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PURIFICATION AND STRUCTURE ELUCIDATION OF CROWN PROCYANIDINDS

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Purification and Structure Elucidation of crown procyanidins

ABSTRACT

The wine phenolic composition will depend on the original grape phenolic profile but also on the extraction and winemaking techniques used. Recently three surprisingly polar tannins oligomers (one tetramer and two pentamers) were detected by Zeng (2015). These oligomers turned out to belong to a new sub class of condensed tannin oligomers, which were never been reported before in wine as well in plant kingdom and was named crown procyanidin. As this kind of compounds has never been identified and detected in grape berries, an extraction method has been developed using acetone/water (70/30, v/v) acidified with 5 g/L of tartaric acid follow by methanol/water (40/60, v/v) with 5 g/L of tartaric acid. Using this extraction method, the crown procyanidin appeared to be tissue specific since they were detected only in the grape skin of Merlot and Cabernet Sauvignon. Moreover, the comparison between the two grape varieties revealed that crown procyanidin tetramer concentration was higher in Cabernet Sauvignon than in Merlot. Besides, the extraction rate of the crown procyanidins during the fermentation process has been estimated. The observed trend revealed that the crown procyanidins are extracted very quickly at the beginning of the vinification which is an opposite trend compare to regular B- type condensed tannins which need ethanol to be extracted. So, the fact that these tannins are very polar, highly soluble in water and exclusively located in skin might be the reason of this specific and unexpected behaviour.

KEYWORDS

Grape, wine, Cabernet-Suavignon, Merlot, Bordeaux

Purification et élucidation sur les tanins couronnes et leur structure

RESUMÉ

La composition phénolique d'un vin dépend de l'origine du raisin et des techniques d'extraction et de vinification utilisées. Durant de nombreuses recherches réalisées sur le vin rouge, trois type de tanins oligomères différents ont été détectés par Zeng en 2015 : un tétramère et deux pentamères. Ces tanins appartiennent à une nouvelle sous-famille des tanins condensés et jusqu'à ce jour, aucune publication n'avait été parue sur ses tanins que ce soit dans le vin ou dans le règne végétal. Ainsi, une nouvelle méthode d'extraction a été déterminée : Acétone/eau (70/30) et Méthanol/Eau (40/60), les deux acidifiées avec 5 g/L d'acide tartrique. En utilisant cette méthode d'extraction, ses tanins couronnes semblent montrer une spécificité de localisation car ils ont été découverts seulement dans la pellicule des cépages Merlot et Cabernet-Sauvignon. De plus, une comparaison entre ces deux cépages a été réalisée et elle démontre que la concentration en tétramère de ses tanins couronnes est plus élevée dans le cépage Cabernet-Sauvignon que dans le cépage Merlot. En parallèle, des mesures de l'évolution et du ratio d'extraction durant la fermentation ont été réalisées. Une tendance apparait démontrant que les tanins couronnes sont extraits plus rapidement au début de la vinification, cette tendance étant en contradiction avec les tendances d'extraction observées pour les autres tanins, la présence d'éthanol étant nécessaire. Enfin, ce comportement spécial ainsi que cette spécificité pourrait provenir de différents paramètres tels que la polarité, la solubilité dans l'eau et la localisation dans les pellicules.

MOTS CLÉS

Raisin, vin, Cabernet-Suavignon, Merlot, Bordeaux

Taninos de Corona: Purificación y Elucidación Estructural

ABSTRACT

En el vino la composición fenólica depende de la propia uva y también de las técnicas de extracción utilizadas en la vinificación. Recientemente se han detectado tres taninos oligoméricos (un tetrámero y dos pentámeros) (Zeng, 2015). Dichos taninos, que nunca antes habían sido identificados en vino ni en el reino vegetal, se clasificaron como taninos condensados oligoméricos y han sido nombrados como taninos de corona.

Como estos compuestos no habían sido identificados ni detectados en uvas, se ha desarrollado un método de extracción utilizando acetona/agua (70/30, v/v) acidificado con 5 g/L de ácido tartárico seguido de metanol/agua (40/60,v/v) acidificado también con 5g/L de ácido tartárico. Utilizando este método de extracción se ha visto que los taninos de corona son específicos del hollejo de uvas de Merlot y Cabernet Sauvignon. Además, si se compara los taninos de corona en estas dos variedades la concentración es mayor en Cabernet Sauvignon que en Merlot.

Durante el proceso de fermentación, se ha observado que el ratio de extracción de los taninos de corona es muy rápido al principio de la vinificación, lo cual es lo contrario de lo que pasa con los taninos tipo B regulares que necesitan de etanol para ser extraídos con lo cual, los taninos de corona no necesitan etanol en la extracción.

El hecho de que estos taninos sean muy polares, con alta solubilidad en el agua y localizados exclusivamente en el hollejo debe ser la razón de este específico e inesperado comportamiento.

Palabras clave

Uva, vino, Cabernet-Suavignon, Merlot, Burdeos

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Abbreviation table

μm	Micrometre
μg	Microgram
A	Anthocyanin
C	Carbon atom
cm	Centimetre
D	Dimension
d	Diameter
DAD	Diode Array Detector
DP	Degree of Polymerization
EIC	Extracted Ion Chromatogram
ESI	Electrospray Ionization
g	Gram
H	Hydrogen
h	Height
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
IPT	Index Polyphenol Total
L	Litre
M	Molaritat
m/z	Mass-to-Charge ratio
mDP	Mean Degree of Polymerization
MeOH	Methanol
mg	Milligram
MHz	Mega hertz
min	Minutes
mm	Millimetre
MS	Mass spectrometry
Nm	Nanometre
NMR	Nuclear Magnetic Resonance
O_2	Oxygen molecular
^oC	Degree centigrade
OD	Optical Density
OH	Hydroxide
QM	Quinone Methide Fission
Q-TOF	Quadruple-Time Of Flight
rpm	Revolutions for minutes
RDA	Retro-Dields-Alder Fission
SO_2	Sulphur Dioxide
T	Tannin
TFA	Trifluoroacetic acid
TSK	TSK gel column
UPLC	Ultra Performance Liquid Chromatography
UV	Light Ultraviolet
V	Voltage

1. INTRODUCTION

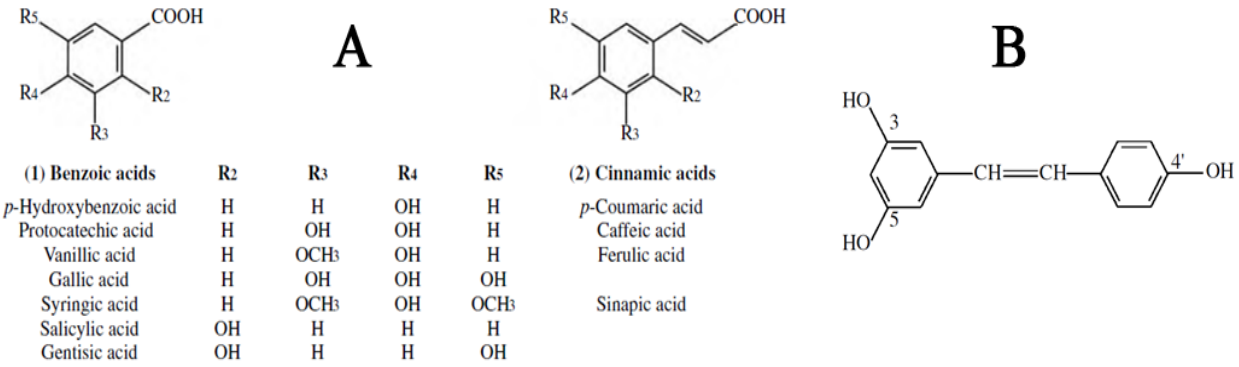
Cabernet Sauvignon (CS) and Merlot (M) are the world's most widely recognized red wine grape varieties. Particularly in Bordeaux vineyard, M represents 62% of the whole area whereas CS represents 25% of the Bordeaux plantation (CIVB, 2006). Indeed, the phenolic nature of CS and M is of paramount interest particularly in this region. Recently Chira *el al*., (2009) started this investigation by reporting the grape variety effect (CS vs. M) on proanthocyanidin composition for two consecutive vintages 2006 and 2007 and then, Lorrain *et al*., (2011) compared the influence of grape variety on proanthocyanidin and anthocyanidin composition for 2009-vintage with 2006, 2007 and 2008 vintages. Grape phenolic compounds (anthocyanidins, condensed tannins) possess various biological properties, including antioxidant (Lopez *et al*., 2008), antibacterial (Jayapraskasha *et al*., 2003), anticancer (Nandakumar *et al*., 2008) and antidiabetic effects (Patel *et al*., 2012). During red wine-making, proanthocyanidins are extracted from grapes seeds and skins. The wine phenolic composition will depend on the original grape phenolic profile but also on the extraction and winemaking techniques used. Proanthocyanidins are of great importance to sensory red wine quality due to their astringent and bitter properties (Lorrain *et al*., 2011) and their role in long-term colour evolution and stability. Condensed tannins are polymers of flavan-3-ols unit such as catechin, epicatechin, epigallocatechin and epicatechin-3-*O*-gallate. In acidic solutions such as wine, proanthocyanidins are very reactive compounds. It is current to notice tannins polymerization and depolymerisation under such acidic conditions. Moreover, ethanal, glyoxylic acid an other aldehydes present in red wine participate as well to the formation of indirect inter-flavanoid linkage which is important for their polymerization (Ribéreau-Gayon *et al.*, 2012). Nowadays, technological improvements in chromatographic techniques, high resolutions mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy facilitate the purification of oligomers/polymers, of condensed tannins as well as their structural characterisation (Lorrain *et al.*, 2013). However, due to the complexity and large diversities of condensed tannins structures presented in red wine, there are still many unknown structures.

Recently, during investigations on red wine condensed tannins three surprisingly polar tannins oligomers (one tetramer and two pentamers) were detected by Zeng (2015). Mercadé Pons (2015) developed a purification method for the tetramer and determinate its structure by high-resolution mass spectrometry as well as NMR. These oligomers turned out to belong to a new sub class of condensed tannin oligomers, which were never been reported before in wine as well in plant kingdom and were named crown procyanidins.

Thus this project has two important objectives:

- The first one was to determine if the crown procyanidin are present in grape and if so in which part of the berries seed or skin and if there are some difference between two grape varieties (Merlot and Cabernet Sauvignon). The extraction rate of these compounds during the alcoholic fermentation was also monitored.

- The second main objective was to separately purified the two crown procyanidins pentamer from red wine in order to characterize their structure by NMR since to date only the structure on the crown procyanidins tetramer has been establish.



(1) Benzoic acids	R2	R3	R4	R5	(2) Cinnamic acids
p-Hydroxybenzoic acid	H	H	OH	H	*p*-Coumaric acid
Protocatechic acid	H	OH	OH	H	Caffeic acid
Vanillic acid	H	OCH_3	OH	H	Ferulic acid
Gallic acid	H	OH	OH	OH	
Syringic acid	H	OCH_3	OH	OCH_3	Sinapic acid
Salicylic acid	OH	H	H	H	
Gentisic acid	OH	H	H	OH	

Figure 1 A: Structure of Phenolic Acids in grapes and wine, B: Structure of Resveratrol (Trihydroxy-3,5,4'-stilben) (Ribéreau-Gayon *et al.*, 2012)

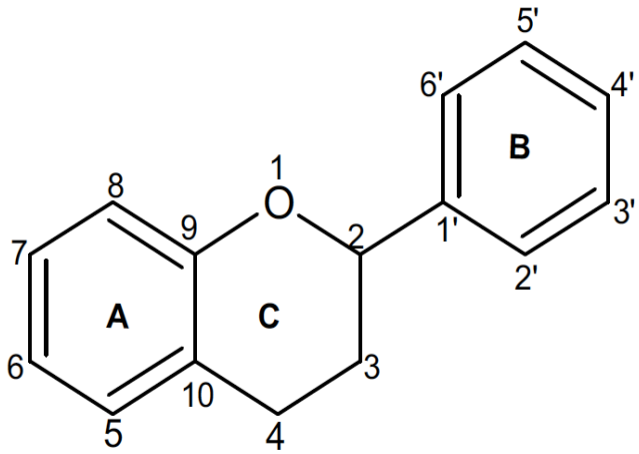


Figure 2: General structure of flavonoids (Chira *el al.*, 2009).

2. BIBLIOGRAPHY

2.1. Non- Flavonoids

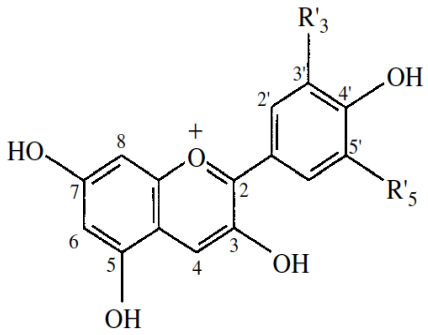
In grape and wine the most important non-flavonoids compounds are phenolic acids and stilbenes. Concentrations of phenolic acids are on the order of 100-200 mg/L in red wine and are divided in two types: benzoic acids (C_6-C_1) and cinnamic acids (C_6-C_3). As is shown in Figure 1.A. the different acids present in wine are differentiated by the substitution of their benzene ring. In grapes, they are mainly present as glycoside combinations, from which they released by acid hydrolysis and esters, but free forms are more prevalent in red wine (Ribéreau-Gayon *et al*., 2012). Phenolic acids play critical roles in the development of the bitterness and astringency properties of wine (Ferreira *et al*., 2016)

Stilbenes have two benzene cycles, generally bonded by an ethane, or possibly ethylene, chain (C_6 - C_2 - C_6). This is a very complex family due to the position of hydroxyls groups, or the sugar or the methoxy substitution on these hydroxyls group as well as the conformation of the double bond (*trans* or *cis*) of the molecule. They are found in grapes, wine and oak wood (Ribéreau-Gayon *et al*., 2012). The most known and abundant is the *trans*-resveratrol (Figure1.B.) and it is well known for its multiple pharmacological activities, such as anti-inflammatory, antioxidant, antimicrobial, anticancer, neuroprotective and cardioprotective effects (Francioso *et al*., 2014).

2.2. Flavonoids

Flavonoids are the most important grape polyphenolic families, which have a high influence on wine quality. They are secondary metabolites from vegetables and they are implicated in several roles: pigmentation, defence against UV, pathogens, and fungi infections, etc. (Benheim *et al*. 2012; Dietrich *et al*., 2009; Prassad *et al*., 2009).

Flavonoids have 15 carbons atoms organized as C_6-C_3-C_6, so two polyhydroxylated aromatic rings, noted as “A” and “B” rings which are connected through a central oxygenated heterocycle ring noted as “C” (Figure 2). There are several flavonoids groups: flovonols, flavones, flavanones, flavanonols, flavanols and anthocyanin. These sub-families are determined due to the saturation of the C-ring as well as the substitution in C_3 and C_4 position of its “C” ring (Chira *et al*., 2009). In wine and grape, the two main flavonoid sub-families are anthocyanin (pigment of grape berries and wine) and flavanols, which are the monomeric units of the condensed tannins.



R'_3	R'_5	Name of aglycone
OH	H	Cyanidin
OCH_3	H	Peonidin
OH	OH	Delphinidin
OH	OCH_3	Petunidin
OCH_3	OCH_3	Malvidin

Figure 3: Structure of anthocyanidins in grapes and wine (Ribéreau-Gayon *et al*., 2012).

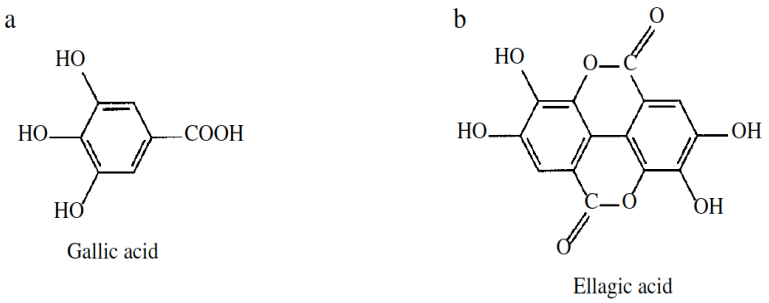
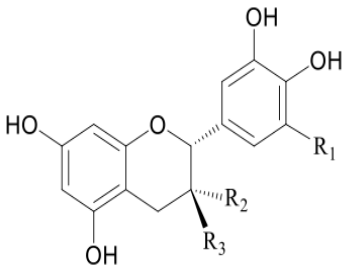


Figure 4: Structure of phenolic acids (A) Gallic acid (B) Ellagic acid. (Ribéreau-Gayon *et al*., 2012).



1. (+)-Catechin — R1=H, R2=H, R3=OH
2. (-)-Epicatechin — R1=H, R2=OH, R3=H
3. (+)-Gallocatechin — R1=OH, R2=H, R3=OH
4. (-)-Epigallocatechin — R1=OH, R2=OH, R3=H
5. (-)-Epicgallocatechin-3-*O*-gallate — R1=H, R2=*O*-gallic acid, R3=H
6. (-)-Epigallocatechin-3-*O*-gallate — R1=OH, R2=*O*-gallic acid, R3=H

Figure 5: Structure of flavan-3-ols monomers (Ribéreau-Gayon *et al*., 2012).

2.2.1. Anthocyanins

Anthocyanins are specific to red varieties and localized in berry skins, except in teinturier varieties that have colored flesh (Ribéreau-Gayon *et al*., 2012). Anthocyanins give the red colour to young red wines and are precursors for the new pigments formed during aging.

The anthocyanins identified in grapes skins and wines from *Vitis vinifera* are the 3-*O*- glucosides, the 3-*O*-*(*6-*O*-acetyl)-glucosides, and 3-*O*-*(*6-*O*-*p*-coumaryl)-glucosides of the five main anthocyanidins (Figure 3). The five anthocyanidin differs from each other by the number, and position of the hydroxyl and methoxyl groups located on the B-ring of the molecule (Moreno-Arribas *et al*., 2009). These molecules are much more stable in their glycoside form (anthocyanin) than in their aglycone (anthocyanidin) form.

Depending of the pH of the solution, anthocyanin can be present under 4 different forms for which three of them are colored. In very acidic condition, anthocyanins are under their flavylium cations form, which is their red form. Because of the positive charge on flavylium cations, this form is not very stable when the pH of the solution increases, it leads to the formation of two neutral species. On the one hand, by acid-base equilibrium flavylium cation can lead to a violet neutral quinone base form and, on the other hand can generate by hydration a colourless carbinol form. Then this carbinol could yield by a tautormeric reaction the yellow chalcones (Brouillard *et al*., 1977; Fulcrand *et al*., 2006).

2.2.2. Condensed Tannins

Tannins are, by definition, substances capable of producing stable combinations with proteins and other plant polymers such as polysaccharides (Riou *et al*., 2002; Ribéreau-Gayon *et al*., 2012) and they are mainly responsible for the astringency, the bitterness and the structure of red wines (Gómez Gallego *et al*., 2011). Tannins present in wine can be divided into hydrolysable tannins and condensed tannins according to their chemical structure (Vivas *et al*., 1996).

Hydrolysable tannins are absent in grapes, and it is generally accepted that they are extractable from oak wood during wine aging in barrels (Clifford *et al*., 2000) or can be added as commercial tannins stocks. Hydrolysable tannins include gallotannins and ellagitannins that release gallic acid and ellagic acid, respectively, after acid hydrolysis (Figure 4).

Condensed tannins are also called proanthocyanidin because they break down to anthocyanidins when they are heated in acid solutions (Keller, 2015). They are polymers of flavan-3-ols units. The basic structural units in grapes and wine tannins are: (+)-catechin, (+)-gallocatechin, (–)-epicatechin, (–)-epigallocatechin and (–)-epicatechin-3-*O*-gallate (Figure 5). These monomeric units differ according to the hydroxyl substitution of the B ring. Moreover, according to this B-ring substitution,

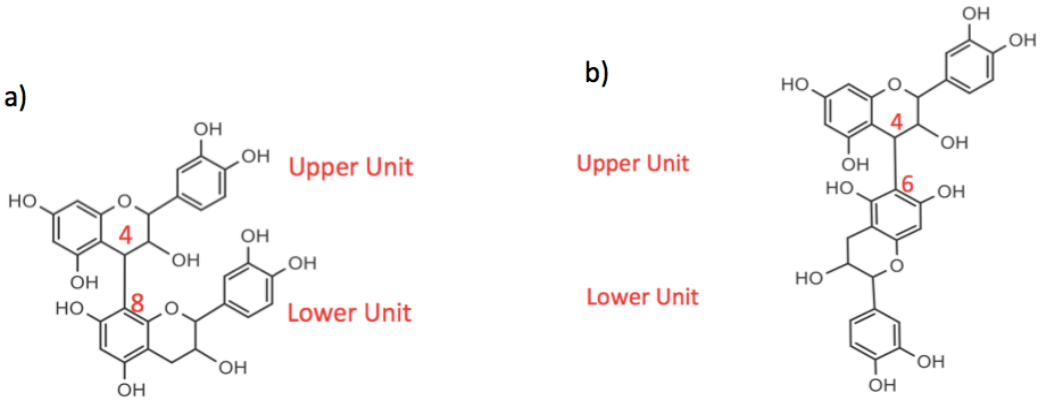


Figure 6: Structure of type-B dimeric procyanidins a) linkage C_4-C_8 b) linkage C_4- C_6 (Ribéreau-Gayon *et al.*, 2012).

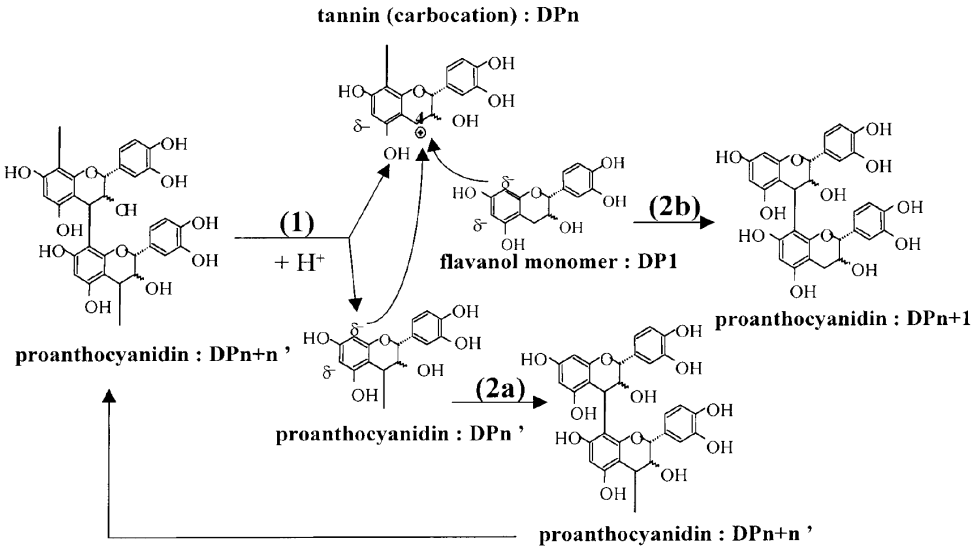


Figure 7: Scheme of the different routes of evolution of the proanthocyanidins (Vidal *et al.*, 2002).

proanthocyanidin can be called procyanidins (H in R1) or prodelphinidines (OH in R1). There are a large diversity of condensed tannins structures in red wine because of different inter-unit (B-type and A-type), linkage, of the different possible flavan-3-ols isomers, and of the fact that there are condensed tannin with various chain length and size (degree of polymerization). Condensed tannins are found in grape skins (procyanidins and prodelphinidins) and seeds (procyanidins only) and are extracted during winemaking. In the skin, tannins are found in vacuole as well as bounded with proteins and polysaccharides of tonoplast and cell walls (Joutei *et al*., 1994). In seeds, they are located it in the outer seed coat (Thorngate *et al*., 1994). The mDP in seed is about 18, whereas it can reach 80 in skins (Prieur *et al*., 1994; Souquet *et al.,* 1996). The proportion of galloyllated units is higher in seeds (13-29%) than in skins (3-6%) (Riou *et al*., 2002).

Condensed tannins are present in all of the solid parts of grapes bunches (skin, seeds, stem), and are dissolved in the wine when it is left on the skins. Concentrations in red wine vary according to grape variety and, to an even greater extent, according to the winemaking methods. After their extraction during the wine making process, condensed tannins are constantly evolved through oxidation, chemical rearrangement and condensation reactions leading to a large diversity of structure and new inter-unit linkage. These structural modifications during wine aging are responsible of the observed organoleptic evolution of the wine.

2.2.2.1. B-type inter-flavonoid linkage

B-type procyanidins are dimers resulting from the condensation of two units of flavan-3-ols. The linkage takes place between the C_4 of the upper unit and the C_6 or C_8 of the lower unit (Figure 6). These kinds of linkages (C_4-C_6 or C_4-C_8) are also called direct linkage and are the main inter-unit linkage in grape procyanidins.

During the aging of the wine and due to the acidity of the wine, procyanidins undergo reactions of polymerization and depolymerisation (Haslam, 1980). Procyanidins polymerization through B-type linkage is due to the electrophilic character of the carbon C_4 and to the nucleophilic character of the carbon C_6 and C_8 (Fulcrand *et al*., 2006). Under mild acidic conditions such as in wine, proanthocyanidin undergo spontaneous cleavage of their interflavanyl bonds (Figure 7; 1), which lead to the formation of a carbocation in the C_4 position of the upper unit. This carbocation can suffer the nucleophilic attack by the nucleophilic carbon C_8 or C_6 from a monomer (Figure 7; 2b) or other polymer (Figure 7; 2a) decreasing or increasing the degree of polymerization, respectively (Vidal *et al.*, 2002). These reactions can change the astringency and bitterness of the wine during aging.

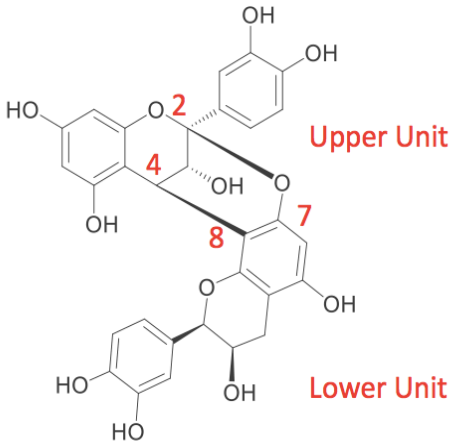


Figure 8: Structure of the A-type linkage (Ribéreau-Gayon et al., 2012)

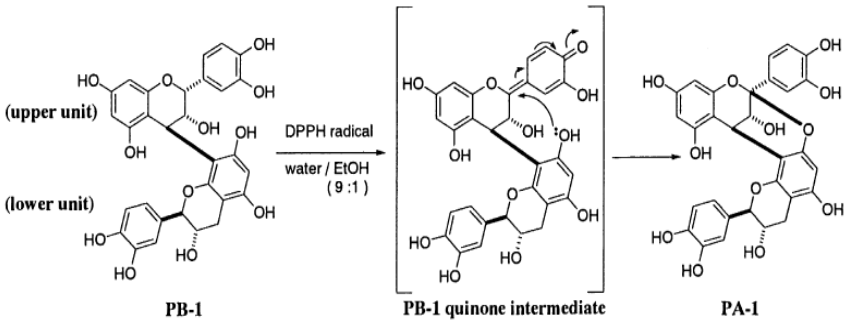
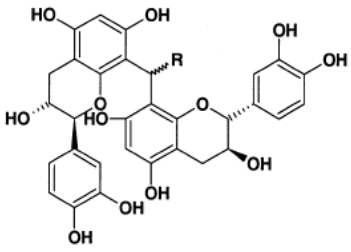


Figure 9 A-type bound formation via methylene quinone intermediate (Kondo *et al*., 2000)



1. Acetaldehyde	R=CH3
2. Glyoxylic acid	R= COOH
3. Furfural	R1=H

Figure 10: Representation of an indirect procyanidins linkage (Es-Safi et al., 2002)

2.2.2.2. A-type inter-flavonoid linkage

A-type linkage has the same kind of linkage as B- type (C_4-C_6 or C_4-C_8) and also has an ether linkage between the oxygen of the carbon C_5 or C_7 of the lower unit and the carbon C_2 of the upper unit (Figure 8). It is known that B-type linkage can be converted into A-type linkage by an oxidation mechanism (Ribéreau-Gayon *et al*., 2012; Kondo *et al*., 2000). This oxidation could be done by a quinone intermediate using DPPH (1,1-diphenyl-2-picrylhydrazyl) or any other radical oxidant in neutral or acidic conditions. This reaction starts with a double oxidation and with two hydrogen deprotonation one on the carbon C_2 of the upper unit and the second deprotonation take place on the hydroxyl of the B-ring of the upper unit. The upper flavanol unit with these two radicals undergo rearrangement in order to create a methylene quinone. Then this quinone intermediary undergo intra molecular nucleophilic attack of the oxygen in C_5 or C_7 position of the lower unit leading to the formation of the ether linkage (Figure 9). This transformation happens in plants by a natural enzymatic oxidant, which can be lacasse produced by *Botrytis cinerea* (Osman *et al.,* 2007). Finally, these reactions could be also performed by oxidases (PPO: Polyphenol Oxidase and XO: xanthine oxidase) as described by Chen *et al.,* (2014). Procyanidins trimers with two interflavanyl bonds, one A-type and one B-type have been noted as D-type procyanidins (Ribéreau-Gayon *et al*., 2012).

2.2.2.3. Indirect Procyanidin linkage

An indirect type of linkage also exists between flavan-3-ols and tannin unit that usually happens during aging. Several studies showed that ethanal, glyoxylic acid, furfural and 5-hydroxymethylfurfural can be intermediates for this linkage (Figure 10) (Es-Safi *et al.,* 2002). However, the most important intermediate for this linkage formation is the acetaldehyde (Drinkine, 2005). In wine ethanal can originate from two different pathways: one by chemical pathway due to the oxidation of ethanol in ethanal and, the other one by biochemistry pathway due to yeast fermentation *Saccharomyces cerevisiae* (Romano *et al.*, 1994; Drinkine *et al.,* 2005). This reaction is done in acidic media due to the presence of ethanal under its enolic form, which receives a nucleophilic attack from the carbon C-6 or C-8 of two flavanols units (Ribéreau-Gayon *et al.,* 2012) in order to form an ethyl bridge linkage between the two units. The presence of tannins link through ethyl bridge linkage in red wine was reported by Saucier *et a*l. (1997).

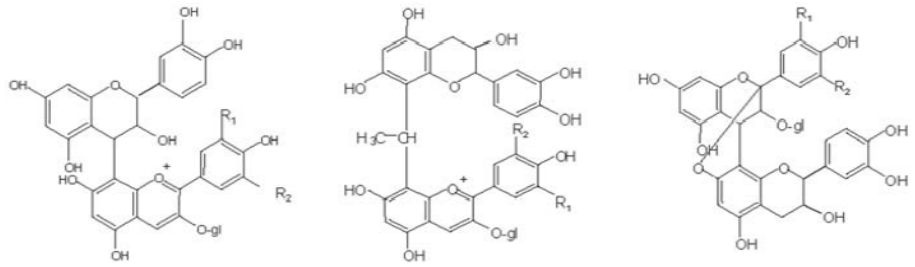


Figure 11: Examples of Anthocyanin - Tannin derivatives detected in wine *(Adapted from Cheynier et al.* 2006)

2.2.2.4. Interaction of tannins with other compounds during wine ageing

In wine, tannins and anthocyanins undergo a lot of structural changes, reactions and interactions between themselves or other molecules presents in the media. Those modifications lead to the colour stabilisation and to the modification of the astringency of red wine (Ma *et al*., 2014). It is known that at red wine pH (3.4 – 4) some anthocyanins are under their flavylium form and flavanols can be under their cationic form due to the process of polymerization and depolymerization. These forms are able to produce the tannin – anthocyanin interactions (Fulcrand *et al*., 2006). These new pigments are more resistant to decolourization by SO_2 , pH changes and presents new colour properties. Two mechanisms have been postulated to the formation of tannin–anthocyanin ($T\text{-}A^+$) or anthocyanin–tannin ($A^+\text{-}T$). In the first mechanism ($A^+\text{-}T$), the C_6 or C_8 of flavanols unit lead a nucleophilic attack on the C_4 of A^+ flavylium form. The second mechanism ($T\text{-}A^+$), under the hydrated hemiketal form, anthocyanins produce a nucleophilic attack to C_4 of carbocation of a flavanols. Direct or indirect (via ethyl-bridge) and A-type linkages exist too in this kind of interactions (Figure 11) (Salas *et al*. 2003; Cheynier *et al*. 2006).

In wine, other molecules as proteins and polysaccharides are also found. Polyphenols like tannins are bulky and are able to form stable combinations with proteins and polysaccharides. Colloidal particles can be formed by tannin aggregations. Those particles are responsible of part of hazes and precipitates in wine. Related with this, polysaccharides are considered as protective molecules to prevent this aggregation and stabilize the wine (Spencer *et al.* 1988). They can interact with tannins and prevent aggregate growth, but this function is still unclear (Riou *et al*. 2002). Tannin/proteins interaction occurs mainly by hydrogen bounds, hydrophobic interactions and ionic bounds. Proline-rich proteins presents in saliva are more reactive against tannins thanks to their high concentration in proline amino acid, this fact is associated with wine astringency (Zanchi *et al*. 2008; Ribéreau-Gayon *et al*. 2012).

2.3.Crown Procyanidins

Recently, for the first time in red wine as well as in the plant kingdom, Zeng (2015) detected, purified and structurally characterised a new macrocyclic procyanidin sub-class that was named “crown procyanidin”. These crown procyanidin exhibited very specific chromatographic characteristics (much higher polarity and partial resistance to phloroglucinolysis depolymerisation conditions) compared to regular B-type procyanidins.

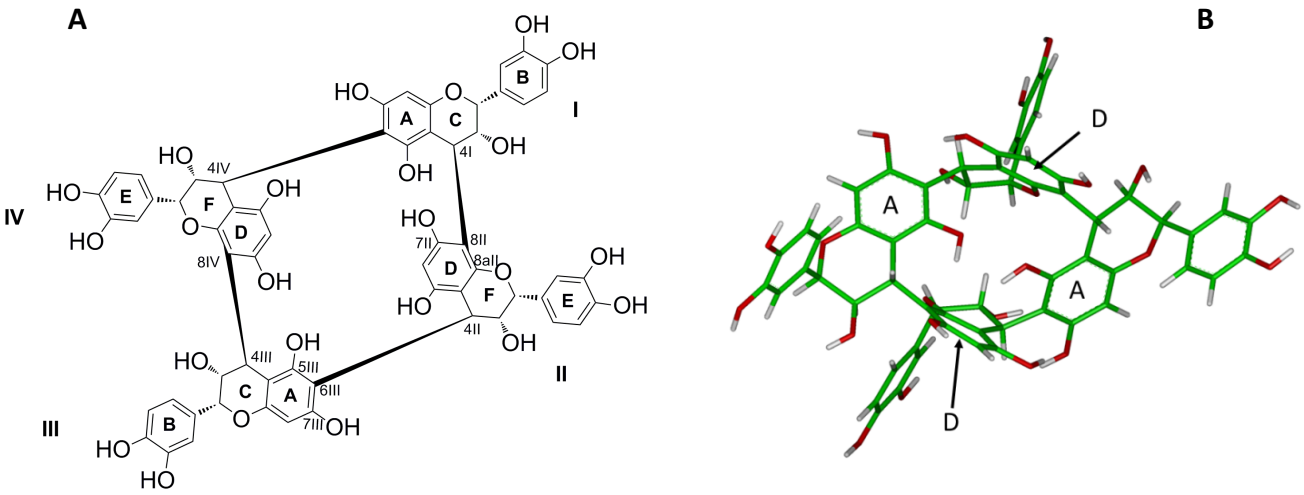


Figure 12. A. Structure of Crown procyanidin tetramer (Pons Mercadé, 2015). B. 3D Structure of Crown procyanidin tetramer (Zeng, 2015).

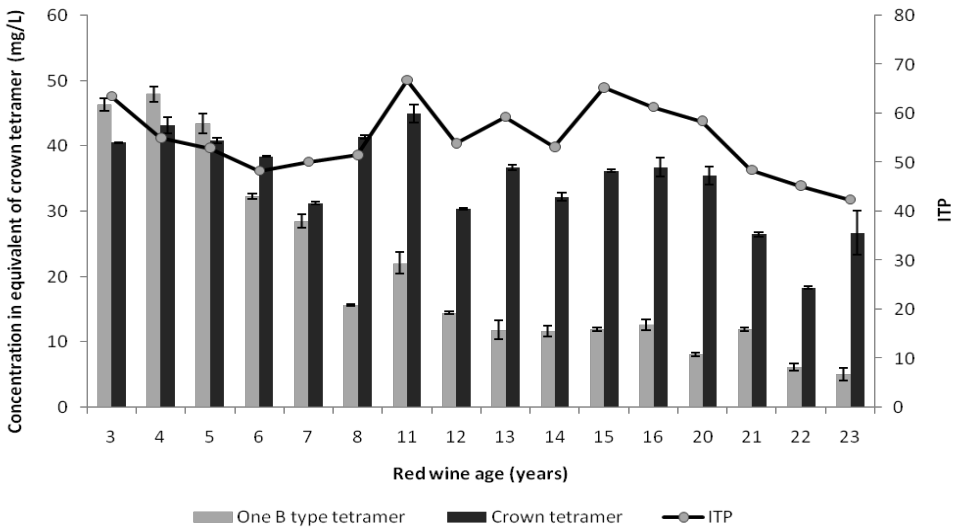


Figure 13: Concentration evolution of crown procyanidin tetramer and B-type tetramer for 16 vintages as well as IPT values (Zeng, 2015).

The high-resolution mass spectrometry Q-TOF analysis showed that these molecules possessed a molecular ion mass of m/z of 1153.2615, which indicates that the molecule has a molecular formula of $C_{60}H_{48}O_{24}$. After a three-step purification procedure (C-18 SPE fractionation, TSK-50F column and HPLC semi-preparative), the crown procyanidin tetramer was obtained a white powder and structurally characterized by NMR analysis. The structure of the crown procyanidin tetramer has been determined to be a symmetric procyanidin tetramer with four (−)-epicatechin linked together by β-orientated B-type interflavanyl linkage in the following sequence of Unit 1-(4β→8)-Unit 2-(4β→6)-Unit 3-(4β→8)-Unit 4-(4β→6)-Unit 1 (Figure 12a). The 3D structure of the crown procyanidin tetramer determined by molecular modelling under NMR constraints, reveals an important cavity in the middle of the structure. Indeed the two aromatic D rings of the units II are parallel and distant of 5.54 Å while on the other side the two aromatic A ring of the unit II are distant of 4.97 Å. Due to the high electronic density with the four aromatic rings, such cavity can be a putative site for chelation (Figure 12b).

The crown procyanidin tetramer evolution was monitor in red wine having several aging time (from vintage 1991 to vintage 2011) and compared to regular B type tetramer. The concentrations of these two compounds showed different evolution profiles during aging. The concentration of the B type tetramer decreases during aging, while the concentration of the crown procyanidin tetramer appeared to remain stable during aging. Moreover, the concentration evolution of the crown procyanidin tetramer was found to be correlated with the total polyphenolic content of the wine (ITP), which means that the concentration variation of the crown procyanidin tetramer was rather due to the vintage effect rather than aging effect (Figure 13). Moreover, a strong correlation between the astringency intensity and the crown procyanidin tetramer concentration has been observed during red wine aging.

Together with the crown procyanidin tetramer, two compounds with an m/z of 1441.3200 which indicate that the molecule has a molecular formula of $C_{75}H_{60}O_{30}$ (-2.91 ppm difference with calculate mass 1441.3242) were detected. These two compounds, which exhibited similar chromatographic characteristic as the crown procyanidin tetramer, correspond to two crown procyanidin pentamer, however their structure has not been characterized yet.

3. MATERIALS AND METHODS

3.1. Reagents

Deionized water was purified with a Milli-Q water system (Millipore, Bedford,MA). Methanol (MeOH, analytical grade), ethanol (HPLC grade), methanol (HPLC grade), acetic acid, L-ascorbic acid and hydrochloric acid were purchased from Prolabo-VWR (Fontenays/Bois, France). Water (Optimal® LC/MS), methanol (Optimal® LC/MS) and formic acid (Optimal® LC/MS) used for high resolution mass spectrometry analysis from Fisher Scientific (Geel, Belgium). Pholoroglucinol were obtained from Extrasynthese (Z.I. Lyon, Nord, France). Red grape (Cabernet Suavignon, Merlot) were obtained from seven vineyards located in the Bordeaux vine-growing region in southwestern France. They are located in Medoc, Graves, Entre deux Mers, Libournais, Blaye Bourg. The vineyards are all planted with *Vitis vinefera* L. cv. Cabernet Sauvignon (CS) and Merlot (M) and the grape were harvested at maturity in September 2015.

3.2. Analysis on UPLC-Q-TOF

The UPLC-MS system used was an Agilent 1290 Infinity equipped with an ESI-Q-TOF-MS (Agilent 6530 Accurate Mass). Chromatographic separation was carried out on an Eclipse Plus C18 column (2.1 x 100 mm, 1.8 μm). The solvents used were: water with 0.1% formic acid for solvent A and methanol with 0.1% formic acid for solvent B at a flow rate of 0.3 mL/min. The gradient of solvent B for red wine analysis and phloroglucinolysis was as follows: 6% of B during 0.5 min; from 6 to 95% of B in 13.5 min; 95%of B during 4 min and UPLC column was equilibrated for 3 min using the starting condition. The gradient of solvent B for oligomers analysis was as follows: 4% of B during 10 min; from 4 to 95% of B in 4 min; 95% during 4 min and UPLC column was equilibrated for 3 min using the starting condition. ESI conditions were as follows: gas temperature and flow were set at 300ºC and 9 L/min respectively; sheath gas temperature and flow were set at 350ºC and 11L/min respectively; capillary voltage was set at 4000 V. The fragmentor was always set at 200 V. The data's obtained were treated using Mass Hunter Qualitative Analysis software.

The quantifications of the crown procyanidin tetramer and pentamer were realized with UPLC-Q-TOF using the red wine analysis gradient. A calibration curve using previously purified crown procyanidin tetramer was established for the quantification of crown procyanidin tetramer, and the crown procyanidin pentamer was expressed as mg/L eq. crown procyanidin tetramer.

3.3. Pre-purification of one tetramer and two pentamers

15 mL of red wine was evaporated to dryness and dissolved in 15 mL of water acidified with 0.1% formic acid. A C-18 column (d: 3 cm, h: 4.5 cm) was activated with 50 mL of methanol and washed with 100 mL of water to remove methanol. 15 mL of sample dissolved in water was applied to the column prior to successive elutions by 1) 150 mL of methanol / water (5/95, v/v) to retrieve the tetramer and the pentamers, 2) 100 mL of methanol to wash the column and 3) 100 mL of water to re-equilibrate the column. All the solvents used were acidified with 0.1% formic acid. The fraction eluted by methanol / water (5/95, v/v) containing the tetramer and the two pentamers was evaporated to dryness, re-dissolved in methanol (4 mL) for the next fractionation step.

This methanolic solution was centrifuged for 3 min at 4500 R/min the supernatant was saved and the precipitate (insoluble compounds) was re-dissolved with 1 mL of methanol and centrifuged again. Then the supernatant containing the tetramer and two pentamer was again evaporated and re-dissolved in 1 mL of water acidified with 0.1% formic acid. Finally, this solution was subsequently loaded on a column packed with Toyopearl HW-40S gel (d: 1 cm, h: 37.5 cm) and eluted with methanol with a flow rate of 0.8 mL/min. Fraction eluted between 15 to 16.5 h mainly contains the two pentamers while the fractions eluted between 16.5 to 22 h contains mainly tetramer. Both fractions were evaporated separately and re-dissolved in 300 μl of water acidified with 0.1% formic acid to be purified by semi-preparative HPLC-UV.

3.4. Semi–preparative HPLC for final purification of the two pentamers

Semi–preparative HPLC-UV was performed on an Agilent system containing a quaternary pump (1260 Infinity), a compartment thermostat column (1290 Infinity), a sample injector (1260 Standard Autosampler) and a diode array detector (1260 DAD VL+). A 500 μL external injector was added to the system. The semi–preparative column is a Prontosil column (250 x 8 mm, 5 μm). The solvents used were: water/TFA (99.975: 0.025, v/v) for solvent A and methanol/TFA (99.975: 0,025, v/v) for solvent B. The flow rate was 2.5 ml/min. The gradient was as follows: 0% of B for 5 min, 0 to 4% of B in 30 min, 4 to 96% of B in 2 min, then 96% of B for 5 in. The column was re-equilibrated to initial conditions for 5 min after each injection. UV absorption spectra were recorded at 280 nm. The system was controlled by the LC Open Lab software. Collected semi-preparative fractions were evaporated, dissolved in water, frozen and lyophilized to yield pure compounds a light brown powder.

3.5. Crown procyanidins extractions from tannins seed, skin and bunch stem from grapes

The study was carried out with samples from seven vineyards located in the Bordeaux vine-growing region in southwestern France. They are located in Medoc, Graves, Entre deux Mers, Libournais, Blaye Bourg. The vineyards are all planted with *Vitis vinefera* L. cv. Cabernet Sauvignon (CS) and Merlot (M). One grape sample (2 kg) from each vineyard of each grape variety was collected at maturity in September 2015. In addiction it was collected at the same time two more samples: one from conventional cropping and one from ecological cropping.

Seeds, skins, and bunch stem were removed by hand from grapes, washed with distilled water, frozen and lyophilized for 2 days. Seeds, skins, and bunch stem were finally the grounded to powder with a mortar.

0.5 g of each obtained powder was extracted using 5 mL of acetone/water (70:30, v/v) acidified with 5 g/L of tartaric acid during 15 h under mechanical agitation. Then, the solid part was separated from the solvent by centrifugation (15 min, 1500 rpm). Solid part was re-extracted with methanol/water (40:60, v/v) acidified with 5 g/L of tartaric acid during 15 h under mechanical agitation. The liquid supernatant was collected again by centrifugation (15 min, 1500 rpm) and mixed with the previous one. Both supernatant were evaporated to remove organic solvents, and then the residue was dissolved in 2 mL of water, freeze, and freeze-dried to obtain a crude tannin extract. Finally, the crude tannin extract was re-dissolved in 1 mL of water, filter (0.20 µm) and analysed by HPLC-UV-QTOF.

3.6. Index of total polyphenols (IPT)

A water dilution of 1/100 was applied to wine. The optic density (OD) was measured at a wavelength of 280 nm. A quartz cuvette was used with 1 cm of diameter. The result was treated with an index (I): I = OD x dilution.

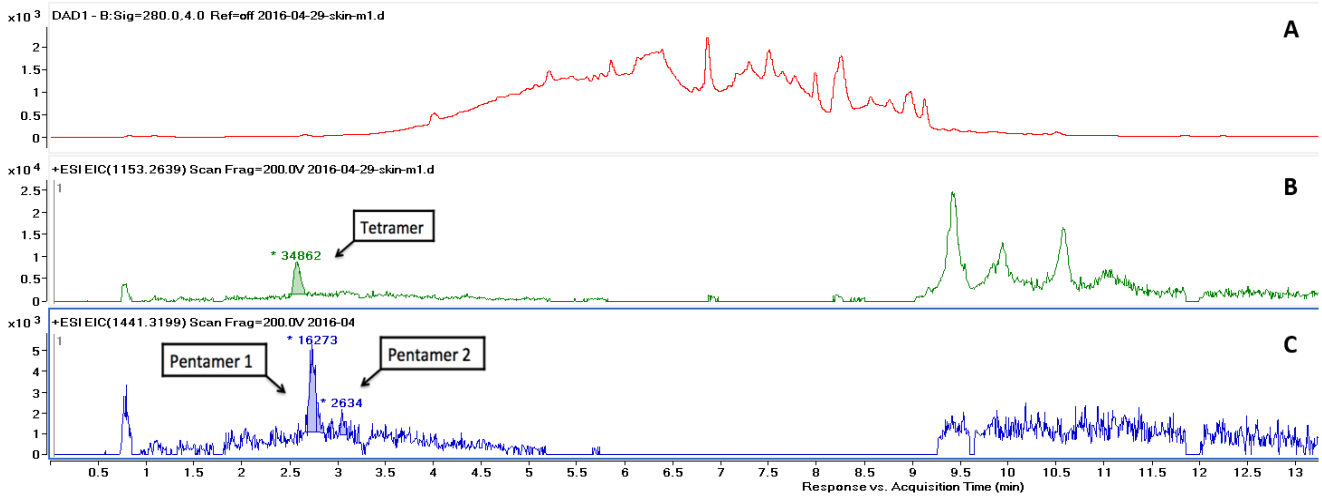


Figure 14: UPLC-Q-TOF chromatograms of skin extract obtain by Acetone/water; 70/30 – Methanol/water; 40/60). A: λ: 280 nm; B: extracted mass chromatogram m/z : 1153.2608 (tetramer); C: extracted mass chromatogram m/z : 1441.3242 (pentamer).

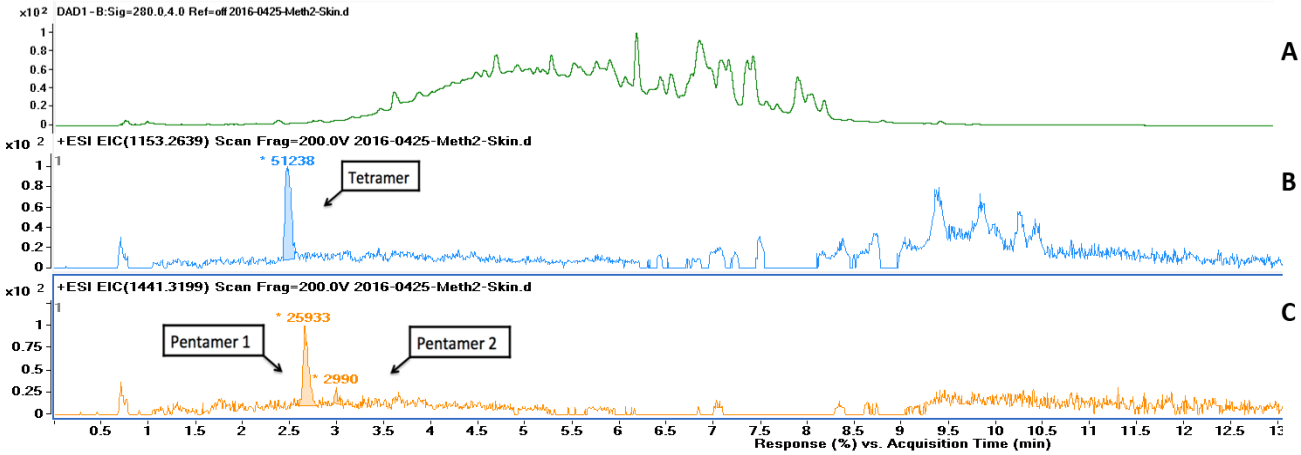


Figure 15: UPLC-Q-TOF chromatograms of skin extract obtain by method 2 (Acetone/water; 70/30 acidificated – Methanol/water; 40/60 acidificated). A: λ: 280 nm; B: extracted mass chromatogram m/z: 1153.2608 (tetramer); C: extracted mass chromatogram m/z : 1441.3242 (pentamer)

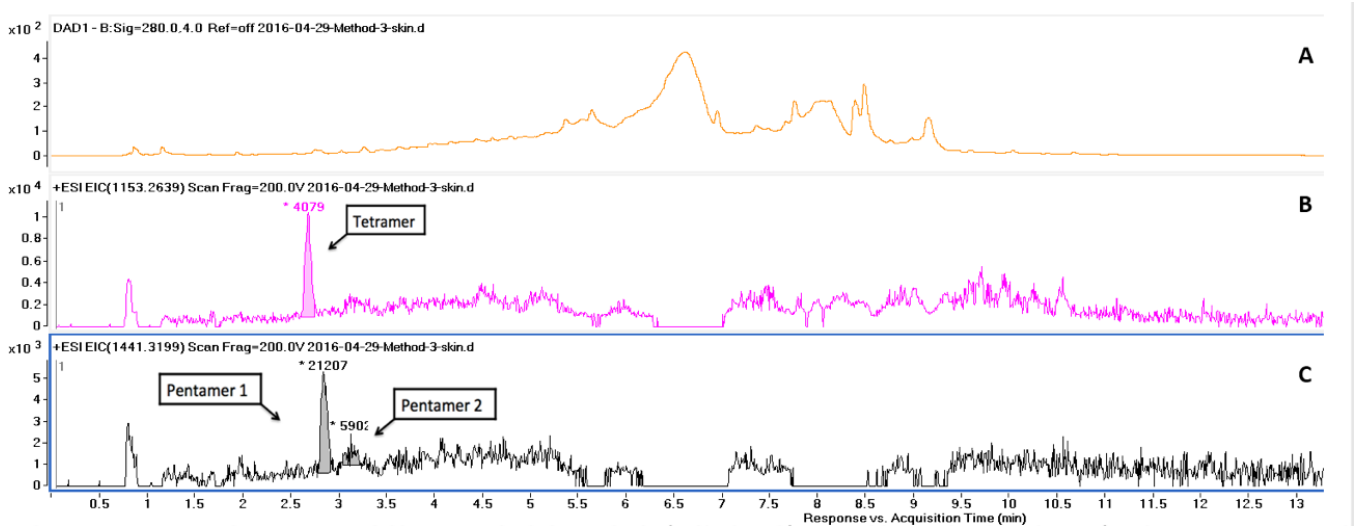


Figure 16 UPLC-Q-TOF chromatogram of skin extract obtain by method 3 (12% Ethanol/ water with tartaric acid and pH 3.5). A: λ: 280 nm; B: extracted mass chromatogram m/z : 1153.2608 (tetramer); C: extracted mass chromatogram m/z : 1441.3242 (pentamer).

4. RESULTS AND DISCUSSION

4.1. CROWN PROCYANIDIN EXTRACTION AND CONTENT IN GRAPES

4.1.1. Adaptation of an extraction method of the crown procyanidin from grapes

Prior to the extraction, skin, seed and bunch stem from grape berries were delicately separated and peeled by hand from the pulp. Then they were washed with distilled water to remove all the sugar form the rest of the pulp, frozen, and freeze-dried for 24 h. Once, seed and skins were dehydrated, they were finally grounded to powder with a mortar in order to be submitted to extraction solvent.

Various polyphenols extractions methods from skin and seeds were described previously in literature. Most of them use organic solvents such as ethanol, methanol or acetone with water. As crown procyanidins has never been identified and detected in grape berries (seed or skin) there are no extraction method described or validated. Thus together with the regular extraction procedure (Acetone/water, 70/30 v/v) it has been decided to use two other extractions solvent. The used extraction condition were as follow:

1. Acetone/water (70/30, v/v) follow by methanol/water (40/60, v/v).
2. Acetone/water (70/30, v/v) acidified with 5 g/L of tartaric acid, follow by methanol/ water (40/60, v/v) acidified with 5 g/L of tartaric acid.
3. 12% Ethanol/ water with 5 g/L of tartaric acid and the pH was adjusted to 3.5.

In order to select the most efficient extraction solvent, the three conditions above were tested on a skin materiel from cabernet sauvignon. 0.5 g of the grounded skin were submitted to the solvent extraction during 15 h. Then, the supernatant was removed from solid part by centrifugation (15 min, 4500 rpm). The liquid part was keep and the solid part was extracted again for 15 h with the second solvent or with the same solvent depending of the extraction procedure

Both liquids parts were mixed and evaporated to remove organic solvents, then they were re-dissolved in 2 mL of water, frozen (24 h) and lyophilized (2 days) to obtain a crude tannin extract. Finally, the crude tannin extract was re-dissolved in 1 mL of water, filter and analysed by UPLC-UV-QTOF.

The UPLC-UV-QTOF analysis of the crude extract from the extraction method 1, shows a very small peak of crown procyanidin tetramer (m/z of 1153.2615) as well as a very small peak of crown procyanidin pentamer (m/z of 1141.3242). Both peaks are very close to noise level of the extracted chromatogram and one crown procyanidin pentamer peak was even missing since in red wine two crown procyanidin pentamer has been previously detected (Figure 14).

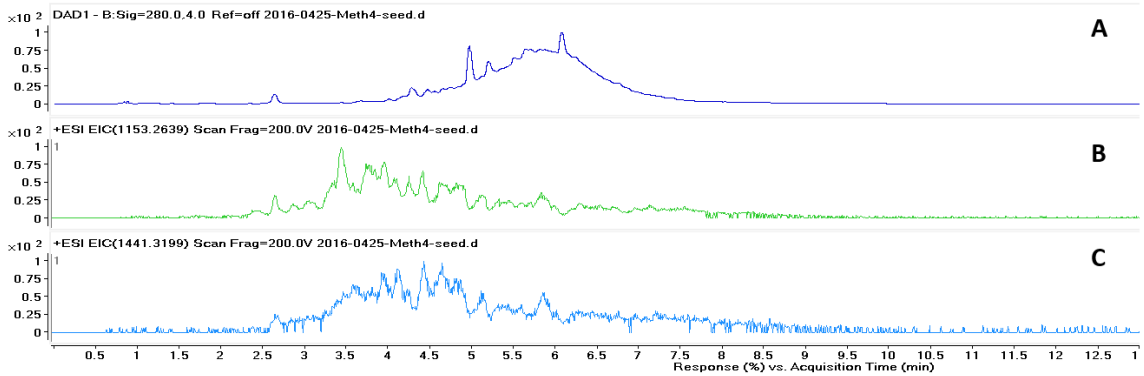


Figure 17: UPLC-UV-QTOF chromatogram of seeds extract. A: λ: 280 nm; B: extracted mass chromatogram m/z : 1153.2608 (tetramer); C: extracted mass chromatogram m/z : 1441.3242.

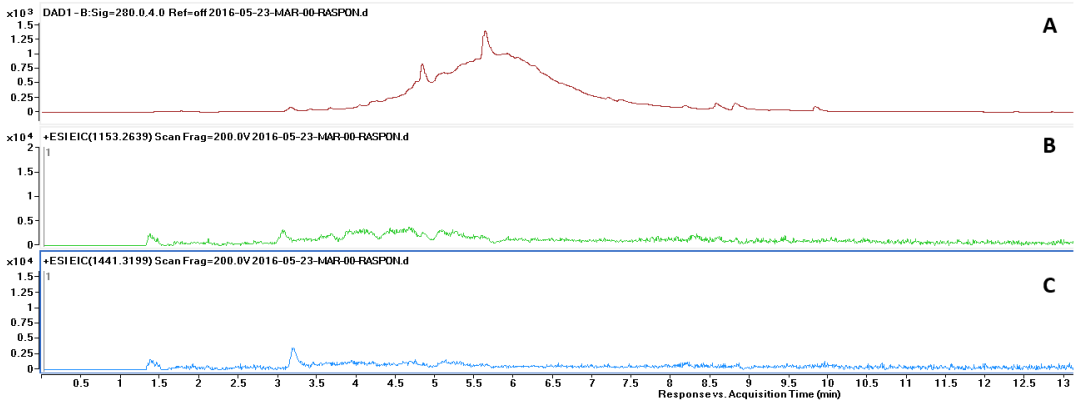


Figure 18: UPLC-UV-QTOF chromatogram of bunch stem extract. A: λ: 280 nm; B: extracted mass chromatogram m/z : 1153.2608 (tetramer); C: extracted mass chromatogram m/z : 1441.3242.

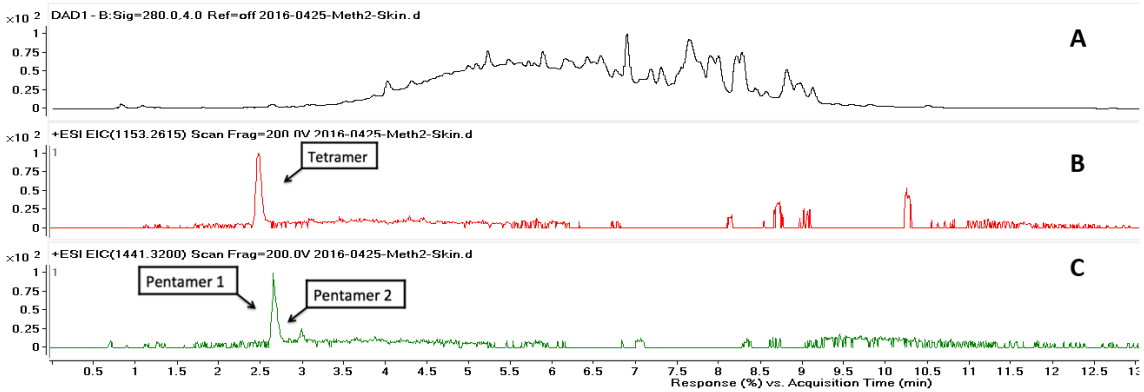


Figure 19: UPLC-UV-QTOF chromatogram of skin extract. A: λ: 280 nm; B: extracted mass chromatogram m/z : 1153.2608 (tetramer); C: extracted mass chromatogram m/z : 1441.3242.

Since the extraction solvent used in the extraction method 1, which is the commonly used extraction solvent for condensed tannin didn't give good extraction yield, it has been decided to acidified the extraction solvent to mimic the acidity of the must. The acidification was done with 5 g/L of tartaric acid, which is the main organic acid in grapes and must.

The UPLC-UV-QTOF analysis of the crude extract from the extraction performed with acidified (5 g/L tartaric acid) acetone/water (70/30, v/v) follow by an extraction with acidified (5 g/L tartaric acid) methanol/water (40/60, v/v) exhibits very strong and intense peaks for the crown procyanidin tetramer as well as both crown procyanidin pentamer (Figure 15). It clearly appeared that using acidified solvent improved the yield of extraction by 20 order of magnitude. Moreover, in this case it was possible to detect the two crown procyanidin pentamer, which was not possible with unacidified solvent.

Finally, it has been decided to also try a "wine model solution" for the extraction composed of 12% ethanol in water, 5 g/L of tartaric acid and the pH was adjusted to 3.5 as in wine medium conditions. The UPLC-UV-QTOF analysis (Figure 16) of the obtain extract using this solvent exhibit a higher extraction yield than the extraction method 1 but a lower yield than the extraction method 2. Overall, it has been noticed that the use of acidified solvent increase the extraction yield of the crown procyanidin tetramer and pentamer. This difference may be due to the specific localization in the skin tissue of the crown procyanidin. Moreover, since the extraction procedure 2 was the best extraction method, it has been used to all the further extraction reported below.

4.1.2. Study of the localization of crown procyanidins in the grape

As describe in the bibliography section, the crown procyanidin has been reported in red wine made from various grape verities (Merlot, Cabernet Sauvignon, Syrah) (Pons Mercadé, 2015). However it has never been detected in grape before and the tissue localization in grape (seed, skin, bunch stem or in all) is also unknown. Thus in order to understand from which grape berries part the crown procyanidin detected in red wine came from, 0.5 g of dry seed, skin and bunch stem from both Merlot and Cabernet Sauvignon collected from the 7 different studied vineyard has been submitted to the extraction procedure 2 as describe above.

The UPLC-UV-QTOF analysis of the crude seeds extract (Figure 17) did not show any defined peaks for crown procyanidin tetramer (m/z of 1153.2615) and pentamer (m/z of 1141.3242). This extraction on seed has been repeated three time for both varieties (Cabernet sauvignon and Merlot) from the 7 studied vineyards from Bordeaux, and the crown procyanidin couldn't be detect in any of

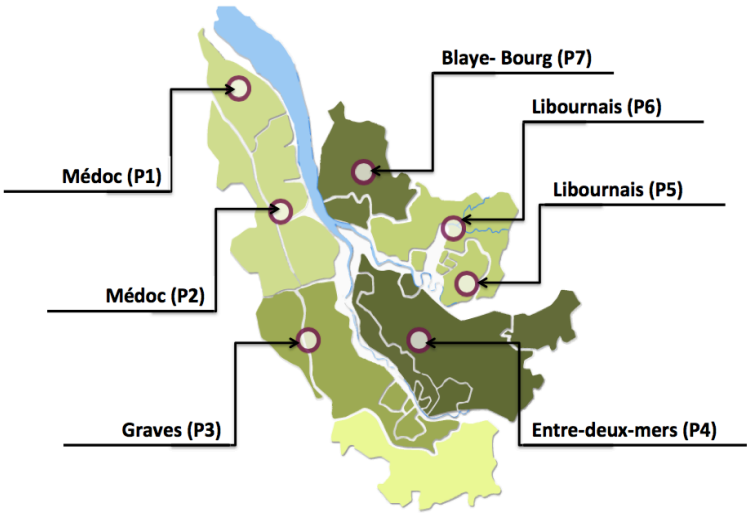
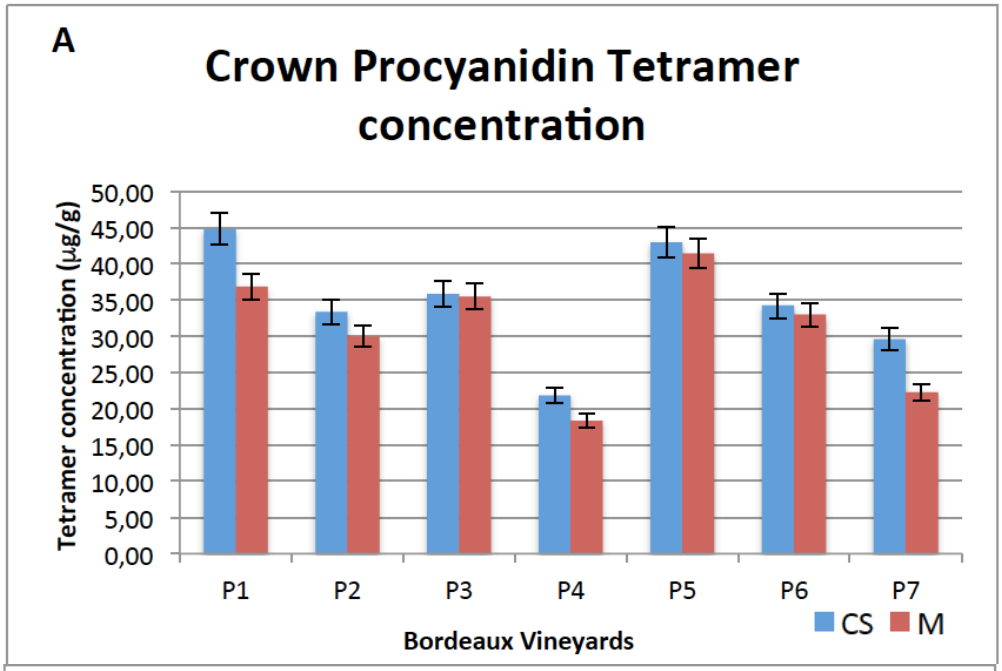


Figure 20: Geographical location of the plots studied



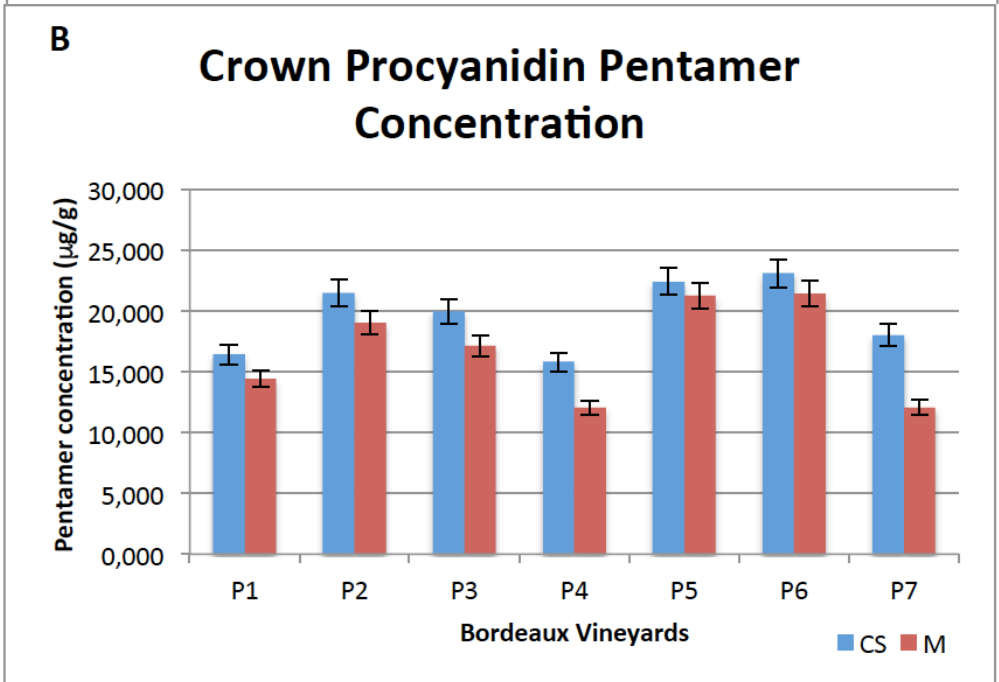


Figure 21: A) A) Tetramer crown procyanidin concentration (µg/g) in skin in the studied parcel. B) Pentamer crown procyanidin concentration (µg/g) in skin in the studied parcel.

the analysed seed extract. Thus it is possible to conclude that there are no crown procyanidins in the seed of these two grape varieties. Similar extractions and analysis were then performed on the bunch stem from both grape varieties from the 7 different vineyards and like for the seed, the crown procyanidin couldn't be detected in any of the analysed bunch stem extract (Figure 18).

Finally, the UPLC-UV-QTOF analysis of the skin extract (Figure 19) shows totally different chromatogram compared to seed and bunch stem. On the selected ion chromatogram of m/z 1153.2615 (crown procyanidin tetramer) and m/z 1141.3242 (crown procyanidin pentamer) three very well define peaks with strong intensities were observed. Moreover, the comparison of the retention time and MS/MS analysis with the previously report data on crown procyanidin tetramer and pentamer (Zeng, 2015) confirmed the assignation of this peaks. These three crown procyanidin has been detected in the 14 skin extracts from both grape varieties (Merlot and Cabernet Sauvignon) collected from the 7 different studied vineyards.

Surprisingly, the crown procyanidin appeared to be tissue specific since they were detected only in the grape skin of Merlot and Cabernet Sauvignon and not in seed an bunch stem. Such specificity is very surprising and raises a lot of questions regarding the biosynthesis of these molecules as well as their localisation in the skin tissue.

4.1.3 Crown procyanidin content in grape skin

Following the first identification of the crown procyanidin tetramer and pentamer in grape skin as describe above, their content in the two studied grape varieties was estimated and compared. The study was carried out with the samples collected from seven vineyards parcel located in the Bordeaux vine-growing region in southwestern France (Figure 20). They are situated in Medoc (parcel 1 and parcel 2), Graves (parcel 3), Entre deux Mers (parcel 4), Libournais (parcel 5 and parcel 6), Blaye Bourg (parcel 7). The vineyards are all planted with *Vitis vinifera* L. cv. Cabernet Sauvignon and Merlot. One grape sample of each vineyard for each variety was collected at maturity in September 2015 (Annex 2). As previously describe, the skin was manually separated from the rest of the berries, freeze-dried and then, 0.5 g was extracted using the extraction method 2 (as observed in section 5.1.1.). Each skin samples were extracted in duplicate and analysed by UPLC-UV-QTOF for the quantification of the crown procyanidin tetramer and pentamer.

First, an important concentration variability for the same grape varieties ware observed for both crown procyanidins. Indeed, for example the concentration of the crown procyanidin tetramer in parcel P4 appeared to be twice lower than in parcel P1 or P5. Similar patterns were observed for both grape varieties on the same vineyard parcels. Moreover, the comparison between the two

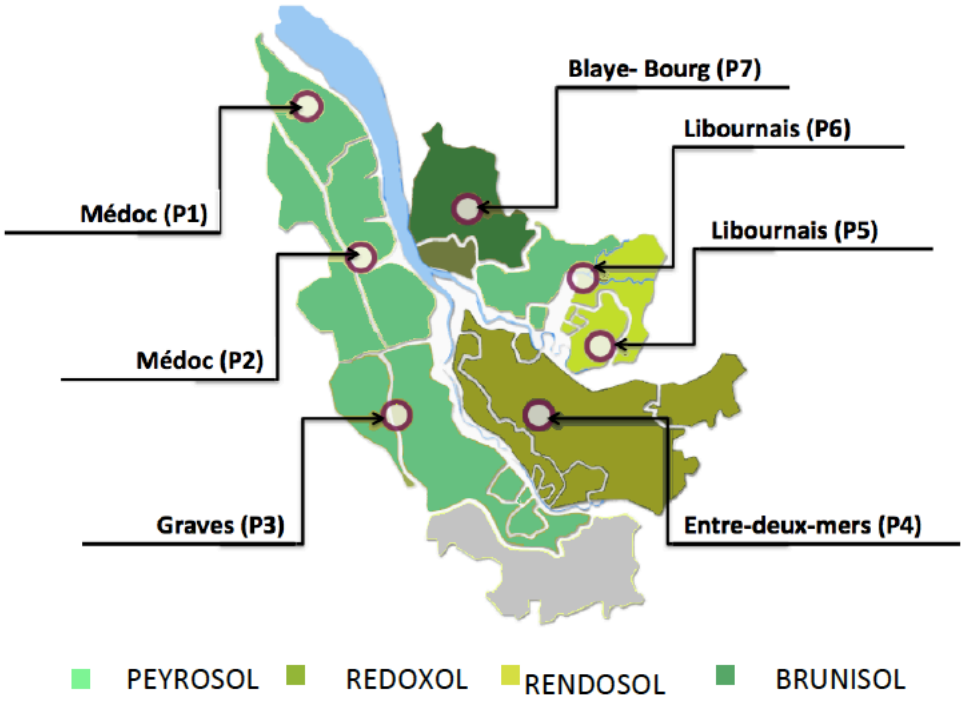


Figure 22: Geographical localization of the plots studied and type of soil.

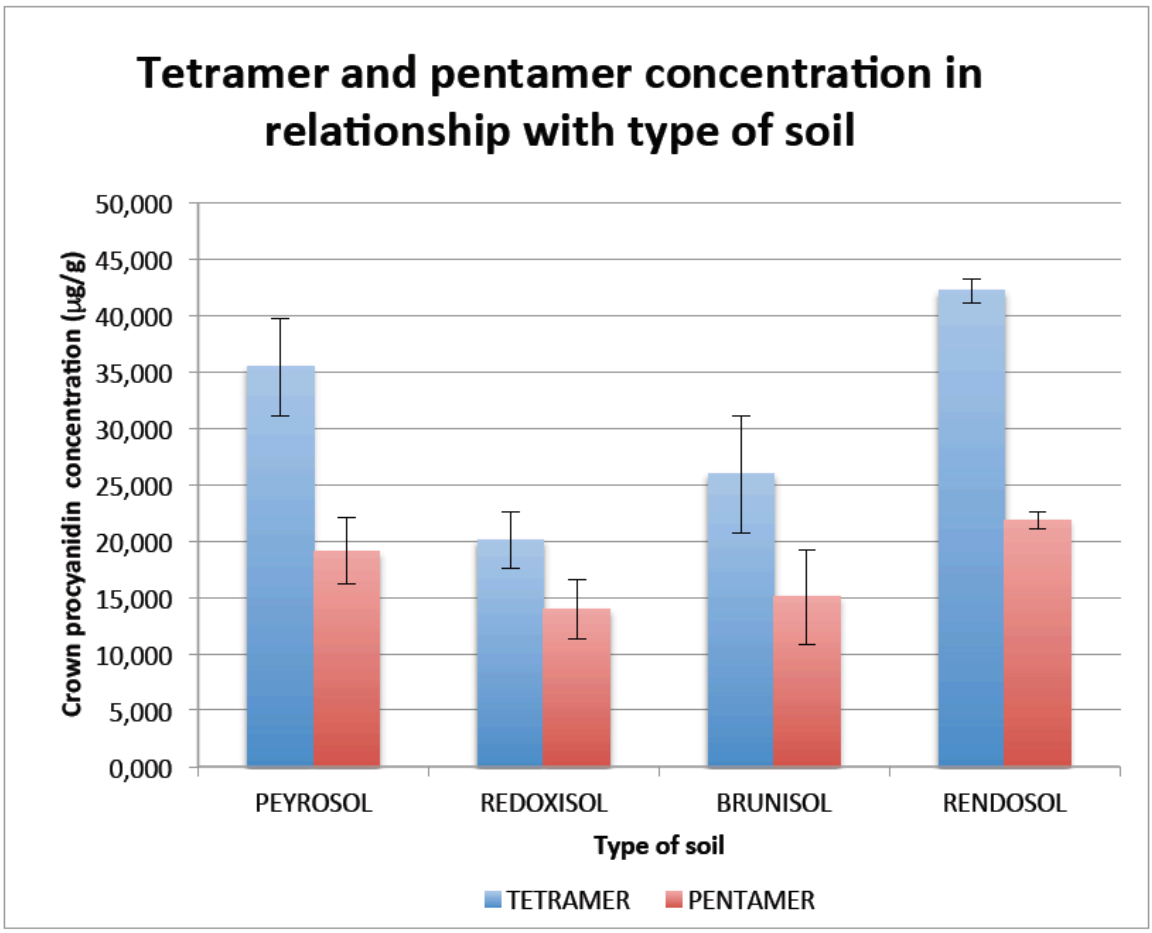


Figure 23: Crown procyanidin tetramer and pentamer concentration (µg/g) in skin according to the type of soil.

grape varieties revealed that crown procyanidin tetramer concentration (Figure 21.A.) is all the time higher in Cabernet Sauvignon than in Merlot for all the analysed vineyard parcel. Similar pattern was also observed for the crown procyanidin pentamer concentration (Figure 21.B), which appeared to have a higher concentration in Cabernet Sauvignon than in Merlot in all the analysed vineyard parcel. Moreover, the concentration of the crown procyanidin pentamer was all the time lower than the crown procyanidin tetramer. Such difference was expected since in all the red wine analysed to date, the concentration of crown procyanidin tetramer was higher than the pentamer. However the concentration difference between the crown procyanidin tetramer and pentamer depends more of the vineyard parcel than of the grape verities.

Similar investigation on other vintages should be performed in order to confirm the observed differences between Merlot and Cabernet Sauvignon and especially the specificity that Cabernet Sauvignon has higher crown procyanidin concentration than Merlot. Similarly it would be interesting to compare crown procyanidin concentration found in Merlot and Cabernet Sauvignon with other grape varieties.

4.1.4 Relationship between the type of soil and crown procyanidin concentration

There are four types of soil in the Bordeaux vine-growing region: PEYROSOL (gravelly soil) is the most frequent soil type in these estates (45% of the total mapped area; 4 of the studied parcels), soils with temporary waterlogging (REDOXISOL), heavy clay soils (RENDOSOL) and sandygravelly soils (BRUNISOL) covered around 10% of the mapped area each (Renouf et al., 2010).

The quantifications of crown procyanidins were realised by mass spectrometry (UPLC-Q- TOF) on the skin extract from the collected grapes. The study was carried out with the same quantification samples that were used in section 4.1.3. and reorganized by the type of soil. Parcel 1, 2, 3 and 6 were Peyrosol, parcel 4 was Redoxisol, parcel 5 was Rendosol and parcel 7 was Brunisol (Figure 22).

The UPLC-UV-QTOF analysis revealed that the highest concentration (Figure 23) was observed on Rendosol and the smaller concentration was obtained with Redoxisol. These data's revealed different trends compare to the regular B-type condensed tannins concentration determined for the vintage 2006 and 2007. These difference needs to be confirmed with both quantification on the same vintage. However difference between rendosol and redoxisol has already been observed in the study realized by Renoulf *et al.*, 2010 that determinate better wine quality was obtained with Rendosol soil, and lower with Redoxisol. The hypothesis could be that the formation of this new kind of procyanidins could be favoured when the pH is lower due to Rendosol has pH more acidity than Redoxisol, but other conditions could affect to the concentration of crown procyanidins as plot

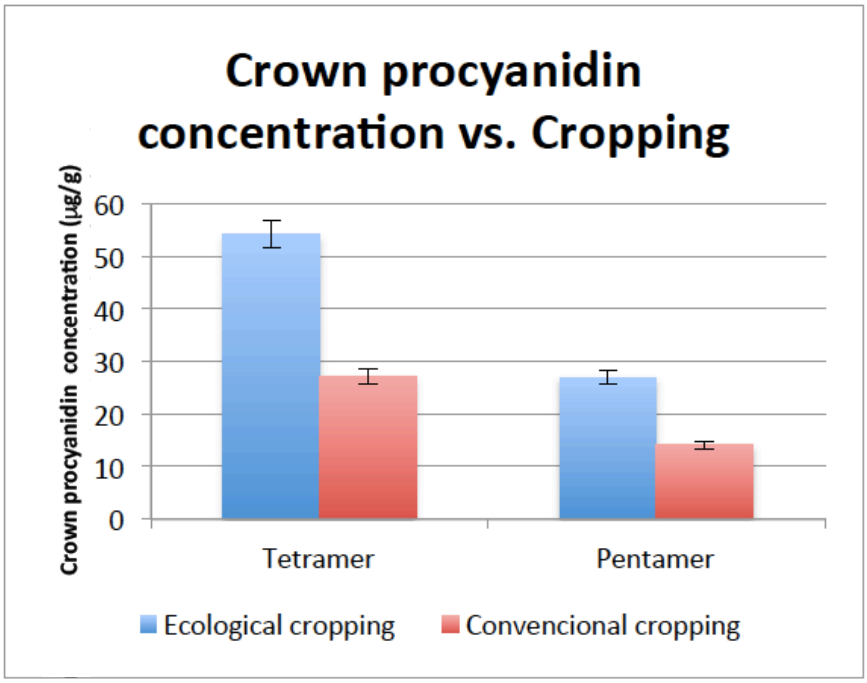


Figure 24: Comparison crown procyanidin tetramer and pentamer concentration (µg/g) according of the cropping.

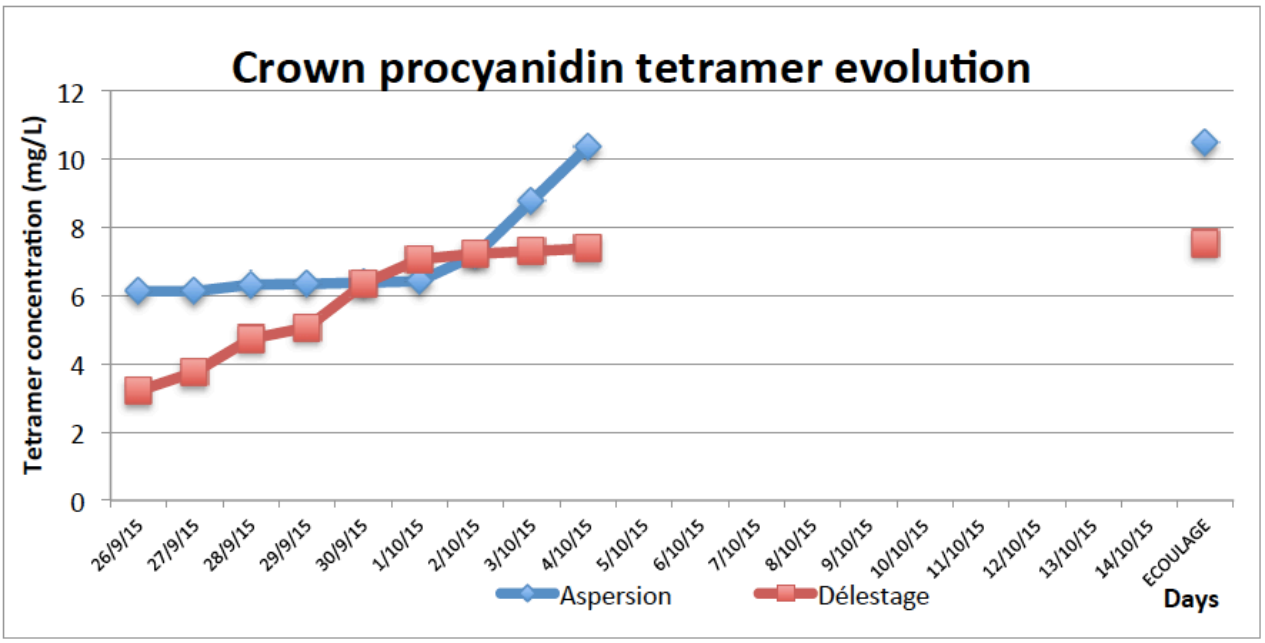


Figure 25: Crown procyanidin tetramer evolution (mg/L) during fermentation process

orientation, microbiology of the soil, UV exposure, pest, phytosanitaries… However, all these affirmations and hypothesis have to be studied on different vintages to prove this difference.

4.1.5. Comparison between crown procyanidin concentration versus type of cropping.

The comparison was carried out with only one Merlot sample from the same vineyard with on parcel conducted under conventional cropping and a second parcel conducted under ecological viticulture practice. Both grapes were collected at maturity in September 2015. Crown procyanidins extraction was done with the same method 2 as described in section 4.1.3. Quantifications of tetramer and pentamer were realised by mass spectrometry (UPLC-Q-TOF). Results from the quantification are represented in figure 24 clearly show that the both crown procyanidin tetramer and pentamer concentration are two time higher in ecological viticulture practice than in convention. . Even if such results are very surprising and very interesting especially for a family of compounds which are specifically located only in grape skin, they raise again a lot of question regarding their biosynthesis and if they could be produce by the plant to protect itself. However all of these hypothesis they must be confirmed on consecutive vintages as well as by studying more samples from ecological cropping and even from biodynamic cropping in different maturation stages. That will help to understand why crown procyanidin are highly formed in such conditions

4.1.6. Evolution kinetics of the crown procyanidins during fermentation process

The objective of this preliminary study was to measure the evolution and extraction rate of the crown procyanidins during the fermentation process. To realize this goal, samples for another laboratory project were analysed by UPLC-UV-QTOF. In this project two extraction methods during the vinification was studied: one was by aspersion of the marcs and the other one by délestage. The fermentation in both cases was conducted at 27^{o}C during 9 days. Monitoring of the fermentation kinetic are described in Annex 3..

The quantification by UPLC-UV-QTOF allows us study the presence and the evolution of crown tetramer and pentamers during fermentation process (Figure 25 and Figure 26). The concentration of the crown procyanidins tetramer (Figure 25) showed an increase tendency during fermentation process. In the aspersion method the tetramer evolution is very stable in the first couple of days and then from day 5 of the fermentation the increase is very fast and reach values greater than 10 mg/L at the end. On the other hand, with délestage method the contrary happened: the increase phase was more at the beginning of the fermentation process and then tetramer concentration remain more or less stable. This difference could be that délestage method allows higher solubilisation of skin tannins in the first couple of days rather than aspersion method.

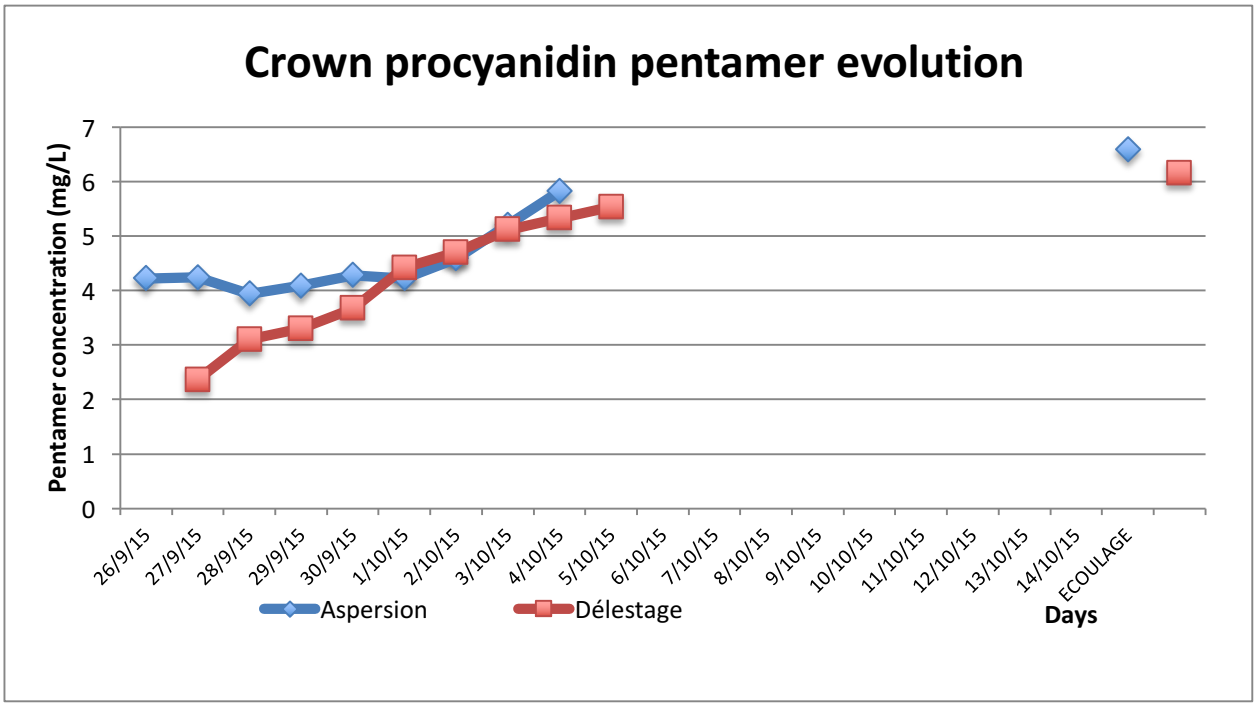


Figure 26: **Crown procyanidin pentamer evolution (mg/L) during fermentation process**

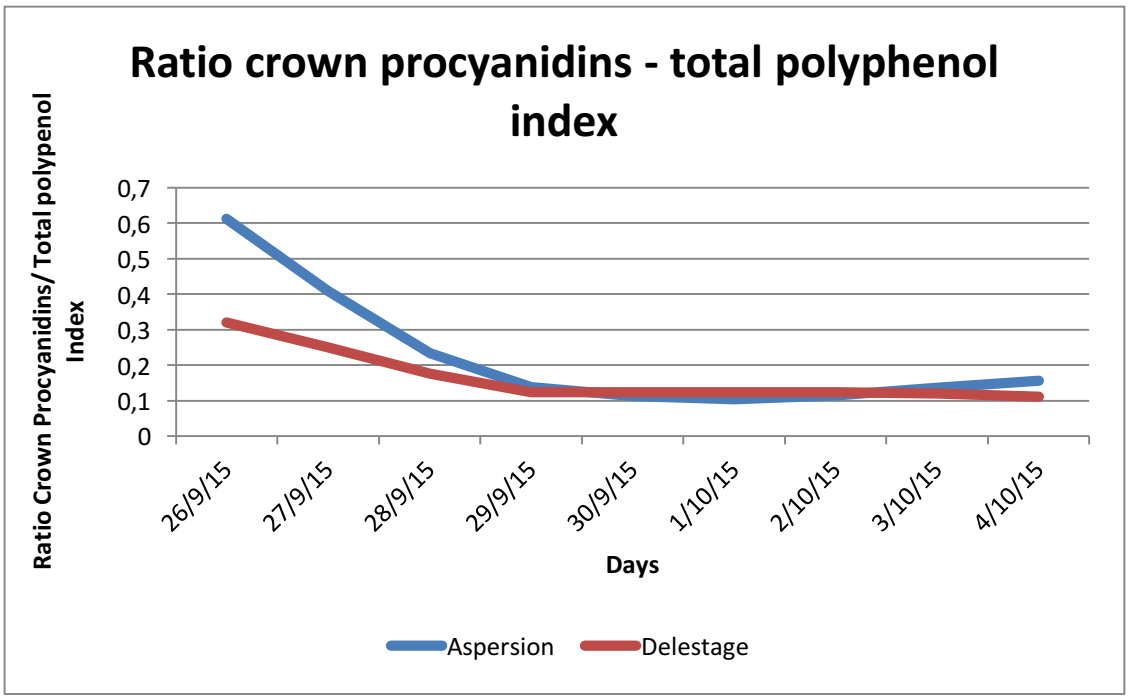


Figure 27: **Ratio between Crown procyanidins concentration and total polyphenol index**

Besides, it was observed that higher concentration of crown procyanidins tetramer were obtained for the aspersion method while similar concentration were obtain for the for the crown procyanidins Besides, it was observed that higher concentration of crown procyanidins tetramer were obtained for the aspersion method while similar concentrations were obtain for the crown procyanidin pentamer. In contrast with crown procyanidins tetramer, crown procyanidins pentamer showed a more linear increasing tendency during the fermentation process (Figure 26). Moreover for both crown procyanidins it was noticed that their concentration at the ends of the alcoholic fermentation was the same than in the free-run wine (after écoulage) after 10 days of post-fermentation maceration.

Looking at the ratio between the crown procyanidins concentration and the total polyphenolic index (Figure 27), it was observed that this ratio decrease very quickly with time. Such trend revealed that the crown procyanidins are extracted very quickly at the beginning of the vinification which is an opposite trend compare to regular B-type condensed tannins which need ethanol to be extracted. The fact that these tannins are very polar, highly soluble in water, and exclusively located in the skin might be the reason of this specific and unexpected behaviour.

However, these results are preliminary, a longer observation time and more conditions with replicates have to be tested to confirm the extraction rate and trend of the crown procyanidins.

4.2. CROWN PROCYANIDIN PURIFICATION

4.2.1. Procyanidin tetramer and pentamers purification

The second main objective of this project was to purify the two crown procyanidins pentamer from red wine in order to characterize their structure by NMR. A three steps purification methodology established by Pons Mercadé (2015) was used for this purification. This purification method is composed of as the first step of a C-18 solid phase extraction, follow by a second step of a TSK-40S gel filtration chromatography and the last step was a C-18 HPLC semi-preparative.

The purification was done with a Bordeaux Cabernet Sauvignon red wine that was previously checked for crown procyanidins contents by the HPLC-UV-QTOF. UPLC-UV-QTOF chromatogram of this red wine (Figure 28) shows well-defined picks, for both tetramer and pentamer (Figure 28 B, C) which are present in high concentration in this wine.

Since the crown procyanidins (tetramer or pentamer) exists higher polarity than regular B-type procyanidins and, the first fractionation step on C-18 reversed-phase cartridge was used to remove most of the lower polarity compounds. The red wine was first evaporated to dryness and re-dissolved in acidified water (i.e., 0.1% formic acid), the sample was loaded on the activated C-18

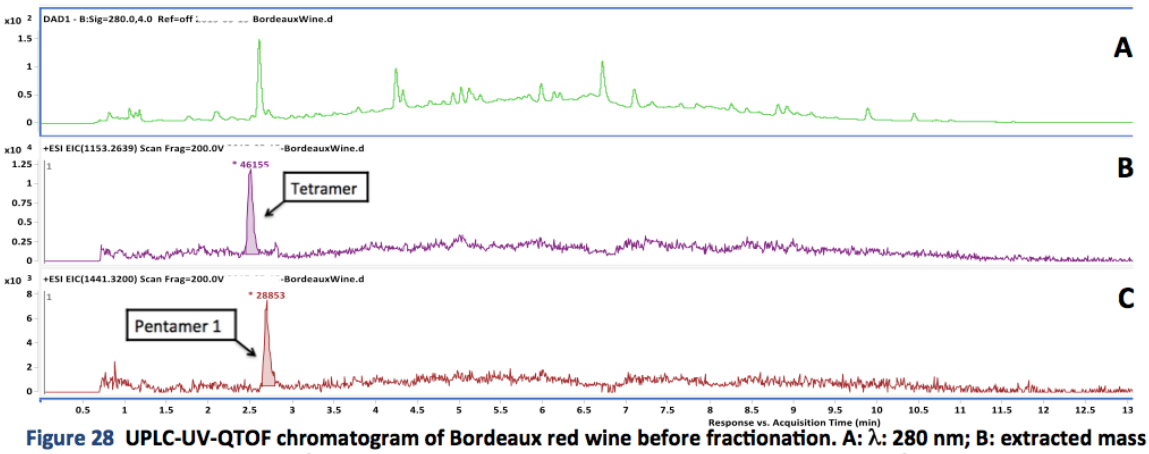


Figure 28 UPLC-UV-QTOF chromatogram of Bordeaux red wine before fractionation. A: λ: 280 nm; B: extracted mass chromatogram m/z : 1153.2608 (tetramer); C: extracted mass chromatogram m/z : 1441.3242.

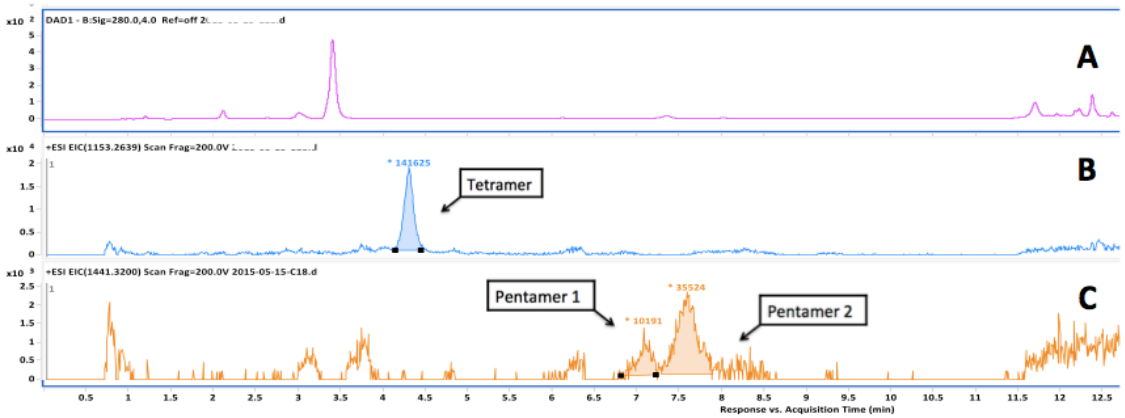


Figure 29: UPLC-UV-QTOF chromatogram of the C-18 fraction. A: λ: 280 nm; B: extracted mass chromatogram m/z : 1153.2608 (tetramer); C: extracted mass chromatogram m/z : 1441.3242.

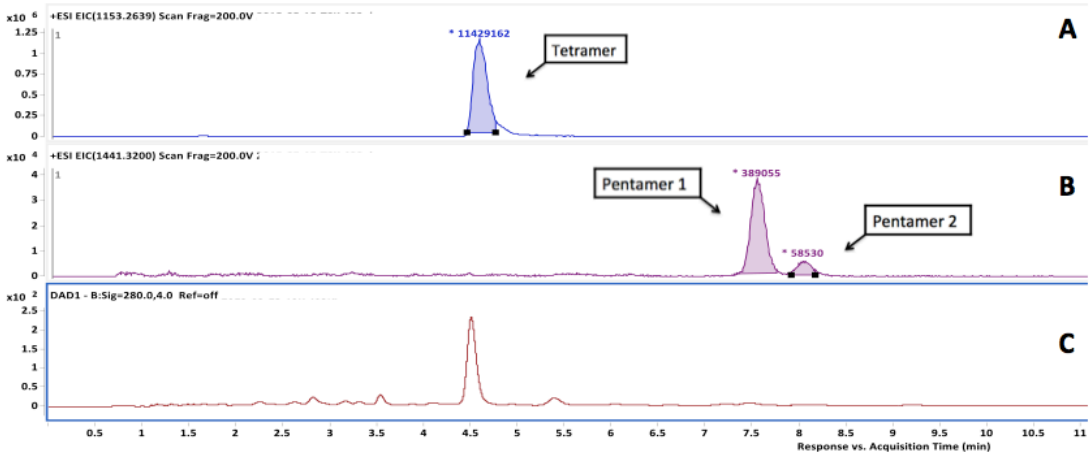


Figure 30: UPLC-UV-QTOF chromatogram of the TSK fraction before semi-preparative HPLC.; A: extracted mass chromatogram m/z : 1153.2608 (tetramer); extracted mass chromatogram m/z : 1441.3242). C: λ: 280 nm

reversed-phase cartridge, and eluted with acidified methanol/water (5/95, v/v). Then, the column was washed with pure methanol and re-equilibrate with water prior to be re-used. The tetramer and pentamers were eluted only with 5% methanol fraction together with impurities and few anthocyanins. Figure 29 demonstrates that the crown procyanidins value increases after C-18 column (Figure 29 A, B) meanwhile wine impurities decrease.

Secondly, TSK HW-40S gel filtration chromatography was applied to separate the tetramer and the two pentamers from each other and from the others low molecular weight compounds presented in the fraction issued from the first step. TSK-40S gel is originally used for protein separation based on size-exclusion chromatography principle. However, it has been shown that the separation of flavanoid compounds on this kind of column is based on their affinities to gel which increase with their sizes (Xiao et al., 2008; Derdelinckx and Jerumanis, 1984). Therefore, higher degree of polymerization signifies more affinity that means more difficulties to elute the compound (De Freitas, 1995). Anthocyanins have more affinity to the mobile phase (methanol) and go out from the column in the first minutes, then little procyanidins run more quickly and are then followed by crown procyanidins tetramer and pentamer. After loading the C-18 fraction on the column, a fraction containing both the crown procyanidins tetramer and pentamers were eluted between 15 h and 16.5 h (Figure 30 B) while a fraction with only the crown procyanidins tetramer were eluted between 16.5 h and 22 h (Figure 30 A). The collected fractions were evaporated and the presence of the expected compounds in each fraction was confirmed by UPLC-Q-TOF analysis (Figure 30). The chromatograms show an increase of the relative concentrations values for the tetramer and the pentamers respectively.

Then, the fraction collected between 15 h and 16.5 h from the TSK HW-40S column was evaporated, re-disolved in acidified water prior to be submitted to HPLC semi-preparative. The third step of purification using C-18 HPLC semi-preparative technique permits to obtain the pure crown procyanidins pentamers. The column used was a C-18 and in this case the gradient of the solvent B (methanol) increase slowly taking 30 min to go from 0% B to 4% B. This very slow gradient allows the easy separation and differentiation of the peaks of two crown procyanidins pentamers and of the crown procyanidins tetramers. The tetramer peak was observed and collected between 18-19 minutes and the peaks of pentamer 1 and 2 were separately collected between 27-32 min.

Overall, the fractionation through the 3 steps of 1 L of red wine yielded 2.1 mg of tetramer, 1.1 mg of pentamer 1 and 1.7 mg of pentamer 2. Unfortunately due to the time frame of the internship, we were not able to run NMR analysis to characterize the structure en 3D configuration of the two collected crown procyanidins pentamers.

5. CONCLUSIONS

During this project crown procyanidin concentration in grape from two different grape varieties, (Cabernet Sauvignon and Merlot) was studied. For the first time it has been proved that crown procyanidin (tetramer and pentamer) detected previously only in red wine are also present in grape berries and are not necessary form during red wine aging. Moreover, the crown procyanidin appeared to be located only grape skin and totally absent from the grape seed and bunch stem. This observation was made in both Merlot and Cabernet Sauvignon varieties collected from the 7 different vineyards around Bordeaux. Such localization specificity is very surprising and raises a lot of questions regarding the biosynthesis of these molecules as well as their role in plant.

Moreover, an important content variability for the same grape varieties was observed for both crown procyanidins according their vineyard origin. In some case the concentration can be two times higher for the same grape varieties. However, in all the analysed sample, the crown procyanidin concentration was higher in Cabernet Sauvignon than in Merlot for all the analysed vineyard parcel. However, similar investigation on other vintages should be performed in order to confirm the observed differences between grape varieties and between vineyard origins.

The monitoring of the crown procyanidins extraction rate during the alcoholic fermentation reveals once again an unusual trend compare to regular B-type tannins. Indeed, the crown procyanidins are extracted very quickly at the beginning of the vinification, which is an opposite trend, compare to regular B-type condensed tannins which need ethanol to be extracted. This specific behaviour might be due to the fact that these tannins are very polar, highly soluble in water, and exclusively located in the skin. However, these results are preliminary and never reported before and thus needs to confirm on different vintage and with replicate.

Moreover, both crown procyanidin pentamer has been purified by a “three steps-two gels” strategy” yielding 1.1 mg of pentamer 1 and 1.7 mg of pentamer 2. However, their structural characterisation by NMR couldn’t be done in the time frame of the internship and will be done in the future.

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7. ANNEX

7.1. Annex I: Anthocyanidins

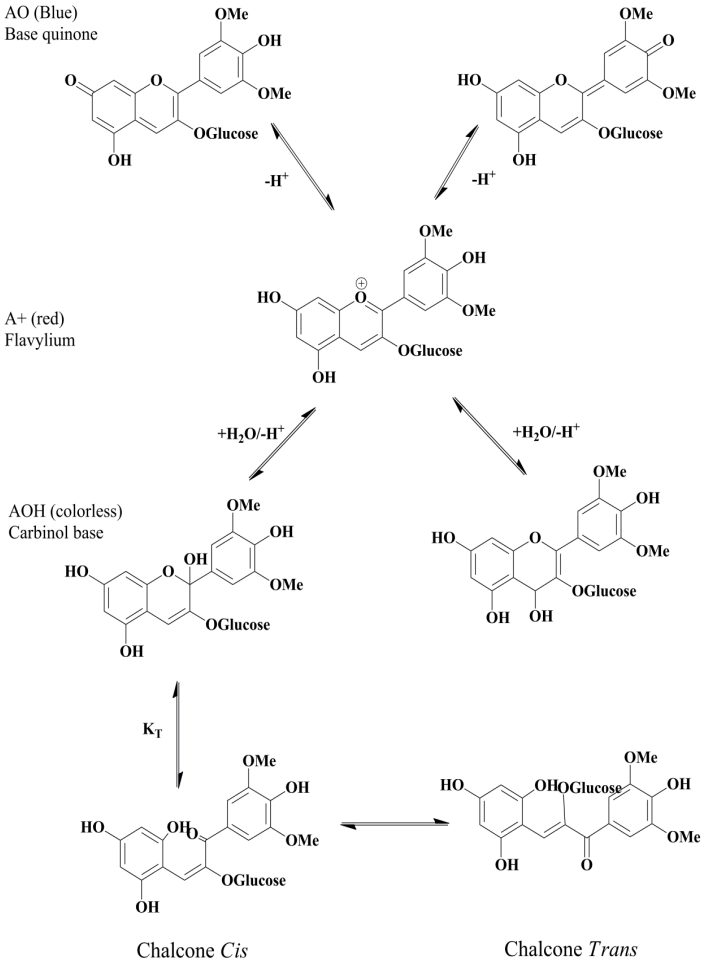

Figure 1 Structure transformations of anthocyanins in acid aqueous solution represented with malvidin-3-*O*-glycosidic. (Ribéreau-Gayon et al., 2012)

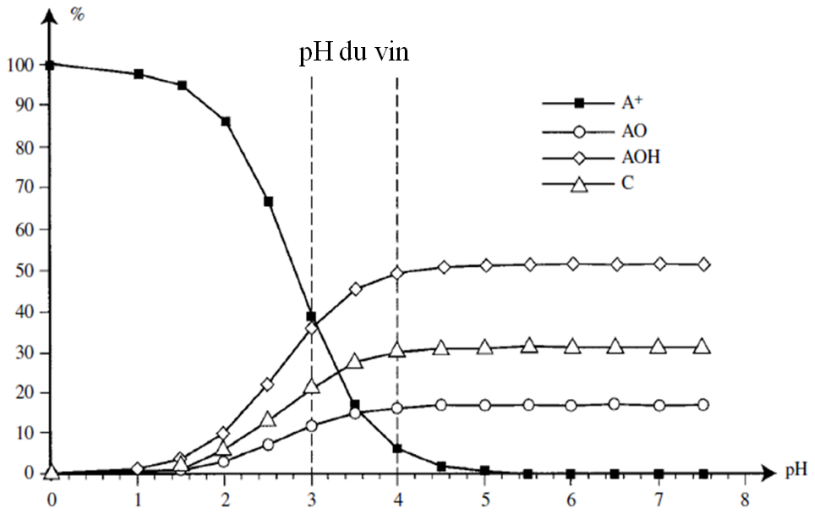

Figure 2: Changes in the proportion of different forms of anthocyanins according to pH. (Ribéreau-Gayon et al., 2012

7.2. Annex II: Results of maturation grape in harvest 2015

	Results of maturation grape in harvest 2015						
	Weigh of 100 berries (g)	Vol juice for 100 berries (mL)	pH	AT (g/L) tartaric ac	AT (g/L) H_2SO_4	Malic ac (g/L)	Sugar (g/l)
	Merlot						
Plot 1	167,7	90,0	3,25	5,10	3,33	2,22	223,2
Plot 2	145,3	74,0	3,30	3,30	3,23	1,35	260,4
Plot 3	103,3	53,3	3,54	3,60	2,35	1,18	245,0
Plot 4	133,3	66,7	3,47	4,50	2,94	1,91	266,0
Plot 5	141,7	71,3	3,28	5,25	3,28	1,18	260,4
Plot 6	151,3	66,0	3.41	4,35	2,84	1,44	266,0
Plot 7	165,3	78,7	3,44	4,50	2,94	1,82	266,0
	Cabernet Sauvignon						
Plot 1	97,3	49,3	3,36	3,50	3,43	2,69	250,0
Plot 2	136,3	70,7	3,24	4,20	4,12	2,86	250,0
Plot 3	109,7	54,7	3,34	5,40	3,53	2,6	250,0
Plot 4	138,0	69,3	3,29	6,60	4,31	3,28	240,0
Plot 5	100,3	49,3	3,27	5,25	3,43	1,37	250,0
Plot 6	134,3	68,0	3,27	5,25	3,43	1,98	250,0
Plot 7	155,3	78,7	3,29	5,85	3,82	2,53	238,0

Table 1: Results of maturation grape in harvest 2015.

7.3. Annex III: Results essay extraction 2015

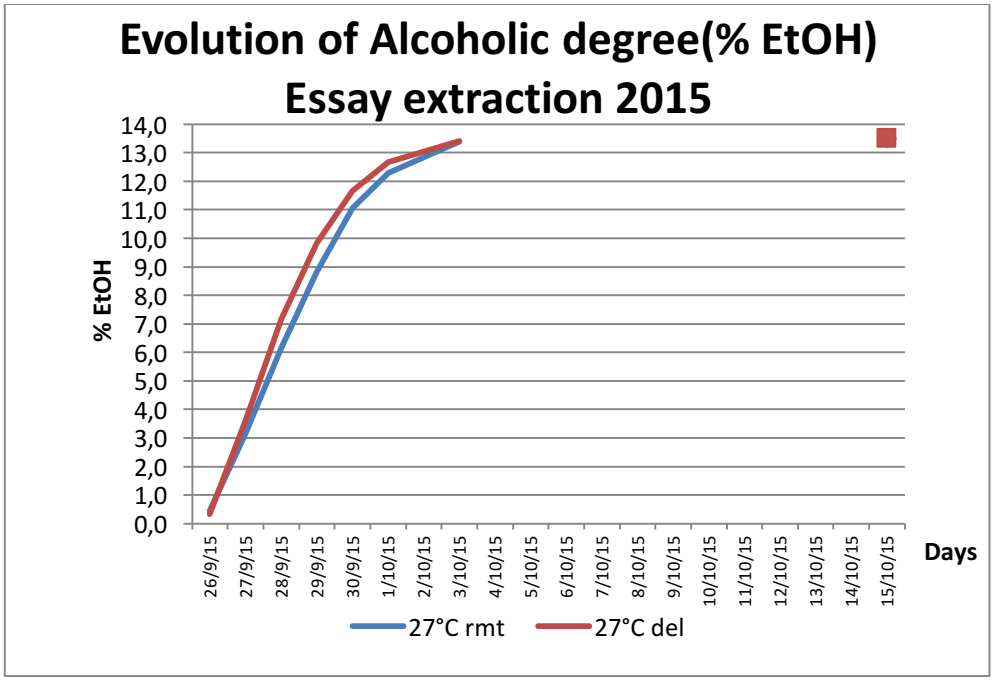

Figure 1: Evolution of alcoholic degree (%EtOH). Results from "Essay Extraction 2015". Source "Institud des sciences de la Vigne et du Vin".

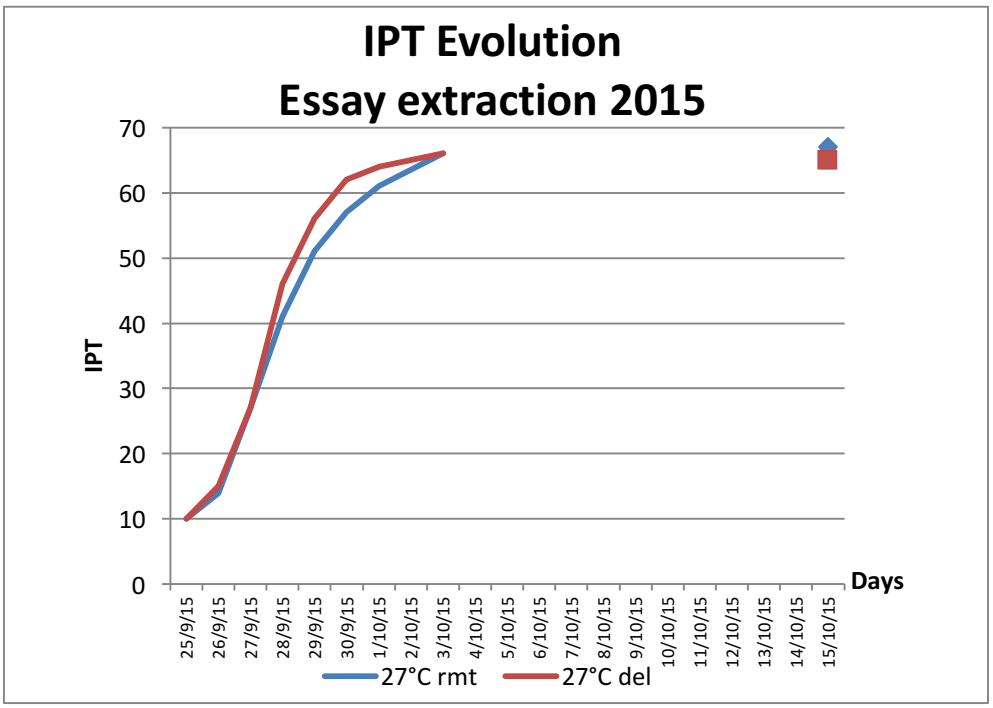

Figure 2: IPT Evolution. Results from "Essay Extraction 2015". Source "Institud des sciences de la Vigne et du Vin".

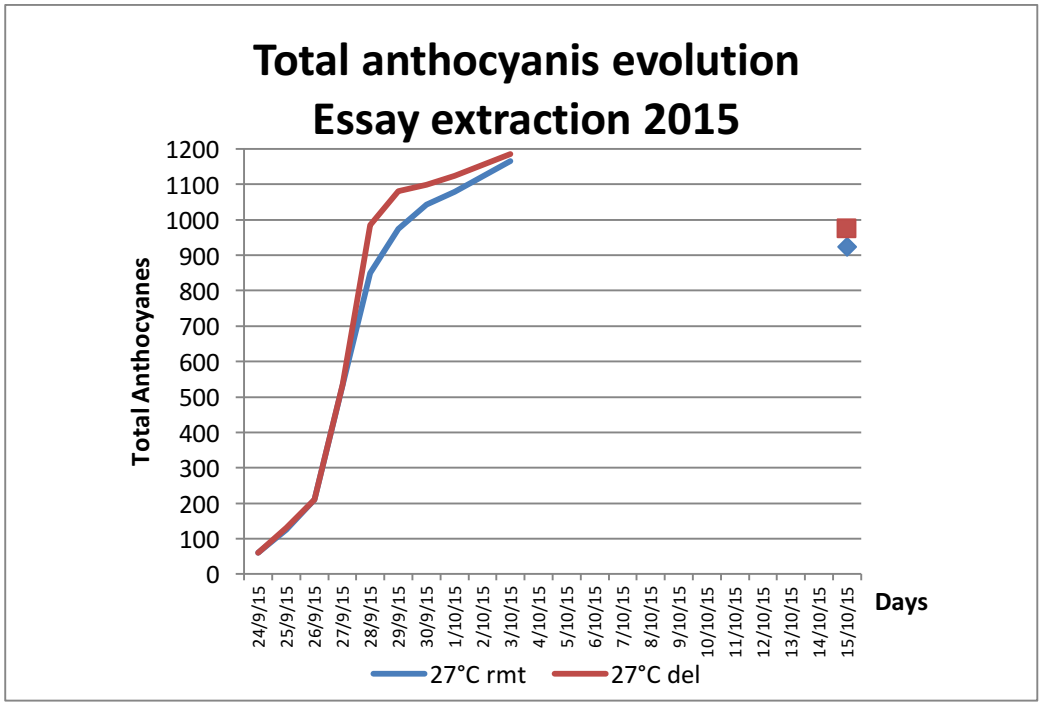

Figure 3: Total Anthocyanins evolution. Results from "Essay Extraction 2015". Source "Institud des sciences de la Vigne et du Vin".