso è stato proposto come tecnologia innovativa al fine di controllare lo sviluppo della flora microbica e fungina e perciò aiutare nella riduzione dell'aggiunta di anidride solforosa. L'influenza del trattamento sulla composizione e estrazione di flavanoli e antociani durante la macerazione è stato valutato, tenendo conto delle modificazioni delle pareti cellulari delle bucce. Nell'uva post-raccolta, l'ozono può influenzare l'estrazione di composti fenolici durante la macerazione delle bucce in entrambe le varietà, portando ad una maggiore estrazione nel Nebbiolo ma più bassa nella Barbera. L'ozono non provoca differenze nell'estrazione delle antocianidine, rispettando il profilo

varietale. Durante l'appassimento, un andamento opposto è stato riscontrato: nel Nebbiolo non sono state riportate differenza significative nel contenuto nelle uve, ma l'estraibilità è diminuita. Invece, nonostante un contenuto maggiore di antociani sia stato riscontrato nella Barbera, il trattamento non ha influito sull'estrazione. I flavanoli polimeri e oligomeri sono meno soggetti a modificazioni indotte dal trattamento con ozono. Solo nel Nebbiolo la loro estraibilità è aumentata dopo il trattamento post-raccolta, mentre una lieve diminuzione è stata riscontrata dopo il trattamento durante l'appassimento. Le modificazioni indotte dal trattamento sulle pareti cellulari, insieme ai parametri di durezza della buccia posso predire l'estraibilità di antociani e flavanoli oligomeri e polimeri grazie alle tecniche di statistica multivariata. In base ai nostri risultati, il trattamento con ozono deve essere modulato in base alla varietà e al prodotto finale desiderato.

Nella seconda parte, le antocianidine sono state estratte dale bucce d'uva, e sono state frazionati con successo in base alla loro acilazione in glucosidi, acetilglucosidi e cinnamoilglucosidi grazie all'utilizzo di technique di Centrifugal Partition Chromatograpy (CPC) e HPLC preparativa. Sugli estratti analisi chimiche per determinare l'astringenza e analisi sensoriali sono state fatte. Gli antociani regiscono con le proteine come la BSA e le proteine salivari, con le seconde in particolare, dato che riduzioni significative sono state riscontrate sia su estratti totali, che sulle frazioni di glucosidi, acetilglucosidi e cinnamoilglucosidi. Gli ultimi sono i più reattivi con le proteine salivari. La soglia di percezione, calcolata come "Best estimate threshold" (BET) degli antociani è coerente con le quantità trovate in vino. Questi risultati confermano che gli antociani possono essere considerati come contributori delle percezioni gustative, e l'importanza del lor contributo è correlata all'acilazione.

Parole chiave: Antociani, Flavanoli, Uva, Ozono, Proprietà sensoriali, Vino

Caractérisation des anthocyanes et des tanins condensés du raisins et leur incidence sur les caractéristiques sensorielles de l'astringence et l'amertume dans les vins

Dans le vin rouge, les composés phénoliques sont souvent associés à la qualité du produit. Parmi eux, les anthocyanes sont resonsables de la couleur du vin rouge et chaque cépage possède un génome spécifique déterminant le profil anthocyanique de chacun. Les pratiques culturales peuvent influencer l'accumulation de ces molécules dans la baie de raisin, tandis que les différentes techniques fermentaires peuvent modifier leur extraction. Les flavanols monomères, oligomères et polymères des pépins et des pellicules contribuent à l'amertume et l'astringence des vins. Pendant la vinification et l'élevage, ceux-ci réagissent avec les anthocyanes modifiant les propriétés du vin. De nombreuses publications concernant les caractéristiques sensorielles des flavanols ont été réalisées, alors que la contribution sensorielle des anthocyanes est encore mal connue.

Cette thèse se décompose en deux parties. La première partie s'intéresse à l'étude de l'utilisation d'ozone gazeux comme technique pré-fermentaire sur les cépages rouges Nebbiolo et Barbera après récolte et pendant le passerillage. L'ozone est une technique innovante pour la réduction des contaminations microbiennes et la réduction du dioxyde de soufre. L'influence de cette technique sur les parois cellulaire des pellicules, sur l'extraction, la concentration en flavanols et en anthocyanes pendant la macération a été évaluée. L'étude montre que l'ozone possède un impact sur la macération des raisins, induisant une extraction plus importante des anthocyanes dans le Nebbiolo. En revanche, l'ozone n'influence ni les anthocyanes moléculaires, ni le profil variétal de chaque cépage. Pendant le passerillage, l'inverse a été étudié. L'extraction des anthocyanes dans le

Nebbiolo est diminuée. A l'inverse, bien que moins d'anthocyanes soient retrouvées dans le raisin de Barbera, le traitement ne possède aucun impact sur l'extraction de celles-ci. Concernant les flavanols, l'extraction est moins influencée par le traitement à l'ozone. Des différences ont été retrouvées dans le cépage Nebbiolo. Les flavonols sont plus concentrés après le traitement avec l'ozone sur les raisins post-récolte, alors qu'ils diminuent pendant le passerillage. La modification causée sur la paroi cellulaire par l'ozone ainsi que la dureté des pellicules pourraient prédire l'extraction des anthocyanes et des flavanols, grâce à des analyses multi-variées. En conséquence, le traitement avec l'ozone doit être adapté en fonction du cépage et du produit final désiré.

Dans la deuxième partie, les anthocyanes des raisins ont été extraites à partir des raisins et ces extraits ont été fractionnés en trois fractions, glucoside, acetyl-glucoside et cinnamoyl-glucoside, par Chromatographie de Partage Centrifuge (CPC) et CLHP (Chromatographie Liquide Haute Performance) préparative. L'évaluation de l'astringence a été réalisée par des analyses de précipitation protéique avec une protéine modèle BSA (Bovine Serum Albumin) et des protéines salivaires, les anthocyanes réagissant avec ces dernières. Les analyses sensorielles et l'évaluation de l'astringence ont été combinées. La concentration en anthocyane diminue après le traitement avec les protéines salivaires, dans l'extrait total et dans les fractions, en particulier les cinnamoyl-glucosides apparaissent comme les plus réactives avec les protéines salivaires. Les seuils de perception gustatifs ont été calculés avec la méthode "Best estimate threshold" dans le vin modèle. Les fractions acétyl-glucosides et cinnamoyl-glucosides, suivies de la fraction glucoside, possédent des seuils de perception plus bas aux concentrations retrouvées dans les vins. Les descripteurs associés à ces fractions sont l'amertume et l'astringence. Ces résultats démontreraient que les anthocyanes apportent une contribution sensorielle dans la perception du vin en bouche, corrélée à l'acétylation des molécules.

Mot clés : Anthocyanes, Flavanols, Raisins, Ozone, Caractéristiques Sensorielles, Vin

Experimental Section

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Chapter I reports the bibliographic research to approach the experimental sections. A first part is composed by a summary on phenolic compounds present in wine grapes, and their evolution is briefly introduced considering their biosynthesis, chemical properties, and their relevance, concentration and evolution in wine. In the second section, a special focus is given to the extractability of flavonoids from grape skins into wine during the maceration process, analysing the factors, such as the grape localization, their structure, the grape cell wall composition, which represents the main obstacle to their diffusion. In 1.3 a brief summary of grape pre-maceration treatments and winemaking techniques influencing the phenolic compounds extractability is explained. In particular, the use of ozone is resumed to introduce to the experimental parts conducted during the PhD and detailed in Chapters 3 and 4. The forth part, 1.4, is dedicated to sensory properties of flavonoids in wine and physiology and mechanism of in-mouth sensory properties, to approach the second experimental part of the PhD, reported in Chapter 5.

Chapter 2 explain the aim of the study of the three years of PhD. The work, is a collection of papers produced from these researches, and reported here as follow in Chapters 3, 4 and 5. The first two are the result of a first part of the PhD conducted at University of Turin and deal with the technological application in oenology, in particular the use of ozone on grapes, and phenolic compounds changes has been investigated. The latter, is the result of the second part of PhD conducted in University of Bordeaux, and it is related with the phenolic compound -anthocyanins- isolation, purification and involvement in sensory properties.

Chapter 3 reports a study published on the use of gaseous ozone on red wine grapes as fast treatments to understand its effect on phenolic compounds extractability. The aim of this study was to investigate possible indirect physico-chemical effects of ozone treatment on berry skin phenolic composition and

extractability. *Vitis vinifera* L. cv. Nebbiolo and Barbera, chosen for their different anthocyanin profiles, were post-harvest treated for 24 and 72 hours with gaseous ozone (30 μL/L). Skin anthocyanin and flavanol extractability was assessed during maceration using a wine-like solution. From our results, the use of ozone as sanitizing agent in red varieties prior to winemaking process can be considered because it did not negatively affect the extractability of skin anthocyanins and flavanols. Considering these results, further experiments were conducted, and **Chapter 4** presents the results. Over phenolic compounds composition and extractability, also cell wall material was investigated. Moreover, these parameters were evaluated for ozone treatment during wine grapes dehydration (10 and 20% weight loss) for Nebbiolo and Barbera, compared with a controlled withering in air atmosphere. The results showed that the ozone effect depends on the profile and content of anthocyanins and flavanols. In addition, using multivariate analysis, the extractability was correlated with skin cell wall composition and mechanical properties.

Chapter 5 deals with the sensory properties of anthocyanins, which is the second aim of this PhD. Anthocyanins are well-known pigments and their role in wine colour was widely investigated. Moreover, antioxidant activity of anthocyanins determines their contribution to human health. Although their colour and nutritional benefits, their contribution in sensory properties of foods hasn't been largely investigated. Among food, wine preference is strongly connected with sensory quality, such as colour, aroma, taste and mouth-feel attributes. In this part, investigation of sensory properties of grape anthocyanins was carried out throughout chemical analyses as reactivity towards bovine serum albumin and salivary proteins and tasting sessions to assess anthocyanins detection threshold in model-wine solution. This new knowledge about anthocyanins in-mouth sensory properties contribute to understand the perceived food quality and preference.

"Il mio lavoro giovanile era la paura di cadere. Poi è diventata l'arte di cadere. Cadere senza farsi male. Infine, l'arte di non mollare."

"In the beginning, my work represented the fear of falling. Afterwards, it became the art of falling. How to fall without being hurt. Then, the art of being here, in this place."

"Au départ, mon travail c'est la peur de la chute. Par la suite c'est devenu l'art de la chute. Comment tomber sans se faire mal. Puis, l'art d'être ici, en ce lieu."

Louise Bourgeois

1.1 Grape phenolic compounds

Phenolic compounds are among the main secondary metabolites present in grape and in wine. From the grapes maturation till the final wine, passing for the steps of winemaking, several changes occur with the formation of new compounds and disappearance of others. These compounds own peculiar properties: sensorial characteristics, from colour to taste, in-mouth sensations, and health involved features, such antioxidant, anti-cancer, and anti-inflammatory influences. Therefore, their composition in grapes, extraction from berry to must, and evolution in wine has been deeply investigated. Post-harvest practices -before grape crushing- or operations such as maceration, alcoholic and malolactic fermentations, precipitation phenomena, oxidation or adsorption, together with enzymes activity and clarification with fining agents can influence the levels of phenolic compounds during the winemaking process (Balík *et al.* 2008; Kennedy, 2008; Saucier, 2010; Ribéreau Gayon *et al.* 2006a; Garrido *et al.* 2013). This introduction summarizes the basic knowledge about non-volatile phenolic compounds throughout winemaking.

All phenolic compounds are synthesized from the amino acid phenylalanine through the phenylpropanoid pathway. Phenylalanine is in turn a product of the shikimate pathway, which links carbohydrate metabolism with the biosynthesis of aromatic amino acids and secondary metabolites. The general phenylpropanoid

pathway is shown in **Figure 1.1** Two main classes of compounds can be produced: flavonoids (by chalcone synthase) and stilbenes (by stilbene synthase).

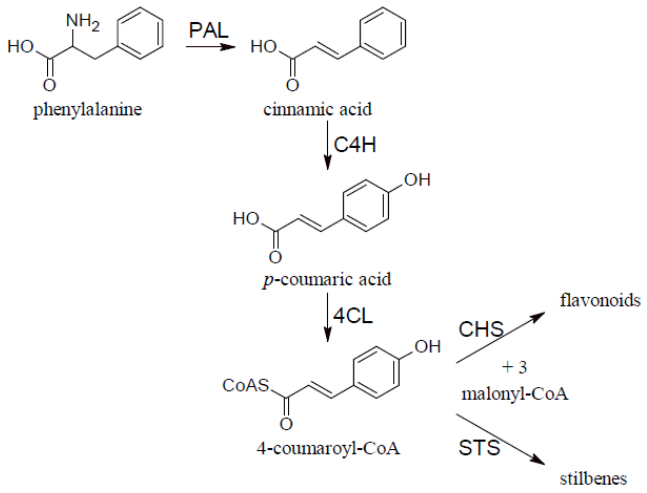


Figure 1.1 General phenylpropanoid pathway. **PAL**, phenylalanine ammonia lyase; **C4**H, cinnamate-4-hydroxylase; **4CL**, 4-coumaroyl-CoA-ligase; **CHS**, chalcone synthase; **STS**, stilbene synthase. *From Flamini et al., 2013.*

Flavonoids are synthesized by two parallel pathways in the grape berry (Winkel-Shirley, 2001). The one is a flavonoid 3′-hydroxylase (F3′H)-mediated branch pathway by which the 3′ position of the B-ring of flavonoids is hydroxylated to produce 3′,4′-hydroxylated flavonoids (also named di-substituted compounds); and the other is a flavonoid 3′,5′-hydroxylase (F3′5′H)-mediated branch that produces 3′,4′,5′-hydroxylated flavonoids (also named tri-substituted compounds) . Di-substituted flavonoids are composed mainly of quercetin-type flavonols, cyanidin-type anthocyanins, as well as catechin (C), epicatechin (EC) and (−)-epicatechin-3-O-gallate (ECG), whereas tri-substituted flavonoids include myricetin-type flavonols, delphinidin-type anthocyanins and epigallocatechin (EGC). The percentage of flavonoids from the two branch pathways determines the sensory attributes of the wine to a certain extent. Except for the genetic factors, the accumulation of flavonoids in grapes is influenced by several external factors.

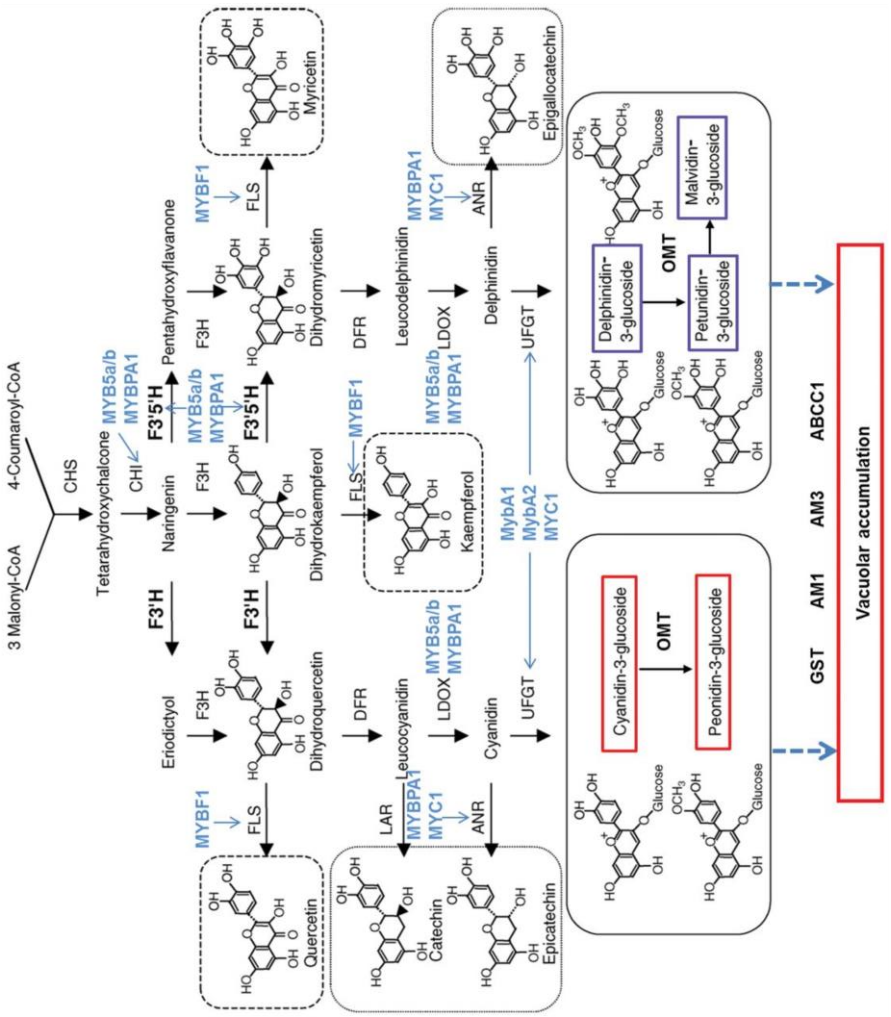


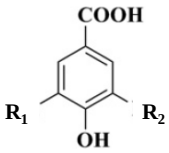
Figure 1.2 Simplified pathways of flavonoid biosynthesis and its regulation in grape. **CHS**, chalcone synthase; **CHI**, chalcone isomerase; **F3H**, flavanone 3-hydroxylase; **F3′H**, flavonoid 3′-hydroxylase; **F3′5′H**, flavonoid 3′5′-hydroxylase; **DFR**, dihydroflavonol 4-reductase; **LDOX**, leucoanthocyanin dioxygenase; **UFGT**, UDP glucose:flavonoid 3-O-glucosyltransferase; **OMT**, O-methyltransferase; **FLS**, flavonol synthase; GST glutathione S-transferase; **AM1/AM3**, anthocyanin multidrug and toxic extrusion transporters; ABCC1, ATP-binding cassette transporter. **ABCC1**, ATP-rusion transporters; **ABCC1**, ATP-binding cassette transporter. Dotted arrows indicate anthocyanin transport by the transporter-mediated model. *From Kuhn et al., 2013.*

1.1.1 Non-flavonoids

The major non-flavonoids compounds found in grape and wine belong to two classes: the phenolic acids and the stilbenes.

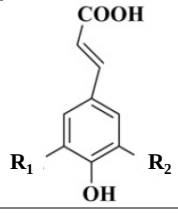
Regarding phenolic acids they are mainly present in grape pulp and can be divided in cinnamic acid derivatives, which owned a structure C6-C1, and benzoic acid derivatives, with a structure C6-C3 (**Table 1.1** and **1.2** for benzoic and cinnamic acids derivatives, respectively). These compounds exist predominantly as hydroxybenzoic and hydroxycinnamic acids and can be found as free or conjugated forms. In grapes, hydroxycinnamic acids are mainly present esterified with tartaric acid, whereas hydroxybenzoic acids are mainly presents as heteroside conjugates. In wine, they can be found in concentration ranging from 100 to 200 mg/L in red wine and from 10 to 20 mg/L in white wine (Ribéreau-Gayon *et al.* 2006b).

Table 1.1 Benzoic acid structure and common derivatives



Compound Name	R_1	R_2
p-hydroxybenzoic acid	H	H
protocatechuic acid	OH	H
vanillic acid	OCH_3	H
gallic acid	OH	OH
syringic acid	OCH_3	OCH_3

Table 1.2 Cinnamic acid structure and common derivatives

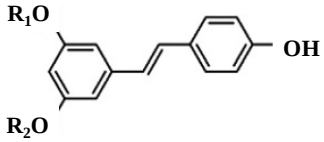
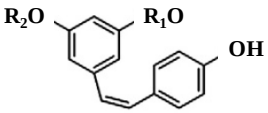


Compound Name	R_1	R_2
p-coumaric acid	H	H
caffeic acid	OH	H
ferulic acid	OCH_3	H
sinapic acid	OCH_3	OCH_3

Stilbenes are produced from several plants as response to biotic, such as fungal disease, or abiotic stresses, such as UV irradiation (Langcake & McCarthy, 1997, Wang *et al.* 2010). They belong to the class of phytoalexin, which are related to diseases resistance. The main stilbenes found in wine is resveratrol, which is a

monomer, as *cis* and *trans* forms, (with the latter form prevalent on the other one), whereas in grape only *trans*-resveratrol has been detected (Mattivi *et al*., 1995; Bavaresco *et al.*, 2002), as well as its glucosylated form, the piceid. Nevertheless, dimer and polymerized forms, the so-called viniferins, can occur in plants and **Table 1.3** shows the main stilbenes compounds in grape (Castellarin *et al*., 2012).

Table 1.3 Stilbene monomers structure.

	Compound Name	**R_1**	**R_2**
	*trans*-resveratrol	H	H
	trans-piceid	H	Glu
	trans-pterostilbene	CH_3	CH_3
	*cis*-reveratrol	H	H
	cis-piceid	H	Glu

Stilbenes are presents in several plant parts, as stems and roots; in grape berry, even if their presence has been reported in both seeds and pulp, they are mainly located in grape skin and extracted during winemaking in wine. Therefore, winemaking process taking into maceration whole bunches (not destemmed) increases the concentration in wine (Bavaresco *et al.* 1999; Sun *et al.* 2006;). High content of stilbenes is auspicable since they have been proved to be highly anti-cancerogenic, anti-oxidant, and anti-inflammatory, and to protect against cardiac diseases as reported by several *in vitro* studies (Baur *et al.* 2006; Richard *et al*. 2011; Anastasiadi *et al.* 2012; Nassra *et al.* 2013).

1.1.1 Flavonoids

Flavonoids represents the most abundant class of phenolic compounds in grape and wine and they strongly contribute to the organoleptic and visual quality of wine. All flavonoids share a C6-C3-C6 skeleton consisting of two phenol rings (named A and B), linked together by a heterocyclic pyran ring (C-ring) (**Figure**

1.3). Among them several classes can be distinguished on the basis of the oxidation state of the C-ring.

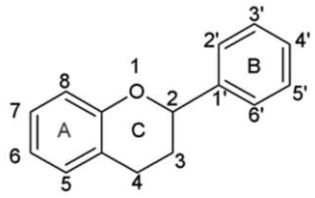


Figure 1.3 Flavanoids general structure: the C6-C3-C6 skeleton.

The main classes of interest in wine are anthocyanins, flavanols or procyanidins, and flavanols (**Figure 1.4**). Nevertheless, in a lesser extent, flavones and flavanononones have been found in minor concentration in grape and wine (Fang *et al.* 2008; Zoecklein *et al.* 1995; Jandera *et al.* 2005; De Sanctis *et al*. 2012). In the next section a deeper insight will be given to the major flavonoid classes involved in wine quality.

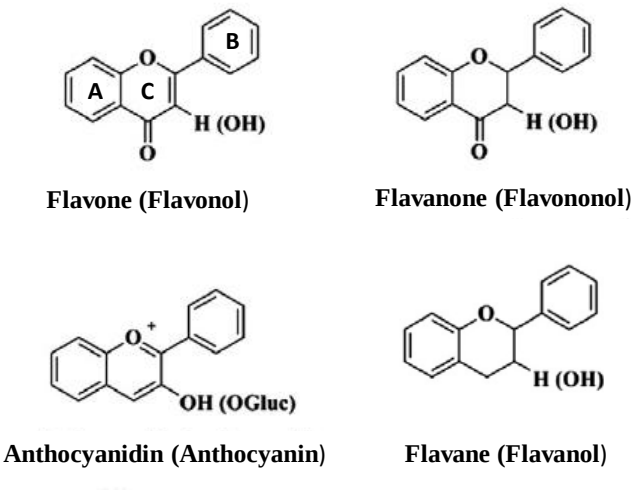
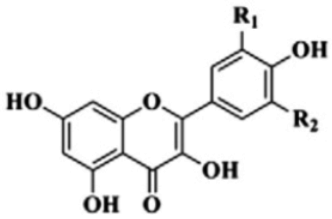


Figure 1.4 Skeleton of the main flavonoids in grape.

1.1.1.1 Flavonols

Flavonols are widespread in the plant kingdom, and their role is connected to UV and photo-protection because of their ability to absorb both UV-A and UV-B wavelengths (Price *et al.* 1995; Haselgrove *et al*. 2000). Flavonols are C6-C3-C6 compounds in which two hydroxylated benzene rings (A and B) are joined by a chain of three carbon which is part of the heterocyclic C ring with a 3-hydroxyflavone backbone, and a double bond. They differ by the number and type of substitution of the B ring, and **Table 1.4** show the main flavanols found in grape and wine. All these compounds are found in grape skins as 3-*O*-glycosylated form, where the sugar can be represented by a glucoside, galactoside, rhamnoside, rutinoside and glucoronide, where the former is largely the most abundant (Cheynier & Rigaud, 1986). Bigger molecules, as flavonols diglucosides are often found in grape.

Table 1.4 Flavonol structure and common derivatives



Compound Name	R_1	R_2
Quercetin	OH	H
Kaempferol	H	H
Isorhamnetin	OCH_3	H
Myricetin	OH	OH
Laricitrin	OCH_3	OH
Syringetin	OCH_3	OCH_3

Regarding their individual concentration, it depends from the grape varieties, and white wine grapes lack of trihydroxylated flavanols, i.e. myricetin, laricitrin, and syringentin since their synthesis enzymes are not present in the white winegrapes flavonoids pathway (Downey *et al.* 2003; Castillo-Munoz *et al.* 2007; Castillo-Munoz *et al.* 2010; Mattivi *et al.* 2006). Regarding red wine grapes varieties, a higher content of quercetin was found in Nebbiolo, Sangiovese, and Pinot Noir,

whereas a higher content of myricetin is usually found the other red wine grapes (Mattivi *et al.* 2006). Methylated derivatives are in general less abundant. Total concentration of flavonols in grape can reach up to 80 mg/Kg and they are presents in the outer layer of skin (Flamini *et al*. 2013), and even if the genomes control the profile, the concentration can be strongly affected by cultural practice, above all the ones which influence the sunlight exposure.

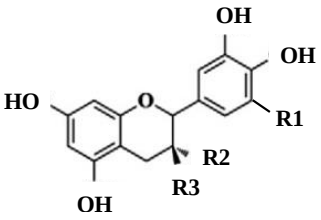
Flavonols are characterized by yellow colour, which is considered to be directly involved in white wines, whereas is masked in red wine by the presence of anthocyanins. Nevertheless, they cover a relevant role in the anthocyanins copigmentation: phenomena given by the interaction between anthocyanins and other compounds (in particular flavanols and flavonols) that can lead to an enhancement of wine chromatic characteristics (Boulton, 2001).

1.1.1.2 Flavanols

With the terms flavanols are generally defined the monomer constituting the condensed tannins. These molecules own great relevance in the vegetal kingdom and arise uprising interest in the scientific fields for their health-benefit and sensorial properties.

Flavanols can be found as monomer or polymerized as oligomer (2-5 units) and polymer (more than 5 units) and they can be differentiate depending on the subunits, the subunits position, subunits orientation, the linkage type and the presence of a subunit not belonging to flavan-3-ols. The monomers are (+) catechin, (-) epicatechin, (+) gallocatechin, (-) epigallocatechin and (-) epicatechin-3-*O*-gallate (**Table 1.5**). These are the main components of grape proanthocyanidins (+) catechin and (-) epicatechin are common called procyanidins, whereas (+) gallocatechin and (-) epigallocatechin are called prodelphinidins, since their depolymerization in acid condition at high temperature gives an anthocyanin, namely cyanidin and delphinidins for procyanidins and prodelphinidins, respectively (Bate-Smith, 1954).

Table 1.5 Flavanol structure and common derivatives.



Compound Name	R_1	R_2	R_3
(+) Catechin	H	H	OH
(-) Epicatechin	H	OH	H
(+) Gallocatechin	OH	H	OH
(-) Epigallocatechin	OH	OH	H
(-) Epicatechin-3-*O*-gallate	H	-*O*-gallic acid	H

The flavan-3-ols units can be linked through two type of linkage: the "Type B linkage", when the C4 carbon of the upper unit is linked to the C8 or C6 carbon of the lower units, or "Type A linkage", when in addition to this, there is a bond between the upper unit C2 carbon of cycle C and the lower unit C7 or C5 of the cycle A (**Figure 1.5**) (Creasy & Swan, 1965; Jacques *et al.* 1973; Appeldorne *et al.* 2009).

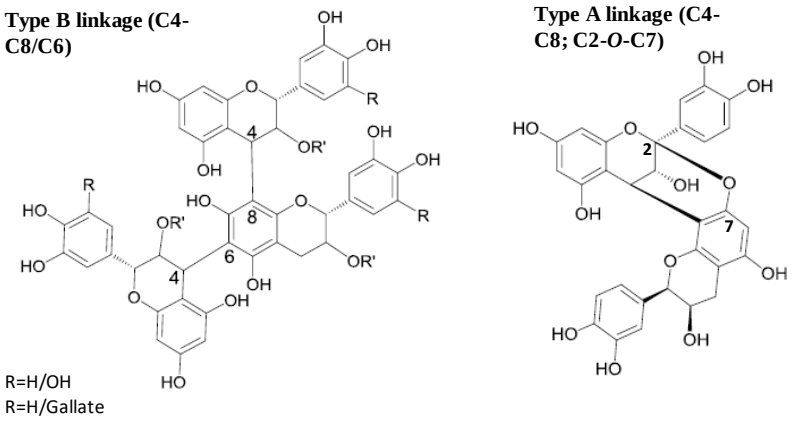


Figure 1.5 Type B and Type A linkage between flavanol monomers.

Condensed tannins structure identified by literature is mainly composed by linear, more or less branched structures and polymers up to 20 units have been recently

identified in seeds (Ma *et al.*, 2018). Nevertheless, recently, also cyclic tannins, the so-called "crown" proanthocyanidins, from tetramer up to hexamer have been discovered in grape skins and wine (Zeng, 2015; Longo *et al*. 2018).
Proanthocyanidins are located in the cell wall (the most) and vacuole (in lesser extent) of skins (Gagné et al. 2006). On one hand, in grape seeds prodelphinidins are not present, whereas is very common the presence of the galloylated group. On the other hand, prodelphinidin are present in grape skins and is uncommon the presence of galloylated units (Kennedy *et al.* 2001; Gagné *et al.* 2006). Moreover, skins proanthocyanidins own a higher mean degree of polymerization with respect to seed proanthocyanidins (Prieur *et al.* 1994; Souquet *et al.* 1996).

In general, condensed tannins are extracted during the alcoholic fermentation and the maceration, and the concentration of condensed tannins in red wine ranges from 1 to 4 g/L, whereas in white wines it ranges from 100 to 300 mg/L (Ribéreau-Gayon *et al.* 2006b). Nevertheless, winemaking techniques can strongly influence the concentration and the characteristic of condensed tannins extracted in wines, as well as the aging conditions.

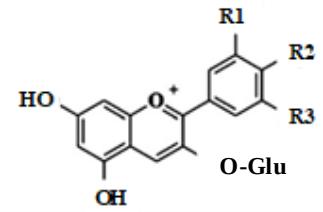
1.1.1.3 Anthocyanins

Anthocyanins are natural pigments responsible for the red, purple, blue and cyan colour of several flower, fruits, and in lesser extent of other plant tissue. Thanks to their attractive colour they are widely used in food industry as colorants, moreover they are related to several health benefit properties (Andersen *et al.* 2006; He & Giusti, 2010). They are natural antioxidant and they act against chronic inflammation and cardiovascular hypertension, and contribute at cancer prevention; for these reasons their application in health beneficial products have been widely investigated (Scalbert *et al.* 2005; Nichenamela *et al.* 2006; Tucker *et al.* 2008).

In plant kingdom, anthocyanins play roles in UV protection, pollinator attraction and seed dispersal agent attraction (Pecket & Small, 1980; Moskowitz &

Hrazdina, 1981). In grape, they are presents in the vacuole of skin cell, and in the *teinturier* varieties, in the pulp cell as well.

Table 1.6 Grape anthocyanins.



Compound Name	R_1	R_2	R_3
Cyanidin	OH	OH	H
Peonidin	OCH_3	OH	H
Delphinidin	OH	OH	OH
Petunidin	OCH_3	OH	OH
Malvidin	OCH_3	OH	OCH_3
Pelagordin	H	OH	H

Structurally, grape anthocyanins are heterosides of an aglycone (anthocyanidin) differentiated among themselves on the number of hydroxylated and methoxylated groups in the anthocyanidin, the nature and the number of bonded sugars in their structure, the aliphatic or aromatic carboxylates bonded to the sugars in the molecule and the position of this bond. The main anthocyanins present in red wine grapes form *Vitis vinifera* L. are delphinidin, cyanidin, petunidin, peonidin and malvidin, which are present as monoglucoside, acetyl-monoglucoside, caffeoyl-monoglucoside and *p*-coumaroyl-monoglucoside derivatives (**Table 1.6**), where the individual anthocyanidins and esterification can strongly influence their colour features, reactivity and stability in wine. Rarely, anthocyanins acetylated with other organic acids, such as the lactic or ferulic acids, have been reported in literature (Alcade-Eon *et al.* 2006; Castaneda-Ovando *et al.* 2009; Valls *et al*. 2009).

Anthocyanins are derivatives of a flavylium ion and have positive charge: considering the double bond in the molecule, the charge is delocalized on the cycle stabilizing it for resonance. Four different anthocyanins structure exist in equilibrium depending on the medium pH: the flavylium cation (red, pH<2), the

quinoidal base (blue, pH 2-7), the hemiketal or carbinol pseudo-base (colourless, pH 4.5-6), and chalcone (colourless) (Brouillard & Dubois, 1977) (**Figure 1.6**). Clearly, for higher pH, higher is the contribution of the chalcone form, leading to a less coloured wine. At wine pH (between 3.2 and 4.5), the four form coexists: the colourless form (quinoidal base) represents from 40 to 60% of anthocyanins, the flavylium cation represents from 5 to 35%, and the the hemiketal from 8 to 15% of anthocyanins (**Figure 1.7**).

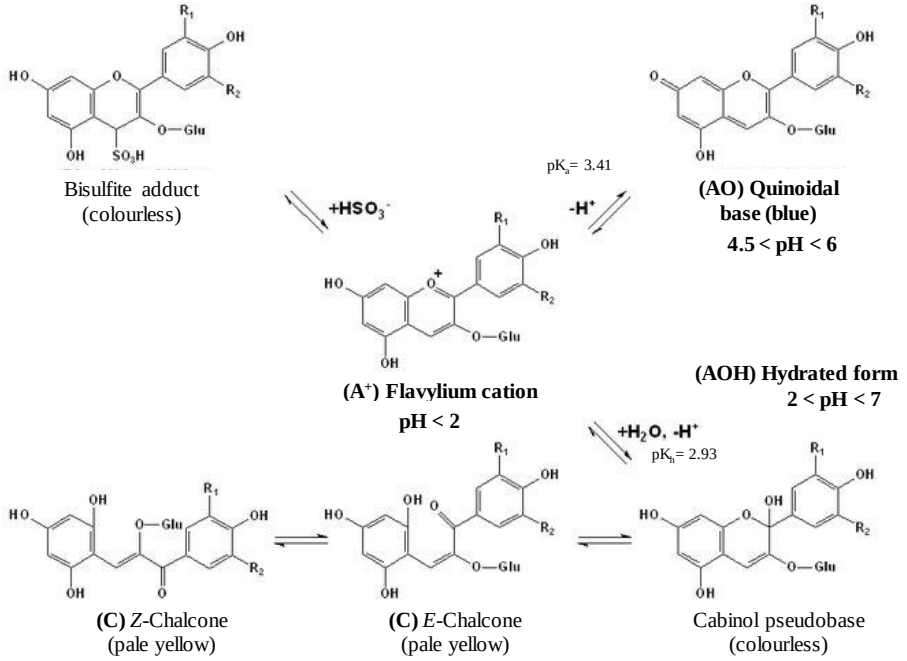


Figure 1.6 Different anthocyanins forms depending on the solution pH.

Anthocyanins in flavylium form are attachable from sulphur dioxide (SO_2) which at wine pH sulphur dioxide is largely presents as anion HSO_3^- leading to a colourless adduct (**Figure 1.6**). During ageing anthocyanin complex formation give the possibility of a stable colour since these structures are not anymore attachable by sulphur anion, leading to a colour stability (Berké *et al.* 1998).

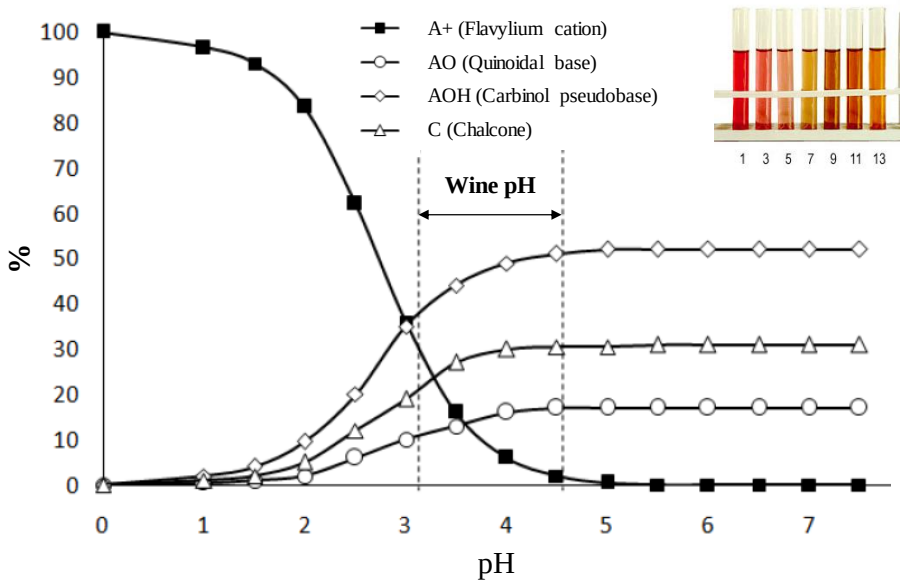


Figure 1.7 Proportion of the different anthocyanins forms depending on the solution pH. *Adapted from Zeng, 2015.*

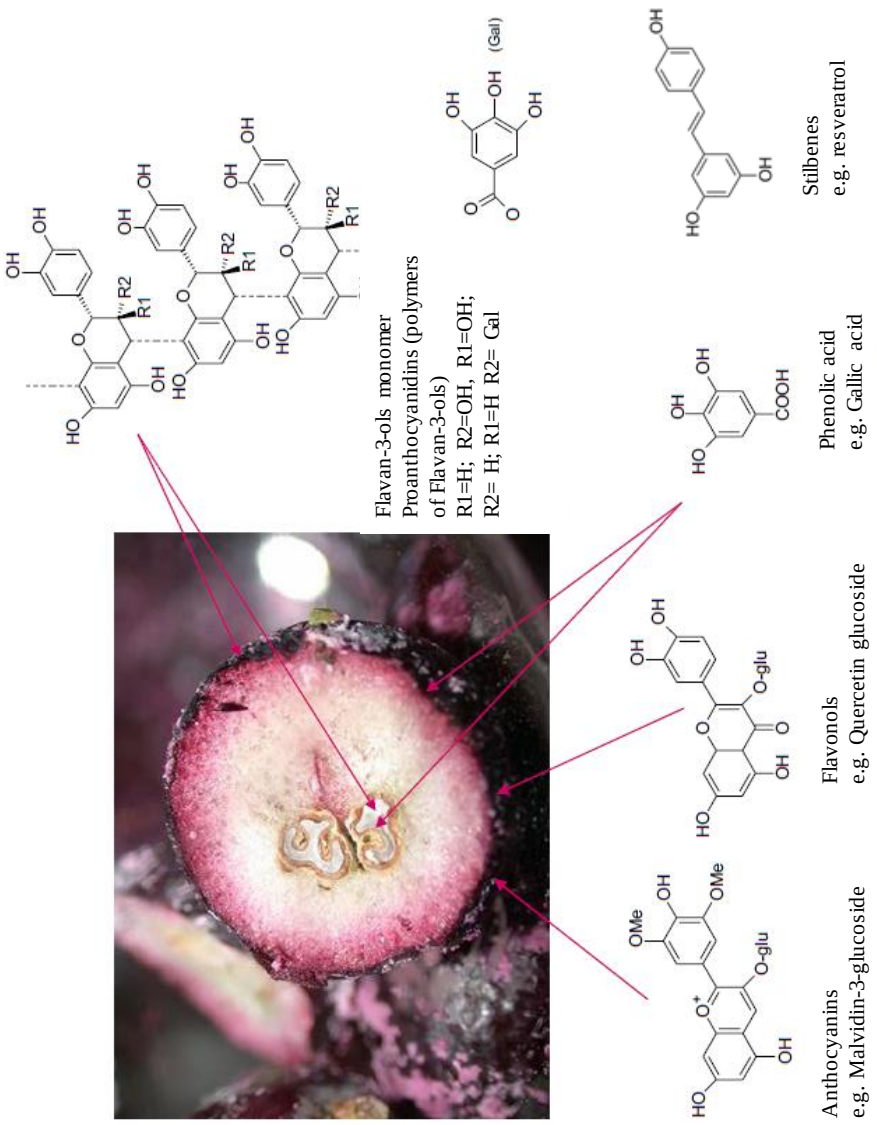


Figure 1.8 Cross-sectional picture of a red grape berry and phenolic compounds present in skin, pulp, and seed. *Adapted from Pinelo et al. 2006.*

1.2 Flavanoids, from grape to wine

1.2.1 Flavonoids accumulation during ripening

Flavanols start their accumulation from the beginning of fruits formation, reaching their maximum accumulation at veraison. After, modification in their degree of polymerization occurs. Regarding flavonols, their synthesis begins in the flower buttons and the highest concentrations is found a few weeks after veraison. Then, it stabilizes during early fruit development and decreases as the grape berries increase in size. From veraison, also anthocyanins are synthetized in the cytosol and translocated into cell vacuole, thanks to tonoplast transported or through vescicular trafficking, where they are stored (Ribérau-Gayon *et al*. 2006b; Serrano *et al*. 2017). The branch of the flavonoid pathway leading to flavonol and anthocyanins biosynthesis has been suggested to be light dependent (Martínez-Lüscher *et al.* 2014). Anthocyanins synthesis is also stimulated by exogenous elicitors such as ormons, i.e. abscisic acid (ABA), jasmonate compounds, ethylene, and salycilic acid, as well as other compounds such as chitosan, yeast derived products containing oligosaccharides (Flamini *et al.* 2013).

Phenolic compounds concentration and profile depends mainly on the grape variety but the degree of ripeness, the growing region, seasonal features and vineyards practice can modify them (Kuhn *et al*. 2013; Massonnet, *et al*. 2017).

Among environmental factors which can influence polyphenols accumulation sunlight, temperature, and water management are very important. Intense sunlight causes excessive sunburn in exposed berries and reduces the anthocyanin accumulation, and if associated with high temperature can also inhibit the colour development. Thus, for the maximum production of anthocyanins in grape berries, moderate sunlight exposure is necessary, but the extent varies among different cultivars. It has been demonstrated that UV irradiation can stimulate the expression of the genes involved in the anthocyanin biosynthesis and hence result in the enhancement of anthocyanin accumulation (Berli *et al*. 2010). Generally, low temperatures, such as 25 °C, favour the anthocyanin biosynthesis, whereas high temperatures, such as 35 °C, are associated with anthocyanin degradation and inhibition of anthocyanin accumulation (Mori *et al*. 2005). Water status is an important environmental factor that can influence anthocyanin biosynthesis: during ripening. In fact, under water deficit conditions, anthocyanin biosynthesis can be greatly stimulated resulting in enhanced anthocyanin accumulation (Castellarin *et al*. 2007).

1.2.1.1 Flavonoids change during grape overripening and dehydration

Grapes dehydration is a widespread technique used in wine industry in order to produce high quality dry and sweet wines. On the contrary of drying process, where fast water removal avoids grapes over-ripening and senescence metabolism, dehydration involves slow water removal and, as consequence, grape berry composition changes in function of metabolic response to the water stress. Dehydration, called “withering” in wine field, can be classified according to the environmental condition as: “on-vine withering”, when grape bunches are attached to the plants and over-ripening process occurs; or in detached bunches as “natural withering”, if dehydration occurs without controlled environmental condition or “forced withering”, better defined as “controlled”, since the environmental conditions such as temperature, relative humidity and air flow are controlled using technology during the process (Mencarelli & Tonutti, 2013).

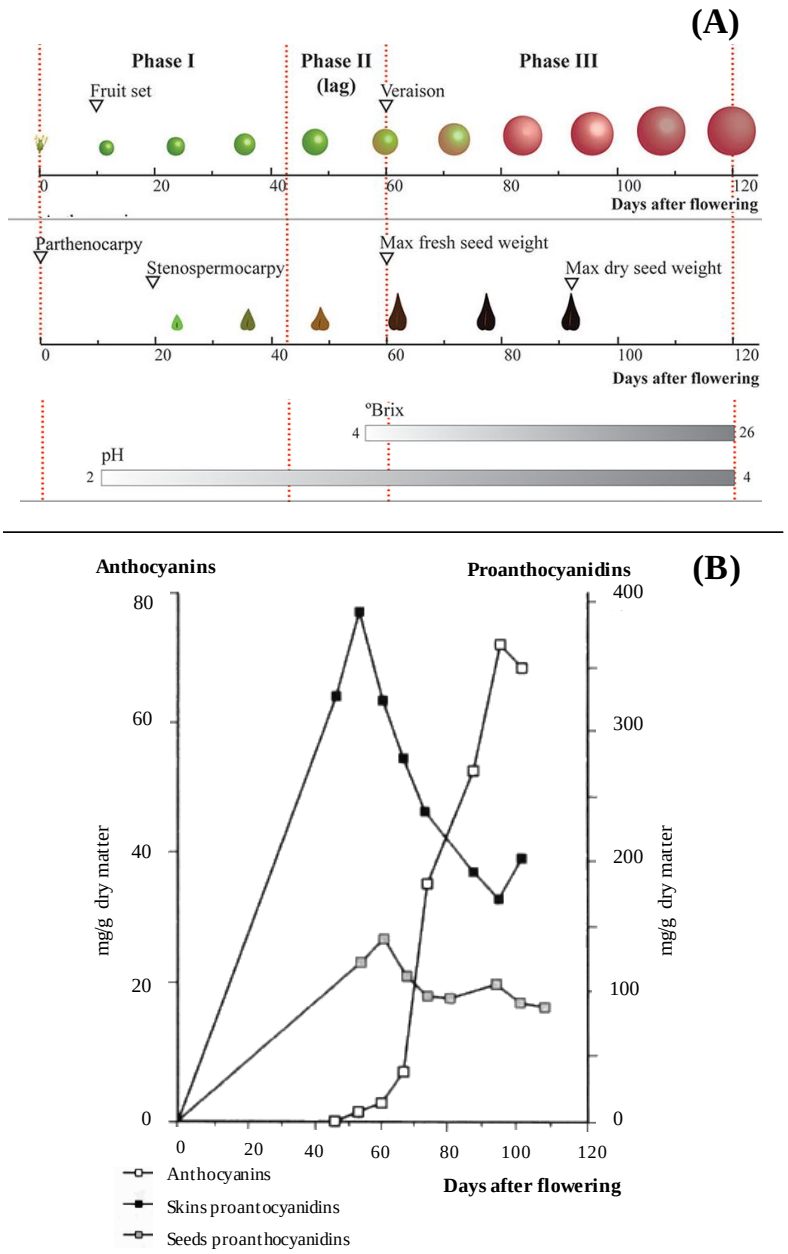


Figure 1.9 Scheme of the most important changes that berries and seeds undergo during development. **(A)** Changes in size, color, brix degree, and pH during berry ripening and seed development. Adapted from Serrano *et al.* (2017). **(B)** Accumulation of anthocyanins and proanthocyanidins during grape berry development. From Ribéreau-Gayon *et al.* (2006)a.

Detached bunches continue to function metabolically, but metabolism is different from that of in-vine ripening/over-ripening, given by lack of water, mineral, and energy supplies. In particular, grape response during withering is strongly correlated with the metabolism response to the water stress: if concentration of cell solutes is common for all the varieties, the metabolic response may vary in relation to genotype, therefore the variety, and environmental condition, in particular the intensity and rate of stress.

Several genes related to withering have been identified in order to understand grape berry metabolism (Zamboni *et al.* 2008; Rizzini *et al.* 2009; Zamboni *et al.* 2010; Versari *et al.* 2001; Bonghi *et al.* 2012). The stress response covers a wide range of metabolic pathways: hexose metabolism, cell wall and lignification, and in particular secondary metabolism with aroma and polyphenols modification. Regarding the latter, several changes can occur on two different sides: on one hand the decrease given by oxidation (due by an increase of enzymes related to stress such as peroxidase, laccase, polyphenols oxidase), and on the other hand by the up-regulation of some genes of phenylpropanoid pathway. Among polyphenols, stilbenes and flavonols genes are up regulated, leading to an increase of these compounds in withered berries (Versari *et al.* 2001; Bonghi *et al.* 2012). On the contrary, a marked decreased of flavanols, in particular monomeric forms, such as catechin, has been observed since there is no induction in neo-synthesis and they are easily oxidable (Bonghi *et al*. 2012; Rolle *et al.* 2013, Torchio *et al.* 2016). As well, changes in the mean degree of polymerization (mDP) of proanthocyanidins has been reported, leading to a decrease in the average mDP (Moreno *et al.* 2008; Ossola *et al.* 2017). Regarding anthocyanins, controversial datas were found. In general, no changes in UFGT gene has been found (Tonutti *et al.* 2004; Zamboni *et al.* 2010; Bonghi *et al.* 2012), therefore no evidence of neo-synthesis in detached berries has been found. Increase content of anthocyanins in withered grapes (Mencarelli *et al.* 2010), can be due to concentration, but on the other hand results showing a decrease of this class as well as no significant changes (Moreno *et al.*, 2008; Bellincontro *et al.* 2009;

Bonghi *et al.*,2012; Torchio *et al*. 2016; Ossola *et al.* 2017), can lead to the hypothesis that can be oxidized during the process depending on the variety, the variety anthocyanins profile, the dehydration techniques, the rate and intensity of dehydration. One important point to preserve pigments is therefore the dehydration condition, in particular low temperature preserve the grape anthocyanins (Del Caro *et al.* 2004), as well as harvesting health grape berries and maintain their status integer throughout dehydration.

1.2.2 Phenolic compounds extractability

During the maceration phase of red grapes winemaking, the grape solids remain in contact with the juice, and phenolic compounds are extracted from skins and seeds through a diffusive process which can be influenced by several factors. For anthocyanins, which are located in cell vacuole, fast extraction occurs from the beginning, followed by a concentration decrease (Boulton *et al*. 1996; Setford *et al*. 2017). For flavanols, extraction is different since there are mainly located in cell wall structure, therefore the disruption of cell integrity is more impactant: in particular an initial lag phase is observed, followed by an increased extraction favoured by the ethanol production by the alcoholic fermentation (Boulton *et al*. 1996). The ethanol content is particularly important for seed flavanols since their extraction needs a disorganization of outer lepidic cuticle surrounding the seeds (Hernández-Jiménez *et al*. 2012). Regarding the skin, endogenous enzymes activities favour the phenolic compounds extractions (Pardo *et al*. 1999; Bautista-Ortín *et al*. 2005). Several winemaking techniques have been developed to improve and control the phenolic compounds extraction, concerning the solid-liquids contact and movement, the use of temperature, addition of exogenous enzymes and tannins, and the management of sulphur dioxide addition. The main goal of these techniques is both enhancing the extraction than the preservation of extracted compounds.

1.2.2.1 Grape skin layers

Grape skin is composed mainly by three layers: 1) the outermost layer, the cuticle, composed by hydroxylated fatty acid and cover by hydrophobic waxes. This is followed by 2) an intermediate epidermis, composed by one or two layers, which is characterised by a regular tilling of cells. The 3) inner layer is the hypodermis, which is composed by several cell layers that contained the major parts of phenolic compounds of grape skin (Lecas & Brillouet, 1994). **Figure 1.10** shows this structure.

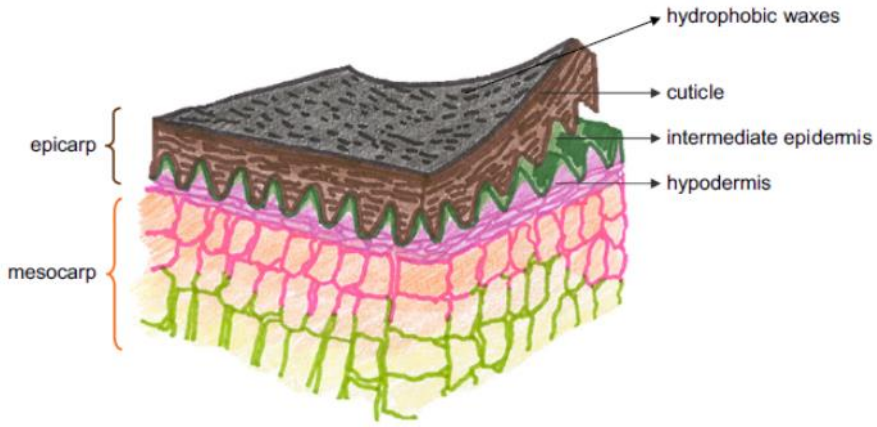


Figure 1.10 Different layers of the grape skin (From Pinelo *et al.* 2006).

Regarding the cell structure, we can identify in general three layers (Raven *et al*. 1999). The first, the middle lamella, which bind the cells together and it is composed mainly by pectic material. Then, the primary cell wall, which is thicker than middle lamella and consists of three parts: 1) fundamental cellulose-xyloglucan framework, 2) a pectin polysaccharides matrix, 3) structural proteins (Bidlack, 1992; Carpita & Gibeaut, 1993).

The third part is represented by the secondary cell wall, thicker than the primary, consisting of cellulose microfibrils, hemicellulose, pectins, and lignin (Bidlack, 1992). The secondary cell wall is formed when the cell has stopped growing, and

it is a derived by the primary cell wall by thickening and including lignin in the structure (Raven *et al.* 1999) While in the grape seeds this is formed during the seed coat formation (Haughn & Chaudhury, 2005), it is unclear its presence in grape berry cell. Nevertheless, the skin softening during ripening it is directly in contrast with this hypothesis (Hanlin *et al*. 2010).

Since the most of skin phenolic compounds are entangled in cell wall, it is surely to be considered as the first barrier to their diffusion. In the next section, the composition of primary cell wall will be detailed.

1.2.2.2 Grape skin cell wall

Grape berry cell wall (CW) structure is based on the type I model of primary cell walls (Carpita & Gibeaut, 1993). Therefore, grape skin CW is mainly formed by cellulose microfibrils, these are embedded in a matrix of pectins, hemicelluloses (generally defined as cell wall polysaccharides) and structural proteins, whereas phenolic compounds are entangled or linked into the matrix (Pinelo *et al.* 2006).

Cellulose is formed by linear chain of β (1→4) linked D-glucosyl residues associated by hydrogen bonds to form microfibrils. This cellulose network is embedded in xyloglucans consisting in a backbone of β (1→4) D- glucosyl with side chains of xylosyl, galactosyl, and fucosyl residues (Albersheim, 1975; de Vries & Visser, 2001).

Regarding the pectin matrix, can be divided in acid and neutral pectins (de Vries & Visser, 2001; Arnous & Meyers, 2009). Acid pectins are composed by three major components: 1) homogalacturonans, consisting of linear homopolimers of galacturonic acid partially esterified with methanol, and 2) rhamnogalacturonans type I, which are polymer of rhamnose and galacturonic acid inserted in homogalacturonans network, and 3) rhamnogalacturonans type II, which are very complex polymers of galacturonic acid with branches of rhamnose and other monosaccharides. Neutral pectins (5%), consist in 1) arabinans, small linear polymers of arabinose, 2) arabinogalactanes type I and 3) arabinogalactanes type

II which are polymers of galactose with ramification of arabinose, the former, and linked with proteins, the latter. Among these, homogalacturonans represents the 80% of total pectins.

Hemicellulose is formed by several polymeric structures in which xyloglucan - backbone of cellulose with side chains of xylose, galactose and fucose residues- is the most abundant (de Vries & Visser, 2001).

Pectins and hemicelluloses formed cross linked complex, tightly linked with a non-polysaccharidic components of cell wall: lignin, which is the result of the enzymatic polymerization of phenols monomer (p-coumaric, ferulic, diferulic, synaptic, cinnamic, and p-hydorxybenzoic acids) between themselves and between lignin monomers and polysaccharides providing structural rigidity to cell wall (Jung, 1989; Bidlack, 1992). In fact, lignification is the results of an oxidative coupling of phenols monomer producing free radicals that react spontaneously to form lignins and additional cross-links between lignin and cell wall polysaccharides. In this complex, phenolic compounds are bound or entangled in the lignin-polysaccharides matrix of the skin CW material.

Cell wall composition is variety-dependant (Ortega-Regules *et al*. 2006b; Apolinar-Valiente *et al*. 2015), but ripeness degree can strongly influence the polysaccharides degradation, since as higher the ripening the higher decomposed are the pectins, due to berries enzymes activities (Nunan *et al.* 2001).

In general, the CW composition is (Lecas & Brillouet, 1994):

- 30% neutral polysaccharides
- 20% acid pectin substances
- 15% insoluble proanthocyanidins
- 5% structural proteins

1.2.2.3 Factors affecting extractability of phenolic compounds during maceration

During winemaking process phenolic compounds are extracted from berry solids parts (skins and seed) into juice through two main mechanisms, an instantaneous leakage from the broken skins cell at crushing, and a second slower concentration-driven diffusion that occurs from solids to liquid during maceration (Setford *et al*. 2017). This diffusion process can be influenced by several factors. Among them the more important are explained below.

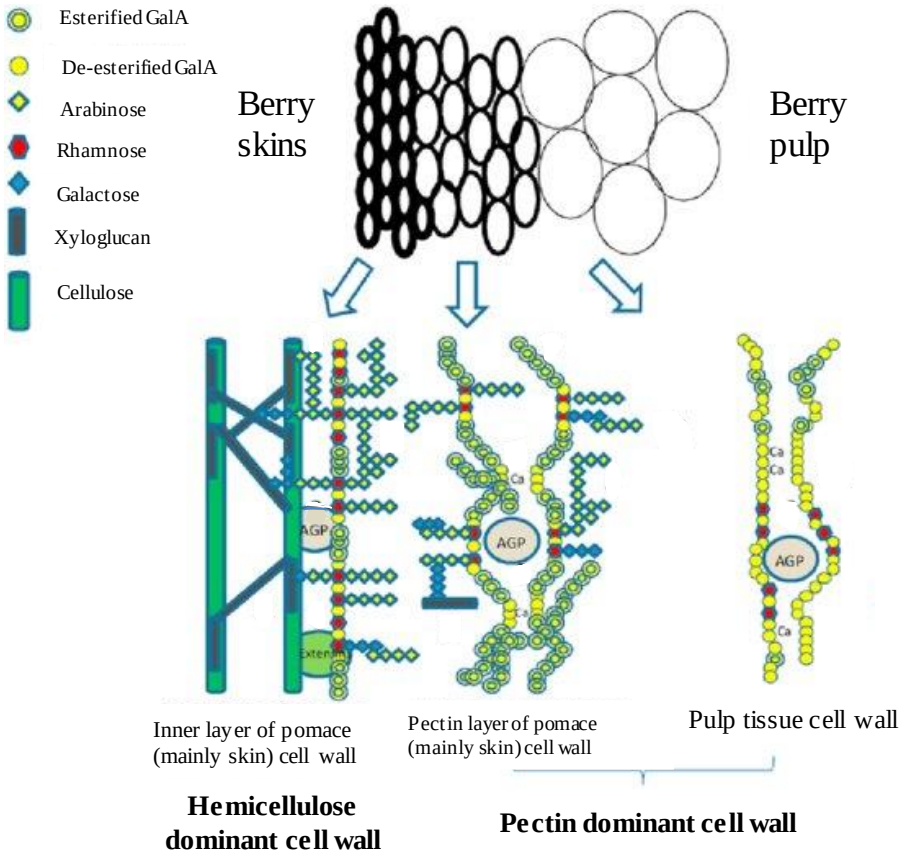


Fig. 1.11 A proposed model of the grape berry cell wall. *From Gao et al. (2016).*

- ***Temperature***

Temperature plays an important role since it influences the permeability of the cell membrane in the grape solids (Koyama *et al*. 2007). Moreover, it influences strongly the rate of fermentation and therefore to the production of ethanol. Therefore, several methods are employed from winemakers involving the use of more extreme temperatures to promote the extraction of phenolic compounds from the grape solid parts. Among them, techniques which imply high temperatures reported are thermovinification, microwave maceration, and flash release (Aguilar *et al*. 2015; El Darra *et al*. 2013; Carew *et al.* 2014; Doco *et al.* 2007; Morel-Salmi *et al.* 2006; Smith *et al.* 2015). On the contrary, other techniques involving cooling or freezing grape berry/must are reported, given by the ability of freezing to damage cell membrane and improved extraction and to inhibite oxidation prior alcoholic fermentation (Koyama *et al*. 2007; Sacchi *et al.* 2005; Gil-Muñoz *et al*. 2009).

- ***Endogenous and exougenous enzymes***

Pectin polysaccharides undergo major changes during fruit ripening since are extensively decomposed by enzymes activities. The main grape enzymes involved are the pectinase: pectin lyase, pectin methyl esterase and polygalacturonase. Their role in maceration is the breaking down of the grape berry cell walls favouring the phenolic extraction first stages of maceration due to the increase and speed up the breakage of skin cell walls (Pardo, 1999; Bautista-Ortín *et al.* 2005; Benucci *et al*. 2017). Other enzymes may facilitate extraction, such as cellulase and β-galactosidases (Pardo, 1999).

- ***Grape post-harvest treatments***

After harvest, fruits remain metabolically active and react to internal and external stimuli and stresses until death occurs, resulting in compositional changes. Postharvest strategies are generally aimed at reducing metabolic activity and at

maintaining the physicochemical properties of fruit at harvest. In case of grapes dehydration, together with metabolites concentration, physical-chemical changes positively affect the metabolic content (Schreiner & Huyskens-Keil, 2006). As well, several post-harvest treatments aim to preserve grape berry status or to reduce or modify the berry microbiota. Among post-harvest treatments, recently applied there are the use of gaseous ozone, electrolyzed water, methyl jasmonate or ethylene treatments, the control of temperature (cold or heat treatment) or the application of altered atmosphere such as nitrogen (Bellincontro *et al*. 2006; Ruiz-Garcia *et al*. 2012; Carbone & Mencarelli, 2015; Botondi *et al*. 2015; Cravero *et al.* 2016 Modesti *et al.* 2018).

- ***Maceration techniques***

During fermentative maceration, the grape solids parts rise to the top of the vessel and form a cap resulting from the upward force of the produce carbon dioxide. This involve a minor contact between solids and liquids (Sacchi *et al*. 2005). Several techniques are used to manage the cap: pumping liquid from the bottom to the top of the cap ("pump-over", or *delestage* if the liquid is completely removed), punching down the solid parts (manually or mechanically), or using rotatory tank (Sacchi *et al*. 2005; Ribéreau-Gayon *et al.* 2006b). Also, temperature management, as explained above, can be considered as a maceration technique as well as the maceration time.

- ***Sulfur dioxide and ethanol content***

Increasing ethanol content results in a greater extraction of anthocyanins and proanthocyanins (Canals *et al.* 2015). This is particularly important regarding the extraction of seeds proanthocyanidins. Regarding the content of sulphur dioxide (SO_2) at normal levels associated with winemaking appear to have lower effects. In fact, no significant differences were found in phenolic compounds extraction using SO_2 concentration from 0.5 to 100 mg/L (Watson *et al.* 1995).

1.2.3 Phenolic compounds evolution in wine

1.2.3.1 The fate of anthocyanins in wine

- ***Copigmentation***

Copigmentation is given by molecular association between anthocyanins and other (usually non-coloured) organic molecules in wine, called "cofactors" (Boulton, 2001). Usual cofactors involved in intermolecular copigmentation belong to phenolic acid, i.e. ferulic and caffeic acids, chlorogenic acid, hydrolysable tannins, and flavonoid belonging molecules, in particular flavanols, i.e. catechin, and flavonols, i.e. quercetin (He *et al.* 2012; Trouillas *et al.* 2016). As well, self-association between anthocyanins themselves can occur. In both the cases, anthocyanins can undergo in copigmentation complex formation as both flavylium (A+) and quinoidal base (AO) forms thanks to non-covalent bond such as Van der Waals and hydrophobic interactions leading to π-π stacking. This phenomenon leads to a hyperchromic effect (higher absorption) and a bathochromic shift (maximum absorbance moves to higher wavelength)(Boulton, 2001). It is generally assumed that in young wine, copigmentation can influence up to the 50% of wine colour contribution (Boulton, 2001; Cavalcanti *et al*. 2011).

- ***New adducts formation***

Since from the beginning of maceration, together to an increase in anthocyanins, an increase of polymeric pigments is observed, this is given by the simultaneous extraction of other compounds, i.e. flavanols, or yeast metabolites able to react with them, i.e. pyruvic acid and acetaldehyde. Those reaction leads to more stable molecules, coloured since not more attachable by SO_2. During wine ageing the molecular anthocyanins are decreasing in favour of the formation of these compounds leading to a colour stabilization (**Table 1.7**).

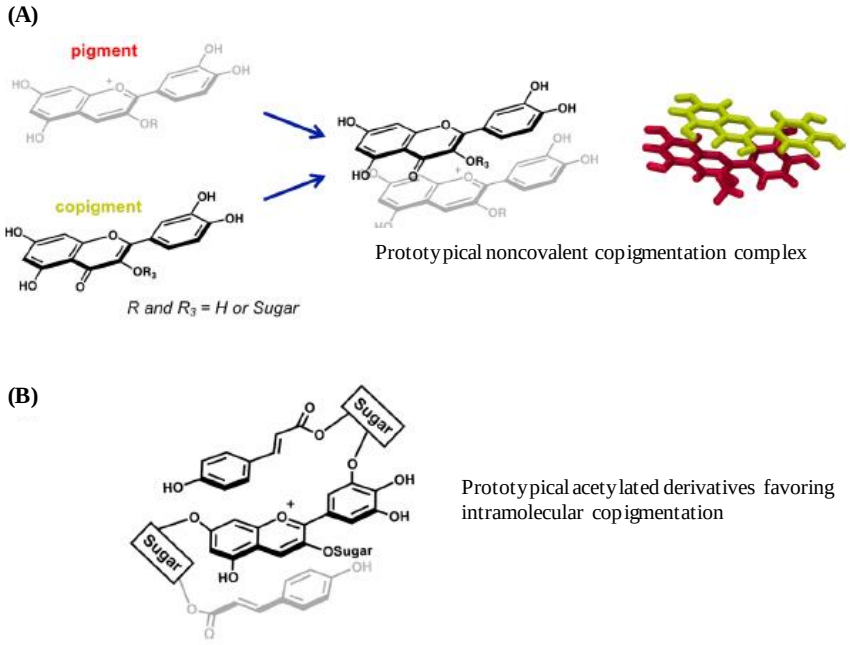


Figure 1.12 Prototypical copigmentation complexes. **(A)** Noncovalent association of anthocyanin pigment and flavanoid copigment (intermoleculare copigmentation). **(B)** Acylated derivatives allowing copigmentation between anthocyanin moiety and two phenolic acids covalently linked (intramolecular copigmentation). *Adapted from Trouillas et al. (2016).*

Anthocyanins can act as both nucleophiles through their C6 and C8 in A ring (hemiacetal form), and electrophiles through the C2 and C4 in C ring (flavylium cation form). The main reactions that can occur are 1) direct condensation, 2) indirect condensation mediate by acetaldehyde, and 3) cycloaddition.

1) Direct condensation can be anthocyanins-flavanol (A^+-F) or flavanol-anthocyanins (F-A^+), where in first case anthocyanins act as electrophile and in the second as nucleophile. In the A^+-F condensation nucleophilic addition of flavanols on anthocyanins lead to a colourless A-F complex, which can undergo to cyclization with an A-type linkage giving a colourless compound, or undergo to an oxidation giving back the red coloured A^+-F (Jurd, 1969; Liao *et al.* 1992; Somers, 1997). In the F-A^+,

is the flavanol to give a carbocation intermediate acting as electrophile reacting with an anthocyanin in hemiketal form (AOH) giving a colourless dimer (F-AOH), which with a loss of a molecule of water can give back the coloured form F-A^+. Following the same mechanism, also dimeric anthocyanins can be formed, between an anthocyanin in hemiacetal form and one in flavylium form, reacting as nucleophile and electrophile, respectively, giving coloured A-A^+ dimer.

2) During winemaking, anthocyanins can react with flavanols through acetaldehyde bridge: the acetaldehyde is bonded in C8 of flavanol, generating a carbocation which can react with anthocyanins in hemiketal forms giving a red-purple pigment (Timberlake & Bridle, 1976; Escribano-Bailón *et al.*, 1996.)

3) By cycloaddition, pyranoanthocyanins can be formed and they are responsible for a gradual change of colour from red-purple to a stable orange hue. The pigments are the result of a nucleophilic substitution in C-4 position on the anthocyanin moiety, leading to the cyclization and subsequent formation of an additional ring between the OH group at C-5 and the C-4 of the anthocyanin pyranic ring (de Freitas & Mateus, 2011; Marquez *et al*. 2013). Main molecules formed are vitisin A-type, vitisin B-type, and methyl-pyranoanthocyanins, formed by cycloaddition of pyuvic acid, acethaldeyde, and acetoacetic acid respectively (Bakker *et al*. 1997; Fulcrand *et al.* 1998; He *et al*. 2006), formed by yeasts metabolite during fermentation. Reaction with hydroxycinnamic acids has been reported giving the so-called pinotins (Schwarz *et al.* 2003a). Flavanol-pyranoanthocyanins are formed by the cycloaddition between anthocyanins and 8-vinylflavanol adducts initially derived from the cleavage of ethyl-linked flavanol oligomers (Mateus *et al.* 2002). These compounds, during ageing can react further to give more complex molecules (Quaglieri *et al.* 2018).

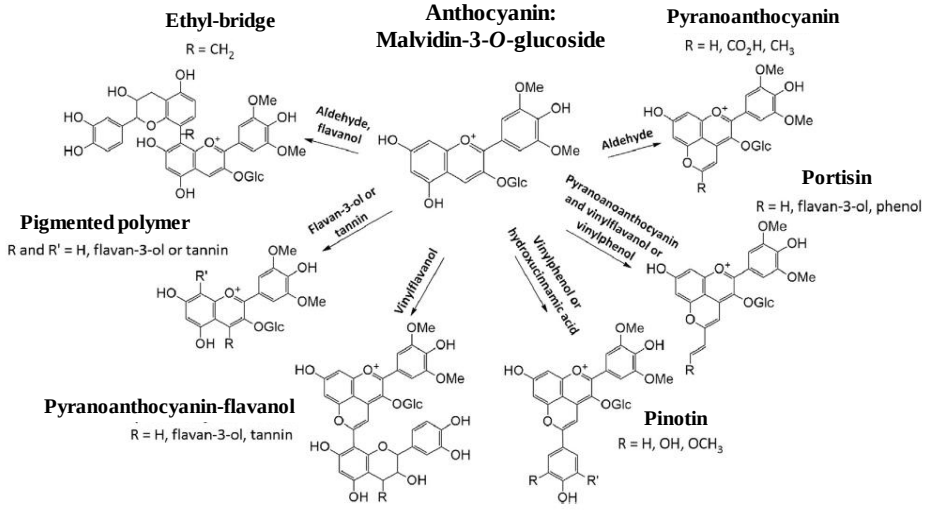


Figure 1.13 Anthocyanin reactions occurring during the winemaking process. *From Setford et al. (2017).*

- ***Anthocyanins losses: oxidation, degradation, and absorption***

Farther their reactivity, other causes lead to the decrease of anthocyanins in wine: in particular degradation reaction and absorption to solid parts presents in young wines, such as residues of fermentation yeast.

Regarding degradation, anthocyanins are sensitive to temperature, oxygen, light exposure, and ketones such as acetone presence, leading to anthocyanins losses (Ribéreau-Gayon *et al.* 2006b).

Losses of both anthocyanins than flavanols can occur for absorption on grapes and yeast cell walls (Hanlin *et al.* 2010). In fact, *Saccharomyces cerevisiae* cell wall are composed by mannoprotein bound to oligopolysaccharides allowing them to absorb molecules such as flavanols and anthocyanins (Morata *et al*. 2013).

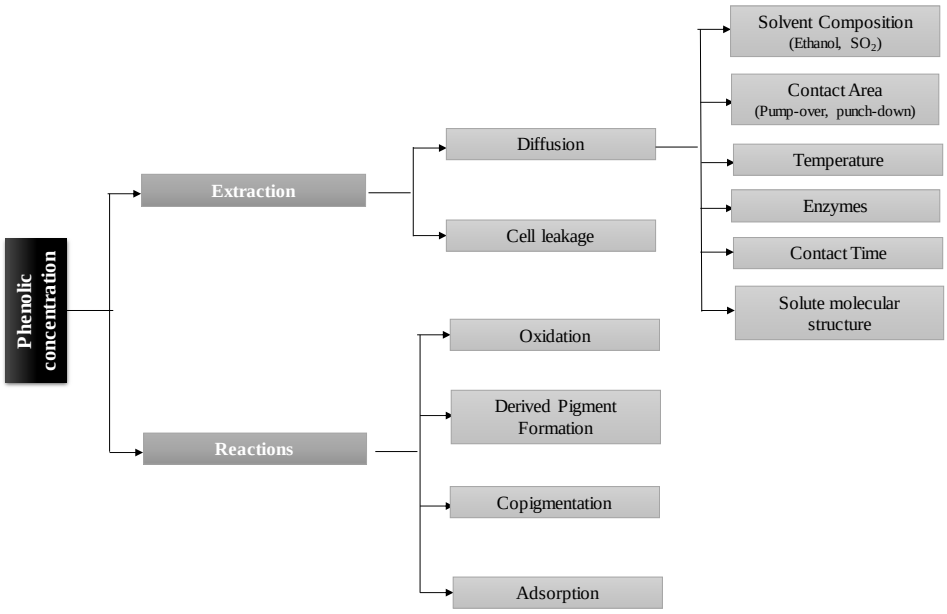


Figure 1.14 Summary of factors affecting phenolic concentration. *Adapted from Setford et al. (2017).*

Table 1.7 Overview of anthocyanin content in winegrapes and monovarietal wines from different areas, vintages, and aging times.

Reference	*Variety*	*Area*	*Vintage*	*Wine*			*Method*	*Glucoside*	*Acetylated*	*Cinnamoylated*
				mg/L Month (n)	**mg/L Month (n)**	**mg/L Month (n)**		**mg/L Month (n)**	**mg/L Month (n)**	**mg/L Month (n)**
Mazza *et al.* 1999	Cabernet Franc	Okanagan Valley (United States)	1996	420(1)	316 (8)	232 (14)	Spect*	-	-	-
			1997	469(1)	337(8)					
	Merlot		1996	412(1)	371 (8)	279 (14)				
			1997	455(1)	338(8)					
	Pinot Noir		1996	340(1)	280 (8)	223 (14)				
			1997	219(1)	171(8)					
	Cabernet Sauvignon		2002	952(2)						
			2001	349(2)						
			2002	563(2)						
	Merlot		2001	226(2)						
			2002	402(2)						
García-Falcón *et al.* 2007	Mencia	Ribeiro (Spain)	-	205 (End MLF)	129 (3)	57 (12)	HPLC-DAD	156 (end MLF); 98 (3); 40(12)	25 (end FML); 17 (3); 8 (12)	24(end MLF); 14(3); 3(12)
	Brancellao			66 (End MLF)	53 (3)	10 (12)		60 (end MLF); 48 (3); 5(12)	6 (end FML); 5 (3); 1 (12)	1(end MLF); 0.8(3); n.q.(12)
Perez-Magariño *et al.* 2004	Tinto Fino	Ribeira del Duero (Spain)	-	343 (End AF)	-	-	HPLC-DAD	304	16	23
				401 (End AF)				358	23	20
	Cabernet Sauvignon			367 (End AF)				322	20	25
				561 (End AF)				375	153	33
				576 (End AF)				401	151	24
				593 (End AF)				397	159	37

Ferrandino *et al.* 2012	Barbera	Piedmont (Italy)	2006	-	-	-	HPLC-DAD	*79.2%*	*10.1%*	*10.7%*
			2007					*78.9%*	*11.3%*	*9.8%*
	Nebbiolo		2006					*92.2%*	*2.5%*	*5.3%*
			2007					*90.9%*	*4.5%*	*4.6%*
	Cabernet Sauvignon		2006					*67.7%*	*25.4%*	*6.9%*
			2007					*67.3%*	*24.7%*	*8.0%*
	Barbera		2001	195(0.5)				*93.6%*	*5.8%*	*0.6%*
			2000	200(0.5)				*93.2%*	*6.7%*	*0.1%*
			2001	231(0.5)				*92.0%*	*6.9%*	*1.1%*
			2000	149(0.5)				*93.0%*	*5.7%*	*1.3%*
			2001	168(0.5)				*72.9%*	*24.9%*	*2.2%*
			2000	454(0.5)				*78.8%*	*21.0%*	*0.2%*
			2001	707(0.5)				*81.9%*	*12.6%*	*5.5%*
			2000	519(0.5)				*75.0%*	*24.7%*	*0.3%*
			2001	547(0.5)				*76.7%*	*19.0%*	*4.3%*
Lingua *et al*. 2016	Syrah	San Juan (Argentina)		334.65 (End AF)	154.85 (FW)			456.97 (Grapes); 185.48 (End AF); 92.15 (FW)	909.03 (Grapes); 107.42 (End AF); 52.59 (FW)	340.76 (Grapes); 41.75(End AF); 10.05 (FW)
	Merlot		-	271.68 (End AF)	72.76 (FW)	-	HPLC-DAD-MS	344.17 (Grapes); 171.56 (End AF); 50.87 (FW)	329.95 (Grapes); 76.03 (End AF); 16.49 (FW)	83.22 (Grapes); 24.09 (End AF); 5.39 (FW)
	Cabernet Sauvignon			119.79 (End AF)	96.52 (FW)			355.33 (Grapes); 85.06 (End AF); 70.19 (FW)	584.16(Grapes); 25.19 (End AF); 22.36 (FW)	86.05 (Grapes); 9.50 (End AF); 3.97 (FW)

Alcalde Eon *et al.* 2006	Tannat	Cerro Chapeu (Uruguay)	2003	762.4 (3)†	-	-	HPLC-DAD-MS	*45.20%*	*27%*	*14.10%*
	Caladoc		2003	469.1 (3) †				*42.60%*	*28.70%*	*16.40%*
	Marselan		2003	445.4 (3) †				*40.60%*	*30.90%*	*12.30%*
	Marzemino		2003	497.1 (3) †				*33.70%*	*33.60%*	*9%*
	Chevenasco		2003	214.5 (3) †				*46.90%*	*10.30%*	*8%*
García-Marino *et al.* 2010	Tempranillo	La Rioja (Spain)	nd	993.63 (1)	608.95(13)	310.61 (27)	HPLC-DAD	794.54(1); 452.27(13); 171.50(27)	163.23(1); 121.04(13); 111.29(27) ‡	
	Graciano		nd	1217.83 (1)	668.78(13)	380.77 (27)		942.01(1); 485.76(13); 230.37 (27)	239.64(1); 147.39(13); 120.64(27) ‡	
Fanzone *et al.* 2012	Malbec	Mendoza (Argentina)	2010	1044.5†; 551.2§ (End MLF)	-	-	Spect* (Total Pigments)	405.8 (*69.1%*)	97.8 (*16.7%*)	47.6 (*8.1%*)
	Bonarda		2010	739.8†; 285.5§ (End MLF)				212.8 (*69.2%*)	43.9 (*14.3%*)	28.8 (*9.4%*)
	Cabernet Sauvignon		2010	681.8†; 269.6§ (End MLF)				182.8 (*63.2%*)	72.1 (*25%*)	14.7 (*5%*)
	Merlot		2010	644.1†; 273.7§ (End MLF)			HPLC-DAD-MS (Monomeric Anthocyanins)	183.8 (*62.5%*)	66.3 (*22.5%*)	23.6 (*13.5%*)
	Shiraz		2010	301.4†; 168.3§ (End MLF)				97.4 (*54.8%*)	47.1 (*26.5%*)	23.8 (*13.5%*)
	Tempranillo		2010	717.6†; 306.5§ (End MLF)				242.9 (*75.8%*)	27.7 (*8.7%*)	35.9 (*11.2%*)
			2004	289(24)						
			2005	299(12)						

Romero-Cascales *et al.* 2005	Monastrell	Jumilla (Spain)	2003	361(0.5)	-	-	HPLC-DAD-MS	301.2 (*83%*)	59.8 (*17%*)
	Cabernet Sauvignon			354.2(0.5)				211.84 (*58.6%*)	142.36 (*41.4%*)
	Syrah			350.8(0.5)				216.75 (*61.4%*)	134.05 (*38.6%*)
	Merlot			225.5(0.5)				146.8 (*64%*)	78.6 (*36%*)

Table 1.7 Overview of anthocyanin content in winegrapes and monovarietal wines from different areas, vintages, and aging times.

Legend *Footnotes and abbreviations legends: * Indicates spectrophotometric measure, as total anthocyanins; Italic indicates % of glucoside, acetylated and cinnamoylated on total anthocyanins; † indicates total pigment content; ‡ indicates acylated as sums of acetylated, p-coumaroylated, and caffeoylated derivatives; § monomeric anthocyanins analysed through HPLC-DAD. MLF = malolactic fermentation; AF= alcoholic fermentation.*

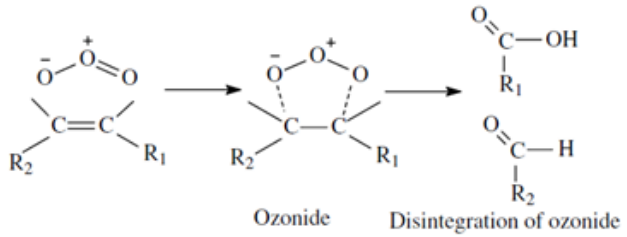
1.3 Grape post-harvest treatments

In the recent literature, several treatments, applied on grape before and after harvest, were found to be able to modify phenolic compounds biosynthesis, accumulation, or degradation. Among these innovative techniques (briefly listed in 1.2.2.3), ozone treatment will be described below.

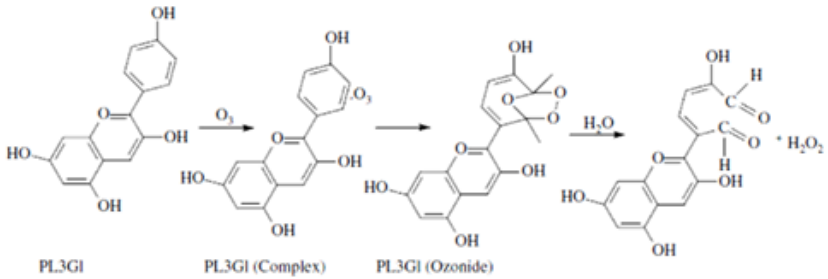
1.3.1 Ozone: a case of study

Ozone has been proposed for the treatment of table and wine grapes because of its several advantages. Ozone for industrial purpose is generated by the passage of air, or oxygen gas, through a high-voltage electrical discharge or by UV light irradiation, at 285 nm (Mahapatra *et al.* 2005). Ozone is currently used in food industry as both gaseous and aqueous forms, and since it decomposed quickly in O_2, leaving no residues, has been recognizes as eco-friendly additive. The half-life of ozone in distilled water at 20°C is about 20-30 min (Khadre *et al.* 2001). Ozone is nowadays recognized from US EPA (United States Environmental Protection Agency) as a safe agent for food contact, it was as well insert in the GRAS (General Recognize as Safe) list in 2001 by the US FDA (United States Food and Drug Administration) for the direct application on foods.

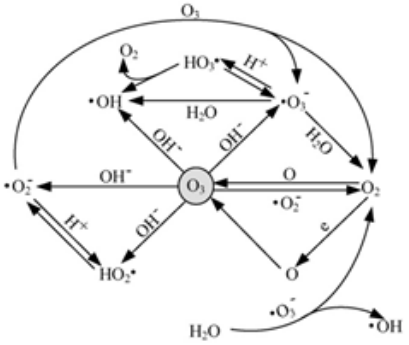
1 **Direct reaction of ozone with the formation of ozonide**



Example of degradation of pelagordin-3-glucoside (PL3Gl)



2 **Formation and transformation of intermediate radicals**



High-reactive species	Oxidantion potential (V)
Hydroxyl radical (•OH)	2.80
Atomic oxygen (O)	2.42
Ozone in alkalescent (O_3)	2.07
Perhydroxyl radical (HO_2•)	1.70
Ozone in acid (O_3)	1.25

Figure 1.15 Activity of ozone: **(1)** Direct reaction of ozone with the formation of ozonide –Criegee reaction- and degradation mechanism of anthocyanin pelagordin-3-glucoside (*From Cullen et al. 2009*); **(2)** Schematic diagram of inter-transformation among primary high-reactive species and their oxidation potential (*From Xue et al. (2008).*

First, ozone (O_3) is the triatomic form of oxygen and it is an instable compound, rapidly decomposing itself spontaneously giving O_2 or hydroxyl radicals, or in contact with oxidable surfaces (**Figure 1.8**). Ozone because of its strong oxidative potential is used as an antimicrobial agent on a wide spectrum of bacteria and fungi, and it is used also for its capacity to destroy pesticides and chemical residues (Khadre *et al.* 2001; Mahapatra *et al*. 2005). Several scientific researches reviewed its application on vegetables and fruits, and its ability to contrast microorganisms. Ozone is used for the postharvest treatments of fresh fruits and vegetables in both air and water solutions, and it can be added as a continuous or intermittent treatment during storage or transportation, as well as used as shock treatment on harvested fruits and vegetables (Horvitz & Cantaleyo, 2014). Regarding grapes, ozone has been studied for storage, packaging atmosphere (Sarig *et al.* 1996; Cayeula *et al*. 2009; Mlikota-Gabler *et al*. 2010; Artés-Hernández *et al*. 2003; Artés- Hernández *et al*. 2004; Artés- Hernández *et al*. 2007; González-Barrio *et al.* 2006), and, for wine grapes, as shock treatments on fresh grapes or as shock or continuous treatment during dehydration with the aim to obtain withered grapes for *passito* or *sfursat* wines (Carbone & Mencarelli, 2015; Botondi *et al.* 2015; Cravero *et al.* 2016; Bellincontro *et al.* 2017; Cisterna *et al.* 2018; Modesti *et al.* 2018; Guzzon *et al.* 2018).

1.3.1.1 Ozone in wine grapes industry

Ozone is often used for winery equipment sanification, in particular hoses, tanks, and barrels cleaning. Guzzon *et al.* (2011) suggested high sensitivity to ozone of some spoilage microorganism typical of the wine environment. The first studies on ozone treatments applied directly on grapes concerned its application in storage of table grapes against the native superficial microflora, which was responsible of its decay (Palou *et al.* 2002; Tzortzakis, *et al.* 2007; Cayeula *et al.* 2009; Mlikota-Gabler *et al.* 2010; Feliziani *et al.* 2014). Short treatments of gaseous ozone showed effective prevention against grey mould, as well, technological parameters, firmness, and secondary metabolites such as aromas and phenolic compounds were investigated to guarantee the product quality.

Ozone showed to do not affect and frequently to increase some classes of phenolic compounds, such as stilbenes (Sarig *et al.*1996; González-Barrio *et al.* 2006; Artés-Hernández *et al.* 2003).

These positive features, such as the reduction of microorganisms and the increase of phenolic compounds, leaded to apply ozone also in wine grapes. Three main objectives were desired: 1) maintaining grape berry health status, on fresh grapes for yeasts - particularly, Non-*Saccharomyces* yeasts and *Brettanomyces bruxellensis* spoilage-, and 2) guarantee the grape preservation against moulds during dehydration, and 3) increasing of desirable phenolic compounds.

Regarding the effectiveness of ozone treatment on fresh grapes, studies found it to reduce and change the yeast population present on the grapes and in the first step of the fermentation independently to the type of treatments, concentration of the active ingredient, contact time of the treatment and to the form (aqueous and gaseous) (Cravero *et al.* 2016; Guzzon *et al.* 2018). In particular, the resulting wines showed lower acetic acid (product from Non-*Saccharomyces* yeasts). As well, when ozone was applied on wine grapes to control *B. bruxellensis* from its surface, a decrease of *B. bruxellensis* was found and the treatment also reduced the concentration of ethyl phenols in wines (Cravero *et al.* 2018). These results, suggested that ozone can be suitable in case of inoculated fermentation, to help the chosen yeast growth, or to prevent *B.bruxellensis* spoilage of the winery.

The microbiota control becames very important in case of grape dehydration. In fact, during dehydration the high relative humidity around berries together with cracks in the berry skin, which can occur with manipulation, can bring to mould infection, which is a danger to the wine quality and can lead to a production loss, such as the *Botrytis cinerea* contamination (Mencarelli & Tonutti, 2013). Another case is the proliferation of rot, given by an increasing content of acetic bacteria, problem increased by the proliferation of insects, such as *Drosophila* contamination. Moreover, fungi development can cause the formation of compounds dangerous for human health, in particular some fungal species

belonging to *Aspergillus* genus are responsible for ochratoxin A (OTA) contamination (Torelli *et al.* 2006; Valero *et al.* 2008). Nowadays, the control of environmental conditions -i.e. using conditionated and ventilated rooms- and the use of sulphur bentonite are the possible solutions to reduce the pathogen attack on berries (Mencarelli & Tonutti, 2013). However, the first is not suitable for small winery, and the second causes bleaching on red wine grapes. Moreover, strategy reducing the use of sulfur dioxide in the winemaking production are recommended nowdays because of its allergenic effects (Simon, 1986; Taylor *et al.* 1986).

Regarding phenolic compounds, one point must be considered: in contrast with table grapes, which is a final product, wine grapes is just the starting point, therefore also modification of extractability can occur. Therefore, also ozone effect on factors other than phenolic compounds accumulation can influence wine final composition, i.e. the modification of the technological parameters, cell wall and its enzymes activities.

- ***Ozone effect on phenolic compounds***

Total polyphenols changes during ozone treatments are variety and dose/time exposure dependent. Nevertheless, a general activation of phenylpropanoid pathway in ozone-treated fruits and vegetables is known (Howell & Kremer, 1973; Keen & Taylor, 1975¸ Rosemann *et al.* 1991; Eckey-Kaltenbach *et al.* 1994; Booker *et al.* 1996), but together with the new formation of certain classes of polyphenols, also their consumption is observed, given by the oxidant capacity of ozone. Artés-Hernández *et al.* (2007) found an increase of total polyphenols in both continuous and intermittent treatment with ozone in Autumn seedless table grapes, in contrast in wine grapes Pignola (red) and Grechetto (white) a where a decrease was found to different extent depending on the dose/time exposure (Botondi *et al.* 20015, Carbone & Mencarelli, 2005).

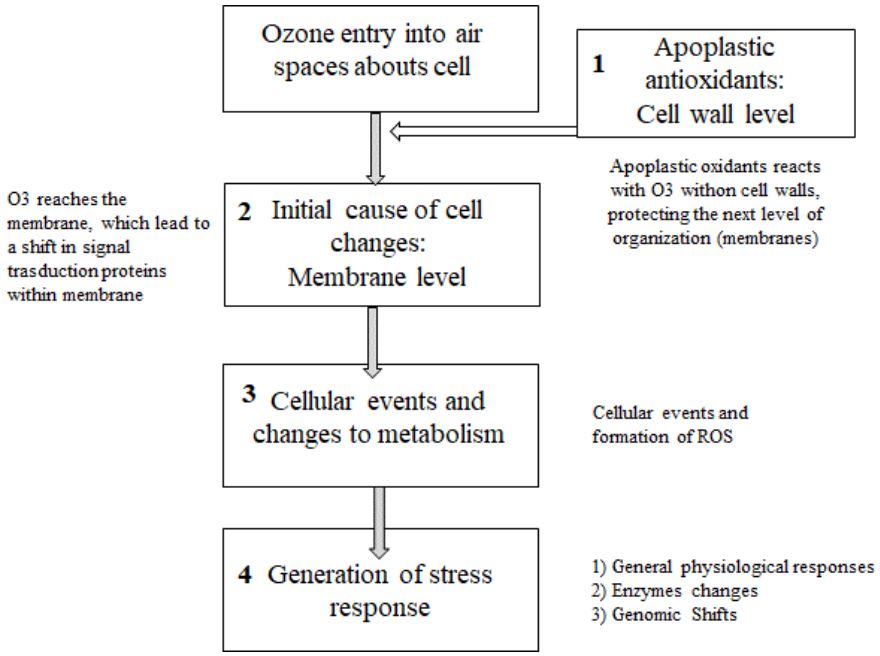


Figure 1.16 Scheme of ozone effects in plant tissue. *Adapted from Heat, 2008*.

Anyway, different polyphenol classes appear to be differently affected by ozone exposure. For example, the increase of stilbenes when ozone was applied in shock or in intermitted treatments was found whereas a decrease is found during long treatments, leading to the supposition that the ozone may induce the stilbene pathway, but continuous treatments can also consume, because of its oxidative capacity, the newly produced resveratrol (Gonzales Barrio *et al*. 2006, Artes Hernandez *et al*. 2003; Sarig *et al*. 1996). In contrast moderate treatments can induce stilbene biosynthesis but avoid its depletion (Cayuela *et al.* 2009, Triska & Howska, 2012). Similar results are found for anthocyanins: dose/time treatment can strongly influence their concentration. As well, anthocyanins substitution, which determine their attitude to oxidability (Cheynier *et al.,* 1994), bring to different final concentration depending on the anthocyanins pattern of the variety. Increase in anthocyanins concentration has been found in industrial-scale vinification, after short treatments on Petit Verdot grapes (Bellincontro *et al.* 2017). If applied during withering, ozone treatment and withering conditions

as well as grape varieties can influence the anthocyanins behaviour. Botondi *et al.* (2015) reported a higher anthocyanins concentration in Pignola grapes after a shock treatment of ozone before withering, whereas when the treatment was longer, the anthocyanins decreased during the withering. A decrease of hydroxycinnamic acids when a short treatment was applied on Grechetto grapes was found (Carbone *et al*. 2015). On the contrary an increase of flavonols and catechin was reported (Carbone *et al.* 2015). Generally, ozone seems to induce phenolic synthesis in grapes after short treatments (Artes-Hernandez *et al.* 2007; Mencarelli *et al*. 2011), while for long time exposure can cause a significant decrease, especially when dehydration is applied, given by the oxidation of these compounds. As well, a strong variety-dependent effect seems to be present, mainly related to the phenolic profile of the variety, in particular for flavanols and anthocyanins classes.

1.4 Sensorial properties of phenolic compounds

1.4.1 In-mouth sensories properties

In-mouth sensory properties of wines involve several sensations, which can be related to taste and to non-taste sensations, generally defined as "mouthfeel", and aroma and flavour features. The term "mouthfeel" identifies different sensations such as astringency, body, burning, irritation, warmth, and viscosity (Jackson *et al.* 2009; Gawel *et al*. 2000), therefore it classifies sensations given by a tactile response in mouth. In mouth, different papillae coexist: filiform, fungiform, foliate, and circumvallate, among them, the non-taster papillae, i.e. the filiform, are considered to be the responsible for mouthfeel perception. Filiform papillae are highly innervated and respond to mechanical and thermal stimulus.

Saliva is the other main component relate to the non-taste stimulus. Human saliva is mainly composed by water (95%), proteins (proline-rich proteins, mucins, histatins; 0.3%), and other minor substances. Saliva film (70-100 μm thicker) protect mouth surfaces, and the ingestion of astringent components, such as phenolic compounds, may changes its composition triggering the sensation (Laguna *et al.* 2017).

In-mouth sensory properties are investigated mainly throughout sensory analysis, also because of their complexity. Anyway, several methods for analytical

determination have been proposed. Here below, a brief description of relevant in-mouth sensation induced by phenolic compounds and their determination methods are resumed.

1.4.1.1 Astringency mechanism and chemical determination

According to the definition of American Society for Testing and Materials, astringency refers to "the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins" (ASTM, 2004).

The first explanation for astringency is the interaction between the salivary proteins and the phenolic compounds: salivary proteins covalently bind to the oral mucosal cells and form a layer surrounding the soft structure of the mouth. When the phenolic compound pass by, they bond to proteins to form insoluble tannin-protein precipitates (Baxter *et al.* 1997). This can be described as a three steps phenomenon as reported by Charlton *et al.* (2002) for tannin:

1. hydrophobic associations occur between the planar surfaces of the tannin aromatic rings and hydrophobic sites of proteins. Simultaneously, hydrogen bonding effect assists to stabilize the complexes, occurring between the hydroxyl group of tannin and H-acceptor sites of proteins.
2. Next, the protein-tannin complexes self-associate via further hydrogen bonding to produce soluble larger protein-tannin complexes and then aggregate.
3. Finally, the aggregated complexes are large enough to form insoluble sediment and precipitate from solution.

Several analytical methods exploit this ability of protein to precipitate with phenolic compounds to assess wine or phenolic compounds solution astringency, such as the use of putative protein (Bovine Serum Albumine – BSA, giving the "tannin specific activities"; Hagerman & Butler, 1981). Salivary proteins (Saliva Precipitation Index) were used to evaluate the astringency intensity of tannin by

SDS-PAGE electrophoresis and dynamic light scattering methods (Pascal *et al.* 2007; Rinaldi *et al*. 2010). Another way, is measuring the different content of a phenolic solutions with and without the addition of putative or salivary proteins (Ma *et al.* 2016; Schwarze & Hofmann, 2008; Ferrer-Gallego *et al*. 2015a, Quijada-Morin *et al.* 2016), using the more suitable methods for the analytes (Usually HPLC-UV-MS techniques).

Anyway, several studies reported the occurrence of astringency perception and interaction between salivary proteins with other compounds, mainly phenols, which creates soluble aggregate, and therefore do not precipitate at all (Kallithraka *et al.* 1998; Schwarz & Hofmann; 2008; Scharbert *et al.* 2004). This kind of interaction has been investigated throughout Saturation-Transfer Difference NMR spectroscopy (STD-NMR) for several phenolic compounds such as anthocyanins and flavonol glycosides (Ferrer-Gallego *et al.* 2015a; Ferrer-Gallego *et al*. 2017) showing the formation of soluble aggregates leading to the supposition that a "free" astringent stimulus may be involved in sensory perception of astringency.

In minor part, astringency is linked to the sensation of "friction" (Rossetti *et al*. 2009) as results of a disruption of oral lubricating coatings that contribute to the development of astringency, confirmed by the founding by Lee *et al*. (2012) demonstrating that mucins - which constitute the coating of epithelium tissues- were able to precipitate alum salts. Therefore, depletion of the protective salivary film, could also be an explanation for the dry mouth perception usually associated with the astringent mouthfeel.

A third contributor in astringency is the precipitation of dead cells and other mouth debris in the mouth leading to an increased sense of particles in the mouth, without the participation neither of mucins nor PRPs to the sensations (De Wijk & Prinze, 2006).

Wine is a complex matrix, in which the presence of compounds other than

astringency elicitors can modify the interaction. Among them, presence of acids, sugars, mannoproteins can influence the astringency sensations (Laguna *et al.* 2017). As well, modification of the astringency sensation can be given also by the ethanol concentration, the solution pH, temperature, and viscosity (Ma *et al*. 2014).

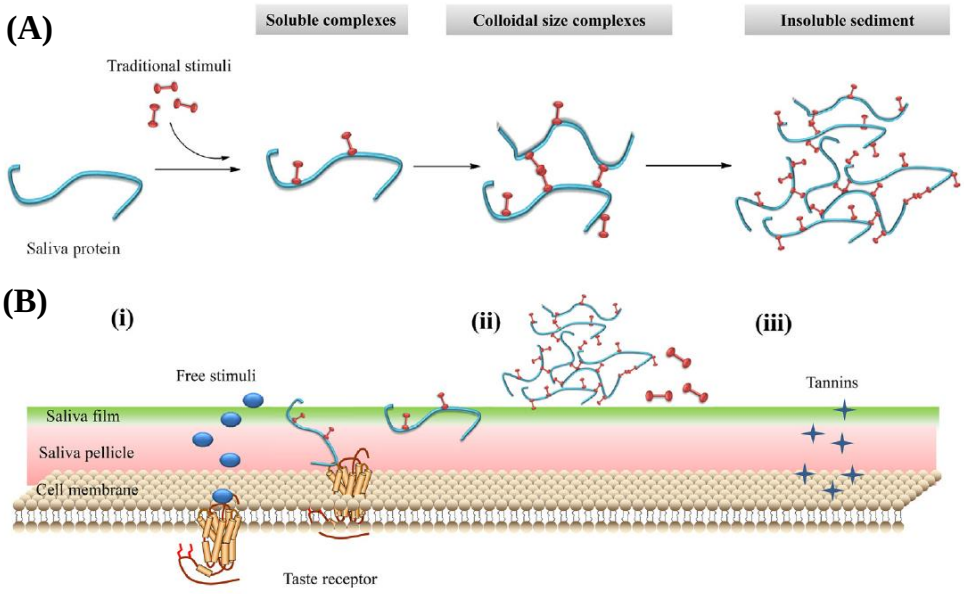


Figure 1.17 Schematic representation of possible astringency mechanisms: **(A)** A 3-stage model of the interaction between stimuli and proteins; **(B)** Astringency stimulation: **(i)** "Free" stimuli and soluble stimuli-protein complexes deplete the protective salivary film and eventually bind to the pellicle or even to the receptors exposed; **(ii)** Insoluble stimuli-protein complex and traditional stimuli are rejected against salivary film. Insoluble stimuli-protein complexes trigger astringency sensation via increasing friction. **(iii)** Tannins interact with oral cavity membrane. *From Ma et al. (2014)*

1.4.1.2 Bitterness and chemical determination

Bitterness, together with saltiness, sweetness, umami and acidity belong to basic tastes. Bitterness perception is a taste recognition mediated by taste buds presenting in the taste papillae (fungiform, foliate and circumvallate) on the

tongue. Each taste bud consists of approximately 50-100 taste receptor cells (TRC) and is innervated by multiple taste fibers that transmit nervous signals to brain (Montmayeur & Matsunami, 2002). Human bitter receptor cell contains approximately 25 bitter G protein-coupled receptors (GPCR) encoded by a TAS2Rs gene family.

Soares *et al.* (2013) showed that different phenolic compounds activate distinguished combination of TAS2Rs: epicatechin stimulated three receptors (TAS2R4, TAS2R5, and TAS2R39) while pentagalloylglucose activated two receptors (TAS2R5 and TAS2R39). Only one receptor was responded to malvidin-3-*O*-glucoside and procyanidin trimer. Using receptors is the best analytical method to determines bitterness, even if a limitation of this techniques is that only the 1% of ethanol can be used, therefore it does not represent totally the wine condition. Farther the chemical analysis, sensory analysis is usually approached to determine bitterness. However, bitterness perception is dependent on individual features, as showed by different sensitivity to PROP (6-n-propylthiouracil), which can influence the bitterness perception elicited by red wines. Phenotypic responses to PROP vary considerably among individuals, from 'taste blindness' to PROP bitter taste (Non-Taster: NT) to a wide range of perceived bitterness intensity (taster) (Bartoshuk, 2000). PROP tasters are further classified as medium (MT) and super tasters (ST), who perceive PROP as moderately and extremely bitter, respectively (Bartoshuk, 2000). The polymorphisms in the gene TAS2R38 mainly explain the observed phenotypic variation. This variation influence wine preferences by consumer with different PROP status (Pickering *et al.* 2004).

2. Aim of the PhD

Wine quality is a complex mix of parameters which involves chemical characteristics on aroma, mouth sensations and visual features. Phenolic compounds are strictly connected with these characteristics as explain in Chapter I, and in particular, a strong relationship with flavonoids content and composition.

This PhD was focused on two main aims:

1. The composition and content of flavonoids in grape is of fundamental importance for the final wine quality, and their initial content must be maximized, since "*good grapes make good wine*". As well, the extraction of these compounds requires the correct attention, because not all the compounds present in grapes are necessarily extracted in wine, since extractability is dependent also from the berry integrity - which is related to ripeness and health status -, the skin mechanical properties, and cell wall composition. The evaluation of an innovative post-harvest technique was studied: the ozone treatment on grapes post-harvested and during dehydration, in order:

    - Gaseous ozone treatment was tested on fresh grape, since recent studied found its elicitor effect on phenolic compounds accumulation together with is antimicrobial effect, which can lead to a reduction of the use of sulphur dioxide in winemaking process. Two different treatment (24 and 48 hours) on two different cv., i.e. Nebbiolo and Barbera, which owned

a different phenolic compounds profile and content, were carried out. The anthocyanins and flavan-3-ols oligomers and polymers extractability was assessed using simulated maceration and compared to a control.

- As well, ozone treatment was applied during grape dehydration. “Withering”, i.e. controlled dehydration in chamber, is a technique widespread to produce high quality wine, resulting in concentration of sugar and desirable metabolites, such as phenolic compounds and aromas. Ozone can prevent the microorganism-caused berry decay during the process, avoiding the loss of product for rot and moulds infection. Moreover, phenolic compounds induction can be found. Therefore, Nebbiolo and Barbera were dehydrated (10 and 20% weight loss) under ozone compared to an air atmosphere. In this case, anthocyanins and flavan-3-ols oligomers and polymers, as well as technological parameters were tested in the grape samples. Skin cell wall composition was also analysed together with skin mechanical properties, to compare the treatments. Simulated macerations were done, and a correlation between these parameters was investigated in order the find a regression equation between extractability and grape parameters.

2. Monomeric, oligomeric and polymeric flavan-3-ols from skins and seeds contribute to astringency and bitterness, and together with anthocyanins are involved in aged wine colour. Regarding wine colour, young wines are strongly influenced by the native grape anthocyanins, extracted from grape skins during the maceration process. Anyway, if flavan-3-ols involvement in sensorial properties has been deeply investigated throughout chemical ad sensorial analyses, the role of anthocyanins is still discussed. Therefore, extraction of anthocyanins form grape skin was performed, and isolation was carried out by Centrifigual Partition Chromatography (CPC) to fractionate and purify glucoside, acetylated, and cinnamoylated anthocyanins. Those extract were used to investigate anthocyanins involvement in in-mouth

sensory properties. Chemical analysis of determination of astringency, i.e. reaction with bovine serum albumin and salivary proteins, were attempted, and sensorial analysis to determine perception thresholds were made.

Experimental Section

3. Impact of post-harvest ozone treatments on the skin phenolic extractability of red winegrapes cv Barbera and Nebbiolo (*Vitis vinifera* L.)

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3.1 Introduction

Wine industry is looking forward for innovative, safe for human health and environment, antimicrobial products allowing chemical treatment reduction in the winemaking process and not negatively affecting the quality of the final product. Ozone has been tested in food industry, as used in both ozonized water and gaseous form, giving good results in preventing fungi and bacteria growth on a wide spectrum of vegetables and fruits, due to its oxidant activity, and leaving no chemical residues on foods decomposing itself rapidly into oxygen (Glowacz *et al.* 2015; Khadre *et al.*; Sengun 2014). Gaseous ozone has been already tested for table grapes storage in order to contain fungi responsible for berry decay (i.e. *Botrytis cinerea*, *Aspergillus* spp., *Fusarium* spp), to maintain the product's visual, sensory, textural and nutritional quality, and to reduce pesticide residues (Artés-Hernández *et al.* 2003; Cayuela *et al.* 2009; Feliziani *et al.* 2014; Gabler *et al.* 2010). As well, ozone fumigation has been used on winegrapes during the withering process, as an alternative to sulphur derivates in order to both prevent moulds development and to reduce indigenous yeast population (Botondi *et al.*; Carbone & Mencarelli, 2015). In particular, the viability reduction of *Brettanomyces bruxellensis*, which is related with off-flavours production in wine (Kheir *et al.* 2013), would be advantageous.

In addition to improve fresh product quality, ozone has been confirmed as phenolic compounds elicitor, stimulating chemical defence responses such as the synthesis of polyphenols, in particular increasing up to 4-fold resveratrol content, and keeping stable anthocyanin content during the storage of red table grapes cv Napoleon (Artés-Hernández *et al.*, 2003). Nevertheless, ozone applied in post-

harvest can permeate inside fruits through lenticels and in damaged grapes through cuts or cracks in the cuticle (Forney, 2003), and reacts with grape compounds. In fact, ozone has a high oxidant potential acting both directly and indirectly, attaching itself to the double bound of organic compounds and by its intermediate radicals, which can react with a wide range of grape molecules (Criegee 1975; Cullen *et al.* 2009). Among them, flavonoids can be susceptible to both degradation reactions, depending on the electrochemical stability of the B ring substituent. In particular up to 99% anthocyanin degradation has been reported in less than 10 minutes in grape juice treated with ozone, to different extents according to individual anthocyanin reactivity (Tiwari *et al.* 2009a).

Phenolic compounds are strictly associated with red wine quality; among them, anthocyanins extracted from skins are responsible for young wine colour. The grapevine genome determines the anthocyanin profile, but several factors such as vineyard practices, climate, soil features, and seasonal conditions can influence anthocyanin accumulation during grape ripening (Ortega-Regules *et al.* 2006a). Monomeric, oligomeric and polymeric flavan-3-ols from skins and seeds contribute to astringency and bitterness, and together with anthocyanins are involved in aged wine colour. Their contribution on the organoleptic properties of wine depends on their content and structural features, such as stereochemistry, hydroxylation pattern, position of the linkage, and in particular the degree of polymerization (Chira *et al.* 2008; Kennedy & Jones, 2001; Mattivi *et al.* 2009; Peleg *et al.*, 1999; Vidal *et al.* 2003).

Phenolic compounds extraction depends on grape composition, extraction technique and cell wall degradation. During ripening and post-harvest treatment, differences in cell wall composition could be responsible for different anthocyanin extractability, and together with cell porosity, for flavanol extractability (Bindon *et al.* 2012; Ortega-Regules *et al.* 2006b; Quijada-Morín *et al.* 2015). Moreover, phenolic compounds have different propensity to be retained by the cell wall depending on their structure. The mechanical resistance

of cell walls to phenols release has permitted to predict phenolic compound extractability from berry physical properties. In fact, texture analysis has been proved to be a reliable tool to relate extractability and skin mechanical properties. In particular, a significant correlation has been found between skin hardness and the extraction of anthocyanins and flavanols with low and high molecular mass (Rolle *et al.* 2008; Río Segade *et al.* 2014). Recently, Laureano *et al.* (2016) demonstrated increased berry skin hardness for table and wine grapes after post-harvest gaseous ozone exposure (30 µL/L) for 24 hours, evidencing a role of the ozone exposure on the berry skin mechanical features. Therefore, it may affect the extraction of phenolic compounds from the skins.

The impact of post-harvest ozone treatments on the phenolic compounds extractability of winegrapes has not been studied nowadays. Therefore, in this work skin phenolic compounds extractabilities were evaluated in red grape berries exposed to continuous ozone treatment for 24 and 72 hours, and then compared to berries exposed to atmospheric air. Extraction kinetics of anthocyanins, low and high polymeric mass flavanols were tested through simulated maceration using a wine like solution in order to understand ozone related effects. Highly cultivated varieties of North-West Italy producing renowned worldwide wines, *Vitis vinifera* L. Nebbiolo and Barbera, were chosen for their different phenolic profiles. Nebbiolo grapes have a profile composed mainly by di-substituted anthocyanins and high flavanol content, whereas Barbera is characterized by tri-substituted anthocyanin prevalence and low flavanol concentration (Lambri *et al.*2015; Río Segade *et al.* 2014).

3.2 Materials and Methods

3.2.1 Grape samples

Whole bunches of *Vitis vinifera* L. cv Nebbiolo and Barbera were harvested once reached 24°Brix at experimental vineyards located in North-West Italy, Piedmont Region, in 2014. Once in the laboratory, for each variety a subsample of berries with short attached pedicels was taken from different bunch parts (shoulders, middle, and bottom). Berries were sorted by flotation as described by Rolle *et al.* (2012) using different saline solutions with sodium chloride contents ranging from 130 to 170 g/L, with the aim to increase intersample homogeneity (**Figure 3.1**). The most representative density class (1107 kg/m^3) was chosen for both varieties, which represented about 33% and 57% (w/w) of total pre-sorting berry weight for Nebbiolo and Barbera, respectively. Sorted berries were then washed with water, visually inspected, and those with damages on the skin were discarded prior to be disposed in boxes (30 × 20 cm) in a single layer for experimental treatments.

Figure 3.1 Berries sorted by flotation.

Three sample boxes for each variety were exposed for 72 hours to atmospheric air at 20°C (control). Other three boxes were introduced into a sealed chamber, where they were exposed to a continuous 30 µL/L ozone concentration for 72 hours (OZ72) at 20°C. Other three boxes were exposed for 24 hours to ozone at 20°C and for 48 hours to atmospheric air condition (OZ24). In all cases, the average relative humidity was 70%. The ozone was supplied by an ozone generator (C32-AG, Industrie De Nora Spa, Milan, Italy) with a nominal production capacity of 32 g O_3/h. Ozone concentration in the chamber was continuously monitored by recirculation of the ozone-enriched air (120 m^3/h flow) from the chamber with a BMT 964 UV-photometric ozone analyzer (BMT Messtechnik Gmbh, DE) that controlled the ozone generator output. The relative humidity in the chamber was controlled by dehumidifiers, and the thermohygrometric conditions were constantly monitored and recorded using a data logger (HOBO H8 RH/Temp, Onset Computer Corporation, Bourne, MA).

3.2.2 Assessment of phenolic compound extractability

Three replicates of 40 berry skins for each treatment and for the control were used to study the phenolic compounds extractability as previously reported by Río Segade *et al.* (2014). The skins were carefully manually removed from the pulp using a laboratory spatula, weighed, and quickly immersed in 100 mL of a hydroalcoholic buffer solution at pH 3.2 containing 12% ethanol, 5 g/L tartaric acid and 100 mg/L sodium metabisulfite (solution A). Extractability solutions were kept at 25°C for 10 days and solution A samples were taken at 6, 24, 48, 96, 168 and 240 hours for phenolic compounds determination (**Figure 3.2**). After 240 hours the skins were removed from the solution A and quickly immersed in 100 mL of a hydroalcoholic buffer with higher sodium metabisulfite content, i.e. 2 g/L (solution B) (**Figure 3.3**). Afterwards, the skins were homogenized using an Ultra-Turrax T25 high-speed homogenizer (IKA Labortechnik, Staufen, Germany) for 1 min at 8000 rpm, and subsequently centrifuged for 15 min at 3000 × *g* at 20°C using a PK 131 centrifuge (ALC International, MI, Italy). The

supernatant was collected and used to determine non-extracted phenolic compounds (Río Segade *et al.* 2014).

To calculate the extraction percentage, phenolic compounds were determined in the skins from three sets of 10 fresh grapes berries (3 replicates) following the extraction protocol described for non-extracted phenolic compounds but the skins were directly immersed in the solution B.

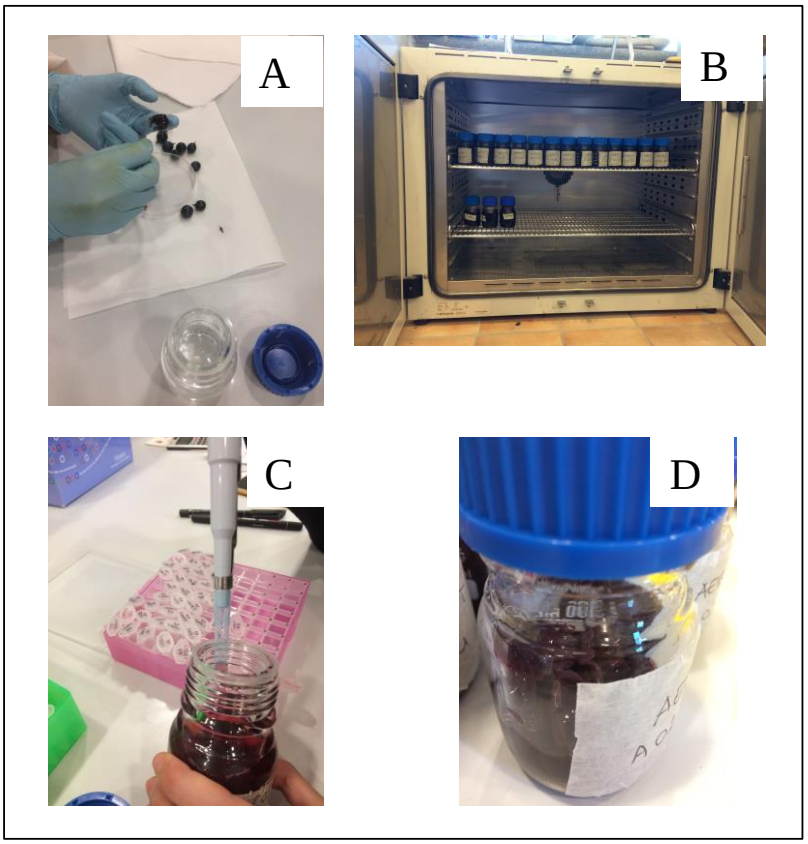

Figure 3.2 Simulated maceration steps. **A)** Berry are peeled and putted in the 100 ml extraction solution; **B)** controlled-temperature hoven; **C)** sampling; **D)** skins left at the end of maceration.

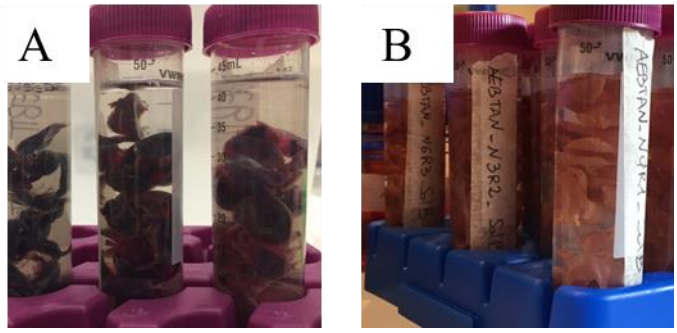

Figure 3.3 Skins immersed in solution B for total extraction: **A)** at the beginning and **B)** at the end of the extraction.

3.2.3 Chemical analysis

3.2.3.1 Reagents and standards

Solvents of HPLC-gradient grade and all other chemicals of analytical reagent grade were purchased from Sigma (Milan, Italy). The solutions were prepared in deionized water produced by a Purelab Classic system (Elga Labwater, Marlow, UK). About standards for calibration curves, malvidin-3-glucoside chloride was supplied by Extrasynthèse (Genay, France), whereas cyanidin chloride and (+)-catechin were purchased from Sigma. For identification purposes, anthocyanin malvidin-3-glucoside chloride, peonidin-3-glucoside chloride, and cyanidin-3-glucoside chloride) were purchased from Extrasynthèse.

3.2.3.2 Technological parameters determination

At harvest, three replicates of 100 fresh berries were manually crushed, and the standard physicochemical parameters were determined in the grape juice obtained by centrifugation. Organic acids (citric, tartaric, and malic acids, g/L) and reducing sugars (glucose and fructose, g/L) were quantified using an HPLC system equipped with a DAD set to 210 nm and a refractive index detector,

respectively, as described by Giordano, Rolle, Zeppa and Gerbi (2009). Chromatographic separation was performed using a 300 mm × 7.8 mm i.d. Aminex HPX-87H cation exchange column and a cation H^+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA) at 65°C. The mobile phase was 0.0065 mol/L H_2SO_4 at 0.8 mL/min flow-rate. Titratable acidity was estimated as g/L of tartaric acid following the OIV method (OIV, 2008), and pH was determined by potentiometry using an InoLab 730 pHmeter (WTW, Weilheim, Germany).

3.2.3.3 Phenolic compounds determination

Phenolic compounds were determined by spectrophotometric methods (Rigo *et al.* 2000; Torchio *et al.* 2010) using an UV-1800 spectrophotomer (Shimadzu Corporation, Kyoto, Japan). Total anthocyanins (TA) were expressed as malvidin-3-glucoside chloride, flavanols reactive to vanillin (FRV) as (+)-catechin, and proanthocyanidins (PRO) as cyanidin chloride. Proanthocyanidins were transformed into cyanidin by acid hydrolysis at 100°C with a ferrous salt ($FeSO_4$) as catalyst (Bate-Smith reaction). Extracted phenolic compounds for each sampling point (6, 24, 48, 96, 168, 240 hours) (solution A), non-extracted phenolic compounds (solution B), and total phenolic compounds (fresh berry skins) were calculated as mg/g of skins, allowing to minimize the effect of berry weight, and then expressed as extraction yield. For each type of phenolic compounds, the extraction yield was estimated as the content in the solution A at each sampling point divided by the content in fresh berry skins, whereas the percentage of non-extracted phenolic compounds from skins was estimated as the content in the solution B divided by the content in fresh berry skins.

For the determination of the anthocyanin profile, berry skin extracts were filtered through 0.45 µm PTFE membrane filters (Pall Corporation, Port Washington, NY, USA) and injected (50 µL) in the HPLC-DAD system. The HPLC-DAD system and chromatographic conditions were previously reported in the literature (Río Segade *et al.*, 2014). Briefly, a LiChroCART column (25 cm × 0.4 cm i.d.)

purchased from Merck (Darmstadt, Germany) and packed with LiChrospher 100 RP-18 (5 μm) particles supplied by Alltech (Deerfield, IL, USA) was used. The mobile phases were A=formic acid/water (10:90, v/v), and B=formic acid/methanol/water (10:50:40, v/v), working at 1 mL/min flow-rate. The free forms of anthocyanins were identified by comparing the retention time of each compound with that of pure standard, whereas the tentative identification of the acylated forms was done by comparing the DAD spectrum and retention time of each chromatographic peak with those available in the literature (Pomar *et al.* 2005). The amounts of individual anthocyanins were expressed as percentages.

3.2.4 Statistical Analysis

Statistical analyses were performed using the SPSS statistics software package (version 19.0; IBM Corporation, Armonk, NY, USA). One-way analysis of variance (ANOVA) was carried out and Tukey-b ($p < 0.05$) test was used to establish significant differences.

3.3. Results and discussion

3.3.1 Grape composition at harvest

The most represented density class was 1107 kg/m^3, corresponding to a reducing sugar content of about 250 g/L in both cultivars, and therefore it was chosen. Complete technological ripeness data, skin phenolic composition and anthocyanin profiles of grapes at harvest are reported in Table 3.1. Anthocyanin profiles of Nebbiolo and Barbera berries were in accordance with those reported in literature for these varieties (Lambri *et al.* 2015; Mattivi *et al.* 2006; Torchio *et al.* 2010). Barbera is characterized by a high tri-substituted anthocyanins percentage with a malvidin-3-glucoside prevalence, whereas Nebbiolo is rich in di-substituted anthocyanins with a predominance of peonidin-3-glucoside. Barbera grapes presented lower FRV and PRO contents, whereas they were more abundant in TA compared to Nebbiolo in accordance with previous results (Río Segade *et al.* 2014; Rolle *et al.* 2012; Torchio *et al.* 2010).

3.3.2 Anthocyanin extraction kinetics

Anthocyanin extraction kinetics, expressed as extraction yield, is reported in **Figure 3.4**. Ozone treatments of Barbera grapes did not show significant effects on final extraction yield, although some differences were found at the beginning of maceration. In fact, the anthocyanin extractability of the control sample was higher than that of ozone-treated grapes: control sample showed a significantly different extraction yield (p<0.05) from OZ24 grapes only at 6 hours of maceration (+2.68%), whereas significant differences (p<0.05) were found compared to OZ72 grapes at 6, 24 and 48 hours of maceration (+4.03%, +8.93%, +9.48%, respectively). At 48 hours of maceration, for both control and OZ24 grapes, the maximum extraction was reached (71.67% and 66.17%, respectively), whereas for OZ72 grapes it was achieved at 96 hours of maceration (63.04%). Probably, these differences are due to a slowest anthocyanin extraction in long

ozone-treated samples. After reaching the maximum extraction yield, it decreased progressively for all the trials as maceration progressed because released anthocyanin compounds can suffer chemical reactions and also be fixed again onto the skins. Nevertheless, this decrease was lower in OZ72 grapes, and so that the differences were shortened. No significant differences were found after 48 hours among the different treatments, and at the end of maceration the final yield was 63.44%, 59.87%, and 59.69% in control, OZ24 and OZ72 samples, respectively.

The ozone treatment effect was more remarkable in Nebbiolo grapes, where the anthocyanin extraction occurred faster than in Barbera grapes.

Table 3.1 Chemical composition of Barbera and Nebbiolo winegrapes at harvest before ozone treatments (fresh grapes).

	Barbera	**Nebbiolo**
Reducing sugars (g/L)	249 ±1	248±1
Total acidity (g/L tartaric acid)	9.71±0.69	7.13±0.11
pH	3.21±0.01	3.18±0.01
Tartaric acid (g/L)	8.14±0.06	8.20±0.11
Malic acid (g/L)	3.17±0.11	2.38±0.02
Citric acid (g/L)	0.42±0.05	0.31±0.05
FRV (mg (+)-catechin/g skin)	1.94±0.17	6.27±0.31
PRO (mg cyanidin chloride/g skin)	10.22±1.09	14.82±0.36
TA (mg malvidin-3-glucoside chloride/g skin)	12.13±1.33	4.85±0.33
Dp-3-G (%)	14.77±0.31	4.50±0.26
Cy-3-G (%)	8.27±0.80	17.95±0.40
Pt-3-G (%)	12.99±0.23	3.44±0.11
Pn-3-G (%)	8.49±0.41	51.04±0.42
Mv-3-G (%)	35.50±0.88	14.52±0.30
∑Acetyl-G (%)	11.72±0.77	2.82±0.15
∑Cinnamoyl-G (%)	8.25±0.12	5.73±0.07

Legend All data are expressed as average value ± standard deviation (*n*= 3). FRV= flavanols reactive to vanillin, PRO= proanthocyanidins, TA= total anthocyanin, Dp-3-G= delphinidin-3-glucoside, Cy-3-G= cyanidin-3-glucoside, Pt-3-G= petunidin-3-glucoside, Pn-3-G= peonidin-3-glucoside, Mv-3-G= malvidin-3-glucoside, G= glucoside.

The highest extraction yield was reached at 24 hours of maceration with values of 90.16%, 86.88%, and 78.65% in OZ24, OZ72, and control grapes, respectively. From early stages of maceration (6 hours), significant differences were found between ozone-treated and control samples ($p < 0.01$), but not between the two ozone treatments. Nevertheless, at the end of maceration, when the extraction yield for control, OZ24 and OZ72 samples was 59.91%, 68.62%, and 64.23%, respectively, significant differences among all the samples were found ($p < 0.01$). At any maceration time, OZ24 sample gave the higher anthocyanin extraction yield, followed by OZ72. Ozone treatments facilitated the anthocyanin release from the skins into the wine-like solution without increasing the loss of released anthocyanins.

In Barbera grapes, longer maceration times seemed to reduce the initial differences in anthocyanin extractability among treatments, on the contrary in Nebbiolo the differences among treatments increased towards the end of the maceration period. Ozone can interact with the cell wall through disassembly phenomena leading to a decrease in pectin solubilization (Rodoni *et al.* 2010). Even if pectin solubilization is a required process to allow anthocyanin extraction, harder berry skins could be connected with a greater cell wall fragility allowing an easier phenolic compounds release in the medium (Río Segade *et al.* 2014). Laureano *et al.* (2016) found an increase in skin hardness in different table and wine grape varieties after ozone treatment (probably as occurred for Nebbiolo in this work). However, the skin hardening grade was variety dependent. In particular, in Barbera grapes with densities lower than 1119 kg/m^3, no significant increase of skin break energy (W_{sk}) was found, justifying the absence of significant differences except for the early maceration stages. Studies on cell wall composition showed some differences in the contents of uronic acids, cellulosic glucose, proteins, lignin and polyphenols among varieties, which can strongly influence the anthocyanin extractability (Hernández-Hierro *et al.*, 2014; Ortega-Regules *et al.* 2006b).

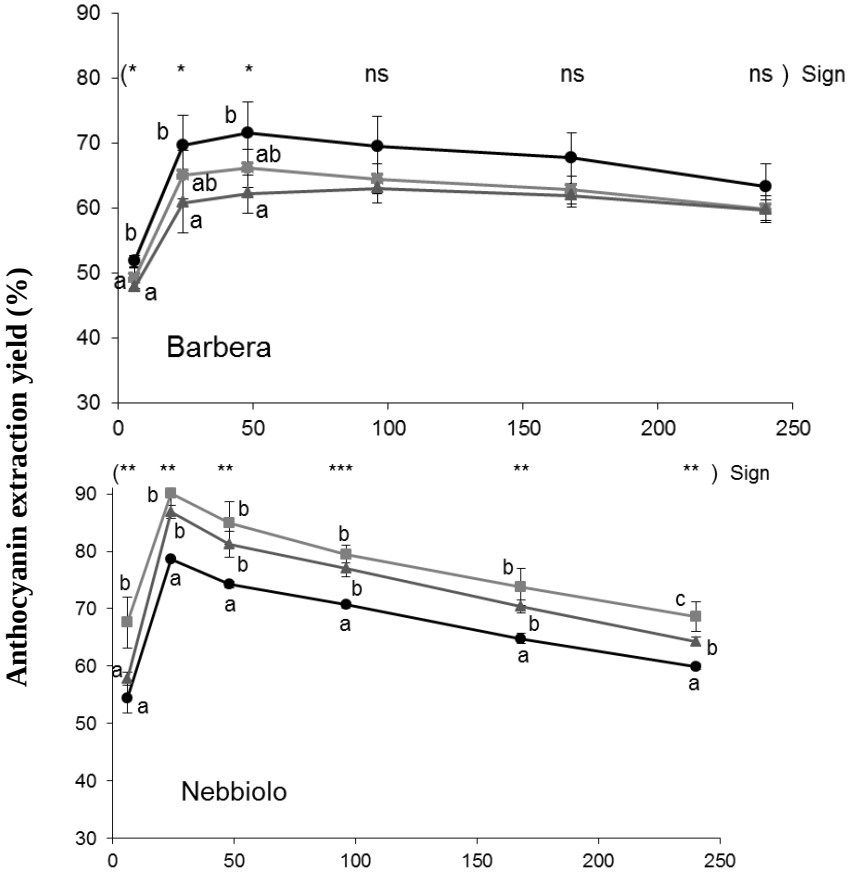

Figure 3.4 Effect of ozone treatment on the anthocyanin extraction during maceration for Barbera and Nebbiolo winegrapes.

Legend *All data are expressed as average value ± standard deviation (n= 3). Sign: *, **, *** and ns indicate significance at p < 0.05, 0.01, 0.001 and not significant, respectively, for the differences among treatments (●, control; ■, ozone treatment during 24 h; ▲, ozone treatment during 72 h) for each maceration time. Different letters indicate significant differences according to the Tukey-b test (p < 0.05).*

3.3.3. Anthocyanin profiles

Barbera and Nebbiolo anthocyanin profiles are shown in **Tables 3.2** and **3.3**, respectively. In all Barbera samples, malvidin-3-glucoside was the most abundant compound, reaching the maximum relative abundance at the end of maceration (48.27%, 49.20%, and 47.27% for control, OZ24, and OZ72, respectively). However, at the same maceration time, no significant differences in the anthocyanin profiles were found among the treatments, except for the non-extracted peonidin-3-glucoside fraction between control and OZ72 samples, showing a significantly higher concentration in control (+ 1.03%) than in OZ72 samples. No significant effect of ozone treatments was found on Nebbiolo anthocyanin profile, which is characterized by a high content of di-substituted anthocyanins: peonidin-3-glucoside was the main compound along maceration in all samples with a relative abundance of 50.59%, 51.46%, and 50.48% for control, OZ24, and OZ72 samples, respectively, at the end of maceration.

In both varieties higher differences in the anthocyanin profile were given by the maceration time. The extraction kinetics of individual anthocyanins was constant for all the treatments, confirming that it is dependent on each individual anthocyanin form (González-Neves *et al.* 2008). Generally, for Barbera grapes in all the treatments, di-substituted anthocyanins (cyanidin-3-glucoside and peonidin-3-glucoside) were extracted first, reaching the highest percentage at 6 hours of maceration and then decreased progressively. Cyanidin-3-glucoside was released at the beginning of maceration in Nebbiolo grapes, decreasing afterwards along maceration.

Cyanidin-3-glucoside is extracted early during vinification (González-Neves et al., 2008), but the higher contribution of this form to the anthocyanin profile (and therefore to the content) for Nebbiolo than for Barbera explains the faster extraction of total anthocyanins in Nebbiolo samples. In fact, cyanidin is

considered as the easiest anthocyanin to be extracted but the fastest form to decrease in grape juice. This is due to its oxidation during the early stages of winemaking when oxidative enzymes are more active and more oxygen is dissolved, and to the higher oxidability rate of the catechol substituent respect to the other anthocyanin substituents (Sarni *et al.* 1995). In the present study, simultaneously to the significant decrease of cyanidin-3-glucoside, as maceration progressed, a higher contribution of malvidin-3-glucoside on the total anthocyanins was observed for Nebbiolo and Barbera grapes in all samples.

In Barbera, petunidin-3-glucoside and delphinidin-3-glucoside reached the highest extraction percentage at 48 hours, although it was only significantly different for petunidin-3-glucoside in OZ72 samples. However, their relative abundances decreased afterwards in all samples, achieving the lowest percentages at the end of maceration. On the other hand, malvidin-3-glucoside increased continuously until the end of maceration. Conversely, in Nebbiolo, delphinidin-3-glucoside contribution was stable during maceration and petunidin-3-glucoside increased slightly at the end of maceration in control and OZ72 samples. Malvidin-3-glucoside also increased continuously during maceration representing the second most abundant anthocyanin form after 96 hours of maceration. The different kinetics of malvidin-3-glucoside and peonidin-3-glucoside can explain the differences among the two varieties at the point of highest extractability for total anthocyanins, where the peonidin prevalent-variety reached the highest extraction percentage before the malvidin-prevalent variety, as a consequence of the different affinity of anthocyanins to be released in the medium (Di Stefano *et al.* 1994). At the end of maceration, the Barbera and Nebbiolo extracts showed the highest percentages of mono-hydroxylated B-ring forms (malvidin and peonidin), which are less prone to oxidation leading to greater colour stability. Delphinidin, cyanidin, and petunidin are more oxidable and their concentration decreases more rapidly (Cheynier *et al.* 1994).

Table 3.2 Anthocyanin profile of berry skins during maceration for untreated and postharvest ozone treated Barbera winegrapes.

Treatment	Maceration time (h)	Di-substituted B-ring		Tri-substituted B-ring			∑Acetyl-G (%)	∑Cinnamoyl-G (%)
		Cy-3-G (%)	Pn-3-G (%)	Dp-3-G (%)	Pt-3-G (%)	Mv-3-G (%)		
Control	6	8.97±0.16c	10.15±0.34b	10.86±0.25c	11.18±0.37ab	40.46±0.39a	11.92±0.26	6.46±0.08b
	24	8.04±0.25bc	9.14±0.25a	11.23±0.39c	11.22±0.29ab	41.18±0.43a	12.20±0.29	6.98±0.04c
	48	7.83±0.29b	9.08±0.23a	11.62±0.45c	11.88±0.21b	40.73±0.76a	11.98±0.35	6.88±0.38bc
	96	7.37±0.45ab	8.91±0.16a	10.87±0.44c	11.57±0.29b	42.34±0.84a	12.02±0.35	6.91±0.04bc
	168	6.66±0.57a	8.73±0.18a	9.88±0.42b	11.29±0.29ab	44.74±0.94b	12.11±0.33	6.59±0.09bc
	240	6.40±0.62a	8.90±0.27a	8.26±0.41a	10.73±0.30a	48.27±1.32c	11.46±0.70	5.98±0.18a
	Sign[a]	***	***	***	**	***	ns	***
	Non-extracted	7.12±1.22	9.30±0.33β	6.29±1.16	11.03±1.14	43.56±2.07	11.09±0.67	11.60±0.51
OZ24	6	8.29±0.92d	9.78±0.55b	10.09±0.81bc	10.88±0.41ab	42.21±1.40a	12.18±0.78	6.57±0.46ab
	24	7.46±0.53cd	8.66±0.23a	11.06±0.39c	11.19±0.16ab	41.87±0.97a	12.64±0.25	7.13±0.30b
	48	7.14±0.51bcd	8.54±0.26a	11.27±0.31c	11.64±0.14b	41.67±0.83a	12.48±0.23	7.26±0.28b
	96	6.67±0.50abc	8.37±0.33a	10.40±0.37bc	11.35±0.14b	43.62±0.93ab	12.57±0.13	7.03±0.23b
	168	5.84±0.47ab	8.07±0.30a	9.54±0.55b	11.11±0.25ab	46.03±1.11b	12.63±0.22	6.78±0.14b
	240	5.49±0.45a	8.14±0.32a	8.02±0.75a	10.54±0.48a	49.20±1.64c	12.53±0.22	6.08±0.05a
	Sign[a]	***	***	***	*	***	ns	**
	Non-extracted	5.99±0.52	8.84±0.09αβ	5.60±0.78	10.34±1.03	45.34±1.52	11.71±0.35	12.18±0.59

continues

OZ72	6	8.83±0.38c	9.95±0.34b	10.30±0.33b	11.18±0.19ab	41.82±0.55ab	11.63±0.45	6.28±0.20a
	24	7.85±0.50bc	8.94±0.39ab	11.21±0.26c	11.40±0.15bc	41.61±0.60ab	12.07±0.26	6.94±0.22b
	48	7.73±0.41bc	8.92±0.27ab	11.51±0.27c	11.84±0.22d	41.06±0.20a	11.96±0.16	6.97±0.21b
	96	7.14±0.59ab	8.54±0.46a	11.10±0.15c	11.77±0.13cd	42.48±0.49b	11.93±0.31	7.03±0.18b
	168	6.50±0.65ab	8.38±0.52a	10.18±0.10b	11.41±0.07bc	44.65±0.54c	12.09±0.39	6.79±0.21ab
	240	6.18±0.70a	8.42±0.61a	8.84±0.37a	10.86±0.16a	47.27±0.13d	12.13±0.40	6.30±0.31a
	Sign[a]	***	**	***	***	***	ns	**
	Non-extracted	6.34±0.65	8.27±0.50α	7.21±0.64	12.31±0.69	43.41±1.05	10.59±0.54	11.87±0.86
	Sign[b]	ns,ns,ns,ns, ns,ns,ns	ns,ns,ns, ns,ns,ns,*	ns,ns,ns,ns, ns,ns,ns	ns,ns,ns,ns, ns,ns,ns	ns,ns,ns,ns, ns,ns,ns	ns,ns,ns,ns, ns,ns,ns	ns,ns,ns,ns, ns,ns,ns

Tables 3.2 Legend

*All data are expressed as average value ± standard deviation (n= 3). [a,b]Sign: *, **, *** and ns indicate significance at p < 0.05, 0.01, 0.001 and not significant, respectively. Different Latin letters ([a]) within the same column indicate significant differences among maceration times for each treatment according to the Tukey-b test (p < 0.05). Different Greek letters ([b]) within the same column indicate significant differences among treatments for each maceration time according to the Tukey-b test (p < 0.05). OZ24= ozone treatment during 24 h, OZ72= ozone treatment during 72 h. Dp-3-G= delphinidin-3-glucoside, Cy-3-G= cyanidin-3-glucoside, Pt-3-G= petunidin-3-glucoside, Pn-3-G= peonidin-3-glucoside, Mv-3-G= malvidin-3-glucoside, G= glucoside.*

Table 3.3 Anthocyanin profile of berry skins during maceration for untreated and postharvest ozone treated Nebbiolo winegrapes

Treatment	Maceration time (h)	Di-substituted B-ring		Tri-substituted B-ring			∑Acetyl-G (%)	∑Cinnamoyl-G (%)
		Cy-3-G (%)	Pn-3-G (%)	Dp-3-G (%)	Pt-3-G (%)	Mv-3-G (%)		
Control	6	18.48±1.41c	50.42±0.99	4.40±0.29	3.15±0.05a	16.11±0.53a	3.01±0.21	4.42±0.17a
	24	17.41±0.99bc	49.64±1.13	4.75±0.26	3.69±0.19b	16.37±0.56a	3.00±0.17	5.14±0.17d
	48	17.00±0.99abc	49.66±0.88	4.78±0.28	3.90±0.05b	16.70±0.45a	2.94±0.15	5.03±0.13cd
	96	16.25±0.98abc	50.09±1.07	4.65±0.27	3.87±0.19b	17.38±0.58a	3.00±0.11	4.77±0.16bc
	168	15.08±1.03ab	50.44±0.98	4.46±0.30	3.71±0.10b	18.74±0.68b	3.06±0.12	4.51±0.07ab
	240	14.51±0.98a	50.59±1.04	4.25±0.28	3.97±0.14b	19.52±0.69b	2.95±0.09	4.22±0.10a
	Sign[a]	**	ns	ns	***	***	ns	***
	Non-extracted	10.50±0.87	53.12±0.87	2.01±0.25	2.77±0.11	17.99±0.56	3.02±0.11	10.59±0.12
OZ24	6	18.18±1.04c	50.84±1.54	4.26±0.31	3.19±0.36	16.06±1.42a	3.12±0.10	4.36±0.21ab
	24	17.50±0.91c	50.42±0.83	4.53±0.24	3.61±0.10	15.92±0.99a	3.00±0.13	5.03±0.16d
	48	17.09±0.87bc	50.54±0.95	4.53±0.23	3.74±0.20	16.22±1.04a	2.98±0.10	4.90±0.23cd
	96	16.31±0.85abc	50.91±0.90	4.40±0.23	3.74±0.18	16.95±1.08ab	3.01±0.05	4.68±0.20bcd
	168	15.17±0.90ab	51.12±1.07	4.19±0.23	3.71±0.31	18.29±1.31ab	3.04±0.06	4.48±0.25abc
	240	14.53±0.75a	51.46±1.16	4.03±0.18	3.79±0.32	19.09±1.16b	2.98±0.10	4.12±0.19a
	Sign[a]	**	ns	ns	Ns	*	ns	**
	Non-extracted	10.92±1.27	53.57±1.83	1.79±0.17	2.32±0.59	17.94±1.37	3.07±0.05	10.39±0.59

Continues

OZ72	6	17.44±0.30e	51.02±1.29	4.24±0.19	3.18±0.22a	16.54±0.91a	3.11±0.10	4.47±0.18b
	24	16.76±0.20d	49.53±0.49	4.68±0.18	3.80±0.05b	17.00±0.46a	3.00±0.07	5.23±0.14e
	48	16.40±0.25d	49.72±0.35	4.70±0.22	3.84±0.04b	17.14±0.52a	3.02±0.06	5.17±0.14de
	96	15.50±0.23c	49.89±0.43	4.59±0.23	3.98±0.05b	18.10±0.52ab	3.07±0.04	4.87±0.10cd
	168	14.38±0.19b	50.04±0.53	4.35±0.26	4.00±0.10b	19.42±0.57bc	3.15±0.02	4.65±0.09bc
	240	13.54±0.11a	50.48±0.39	4.15±0.20	4.04±0.13b	20.59±0.57c	3.05±0.06	4.16±0.16a
	Sign[a]	***	ns	ns	***	***	ns	***
	Non-extracted	9.99±0.20	52.99±0.72	1.62±0.45	2.13±0.03	19.67±0.32	3.11±0.04	10.50±0.14
	Sign[b]	ns,ns,ns,ns, ns,ns,ns	ns,ns,ns,ns, ns,ns,ns	ns,ns,ns,ns, ns,ns,ns	ns,ns,ns,ns, ns,ns,ns	ns,ns,ns,ns, ns,ns,ns	ns,ns,ns,ns, ns,ns,ns	ns,ns,ns,ns, ns,ns,ns

Tables 3.3 Legend

*All data are expressed as average value ± standard deviation (n= 3). [a,b]Sign: *, **, *** and ns indicate significance at p < 0.05, 0.01, 0.001 and not significant, respectively. Different Latin letters ([a]) within the same column indicate significant differences among maceration times for each treatment according to the Tukey-b test (p < 0.05). Different Greek letters ([b]) within the same column indicate significant differences among treatments for each maceration time according to the Tukey-b test (p < 0.05). OZ24= ozone treatment during 24 h, OZ72= ozone treatment during 72 h. Dp-3-G= delphinidin-3-glucoside, Cy-3-G= cyanidin-3-glucoside, Pt-3-G= petunidin-3-glucoside, Pn-3-G= peonidin-3-glucoside, Mv-3-G= malvidin-3-glucoside, G= glucoside.*

In both varieties, acetyl derivatives were not influenced by neither the treatment nor the maceration time, whereas cinnamoyl derivatives seem to be affected by the maceration time. In fact, in Barbera the higher percentage of cinnamoyl derivatives was reached at 24, 48, and 96 hours for control, OZ24, and OZ72 samples, respectively, whereas in Nebbiolo the maximum contribution was observed at 24 hours.

The post-harvest ozone treatments tested did not modify or negatively influence the anthocyanin profiles of grapes. A previous work reported that physical treatments applied on fresh grapes, such as microwave, freezing, and steam blanching, can affect individual anthocyanin extractability (Río Segade *et al.* 2014), but this did not occur with ozone. In accordance with a previous study, the extraction kinetics of individual anthocyanins highlighted that their release during maceration depends on different solubility and structure of individual compound, and their content is influenced by the reactivity in the medium (Cheynier *et al.* 1994). The different affinity of individual anthocyanins for cell wall components conditions their extractability, and once solubilized in the medium, they can undergo reactions leading to losses or adducts neo-formation (Gonzales-Neves *et al.* 2008; Ortega-Regules *et al.* 2006b; Sarni *et al.* 1995). In general, the ratio tri-substituted/di-substituted anthocyanins increased with maceration in both varieties. In fact, an initial peonidin-3-glucoside and cyanidin-3-glucoside diffusion is followed by a higher tri-substituted anthocyanin extraction, in particular malvidin-3-gucoside (Di Stefano *et al.* 1994). This can result in an improvement of wine colour stability, since malvidin-3-glucoside is the most stable form of free anthocyanins.

3.3.4. Oligomeric and polymeric flavanol extraction kinetics

The determination of proanthocyanidins (PRO) using Bate-Smith reaction can estimate high molecular mass flavanols (i.e. ≥ 5 units, polymeric flavanols),

whereas flavanols reactive to vanillin (FRV) account for flavanols of 2-4 units and monomers (oligomeric flavanols) (Vrhovsek *et al.* 2001).

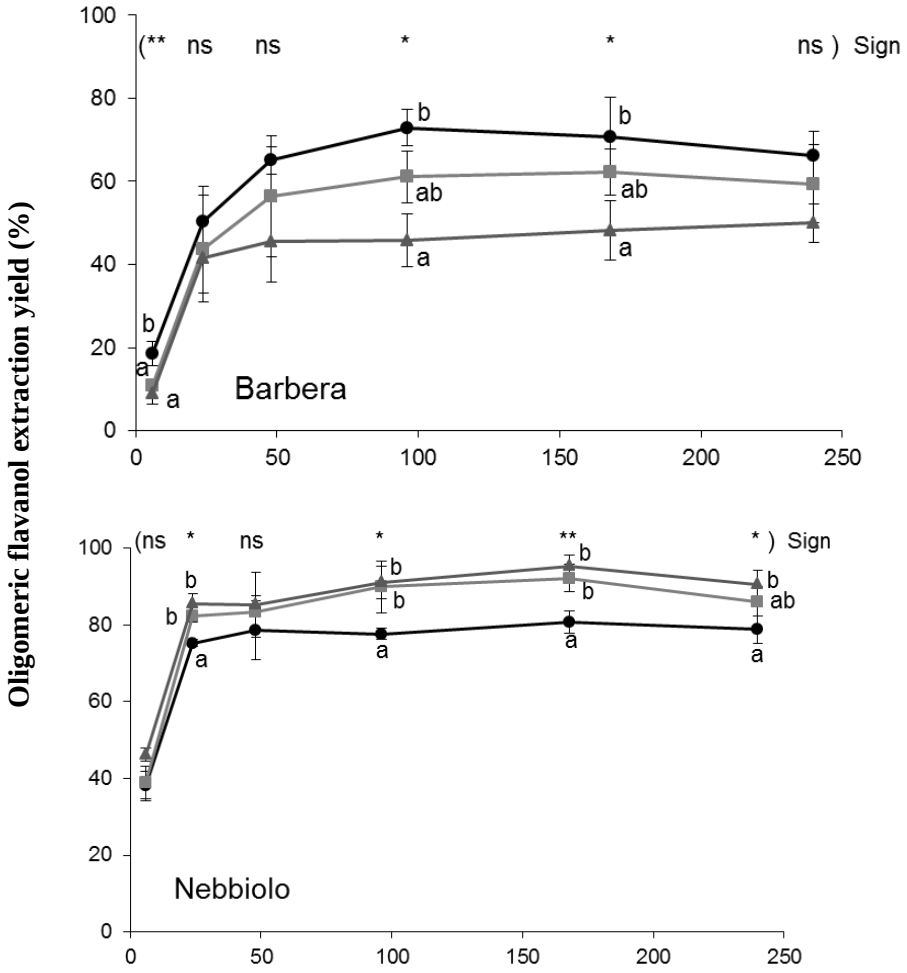

Figure 3.5 Effect of ozone treatment on the oligomeric flavanol extraction during maceration for Barbera and Nebbiolo wine grapes.

Legend *All data are expressed as average value ± standard deviation (n= 3). Sign: *, ** and ns indicate significance at p < 0.05, 0.01 and not significant, respectively, for the differences among treatments (●, control; ■, ozone treatment during 24 h; ▲, ozone treatment during 72 h) for each maceration time. Different letters indicate significant differences according to the Tukey-b test (p < 0.05).*

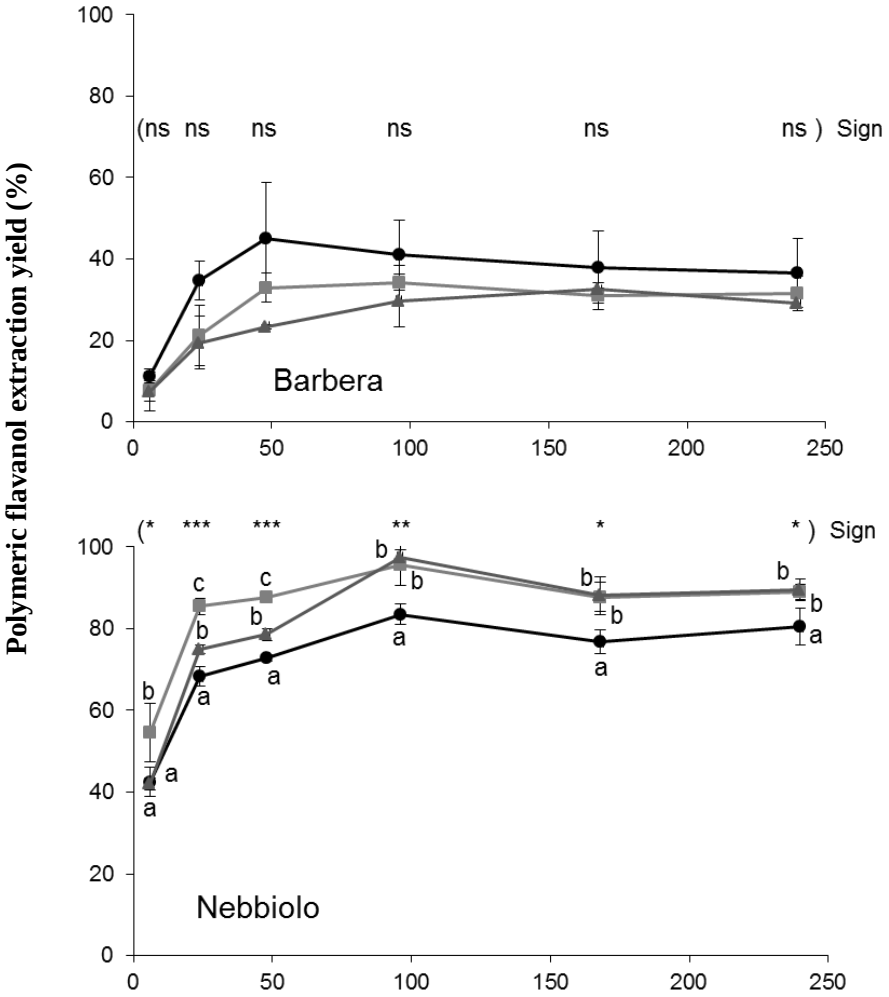

Figure 3.6 Effect of ozone treatment on the polymeric flavanol extraction during maceration for Barbera and Nebbiolo winegrapes.

Legend *All data are expressed as average value ± standard deviation (n= 3). Sign: *, **, *** and ns indicate significance at p < 0.05, 0.01, 0.001 and not significant, respectively, for the differences among treatments (●, control; ■, ozone treatment during 24 h; ▲, ozone treatment during 72 h) for each maceration time. Different letters indicate significant differences according to the Tukey-b test (p < 0.05).*

Figure 3.5 shows the extraction kinetics of oligomeric flavanols (FRV), expressed as extraction yield. Barbera grapes showed, in general, a lower FRV extraction yield than Nebbiolo, particularly in the ozone-treated grapes. In Barbera, as occurred for anthocyanin extraction, in the early maceration stage (6 hours), the two ozone-treated samples achieved significantly lower FRV extraction percentages than the control samples (-7.59% and -9.46% for OZ24 and OZ72, respectively; $p<0.01$). Then, no significant differences between the two ozone treated samples were found, whereas differences between OZ72 and control samples were found during maceration. After 6 hours of maceration, the FRV extractability agreed for OZ24 and control samples, but significantly lower extraction percentages were observed during maceration (i.e. at 96 and 168 hours) for OZ72 samples compared to control samples ($p<0.05$). Ozone treatments resulted in a slower FRV extraction: the maximum yields of 72.94%, 62.27%, and 50.05% were recorded at 96, 168, and 240 hours for control, OZ24, and OZ72, respectively. However, the final FRV extraction yield was not significantly different among treatments (66.10%, 59.38%, and 50.05% for control, OZ24, and OZ72, respectively) because the extraction percentage decreased for control and OZ24 samples after achieving the maximum value whereas it continued to increase in OZ72 samples until 240 hours of maceration.

Regarding Nebbiolo, higher FRV extraction yields were reached at 168 hours for all the trials. Contrarily to ozone-treated Barbera samples, Nebbiolo grapes treated with gaseous ozone had significantly higher FRV yields than the control sample ($p<0.05$) at 24, 96 and 168 hours of maceration for OZ24 and at 24, 96, 168 and 240 hours for OZ72. The final FRV extraction yield was 78.82%, 86.13%, and 90.55% for control, OZ24, and OZ72, respectively. In general, the longer the maceration the smoother the differences among samples, probably due to cell wall degradation phenomena and the ethanol enriched medium which probably facilitated the compounds extraction (Canals *et al.* 2005).

Polymeric flavanols (PRO) extraction kinetics for both Nebbiolo and Barbera winegrapes varieties is shown in **Figure 3.6**. In Barbera, PRO extraction was not significantly influenced by the ozone treatment at any maceration time, probably due to high standard deviations among replicates as well as to low values of extraction yield. As occurred for anthocyanins and oligomeric flavanols, the two ozone-treated samples showed lower PRO extraction percentages than the control samples, particularly OZ72 at maceration times lower than 168 hours. As seen also for oligomeric flavanols, ozone treatments slowed down the extraction kinetics: the highest PRO yield of 45.14%, 34.12%, and 32.53% was reached at 48, 96, and 168 hours for control, OZ24, and OZ72 samples, respectively. On the contrary, Nebbiolo showed significantly different PRO extraction kinetics among treatments. In the early stages of maceration (i.e. between 24 and 48 hours), significantly different PRO extraction yields were found among all three treatments themselves ($p<0.001$), in particular reaching higher extraction percentages in OZ24 samples followed by OZ72. In both ozone-treated samples, similar PRO extraction yields were observed at 96 hours of maceration (95.62% and 97.56% for OZ24 and OZ72 samples, respectively), whereas the control reached significantly lower values of 83.54% ($p<0.01$). These differences were kept along maceration and ozone-treated grapes had significantly higher extraction yields than control samples at 240 hours (80.51%, 89.08%, and 89.59% for control, OZ24, and OZ72 samples, respectively).

In Nebbiolo, ozone-treated grapes showed increased flavanol extraction yield, which was more evident in polymeric flavanols than in oligomers from the early stages of maceration. The oligomeric fraction is more easily extracted than the polymeric, because flavanol extraction becomes more difficult as the polymerization degree increases (Quijada-Morín *et al.* 2015). Polymeric flavanols strongly interact with the components of the skin cell wall, but its porosity also influences the extractability of these compounds. Ozone treatments decrease pectin solubilization and can lead to changes in the affinity degree between the cell wall and high molecular mass flavanols (Quijada-Morin *et al.*

2015; Rodoni *et al.* 2010). Changes in the skin cell wall composition facilitate the adsorption of high molecular mass fractions in relation with enhanced cell wall porosity (Bindon *et al.* 2012). As in grape ripening, the increase in the cell wall porosity can result in a greater adsorption of highly polymerized flavanols in the pores, leading to a slower or decreased extractability (Bindon *et al.* 2012; Quijada-Morín *et al.* 2015). Indeed, as the flavanols concentration increases, the selectivity of cell walls for the adsorption of high molecular mass flavanols decreases due to a concentration-dependent effect (Bindon *et al.* 2014). It partially explains the differences in extraction kinetics between the two varieties. A reduced and slow extraction of polymeric flavanols can be common in varieties with low flavanol contents, as it happened in Barbera. In Nebbiolo, higher skin flavanol concentrations could decrease the membrane selectivity for high molecular mass flavanols, resulting in an easier polymeric flavanols extraction accordingly to the concentration-dependent effect described by Bindon *et al.* (2014). Increased skin hardness after ozone treatment probably also facilitates the release of flavanols during maceration of Nebbiolo grapes (Laureano *et al.* 2016; Río Segade *et al.* 2014). Considering that the amount and structure of extracted flavanols are related to the grape variety (Mattivi *et al.* 2009), further studies should be done taking into account flavanols profiles and interactions with cell walls during ozone treatment to better understand these variations.

3.3.5. Ozone effects on phenolic compounds extractability

Ozone treatment showed different tendencies in the two varieties, leading to an increased skin phenolic compounds extraction in Nebbiolo grapes, while it did not influence the final extraction yield of Barbera grapes. Therefore, the ozone influence on phenolic compounds extractability was variety-dependent. Skin cell wall composition, thickness and hardness, berry weight as well as phenolic composition have a great effect on the extraction kinetics and extraction yield of phenolic compounds. Laureano *et al.* (2016) reported that post-harvest gaseous ozone treatments lead to an increase in skin hardness in all the grape varieties

studied, but the hardening degree is variety-dependent. In detail, higher skin break energy (W_{sk}) values were observed in ozone-treated Barbera only on berries with high level of ripeness (i.e 1,119 kg/m3), while at 1,107 kg/m3, Barbera grape density of this study, no difference were found.

Moreover, Río Segade *et al.* (2014), studying correlations between W_{sk} and phenolic compounds extractability, found an inverse relationship in the varieties studied: W_{sk} is positively correlated with phenolic compounds extractability in Nebbiolo, whereas in Barbera lower PRO, FRV and TA extraction yields were achieved for higher values of W_{sk} in berries belonging to the same density class (1107 kg/m^3). Mechanical properties, such as skin break energy, depend mainly on skin cell wall composition, which varies according to the maturity and to the grape variety (Hernández-Hierro *et al.*, 2014; Ortega-Regules *et al.* 2006b). During grape ripening, berry firmness loss involves complex phenomena associated with the disassembly of the pectin network at the primary cell wall and middle lamella (Ortega-Regules *et al.* 2006b). This degradation is derived from the action of hydrolytic enzymes. Among them, pectinmethylesterase (PME) catalyzes the demethylesterification of pectin residues, releasing sites accessible to polygalacturonase (PG) (Roe & Bruemmer, 1981). Botondi *et al.* (2015) studied PME and PG activities in shock ozone treatments (18 hours, 1.5 g/h) and long treatments (4 hours each day, 0.5 g/h) prior to or during withering, respectively, of wine grapes. They reported that those enzymes are unaffected by the ozone immediately after the treatment, but they showed a decline of PME activity in all samples and of PG activity in untreated berries after dehydration. In other horticultural products like tomatoes ozone fumigated at 10 µL/L for ten minutes, no differences were found in PG and PME activities immediately after the treatment, whereas after 9 days of storage PME showed a 50% decrease in its activity compared to the untreated sample (Rodoni *et al.* 2010).

D'Haese *et al.* (2006) highlighted that ozone-stress responses in *Arabidopsis thaliana* exposed to 150 ng/L ozone for 8 hours a day during two days include

up-regulating genes involved in cell wall stiffening and repressing those related to cell elongation processes. In our experimental conditions, probably there was not enough treatment time and/or maceration time to appreciate this effect, considering that the berries were processed after three days of treatment. Nevertheless, a possible induction of cell wall stiffening could have contributed to skin hardening of Nebbiolo grapes after ozone treatment promoting increased extractability of phenolic compounds.

Other cell defense response to ozone stress is the synthesis of antioxidants, such as flavanols (Artés-Hernández *et al.*, 2003; Carbone & Mencarelli, 2015). In particular, a study on white winegrapes cv. Grechetto showed a significant increase in (+)-catechin concentration after 12 hours of 1.5 g/h gaseous ozone treatment followed by one day of storage, showing a fast response of cells to ozone stress (Carbone & Mencarelli, 2015). However, other studies found no significant differences in total polyphenol and anthocyanin content in red winegrape cv. Pignola (Botondi *et al.* 2015), enforcing the supposition that grapes response to ozone stress could depend on the variety, as well as on the exposure time and ozone concentration.

Regarding the treatment time effect, in our findings OZ72 samples gave lower extractability confronted to the OZ24 samples in Nebbiolo for TA and PRO, whereas no significant differences were found for Barbera. Farther the hypothesis mentioned above, we cannot exclude an oxidation of phenolic compounds in samples treated with longer ozone exposure. Ozone oxidant activity is known, as it is decomposing itself either spontaneously or in contact with oxidable substrates such as phenolic compounds. Through direct reaction, ozone attaches itself to a double bound of organic compounds forming an unstable primary ozonide, which cleaves to form carbonyl compounds. In anthocyanins, the ring-opening is responsible for their degradation, leading to chalcone formation (Criegee 1975; Tiwari *et al.* 2009b). Tiwari *et al.* (2009a) found that gaseous ozone treatment (1.6 % w/w) for 10 minutes in processing grape juice causes

losses of 78%, 95%, and 99% of cyanidin-3-glucoside, delphinidin-3-glucoside, and malvidin-3-glucoside, respectively. Although even small quantities of ozone can strongly compromise the anthocyanin content of juices, no change was observed after ozone shock treatment of grapes (Artés-Hernández *et al.* 2003; Botondi *et al.* 2015). Moreover, ozone plays an important role in the formation of ozone derivative species with high reactivity, such as $^{\bullet}O_2^-$, $HO_2^{\bullet}$, $^{\bullet}OH$, and $^{\bullet}O_3^-$, which facilitates phenolic compounds degradation in a greater extent as their attitude to release electrons increases (based on the B-ring substituent). As a consequence, variety differences in the concentration of anthocyanins and flavanols, and their chemical patterns and degree of polymerization, can influence the extent of ozone effect on phenolic compounds extractability and final content.

3.4 Conclusions

The use of ozone as sanitizing agent has been largely discussed in table grapes storage. Nevertheless, ozone treatment of winegrapes is an innovative technology, which deserves further research. Our study was focused on the post-harvest treatment of winegrapes with short ozone treatments (maximum three days to allow the next production phases) prior to their processing in order to avoid mycobiota spoilage and to limit the use of sulphur dioxide.

Ozone influenced the early stages of skin maceration for both Nebbiolo and Barbera grapes, leading to a higher anthocyanin extraction yield in Nebbiolo grapes and lower in Barbera. This can be due to the faster extraction of di-substituted anthocyanins, hence an improved extraction of total anthocyanins in the peonidin-prevalent variety was observed. The final anthocyanin content was not influenced for Barbera, while it increased for Nebbiolo after treatment. Moreover, ozone did not influence the final individual anthocyanin extractability, respecting the varietal anthocyanin fingerprint. For Nebbiolo, a higher flavanol extraction in ozone-treated grapes, in particular high molecular mass flavanols, can improve wine colour stability during ageing through combinations with anthocyanins. Oligomeric and polymeric flavanol extraction was slowed in both varieties after the ozone treatment, in higher extent as long as the treatment exposure time increased.

Considering these results, the use of gaseous ozone on winegrapes should be considered as a possible tool in winemaking because phenolic compounds extractability is not affected or is enhanced in ozone-treated grapes, mainly depending on the variety and, to a lesser extent, on the exposure time.

4. Winegrapes dehydration under ozone-enriched atmosphere: influence on berry skin phenols release, cell wall composition and mechanical properties

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4.1 Introduction

Grape withering is a widespread technique used in wine industry to produce special wines with peculiar features, such as passiti, reinforced, sfursat and ice wines. Unlike the drying process, where the fast water removal avoids grapes over-ripening and senescence metabolism, dehydration during the withering process involves slow water loss and, as a consequence, grape berry composition changes depending on metabolic responses to water stress and on the susceptibility to fungal attack (Mencarelli & Tonutti, 2013). During "off-vine withering", grape dehydration takes place in detached bunches. "Natural off-vine withering" occurs under uncontrolled environmental conditions, whereas "forced off-vine withering", better defined as "controlled withering", is carried out in controlled thermohygrometric conditions using technology (Mencarelli & Tonutti, 2013).

The metabolism of berries during postharvest dehydration involves primary metabolites changes, such as sugars respiration/fermentation, gluconeogenesis and malate catabolism, and influences secondary metabolism, such as lignin pathway, cell wall composition, aroma and phenolic compounds, as responses to osmotic and oxidative stress (Bonghi *et al.* 2012). The direct consequence of water loss is metabolites concentration, in particular sugars, volatile compounds and polyphenols, although synthesis and loss can also occur (De Rosso *et al.* 2016).

Regarding red grape phenolic compounds, anthocyanins from skins, and monomeric, oligomeric and polymeric flavanols from both skins and seeds strongly influence the quality of final product depending on their contents and

chemical features because they are responsible for colour, astringency and bitterness of the wine (Chira *et al.* 2009; Vidal *et al.* 2004a; Vidal *et al.* 2004c). Grape dehydration leads to wines with increased mean degree of polymerization (mDP) of flavanols and reduced monomeric flavanol contents (Bonghi *et al.* 2012; Moreno *et al.* 2008), whereas controversial results are reported for grape anthocyanins depending on the variety, withering conditions and anthocyanin substitution patterns (Bellincontro *et al.* 2009; Bonghi *et al.* 2012; Toffali *et al.* 2011; Mikulic-Petkovsek *et al.* 2017). The extractability of phenolic compounds depends not only on the grape richness but also on the tendency to yield up them. In berry skins, anthocyanins are located inside cell vacuoles, whereas flavanols are mainly linked to the cell wall (Quijada-Morín *et al.* 2015). Therefore, skin cell wall constitutes the first barrier to phenolic compounds release even though the chemical and structural characteristics of phenolic compounds, such as stereochemistry, conformational flexibility, molecular weight and substitution pattern, together with cell wall composition and porosity can strongly influence their extractability (Bindon *et al.* 2014; Hernández-Hierro *et al.* 2014; Ortega-Regules *et al.* 2015).

Skin cell wall composition is variety-dependent, but postharvest dehydration can strongly influence the polysaccharides degradation because the higher the dehydration the higher the demethoxylation and depolymerization of pectins as a consequence of berry enzyme activities (Zoccatelli *et al.* 2013). This natural degradation of cell wall has a key role in berry skin softening (Yakushiji *et al.* 2001; Rolle *et al.* 2013). In particular, skin hardness parameters determined by instrumental texture analysis, such as berry skin break energy (W_{sk}) and berry skin break force (F_{sk}), have been largely investigated as predictors of the easiness of phenolic compounds to be released from skins to the wine (Río Segade *et al.* 2014).

An important aspect to take into account during dehydration is the microbiological control of grapes: the high relative humidity around berries

together with cracks in the berry skin can bring to mould infection, which is a danger to the wine quality and can lead to a production loss. Moreover, fungi development can cause the formation of compounds dangerous for human health, in particular some fungal species belonging to Aspergillus genus are responsible for ochratoxin A (OTA) contamination (Valero *et al.* 2008). Nowadays, the control of environmental conditions and the use of sulphur bentonite are the possible solutions to reduce the pathogen attack on berries (Mencarelli & Tonutti, 2013). Sulphur bentonite causes blanching of red grapes and could compromise secondary metabolites located in the skin. As an innovative alternative, ozone is a powerful tool to reduce fresh grapes microbiota, leading to satisfactorily healthy berries and resulting in faster and better controlled alcoholic fermentation (Bellincontro, *et al.* 2017; Cravero *et al.* 2016). Moreover, phenolic compounds extractability is not negatively affected or, in some cases, is even enhanced in fresh grapes (Bellincontro *et al.* 2017; Paissoni *et al.* 2017), as well as phenolic compounds content in withered grapes (Botondi *et al.* 2015), depending on the dose/time ratio of the ozone treatments and on the variety.

Nowadays, no studies on the impact of ozone treatments during the winegrape dehydration process on the extractability of the skin phenolic compounds have been made. Therefore, the aim of this work is to evaluate if the use of ozone as a sanitizing tool during grape dehydration affects the final content in withered grapes or the extractability of skin phenolic compounds during simulated maceration in a wine-like solution, as well as to try to justify those effects on the basis of skin cell wall composition and mechanical properties that are studied in withered ozone-exposed grapes also for the first time in this work.

4.2 Materials and methods

4.2.1 Grape samples and dehydration process

Whole bunches of Vitis vinifera L. cv. Nebbiolo and Barbera red winegrapes were harvested at technological maturity (about 24 ºBrix) in vineyards located in Piedmont region (Cuneo province, North-West Italy) in 2015. Once in the laboratory, for each grape variety a set of randomly selected grape berries (about 2 kg) was taken as fresh sample (fresh berries). The other bunches were cut in smaller clusters (5-6 berries each), visually inspected to remove unhealthy or damaged berries and randomly arranged in a single layer into twelve small perforated boxes (20 cm ×30 cm, about 1.5 kg of clusters each) for correct aeration. Six sample boxes were partially dehydrated into an ozone-enriched chamber and the other six boxes into an air chamber (control), taking three boxes at 10% weight loss and three boxes at 20% weight loss for both ozone-treated and control grapes. Weight loss (WL) was monitored daily, and thermohygrometric parameters were continuously recorded using a data logger (HOBO H8 RH/Temp, Onset Computer Corporation, Bourne, MA, USA) to confirm that the environmental conditions were similar in the two withering chambers. Temperature and relative humidity (RH) were controlled at 20 °C and 70% RH (Ossola *et al.* 2017) using dehumidifiers and air conditioning systems. In the ozone-enriched chamber, the ozone was continuously supplied by an ozone generator (C32-AG, Industrie De Nora Spa, Milan, Italy) with a nominal production capacity of 32 g O3/h. Ozone concentration into the chamber was set at 30 µL/L (Paissoni *et al.* 2017) and constantly monitored with a BMT 964 UV-photometric ozone analyzer (BMT Messtechnik GmbH, Stahnsdorf, Germany) that controls the ozone generator output.

4.2.2. Standard chemical parameters

For each variety studied, a first set of three berry subsamples (100 g each) of fresh grapes, as well as of air-treated and ozone-treated grapes dehydrated at 10 and 20% WL, were randomly collected to determine standard technological parameters. For each subsample, grape must was obtained by manual crushing and centrifugation. Reducing sugars (glucose and fructose, g/L), organic acids (tartaric acid, malic acid, citric acid, g/L), ethanol (% v/v) and glycerol (g/L) were determined by high performance liquid chromatography (HPLC) (Agilent Technologies, Santa Clara, CA, USA) using a refractive index detector and a diode array detector (DAD) set to 210 nm (Ossola *et al.* 2017). Titratable acidity (g/L tartaric acid) was estimated according to the International Organization of Vine and Wine method (OIV, 2018). pH was determined by potentiometry using an InoLab 730 pH meter (WTW, Weilheim, Germany).

4.2.3. Phenolic composition

4.2.3.1. Extraction of total phenolic compounds

Total content determination of phenolic compounds in fresh berries, as well as in air-treated and ozone-treated dehydrated berries, was performed as described by Río Segade *et al.* (2014). Briefly, for each grape variety and sample, a second set of three replicates of 10 berries were randomly selected and manually peeled with a laboratory spatula to separate skins from pulps. The berry skins were weighed and quickly immersed into 50 mL of a hydroalcoholic buffer solution at pH 3.2 containing 12% v/v ethanol, 5 g/L tartaric acid and 2 g/L sodium metabisulfite (solution B). The pulps were separately collected into tubes containing 100 mg sodium metabisulfite, weighed and diluted (9:1, m/m) with 5 mol/L sulphuric acid. Afterwards, an Ultraturrax high-speed homogenizer (IKA Labortechnik, Staufen, Germany) was used to homogenize the suspensions (Ultraturrax T25 at 8000 rpm for 1 min for skins, and Ultraturrax T10 at 9500 rpm for 30 s for pulps).

Homogenized suspensions were subsequently centrifuged in a PK 131 centrifuge (ALC International, Milan, Italy) for 15 min at 3000×g at 20 °C. Phenolic compounds were determined in the resulting pulp and skin solutions.

4.2.3.2 Extractability assessment of skin phenolic compounds

A third set comprised three replicates of 20 berry skins for fresh grapes, as well as for air-treated and ozone-treated dehydrated grapes, which were used to study the phenolic compounds extractability during simulated maceration as previously reported by Río Segade *et al.* (2014). For each variety and replicate, the skins were carefully manually removed from the pulp, weighed and quickly immersed into 100 mL of a hydroalcoholic buffer solution at pH 3.2 containing 12% v/v ethanol, 5 g/L tartaric acid and 100 mg/L sodium metabisulfite (solution A). Extractability solutions were kept at 25 °C for 7 days, and samples were taken at 3, 6, 9, 12, 24, 48, 85 and 168 h for phenolic compounds determination. The extraction percentage was calculated as the ratio between phenolic compounds contents in each solution A and in the solution B.

4.2.3.3. Phenolic compounds determination

The spectrophotometric determination of total anthocyanins (TA), flavanols reactive to vanillin (FRV) and proanthocyanidins (PRO) was performed as reported by Río Segade *et al.* (2014) using an UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Solutions A and B from the skins were directly analyzed, whereas the pulp extracts were submitted to reverse-phase solid-phase extraction (RP-SPE) using a 1 g Sep-Pak C-18 cartridge (Waters Corporation, Milford, MA, USA) with methanol as the eluent to remove sugars and organic acids that can interfere with the analysis. The contents for skins were calculated as both mg/kg grape (wet weight) and mg/g skin (lyophilized, dry weight) in order to consider overall changes (dehydration and ozone) in grapes phenolic composition and to underline differences imputable only to ozone treatment, respectively. The contents for pulps were calculated as mg/kg grape.

The results were expressed as malvidin-3-glucoside chloride (Extrasynthèse, Genay, France) for TA, (+)-catechin (Sigma-Aldrich, Saint-Louis, MO, USA) for FRV and cyanidin chloride (Sigma-Aldrich) for PRO.

For the determination of the anthocyanin profile, berry skin extracts (solution B) and C-18 purified pulp extracts were diluted 1:1 with 0.3 mol/L hydrochloric acid, filtered through 0.45 μm PTFE membrane filters (Pall Corporation, Port Washington, NY, USA) and injected (50 μL) in the HPLC-DAD system. The HPLC-DAD system and chromatographic conditions were previously reported (Río Segade *et al.* 2014). The amounts of individual anthocyanins were expressed as percentages.

4.2.4. Cell wall composition

For each variety, a fourth set of 300 berries for fresh grapes, as well as for ozone-treated and air-treated dehydrated grapes, were randomly taken to determine the skin cell wall composition. All berries were peeled using a laboratory spatula. The skins were carefully removed from the pulp, lyophilized and then manually ground to a fine powder with a mortar and pestle.

4.2.4.1. Isolation of cell wall material

The isolation of cell wall material was performed following the procedure proposed by De Vries *et al.* (1981) and adapted by Apolinar-Valiente *et al.* (2010). Briefly, 5 g of lyophilized berry skins were suspended in boiling water for 5 min, homogenized for 1 min at 10,000 rpm and centrifuged for 15 min at 3000×g. The raw alcohol-insoluble solids were obtained after treating the residue several times with fresh 70% v/v ethanol for 30 min at 40 °C, until the Dubois test (Dubois *et al.* 1956) indicated no sugars in the ethanol phase. After centrifugation, the alcohol-insoluble solids (AIS) was washed twice with 96% v/v ethanol and once with acetone, and finally dried overnight under an air

stream at 20 °C. The recovered cell wall material was manually ground and quantified as mg/g fresh skin.

4.2.4.2. Determination of cell wall composition

A set of four AIS replicates (10 mg each) were treated with 72% v/v sulfuric acid for 1 h at 30 °C and subsequently with 1 M sulfuric acid for 3 h at 100 °C for acid hydrolysis. Uronic acids were determined in the resulting solution by the colorimetric 3,5-dimethylphenol assay using galacturonic acid from Sigma-Aldrich as a standard (Bautista-Ortín *et al.* 2016). Neutral carbohydrates were also quantified in this solution as total glucose (Bautista-Ortín *et al.* 2016). Non-cellulosic glucose was determined performing directly acid hydrolysis with 1 mol/L sulfuric acid (Apolinar-Valiente *et al.* 2010) in other set of four replicates (10 mg each). Total glucose and non-cellulosic glucose were determined using an enzymatic kit from R-Biopharm (Darmstadt, Germany). Cellulosic glucose content was calculated as the difference between total glucose and non-cellulosic glucose contents. Klason lignin was determined gravimetrically after indirect acid hydrolysis (72% v/v sulfuric acid for 1 h at 30 °C and 1 mol/L sulfuric acid for 3 h at 100 °C) as described by Apolinar-Valiente *et al.* (2016).

In a third set of four AIS replicates (10 mg each), proteins and total phenols were extracted with 1 mol/L sodium hydroxide for 10 min at 100 °C and then quantified as reported by Apolinar-Valiente *et al.* 2010). Proteins were determined by the colorimetric Coomassie Brilliant Blue assay with Bovine Serum Albumin fraction V from J.T. Baker (Center Valley, PA, USA) as a standard. Total phenols were quantified spectrophotometrically by the Folin-Ciocalteu method using gallic acid from Sigma-Aldrich as a standard. All results were expressed as mg/g AIS cell wall material (mg/g CW).

4.2.5. Mechanical properties

A TA.XT plus texture analyzer (Stable Micro Systems, Godalming, Surrey, UK), equipped with a HDP/90 platform and a 5 kg load cell, was used for skin texture analysis. For each variety and sample, a fifth set composed of three replicates of 20 randomly selected grape berries were manually peeled, and the skins were removed from the pulp using a laboratory spatula. Each skin was individually punctured using a P/2N needle probe (Stable Micro Systems) and a test speed of 1 mm/s (Rolle *et al.* 2008). The skin hardness was experimentally assessed by measuring two parameters: berry skin break force (N, as F_{sk}) and berry skin break energy (mJ, as W_{sk}) (**Figure 4.1**).

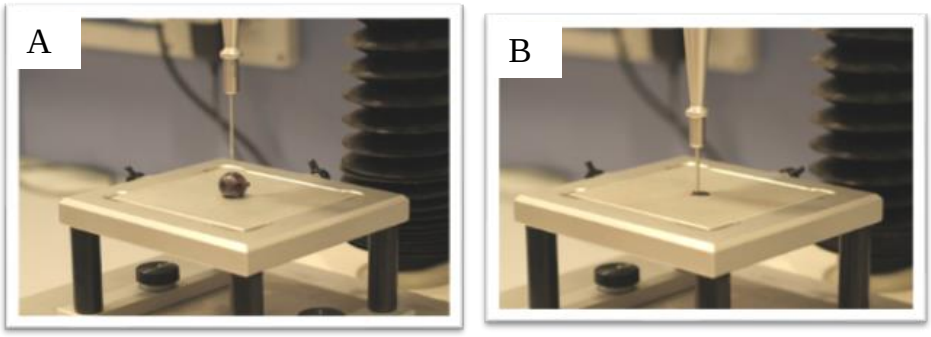

Figure 4.1 Texture analysis: A) berry skin break force and B) berry skin thickness.

4.2.6 Statistical analysis

Statistical analyses were performed using the SPSS statistics software package (IBM Corporation, Armonk, NY, USA). One-way analysis of variance (ANOVA) was carried out to establish significant differences between air and ozone treatments for grapes dehydrated at 10 and 20% WL. Pearson's correlation coefficients were calculated to determine significant relationships between phenolic compounds extractability and skin mechanical properties or cell wall components. Multivariate regression was used to propose a model that can explain better these relationships.

4.3 Results and discussion

4.3.1 Grape berries chemical composition

Grape analyses were performed before the treatment (fresh berries) to characterize the initial grape berries, and on dehydrated berries at 10 and 20% WL under both air and ozone-enriched atmosphere to assess the differences in the content of primary metabolites and phenolic compounds imputable to the treatment during dehydration (ozone-treated and air-exposed grapes). Results are shown in **Tables 4.1** and **4.2** for Barbera and Nebbiolo grapes, respectively.

Regarding standard technological parameters, significantly higher contents of reducing sugars were found in ozone-treated samples with respect to air-exposed berries for Nebbiolo at 20% WL ($p<0.05$) and Barbera at both 10% ($p<0.01$) and 20% WL ($p<0.05$), ranging from +6.8% to +13.7%. Increased sugars contents in ozone-treated fruits, in particular fructose and glucose, were previously reported for the storage of tomato fruit and papaya (Ali *et al.* 2014; Tzortzakis *et al.* 2007). The other technological parameters were not significantly affected by the berries exposure to ozone, except for glycerol where the trend was not evident. In the case of long-term but intermittent ozone treatments of grapes (1.5 g/h continuous flow followed by 0.5 g/h for 4 h each day during dehydration), the malate catabolism, which is responsible for the decrease of malic acid content and titratable acidity value, could be due to a double stress response (gluconeogenesis and respiration by water stress and oxidation by ozone stress) as hypothesized by Botondi *et al.* (2015). However, titratable acidity did not decline during dehydration when the grapes were previously shock-ozone treated at 1.5 g/h continuous flow for 18 h (Botondi *et al.* 2015). Malic acid contents were unaffected by continuous ozone treatment also during tomato fruit storage for six days when compared with air-exposed fruits (Tzortzakis *et al.* 2007). According

to Heath (2008), different metabolic pathways are stimulated by ozone exposure, depending on ozone dose or exposure time regimes.

Regarding phenolic composition, it is important to understand if the changes in partially dehydrated grapes are due to chemical reactivity, degradation phenomena or metabolic induction by ozone exposure. Barbera and Nebbiolo red winegrapes were chosen for this study to evaluate the effects of ozone during the partial dehydration of two varieties with distinctive content and profile of phenolic compounds (Río Segade *et al.* 2014). Taking into account that the diffusion of anthocyanins from the skin to the pulp occurs during grape dehydration due to the structural alterations in the skin (Marquez *et al.*, 2014), phenolic compounds were determined in both berry skins and pulps (**Tables 4.1** and **4.2**). TA contents from skins and pulps were not influenced by the ozone treatment in Nebbiolo at both 10 and 20% WL, whereas significantly lower TA contents (-11.3%, $p<0.05$, and -49.7%, $p<0.001$, for skins expressed as dry weight and pulps, respectively) were found in ozone-treated Barbera winegrapes at 20% WL with respect to control samples. A small TA decrease was observed in the skin of air-treated Barbera winegrapes, but it was partially offset by the increased release of TA to the pulp during dehydration. Ozone-enriched atmosphere favoured this decreasing effect more than the water loss, although it was less balanced by releasing TA to the pulp during dehydration.

It was previously demonstrated that postharvest physical treatments on whole berries can facilitate the anthocyanin release from the skin to pulp (Río Segade *et al.* 2014). On the other hand, Botondi *et al.* (2015) observed that dehydration contributed negatively to the TA content, which was compensated for ozone-exposed Pignola red winegrapes using both shock and long-term but intermittent treatments at 20% WL, but greater TA losses were reported at 35% WL for ozone-treated berries with respect to untreated samples. Tiwari *et al.* (2009)a showed that the degradation of free anthocyanin forms is due to the oxidizing potential of ozone, and it is favoured when long treatments are applied. However, the

metabolic response to ozone stress depends on ozone dose, exposure time and treatment temperature (Heath, 2008) but also on grape variety as shown by our results. In our experimental conditions, ozone did not influence negatively TA content in whole grape berries, and a slight decrease was observed when the 20% WL was reached only for the Barbera variety (about -5% considering together skin and pulp), which is characterized by a high content of anthocyanins.

Regarding individual skin anthocyanins, in both varieties, a significant decrease in the percentage of di-substituted anthocyanins at 10% WL was observed: cyanidin-3-glucoside and peonidin-3-glucoside in ozone-treated Barbera grapes (for both compounds -0.7%, $p<0.05$) and peonidin-3-glucoside in ozone-treated Nebbiolo (-2.8%, $p<0.05$) when compared with control samples. In our case of study, differences in the skin anthocyanin composition between ozone-treated and air-exposed samples could be better justified by chemical reactivity than by release from skins to pulp during the grape treatment. In fact, the anthocyanin profile of the pulp was not significantly different for ozone-treated and control berries dehydrated at 10% WL (**Table 4.1** and **4.2**). This is a positive effect of ozone exposure because di-substituted anthocyanin forms are released faster from the skin during maceration and therefore can undergo more easily oxidation than tri-substituted anthocyanins, particularly cyanidin derivatives (González-Neves *et al.* 2008). At 20% WL, a significant increase of skin tri-substituted anthocyanins was found due to ozone effect: delphinidin-3-glucoside (+0.8%, $p<0.05$) and malvidin-3-glucoside (+3.3%, $p<0.05$) for Barbera and Nebbiolo, respectively. The greater presence of malvidin derivatives can favour a more stable red pigmentation through interaction with flavanols and ethanal (Cheynier *et al.* 1994), and it is particularly important for di-substituted prevalent varieties, such as Nebbiolo.

Considering the pulp, Barbera grapes dehydrated at 20% WL under ozone-enriched atmosphere showed a significantly increased percentage of peonidin-3-glucoside (+3.4%, $p<0.05$) and decreased relative amounts of delphinidin-3-

glucoside and petunidin-3-glucoside (-4.5 and -4.1%, respectively, both $p<0.001$). Tiwari *et al.* (2009)a reported different degradation kinetics for each individual anthocyanin during ozone treatment of grape juice, where malvidin-3-glucoside, delphinidin-3-glucoside and cyanidin-3-glucoside decreased 99, 95 and 78%, respectively, after 10 min at an ozone concentration of 1.6% (w/w). However, in our study, malvidin-3-glucoside derivatives in the skin and pulp were not negatively affected by the ozone treatment.

Regarding flavanols, the response to the ozone treatment was quite similar to that observed for anthocyanins. At 10% WL, no significant differences were found in both skin and pulp monomeric and oligomeric (FRV) and polymeric (PRO) flavanols between ozone-treated and control samples for both the varieties analyzed (Table 4.1 and 4.2). Instead, at 20% WL, FRV showed inverse trends in the two varieties studied: a significantly increased FRV content was observed for ozone-treated samples in Nebbiolo skins (+14.6%, $p<0.05$, for wet berry weight), but a decrease was found in Barbera skins (-21.8%, $p<0.05$, for dry skin weight) and pulps (-42.0%, $p<0.05$). For PRO contents in Barbera winegrapes, a decrease was reported only in the skins with the ozone treatment (-21.4 and -14.0%, both $p<0.05$, for dry skin weight and wet berry weight, respectively). In Nebbiolo, no significant differences were found in PRO content. The different behaviour of the two varieties under the same ozone treatment could be associated with the dehydration effect for Nebbiolo and with a combined effect of dehydration and ozone for Barbera. The varietal differences in the flavanic profile could justify these results.

Carbone and Mencarelli (2015) showed a great reduction of both total flavanols and total phenolics contents for ozone-treated Grechetto white winegrapes (1.5 g/h ozone for 12 h at 10 ºC) when compared to air-exposed fresh berries, whereas Bellincontro *et al.* (2017) observed a significant increase in flavanols for Petit Verdot red winegrapes fumigated at max 20 g/h with 6% (w/w) of ozone at 4 ºC (+8.9%). Botondi *et al.* (2015) reported no significant differences in total

phenolics contents of Pignola red winegrapes just after shock-ozone treatment (1.5 g/h ozone for 18 h at 10 ºC), but they also showed a greater decrease when ozone-treated samples were then dehydrated at 20 and 35% WL under atmosphere enriched for 4 h/day with 0.5 g/h of ozone with respect to dehydration in air atmosphere.

Table 4.1 Chemical composition of fresh berries and partially dehydrated berries under air and ozone atmosphere for Barbera.

Compound	Units	Fresh berries	10% Average berry WL			20% Average berry WL		
			Air	Ozone	Sign	Air	Ozone	Sign
Grape must								
Reducing sugars	g/L	254±2	270±2	307±1	**	307±2	333±4	*
Titratable acidity	g/L tartaric acid	9.1±0.2	8.4±0.0	8.3±0.3	ns	8.6±0.4	8.5±0.2	ns
pH	-	3.06±0.03	3.10±0.01	3.05±0.01	*	3.09±0.01	3.10±0.02	ns
Tartaric acid	g/L	9.83±0.02	9.05±0.01	9.46±0.14	ns	9.97±0.04	10.01±0.11	ns
Malic acid	g/L	1.86±0.09	1.31±0.01	1.44±0.10	ns	1.79±0.82	1.28±0.01	ns
Citric acid	g/L	0.24±0.01	0.27±0.01	0.27±0.01	ns	0.27±0.01	0.31±0.02	ns
Ethanol	% v/v	0.00±0.01	0.14±0.01	0.14±0.02	ns	0.45±0.23	0.26±0.05	ns
Glycerol	g/L	0.05±0.01	0.85±0.09	0.51±0.03	*	1.47±0.01	2.28±0.09	**
Grape skin								
TA	mg malvidin-3-G chloride/g skin (dry weight)	33.0±1.4	26.3±1.3	25.4±0.6	ns	24.0±0.6	21.3±1.1	*
	mg malvidin-3-G chloride/kg grape (wet weight)	1534±95	1491±7	1558±58	ns	1487±170	1441±75	ns
Dp-3-G	%	12.6±0.6	13.2±0.4	13.1±0.5	ns	12.1±0.4	12.9±0.3	*
Cy-3-G	%	3.6±0.1	3.9±0.3	3.2±0.2	*	3.5±0.4	3.7±0.7	ns
Pt-3-G	%	13.4±0.5	13.7±0.3	13.9±0.4	ns	13.2±0.2	13.6±0.2	ns
Pn-3-G	%	4.6±0.4	4.7±0.4	4.0±0.2	*	4.5±0.6	4.6±0.7	ns
Mv-3-G	%	43.9±0.4	44.0±0.3	45.1±1.0	ns	45.9±0.3	45.4±0.9	ns
Σ Acetyl-G	%	10.3±0.3	9.6±0.4	9.6±0.7	ns	8.8±0.3	8.3±0.3	ns
Σ Cinnamoyl-G	%	11.5±0.2	10.9±0.1	11.1±0.2	ns	12.0±0.4	11.5±0.9	ns

Continues

FRV	mg (+)-catechin/g skin (dry weight)	8.54±0.96	8.29±0.67	7.39±1.17	ns	9.68±0.95	7.57±0.14	*
	mg (+)-catechin/kg grape (wet weight)	397±43	486±63	479±56	ns	603±107	512±20	ns
PRO	mg cyanidin chloride/g skin (dry weight)	32.2±2.9	29.1±3.9	28.2±3.6	ns	30.3±3.5	23.8±0.6	*
	mg cyanidin chloride/kg grape (wet weight)	1495±111	1696±165	1825±96	ns	1868±136	1607±64	*
Grape pulp								
TA	mg malvidin-3-G chloride/kg grape (wet weight)	21.2±3.4	31.0±9.9	22.1±1.1	ns	58.5±2.2	29.4±2.9	***
Dp-3-G	%	5.6±0.3	5.1±1.1	4.8±1.0	ns	8.7±0.6	4.2±0.4	***
Cy-3-G	%	12.4±1.7	9.0±1.6	9.5±2.5	ns	7.2±1.0	8.6±0.6	ns
Pt-3-G	%	7.8±0.2	8.2±1.5	7.9±0.6	ns	11.5±0.2	7.4±0.6	***
Pn-3-G	%	20.9±1.3	16.4±3.1	15.3±2.8	ns	11.5±0.8	14.9±1.4	*
Mv-3-G	%	44.7±2.6	52.8±3.4	54.5±5.1	ns	52.6±1.5	56.7±2.1	ns
Σ Acetyl-G	%	0.0±0.0	0.0±0.0	0.0±0.0	-	0.0±0.0	0.0±0.0	-
Σ Cinnamoyl-G	%	8.5±0.4	8.5±0.3	7.9±0.3	ns	8.3±0.8	8.2±0.9	ns
FRV	mg (+)-catechin/kg grape (wet weight)	12.0±1.7	13.4±0.8	12.4±1.2	ns	22.4±1.5	13.0±3.8	*
PRO	mg cyanidin chloride/kg grape (wet weight)	48.0±6.3	48.0±5.7	47.2±7.4	ns	81.3±11.7	52.8±15.2	ns

Table 4.1 Legend *All data are expressed as average value ± standard deviation (n = 3). TA: total anthocyanins, Dp-3-G: delphinidin-3-glucoside, Cy-3-G: cyanidin-3-glucoside, Pt-3-G: petunidin-3-glucoside, Pn-3-G: peonidin-3-glucoside, Mv-3-G: malvidin-3-glucoside, G: glucoside, FRV: flavanols reactive to vanillin, PRO: proanthocyanidins, WL: weight loss. Sign: *, **, *** and ns indicate significance at p<0.05, 0.01, 0.001 and not significant, respectively, for the differences between air and ozone treatments at the same dehydration level.*

Table 4.2. Chemical composition of fresh berries and partially dehydrated berries under air and ozone atmosphere for Nebbiolo.

Compound	Units	Fresh berries	10% Average berry WL			20% Average berry WL		
			Air	Ozone	Sign	Air	Ozone	Sign
Reducing sugars	g/L	258±19	270±2	274±2	ns	307±2	328±2	*
Titratable acidity	g/L tartaric acid	6.4±0.2	5.1±0.0	5.2±0.1	ns	5.3±0.2	5.3±0.1	ns
pH	-	3.16±0.04	3.32±0.02	3.27±0.01	ns	3.25±0.03	3.26±0.03	ns
Tartaric acid	g/L	7.51±0.50	7.05±0.27	6.41±0.20	ns	7.86±0.10	7.36±0.36	ns
Malic acid	g/L	1.40±0.26	1.00±0.06	1.07±0.05	ns	0.94±0.01	1.84±0.57	ns
Citric acid	g/L	0.19±0.07	0.13±0.01	0.14±0.01	ns	0.01±0.01	0.01±0.02	ns
Ethanol	% v/v	0.00±0.01	0.05±0.01	0.03±0.01	ns	0.14±0.07	0.33±0.30	ns
Glycerol	g/L	0.21±0.30	0.18±0.06	0.07±0.03	ns	1.29±0.13	0.83±0.18	ns
Grape skin								
TA	mg malvidin-3-G chloride/g skin (dry weight)	13.3±1.1	13.0±1.2	13.3±0.2	ns	13.4±0.1	13.5±0.4	ns
	mg malvidin-3-G chloride/kg grape (wet weight)	612±36	701±40	710±12	ns	760±25	796±29	ns
Dp-3-G	%	7.1±0.0	7.3±0.6	7.9±0.2	ns	7.2±0.5	6.8±0.4	ns
Cy-3-G	%	8.9±0.7	13.9±0.6	13.1±0.8	ns	12.5±0.6	11.0±1.6	ns
Pt-3-G	%	5.7±0.1	5.4±0.4	5.6±0.2	ns	5.4±0.5	5.3±0.3	ns
Pn-3-G	%	32.8±1.5	36.5±0.5	33.7±0.9	*	35.9±1.3	34.1±0.7	ns
Mv-3-G	%	31.5±1.0	23.9±0.8	25.8±1.5	ns	25.3±1.1	28.6±1.4	*
Σ Acetyl-G	%	4.4±0.4	3.8±0.2	4.0±0.2	ns	3.5±0.1	3.6±0.2	ns
Σ Cinnamoyl-G	%	9.5±1.0	9.3±0.1	9.9±1.0	ns	10.2±1.0	10.6±1.3	ns

Continues

FRV	mg (+)-catechin/g skin (dry weight)	36.0±3.0	35.3±4.1	33.9±2.4	ns	36.7±3.5	40.3±0.7	ns
	mg (+)-catechin/kg grape (wet weight)	1658±99	1907±178	1809±129	ns	2084±200	2389±33	*
PRO	mg cyanidin chloride/g skin (dry weight)	78.3±6.4	81.6±0.9	75.5±5.6	ns	90.2±7.6	84.4±7.6	ns
	mg cyanidin chloride/kg grape (wet weight)	3607±225	4419±138	4026±320	ns	5123±453	5003±456	ns
Grape pulp								
TA	mg malvidin-3-G chloride/kg grape (wet weight)	10.4±1.9	11.0±0.6	9.4±2.2	ns	13.3±0.6	13.1±0.1	ns
Dp-3-G	%	3.7±0.6	4.4±0.5	5.2±0.8	ns	5.3±1.0	4.7±0.5	ns
Cy-3-G	%	33.9±2.0	37.5±5.0	36.9±3.5	ns	31.0±1.7	30.9±3.2	ns
Pt-3-G	%	2.8±0.4	2.9±0.3	3.2±0.5	ns	3.4±0.5	3.1±0.1	ns
Pn-3-G	%	41.6±0.6	38.9±2.5	38.3±1.9	ns	42.0±0.8	41.7±2.3	ns
Mv-3-G	%	15.4±0.5	13.7±2.0	13.4±1.3	ns	14.9±1.4	16.6±1.3	ns
Σ Acetyl-G	%	0.0±0.0	0.0±0.0	0.0±0.0	-	0.0±0.0	0.0±0.0	-
Σ Cinnamoyl-G	%	2.5±0.3	2.7±0.2	2.9±0.5	ns	3.5±0.3	3.0±0.3	ns
FRV	mg (+)-catechin/kg grape (wet weight)	73.4±12.2	92.7±8.0	76.7±21.4	ns	103.4±10.0	98.6±12.7	ns
PRO	mg cyanidin chloride/kg grape (wet weight)	176±23	188±24	161±38	ns	190±5	183±14	ns

Table 4.2 Legend *All data are expressed as average value ± standard deviation (n = 3). TA: total anthocyanins, Dp-3-G: delphinidin-3-glucoside, Cy-3-G: cyanidin-3-glucoside, Pt-3-G: petunidin-3-glucoside, Pn-3-G: peonidin-3-glucoside, Mv-3-G: malvidin-3-glucoside, G: glucoside, FRV: flavanols reactive to vanillin, PRO: proanthocyanidins, WL: weight loss. Sign: *, **, *** and ns indicate significance at p<0.05, 0.01, 0.001 and not significant, respectively, for the differences between air and ozone treatments at the same dehydration level.*

4.3.2. Skin phenolic compounds extractability

In addition to the differences of phenolic compounds content between air-exposed and ozone-treated grapes, TA, FRV and PRO extractabilities were also assessed through simulated maceration of the skins. The results are shown in **Figures 4.2** and **4.3** for Barbera and Nebbiolo, respectively.

Regarding TA extractability, the two varieties showed different kinetics. For Barbera, a significantly higher TA extractability was found for ozone-treated grapes at 20% WL from the beginning up to 48 h of maceration (**Figure 4.2b**). For longer maceration times, the differences were not significant as also occurred for grapes dehydrated at 10% WL throughout the entire maceration process (**Figure 4.2a**). At the end of maceration, the TA extraction yield and extractable content for ozone-treated Barbera grapes were not significantly different from those for air-exposed samples (**Table 4.3**). On the contrary, in Nebbiolo at both 10 and 20% WL, ozone treatment leaded to a significantly lower anthocyanin extraction throughout the maceration process with respect to the air-exposed grapes: the greater the %WL the lower the ozone unfavourable effect (**Figure 4.3a** and **b**). In particular, at the end of maceration, TA extraction yield was reduced by -9.1% ($p<0.05$) and -7.7% ($p<0.001$) for 10 and 20% WL, respectively, and therefore the TA extractable content (**Table 4.3**) for ozone-treated samples also decreased when compared to air-exposed grapes at both 10 and 20% WL (about -11.8%, $p<0.05$, and -12.9%, $p<0.01$, respectively, considering dry skin weight, and -13.0% and -13.9%, respectively, both $p<0.05$ considering wet berry weight). The same significant differences were observed by assessing together anthocyanins released to the pulp during dehydration and those extracted after 168 h of maceration: the TA extractability was 49.0% for Barbera air-treated grapes at 10 and 20% WL, 47.5 and 51.2% for Barbera ozone-exposed grapes at 10 and 20% WL, 67.3 and 56.8% for Nebbiolo air-treated

grapes at 10 and 20% WL, and 58.3 and 49.0% for Nebbiolo ozone-exposed grapes at 10 and 20% WL, respectively.

In fresh grapes, Bellincontro *et al.* (2017) reported a higher anthocyanin extraction during Petit Verdot grapes industrial-scale fermentation after a shock ozone treatment (12 h, max 20 g/h with 6% w/w of ozone). During simulated maceration, Paissoni *et al.* (2017) found an increased anthocyanin extractability in Nebbiolo grapes after shock ozone treatment (24 and 48 h, 30 µL/L), whereas in the same conditions the anthocyanin extractability for Barbera was not significantly affected by the treatment. In the present study on partially dehydrated grapes, an inverse trend was observed for the Nebbiolo variety. This highlights that, in addition to the ozone effect on the TA extractability, the dehydration process can induce changes in the skin cell wall composition and texture as will be reported later.

Regarding the anthocyanin profile at the end of maceration (**Table 4.3**), no significant difference was observed for Barbera grapes dehydrated at 10 or 20% WL under ozone-enriched atmosphere and air exposure, as previously reported by Paissoni *et al.* (2017) in fresh grapes. However, Nebbiolo showed significantly lower di-substituted anthocyanin percentages (-2.4% for cyanidin-3-glucoside and -2.5% for peonidin-3-glucoside, both $p<0.05$) for ozone-exposed grapes only at 20% WL in favour of higher malvidin-3-glucoside amounts (+5.9%, $p<0.01$). This may result in improved colour stability, since malvidin-3-glucoside structure is less prone to oxidation (Cheynier *et al.* 1994).

In Barbera, FRV extractability at the beginning of maceration was significantly higher in ozone-treated grapes than in air-exposed samples at both the dehydration levels (until 168 h for 10% WL and 48 h for 20% WL, as shown in **Figure 4.2 c** and **d**). Although ozone treatment seems to facilitate the FRV extraction in this variety, the extractable content of flavanols at the end of maceration decreased significantly in Barbera grapes dehydrated at 20% WL under ozone-enriched atmosphere (-20.2 and -26.6%, both $p<0.05$ for FRV considering dry skin weight and wet berry weight, respectively, and -18.1%, $p<0.05$, and -25.0%, $p<0.01$, for PRO considering dry skin weight and wet berry

weight, respectively; **Table 4.2**). PRO extraction kinetics was not modified in ozone-treated Barbera grapes (**Figure 4.2 e** and **f**). In Nebbiolo, as it can be seen in **Figure 4.3 c** and **d**, FRV extraction during maceration was not influenced by the treatment (ozone or air), and significant differences were found only at the end of maceration for 20% WL grapes when a lower extraction yield was observed for ozone-treated grapes (-4.6%, $p<0.05$). Nevertheless, Nebbiolo grapes dehydrated at 20% WL under ozone-enriched atmosphere showed increased PRO extractability until the end of maceration (**Figure 4.3 f**), at which time no significant differences were observed in agreement with the extractable PRO contents (**Table 4.3**). Decreased flavanol contents were found in Nebbiolo grapes dehydrated at 10% WL in ozone-enriched atmosphere (-5.1%, $p<0.001$, and -6.0%, $p<0.05$, for FRV considering dry skin weight and wet berry weight, respectively, and -9.0 and -10.1%, both $p<0.05$, for PRO considering dry skin weight and wet berry weight, respectively, both $p<0.05$; **Table 4.3**).

Considering together skin flavanols released to the pulp during dehydration and those extracted after 168 h of maceration, no change was found in the significance of the differences with respect to only extractable skin flavanols. On the one hand, the FRV extractability was 58.2 and 56.8% for Barbera air-treated grapes at 10 and 20% WL, 62.9 and 56.7% for Barbera ozone-exposed grapes at 10 and 20% WL, 87.6 and 68.8% for Nebbiolo air-treated grapes at 10 and 20% WL, and 86.0 and 63.4% for Nebbiolo ozone-exposed grapes at 10 and 20% WL, respectively. On the other hand, the PRO extractability was 57.4 and 55.2% for Barbera air-treated grapes at 10 and 20% WL, 53.2 and 56.7% for Barbera ozone-exposed grapes at 10 and 20% WL, 75.0 and 54.0% for Nebbiolo air-treated grapes at 10 and 20% WL, and 73.5 and 56.0% for Nebbiolo ozone-exposed grapes at 10 and 20% WL, respectively.

The different effect of ozone exposure on the extractability of flavanols, particularly oligomeric forms (FRV), for partially dehydrated Barbera and Nebbiolo red winegrapes with relation to that previously published on fresh grapes (Paissoni *et al.* 2017) confirms the need to relate phenolic compounds extractability with skin cell wall composition and texture.

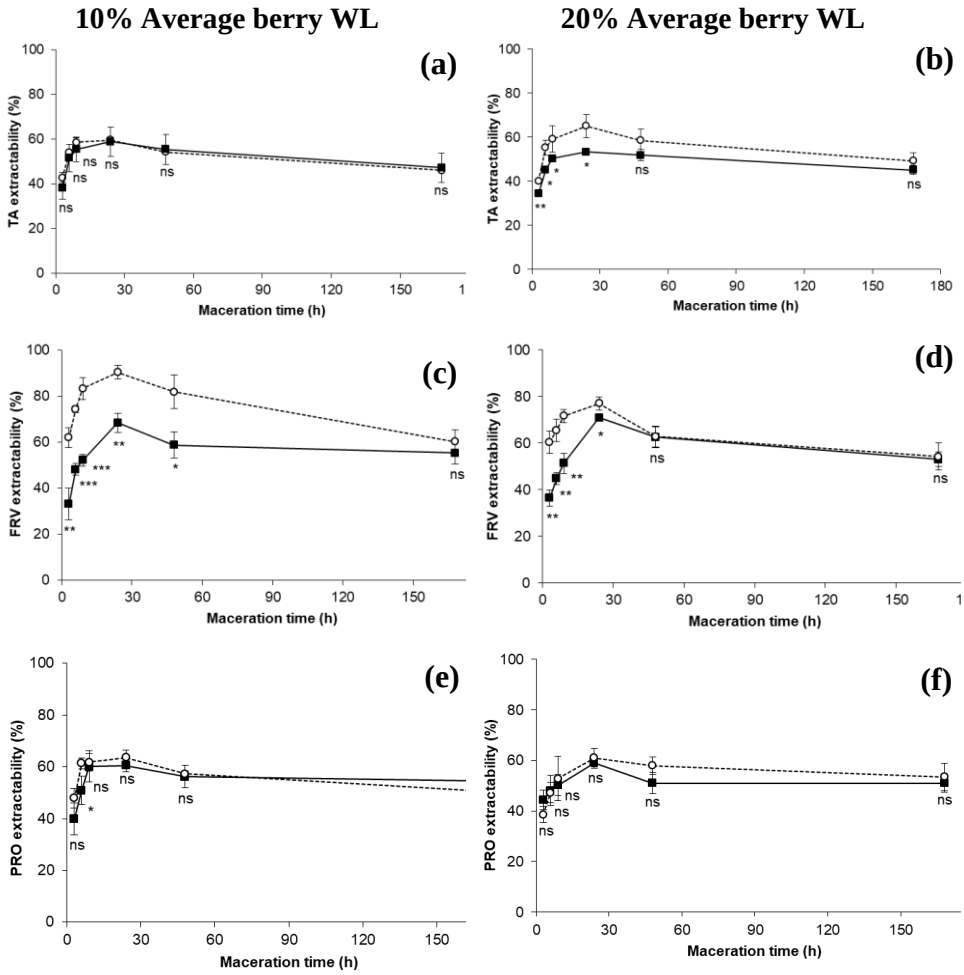

Figure 4.2 Effect of gaseous ozone exposure on the extractability of total anthocyanins (a, b), monomeric and oligomeric flavanols (c, d) and polymeric flavanols (e, f) during maceration for Barbera winegrapes partially dehydrated at 10% WL (a, c, e) and 20% WL (b, d, f).

Legend *All data are expressed as average value ± standard deviation (n = 3). Sign: *, **, *** and ns indicate significance at p< 0.05, 0.01, 0.001 and not significant, respectively, for the differences between air (■) and ozone (○) treatments for each maceration time.*

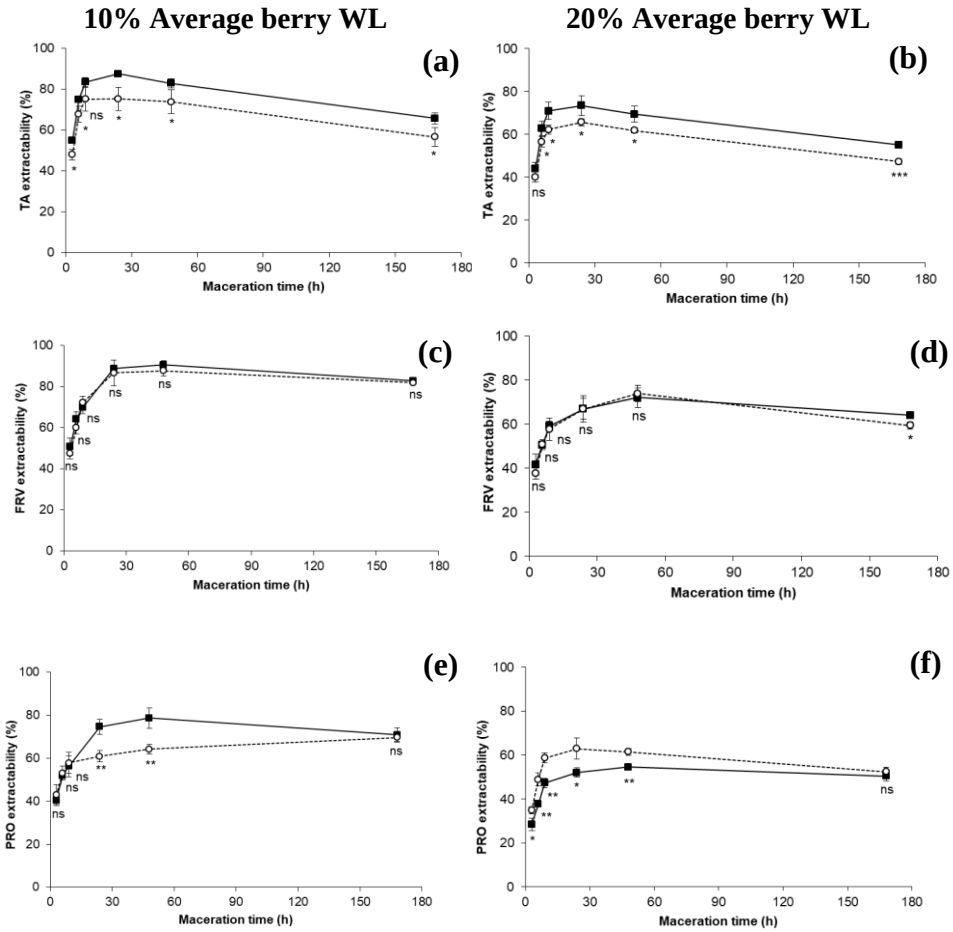

Figure 4.3 Effect of gaseous ozone exposure on the extractability of total anthocyanins (a, b), monomeric and oligomeric flavanols (c, d) and polymeric flavanols (e, f) during maceration for Nebbiolo winegrapes partially dehydrated at 10% WL (a, c, e) and 20% WL (b, d, f).

Legend *All data are expressed as average value ± standard deviation (n = 3). Sign: *, ** and ns indicate significance at p< 0.05, 0.01 and not significant, respectively, for the differences between air (■) and ozone (○) treatments for each maceration time.*

Table 4.3. Extractable content of phenolic compounds in Barbera skins, evaluated after a 7-day maceration, for fresh berries and partially dehydrated berries under air and ozone atmosphere.

Compound	Units	Fresh berries	10% Average berry WL			20% Average berry WL		
			Air	Ozone	Sign	Air	Ozone	Sign
BARBERA								
TA	mg malvidin-3-G chloride/g skin (dry weight)	18.8±0.5	12.4±1.7	11.7±0.1	ns	10.8±0.5	11.2±1.4	ns
	mg malvidin-3-G chloride/kg grape (wet weight)	721±28	660±63	605±15	ns	733±13	692±32	ns
Dp-3-G	%	7.2±0.6	6.7±1.1	5.3±0.2	ns	7.1±0.8	6.4±0.7	ns
Cy-3-G	%	2.9±0.1	3.0±0.6	2.5±0.3	ns	2.9±0.6	3.2±0.5	ns
Pt-3-G	%	10.9±0.3	10.9±0.6	10.2±0.2	ns	10.9±0.4	10.5±0.5	ns
Pn-3-G	%	4.4±0.2	4.9±0.6	4.5±0.4	ns	4.6±0.6	4.9±0.3	ns
Mv-3-G	%	54.6±0.7	57.0±2.4	59.1±0.4	ns	56.5±1.5	58.0±1.6	ns
Σ Acetyl-G	%	12.1±0.4	10.5±0.3	10.9±0.2	ns	10.1±0.3	9.9±0.3	ns
Σ Cinnamoyl-G	%	7.8±0.2	7.0±0.2	7.5±0.5	ns	8.0±0.5	7.1±0.2	ns
FRV	mg (+)-catechin/g skin (dry weight)	6.20±0.82	4.60±0.41	4.46±0.38	ns	5.14±0.31	4.10±0.44	*
	mg (+)-catechin/kg grape (wet weight)	237±33	245±19	231±22	ns	349±18	256±44	*
PRO	mg cyanidin chloride/g skin (dry weight)	20.5±0.5	15.9±1.1	14.3±0.3	ns	15.5±1.1	12.7±1.3	*
	mg cyanidin chloride/kg grape (wet weight)	786±26	847±67	739±26	ns	1047±38	785±39	**

Continues

Compound	Units	Fresh berries	10% Average berry WL			20% Average berry WL		
			Air	Ozone	Sign	Air	Ozone	Sign
NEBBIOLO								
TA	mg malvidin-3-G chloride/g skin (dry weight)	9.03±0.21	8.53±0.34	7.52±0.62	ns	7.37±0.11	6.42±0.17	**
	mg malvidin-3-G chloride/kg grape (wet weight)	304±9	346±11	301±18	*	366±17	315±11	*
Dp-3-G	%	5.5±0.2	3.8±0.8	3.9±0.5	ns	4.3±0.7	3.5±0.1	ns
Cy-3-G	%	9.0±1.0	9.2±0.7	7.9±1.1	ns	10.8±0.8	8.4±0.4	*
Pt-3-G	%	5.4±0.2	4.2±0.5	4.4±0.3	ns	4.3±0.3	4.0±0.1	ns
Pn-3-G	%	34.7±2.2	38.3±2.0	35.1±2.2	ns	38.1±1.0	35.6±0.6	*
Mv-3-G	%	34.5±2.8	32.8±0.9	36.8±2.9	ns	30.0±1.3	35.9±1.4	**
Σ Acetyl-G	%	4.2±0.1	4.4±0.3	4.6±0.1	ns	4.2±0.1	4.3±0.2	ns
Σ Cinnamoyl-G	%	6.7±0.1	7.2±0.4	7.4±0.4	ns	8.3±0.4	8.3±1.1	ns
FRV	mg (+)-catechin/g skin (dry weight)	31.1±2.2	29.3±0.3	27.8±0.1	***	23.5±0.3	24.0±0.7	ns
	mg (+)-catechin/kg grape (wet weight)	1049±74	1185±13	1114±30	*	1166±51	1176±32	ns
PRO	mg cyanidin chloride/g skin (dry weight)	62.2±2.2	57.8±2.7	52.6±1.4	*	45.5±1.9	44.3±1.7	ns
	mg cyanidin chloride/kg grape (wet weight)	2096±78	2344±97	2108±25	*	2258±189	2174±43	ns

Table 4.3 Legend

*All data are expressed as average value ± standard deviation (n = 3). TA: total anthocyanins, Dp-3-G: delphinidin-3-glucoside, Cy-3-G: cyanidin-3-glucoside, Pt-3-G: petunidin-3-glucoside, Pn-3-G: peonidin-3-glucoside, Mv-3-G: malvidin-3-glucoside, G: glucoside, FRV: flavanols reactive to vanillin, PRO: proanthocyanidins, WL: weight loss. Sign: *, ** and ns indicate significance at p<0.05, 0.01 and not significant, respectively, for the differences between air and ozone treatments at the same dehydration level.*

4.3.3. Skin cell wall composition and mechanical properties

Berry skin cell wall (CW) composition and mechanical properties were reported in **Table 4.4**. No significant differences were found when compared ozone-treated and air-exposed samples at the two dehydration levels in both Barbera and Nebbiolo varieties regarding CW total phenols contents, whereas a significantly higher proteins content (+9.3%, $p<0.01$) was observed only in ozone-treated Nebbiolo grapes at 10% WL.

For the two varieties studied, several changes were found in polysaccharides and lignin contents of CW between grapes partially dehydrated under ozone-enriched and air atmosphere. Neutral polysaccharides contents, expressed as total glucose, were significantly reduced in ozone-treated samples for Barbera at 20% WL (-11.6%, $p<0.001$), whereas increased for Nebbiolo at both 10 and 20% WL (+11.5%, $p<0.05$, and +7.2%, $p<0.01$, respectively). In particular, non-cellulosic glucose, which represents the hemicelluloses constituent of CW, was significantly reduced by the ozone treatment in Barbera at 10 and 20% WL (-35.8%, $p<0.01$, and -48.2%, $p< 0.001$, respectively) and in Nebbiolo at 20% WL (-27.3%, $p<0.05$). In the two varieties, cellulosic glucose contents increased with the dehydration process. This increase was significantly higher in ozone-treated Nebbiolo samples at 10 and 20% WL (+10.1%, $p<0.05$, and +11.0%, $p<0.01$, respectively) when compared with air-exposed grapes, whereas no significant differences were found between air-exposed and ozone-treated Barbera grapes.

Higher cellulosic glucose amount could justify a reduced TA extraction from ozone-treated Nebbiolo because a significant negative correlation between cellulosic glucose content and anthocyanin extraction was found ($n= 10$, considering average values for each of two varieties, three sampling points, and ozone and air grapes exposure during partial dehydration; $R= -0.757$, $p<0.05$). This agreed with the findings reported by other authors who highlighted that

samples with the lowest TA extractability are characterized by high contents of cellulosic glucose (Ortega-Regules *et al.* 2006b). In addition, a reduced non-cellulosic glucose content in ozone-treated samples might facilitate the TA and FRV extraction (n= 10, R= -0.661, p<0.05 and R= -0.735, p<0.05, respectively), particularly at the first maceration stages of Barbera, probably as a consequence of its higher non-cellulosic glucose contents in both fresh and partially dehydrated grapes in relation to Nebbiolo. Quijada-Morín *et al.* (2015) also reported a negative correlation between hemicellulosic constituents (i.e. non-cellulosic glucose) of skin CW and flavanol extraction in Tempranillo grapes. Anyway,in the present study, in partially dehydrated Nebbiolo grapes, the decrease of extraction yield for TA under ozone treatment was not observed for FRV and PRO. A higher cellulose presence in the skin CW is related to higher proanthocyanidin extractability (Quijada-Morín *et al.* 2015), and therefore the increased cellulosic glucose content in ozone-treated samples at 20% WL may facilitate the PRO release from skins. In our study, even though Nebbiolo grapes at 10 and 20% WL had similar cellulosic glucose contents, different PRO extraction kinetics were found and they will be justified later.

Although lignin contents increased in all partially dehydrated samples with respect to fresh berries, the lignification process seems to occur more slowly in Nebbiolo for ozone-treated grapes. These showed slightly lower lignin contents at 10% WL than air-exposed samples, but the content increased at 20% WL until achieving significantly higher values with the use of ozone (+17.8%, p<0.05). On the contrary, ozone-treated Barbera samples at 10% WL showed higher lignin contents (+27.3%, p<0.05), but no significant differences were found at 20% WL between ozone-treated and air-exposed berries. Hernández-Hierro *et al.* (2014) have reported that lignin would prevent anthocyanin extraction from skins.Lignin together with cellulose combines to produce a very resistant material to chemical and biological degradation (Düsterhölt *et al.* 1993). This fact may justify the small differences in TA extractability among partially dehydrated Barbera grapes, for which the lowest TA extraction yields corresponded to the highest lignin

contents. As well, it partially explains the lowest TA extraction yield obtained for Nebbiolo grapes dehydrated at 20% WL under ozone-enriched atmosphere (Figure 4.2b), given the higher content in both lignin and cellulosic glucose.

Pectic polysaccharides represent up to the 80% of grape skins polysaccharides, and their degradation strongly influences the phenolic compounds release (Apolinar-Valiente *et al.* 2016). In our study, pectic polysaccharides were evaluated as uronic acids, and a significantly higher content was found in both the two varieties at 10% WL (+29.8%, $p<0.001$, and +18.0%, $p<0.05$, for Barbera and Nebbiolo, respectively) and in Barbera at 20% WL (+48.0%, $p<0.001$) for ozone-treated samples. The dehydration and ozone effects were more evident in Barbera grapes, which also presented a higher quantity of uronic acids than Nebbiolo in fresh berries (**Table 4.4**). This increase could have contributed to facilitate the TA and FRV extraction for ozone-treated Barbera grapes at 20% WL, but only during the first 48 h of maceration, because TA extractability is positively related to the uronic acid content of skin CW (Hernández-Hierro *et al.* 2014; Ortega-Regules et *al.* 2006b). Nevertheless, pectic polysaccharides fraction of skin CW has a high tendency to associate with proanthocyanidins, limiting their release (Quijada-Morín *et al.* 2015). This may explain the lower PRO extraction yield for ozone-treated Nebbiolo grapes at 10 % WL, particularly evident at 24 and 48 h of maceration.

In spite of the differences in CW composition between the dehydration treatments studied, no significant differences were found in the skin mechanical properties of Nebbiolo, whereas both F_{sk} and W_{sk} parameters were significantly higher in ozone-treated samples than in air-exposed ones for Barbera dehydrated at 20% WL (+24.8%, $p<0.01$, and +23.5%, $p<0.05$, for F_{sk} and W_{sk}, respectively; Table 4.4). This increase is directly associated with skin hardening. According to the CW composition, this difference in the texture parameters might be linked to the significant changes in neutral carbohydrates, non-cellulosic glucose and uronic acids contents found in the skin CW of Barbera at 20% WL (**Table 4.4**). Previous

studies performed on Corvina grape berries have highlighted that the skin mechanical properties are negatively correlated with the %WL during partial dehydration (Rolle *et al.* 2013), this correlation being significant for the F_{sk} parameter as observed in the present study for air-exposed Barbera grapes. Nevertheless, Laureano *et al.* (2016) reported an increased W_{sk} value in Barbera fresh grapes after post-harvest ozone treatments (30 µL/L, 24 h) in agreement with the results showed in Table 4 for Barbera dehydrated at 20% WL. Skin hardening has a direct impact on the extractability of phenolic compounds (Rolle *et al.* 2008), although the effect of pre-harvest grape berry treatments on the skin mechanical properties as well as the relationship between these texture parameters and the extraction yield are variety-dependent (Río Segade *et al.* 2014). In our conditions, despite the possible favourable effect of ozone exposure of Barbera grapes at 20% WL on the TA extractability, the W_{sk} parameter was negatively correlated with TA extraction (n= 10, R= -0.645, p<0.05), as well as with FRV extraction (n= 10, R= -0.656, p<0.05).

Table 4.4 Skin mechanical properties and cell wall composition of fresh berries and partially dehydrated berries under air and ozone atmosphere for Barbera and Nebbiolo winegrapes.

Parameter	Units	Fresh berries	10% Average berry WL			20% Average berry WL		
			Air	Ozone	Sign	Air	Ozone	Sign
			BARBERA					
Mechanical properties[a]								
F_{sk}	N	0.987±0.041	0.931±0.144	0.985±0.033	ns	0.824±0.058	1.028±0.011	**
W_{sk}	mJ	0.544±0.056	0.574±0.120	0.618±0.007	ns	0.520±0.056	0.642±0.044	*
Cell wall composition[b]								
Skin CW	mg/g fresh skin	70.5	59.0	58.7	-	62.4	60.9	-
Proteins	mg BSA/g CW	83.1±3.2	84.2±2.5	87.5±1.6	ns	83.3±4.2	83.5±4.3	ns
Total phenols	mg gallic acid/g CW	53.1±2.9	52.9±2.3	62.8±4.7	ns	63.9±2.6	58.4±4.5	ns
Neutral carbohydrates	mg glucose/g CW	204±8	212±7	210±10	ns	250±6	221±6	***
Non-cellulosic glucose	mg glucose/g CW	13±1	23±3	15±1	**	51±2	26±3	***
Cellulosic glucose	mg glucose/g CW	191±8	189±7	196±10	ns	199±4	195±4	ns
Uronic acids	mg galacturonic acid/g CW	229±21	151±11	196±6	***	127±11	188±7	***
Lignin (Klason)	mg/g CW	235±8	253±15	322±28	*	323±28	291±22	ns

Continues

Parameter	Units	Fresh berries	10% Average berry WL			20% Average berry WL		
			Air	Ozone	Sign	Air	Ozone	Sign
			NEBBIOLO					
Mechanical properties[a]								
F_{sk}	N	0.747±0.031	0.853±0.054	0.824±0.054	ns	0.825±0.047	0.839±0.021	ns
W_{sk}	mJ	0.361±0.020	0.475±0.033	0.452±0.033	ns	0.438±0.042	0.447±0.018	ns
Cell wall composition[b]								
Skin CW	mg/g fresh skin	55.5	44.5	49.8	-	50.6	51.7	-
Proteins	mg BSA/g CW	83.0±5.3	80.4±3.0	87.9±0.8	**	85.7±2.3	88.1±4.3	ns
Total phenols	mg gallic acid/g CW	61.1±4.0	64.5±2.8	69.8±3.2	ns	65.1±4.4	66.1±5.8	ns
Neutral carbohydrates	mg glucose/g CW	169±11	174±5	194±8	*	181±5	194±1	**
Non-cellulosic glucose	mg glucose/g CW	2±1	10±1	9±1	ns	16±1	12±3	*
Cellulosic glucose	mg glucose/g CW	167±10	168±6	185±7	*	164±4	182±3	**
Uronic acids	mg galacturonic acid/g CW	139±13	139±7	164±14	*	160±15	151±6	ns
Lignin (Klason)	mg/g CW	336±18	414±39	361±19	ns	359±5	423±16	*

Legend *All data are expressed as average value ± standard deviation. [a]Three replicates of 20 berry skins (n = 3). [b](n = 4). CW: cell wall, BSA: bovine serum albumin, F_{sk}: berry skin break force, W_{sk}: berry skin break energy, WL: weight loss. Sign: *, **, *** and ns indicate significance at p<0.05, 0.01, 0.001 and not significant, respectively, for the differences between air and ozone treatments at the same dehydration level.*

4.3.4 Multivariate analysis

Multivariate linear regression (MLR) was performed to better understand the relationship of skin cell wall (CW) composition and mechanical properties with phenolic compounds extractability (**Table 4.3** and **Figures 4.2** and **4.3**). TA, FRV and PRO extraction percentages were chosen as dependent variable, and CW composition (proteins, total phenols, non-cellulosic glucose, cellulosic glucose, uronic acids and lignin) together with the texture (parameter W_{sk}) were independent variables. The obtained R^2 values (multiple determination coefficient), B (non-standardized regression coefficient) and β (standardized regression coefficient) were calculated. Furthermore, the MLR model was obtained excluding W_{sk}, namely considering only CW composition, but it fitted better (higher R^2 value) taking into account both the skin CW composition and mechanical properties together for all the dependent variables (R^2= 0.948, 0.915 and 0.931 for TA, FRV and PRO models, respectively, considering CW composition alone, and R^2= 0.999, 0.986 and 0.993 for TA, FRV and PRO, respectively, considering CW composition and W_{sk} together).

For TA extractability, proteins, total phenols, non-cellulosic glucose, lignin and W_{sk} resulted to be statistically significant (p<0.001), and the final model is represented by the following equation (1):

Equation (1)

$$\textbf{TA extractability}\ (\%) = 255.262 - 2.558\,[\text{Proteins}] + 0.988\,[\text{Total phenols}] - 0.434\,[\text{Non} - \text{cellulosic glucose}] - 0.071\,[\text{Lignin}] - 28.925\,[W\text{sk}]$$

A negative relationship was found between TA extractability and proteins (β= -0.792), non-cellulosic glucose (β= -0.715), lignin (β= -0.533) and W_{sk} (β= -0.312), whereas CW total phenols were positively correlated (β=0.665). Therefore, the variables that contribute most to the model are proteins and non-cellulosic glucose contents. This model is partially in accordance with that previously reported by Hernández-Hierro *et al.* (2014) where a negative correlation of TA extraction with lignin and glucose contents was also found but, in our study, no significant influence of pectic polysaccharides was observed. Ortega-Regules *et al.* (2006b) showed an opposite influence of the CW composition on TA extractability, where higher non-cellulosic glucose and proteins contents facilitated TA extraction, whereas it was prevented by a higher total phenols quantity. Nevertheless, the contribution of these three parameters to the model was low compared to others such as fucose, galactose and mannose contents. Taking into account what was commented in the previous section (section 4.3.3) and the contribution of each variable to the model, we can hypothesize that lower non-cellulosic glucose and lignin contents in the skin CW after ozone treatment explain the higher TA extraction in the first maceration stages for Barbera grapes at 20% WL. Moreover, lower TA extraction in Nebbiolo can be mainly explained by a higher amount of proteins in ozone-treated grapes at 10% WL. Conversely, at 20% WL, lignin contents became the most influent parameter on the decreased TA extractability in ozone-treated Nebbiolo samples.

For FRV extractability, proteins, total phenols, non-cellulosic glucose, cellulosic glucose, lignin and W_{sk} were statistically significant (p<0.01). The model obtained is defined by the following equation (2):

Equation (2)

$$\textbf{FRV extractability}\ (\%) = 273.744 - 3.800\,[\text{Proteins}] + 2.153\,[\text{Total phenols}] - 0.929\,[\text{Non} - \text{cellulosic glucose}] + 0.423\,[\text{Cellulosic glucose}] - 0.135\,[\text{Lignin}] - 68.228\,[W\text{sk}]$$

As for TA extractability, proteins (β= -0.750), non-cellulosic glucose (β= -0.976), lignin (β= -0.643) and W_{sk} (β= -0.469) were negatively correlated with the FRV extractability, whereas CW total phenols were positively correlated (β= 0.924). In addition, cellulosic glucose contents resulted positively correlated with FRV extractability (β= 0.424). In this case, the variables that contribute most to the model are non-cellulosic glucose, total phenols and proteins. Quijada-Morín *et al.* (2015) found a positive correlation between the cellulose content and monomeric and oligomeric flavanol extractabilities. Therefore, the higher the cellulose content in the CW, the higher the FRV extractabilities. On the contrary, non-cellulosic and pectic polysaccharides showed an opposition to the FRV release. In the present study, lower non-cellulosic glucose contents in the skin CW after ozone treatment explain well the higher FRV extraction in the first maceration stages for Barbera grapes at 10 and 20% WL. In our case, according to the models obtained, uronic acids influenced only polymeric flavanol (PRO) extractabilities, whose negative effect was particularly evident in Nebbiolo grapes partially dehydrated at 10% WL under ozone-enriched atmosphere after 24 and 48 h of maceration. As observed for FRV, the higher the cellulose content in the CW, the higher the PRO extractability but only in Nebbiolo at 20% WL. In fact, regarding PRO extractability, the same parameters defining FRV model resulted to be also statistically significant (p<0.05) with the addition of uronic acids contribution; in detail, proteins (β= -0.864), non-cellulosic glucose (β= -1.235), lignin (β= -0.778), uronic acids (β= -0.396) and W_{sk} (β= -0.501) were negatively correlated with the PRO extractability, whereas CW total phenols (β=

0.787) and cellulosic glucose (β= 0.804) were positively correlated, as reported in the following equation (3):

Equation (3)

$$\textbf{PRO extractability}\ (\%) = 262.772 - 3.579\,[\text{Proteins}] + 1.499\,[\text{Total phenols}] - 0.961\,[\text{Non} - \text{cellulosic glucose}] + 0.656\,[\text{Cellulosic glucose}] - 0.133\,[\text{Uronic acids}] - 0.133\,[\text{Lignin}] - 59.584\,[W\text{sk}]$$

At the end of maceration, the FRV and PRO extractabilities for ozone-treated and air-exposed grapes were not statistically different (**Figures 4.2c-f** and **4.3c-f**), probably due to the long contact time of skins with the hydroalcoholic solution, which facilitates flavanol extraction independently on the initial CW composition or mechanical properties (Bautista-Ortín *et al.* 2016).

Finally, it is important to point out that the varietal differences in the phenolic composition, namely chemical features and molecular mass of flavanols, influence their extractability because different adsorption and chemical interaction phenomena with skin CW are involved (Quijada-Morín *et al.* 2015; Ruiz-Garcia *et al.* 2014). In fact, the ozone treatment in Barbera grapes (richer in anthocyanins but poorer in flavanols; **Table 4.2**) strongly influenced FRV extractabilities (**Figure 4.1 c** and **d**), but no significant changes were found in PRO extractabilities, even if both the skin CW composition and mechanical properties were strongly affected by the treatment (**Table 4.4**). The opposite phenomena were found in Nebbiolo (poorer in anthocyanins but richer in flavanols; **Table 4.3**) where PRO extractabilities were more affected (**Figure 4.3 e** and **f**). A variety-dependence of the ozone influence on phenolic compounds extractability was also observed in fresh grape berries (Paissoni *et al.* 2017).

4.4 Conclusions

New technologies may aid to maintain the berries in good phytosanitary conditions during grape dehydration without negatively affecting the quality of grapes and to preserve the final wine quality. Ozone has been used to prevent moulds and microbiological contaminations, but to date no studies were performed on the influence of ozone sanitizing treatments during winegrape dehydration on the extractability of the skin phenolic compounds. In our findings, ozone has a variety-dependent effect, which can be strongly related to the phenolic profiles of grapes, in particular to anthocyanins. Nebbiolo, which is a di-substituted anthocyanins prevalent variety, reported no change in the content of total anthocyanins just after ozone-assisted dehydration, but their extraction yield was lower with respect to the control at 10 and 20% WL. On the contrary, although lower contents of anthocyanins were found in Barbera grapes (tri-substituted anthocyanins prevalent) just after dehydration at 20% WL under ozone-enriched atmosphere, their extractability was significantly increased during the first 48 h of maceration. Regarding oligomeric and polymeric flavanols, their extractability was less affected by the ozone treatment. Nevertheless, ozone caused changes in the extractability of flavanols in the first hours of maceration, particularly in oligomeric flavanols for Barbera and polymeric flavanols for Nebbiolo. In the case of Nebbiolo, lower extractable contents of polymeric flavanols were found in grapes partially dehydrated at 10% WL under ozone atmosphere, although no significant differences were observed in their content just after treatment. Therefore, the winemaking process should be adapted depending on the variety and on the target wine.

Several factors other than the chemical structure and content of phenolic compounds influenced their extractability, such as the amount and composition of skin cell wall material and skin hardness. In our study, the ozone-induced modification of skin cell wall composition together with skin hardness parameters fitted well in multivariate models to predict anthocyanins, oligomeric flavanols

and polymeric flavanols. As a general trend, higher non-cellulosic glucose contents prevent the phenolic compounds release from skins.

Chemical and sensorial investigation of in-mouth sensory properties of grape anthocyanins

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5.1. Introduction

The in-mouth sensory properties of wine are a complex mixture of taste (e.g. bitterness, acidity, sweetness, and saltiness) and mouth-feel sensations, mostly astringency, and flavour. Bitterness and astringency play an important role in the quality of red wine. Bitterness is a taste correlated with the presence of various structured receptors1 that are activated by a wide range of molecules, while astringency is a sensation of drying and puckering that is considered to be a mouth tactile response (Breslin *et al.* 1993). It is currently accepted that astringent molecules form complexes with salivary proteins due to hydrophobic interactions and hydrogen bonding precipitate the saliva protein, leading to a lack of lubrification in mouth (Ma *et al.* 2014; Laguna *et al.* 2017). In addition, breakdown of the mouth saliva film is detected by increasing activation of mechanoreceptors, and precipitation of dead cells and other mouth debris increases the feeling of particles in the mouth (De Wijk & Prinz, 2006).

In wine, phenolic compounds are the main class of compounds involved in in-mouth sensory properties, in particular monomeric flavanols and their polymerized forms, usually referred as proanthocyanidins. They are the major compounds influencing wine astringency and bitterness, depending on their concentration, degrees of polymerization and galloylation, B-ring hydroxylation, and their stereochemistry (Harbetson *et al.* 2014; Peleg, Gacon *et al.* 1999; Chira *et al.* 2008; Schwarz & Hoffman, 2008). Several methods have been published to quantify tannin astringency based on their ability to react with proteins, such as Serum Bovine Albumin (BSA), gelatine, and salivary proteins (Hagerman & Butler, 1981; Calderon *et al.* 1968; Rinaldi *et al.* 2010). While these methods induce the formation of insoluble complex that may precipitate, the interaction between phenolic compounds and protein in soluble complexes has also been reported (Ferrer-Gallego *et al.* 2015a; de Freitas & Mateus, 2001). Thus, astringency is a complex sensation involving several interactive mechanisms that

are perceived as intensity and persistence in the mouth. Therefore, overall sub-qualities (Gawel *et al.* 2001) can be investigated only by sensorial analysis.

On the other hand, several non-flavanols phenolic compounds have been reported to contribute in in-mouth attributes of wine such as phenolic acids and their derivatives, flavonols, and polymeric pigments formed by the reaction of anthocyanins with flavanols and carboxylic compounds (Hufnagel & Hofmann, 2008; Sáenz-Navajas *et al.* 2017). Among them, anthocyanins are a class a particularly abundant in grape and wine, since their concentration may reach up to 6 g/Kg (Mattivi *et al.* 2006) and can be extracted during winemaking. Structurally, anthocyanins are heterosides of an aglycone (anthocyanidin) differentiated among themselves on the number of hydroxylated and methoxylated groups in the anthocyanidin, the nature and the number of bonded sugars in their structure, the aliphatic or aromatic carboxylates bonded to the sugars in the molecule, and the position of this bond. The main anthocyanins present in red winegrapes form Vitis vinifera L. are delphinidin, cyanidin, petunidin, peonidin and malvidin, which differ in the B ring substitution, and are present as monoglucoside, acetyl-monoglucoside, caffeoyl-monoglucosides and p-coumaroyl-monoglucoside derivatives, where the individual anthocyanidins and esterification can strongly influence their color features, reactivity and stability in wine. Their main role is the contribution to chromatic features of rosé and red wine. They are extracted from grape skins during the first step of the winemaking process and their influence on colour is dependant by the solution pH and by copigmentation. As a fuction of pH, four different forms can be found, e.g. flavylium form (red, pH=1), quinoidal species (blue, pH=2-4), and at higher pH as carbinol pseudobase (colourless) and chalcone (yellow). At wine pH (3.0-4.0), these four species coexist, with a prevalence of quinodal species (Heredia *et al*. 1998). Copigmentation, a phenomenon in which anthocyanins can form non-covalent linked complexes with other organic compounds, the co-factors, or between anthocyanins themselves (self-association) can stabilize the coloured flavilyum cation. In addition, a change in absorption toward higher wavelenght

(bathochromic effect) and higher intensity (hypechromic effect) occurs, and copigmentation is thought to be implicated in up to the 50% of young red wine colour features (Boulton 2001). On the other hand, once they are extracted, anthocyanins can undergo several reactions with grapes and yeast metabolites to produce new pigments. These reactions produce more complex molecules as long as the wine continues to age, and they are responsible of a minor content of monomeric anthocyanins in aged wines (Mazza *et al.*1999; González-Neves *et al.* 2004 ; García-Falcón *et al.* 2007; Pérez-Magariño & González-San José, 2004; Ferrandino *et al.* 2012; Cagnasso *et al.* 2008; Lingua *et al.* 2016; Alcade-Eon *et al.* 2006; García-Marino *et al.* 2010; Fanzone *et al.*2012; Ginjom *et al.* 2010; Romero-Cascales *et al.* 2005; Chira *et al.* 2011), and Table S1 provides an overview of grape and wine contents of pigmented materials. This process is considered to be responsible for the changing sensory properties of wine during ageing, such as the shift of colour from bluish-red to orange and the increasing smoothness of astringency for the complexation of monomeric and polymeric flavanols. Although the role of anthocyanins in wine colour has been widely investigated, their contribution to in-mouth sensory properties is still controversial. Several studies have attempted to explain their involvement in taste and mouthfeel properties, but without any clear consensus. Anthocyanins are reported to have a “mild taste” (Hufnagel & Hofmann, 2008; Sáenz-Navajas *et al.* 2017), and increasing astringency, in particular sub-qualities as “fine grain” (Brossaud *et al.* 2001; Vidal *et al.* 2004a; Oberholster *et al.* 2009; Ferrer-Gallego et al. 2015a). Later, Gonzalo-Diago et al. found the acetylated and coumaroylated anthocyanins contributed to both astringency and bitterness. The chemical determination of astringency as interaction with salivary protein was achieved with glucoside anthocyanins. Notably, malvidin-3-O-glucoside was found to form soluble complexes with salivary proteins (Ferrer-Gallego *et al.* 2015a) and to activate TAS2R7 bitterness receptor (Soares *et al.* 2013). Anyway, Vidal et al. (2004)b found no differences either in model wine added with glucosides or coumaroylated anthocyanins or in slightly unbuffered ethanolic solution (5%),

thereby confirming the in-mouth sensation reported previously as impurities in the isolated fractions.

To date, obtaining pure anthocyanin samples in sufficient quantity has been a problem in characterizing their sensory properties. Centrifugal partition chromatography (CPC) is a liquid-liquid separation technique that allows different solvents to be used as stationary and mobile phase as long they are immiscible, and which can be adapted for injecting several grams of raw extract. Liquid-liquid separation of anthocyanins has been successfully achieved by multi layers and high speed countercurrent chromatography (MLCCC and HSCCC), and centrifugal partition chromatography (CPC) of different capacity (up to 5 L) from fruit extracts and in particular from grape skins, marcs, and wines. (Renault *et al.* 1997; Schwarz *et al.* 2003b; Vidal *et al.* 2004d; Salas *et al.* 2005; Kneknopoulos *et al.* 2011; **Table 5.1**).

The aim of this study was to isolate anthocyanins classes present in wine grapes and to evaluate the sensoactive features by chemical and sensorial analysis. To obtain purified glucoside, acetylated and cinnamoylated (as mix of caffeoylated and coumaroylated derivatives), Vitis vinifera L. c.v. Nebbiolo and Barbera were extracted from skin and fractionated using CPC and preparative HPLC techniques. These two varieties were chosen because they have different anthocyanin profiles, which we expected to provide a different degrees of fractionation. The reactivity of the extract and fractions toward proteins as a marker of astringency was tested by adapting BSA and salivary protein precipitation methods. Sensory analysis was performed in addition to chemical investigation, and a in-mouth detection threshold was estimated for total anthocyanins extract, and for glucosides, acetylated, and cinnamoylated anthocyanin classes.

5.2 Material and Methods

5.2.1 General Information

5.2.1.1 Chemicals

Chemicals Distilled water was obtained from an ELGA system, and Milli-Q (Millipore) water was prepared using a Sarterius-arium 611 system. All solvents were HPLC grade, in detail: methanol, acetonitrile, and ethyl acetate were 99.9% and 1-buthanol was 99.8%. Formic acid and trifluoroacetic acid were ≥ 95% and 99%, respectively. They were purchased from Prolabo-VWR (Fontenays/Bois, France).

5.2.1.2 Ethical Permission

The ethical committee of Laboratory Research Unit USC 1366 Board, Institut des Sciences de la Vigne et du Vin of University of Bordeaux (ISVV) approved the study for saliva collection of volunteers. All participants signed an informed consent form with type of research, voluntary participation and saliva collection protocol by spitting.For sensory analysis, participants were volunteers and signed an informed consent form with type of research, voluntary participation and agreement to taste of extracts produced as in protocol described in section “Total anthocyanins extracts and samples purification”.

5.2.2 Apparatus and Analytical Methods

5.2.2.1 Centrifugal Partition Chromatography (CPC)

The 200 mL CPC was an FCPC 200 provided by Kromaton Technologies (Saintes-Gemmes-sur-loire, France), consisting of a rotor (20 circular partitions disks, total volume capacity of 204 ml; 1320 partitions cells). High-pressure gradient pump (Gilson 321-H1) and high-pressure injection valve (21 mL loop,

Rheodyne) were used for the gradient. The rotor ran at 1000 rpm, at 3 mL/min flow rate. Chromatogram was checked by a Kromaton UV-Vis detector at 280 nm. Fraction were collected every 3 minutes for each tube by a Gilson 204 fraction collector and analysed in analytical HPLC-DAD system. The system allowed the injection of 100 mg for each run in 10 mL of lower phase. Retention of stationary phase was calculated as 74.4%. The 1L centrifugal partition chromatography (CPC) apparatus was an FCPC 1000 provided by Kromaton Technologies (Saintes-Gemmes-sur-Loire, France). It consisted of a rotor (45 circular partition disks; total column capacity of 940 mL; 1440 partition cells), a binary high-pressure gradient pump (Gilson 321-H1), a high-pressure injection valve (50 mL sample loop, Rheodyne) and a Kromaton UV–vis detector. Fractions were collected manually checking the UV-Vis signal at 280 nm and 520 nm. Anthocyanins extract (maximum 2.5 g) were dissolved in lower phase (40 mL) and filtered prior injection (0.45 um). CPC method was the compatible with the system described above, the rotor was running at 1000 rpm, and flow rate was 15 mL/min. Retention of stationary phase was calculated as 76.1%

5.2.2.2 Preparative High-Performance Liquid Chromatography (PREP-HPLC)

PREP- HPLC was performed on a Varian LC machine consisting of a Prostar 210 two-way binary high-pressure gradient pump, a 2 mL loop and a Prostar 325 UV/Vis detector, recording at 520 and 280 nm. The column use was a Nucleosil C18 (21 × 250 mm, 5 µm) and the mobile phase consisted of acidified acetonitrile (Eluent B) and acidified water (Eluent A), both containing 0.1% TFA. The flow rate was 10 mL/min and the gradient was from 15% to 45% of B in 35 minutes, followed by 7 minutes of 100% B and reconditioning at 15% B for 7 minutes. For each injection, 40 mg of fraction compounds were dissolved in 250 µL 50:50 (v/v) methanol/water acidified with 0.1% TFA and manually injected into the system.

5.2.2.3 Analytical High-Performance Liquid Chromatography-Diode Array Detection (HPLC-DAD)

Anthocyanins extracts and fractions analysis were performed on a Thermo-Finnigan Accela HPLC system consisting of an autosampler (Accela autosampler), pump (Accela 600 Pump), and diode array detector (Accela PDA Detector) coupled to a Finnigan Xcalibur data system. Separation was performed on a reversed phase Agilent Nucleosil C18 (250 mm × 4 mm, 5 μm) column. Gradient consisting of water/formic acid (95:5, v/v) (solvent A) and acetonitrile/formic acid (95:5, v/v) (solvent B) was applied at a flow rate of 1 ml/min. Method was slightly modified from Chira (2009) as follow: 10–23% B linear from 0–16 minutes, 23–28% B in 19 minutes, 28-100% B in 6 minutes, 100% isocratic B for 5 minutes, 100% B gradient to initial condition for 6 minutes and re-equilibration of the column for 3 min under the initial gradient conditions. Purity was checked as 520/280 nm detectable peaks. Peaks were previously identified with MS injection[51] and quantification was done on malvidin-3-*O*-glucoside (Sigma–Aldrich, Saint Quentin Fallavier, France) calibration curve.

Table 5.1 Some liquid-liquid chromatography methods reported in bibliography.

Compounds	Extracts Origin	Instrument	Solvent System	Specifics: Eluition Mode	Specifics: Flow rate/ rpm/ column volume	Injection	Notes	Reference
Anthocyanins	Roselle, Red Cabbage, black currant, black chokeberry	HSCCC	TBME/ BuOH /ACN/Water 2:2:1:5 +TFA Stationary phase retention:53-75%	Isocratic H-T	5 ml/min 1000 850 ml	300 mg to 2 g in 1:1 mixture up and low phase		Degenhardt *et al.* (2000)a
		HSCCC	TBME/ BuOH /ACN/Water 2:2:1:5 +TFA	Isocratic H-T	2.5ml/min 800 360 ml			
Pigments	Red Wine	HSCCC	TBME/ BuOH /ACN/Water 2:2:1:5 +TFA 0.1%	H-T	3.5ml/min 800 850 ml	1 g in 20 ml 1:1 mixture up and low phase		Salas *et al.* (2005)
Anthocyanins	Skins from pomace and fresh grape	MLCCC	TBME/ BuOH /ACN/Water 2:2:x:5 + 0.02% TFA A: 2:2:0.1:5 (start with a more polar lower phase) B: 2:2:2.5:5 Stationary phase retention:75%-85%	Gradient T-H	- 800 100-700 ml	100 mg to 2 g in 2/5 ml	4 fractions: -Glucoside -Acetylated -*p*-Coumaroylated -Caffeoylated Stationary phase recovered for analyisis of polymeric pigment (Vidal *et al*, 2004e)	Vidal *et al.* (2004)d

Anthocyanins	Wine-grape skins extract	HSCCC	4 Solvent Systems: -I: medium polar TBME/BuOH/ACN/Water ater+0.1% TFA 2:2:0.1:5 -II: polar EtOAc/BuOH/Water +0.1% TFA 2:3:5 III: non polar EtOAc/Water + 0.1% TFA 1:1 IV- medium polar: EtOAc/BuOH/W +0.1% TFA 4:1:5 Stationary phase retention:45-75%		- - 850ml	300-750 mg	Sample Preparation: Amberlite XAD7 Fractions corrispoding to solvent systems: I: Glucoside (Mv-G e Pn-G) II: vitisin, diGlucoside III: *p*-Coumaroylated -Caffeoylated IV: Acetylated	Deegenhardt *et al*. (2000)b
Anthocyanins	Champagne vintage by-products-Pinot noir skins, stalks, seeds	CPC	BuOH/Acetic acid/Water 4:1:5 Stationary phase retention:75%	Isocratic H-T	3 ml/min 1400 -	1 g in 10 ml stationary phase	Separation of glucosides acetylated, cinnamoylated forms	Renault *et al.* (1997)
		CPC	EtOAc/BuOH/Water 0.2% TFA I mobile: 77:15:8 II mobile :40:46:14 Stationary : 5:5:90 Stationary phase retention:75%	Gradient H-T	3 ml/min 1400 -	1g in 10 ml stationary phase		
		Pilot-CPC	EtOAc/BuOH/Water 0.2% TFA I mobile : 77:15:8 II mobile :40:46:14 Stationary : 5:5:90 Stationary phase retention:75%	Gradient H-T	60 ml/min 1140 5L	24.5 g in 500 ml stationary phase		

Anthocyanins and related compounds	Pinot noir grape skins	MLCCC	TBME/BuOH/ACN/Water +0.01% TFA 2 :2 :0.1-1.8 :5	Gradient T-H	2 ml/min 800 450 ml	250 mg in 5 ml of 1:1 mixture up and low phase	Mobile phase: Glucoside Stationary phase: Anthocyanin oligomers in GRP with Pn-G and Mv-G	Kneknopoulos *et al.* (2011)
Anthocyanins	Red wines, purple heart, purple corn, elderberries	HSCCC	Solvents: 4 runs I: TBME/BuOH/ACN/Water, 2:2:1:5 II: TBME/BuOH/ACN/Water, 2:2:1:5 III: EtOAc/Water, 1:1 IV:BuOH/TBME/ACN/Water 3:1:1:5 All +0.1% TFA	Isocratic H-T	- 1000 850 ml	100 to 500 mg in 20 ml	Solv I: separation of glucosides acetylated, cinnamoylated Solv III: for anthocyanin derivatives Solv IV: diglucosides	Schwarz *et al.* (2003)b

Table 5.1 Some liquid-liquid chromatography methods reported in bibliography.

Legend ***HSCC*** *High Speed Counter Current Chromatography,* ***MLCCC*** *Multi-Layer Counter Current Chromatography,* ***CPC*** *Centrifugal Partition Chromatography;* ***TBME*** *Methyl tert-butyl ether, BuOH Butanol,* ***ACN*** *Acetonitrile,* ***TFA*** *Trifluoracetic acid,* ***EtOAc*** *Etyl Acetate;* ***T-H*** *tail to head= Organic layer is stationary phase, whereas aqueous layer is the mobile phase – Reversed phase;* ***H-T*** *head to tail, aqueous layer is stationary phase, whereas organic layer is the mobile phase - Normal phase;* ***Mv-G*** *Malvidin-3-O-glucoside,* ***Pn-G*** *Peonidin-3-O-glucoside,* ***GRP*** *Grape reaction product.*

5.2.3 Total anthocyanins extracts and samples purification

50 kg of Nebbiolo and Barbera grapes were harvested in Alba (Piedmont, Italy) at full ripeness, cutted in small cluster (5-6 berries each), collected in small boxes of 600 g each and stored at -20°C. For skins processing, one small box at time was taken and skins were removed with a laboratory spatula by frozen berries and washed with water to remove potentially pulp residues. Skins were then freeze-dried for two days and grounded to powder in a ball grinder. Skins powders were stored at -20°C, until extracted. For Nebbiolo, a total of 5161 g of berries were peeled, giving 573 g of fresh grape skins and final lyophilized skins weight was 197.5 g. For Barbera, 5174 g of berries were peeled, giving 536 g of fresh skins and final lyophilized skins weight was 210.1 g. Extraction was performed on 100 g of skin powder in 1L acidified methanol as solvent (0.1% TFA) for two hours two times under stirring. The recovered solvent was filtered to avoid particulate, evaporated and freeze dried. The anthocyanins extract was cleaned from acids and sugars through solid phase extraction (SPE) using Amberlite XAD 16 resin (Sigma–Aldrich, Saint Quentin Fallavier, France). A large-scale column was filled with 1 Kg of resin and samples were washed with acidified water (0.1% TFA) until the eluate was clear (around 2 bed volumes). Anthocyanins were then recovered with acidified methanol (0.1% TFA), evaporated and freeze-dried. The resulting powder was used to CPC fractionation and it is the so-called total anthocyanins extract (TAE) and was stored at -20°C until needed. Purity and composition of Nebbiolo and Barbera TAEs were checked with HPLC-DAD system, slightly modified from Chira (2009).

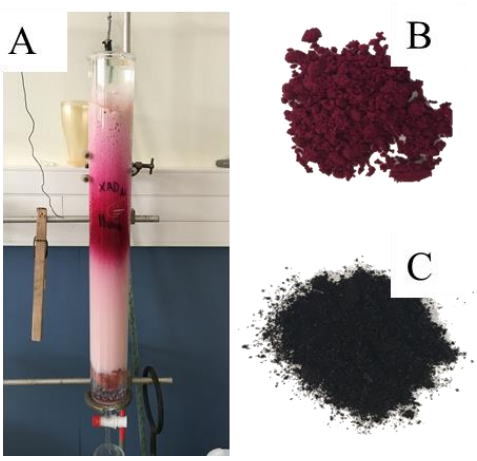

Figure 5.1 (**A)** Purification of anthocyanins extract in XAD 16 resins and resulted powders from **(B)** Nebbiolo and **(C)** Barbera.

Two different CPC equipments were used, 200 mL CPC was used to carried out method improvement and a 1L CPC to obtain the powder designated to sensorial and chemical analysis (details of CPC apparatus are described in “Apparatus and analytical method” section). The CPC system were adapted from Renault *et al.* (1997): apparatus was working in ascending mode where lower phase, as stationary, was composed by Ethyl Acetate:Butanol:Water 5:5:90 (v/v/v), whereas a gradient of two mobile phase was applied using two solvent B system Ethyl Acetate:Butanol:Water 770:150:80 (v/v/v) as initial mobile phase (B1) and Ethyl Acetate:Butanol:Water 400:460:140 (v/v/v) as final mobile phase (B2). The gradient was: 30 minutes 100% of B1, from 100% B1 to 50%B1/50% B2 in 90 minutes, 30 minutes 50%B1/50%B2, to 100% B2 in 60 minutes, and 100%B2 for 90 minutes. Regarding 1L-CPC, the gradient was interrupted after 140 minutes, since the separation of the two first classes occurred in the first part, and the remaining compounds were collected by stationary phase extrusion.

Barbera and Nebbiolo TAEs were injected separately since their anthocyanin profiles is different. Therefore, differences in fractions collection were applied

and a total of 8 and 7 fractions were collected for Barbera and Nebbiolo, respectively.

To fractionate acetylated and coumaroylated anthocyanins, a further purification was needed to achieve a satisfactory level of purity and preparative HPLC was carried out. Chromatographic peaks were collected manually, and the collected fractions were evaporated and freeze-dried twice to avoid the presence of solvents, and stored at -20°C Purity and composition of fractions were checked with the HPLC-DAD system.

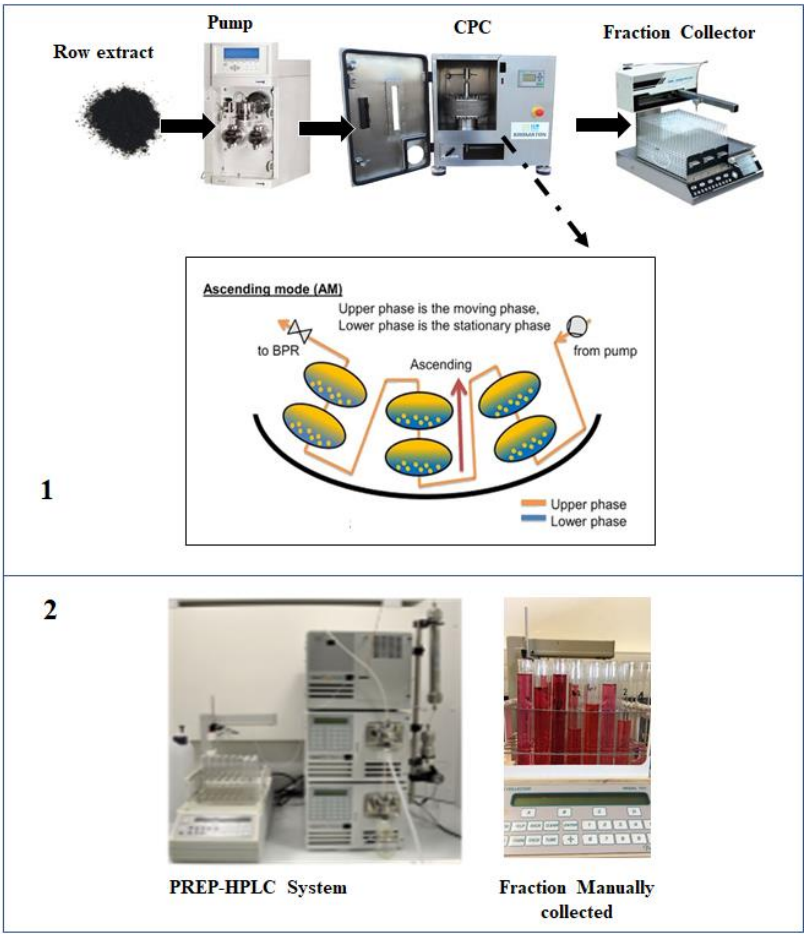

Figure 5.2 CPC separation scheme (1) and Prep-HPLC (2).

5.2.4 Anthocyanins-Protein binding test

5.2.4.1 BSA test

The bovine serum albumin (BSA) method for predicting astringency of tannins was modified for the analysis of anthocyanins. The method was described by Boulet et al. (2016) for wine and was modify in order to achieve repeatability of results, testing different amount of Bovine Serum Albumin Fraction V (Sigma–Aldrich, Saint Quentin Fallavier, France, 2 and 4 mg/mL), and of anthocyanins extract (0.5, 1 and 2 mg/L) and reaction time (15, 30, and 40 minutes) and waiting time after centrifugation (0, 15, 30 minutes). Variation between treated and untreated samples were checked by spectrophotometric lecture at λ=520 nm after 10 dilutions with 2% HCl solution (V-630 UV–vis spectrophotometer, JASCO, Japan) and direct HPLC-DAD injection. Finally, good coefficient of variation (<5%) was achieved using the following protocol. Barbera TAE and CPC fractions GF, AF, and CF were dissolved in wine-like solution (12% ethanol, 4 g/L tartaric acid, 3.5 pH) at a concentration of 1 g/L and centrifuged at 13500g for 10 minutes to eliminate all the insoluble material. BSA was dissolved at concentration of 4 mg/mL in pH 4.9 buffer solution and 0.5 ml were added to 2 ml anthocyanin solution samples (BSA) and buffer solution without BSA were added to 2 ml anthocyanin solution samples (control). Samples were left under slight agitation for 30 minutes before being centrifuged 13500 g for 5 minutes. The supernatant was filtered through a 0.45 µm filter and inject in HPLC-DAD system as described before for quantitative analysis. Each analysis was performed in triplicate. Reactions with BSA were then measured as the difference (delta) between the sample without BSA (control) and sample with BSA (BSA).

5.2.4.2 Saliva test

Saliva collection was performed from 18 volunteers (6 males and 12 females aged 20 to 35 years old) from 10 to 12 a.m. to follow circadian rhythm (Dawes, 1972).

Volunteers were asked to avoid eating and drinking beverages for at least one hour before sampling. Saliva was collected in 5 ml Eppendorf tubes, pooled together and immediately stored at -20°C before freeze-drying. Lyophilized saliva was dissolved at 10 mg/L -corresponded to one/third concentration as reported by Ma et al. (2016) in phospate buffer at pH 6.8 and centrifugated 8000g for 5 min at 4°C by a Jouan MR22 refrigerated centrifuge and the supernatants used as salivary protein sample. The method was that of Schwarz and Hoffman, (2008) with some modifications. Barbera TAE, and CPC fractions GF, AF, and CF were dissolved 1 mg/ml in wine-like solution (12% ethanol, 4 g/L tartaric acid, pH 3.5). A target compounds solution (300 μL) was mixed with 700 μL of prepared saliva sample or phosphate buffer as control and incubated at 37°C for 5 min. After incubation, an aliquot (400 μL) of the mixture was moved to a 3k Da centrifugal filter (Amicon Ultra-0.5 Centrifugal Filter 3k Devices, Merck Millipore) and centrifuged at 18,000 rpm for 5 min at 37°C. The filtrate in the bottom was injected into the HPLC-DAD system for quantitative analysis. Each analysis was performed in triplicate. Reactions with saliva were then measured as the difference between the sample without salivary protein (control) and sample with salivary protein (saliva).

5.2.5 Statistical analysis

Statistical analyses were carried out using R Statistics software version 3.4.0 (R Core Team, 2017) for one-way analysis of variance (ANOVA) and correlation. Levene’s and Shapiro-Wilk’s tests were used for assessing the homogeneity of variance and normality of ANOVA residuals, respectively. Correlation between anthocyanins decrease (treated-untreated samples as delta) and anthocyanins concentration was carried out depending on anthocyanidins substitution and anthocyanins esterification. Shapiro-Wilk’s test for normality of distribution was carried out and correlation was calculated by Pearson or Spearman correlation formula if normally or not normally distributed, respectively.

5.2.6 Sensory Analysis

Sensory analyses were conducted in a tasting room at our oenology research unit (ISVV, France) corresponding to the ISO 8589:2007 standards for this type of equipment (sound insulation, constantly regulated temperature).

5.2.6.1 Panel selection

All of the judges came from ISVV and are experienced with wine tasting. Judges were tested for determine if they can determine the interested sensory properties, i.e. basic taste found in wine, and astringency by tasting standard solutions: aluminium sulphate 2 g/L for astringency, quinine sulphate 15 mg/L for bitterness, tartaric acid 5 g/L for acidity, catechin 1 g/L for astringency and bitterness together. In order, two test were carried out: triangular test and identification of the the descriptors. In triangular test, equal number of the six possible combinations (ABB, BAA, AAB, BBA, ABA, and BAB, where A is the wine-like solution and B is the wine-like spiked with the molecule of interest) were proposed and judges were asked to recognize the different sample in the series. For identification test, the four spiked wine-like solutions were proposed and was asked to identify and describe the in-mouth sensation perceived. Judges who could not recognize the descriptors were not include in the panel. The final panel consisted of 18 judges, 12 females and 6 males aged 20-45.

5.2.6.2 In-mouth detection thresholds

In all experiments, black glasses filled with 8 mL of solution were labelled with three-digit random codes and presented to the panellists in random order for each presentation (following the scheme AAB, ABA, BAA where A is the wine-like solution and B is the wine-like spiked with the extract/fraction of interest), and presentation were randomized as well so to have an equal number of the possible combinations. Solutions, at room temperature, were presented in black glass in order to avoid colour influence, and judges were also instructed to spit in a black

glass to avoid seeing the difference meanwhile expectoration. Each judge was asked to sip the total glass volume, for avoiding differences given by the quantity tasted. Between each sample, judges were asked to take a 30 seconds rest, and water and cracker were provided for each presentation. In-mouth detection thresholds of the Barbera total anthocyanins extract, and CPC fractions GF, AF, and CF in wine-like solution (12% ethanol, 4 g/L tartaric acid, pH 3.5) were estabilished. The detection threshold was determined using the three alternative forced-choice presentation method 3-AFC (ISO 13301:2002) at concentration representative of the real wine concentration, i.e for total anthocyanins from 62.5 to 2000 mg/L, glucoside fraction from 31.25 to 1000 mg/L, for both acetylated and cinnamoylated fractions from 3.125 to 100 mg/L. A dilution factor of 2 for 6 total presentations was applied. The concentration were chosen because of the content of anthocyanins in wine and after a preliminary essay (triangular test, n=7) as suggested by Meilgaard et al (1999). Four tasting sessions were performed for total anthocyanins extract, glucoside, acetylated and cinnamoylated fractions, respectively. In each session, samples were presented following increasing concentration for each presentation as reported above. Judges were asked to specify one or more descriptors belonging to in-mouth properties that allowed the sample to be discriminated. The corresponding detection threshold was calculated as best estimated threshold (BET) (Meilgaard *et al*. 1999). The individual BET was determined as the geometric mean of the highest concentration missed and the next higher concentration. For judges who were correct at the lowest concentration, their individual BET was estimated as the geometric mean of the lowest concentration and the hypothetical next lower concentration that would have been given. For judges who failed to correctly identify the highest concentration, their individual BET was estimated as the geometric mean of the highest concentration tested and the next higher concentration that would have been given had the series been extended. The group BET was calculated as the geometric mean of the individual BET. Standard deviation log10 provided a measure of the group's variation.

TEST SENSORIEL

G1 Prenom *Nom* *Date*

Vous dégustez les échantillons en bouche. Vous dégustez tout le volume contenu dans le verre, une seul fois, en suivant l’ordre reporté en-dessous. Crachez dans le verre noir vous donne au début. Entourez l’échantillon qui vous semble différent des deux autres. Indiquez-le(s) descripteur(s) qui vous ont permis de différencier l’échantillon. Vous donnez une réponse même si vous n'êtes pas certain.

Poste 1			
Poste 2			
Poste 3			
Poste 4			
Poste 5			
Poste 6			

Descripteur(s)_____

Figure 5.2 Tasting sessions protocol.

5.3 Results and discussion

5.3.1 Anthocyanins extraction and purification

Extraction of grape skin anthocyanins produced compounds of 7.2% and 5.8% for Barbera and Nebbiolo, respectively, on the total skin powder weight (w/w). As expected by the total anthocyanins concentration of the variety, Barbera produced higher quantity than Nebbiolo (Mattivi *et al.* 2006; Río Segade *et al.* 2014). The latter, has a particular anthocyanin profile because it is a disubstituted prevalent variety, so peonidin and cyanidin derivatives are particularly abundant accounting for the 51.67% of the total anthocyanins. Barbera is, as usual in Vitis vinifera L, a malvidin-prevalent variety, so trisubstituted anthocyanins accounted for the 90% of all anthocyanins (chromatographic profiles are reported in **Figures 5.5** and **5.6**). Regarding esterification, 79.7% and 72% were glucoside, whereas 8% and 14.3% were acetylated and there were 12.3% and 13.7% p-coumaroylated and caffeoylated derivatives for Nebbiolo and Barbera, respectively. Purity of total anthocyanins extracts (TAEs) was calculated from the peak visible at 520 nm and 280 nm chromatograms and is reported as percentage. It was higher than 95% for both Nebbiolo and Barbera. Fast 4-hour extraction partially avoided the extraction of other phenolic compounds which may interfere with in-mouth chemical and sensorial analysis, especially oligomeric and polymeric flavanols. Regarding monomeric flavanols, neither catechin nor epicatechin were detected in TAEs. The main impurities in the extract were flavonol that were detected at 365 nm.

A first attempt at separation was carried out using a 200 ml CPC according to an already published method (Renault *et al.* 1997). Since the separation was satisfactory, the system was then applied to a larger apparatus (1L). Normal-phase CPC was conducted, so the stationary phase was constituted by the aqueous and the mobile phase corresponding to the organic solvents of low (B1) and high polarity (B2) in gradient. By doing so, the less polar cinnamoylated (p-

coumaroylated and caffeoylated) anthocyanins eluted first during the isocratic phase of solvent B1, followed by acetylated, eluted with the gradient up to 50% of solvent B2, and then glucosides during the gradient to 100% B2. **Figure 5.6b** and **5.7b** shows the chromatogram of 200 ml CPC for Nebbiolo and Barbera, respectively. The 1L-CPC chromatogram is equivalent, although it finishes at 140 minutes since it was stopped after acetylated separation, and glucosides were collected with extrusion of the stationary phase. The fractions collected in CPC 1L are shown in Figure 5.6a and 5.7a, reported as percentage of cinnamoylated (caffeoyl and p-coumaroyl derivatives), acetylated, and glucosilated forms found in each fraction by 1L CPC separation, for Nebbiolo and Barbera, respectively. Regarding glucoside anthocyanins, separation was in accordance with previous reports since they eluted according to the hydroxylation/methoxylation substitution: thus, cyanidin and peonidin, which are disubstituted anthocyanins, are eluting first than delphinidin, petunidin, and malvidin, which are trisubstituted (Renault 1997; Schwarz *et al.* 2003; Vidal *et al.* 2004d). This elution, did not provide fractions that differed between varieties. Only peonidin-3-O-glucoside in Nebbiolo could be extracted as almost pure compound (F5-F6, **Figure 5.4**). CPC allowed for good separation depending on the esterification of the glucoside moiety, although it was not able to completely avoid the presence of other derivatives. Notably, the most abundant anthocyanin, i.e. malvidin, is present in acetylated fraction as p-coumaroylated form, and in acetylated as its glucoside form. The great advantage of this technique is the quantity obtained and in particular the possibility to collect sufficient amount of acetylated and cinnamoylated derivatives by extruding the most abundant glucosides. For Nebbiolo (**Figure 5.4**), p-coumaroylated and caffeoylated anthocyanins eluted in the first two fractions, and acetylated were eluted in fraction 3, 4 and 5. Although glucosides were abundant from F4, particularly in Nebbiolo, given the abundance of di substituted glucoside which eluted first. Finally, F1 and F2 were collected as a cinnamoylated fraction (CF), F3 as an acetylated fraction (AF) and F8 as a glucosides fraction (GF) which corresponded to the 12.98%, 3.86%, and 83% of

the total amount injected. Separation was similar for Barbera (**Figure 5.5**) where F1 and F2 were collected as CF, F3 and F4 as AF, and F8 as GF, but higher proportion of esterified anthocyanins was found accounting 31.82%, 10.84% and 57.33% for CF, AF and GF, respectively.

Since there were no interesting differences between the two varieties, the fractions collected from them were mixed together for chemical and sensory analysis, producing a final amount of 820.8 mg of CF, 303.3 mg of AF and 3016 mg of GF.

TAE impurities (i.e. other phenolic compounds detected at 280 nm, mainly flavonols) were eluting in the beginning of the separation, in particularly in CF and AF fractions, with 60.86% and 66.7% of anthocyanins detected respectively, whereas high purity was achieved for GF directly from CPC extrusions (98.55%). Therefore, a preparative HPLC separation was performed to remove the impurities and to isolated anthocyanins not belonging to the same derivatives class for F4 and F5 for Nebbiolo and F5 for Barbera, in order to recover acetylated anthocyanins. Both F1 and F2 (CF) were purified to extrude impurities. The final purity achieved was 91% for CF and 85% for AF. Purification of acetylated fractions was very difficult because of the presence of peaks at 280 nm co-eluting with anthocyanins, and above all to a loss of acetic acid moiety during fraction evaporation which gave the respective simple glucoside anthocyanins. Therefore, AF purification was conducted several times, which leaded to a great loss of compounds. The level of 85% purity of acetylated fraction was reached, and where a 5% of impurities corresponded to their relative glucosides. Final fractions obtained are shown in **Figure 5.8b**, **c**, and **d**.

Table 5.2 Composition of Barbera TAE and derived glucoside, acetylated and cinnamoylated fractions expressed as percentage on the HPLC chromatogram at 520 nm.

Peak	Compounds	Composition			
		Total Anthocyanins (%)	Glucoside Fractions (%)	Acetylated Fractions (%)	Cinnamoylated Fractions (%)
1	Delphinidin-3-*O*-glucoside	10.7	12.96		
2	Cyanidin-3-*O*-glucoside	3.19	13.58		
3	Petunidin-3-*O*-glucoside	12.73	10.82		
4	Peonidin-3-*O*-glucoside	4.16	22.04		
5	Malvidin-3-*O*-glucoside	43.24	40.6		
6	Delphinidin-3-*O*-acetylglucoside	1.25		2.34	
7	Cyanidin-3-*O*-acetylglucoside	0.45		3.73	
8	Petunidin-3-*O*-acetylglucoside	2.4		7.7	
9	Peonidin-3-*O*-acetylglucoside	0.45		17.57	
10	Malvidin-3-*O*-acetylglucoside	10.38		66.55	
11	Delphinidin-3-*O*-coumaroylglucoside	0.33			10.56
12	Malvidin-3-*O*-caffeoylglucoside	0.56			4.12
13	Cyanidin-3-*O*-coumaroylglucoside	nd			2.13
14	Petunidin-3-*O*-coumaroylglucoside	1.79			10.86
15	Peonidin-3-*O*-coumaroylglucoside	0.65			24.77
16	Malvidin-3-*O*-coumaroylglucoside	7.77			47.56

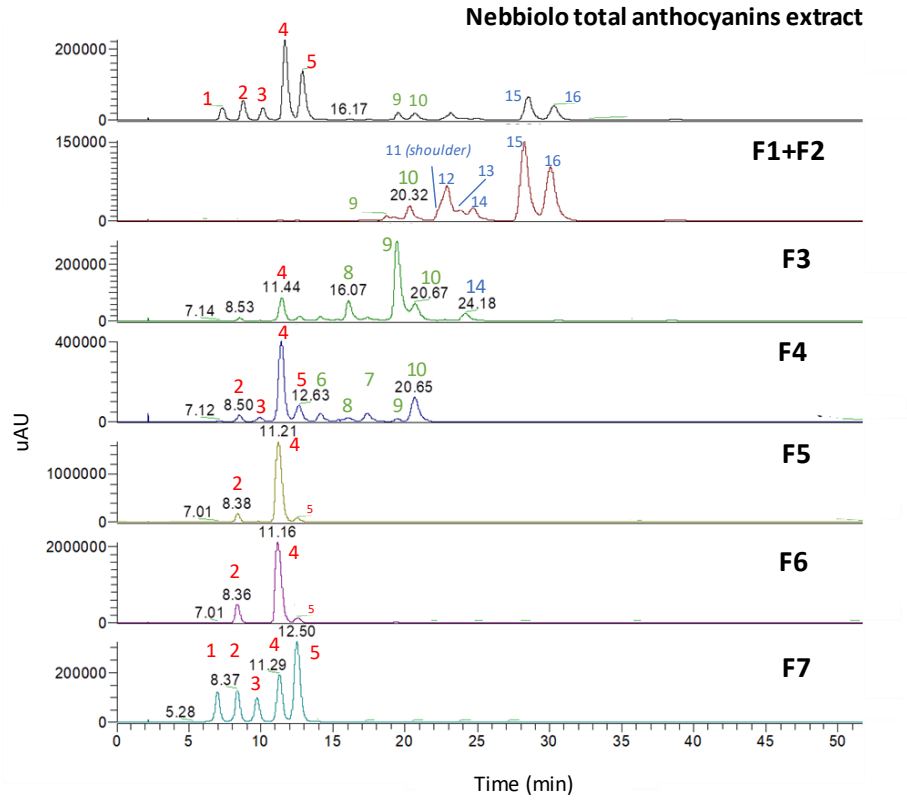

Figure 5.4 HPLC-UV chromatograms of Nebbiolo total anthocyanins extract (TAE) and CPC fractions (λ=520nm). Peak numbers are reported in table 5.2.

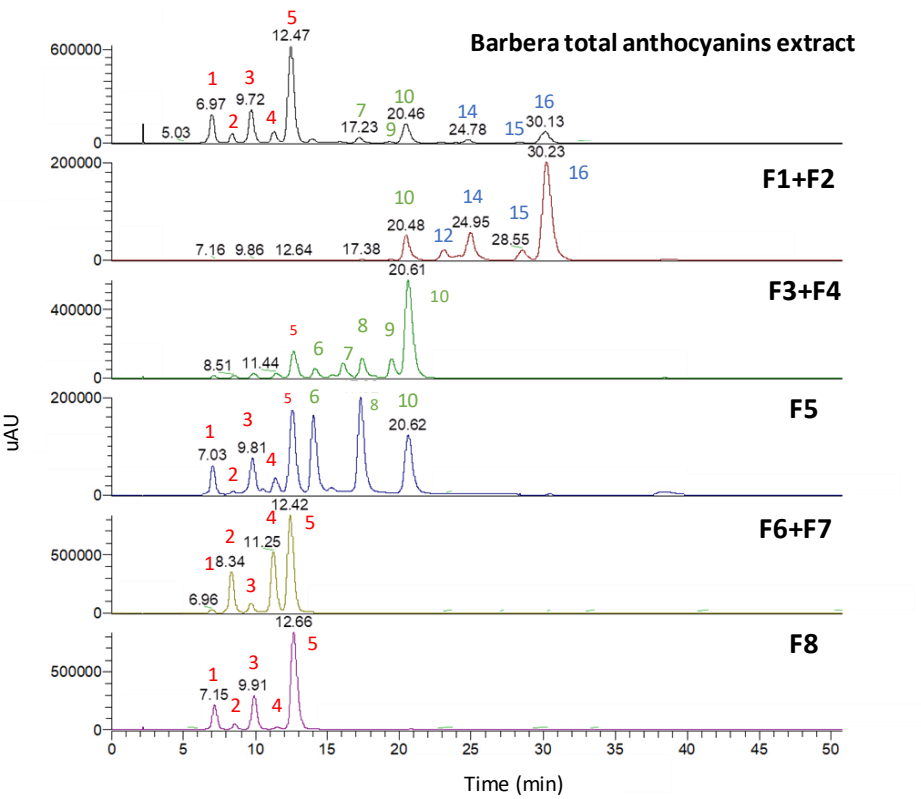

Figure 5.5 HPLC-UV chromatograms of Barbera total anthocyanins extract (TAE) and CPC fractions (λ=520nm). Peak numbers are reported in table 5.2.

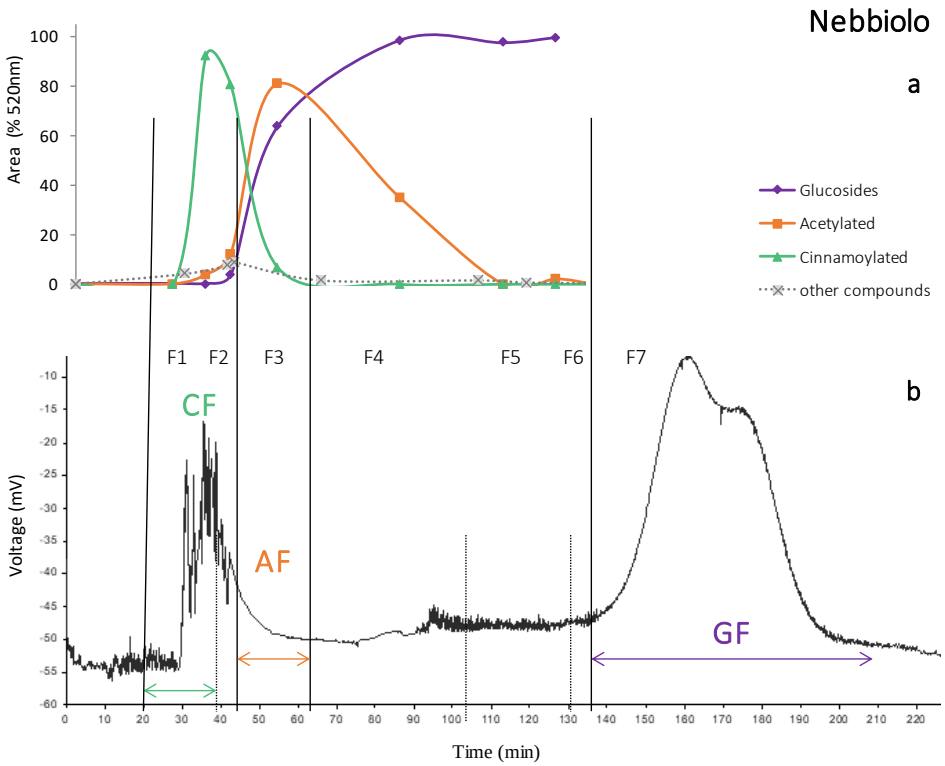

Figure 5.6 Nebbiolo CPC separation results: **a)** 1L-CPC concentration of glucoside, acetylated and *p*-coumaroylated anthocyanins and other compounds as percentage at 520 nm. **b)** 200ml-CPC chromatogram at 280 nm and corresponded collected fraction CF= cinnamoylated fraction, AF= acetylated fraction, and GF= glucoside fraction.

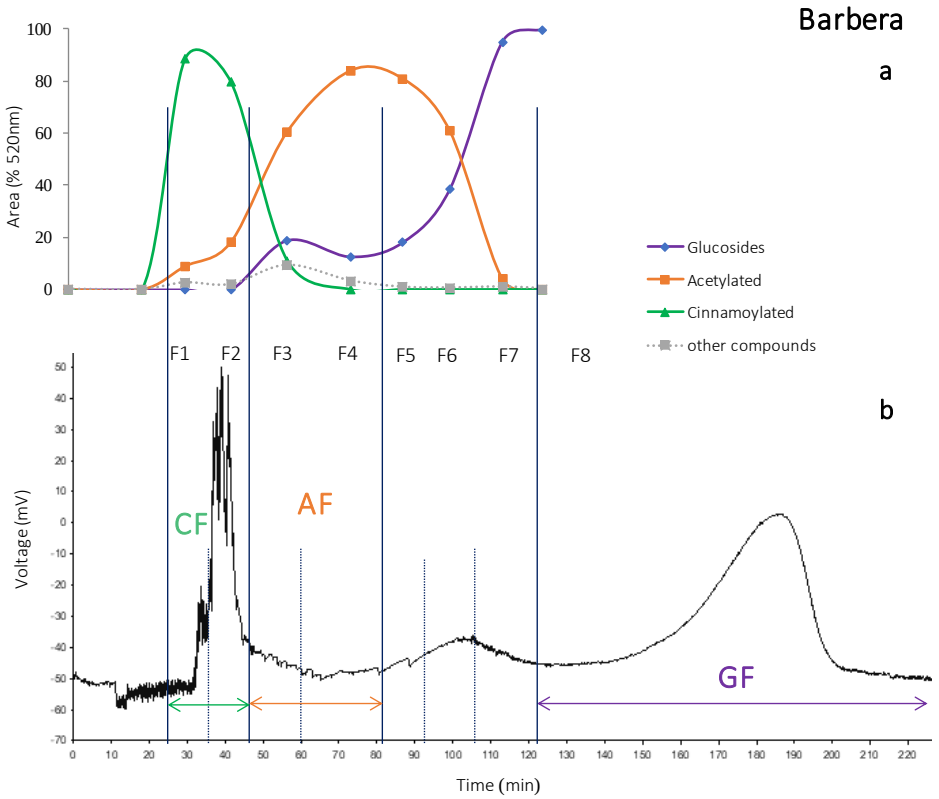

Figure 5.7 Barbera CPC separation results: a) 1L-CPC concentration of glucoside, acetylated and *p*-coumaroylated anthocyanins and other compounds as percentage at 520 nm. b) 200ml-CPC chromatogram at 280 nm corresponded collected fraction CF= cinnamoylated fraction, AF= acetylated fraction, and GF= glucoside fraction.

5.3.2 Chemical evaluation of astringency

While there are numerous methods to quantifiy astringency in wine and tannin extracts, there are fewer for anthocyanins. When the saliva test was coupled with MALDI-TOF to detect anthocyanin glucosides interaction, the proline protein (PRPs) and histatin chromatographic profiles were different with or without anthocyanins (Ferrer-Gallego *et al*., 2015a). In particular, the decrease of PRPs fraction of saliva in the presence of anthocyanins leads to supposition of the formation of precipitable complexes. The strength of the affinity between malvidin-3-*O*-glucoside and PRPs, evaluated as dissociation constant (K_D), was assessed by STD-NMR spectroscopy with success (Ferrer-Gallego *et al*., 2015a). Also, binding between anthocyanins and human serum albumin (HSA) has been reported, and K_D at different pH was determined (Cahyana, & Gordon, 2013). Therefore, saliva and bovine serum albumin (BSA) were assessed on total anthocyanins and fractions by common methods used on other classes of phenolic compounds.

To conduct the experiment of chemical and sensory analysis, CPC fractions combined by Nebbiolo and Barbera were taken and Barbera TAE alone as total anthocyanins extract, since its anthocyanins profile is similar to that of most *Vitis vinifera* cultivars and therefore can be more representative of wine anthocyanins profile.

BSA and Saliva test were first applied to Barbera TAE. BSA showed a significant difference only for malvidin-3-*O*-acetylglucoside (**Figure 5.9a**, -3.74%, $p<0.05$). Saliva test detected a significant difference between the saliva and control samples especially for glucosides (**Figure 5.9b**, cyanidin and petunidin -2.55% and -3.25%, respectively $p< 0.01$; peonidin -3.82% $p<0.05$; malvidin -6.26%, $p<0.001$) and cinnamoylated anthocyanins (for *p*-coumaroylated petudin -1.71%,

$p<0.01$; for caffeoylated malvidin -0.97%, $p<0.001$). Higher reactivity towards saliva than BSA was also found as sums of anthocyanins, since only saliva treated samples had lower values than control (-3.52%, $p<0.01$). The first impression is that since glucosides are the most abundant class in the extract, that they may mask the individual behaviour of the derivatives. The difference between BSA- and saliva-treated samples and their respectively untreated controls (delta) was correlated with initial concentration of individual anthocyanins (n=12, R^2 Spearman= 0.75, $p< 0.01$ and R^2 Spearman= 0.92, $p< 0.001$ for BSA and saliva, respectively).

Therefore, to avoid any concentration effect, analysis was then carried out on CPC fractions (**Figure 5.10**). Although a coloured precipitation occurred with BSA, no significant differences were found in total GF and AF, whereas a significant decrease in total CF was detected (-5.75%, $p<0.05$), i.e caffeoylated malvidin and *p*-coumaroylated petunidin decreased by 12.45% ($p<0.01$) and 16.91% ($p<0.05$), respectively (**Figure 5.10e**). Greater differences were detected with the saliva test: the concentration of anthocyanins is reduced in all samples, with -8.53% and -9.48% ($p <0.05$) for GF and AF, respectively, and -12.82% ($p<0.001$) for CF. **Figure 5.10b** shows that cyanidin and peonidin were decreased of -10.4% and -10.41% respectively ($p<0.01$), and petunidin of -6.91% ($p<0.05$) in GF. Malvidin, the most abundant glucoside, was reduced of 9.17% ($p=0.054$). Among the acetylated form (**Figure 5.10d**), petunidin and peonidin were decreased by -6.52% ($p<0.05$) and -9.13% ($p<0.05$) and malvidin by 10.59% ($p=0.053$). Highly significant differences were found for CF for all compounds except cyanidin (**Figure 5.10f**): *p*-coumaroylated delphinidin and petunidin were decreased by -6.48% and 8.43% with respect to the control ($p<0.01$), whereas *p*-coumaroylated peonidin and malvidin were decreased by 15.67% and 17.54% ($p<0.001$). In addition, saliva-treated caffeoylated malvidin decreased by -14.91% ($p<0.001$) with respect to the control.

Saliva more reliably reproduces the in-mouth anthocyanin behaviour than BSA, since it contains the proline-rich proteins (PRPs) responsible for complexes precipitation, whereas BSA is a common protein substitutive that may not react to form precipitable complexes with anthocyanins. In fact, even if the coefficient of variation between replicates (c.v. <5%) was achieved with an adapted BSA test, a large standard deviation was found among BSA-treated samples, thus confirming the hypothesis that affinity between the protein and anthocyanins is poor. It was previously reported that small phenolic compounds do not form insoluble complexes with protein (Ferrer-Gallego *et al.* 2015a; de Freitas & Mateus, 2001). In our study, a red precipitate was found in BSA-added samples, but in most of the cases there was not significant difference. Moreover, an interaction can occur between proteins and anthocyanins and lead to soluble compounds in wine-like solution, but it was not detectable with the method used. Therefore, a qualitative but not quantitative estimation of anthocyanins-protein interaction is possible. Regarding saliva-anthocyanins interaction, Ferrer-Gallego *et al*. (2015a) reported the formation of soluble complexes between malvidin-3-*O*-glucoside and a peptide sequence of histatin and proline-rich proteins. The latter, which can be considered high molecular weight proteins among salivary protein fractions (Ferrer-Gallego *et al*. 2015a), are thought to form insoluble complexes and therefore to precipitate with several polyphenols[3]. In our study, the CF fraction was the most reactive, perhaps due to the known reactivity of coumaric and caffeic acids with salivary protein (Ferrer-Gallego *et al* 2017) and their involvement in wine astringency (Hufnagel & Hofmann, 2008).

The correlation between anthocyanidin substitutes (i.e delphinidin, cyanidin, petunidin, peonidin, and malvidin) and their delta (difference between control and treated, as marker of the magnitude of the interaction leading to a precipitation) was not significative, except for cyanidin (R^2 Pearson = 0.99, p<0.01). Regarding flavanols (i.e. catechin and gallocatechin), the substitution of B ring strongly influences astringency, in particular the presence of two or three hydroxyl groups, since di-hydroxylated compounds lead to negative sensory

attributes of astringency, such as “dry”, “rough”, and “unripe”, whereas tri-hydroxylated compounds are correlated with the positive attributes “velvety”, “smoothness”, and “viscosity” that arise from different interaction among the molecules and protein (Ferrer-Gallego *et al.*2015b). Further studies, should be conducted on individual anthocyanins since the concentration effect can mask the difference in the reactivity of individual anthocyanins to protein depending on the B ring substitution, owing to the presence of the methyl group in peonidin, petunidin, and malvidin. On the other hand, when the CPC fractions were treated with salivary protein, the glucoside esterification was highly correlated with the precipitate concentration (R^2 Pearson = 0.939, $p<0.05$, R^2 Pearson = 0.999, $p<0.001$, and R^2 Pearson = 0.996, $p<0.001$, for glucoside, acetylated, and cinnamoylated, respectively). Altogether, our results show that reactivity of anthocyanins is mainly dependent on glucoside acylation, with *p*-coumaroyl and caffeoyl moieties increase the interaction between anthocyanins and salivary proteins.

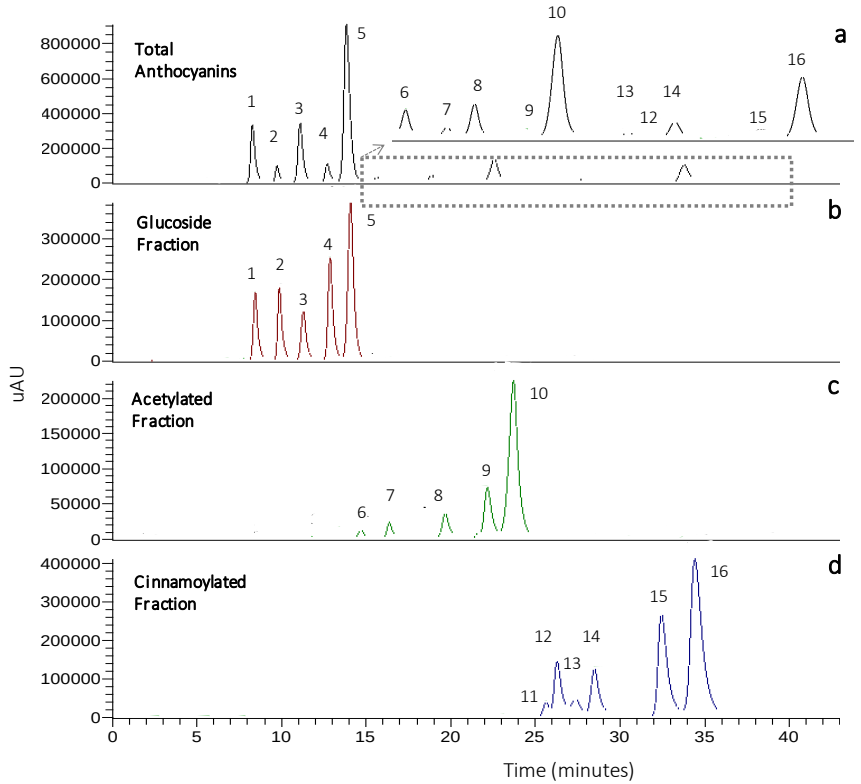

Figure 5.8 Chromatogram of HPLC-DAD analysis at 520 nm of anthocyanins used for chemical and sensorial analysis: a) total anthocyanins extract of Barbera, b) glucoside fraction, c) acetylated, and d) cinnamoylated fractions of anthocyanins. Corresponding molecule identifications are reported in Table 5.2.

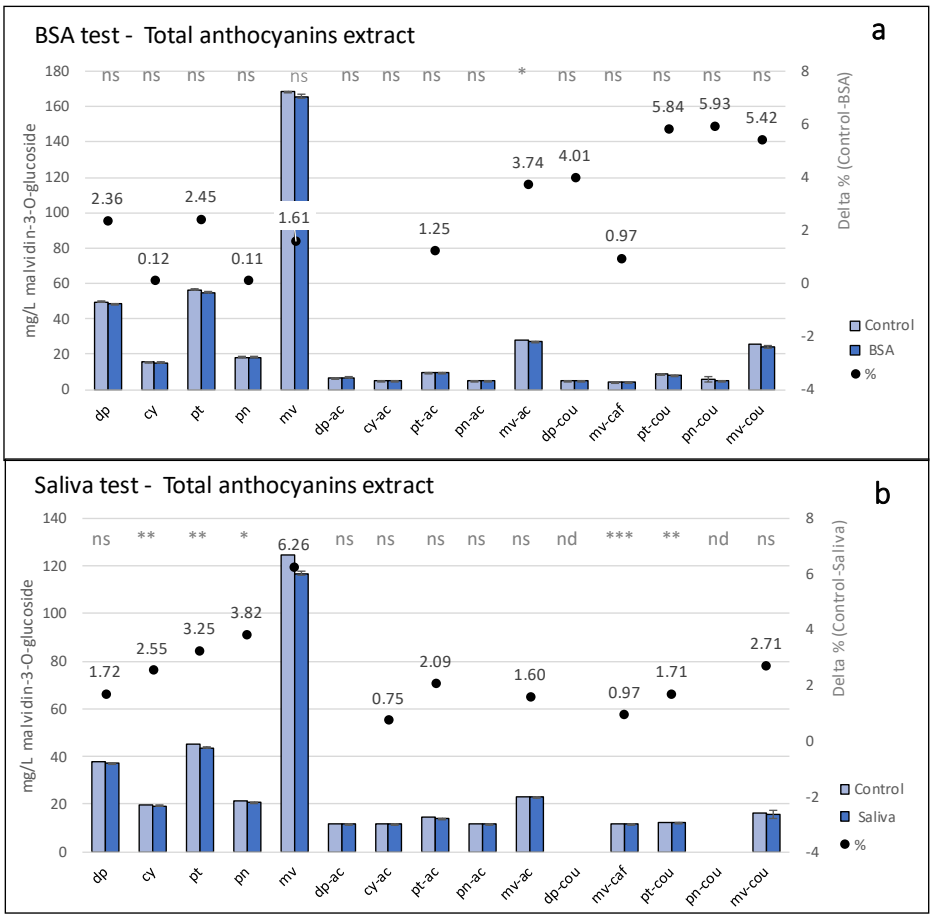

Figure 5.9 BSA (a) and Saliva(b) tests results on total anthocyanins extracts.

Legend *All data are expressed as average value ± standard deviation (n = 3). Sign: *, **, ***, and ns indicate significance at p< 0.05, 0.01, 0.001, and not significant, respectively, for difference between each identified compound for control and treated samples. Delta between treated (BSA and Saliva) and control (•) is reported as percentage for each compound, not reported delta are ≤ 0.*

Legend: dp= delphinidin-3-O-glucoside, cy= cyanidin-3-O-glucoside, pt= petunidin-3-O-glucoside, pn= peonidin-3-O-glucoside, mv= malvidin-3-O-glucoside, dp-ac= delphinidin-3-O-acetylglucoside, cy-ac= cyanidin-3-O-acetylglucoside, pt-ac= petunidin-3-O-acetylglucoside, pn-ac= peonidin-3-O-acetylglucoside, mv-ac= malvidin-3-O-acetylglucoside, dp-cou= delphinidin-3-O-coumaroylglucoside, mv-caf= malvidin-3-O-caffeoylglucoside, pt-cou= petunidin-3-O-coumaroylglucoside, pn-cou= peonidin-3-O-coumaroylglucoside, mv= malvidin-3-O-coumaroylglucoside.

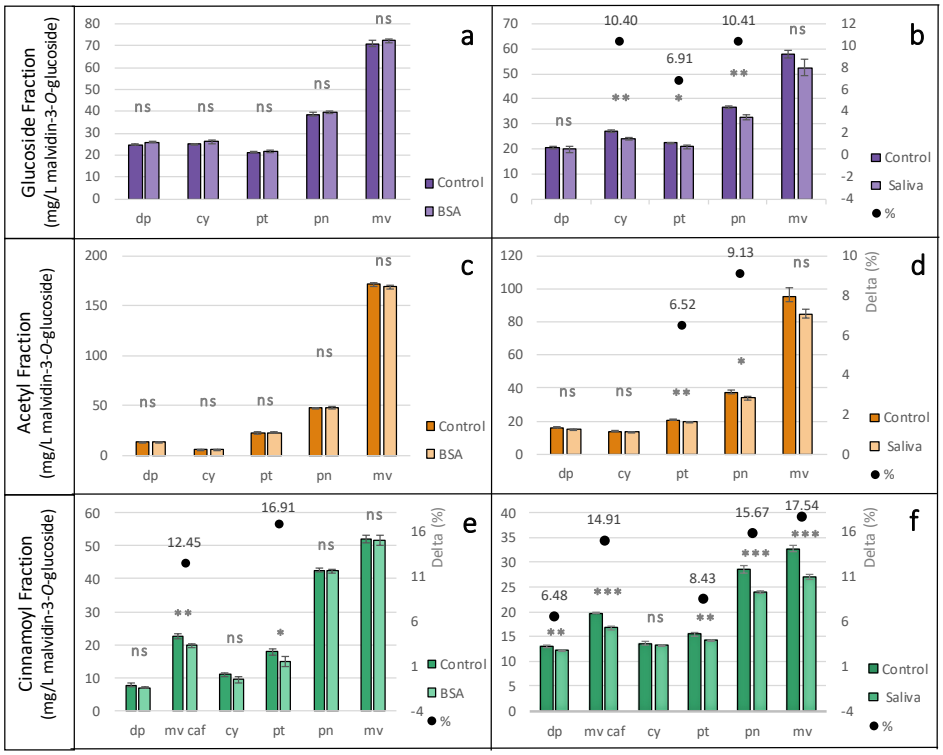

Figure 5.10 BSA and Saliva tests results on glucoside fraction (a and b, respectively), acetylated (c and d, respectively), and cinnamoylated (e and f, respectively) fractions.

Legend *All data are expressed as average value ± standard deviation (n = 3). Sign: *, **, ***, and ns indicate significance at p< 0.05, 0.01, 0.001, and not significant, respectively, for difference between each identified compound for control and treated (BSA and Saliva) samples. Delta between treated (BSA and Saliva) and control (•) is reported as percentage for each compound, deltas is for significantly different compounds reported only.*

For a and b, dp= delphinidin-3-O-glucoside, cy= cyanidin-3-O-glucoside, pt= petunidin-3-O-glucoside, pn= peonidin-3-O-glucoside, mv= malvidin-3-O-glucoside; for c and d, dp= delphinidin-3-O-acetylglucoside, cy= cyanidin-3-O-acetylglucoside, pt= petunidin-3-O-acetylglucoside, pn= peonidin-3-O-acetylglucoside, mv= malvidin-3-O-acetylglucoside; and for e and f, dp= delphinidin-3-O-coumaroylglucoside, mv-caf= malvidin-3-O-caffeoylglucoside cy= cyanidin-3-O-coumaroylglucoside, pt= petunidin-3-O-coumaroylglucoside, pn= peonidin-3-O-coumaroylglucoside, mv= malvidin-3-O-coumaroylglucoside.

5.3.3 Sensory analysis of extract and fractions

As described above, in-mouth properties involve various parameters of which astringency is only one. In addition, bitterness can be tested by using receptors, as done successfully for malvidin-3-*O*-glucoside (Soares *et al*. 2013). However, a limitation of this technique is that only 1% ethanol content can be used owing to its cellular toxicity, so true wine condition is not completely reproduced. To understand whether anthocyanins can be detected in wine, we performed a sensory detection thresholds test for the Barbera total anthocyanins extract (TAE) and the fractions cinnamoylated (CF), acetylated (AF), and glucoside (GF). A wine scale range and wine model solution were chosen to estimate the detection threshold. The best estimate threshold method (BET) was used since it is very difficult to obtain a sigmoid curve in taste threshold. Several factors should be taken into account, especially variability in taster (Bartoshuk *et al.* 2000). The quality and quantity of the multiple cellular mechanisms associated with bitter taste varies considerably from one person to another. Moreover, receptors saturation during tasting with several presentations can occur.

Detection threshold test results are shown in **Table 5.3**, with the concentrations used. Sensory results were in agreement with chemical data, since CF, which was the most reactive towards protein, was also the fraction with the lowest perception threshold (BET= 58 mg/L), followed by AF (BET= 68 mg/L). Moreover, the higher perception threshold of anthocyanins glucosides alone (BET= 297 mg/L) than TAE (BET= 255 mg/L) suggested that the presence of a percentage of acetylated and cinnamoylated anthocyanins on the total extract had a higher impact on sensory properties, thereby lowering the BET of total anthocyanins extract. Judges were asked to express one (or more) in-mouth descriptor(s) that helped them to discriminate the samples, and Table 2 shows descriptors only for those judges who correctly discriminate the sample over the BET. The common

descriptors were astringency and bitterness for all the anthocyanins tasted, and saltiness was reported for glucoside and total extract. Saltiness was described as a tingling sensation on the tongue. Taster were not trained for astringency sub-qualities for the detection threshold test, so the “saltiness” descriptor could be misunderstood as a mouthfeel sensation such as irritation or the particulate in mouth of astringency, as proposed by Gawel *et al.* (2000). This is in accordance with previous studies which reported a “mild taste” of anthocyanins in solution and in particular an increase in astringency (particularly “fine grain” attribute) descriptor (Brossaud *et al.* 2001; Vidal *et al.* 2004a). Previous research also estabilished a relationship between the presence of anthocyanins (especially acetylated and *p*-coumaroylated) and the perceived bitterness of wine (Gonzalo-Diago *et al.* 2014). Under our conditions, all the solutions tasted were described as bitter tasting. It seems that anthocyanins are involved in in-mouth sensory properties at wine concentration and that their influence depends on the esterification of glucosides.

Table 5.3 *BET (Best estimated threshold) of total anthocyanins extract of Barbera and of CPC Fractions, with tasted concentrations. †Descriptors are reported only for correct answers over the BET.

Group	BET* (mg/L)	Log_{10} BET	Log_{10} St. Dev.	Concentration (mg/L)	Descriptors† (n)
Total Anthocyanins (mg/L)	**255**	2.41	0.75	2000	Astringency (8); Bitterness (8); Saltiness (5)
				1000	Astringency (7); Bitterness (6); Saltiness (3)
				500	Astringency (6); Bitterness (5); Saltiness (2)
				250	
				125	
				62.5	
Glucosides (mg/L)	**297**	2.47	0.50	1000	Astringency (10); Bitterness (5); Saltiness (4)
				500	Astringency (6); Bitterness (8); Saltiness (4)
				250	
				125	
				62.5	
				31.125	
Acetylated (mg/L)	**68**	1.81	0.34	100	Bitterness (7); Astringency (4)
				50	Bitterness (9); Astringency (9)
				25	
				12.5	
				6.25	
				3.125	
Cinnamoylated (mg/L)	**58**	1.76	0.42	100	Bitterness (4); Astringency (3)
				50	Bitterness (4); Astringency (4)
				25	
				12.5	
				6.25	
				3.125	

5.4 Conclusion

Anthocyanins are well-known for their contribution to wine colour and chromatic features, and several vineyard and winemaking strategies are exploited to ensure the maximum anthocyanins accumulation and extraction for improve wine’s visual quality. On the other hand, understanding of their influence on in-mouth sensory properties is only partial, in particular regarding astringency, mouthfeel attributes, and bitter taste. Wine in-mouth sensation variability is recognized to be strongly connected to flavanols concentration and characteristics, however several molecules can contribute to in-mouth sensations and implicate wine in-mouth complexity. Full understanding of these different factors can help in the definition of winemaking strategy. Therefore, in this study, grape anthocyanins were extracted from skins and fractionated in classes depending on their substitution, i.e. glucoside, acetylated and cinnamoylated, by a combination of liquid–liquid chromatography (CPC) and preparative-HPLC. Yield and purity were of sufficient quality and quantity to investigate their sensory properties, in particular regarding glucoside anthocyanins whereas acetylated and cinnamoylated anthocyanins contained some impurities. These compounds, that were mainly detected at 365 nm, were considered belong to flavonols classes, which are involved in in-mouth sensory properties and therefore may have influenced sensory analysis results. Taste detection thresholds of these compounds as previously reported (Vidal *et al.* 2001) and trace level detected lead to exclude this hypothesis. Both chemical and sensory analyses were performed. Additionally, this is the first time that acetylated anthocyanins have been tasted, and the interaction of acetylated and cinnamoylated anthocyanins with protein assessed. Anthocyanins reacted with both BSA and salivary protein, but to different extents, as the saliva test gave higher response between anthocyanins and salivary protein. Importantly, the saliva test revealed a significant reduction of anthocyanins, both in the total extract and when

fractionated in glucoside, acetylated, and cinnamoylated. The latter in particular is the most reactive to salivary protein. These results are confirmed by sensorial analysis carried out by detection threshold test. Best estimated threshold (BET) of anthocyanins were in wine range scale, and acetylated and cinnamoylated thresholds were below the glucoside threshold. This was confirmed by the lower BET of total extract compared to the glucoside fraction alone. Therefore, anthocyanins can be detected as contributors to in-mouth properties, and the degree of their involvement is related to their acylation. Indubitably, anthocyanins concentration in wine must be considered: BETs concentration as hereby reported are founded in young and anthocyanins-rich wines, therefore the presence of other well-known eliciting compounds, such as monomeric and polymeric flavanols, is still to assume as the major contribution to wine astringency and bitterness. Interaction between anthocyanins and other phenolic class compounds are reported in studying wine colour, such as copigmentation. These non-covalent reactions may influence affinity of both cofactor and anthocyanins for salivary protein, as recently reported by Soares *et al.* (2018) for malvidin-3-*O*-glucoside and epicatechin. Therefore, in addition to the individual compound concentration, the interaction with other sensoactive compounds is also relevant from several points of view, including the direct interaction with salivary proteins or the bitter receptors, the interaction between the compounds themselves, and the competition to elicite the sensation. Further sensory analysis should be carried out with a panel trained in mouthfeel descriptors to investigate the in-mouth descriptors of anthocyanins in more complex solutions. In particular, evaluation of pH and ethanol content, and the interaction with other taste compounds will be useful in order to achieve a deeper understanding of the wine in-mouth complexity.

6. General Conclusion and Future Perspective

This PhD was focused on two main aims:

1. The evaluation of an innovative post-harvest technique was studied: the ozone treatment on grapes post-harvested and during dehydration, in order:

    - Gaseous ozone treatment was tested on fresh grape;

    - Gaseous zone treatment was applied during grape dehydration.

2. Extraction of anthocyanins form grape skin and isolation by Centrifigual Partition Chromatography (CPC) to fractionate and purify glucoside, acetylated, and cinnamoylated anthocyanins in order to investigate anthocyanins involvement in in-mouth sensory properties.

Regarding the first part, ozone in winemaking industries has been demonstrated an interesting tool for several points of view, in particular regarding the possibility to reduce the use of sulphur dioxide since it can reduce mould infection and yeast population, leading on one hand to preserve berry health status, on the other hand to conduct easily controlled fermentation. The second important role of ozone

that was investigated is related to its elicitors effect on phenolic compounds accumulation. For wine grapes, where flavanols and anthocyanins play the major role in organoleptic qualities, its capacity to induce phenolic production must be balanced with its strong oxidant activity. Therefore, its application must be controlled and adapted to the variety features and winemaking technology, anyway short treatments seems to be promising in wine industry, above all for dehydration technology. In this case, the risk of mould infection is clearly higher than fresh product and ozone can be an important tool to preserve the grape until winemaking. The more sensitive compounds are surely anthocyanins. If in fresh grapes, a higher or similar final content of anthocyanins with respect of control was achieved, in withered berry the final content was reduced as much as the dehydration proceed. Therefore, the balance induction/depletion must be taken into account. Moreover, ozone can modify cell-wall composition and skin mechanical properties leading to a different extraction farter the initial phenolic compounds content. Ozone showed different behaviour in the first and in the second experiments, therefore depending on the different strategy of application. Moreover, a strong variety influence was found, so not the same trends were found in Barbera and Nebbiolo, therefore variety features, such as phenolic compounds composition and content, skin properties, and cell wall composition are strongly influencing the extraction of phenolic compounds. These results lead to the conclusion that, if in Barbera moderate dose of ozone is not affecting negatively phenolic compounds content and extractability, its application should be adapted in Nebbiolo considering dose, time, and modality of the treatment. In this case, if in fresh grape ozone had a positive effect in enhancing phenolic compounds extraction, during dehydration a decrease was found.

Anthocyanins are surely involved in wine quality, since the visual component is very important to consumers. The second part of this PhD wanted to understand if they are involved also in in-mouth quality. Surely, anthocyanins are among the most abundant secondary metabolites in grape/wine and enhancing anthocyanin extraction has been one of the major topic in wine research. In our study, we

underlined a role of anthocyanins in in-mouth perception, and it is correlated with astringency and bitterness sensation. Detection thresholds (BET) were established and the acylation seems to be the key of this difference in sensory properties, since cinnamoylated derivatives own the lower BET, followed by acetylated, and glucoside, at last. As well, the same trend was observed with regards to the interaction with salvary proteins, as a marker of astringency. Although BETs are in wine range concentration, they are higher with respect to other phenols, therefore it will be interesting in future research to evaluate the perception in wine matrix and the possible interaction with other wine sensoactive compounds, form phenolic compounds to polysaccharides, well-known for their impact in mouthfeel sensations. In this way, a double way investigation could be followed, one, given by the direct interaction of anthocyanins as sensoactive compounds, since they were proved to react with salivary protein and be perceived in in wine-like solution. On the other hand, anthocyanins are able to react giving new pigments, but most interesting, to interact non-covalently with high sensory eliciting compounds, such as flavanols and flavonols, throughout copigmentation, which may lead to a change to the cofactor ability to interact with salivary protein and taste receptors, therefore modifying other phenolics sensory properties.

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