Anthocyanins and tannins extraction in red winemaking: study of certain mechanisms and impact of the grape composition.

Elissa Abi-Habib

THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'INSTITUT AGRO - MONTPELLIER SUPAGRO

En Sciences des Aliments et Nutrition

École doctorale GAIA – Biodiversité, Agriculture, Alimentation, Environnement, Terre, Eau

Portée par

Unité de recherche UMR Sciences Pour l'Œnologie INRAE

Extraction des anthocyanes et des tanins en vinification en rouge : étude de certains mécanismes et impact de la matière première

Présentée par Elissa ABI HABIB

Le 02 décembre 2020

Devant le jury composé de

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Mme Chantal MAURY, Enseignant-Chercheur, Ecole d'Agricultures d'Angers	Rapporteur
Mme Catherine RENARD, Directrice de recherche, INRAE Nantes	Président
Mme Aude VERNHET, Professeur, Montpellier SupAGRO	Co-Directeur
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Résumé

Extraction des anthocyanes et des tanins en vinification en rouge: étude de certains mécanismes et impact de la matière première

Résumé. Les polyphénols du raisin, principalement localisés dans la pellicule et les pépins, jouent un rôle clé dans le goût et la couleur des vins rouges. Leur extraction par diffusion a lieu pendant la macération. Elle est partielle, et modulée par plusieurs facteurs pouvant la favoriser ou au contraire la limiter. Après diffusion, les polyphénols subissent d'autres modifications liées à leur réactivité chimique. Il est par conséquent difficile de prévoir la composition finale d'un vin à partir de celle du raisin. Les objectifs de ce travail étaient d'identifier les facteurs impactant l'extraction des polyphénols. Dans ce but, deux variétés contrastées ont été étudiées : le Grenache et le Carignan. Les compositions en polyphénols des différents compartiments ont été déterminées, et l'analyse du matériel pariétal des pulpes et pellicules réalisées. Des études en milieux modèles ont été réalisées : diffusion des polyphénols à partir des pellicules et des pépins, pris ensemble ou séparément et en présence ou non des constituants insolubles de pulpe (CIPs), adsorption d'anthocyanes et de tanins purifiés sur les CIPs. Elles ont été comparées à des microvinifications. Les résultats obtenus ont permis de montrer que : i) l'extraction des anthocyanes est dépendante de la proportion d'anthocyanes *p*-coumaroylées ; ii) la diffusion des tanins à partir des pellicules et leur adsorption sur les CIPs sont influencées par la composition des parois cellulaires, notamment en termes d'extensines et d'AGP ; iii) cette adsorption est essentiellement irreversible, sélective (tanins de plus haut DPs et galloylés) et peu influencée par la présence des anthocyanes ; iv) les diffusions de tanins observées à partir des pépins seuls sont importantes mais dès qu'ils sont en présence de parois cellulaires de pellicules/des CIPs et/ou d'anthocyanes, leur concentration chute fortement, en lien avec de l'adsorption et/ou des précipitation et/ ou des réactions chimiques; v) si les CIPs peuvent adsorber des quantités importantes de tanins, cette adsorption déplace les équilibres solide/liquide en faveur de la diffusion. Ces résultats ont permis de rendre compte des différences observées en microvinification entre ces deux cépages et de progresser dans la compréhension de l'impact de la composition de la matière première.

Mots-clés : raisin, anthocyanes, tanins, extraction, polysaccharides et protéines pariétaux.

Abstract

Anthocyanins and tannins extraction in red winemaking: study of certain mechanisms and impact of the grape composition

Abstract. Grape polyphenols, mainly located in skins and seeds, play a key role in the taste and color of red wines. Their extraction occurs by diffusion during maceration. It is partial and modulated by several factors that can favour it or on the contrary limit it. After diffusion, they undergo other modifications linked to their chemical reactivity. It is therefore difficult to predict the final composition of a wine from that of grapes. The objectives of this work were to identify the factors impacting polyphenol extraction. To this end, two contrasted varieties were studied: Grenache and Carignan. The polyphenol compositions of the different compartments were determined and the analysis of the cell walls of the fleshes and skins was carried out.Studies in model solutions were performed: diffusion of polyphenols from skins and seeds, taken separately or together and in the presence or not of flesh water-insoluble materials (FWIM); adsorption of anthocyanins and tannins on FWIM. They were compared to microvinifications. Results showed that: i) the extraction of anthocyanins is dependent on the proportion of *p*-coumaroylated anthocyanins; ii) the diffusion of tannins from the skins and their adsorption on FWIM are influenced by the composition of the cell walls, in particular in terms of extensins and AGP ; iii) this adsorption is essentially irreversible, selective (tannins of higher DPs and galloylated) and little influenced by the presence of anthocyanins ; iv) the tannin diffusions from the seeds alone are important but their concentration drops sharply as soon as they are in the presence of skins/fleshes cell walls and/or anthocyanins, in relation to adsorption and/or precipitation and/or chemical reactions; if the pulp insolubles can adsorb large amounts of tannins, this adsorption shifts the solid/liquid equilibrium in favour of diffusion. These results made it possible to account for the differences observed in microvinification between the two grape varieties studied and to progress in the comprehension of the impact of grape composition.

Keywords: grape, anthocyanins, tannins, extraction, cell wall polysaccharides and proteins

Publications and Communications

Publications

Impact of grape variety, berry maturity and size on the extractability of skin polyphenols during model wine-like maceration experiments. Elissa Abi-Habib, Céline Poncet-Legrand, Stéphanie Roi, Stéphanie Carrillo, Thierry Doco and Aude Vernhet
Journal of the Science of Food and Agriculture, sous presse, (2021) https://doi.org/10.1002/jsfa.10955

Mechanical tests and definition of new indexes of grape berry firmness. Evolution of berry skin hardness during alcoholic fermentation. Philippe Abbal, Aude Vernhet, Elissa Abi-Habib, Stéphanie Carrillo, Marie-Agnès Ducasse and Céline Poncet-Legrand*, Vitis 59, 163–168,* (2020). *Doi : 10.5073/vitis.2020.59.163-168*

Communications

OenoIvas 2019, Bordeaux, France. Topic: Analysis and composition of grapes, wines, wine spirits (Poster).
Title: Skin polyphenol extraction during maceration: a quite complex problem.

F&V Processing 2020-Third Symposium on Fruit and Vegetable Processing, Avignon, France. Topic: Processing and reactivity of F&V (Oral communication).
Title: Impact of grape variety, berry maturity and size on the extractability of skin polyphenols during model wine-like maceration experiments

Abbreviations

mDP: mean degree of polymerization

AGP: arabinogalactan proteins

AIS: alcohol insoluble cell wall solids

car: carignan

CBMs: carbohydrate-binding modules

CDTA: cyclo-hexane-diamino-tetra-acetic acid

CoMPP: Comprehensive Microarray Polymer Profiling

DE: degree of methylesterification

Deg: density or degree of maturity

FWIM: fresh flesh water-insoluble material

gre: grenache

HG: homogalacturonan

HPLC: High-Performance Liquid Chromatography

HPSEC: High-Performance Size Exclusion Chromatography

ISP: initial skin polyphenols

MAbs: monoclonal antibodies

MRM: Multiple Reaction Monitoring

NESP: non-extracted skin polyphenols

non-acyl. antho: non-acylated anthocyanins

***p*-coum antho:** *p*-coumaroylated anthocyanins

PRAGs: polysaccharides rich in arabinose and in galactose

PSP: srecipitated Skins Polyphenols

RESP: residual extractable polyphenols in wine-like solvents

RGI: rhamnogalacturonan I

SSP: soluble Extracted Skin Polyphenols

TPI: Total polyphenols Index

TRP: total red pigments

UHPLC-QqQ-MS: Ultra High-Performance Liquid Chromatography coupled to triple-quadrupole Mass Spectrometry

Vol: volume or size

Table of Contents

Contexte et présentation du sujet

Cette thèse s'inscrit dans le cadre du projet Interfaces financé par Agropolis Fondation, qui vise à mieux comprendre et valoriser la variabilité et l'hétérogénéité de la matière première tout au long des étapes de culture pour optimiser en fonction les procédés de transformation.

La qualité des vins rouges dépend fortement de leur composition en polyphénols, qui joue un rôle déterminant dans leur goût (astringence, amertume) et leur couleur. Ces différentes propriétés organoleptiques sont principalement liées aux anthocyanes et aux tanins, deux familles majeures de flavonoïdes.

Les polyphénols des baies de raisin sont principalement localisés dans les pellicules (anthocyanes, tanins, acides hydroxycinnamiques, flavonols), les pépins (tanins) et en très petites quantités dans la pulpe. Leur extraction se réalise pendant l'étape de macération de la vinification. Elle nécessite leur diffusion depuis les cellules des pellicules et des pépins vers la phase liquide (moût en fermentation).

En pratique, il est souvent difficile de trouver une relation simple et directe entre la composition en polyphénols des baies et celle des vins à la fin de la vinification. Il s'agit d'un système complexe. Cette difficulté peut être lié à des différences dans les procédés de vinification appliqués, qui sont très variables en œnologie : température, durée et modalités de contact entre les pellicules, les pépins et les moûts en fermentation, souche de levure utilisée, ajout d'enzymes pectolytiques, etc... Par exemple, une étape de chauffage préfermentaire des baies fragilisera les parois cellulaires, permettra une meilleure extraction des polyphénols, et est susceptible de favoriser certaines réactions chimiques, alors qu'au contraire une macération à froid et à l'abri de l'oxygène limitera la diffusion et les réactions. En effet, les polyphénols sont des composés très réactifs d'un point de vue chimique et donnent naissance à de nouveaux composés dont les structures chimiques sont différentes.

Même en utilisant les mêmes procédés (ou très proches), on peut observer des différences marquées, notamment liées à la matière première lorsqu'on compare des cépages contrastés. Ainsi, en fonction de la matière première et des itinéraires technologiques suivis, les vins auront des caractéristiques qualitatives et quantitatives très différentes. En effet, d'autres paramètres liés à la matière première peuvent influencer la composition finale en polyphénols des vins : la concentration finale en éthanol, liée à la teneur initiale en sucre des baies, et la structure et la composition des constituants insolubles du raisin, principalement les parois

cellulaires de pulpe et de pellicule. Le rôle joué par les constituants insolubles des pellicules et de la pulpe a été mis en évidence dans plusieurs études. En effet, pour être extraits, les composés phénoliques doivent diffuser des vacuoles des cellules de la pellicule vers le moût et donc traverser les parois cellulaires qui constituent une barrière à cette diffusion. Le rôle limitant des parois est principalement attribué aux interactions qu'elles ont avec les composés phénoliques, en particulier les tanins de haut poids moléculaire. Une fois extraits, les polyphénols peuvent également être adsorbés sur les débris solides des cellules de la pulpe. La composition et la structure (réseau tridimensionnel) des parois cellulaires de la pellicule et de la pulpe peuvent varier en fonction du cépage et de son degré de maturité, influençant ainsi l'extraction et la composition finale en polyphénol dans le vin.

Bien que moins étudiés, ces mêmes paramètres peuvent également avoir un impact sur la diffusion des tanins à partir des pépins de raisin. Une fois extraits, les tanins des pépins peuvent également être adsorbés par les résidus insolubles suspendus dans le moût. De plus, au cours de la fermentation, l'augmentation progressive de la teneur en éthanol du milieu est susceptible de modifier ces interactions.

Enfin, la levure, microorganisme responsable de la fermentation, intervient également à plusieurs niveaux : elle produit des métabolites qui réagissent avec les polyphénols et créent de nouvelles molécules aux propriétés différentes, elle aussi adsorbe des composés phénoliques sur leurs parois.

Les objectifs de la thèse sont : i) de caractériser l'impact de la variété de raisin, de la taille des baies et de la maturité sur l'extraction et l'évolution des anthocyanes et des tanins pendant la macération pelliculaire ; ii) d'identifier les principaux facteurs impliqués dans les phénomènes de diffusion et d'adsorption des polyphénols et leur impact sur la composition phénolique finale dans le vin ; iii) d'examiner les liens entre ces phénomènes et la composition des parois cellulaires de pulpe et de pellicule afin d'en identifier les marqueurs les plus pertinents.

Afin de répondre aux objectifs de cette thèse, la démarche expérimentale qui en résulte est constituée de différentes étapes. Pour prendre en compte l'effet hétérogénéité du matériel végétal et éviter les biais liés aux conditions climatiques et au mode de culture, chaque cépage a été récolté à une date donnée sur une même parcelle. Les baies d'une même récolte de deux variétés de raisin ont été triées en fonction de leur taille et stade de maturité (tri densimétrique). La matière première raisin se caractérise par la présence, pour une maturité moyenne et une récolte donnée, d'une grande hétérogénéité. A partir de ces matières premières

il a été prévu différentes approches, en solutions modèles et en milieux réels. En solutions modèles, nous nous sommes focalisés sur la diffusion des polyphénols à partir des différents compartiments de la baie et leurs interactions avec les parois insolubles de la pulpe. En milieu réel, une méthode de vinification a été développée, grâce à laquelle nous avons étudié les liens entre les caractéristiques de la matière première et la diffusion des composés phénoliques en vinification en phase hétérogène et la composition en polyphénols et leurs propriétés en fin de fermentation alcoolique (FA). Les composés principalement analysés sont les anthocyanes et les tannins.

La première partie de ce manuscrit (Chapitre 1) est une synthèse bibliographique relative aux différents points abordés dans ce travail, qui concernent les baies de raisin (structure, composition…), la diffusion phénolique et l'interaction de ces deniers avec les débris insolubles de raisin. Dans le Chapitre 2, les expériences ont été effectuées dans des conditions modèles mimant une macération, pour deux variétés contrastées en termes de rapports anthocyanes/tanin (Carignan et Grenache). Les expériences de diffusion ont tout d'abord été réalisées à partir de la pellicule fraiche, en absence de pulpe pour éviter l'impact d'adsorption/précipitation de polyphénols non liés à la composition de la pellicule, et de fermentation pour éviter les changements chimiques liés aux réactions avec les métabolites de la levure et l'adsorption par les parois de levure. Le Chapitre 3 traite les interactions pulpe-polyphenols. Nous avons caractérisé et quantifié l'adsorption de deux familles de polyphénols (anthocyanes et tanins) sur des parois de pulpe de raisin. Ces interactions ont été étudiées dans un système modèle vin, toujours en l'absence de levures. Le Chapitre 4 est consacré à une complexification progressive du modèle : des macérations ont été réalisées avec les pépins, les pulpes et les pellicules, seuls et en mélange, afin de comparer les résultats de ces macérations avec les vins obtenus lors de microvinifications. L'ensemble de ces résultats fait l'objet d'une discussion générale.

Chapter 1:

Literature review

Chapter 1: Literature review - Part A: The grape berry

I - The morphology and physiology of grape berries

I.1 The berry

Grape is a non-climacteric fleshy fruit, classified in the category of berries. Berries develop as clusters, with each berry attached to the bunch stem via the pedicels. Grape berries are divided into three compartments (Figure 1): the skin (or exocarp), the pulp (mesocarp), and the seeds. The pericarp is formed by the exocarp, the mesocarp, and the endocarp.

The skin consists of outer layers of thick-walled cells (6 to 8 layers, 100-250 µm thick), covered with the cuticle, a wax-like coating outer membrane (cutin). The outermost cells are small and thick-walled while the innermost cells are larger and thinner-walled (Cadot *et al.,* 2006). The parenchymal cells of the flesh or pulp make up most of the berry volume. The pulp, which is the main compartment of the berry, can be dived in three zones: an external zone, not very thick, which covers the inner face of the skin; an intermediate zone that mostly releases the juice first; an internal zone where the seeds are localized (Conde *et al.,* 2007; Ollat *et al.,* 2002). At maturity, pulp cells are largely occupied by a vacuole containing water and soluble substances such as sugars (glucose and fructose) and organic acids (Diakou & Carde, 2001). It is composed of 25 to 30 layers of large (up to 400 µm) thin-walled cells. Pulp cell walls are thin and fragile, susceptible to rapid rupture and juice release when berries are pressed. The berry contains one to four seeds. The grape seed consists of an epidermis surrounded by a cuticle of lipidic nature and three envelopes that surround the albumen and the embryo: an outer parenchymal and soft envelope; a sclerified envelope; an inner envelope formed by three layers of soft cells.

I.2 Physiological stages of grape berry development

A thorough description of the development and ripening a grape berry can be found in different reviews (Ollat *et al.,* 2002; Conde *et al.,* 2007; Kennedy, 2002) The grape berries development from anthesis to maturity follows a double sigmoid (S-shape) growth pattern (Coombe *et al.,* 1987, Coombe, 1992; Coombe & McCarthy, 2000). In general, development consists first of a cell division (green) phase followed by a cell expansion (ripe) phase. The curve is generally subdivided into three phases or stages (Figure 2).

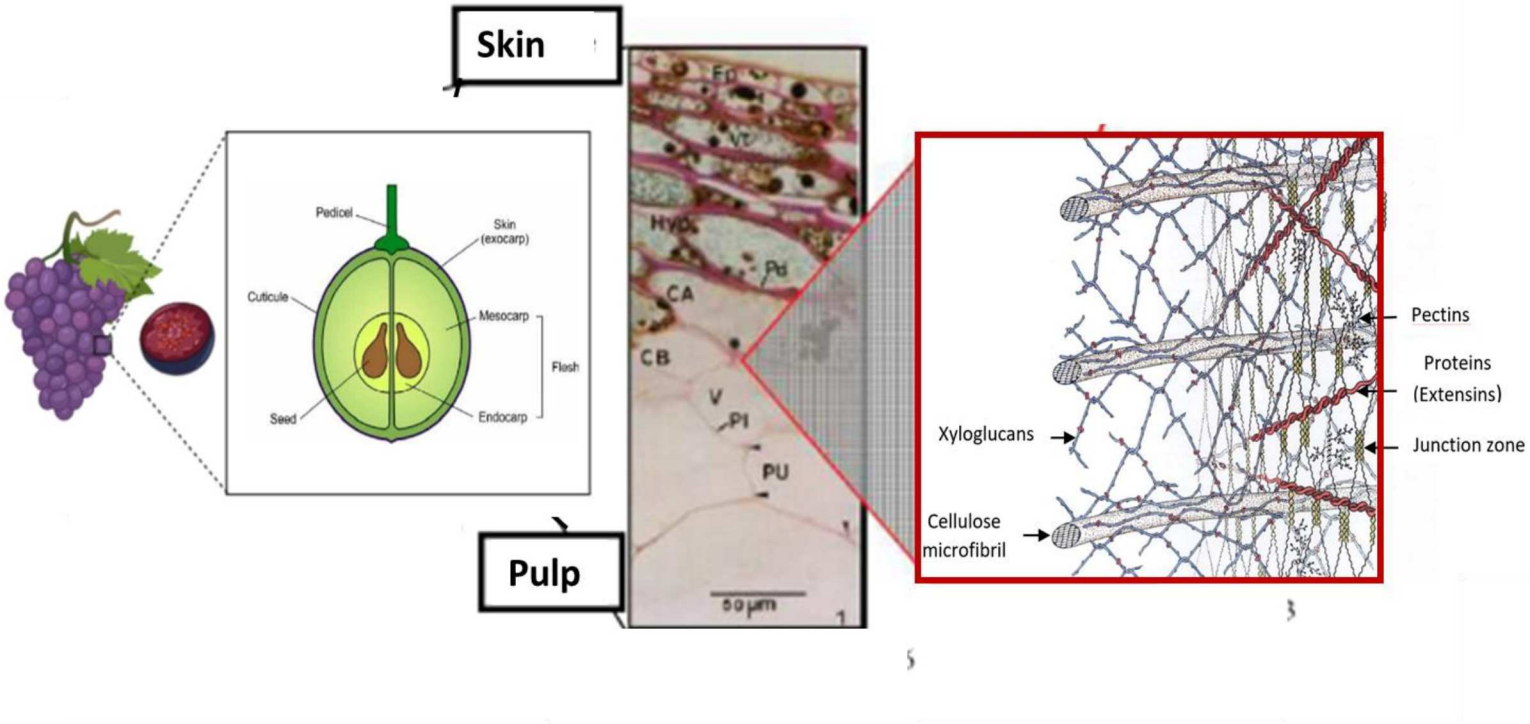


Figure 1. Section of a berry and tissue organization (Coombe *et al.,* 1987; Fougère-Rifot *et al.,* 1996). Model structural organisation of the primary plant wall (Carpita *et al.,* 1993).

Phase 1 is known as the herbaceous or green stage. It starts after anthesis, and can last up to 60 days. It is characterized by the rapid growth of berries and seeds. Growth occurs first by cell division of the pericarp (10 to 25 days) and later by cell expansion until the end of phase 1, promoted by endogeneous hormones (auxins, cytokinins and gibberellins). The growth of berries during this phase is also due to the accumulation of water and organic acids in the cell vacuoles. Several other components of importance for wine quality accumulate during this period. Among them are hydroxycinnamic acids in flesh and skin cells, (Romeyer *et al.,* 1983), flavan-3-ols monomers and polymers (tannins) in the skin and seed tissues (Kennedy *et al.,* 2000a; Kennedy *et al.,* 2000b; Kennedy *et al.,* 2001; Cadot *et al.,* 2006; Downey *et al.,* 2003), aromas such as methoxypyrazines (Hashizume & Samuta, 1999), minerals, proteins, …. The first growth phase is followed by a lag phase.

Stage 2, which is also known as the "lag" phase, is characterized by the decrease or stop of berry growth (herbaceous threshold). It can last from 8 to 48 days within the same variety, depending on the temperature and the time of flowering. At this stage, skin cell walls reach their maximum thickness due to the synthesis of structural polysaccharides (mainly pectins) and the hydration of the cell walls. During phase 1, cellular expansion proceeds throughout all tissues, while during the

transition between phases 2 and 3 only the exocarp cells expand, and during phase 3 (berry ripening) only the expansion of the mesocarp cells occurs. The loosening of mesocarp cell wall allows for the accumulation of soluble sugars and takes place prior to the loosening of exocarp cell wall.

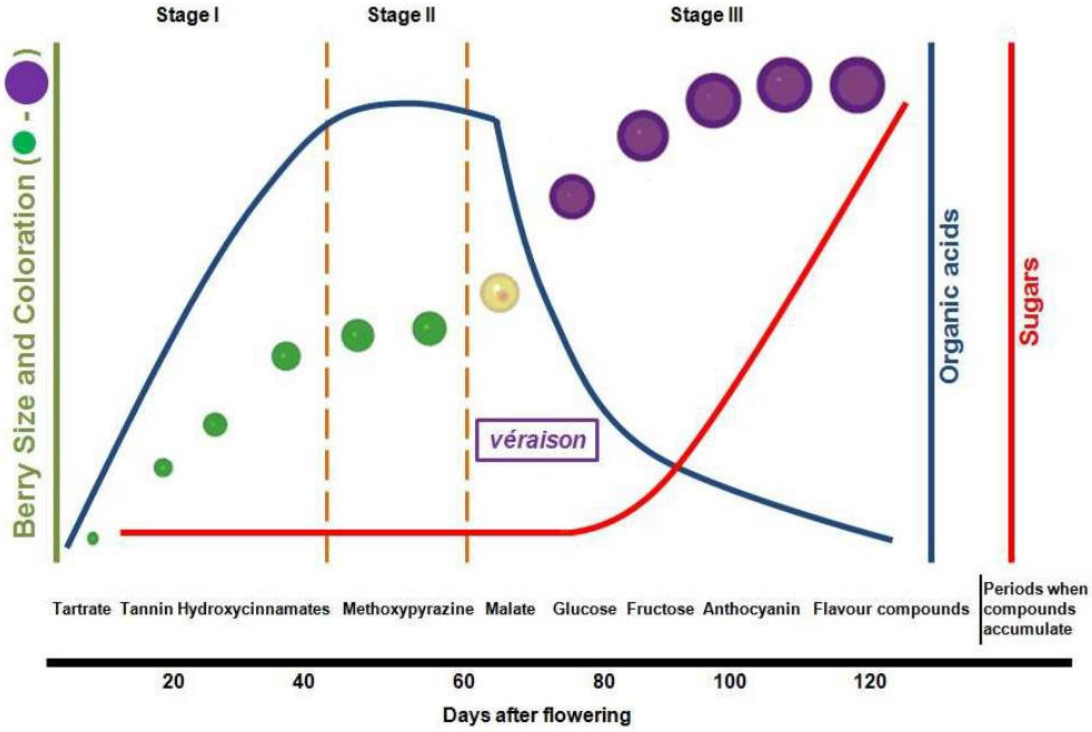


Figure 2. Grape berry development and ripening: Stage 1: early fruit development; Stage 2: lag phase; Stage 3: berry ripening. The main compounds that accumulate in the fruit, are indicated in the bottom of the curves (Noronha, 2017).

During phase 3, known as the maturity or ripening phase, a second and rapid growth of the berries takes place. The beginning of this phase is called veraison, a term used to describe the change in skin color and the beginning of berry ripening. It is marked by the initiation of sugar accumulation, a decrease in organic acids, color development, berry expansion and fruit softening. The time of veraison varies between different vintages, depending on the vineyard, the vines, the bunches of grapes and from one berry to another from the same bunch. This underlines the heterogeneity of the raw material within the same harvest. Phase 3 can last from 40 to 50 days. Berries change from a status where they are small, hard and acidic, with little sugar to a status where they are larger, softer, sweeter, less acidic, flavored and colored. The anthocyanin content of grape skins increases during the first 2 to 3 weeks after veraison, followed by a stabilization phase before a decrease at the end of

ripening (Roggero *et al.,* 1986; Mazza *et al.,* 1999; Kennedy, 2002; Kennedy, 2008). The composition and content of anthocyanins in red grapes vary according to several factors among which are: the climatic conditions, the terroir, the vineyard practices, the sunlight and the degree of ripeness (Kennedy, 2002). The physiological maturity corresponds to the point where the berries reach their largest diameter and maximum sugar content. When berries are over-ripened, they consume their own reserves, lose water and the must becomes concentrated. The parameters that are followed during berry ripening are first the sugar content, the titrable acidity and the pH.

II – Cell Walls structure and composition

II.1 Cell wall structure

The primary cell wall of the berry skin and pulp cells is a complex and dynamic structure composed of high molecular weight polysaccharides and proteins, that represent about 90 and 10% of its dry weight, respectively (Carpita & Gibeaut, 1993). Cell walls constitute a thin unlignified flexible layer that provides mechanical strength, maintains cell shape, controls cell expansion, regulates transport, and provides protection against pathogens attack (Carpita & McCann, 2000). Their structural properties determine their mechanical resistance and texture. It may affect the extraction of some of the skin cell constituents that are decisive for the quality of the wine, such as polyphenols (Hanlin *et al.,* 2010). It is also a source of pectic oligosaccharides and polysaccharides in must and wine since the degradation and solubilization of pectic polysaccharides are involved in the softening of the fruit.

The primary cell walls consist of three domains of independent and interconnected structures. The first one is constituted by a network of cellulose-hemicellulose which represents more than 50% of the total primary cell wall material, in blue in the Figure 3. The cellulose, arranged in microfibrils, are crosslinked by hemicellulose, primarily xyloglucan (XG), which is hydrogen bonded to the cellulose fibrils. This latter is considered to be the backbone of primary cell walls and can represent 20 to 40% of the polysaccharide cell wall fraction. This first domain is embedded in the second domain, constituted by a pectin matrix, which accounts for 25 to 40% of the cell wall. For example, Rhamnogalacturonan I (RGI) is aligned with the cellulose microfibrils (Figure 3). Homogalacturonan (HG), arabinans (Ara), galactans (Gal) and rhamnogalacturonan II (RGII) are side branches attached to a high molecular weight RGI backbone. RGII molecules form crosslinks between different pectin molecules through borate esters (not shown) (Figure 3). In addition, calcium ions form extensive crosslinks between the acid moieties of non-esterified HG molecules.

The whole is locked into shape by the third domain represented by cross-linked structural proteins (extensins) (Fougere-Rifot *et al.,* 1996), which range in content from 1 to 10% of the total composition.

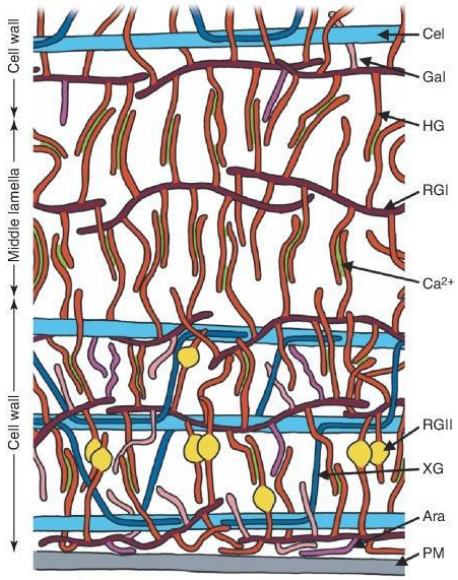


Figure 3. Model to show the organization of polysaccharides in plant cell walls. PM indicates the plasma membrane. (Vorwerk *et al.,* 2004).

II.2 Cell wall polysaccharides

Parietal polysaccharides represent respectively 50 and 90% of the weight of the exocarp and mesocarp cell walls at berries maturity. Neutral polysaccharides (cellulose, hemicelluloses and neutral side chains of pectins) account for 30-35%, while acidic pectin substances (of which 62% are methylesterified) account for 20% in skin cell wall (Lecas & Brillouet, 1994; Goulao *et al.,* 2012).

II.2.a) Cellulose

Cellulose, in the cellulose-xyloglucan network, is composed of linear chains of β-(1➔4) linked D-glucosyl residues. These chains are associated together by hydrogen bonds to form cellulose microfibrils, which also are associated together to form fine threads (Mueller *et al.,* 1976; Emons, 1988). Cellulose microfibrils play a determinant part in the cell wall mechanical strength (Cosgrove, 1997). They are crosslinked by a matrix of xyloglucans through intermolecular non-covalent hydrogen bonds (Carpita &Gibeaut 1993; McCann *et al.,* 1990; Pauly *et al.,* 1999).

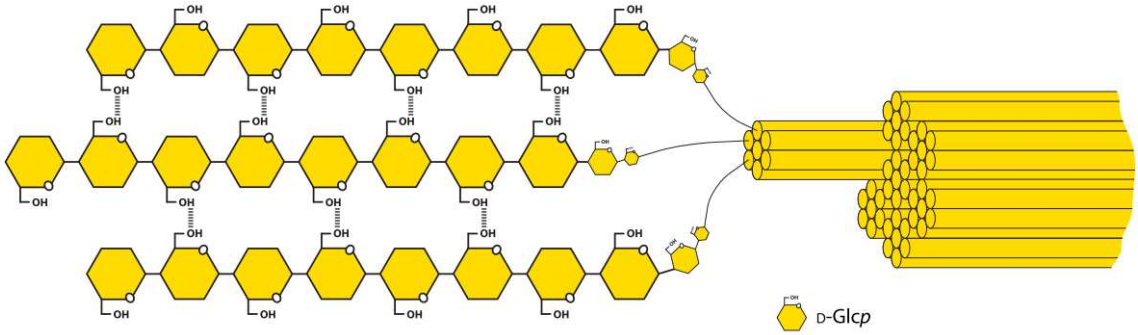


Figure 4. Primary sequence of cellulose and hydrogen bonds between two cellulose chains to form crystalline microfibrils (Fangel, 2013).

II.2.b) Hemicellulose

Cell walls contain 10 to 15% hemicelluloses, more abundant in the pulp than in the skin. Xyloglucans represent the major fraction of hemicelluloses, followed by mannans (glucomannans, galactomannans and galactoglucomannans), xylans (arabinoxylans, glucuronoarabinoxylans). Xyloglucans consist of D-glucosyl residues linked through β-(1➔4)-glycosidic linkages. Up to 75% of the glucosyl backbone residues of xyloglucan are branched with D-xylose residues through an α-(1→6) linkage. Other side-chain sugars, such as β-D-galactose, α-L-arabinose and α-L-fucose are sometimes found linked to the xylose residues (Carpita & Gibeaut 1993; Barnavon *et al.,* 2000). It has been suggested that xyloglucans can be covalently linked to pectic and other polysaccharide fractions.

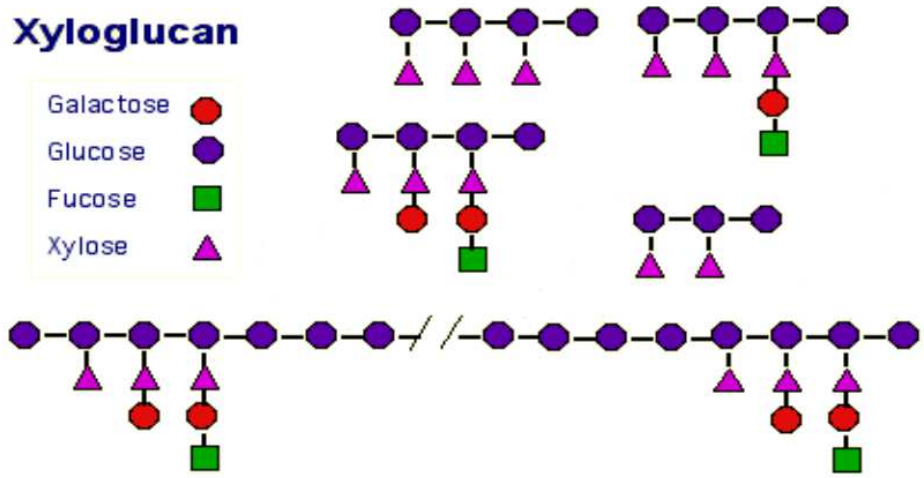


Figure 5. Shematic representation of Xyloglucans (Ducasse *et al.,* 2009).

II.2.c) Pectic polysaccharides

Pectic polysaccharides are one of the major components of primary walls. Pectins are a family of complex polysaccharides that contain 1,4-linked α-D-galactosyluronic acid residues. Three pectic polysaccharides have been isolated from plant primary cell walls and structurally characterized in Figure 6. These polymers together form the pectic matrix, which fills the gaps or interstices of the cellulose-xyloglucan network. They are homogalacturonans (HGs), substituted galacturonans (apiogalacturonans and xylogalacturonans) and the rhamnogalacturonan (RG) polymers (O'Neill, 1990; Doco *et al.,* 1995).

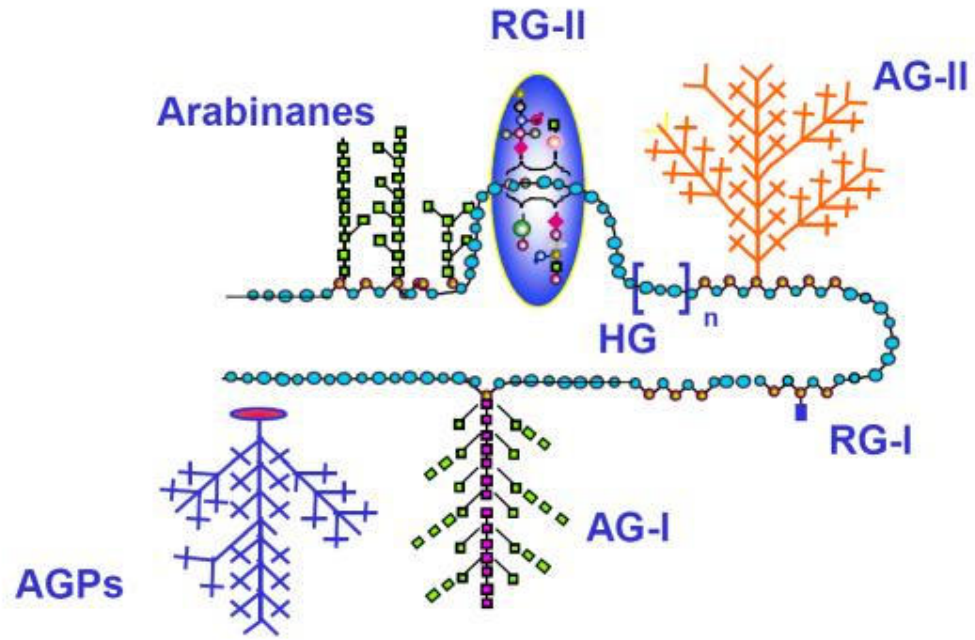


Figure 6. Schematic representation of grape berry pectins (Doco *et al.,* 1995).

Pectins in the cell walls are characterized by smooth homogalacturonic (HG) regions linked to rhamnogalacturonic hairy regions (RG) carrying arabinan and arabinogalactan type I side chains. The grape pectic fraction is composed of 65% HG, 10% RG-I, 2% RG-II and 23% neutral side chains (Nunan *et al.,* 1997; Vidal *et al.,* 2001). Arabinans and AGI contribute from 4-6% to the pectic polysaccharides (Nunan *et al.,* 1997). They are mainly realeased during the maceration steps by enzymatic degradation.

Homogalacturonan (HG) consists of α-1,4-linked D-galacturonic acid (GalA) backbone with no side chain sugars, which forms a homopolymeric chain. They can account for more than 60-65% of the total plant pectins. The degree of methylesterification of parietal homogalacturonans can vary from 40 to 80% depending on the variety, the stage of development and the tissue under consideration. The degree of methyl esterification of homogalacturonan carboxyl groups has a major influence the ability of HG to form gels that control cell wall porosity, to contribute to intra-cellular adhesion and to cell wall strength (Knox, 1992; Carpita & Gibeaut 1993; O'Neill, 1990). De-esterification of homogalacturonan generates carboxylate ions that can bind cations such as calcium to form cross-linking bridges between galacturosyl residues.

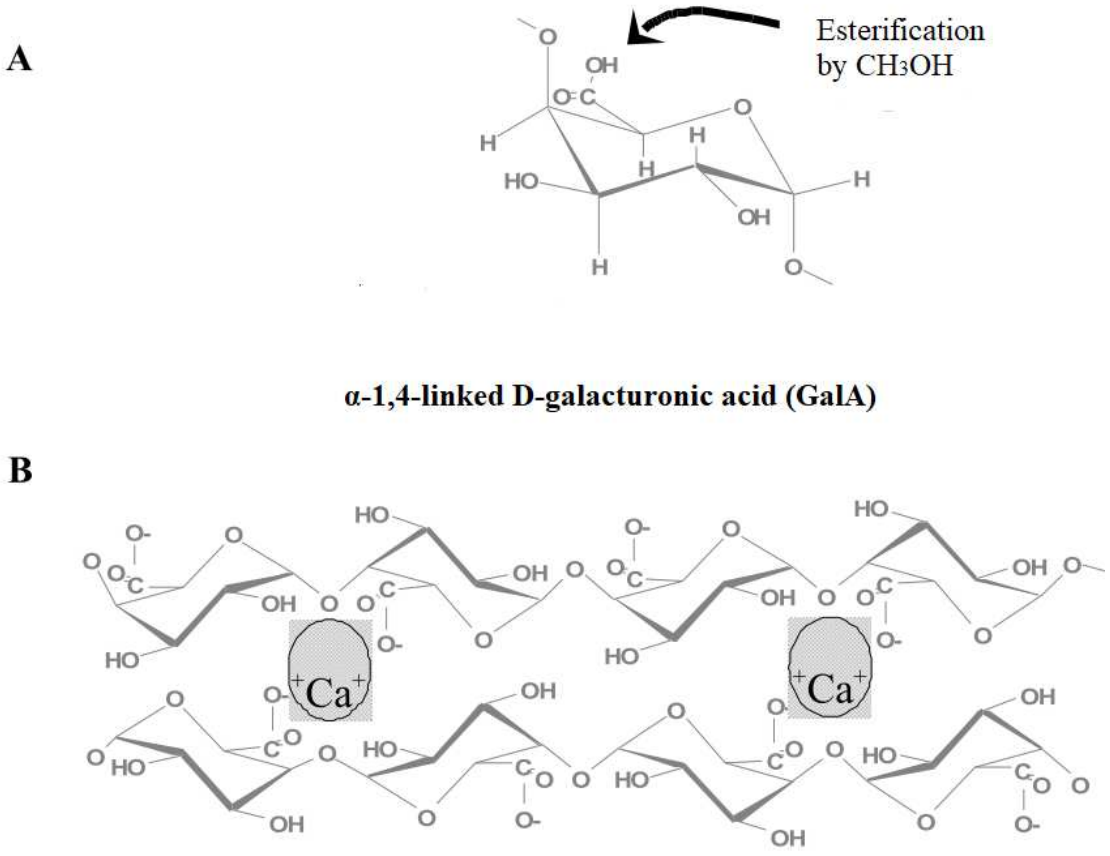


Figure 7. A) Scheme of esterification by methanol of α-D- galacturonic acid. B) Complexation of calcium ions and formation of gels by de-esterified homogalacturonic chain.

Rhamnogalacturonans differ from homogalacturonans by the high number of branched side chains containing arabinosyl, galactosyl and arabinogalactosyl residues. Rhamnogalacturonan I (RGI) has a backbone of repeating disaccharide units of rhamnose (Rha) and galacturonic acid

(GalA). The rhamnoses can be substituted with side chains, including arabinans, galactans, arabinogalactans (type I and II).

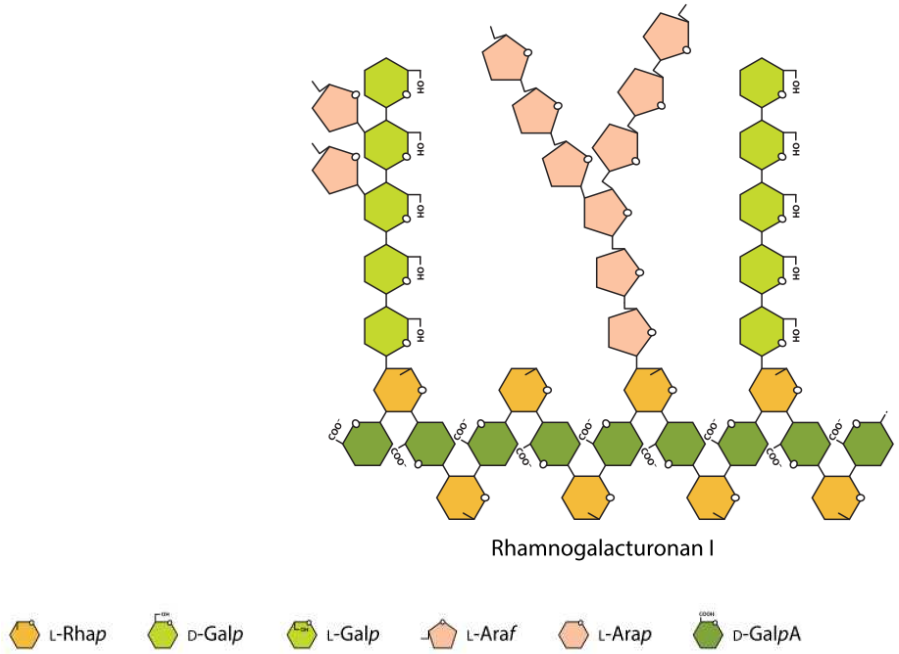


Figure 8. Shematic representation of rhamnogalacturonan I found in pectin polymers (Fangel, 2013).

Arabinans consists a skeleton of α-L-(1→5) arabinofuranose chains with 2- and 3-linked α arabinose branching.

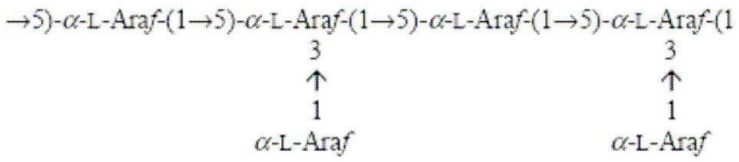


Figure 9. Primary sequence of arabinanes (Flanzy, 1998).

Arabinogalactan type I contains β-D-(1→4)-Galactose chains with α-(1→3)-L-Arabinose branching. AG-I are present in the cell walls of the grape berries. AG-I represents the predominant form of AGs in the skins, in which AG-II is in the minority (Flanzy, 1998).

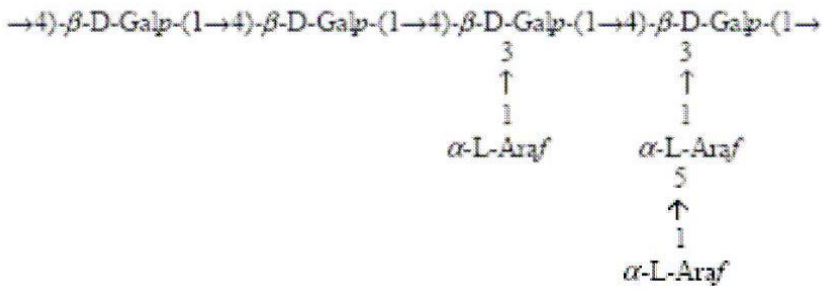
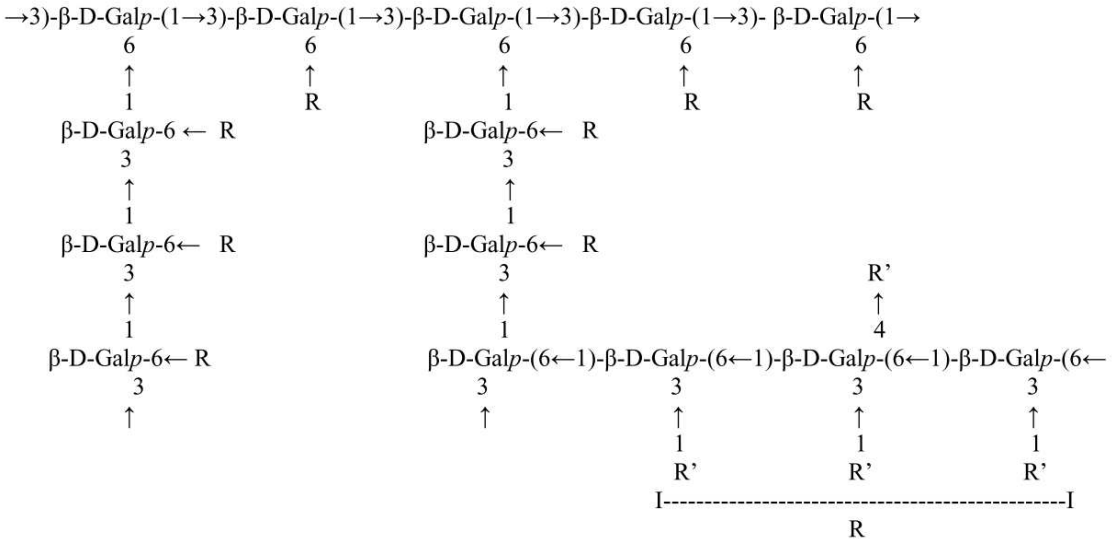


Figure 10. Primary Sequence of Arabinogalactans Type I (AG-I) (Flanzy, 1998).

Arabinogalactans of type II (AG-II) consist of a main chain of β -D-galactopyranose linked at (1→3), branched at O-6 by short chains linked at (1➔6) D-galactopyranose which may themselves be substituted at O-3 or O-4 positions with α -L-arabinofuranose linked at (1➔4). Other oses are also detected: L-rhamnose, D-mannose, D-xylose, D-glucose, D-glucuronic and D-galacturonic acids and their respective 4-O-methyl derivatives (Vidal *et al.,* 2003). Type II arabinogalactans are often bound to a protein characterized by the presence of hydroxyproline and thus form arabinogalactan proteins (AGP). AGP are either free and soluble or associated with pectins at the RG-I level.



R' = α-L-araf, α-D-Glcp A, α -L-Rhap-(1 4)-α-D-Glc A

Figure 11. Primary Sequence of Arabinogalactans Type II (AG-II) (Flanzy, 1998).

Rhamnogalacturonan type II (RGII) structure differs from rhamnogalacturonan I as it is composed of an homogalacturonic part (8 to 10 acid residues of galacturonosyls linked in α-(1➔4)) connected by 4 main side chains (A, B, C and D) as indicated in figure 12 (Penhoat *et al.,* 1999). It is usually a side chain attached to HG and consists of 12 different rare sugars such as the β-D-apiofuranose, the 2-O-α-L-methylfucose, the 2-O-α-D-methylxylose, the DHA (3-deoxy-β-D-lyxo-heptulosaric acid), the KDO (3-deoxy-α-D-mannooctolosonic acid), and the α-L-aceric acid (3-C-carboxy-5-deoxy-L-xylose acid). This last one gives a strong indication about the presence of RG-II. Due to its particular structure, RG-II is highly resistant to enzymatic degradation. Under the action of endo-polygalacturonase, it is released in wine by degradation of smooth areas of pectins. It exists in monomeric form (4.5-5 kDa) and dimeric form (9.5-10 kDa), linked by borate-diol diesters and calcium. In wine, RG-II is mainly found in dimeric form.

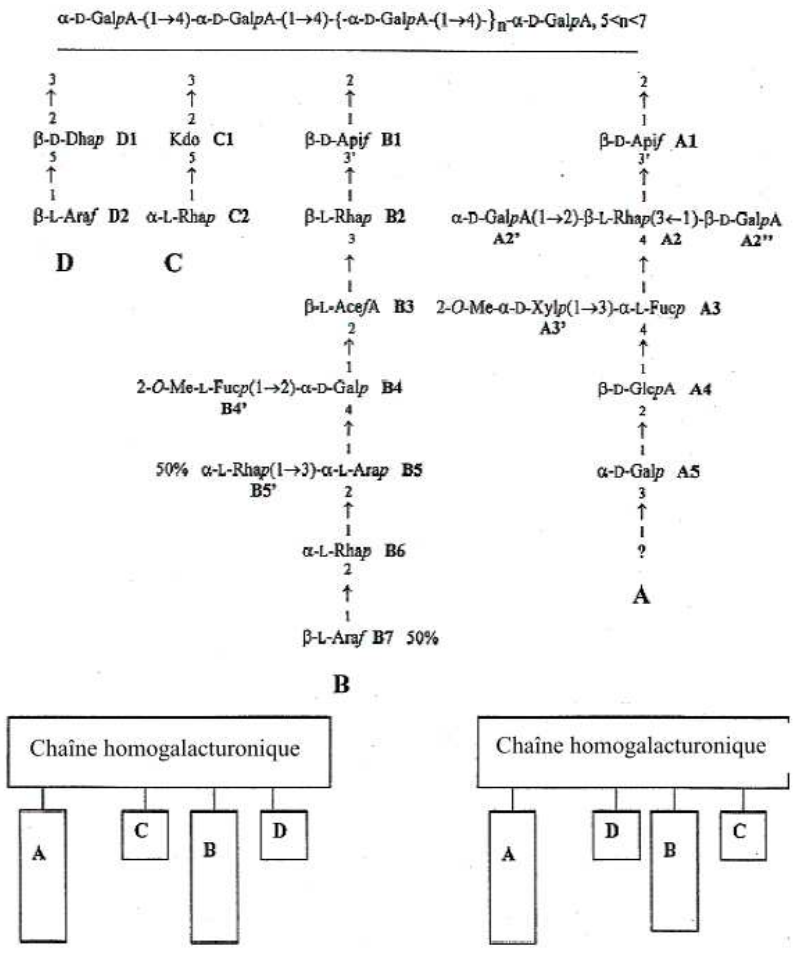


Figure 12. Shematic representation of RG II with the two possible arrangements (determined by RMN) (Penhoat *et al.,* 1999).

II.3 Parietal proteins

The shape of the primary cell wall is maintained by structural proteins, that act as a support to the polysaccharide chains. The cross-linking mechanism of proteins with the cellulose-xyloglucan network is not known. Proteins in cell walls can be divided into different types mainly based on their dominating amino acids : glycoproteins rich with hydroxyproline-rich structures (HRGPs) (including extensins), proline-rich proteins (PRPs), the glycine-rich proteins (GRPs) and proteins associated with arabinogalactans (AGPs) (Table 1).

The main cell wall proteins are extensins (Cosgrove, 2001), which are rich in hydroxyproline amino acid residues. They consist of a protein backbone containing strongly basic repetitive peptide units rich in proline, hydroxyproline, tyrosine, lysine, serine, histidine, valine (Lamport *et al.,* 2011), glycosylated by short side chains (96% arabinose and 4% galactose). They adopt a type II polyproline helix conformation. They have a role in the extension of the plant cell wall but also a role of defence against pathogens.

The next most abundant proteins are glycine- and proline-rich proteins (GRPs and PRPs) and arabinogalactan-proteins (AGPs) (Carpita & Gibeaut 1993; Carpita *et al.,* 2000). Arabinogalactan-proteins are considered as proteoglycans as the majority of their molecular structure is composed of polysaccharides rich in galactose and arabinose (Johnson *et al.,* 2003).

Table 1. Global composition of the main glycoproteins found in plant cell walls. Table taken from (Chen, 2015).

	EXTs	AGPs	PRPs
Protein composition	About 45% protein backbone, 55% sugar.	About 1-10% protein backbone, 90-99% sugar.	About 80-100% protein backbone, 0-20% sugar.
Glycosylation	Gal-Ser and Ara_n-Hyp (n=2,3,4).	Complex sugar chains contain Ara, Gal, Fuc, and Rha… to Hyp.	Some Ara-Hyp.
Abundant amino acids	Hyp (O), Ser (S), Lys (K), Tyr (Y), Val (V) and His (H).	Hyp (O), Ser (S), Ala (A), Pro (P) and Thr (T).	Hyp (O), Pro (P), Val (V), Tyr (Y) and Lys (K).
Major peptide repeats	$S(O)_n$, n=2,3,4; YXYK; VYK.	SOOAPAP, AO, SO.	POVYK, POVEK and variants.

II.4 Cuticle

The cuticle is the outermost layer of the skin; it may remain intact during ripening or may become thinner from anthesis to veraison depending on the variety. It is essentially composed of

layers of cutin (lipidic polyesters) covered with waxes and acts as a barrier against pathogenic attacks, reduces water.

II.5 Changes in cell walls during berry development and ripening

During the development of the fruit, and particularly during ripening, the primary cell walls of skin cells undergo numerous changes that cause softening, tissue deterioration and increased susceptibility to pathogenic attacks. The solubilization and reduction of the parietal polysaccharides appear to play a key role in the berry softening. The process is regulated by the expression of genes coding for enzymes (cellulases, polygalacturonases, pectin methylesterases, ...) that catalyze their hydrolysis, leading to the disorganization of the walls (Nunan *et al.,* 1998). Grape berries begin to soften at veraison (Maury *et al.,* 2009) and the degree of softening at maturity is determined largely by the cultivar (Letaief *et al.,* 2008).

In general, during the ripening stages of grape berry, the cell walls of the mesocarp experience a decrease in both pectic and hemicellulosic polysaccharides and a reduction in their cellulose content (Huang *et al.,* 2005; Goulao & Oliveira, 2008; Yakushiji *et al.,* 2001). It is speculated that the depolymerization process starts from pulp tissue and progressively continue into the skin tissue (Gao *et al.,* 2016). At the end of maturation, the cell wall becomes thinner both in mesocarp and exocarp. This can explain the lower amount of isolated cell wall material as ripening progresses, particularly during the last weeks of grape development (Barnavon *et al.,* 2000).

The most significant change in sugar composition is the decrease in galactose content (Nunan *et al.,* 1998), corresponding to a significant loss of AG-I from side chains of pectic polysaccharides, from before veraison to ripe berries (Barnavon *et al.,* 2000; Barnavon *et al.,* 2001). In parallel, a decrease in the degree of methylesterification of the pectins (Ortega *et al.,* 2008), and an increase in the soluble fraction of polysaccharides is observed (Nunan *et al.,* 1998; Vicens, 2007). Higher solubility of galacturonic acid, AG-II and arabinan is noticed as ripening progresses and the grapes soften (Silacci *et al.,* 1990).

The partial loss of wall structural polysaccharides is compensated by the incorporation of structural proteins and formation of phenolic cross-linkages that happen at the end of the maturation period especially in the walls of epidermis and sub-epidermis cells (Huang *et al.,* 2005 ; Nunan *et al.,* 1998)

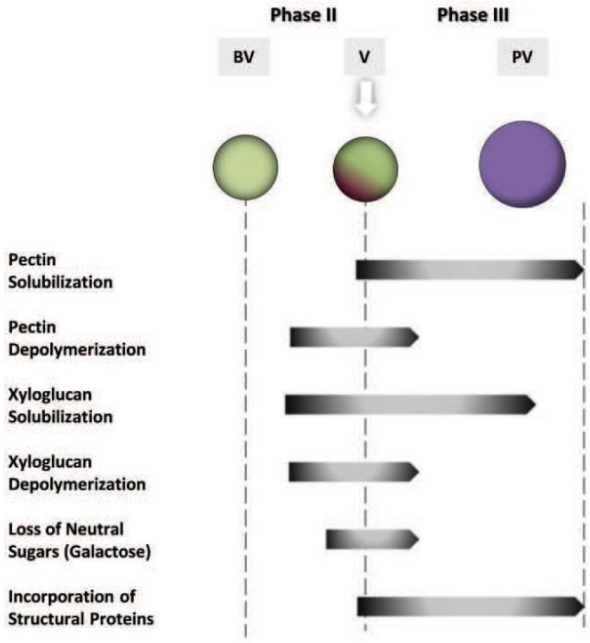


Figure 13. Cell wall modification during berry growth and ripening (Goulao *et al.,* 2012).

III- Polyphenols

Phenolic compounds are important constituents of grapes and wine, that play a fundamental part in determining the colour and taste of red wines.

Polyphenols are secondary plant metabolites that are not essential for the survival of the species (primary metabolites) but have antibacterial, antifungal and antioxidant properties. Polyphenols are molecules characterized by the presence of one or more benzene rings carrying one or more hydroxyl groups. They are divided into two classes: non flavonoid compounds (phenolic acids, stilbenes) and flavonoids (anthocyanins, tannins, flavonols, flavanols), see in Figure 14.

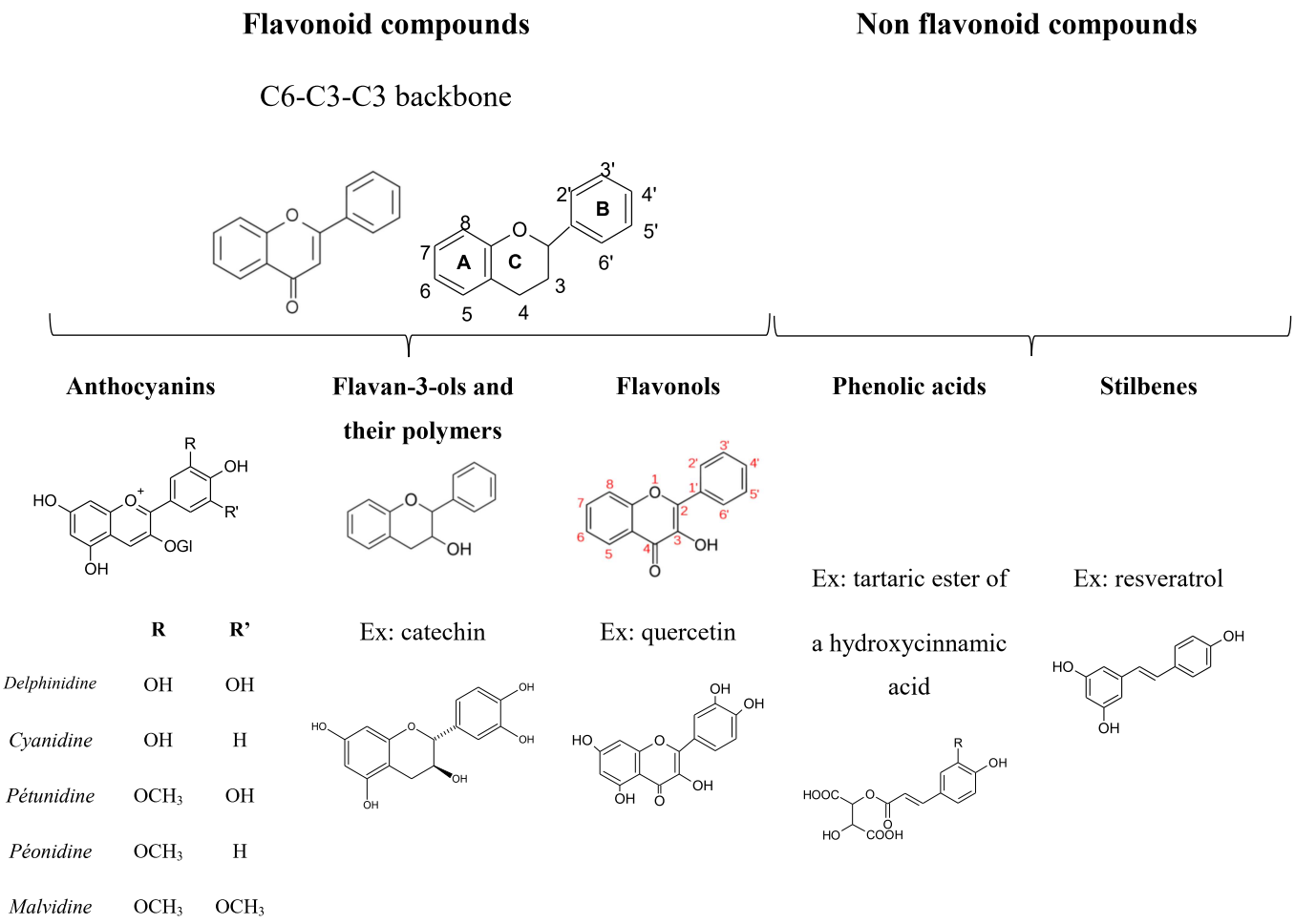


Figure 14. Phenolic coumpounds in grapes and wines

III.1 Non Flavonoid compounds

Non-flavonoid phenolic compounds in grape are phenolic acids and stilbenes. They are located in the vacuoles of the pulp and skin cells. Phenolic acids are divided into two main groups: the benzoic acid and the hydroxycinnamic acid groups.

Hydroxycinnamic acids are characterized by a C6-C3 skeleton. In grapes and wines, they are mainly found under the form of esters of tartaric acid: caftaric acid, *p*-coutaric acid and fertaric acid (Flanzy, 1998). They can be found in trans and cis configuration, however the trans is predominant due to its stability.

Benzoic acids are characterized by a C6-C1 skeleton. One of the most commonly found in high concentrations in wine is gallic acid (Flanzy, 1998). Benzoic acids are involved in enzymatic oxidation phenomena that lead to the browning of grape juices (Singleton, 1987).

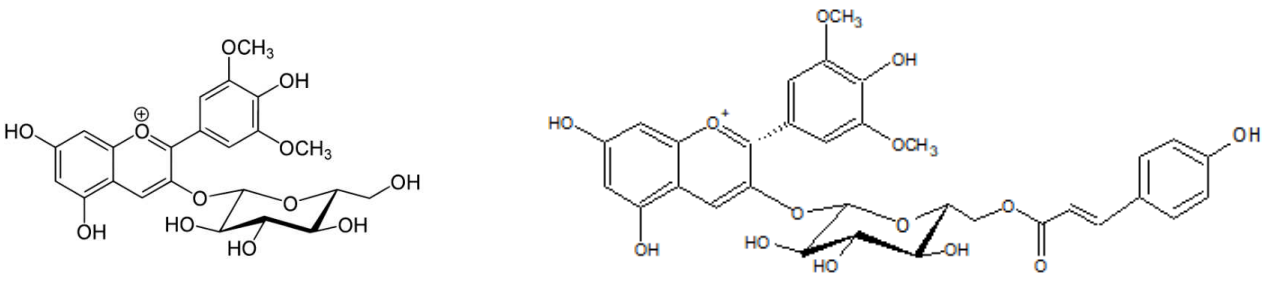
Stilbens are biosynthetised in grape as a defense response to stress such as UV radiation or microbial infection. They are characterized by two aromatic rings linked by a double bond (C6-C2-C6). The most abundant is transveratrol and its glycosylated derivative, piceid (Waterhouse & Lamuela-Raventos, 1994), mainly located in the grape skins.

III.2 Flavonoids

Flavonoids are characterized by a 15-carbon (C6-C3-C6) base skeleton corresponding to the structure of 2-phenyl-benzopyrone. In grapes and wine, we find mainly anthocyanins (red pigments) and flavan-3-ols monomers and polymers (tannins), to which are added the families of flavonols (yellow pigments) and flavanonols. Anthocyanins and flavanols are the major phenolic compounds found in red wines. These compounds have two phenolic rings (A and B) linked together by a heterocyclic pyran ring (C-ring). They are distinguished by the oxidation degree of the pyran ring.

III.2.a) Anthocyanins

Anthocyanins are the red pigments responsible for the red colour in grapes and wines. They consist of two phenolic rings A and B linked by a flavylium heterocycle. The variation in the degree of hydroxylation or methoxylation (R and R‘) of the B ring leads to the five aglycones, or anthocyanidins found in Vitis: delphinidin, cyanidin, petunidin, peonidin and malvidin (Figure 14). The aglycone is the chromophore group of the pigment. These latters are linked to one or two glucoses known as 3-monoglucoside or 3,5-diglucoside anthocyanins. The sugars best known to be bound to anthocyanidins are glucose, rhamnose, arabinose and galactose (Mazza & Miniati, 1993). Glycosylated malvidin is the most common anthocyanidin in *Vitis vinifera*, however each variety has its own composition. Anthocyanins are also present in grapes in acylated form: glucose can be acylated in position 6 by acetic acid, cafeic acid or *para*-coumaric acid (Figure 15). Anthocyanins are located in the skin upper cellular layers of the hypodermis, specifically in the vacuoles of the skin cell wall of red grapes in a free noncomplex form (Amrani Joutei *et al.,* 1994).



a) b)

Figure 15. Structures of a) malvidin-3-glucoside, b) malvidin-3-coumaroyl glucoside

III.2.a.i Equilibrium of anthocyanins in aqueous medium

In solution, anthocyanins exist in various equilibrium forms depending on the pH (Figure 16 and 17). The flavylium forms (A^+), red colored, are predominant only in acidic medium and are subject to deprotonation and hydration reactions as pH increases (Brouillard & Dubois, 1977). The proton exchange kinetic is carried out rapidly. It leads to quinonic forms (AO) of violet color. However, it is the colorless hemiacetal forms (AOH) that predominate during the increases of pH. Thus, at the pH of the wine (pH=3.5), the flavylium forms represent only between 10 and 30% (Ribéreau-Gayon, 1982), while the colorless form represents between 60 and 80% of the forms in equilibrium. The colourless hemiacetal form (AOH) by a tautomerization reaction (cycle opening) gives the chalcone (pale yellow in cis and trans forms).

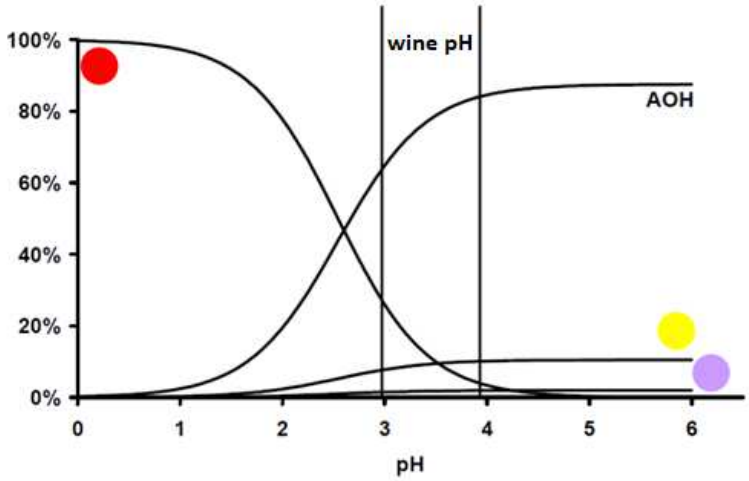


Figure 16. Forms of malvidin-3-O-glucoside in equilibrium as a function of pH (Brouillard & Dubois, 1977)

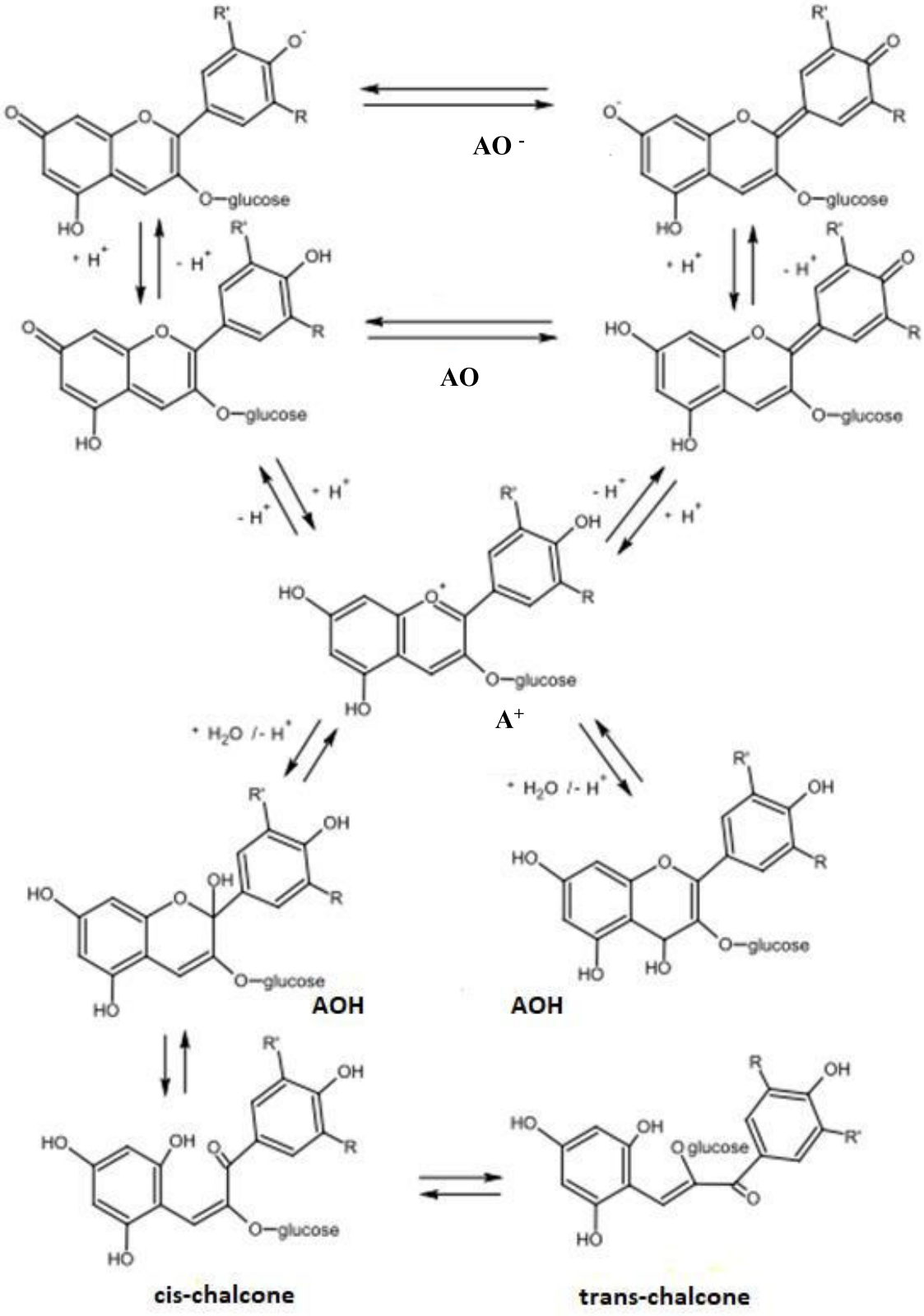


Figure 17. Forms of mavidin-3-O-glucoside in equilibrium in acid solution. A^+=flavylium cation (red), AO=quinone base (violet), AO^{--}=anionic quinone base (blue-violet), AOH=hemiacetal form (colorless), cis-chalcone and trans-chalcone (pale yellow) (Brouillard & Dubois, 1977)

III.2.a.ii Copigmentation

- Intermolecular copigmentation

It results from the vertical stacking between the electron-rich flat part π of the copigment and the coloured forms of the anthocyanin (Brouillard *et al.,* 1989; Cai *et al.,* 1990; Dangles & Brouillard, 1992a; Dangles & Brouillard, 1992b).

Unlike colorless forms, the colored forms (A+, AO) have rather flat structures with a strong delocalization of the electrons π allowing a stacking of the type π- π with the copigment. Copigmentation is therefore a selective phenomenon of colored shapes. The copigment competes with water to interact with the flavylium chromophore and shifts the hydration equilibrium towards the formation of the flavylium cation resulting in an intensification of color (Brouillard & Dangles, 1993).

This molecular association results in a hyperchromic and bathochromic effect on the absorption in the visible. The hyperchromic effect is essentially due to the increase in the concentration of colored molecules while the bathochromic effect can be explained in part by a decrease in the polarity of the medium of the flavylium chromophore due to its hydrophobic association with the copigment.

This phenomenon would be the first step in the reactions between anthocyanins and tannins. (Brouillard & Dangles, 1994)

- Intramolecular copigmentation

It involves two parts of the same molecule: one plays the role of copigment, the other being the chromophore (Goto & Kondo, 1991; Brouillard & Dangles, 1993). For example, the phenolic nucleus of the acylated part substituting glucose and the chromophore of the acylated anthocyanins: The intramolecular copigmentation result in anthocyanin folding on itself. This will allow the chromophore to be surrounded by the phenolic nucleus, thus stabilizing the anthocyanin.

III.2.b) Flavan-3-ols or flavanols

Flavanols are commonly called flavan-3-ols due to their hydroxylation in the third position. They are found as monomers, oligomers or polymers. They contribute to wine taste, especially astringency and bitterness, and are also involved in the formation of oxidative browning and of hazes. They are mainly localized in the skin and the seed of grape berries. Only traces have been detected in the pulp (Kennedy *et al.,* 2001). The main monomers in grapes are catechin (C), epicatechin (EC), epigallocatechin (EGC) and epicatechin-3-gallate (ECG). They differ by the presence of galloyl substitute on the carbon C2 of the C-ring and by the hydroxylation degree of the ring B, Figure 18.

Condensed tannins are oligomers or polymers of flavan-3-ols units linked via interflavan bonds between C-4 and C-8 and less commonly between C-4 and C-6 (Cheynier *et al.,* 1997; Kennedy *et al.,* 2000a; Downey *et al.,* 2003). In addition to the nature of the constituent units the tannins differ in the number of units, called degree of polymerization (DP), and in the type and position of the inter-monomeric bonds. Proanthocyanidins of type B are characterized by an inter-monomeric bond between carbon 4 (C4) of the upper unit and carbon 6 (C6) or carbon 8 (C8) of the lower unit, of trans configuration in regards to the hydroxyl in the C3 position. Proanthocyanidins of type A have an additional ether linkage between the C2 carbon and the hydroxyl 5 or 7 of the lower A nucleus (Figure 18).

Condensed tannin composition differs between grape seeds and skins, with seed tannins generally having a higher concentration of galloylated subunits, while skin tannins contain epigallocatechin subunits, which are generally not observed in seed tannins (Cheynier *et al.,* 1997, Downey *et al.,* 2003, Kennedy & Taylor, 2003). The mean degree of polymerization (mDP) of seed tannins is in order of 10. On the other hand, skin tannins consist of the units catechin, epicatechin and epigallocatechin (prodelphinidin). Their mean degree of polymerization, about 30 units (Souquet *et al.,* 1996), is much higher than that of the seed tannins. Furthermore, they have lower proportions of gallates than seed tannins.

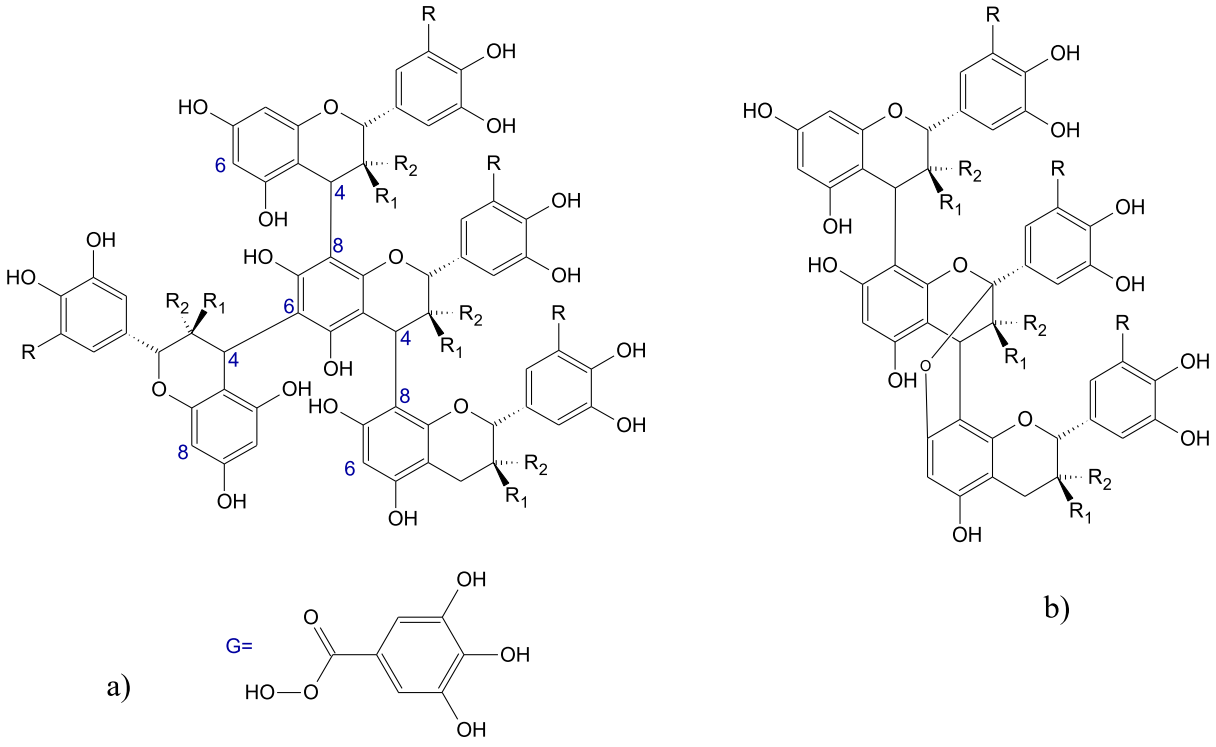


Figure 18. Examples of a) condensed tannins made up of catechin units C (R=H, R_1=OH, R_2=H), epicatechin E (R=H, R_1=H, R_2=OH), epicatechin gallate ECG (R=H, R_1=H, R_2=OG), epigallocatechin EgC (R=OH, R_1=H, R_2=OH). Condensed grape tannins are linked together by C4-C8 (majority) or C4-C6 carbons (a), or (b) by a type A bond (i.e. two monomers linked by a C4-C8 bond and a C2-O-C7 ether bond).

Skin and seed tannins have also different locations: skin tannins are found to be mainly located in the different cell wall compartments: vacuolar as a free compound, bound to plasma membrane as well as bound to the skin cell wall. Seed tannins are located in the inner and outer shells (Cadot *et al.,* 2006).

III.2.c) Flavonols

Flavonols are the yellow pigments found only in the skins and leaves of the grape berry. They are characterized by an absorption maximum at a wavelength of 360 nm. They are in the form of mono and diglycosides in position 3. The glycosyl forms are the most abundant. Other sugars encountered are galactose, xylose, arabinose, especially in the terminal position of the diglycosides. Four glycosyl flavonols derived from four aglycones (kaempferol, quercetin, myricetin and isorhamnetin) are mainly present in grapes and wines.

Part B: Winemaking: From berries to red wines

In the previous section, we detailed the structure of the grape berry and its biochemical composition. In part B, we will discuss the winemaking process and the parameters that can have an impact on the extraction of phenolic compounds during maceration.

I- Red winemaking process

Wine is defined by the International Organisation of Vine and Wine (OIV) (CE n°1493/1999) as: “Wine is the beverage resulting exclusively from the partial or complete alcoholic fermentation of fresh grapes, whether crushed or not, or of grape must. Its actual alcohol content shall not be less than 8.5% vol”. Winemaking is described as the succession of spontaneous or induced transformations of the grape must into the wine. These transformations combine simultaneously fermentative (biological) and non-fermentative (physico-chemical and chemical) phenomena.

Red wine is an acidic (pH between 3 and 4) hydro-alcoholic solution with a complex composition. The second most abundant constituent of wine after water is ethyl alcohol, which, according to the type of wine, varies from 8% to 15% (v/v) or more. In dried red wines, the dry extract represents between 20 and 30 g/L. It is composed of different families of compounds including organic acids, salts, oses (monosaccharides, oligosaccharides, and polysaccharides), nitrogen compounds (amino acids, peptides, proteins), polyphenols (phenolic acids, anthocyanins, tannins, and stilbenes), and aromas. Among these various constituents, anthocyanins and tannins, mainly present in skins and seeds, are the molecules most directly responsible for the color and mouthfeel of red wines. Their extraction from the grape berry is therefore one of the objectives of the winemaking process. This extraction is carried out during the maceration step of the winemaking process, in other words, during the contact between the must or wine with the solids parts of the berry.

The different steps of a traditional red winemaking process are summarized in Figure 19. At first, the grapes are sorted (elimination of undesirable grapes and foreign bodies) and destemmed (separation of the berries from the stalk). The berries are then crushed before being sent to the fermentation/maceration vat. Crushing is a mechanical process that consists of bursting berries to favor the extraction of the juice from the vacuole of flesh cells. Flesh cells are relatively easily broken up due to their relatively thin cell walls and juice release starts during crushing and tank filling. In contrast, skins are much more resistant to mechanical disruption. SO_2 is usually added during the prefermentative step to prevent oxidation (chemical oxidation and inhibition of enzymes) and the development of undesirable micro-organisms (selection between yeasts and bacteria). For a healthy harvest, the doses used are of the order of 30-50 mg/L.

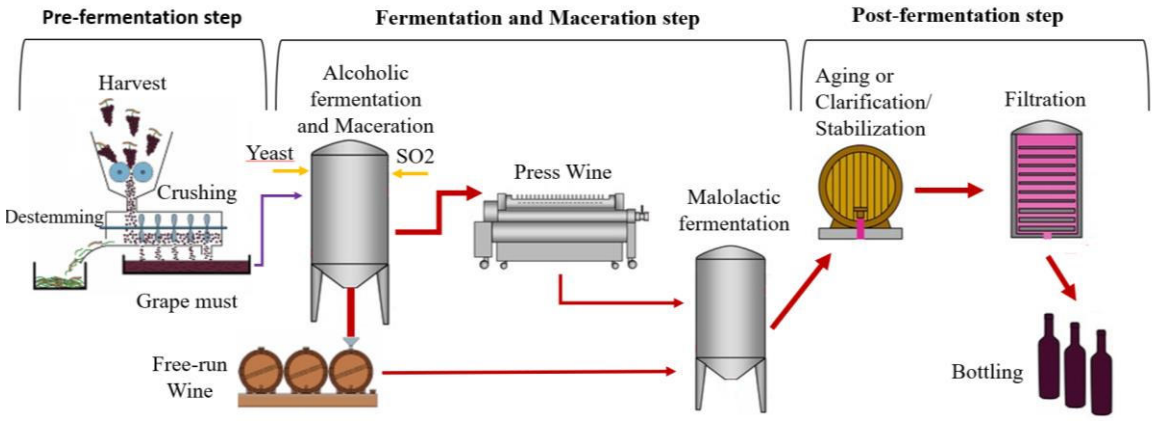


Figure 19. Schematic representation of a classic red wine making process.

The first biological transformation is the alcoholic fermentation (AF) of the must by the yeasts *Saccharomyces cerevisiae* at temperatures between 20 and 28°C. Fermentation can be carried out by indigenous yeasts but most often winemakers use commercial selected strains. During this process, fermentable sugars in the must are converted to ethanol, carbon dioxide, and other by-products (glycerol, organic acids, higher alcohols, esters, and ethanal). The total duration for a complete fermentation varies from 4 to 20 days. In parallel, the constituents of solid parts are extracted progressively by maceration. Quite rapidly, the CO_2 release related to fermentation induces the separation of the solid parts (skins, cell debris, seeds, …) from the whole fermenting juice and the formation of a cap at the top of the tank. This largely restricts the contact area between solids and juice and thus extraction. To promote diffusion and extraction, it is necessary to wash or break this cap and to homogenize the juice. Homogenization is obtained by different techniques (punching down, pumping over, submersion, …), implemented and adapted according to the desired extraction level.

When the extraction is sufficient, the free-run wine is collected by gravity at the bottom of the tank while the pomace is pressed to give the press wine. Press and free-run wine, whether mixed or not, are then placed in other tanks for malolactic fermentation. This latter is an optional step carried out by *Oenococcus oeni* lactic bacteria, which convert L-malic acid into L-lactic acid and carbon dioxide. This helps to reduce the acidity of the wine and enhances its aromatic complexity. After fermentations, the wine can be aged in tanks or barrels, depending on the characteristics wanted. The objective is to favor transformations leading to a modification of the structure and the olfactory and gustatory characteristics of the wine. Wines are then clarified and stabilized before bottling.

The maceration and extraction of polyphenols from the skins and seeds is a key stage in the production of red wines. This extraction is dependent on the cap-management practices, on the temperatures, and the maceration length. However, it is only partial and not proportional to initial grape contents, influencing the wine polyphenol profile and therefore leading to a different quality of wines. Several authors report this lack of correlation between the berry and the final wine composition, highlighting a variety effect (Adams & Scholz, 2008; Ortega Regules *et al.,* 2006), and a ripening effect (Amrani & Glories, 1994). This is attributed to different factors, among which are physicochemical interactions of polyphenols with other soluble and insoluble compounds in the medium. All of these potential factors will be discussed below.

II- Existing Models of the extraction of polyphenols during wine-making

When we talk about the extraction of polyphenols during winemaking, it is the quantity of a given molecule or class of molecule (e.g. anthocyanins or tannins) that is found in the final product in relation to what was in the raw material. What is extracted is what diffuses mainly through the skin, pulp, and seed cells, minus what is re-adsorbed on solid parts (grape cell walls, yeasts, membranes…), due to favorable interactions between polyphenols and molecules of these solid parts, minus what precipitates, and minus what is chemically modified.

Due to the localization of phenolic compounds in the solid part of the berries, the phenomenon of diffusion must be considered first. Diffusion is a mass transfer process by which a compound moves from a medium of high concentration to a medium of lower concentration (i.e. from the plant cell to the juice or wine). When dealing with plant tissues, mass transfer mechanisms are difficult to model because of their complex morphology and to the localisation of components of interest in these tissues. Depending on the family of polyphenols considered (anthocyanins, tannins), it can be modelled in a more or less simple way.

II.1 Anthocyanins

Anthocyanins diffuse rapidly due to their solubility. If their extraction was simply based on their diffusion from grape skins, an exponential approach to the final level would be expected. This is not observed in practice. During red winemaking, anthocyanin concentration peaks after a few days of extraction and then decreases, when their rate of disappearance exceeds their rate of diffusion (Figure 20).

This process has been described by a two-term extraction model with an initial fast extraction followed by a slow decrease to its final value (Boulton *et al.,* 1996; Somers & Evans, 1979) and described by Equation 1:

$$\frac{d[A]}{dt} = k_1[A_1 - A] - k_2[A - A_2] \qquad \text{Equation (1)}$$

where [A] is the concentration of anthocyanins at time t, A_1 and k_1 are the maximum value and rate constant for the initial irreversible extraction, and A_2 and k_2 are the equilibrium value and rate constant for the second equilibrium stage resulting from a series of physical and chemical reactions. The wide variation of the different constants (diffusion rate and equilibrium concentrations) suggests that they are not only related to molecular diffusion and indicates that other factors are involved in the extraction of total anthocyanins from grapes to wine such as the chemical changes that will be described in section III.3.

II.2 Tannins

Skin tannins are extracted with the anthocyanins at the beginning of the maceration, but extraction continues for a longer period, due to their location in the skin cells. In addition, tannins are also extracted from seeds. Modelling of tannin concentration during winemaking indicates there is a diffusion process dependent on the concentration of tannin already in the wine as well as a dissolution process that is independent of concentration. Experimental data can be accurately fitted with a two-term extraction model with first and zero-order terms (Boulton *et al.,* 1996) (Figure 20)). Mathematically, this two-term extraction model can be represented by Equation 2 :

$$\frac{d[T]}{dt} = k_3[T_1 - T] + k_4 \qquad \text{Equation (2)}$$

where [T] is the concentration of tannin in the wine at time t, T1 is the equilibrium concentration of the diffusion extraction step, and k_3 and k_4 are the rate constants for the diffusion and dissolution steps, respectively. The apparent existence of two separate extraction processes has been attributed to tannin being extracted from both the grape skins and the seeds (Boulton *et al.,* 1996). Phloroglucinolysis, which is an analysis of the depolymerized tannins confirmed that the diffusive term could be attributed to extraction from the skins and the dissolution term attributed to extraction from the seeds (Cerpa-Calderon & Kennedy, 2008). Gonzalez-Manzano *et al.,* (2004) found a similar pattern when extracting flavan-3-ols from grape skin and seeds into a 12.5% ethanol solution. However, it appears that the extraction of tannins is highly variable, depending on the variety, so that measurement of the total tannin content in grape may not always be useful for predicting their content in the resulting wine.

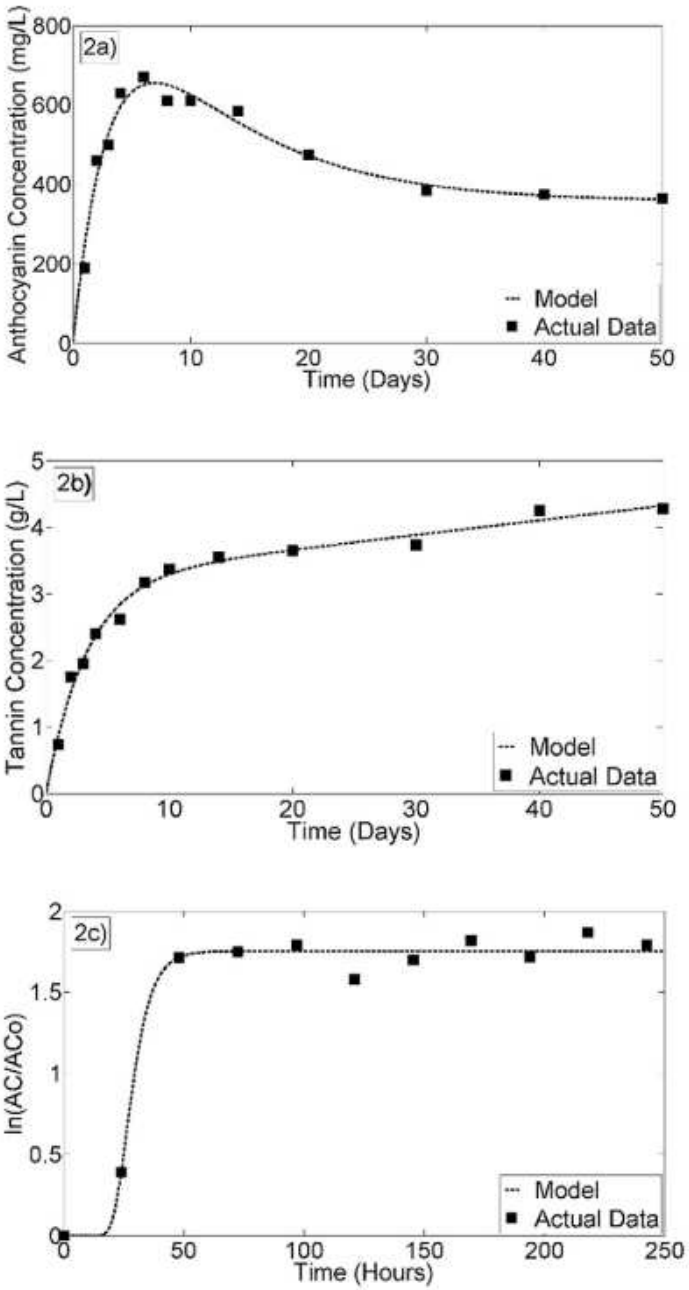


Figure 20. Examples of commonly used regression models to fit red wine maceration data for the evolution of (a) anthocyanins and (b) proanthocyanidins. (c) formation of polymeric pigments: AC = derived pigment content at time t, AC_0 = derived pigment content at t= 0). (Setford *et al*., 2017)

In summary, anthocyanins diffuse more rapidly than skin tannins, which themselves diffuse more rapidly than those coming from the seeds. The extraction of tannins from the seeds starts during the maceration phase when the alcohol content increases. The reason for seed tannin extraction presenting itself as a dissolution process (or one which exhibits an initial lag phase) is likely due to the required disorganisation of the outer lipidic cuticle surrounding the seeds, which is assisted by higher alcohol concentrations (Hernandez- Jimenez *et al.,* 2012).

II.3 Derived Pigments

In addition to the evolution of anthocyanin and tannin concentrations, an increase in derived pigments is also observed during fermentation. This increase of derived pigments is not the result of solid-liquid extraction but that of chemical changes. Different types of derived pigments are formed, as summarized in Figure 21. An important group is oligomeric and polymeric pigments formed by direct addition reactions between anthocyanins and tannins (T-A adducts) (Cheynier *et al.,* 2006; Singleton & Trousdale, 1992). Oligomeric and polymeric pigments may also be formed by acetaldehyde-mediated condensation reactions (T-ethyl-A adducts, A-ethyl-A adducts). T-ethyl-A pigments are usually unstable in wines and evolve towards flavanyl-pyranoanthocyanins. Another important group, formed through reactions involving yeast metabolites, are pyranoanthocyanins and phenylpyranoanthocyanins. The former result from reactions between anthocyanins and acetaldehyde (pyranoanthocyanins) or pyruvic acid (carboxypyranoanthocyanins), whereas phenyl-pyranoanthocyanins result from reactions of anthocyanins either with hydroxycinnamic acids or with vinylphenols (Fulcrand *et al.,* 1998 ; Fulcrand *et al.,* 1996 ; De Freitas *et al.,* 2011). These derived pigments, which are more resistant to SO_2 bleaching and oxidative degradation than grape anthocyanins, are very important to maintain wine colour in the long term. Their formation continues during wine ageing and contributes, beyond a stabilisation of the colour, to an evolution of the hue. As well, the decrease in astringency that occurs during aging has also been attributed to the formation of polymeric pigments (Cheynier *et al.,* 2006). It is important to note however that chemical reactions such as oxidation and condensation reactions also occur with tannins (Guyot *et al.,* 1996 ; Kusano *et al.,* 2007 ; Tanaka *et al.,* 2005; Fulcrand *et al.,* 1996) , without involving anthocyanins, and that these reactions modify interactions between polymeric tannins and proteins (Mac Rae *et al.,* 2010).

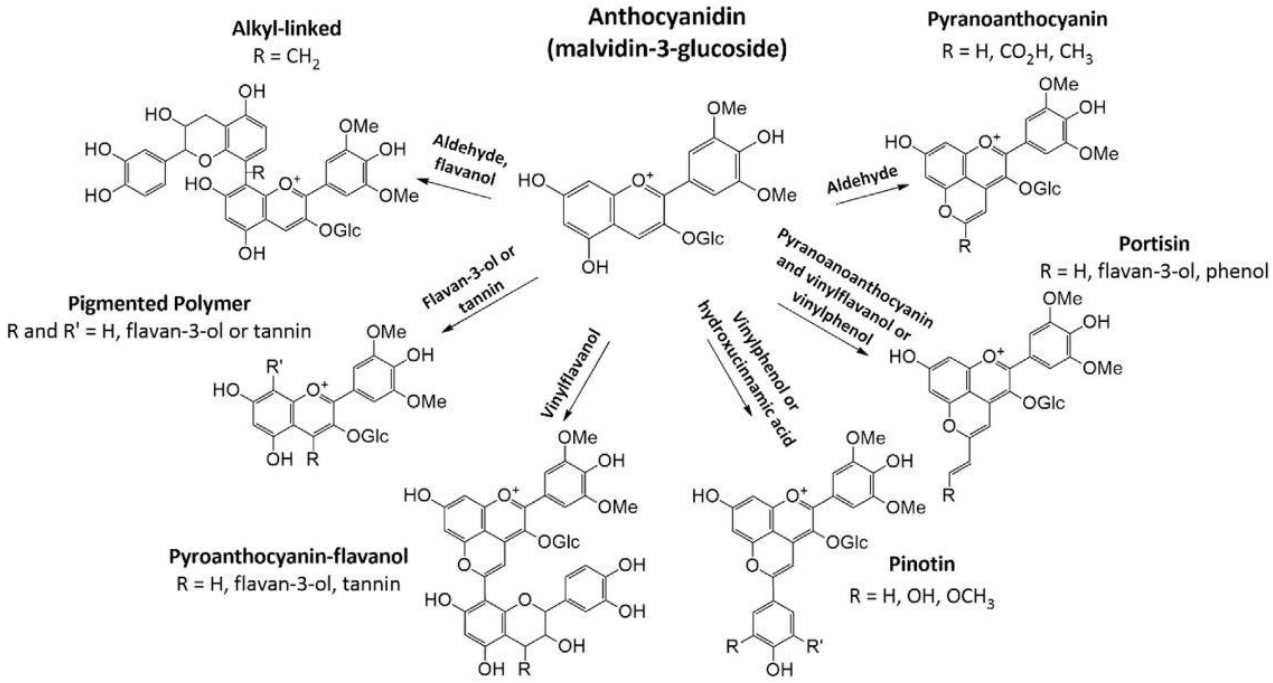


Figure 21. Anthocyanin reactions occurring during the winemaking process. Adapted from Setford *et al.,* 2017

Modelling the extraction and transformation of anthocyanins, Zanoni *et al.,* (2010) found that the formation of derived pigments during fermentation can be described in three phases: a lag phase, an exponential formation phase, and a stationary phase. The kinetics of this reaction can be mathematically described by a sigmoidal kinetic model described Figure 20c.

These kinetic models are very effective at fitting experimental data and are useful for giving an insight into the effect of changing process conditions by comparing the kinetics obtained over a series of fermentations. However, they are limited to quantitatively predict the effect that changing process conditions such as temperature, solvent concentration, mixing operations, and subsequent phenomena (reactions but also interactions) have on the extraction and transformation kinetics of polyphenols and their final concentrations in wine. These transformations are not only related to chemical changes but also interaction phenomena. The main factors that affect the phenolic composition in wines at the end of the fermentation/maceration steps are summarized in Figure 22 and further discussed below.

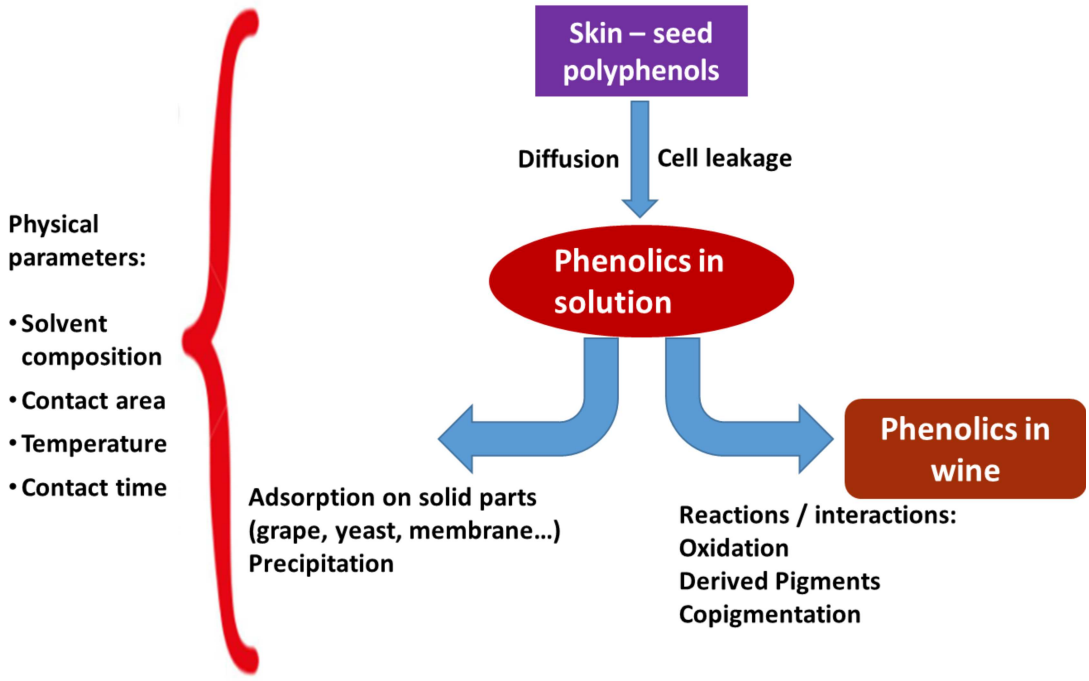


Figure 22. Summary of factors affecting phenolic concentrations during winemaking**.**

III-Factors affecting the phenolic composition in wine

During winemaking, the phenolic compounds are extracted from the solid parts to the must in two stages: a very rapid initial leakage after crushing the grapes, through the broken cell walls, Slower diffusion throughout the maceration process.

Generally speaking, the extraction of a solute through a porous particle in the plant is done in 4 stages (Gertenbach, 2001):

1. Solvent diffusion into the porous solid;
2. Solute dissolution into the solvent;
3. Dissolved solute diffusion to the particle surface;
4. Dissolved solute diffusion from the particle surface to the surrounding solvent.

Step 1 is dependent on the solvent composition, that is to say here its ethanol concentration and the presence of SO_2. Step 2 depends on the chemical nature of the extracted compound and the solvent and step 3 on the interactions between the solute and the walls of the skin or seed tissues (themselves dependent on the solvent and the structure of the cell walls). Step 4 is impacted by the mixing conditions as well as by the chemical reactions undergone by the extracted compounds, their re-

adsorption on other solid surfaces (flesh cell walls, yeast surfaces …), the formation of soluble or insoluble complexes with other molecules.

In summary, the extraction is dependent:

- on the solubility of the compound to be extracted (polyphenol / solvent interactions);
- on solute / "solid" interactions (skin and pulp cell walls, yeasts,…);
- on polyphenols / soluble macromolecules interactions (proteins, polysaccharide, etc...).

All of these parameters are themselves dependent on the composition of the solvent (increasing from 0% ethanol to 12-15% ethanol during fermentation), the temperature, and the surfaces in contact. In the following sections, we will discuss first the interactions and the physical parameters that rule them, and then the chemical reactions that are likely to modify the final concentration of polyphenols in wine.

III.1 Interactions of wine macromolecules

III.1.a) Intermolecular forces

Different intermolecular forces exist between atoms and/or molecules. Their energy and their evolution as a function of the distance of separation R are summarized in Table 2 and compared to those of covalent bonds. Their origin is detailed below.

Table 2. Energy and distance of intermolecular forces. The range of energy of covalent bonds is given as a reference**.**

Force	Energy (kJ/mol)	Dependance/R
Ionic	400-4000	1/R
Ion-dipole	40-600	$1/R^2$
H bond	10-40	~200 pm*
Dipole-dipole	5-25	$1/R^3$
Dipole-Induced dipole (Debye)	2-10	$1/R^6$
Dispersion (London)	0.05-40	$1/R^6$
Covalent bond	150-950	70 – 270 pm*
pm = picometers		

The hydrogen bonding interaction energy is much lower than that of ion-ion or ion-dipole forces. However, it is higher than the van der Waals forces. This strong attraction force can line up nearby molecules. H-bonds develop between a molecule hydrogen bond donor and another hydrogen bond acceptor such as water, ammonia, hydrofluoric acid. The water molecule is used as an example. Many possibilities exist to connect two molecules of water. Indeed, there are two lone pairs of electrons on the oxygen atoms and then the possibility to make two bonds between two molecules. Energetically, the most favorable structure is presented in Figure 23. It is much stable than others due to the orientation of the molecules and underlines the directional behavior of the hydrogen bond.

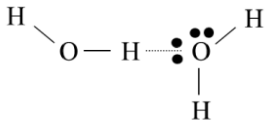


Figure 23. H bond between small molecules (example of water)

Interactions between water molecules are stronger than those of other polar molecules. In the presence of small apolar molecules, water molecules adopt a more ordered configuration than in the bulk. The orientation of water around apolar molecules or in the vicinity of an apolar surface is thus entropically very unfavorable since it disrupts the existing structure of liquid water and requires a more ordered structure. This hydrophobic effect is responsible for a strong attraction between apolar molecules when they are immersed in water, called “hydrophobic” attraction. This interaction, which is difficult to quantify, occurs for separation distances larger than those of the other forces described before. It plays a central part in numerous surface or colloidal phenomena, in the conformation of macromolecules such as proteins, in molecular/macromolecular assemblies …

By contrast, some groups or molecules are highly soluble in water and repel strongly each other in that solvent (“hydrophilic” interaction). Hydrophilic molecules or groups are not necessarily charged or polar. It depends on their geometry and if they contain electronegative atoms capable of forming H-bonds with the H-bond network of water.

In the following parts, we will present the interactions that can occur between polyphenols and other compounds of interest in oenology.

III.1.b) Polyphenol Interactions with cell walls.

III.1.b.i Interactions of tannins

Overall, there are two classes of macromolecules likely to interact non-covalently with phenolic compounds: polysaccharides and proteins. In addition, polyphenols can interact with themselves (copigmentation, stacking). In the context of this study, we will not deal with covalent bonds, which are a minority compared to non-covalent (Rustioni *et al.,* 2014).

The amount of tannins that can be extracted from the grape in given solvent and temperature conditions depends on their association with cell wall material such as proteins and polysaccharides through non-covalent hydrogen bonds and hydrophobic interactions. These interactions lead to a reduced extractability (Amrani Joutei *et al.,* 1994; Hanlin *et al.,* 2010; Hazak *et al.,* 2005*;* Fournand *et al.,* 2006; Le Bourvellec *et al.,* 2004; Bindon *et al.,* 2010 ; Ortega-Regules *et al.,* 2006 ; Ruiz-Garcia *et al.,* 2014; Le Bourvellec & Renard, 2005).

Indeed, tannins are amphipathic molecules having both hydrophobic aromatic rings and hydrophilic hydroxyl groups allowing them to bind simultaneously at several sites on the surface of other molecules (Baxter *et al.,* 1997 ; Haslam, 1998). Cell wall polysaccharides also contain hydroxyl groups as well as glycosidic oxygen atoms. This mechanism is thought to involve the formation of hydrogen bonds between hydroxyl groups of tannins and the oxygen atoms of the glycosidic linkages that interconnect individual monosaccharide residues of cell wall polysaccharides (Le Bourvellec *et al.,* 2004, Le Bourvellec & Renard, 2005), or between hydroxyl groups of tannins and hydroxyl or acetyl groups of polysaccharides. Hydrophobic bonding has also been reported. These interactions are influenced by ethanol, which decreases solvent polarity in an aqueous environment. This leads to disruption of hydrophobic interactions, and thus decreases tannin/cell wall interactions (Le Bourvellec *et al.,* 2004).

The interaction of condensed tannins with cell wall material is also influenced by the nature of the tannins structure involved. It increases with the molecular degree of polymerization and galloylation (Fournand *et al.* 2006; Bautista-Ortín *et al.,* 2014; Bindon *et al.,* 2010, 2011, 2012 ; Le Bourvellec *et al.,* 2004). An exception has been found for very high molecular mass tannins from the skins of red grapes, which appear to be excluded from binding events when cell wall porosity is low (Bindon *et al.,* 2010; Bindon & Kennedy, 2011; Bindon *et al.,* 2012). The adsorption of higher molecular weight tannins may be enhanced by an increased porosity of the cell wall, and thus reduce their extractability.

The binding capacity of the cell wall may also be dependent on the affinity tannins have for different polysaccharides. Using apple cell walls, Renard *et al.,* (2001) showed that tannins had a greater affinity for pectic polysaccharides than xyloglucan and the least affinity for cellulose (Renard *et al.,* 2001).

Among pectic polysaccharides, the acidic ones are able to form stronger hydrogen bonds than the neutral polysaccharides. In addition to the hydrogen bonds, the gel network structure of pectic polysaccharides has hydrophobic domains that may also encapsulate complex tannins more strongly than xyloglucan. The effect of methylation on interactions tends to confirm this: highly methylated homogalacturonans have higher affinities for tannins (Le Bourvellec *et al.,* 2012; Watrelot *et al.,* 2013) . This ranking (pectic polysaccharides interact more than hemicellulosic polysaccharides) was confirmed with grape berries by a study of (Ruiz-Garcia *et al.,* 2014) in a model adsorption experiment with a purified grape skin cell wall.

Only a few studies dealt with the impact of fresh grape mesocarp cell walls on the extraction and composition of tannin under wine-like conditions (Hazak *et al.,* 2005; Bindon *et al.,* 2017). In Cabernet Sauvignon berries, they found that the skin cell-walls and mesocarp material adsorbed more than 22% of tannins present in the grape (Hazak *et al.,* 2005). Bindon *et al.,* 2017 found that the adsorption of tannin by mesocarp facilitated the ongoing extraction of tannin from the skins: seed tannins were selectively adsorbed by mesocarp, and this resulted in higher proportions of skins tannins remaining in wines.

III.1.b.ii Interactions of Anthocyanins

Anthocyanins interact rather less than tannins, either with purified walls or with fresh mesocarps (Bindon *et al.,* 2010, 2014). Adsorption on cell walls depends on the variety (Ortega-Regules *et al.,* 2006), and among other things on the pectins, their degree of methylation, cellulose, and glucan composition of the cell walls. However, only a few differences in uronic acids, neutral sugars, cellulosic glucose, and proteins could be detected.

Interactions are also dependent on the anthocyanin chemical structure. Favre *et al.* (Favre *et al.,* 2019) highlighted the impact of the B-ring substitution pattern of anthocyanins: during winemaking, the *p*-coumaroylated anthocyanins showed the lowest extractability from the skin to wine for the three varieties they worked with.

III.1.c) Impact of the polyphenol composition

It is said in the literature that the anthocyanins/tannins ratio plays an important role in the extraction of phenolic compounds. Working with four batches of Shiraz berries differing by their

anthocyanin/ tannin ratio, Kilmister *et al.,* 2014 found the one with a higher ratio leads to higher tannin extraction during winemaking. It was suggested that anthocyanins may influence interactions between tannins and skin cell walls. It was also suggested that this would be related to the fact that anthocyanin might also increase tannin solubility in the wine and therefore extraction from the solid part.

III.1.d) Interactions with yeasts

Phenolic compounds (such as tannins and anthocyanins) are amphiphilic and able to bind with other molecules with hydroxyl and aromatic (or aliphatic) groups, such as cell wall polysaccharides. *Saccharomyces Cerevisiae* yeast cell walls are composed of exposed mannoproteins bound to oligo/polysaccharides (with different polarities depending on the strain), allowing them to adsorb molecules such as tannins, anthocyanins, and other volatile compounds that are released during the maceration stage of winemaking (Morata *et al.,* 2003). In addition to studies conducted on the adsorption of components responsible for quality attributes (anthocyanins and tannins) by different yeast strains (Lubbers *et al.,* 1994; Morata *et al.,* 2003; Razmkhab *et al.,* 2002, Mekoue *et al.,* 2015) some investigation has been undertaken by Vasserot *et al.,* (1997) on modelling the rate of decoloration at different anthocyanin concentrations. This study demonstrated that the majority of anthocyanin adsorption takes place within the first five minutes of contact and also that strong linear correlations exist between absorbed anthocyanins and the initial anthocyanin concentration at yeast levels ranging from 3 to 30 g/L. The study also revealed that temperature and ethanol concentration strongly affected the adsorption of anthocyanins by yeast lees, with higher temperatures increasing anthocyanin adsorption and higher ethanol concentrations having the opposite effect.

III.1.e) Interactions with other soluble macromolecules

After extraction from seed, skin and pulp cells, and adsorption on solid parts, polyphenols may interact as well with soluble protein and polysaccharides and precipitate, or instead, be stabilised.

Tannins have the ability to interact with proteins. In particular interactions with the proline-rich proteins in saliva produce a sensation of dryness in the mouth, a loss of lubrication, known as astringency (Bate-Smith, 1954). This property of affinity for proteins is also used onenological processes such as protein fining where it leads to the selective precipitation of the tannins with a higher degree of polymerisation. Many studies have focused on these interactions (Haslam, 1974; Pascal *et al.,* 2008 ; Sarni-Manchado & Cheynier, 2002 ; Sarni-Manchado *et al.,*1999 ; Poncet-Legrand *et al.,* 2007 ; Brandão *et al.,* 2017 ; Brandão *et al.,* 2020). Interactions are modulated by size, charge, protein type, structure and tannin concentration as well as by the solvent. Bindon *et al.,* (2016)

highlighted the important effect of soluble components specially of mesocarp on the interaction with polyphenol and confirmed a role for grape mesocarp proteins in the precipitation of tannins.

Wine soluble polysaccharides can be divided into two main groups: those originating from the grape cell wall and those originating from yeast. The polysaccharides present in wine may also have the ability to enhance or inhibit tannin aggregation. A study by Riou *et al.,* (2002) showed that mannoproteins and acidic arabinogalactan-proteins strongly inhibited tannins aggregation, while rhamnogalacturonan II dimers strongly enhanced colloidal particle size suggesting co-aggregation between polysaccharides and tannins in wine. This suggests that polysaccharides could be used either as fining or stabilising agents to influence wine stability and organoleptic properties, depending on their structures (De Freitas *et al.,* 2003 ; Mateus *et al.,* 2004 ; Quijada-Morín *et al.,* 2014).

III.2 Impact of physical parameters

III.2.a) Composition of the solvent

During the fermentation, the concentration of ethanol increases, resulting in a decrease of the dielectric constant (or permittivity ε_r) of the solvent, which in turn has a strong impact on the intermolecular forces (Van der Waals forces, H-bond, hydrophobic interactions). The solubility and extraction of phenolic compounds are thus expected to vary upon ethanol increase. Working with apple cell walls and tannins in model systems, Le Bourvellec *et al.,* (Le Bourvellec *et al.,* 2004) showed that increasing the ethanol concentration led to disruption of hydrophobic interactions, and thus decreased tannin/cell wall interactions.

In real wines, Gonzalez-Manzano *et al.,* (2004) studied the extraction of flavan-3-ols of white grape pomace using simulated maceration of grape skin and seeds at varying ethanol concentrations (0%, 5% and 12.5% ethanol by volume) and showed a positive trend of flavanol extraction rate with increasing ethanol concentration. Nevertheless it was unclear whether ethanol concentration impacted the final equilibrium concentration of flavanols or simply the rate of extraction, because the maceration time was very short.

In red wines, Canals *et al.,* 2005 studied how different grape ripeness levels and ethanol concentrations affect the extraction of color and phenolic compounds from skins and seeds of Tempranillo Grapes at different stages of ripening during simulated maceration assays. Ripeness and ethanol percentage were found to have a significant effect on the extraction of anthocyanins and tannins. The presence of ethanol in the medium facilitates anthocyanin (Medina-Plaza *et al.,* 2019) and tannin extraction from skins and seeds (higher with skin PAs) (Sherman *et al.,* 2017). Medina-Plaza *et al.,* (2019) showed that with a high ethanol percentage (15%) the adsorption percentage of

anthocyanin to skin cell wall decreased potentially due to the increase of the solubility of the pigments in the model wine.

III.2.b) Temperature

Temperature, which usually varies between 20 and 30°C during red wine fermentation, plays a significant role in the extraction of phenolic compounds during fermentative maceration for several reasons:

i) it influences the permeability of the cell membranes in the grape solids (Koyama *et al.,* 2007);
ii) it enhances the solubility of phenolic compounds, thus affecting the internal diffusion coefficient (Cacace & Mazza, 2003) ;
iii) it impacts on the rate of fermentation (and thus the rate of ethanol production) (Boulton, 2001; Coleman *et al.,* 2007; O'Neill *et al.,* 2011).

Thus several methods are employed by winemakers to promote the extraction of phenolic compounds from the grape solids, such as thermovinification (Aguilar *et al.,* 2015; El Darra *et al.,* 2013; El Darra *et al.,* 2016; Gao *et al.,* 1997), or microwave maceration prior to fermentation (Carew *et al.,* 2014). Another heating process called flash release is used by winemakers to assist phenolic extraction. It involves heating the must at atmospheric pressure to around 95 °C before applying a strong vacuum. This results in the rapid cooling of the berry skin cell walls, which fragilizes them and results in higher tannin extraction (Doco *et al.,* 2007; Morel-Salmi *et al.,* 2006; Smith *et al.,* 2015). However, the effect of the process was cultivar dependent and when pressing was performed directly after treatment, phenolic substances were lost (Morel-Salmi *et al.,* 2006).

III.2.c) Contact surface

During fermentative maceration, the grape solids (skins, seeds, and stems) rise to the top of the fermenting vessel and form a cap (Weber *et al.,* 2002). The production of a cap results in less contact between the solid and liquid components of the must (Sacchi *et al.,* 2005), and helps insulate the system, which increases the must temperature. In order to increase the solid-liquid contact area and help maintain a constant temperature, winemakers generally “manage” the cap: they pump liquid from the bottom of the tank and spray it over the solids cap; they mechanically punch down the solids into the liquid, or they use a baffled rotary tank that assists in submerging the grape solids back into the liquid.

III.3 Chemical reactions

III.3.a) Reactions of anthocyanins

At the beginning of maceration, most of the anthocyanins are present in free (uncombined) forms. During vinification, they can react with sulfur dioxide added by the winemaker or produced by the yeasts to give colourless compounds. In addition, the oxidation of the anthocyanidins causes a loss of colour in the wine. Di-hydroxylated anthocyanidins (delphinidin, cyanidin, petunidin) are the most oxidizable, which results in a more rapid decrease in their concentrations compared to those of malvidin and peonidin.

The extracted anthocyanins can react with yeast metabolites (acetaldehyde, pyruvate) or compounds formed in wine (vinylphenol, caffeic acid) to form pyranoanthocyans: an additional pyran ring is formed between the C5 and C4 hydroxyl of the starting anthocyanin. Reactions with hydroxycinamic acids (Schwarz *et al.,* 2003a; Schwartz & Winterhalter 2003) give rise to the pinotin family. Yeast metabolites such as acetaldehyde and pyruvate give rise to Vitisin A and B (Bakker *et al.,* 1997, Revilla *et al.,* 1999, Romero & Bakker 2000a; Fulcrand *et al.,* 1998; Benabdeljalil *et al.,* 2000; Mateus *et al.,* 2001).

The pyranoanthocyans formed can subsequently react with flavanols to form flavanylpyranoanthocyans. Alternatively, a vinyl-flavanol might react with anthocyanin.

Anthocyanins can also auto-associate (Boulton, 2001) or participate in direct condensation reactions with flavanols, which gives rise to orange-red pigments which modify the colour of the wine (Salas *et al.,* 2003). They can also undergo polymerisation reactions, either directly or via condensation with acetaldehyde (Atanasova *et al.,* 2002a ; Vidal *et al.,* 2004).

Last, but not least, the degradation of anthocyanins yields colorless, low molecular weight compounds such as syringic acid.

III.3.b) Reactions of tannins

Polymeric pigments are formed when anthocyanins and tannins react to form more stable compounds (Cheynier *et al.,* 2006 ; Salas *et al.,* 2004 ; Salas *et al.,* 2003). In the case of type A-T addition, anthocyanin plays the role of electrophile while tannin plays the role of nucleophile. In the case of T-A type reactions, which concerns only polymeric flavanols, the electrophile is a carbocation released by breaking the interflavanic bonds of the tannins which reacts with carbon 6 or 8 of an anthocyanin or another molecule of flavanols. These two types of reactions depend on the pH, and A-T species are detected only at the higher pH value.

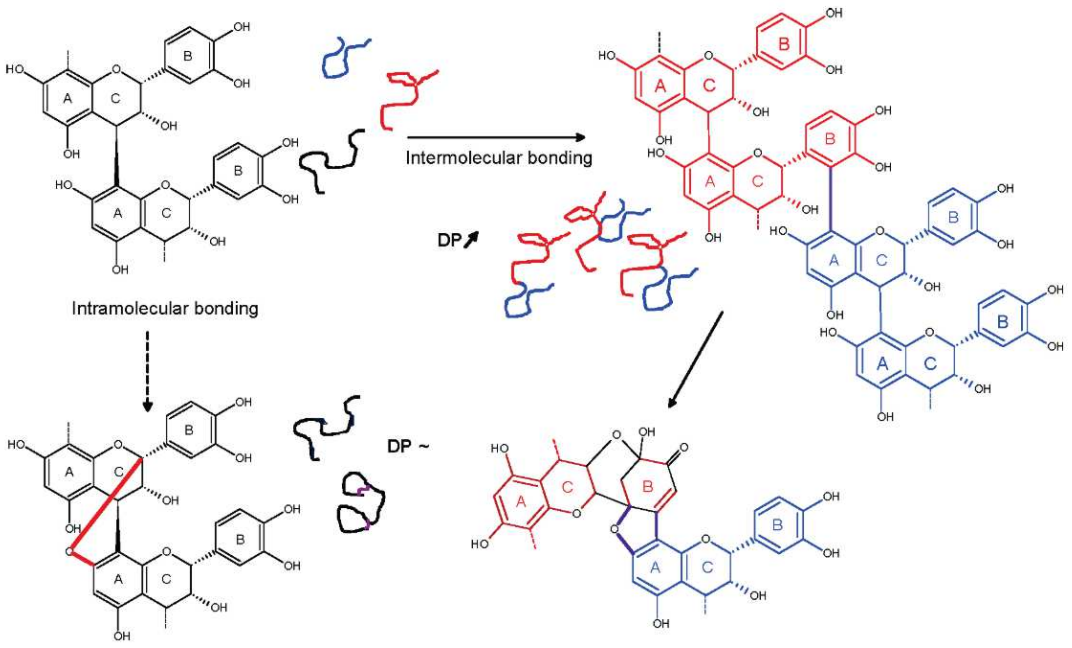


Figure 24. Examples of chemical structures of oxidized tannins. The creation of new bonds may occur on the same macromolecule (intramolecular bonding) leading to the formation of a A-type tannin, or between two macromolecules (intermolecular bonding). When intermolecular reactions occur, the new bond is formed between A and B aromatic rings. Further oxidation may lead to additional cyclization between rings A and B. (Poncet-Legrand *et al.,* 2010)

In addition to condensation reactions with anthocyanins, tannins are also involved in condensation reactions with each other and in polymerization reactions.

Flavanol autoxidation reactions have been particularly studied in the case of monomers and dimers and have revealed the existence of intra- and intermolecular reactions (Guyot *et al.,* 1996; Kusano *et al.,* 2007; Tanaka *et al.,* 2005). In the case of oligomers and polymers, these intra- and intermolecular reactions can also occur (Poncet Legrand *et al.,* 2010), Figure 24. In this case, these reactions can have different consequences depending on the competition between intra and intermolecular reactions and between terminal and extension units. In all cases, these autoxidation reactions will result in modifications of the chemical structure and conformation of the tannins, which may affect their solubility as well as their physico-chemical interaction in solution.

IV-Managing the extraction through the winemaking

The importance of phenolic extraction is defined at first by the winemaking technique (thermo-treatment of the harvest, flash release…) and by the conditions used (enzyme addition, temperature, duration of maceration, frequency, and type of homogenization process: punching of the

cap, pumping over) (Busse-Valverde *et al.,* 2010 ; Busse-Valverde *et al.,* 2012, Busse-Valverde *et al.,* 2011). Various means can be used by winemakers to overcome the obstacles in order to facilitate this extraction and obtain the desired wine profile (Gonzalez-Neves *et al.,* 2013).

Previous studies have shown that for any fermentation, time and temperature are the two critical variables that determine the final amount of polyphenols extracted in the wine. In addition, the presence of ethanol in the fermenting must facilitates anthocyanin and even more tannin extraction. Therefore, the length of maceration can determine the tannin concentration and the astringency of red wines. A short maceration will lead to wines with a low proanthocyanidin concentration and low astringency because seeds and skins have been in contact with a medium rich in ethanol for a short time. On the other hand, a long maceration will lead to wines with a high proanthocyanidin concentration and high astringency because skins and especially seeds have been in contact with a medium rich in ethanol for a long time (Canals *et al.,* 2005).

In the case of anthocyanin extraction, the use of high temperatures in the winemaking process will initially reduce the maceration time and allow maximum anthocyanin concentrations. It also decreases the association between tannins and cell wall material (Le Bourvellec *et al.,* 2004).

V-Conclusion

This “state of the art” shows that the notion of extractability of polyphenols is a complex notion. The aim of this thesis is to highlight the key parameters of this extractability and to distinguish between the different physical and chemical events that occur: diffusion, solubilisation, adsorption, precipitation, chemical reactions, as well as their impact on wine quality.

The bibliographical study has shown a significant impact of maturity and grape variety (if physical parameters are kept constant). We therefore decided to work with two grape varieties that are contrasted in terms of extractability, Grenache and Carignan, harvested at maturity but sorted according to their size and potential alcohol content.

On these freshly harvested berries, experiments of diffusion from the skins to model wines were carried out (Chapter 2), followed by studies of interactions between phenolic compounds and berries pulp cell walls (Chapter 3). In Chapter 4, a comparison was done, between the wine-like model system and wines obtained by microvinification.

Chapter 2:

Impact of grape variety, berry maturity and size on the extractability of skin polyphenols during model wine-like maceration experiments

Abi-Habib E., Poncet-Legrand C., Roi S., Carrillo S., Doco T. & Vernhet A. (2020). Impact of grape variety, berry maturity and size on the extractability of skin polyphenols during model wine-like maceration experiments. *Journal of the Science of Food and Agriculture*, 2021, in press
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Chapter 2: Impact of grape variety, berry maturity and size on the extractability of skin polyphenols during model wine-like maceration experiments.

Abstract

Skin cell walls modulate anthocyanin and tannin extraction from grape skins. However, relationships between the composition of their alcohol insoluble cell wall solids (AIS) and extraction are still unclear. Our objectives were to characterize the impact of variety, berry size, and ripeness on the skin AIS composition (polysaccharides, proteins) and the polyphenol extraction during maceration.

The grape skin composition and its impact on polyphenol extraction were compared for two varieties, Carignan and Grenache, with skins of berries sorted according to their size and density. Extractions were performed in wine-like maceration conditions. Fresh skins had similar contents in polymeric tannins but strongly differed by their anthocyanin contents (higher in Carignan and the ripest berries) and composition (higher proportions in coumaroylated anthocyanins in Carignan). Anthocyanin extraction was proportionally much higher in Grenache, which was not just related to the Carignan's richness in coumaroylated anthocyanins. Chemical changes in solution decreased anthocyanin concentrations (40-50%) in all cases. Tannin extraction was slightly higher but above all faster for Grenache. Skin AISs differed mainly between the varieties and their maturity degree by their carbohydrate composition and protein content. Mass balances performed at the end of the maceration (analyses of precipitates and extracted skins) highlighted differences between the two varieties and between berries with different ripeness.

The differences in anthocyanin and tannin extraction between Carignan and Grenache were attributable to differences in the composition of their skin cell wall AISs and anthocyanins, but also different changes during maceration.

Keywords: grape skins, cell walls, extraction, anthocyanins, tannins.

Introduction

Anthocyanins and condensed tannins (flavan-3-ol polymers, also referred to as proanthocyanidins) are responsible for the colour and mouthfeel of red wines and play a determinant part in their quality. Anthocyanins and tannins in skin cells are mostly located in vacuoles, tannins being also found associated with the cell walls (Ortega-Regules *et al.,* 2006; Amrani Joutei *et al.,* 1994). Tannins are also extracted from seeds, although to a lower extent than from skins. Tannin and anthocyanin extraction from the skins/seeds of grapes during winemaking is not total and several studies have shown that there is no direct relationship between their contents in grapes and those found in the corresponding wines (Hazak *et al.,* 2005; Bautista-Ortín *et al.,* 2016). The latter depends on the extraction conditions (solvent, temperature, maceration length, …) and the cap-management practices (Boulton *et al.,* 1996; Setford *et al.,* 2017) during winemaking, as well as on their ability to cross the barriers represented by cell structure and in particular cell walls (Ortega-Regules *et al.,* 2006; Hernández-Hierro *et al.,* 2014). Other factors that modulate anthocyanin and tannin concentrations in wines are: (i) their adsorption on insoluble pulp debris (flesh cell walls) (Bindon *et al.,* 2010) and on yeasts (Mekoue Nguela *et al.,* 2015); (ii) their interactions with soluble grape compounds (polysaccharides, proteins) extracted during maceration, leading to precipitations (Bindon *et al.,* 2016); iii) their chemical reactivity (Cheynier *et al.,* 2006). Indeed, once extracted, anthocyanins and tannins undergo several chemical reactions that profoundly change their composition throughout the process (Hanlin *et al.,* 2010). Not all the compounds formed can be identified and quantified, which may contribute to the lack of relationship observed between the polyphenol composition of grapes and that of red wines at the end of the maceration. This relationship, related to several factors, is difficult to model, even under constant process conditions and even though this knowledge would be of great interest for the selection of new grape varieties or the adaptation of winemaking techniques to different raw materials. Experiments under model conditions, associated with the characterisation of grapes and wines are therefore necessary in order to compare the varieties, dissociate the various phenomena, and highlight those factors likely to play a predominant role in the polyphenolic composition of wines.

When dealing with polyphenol extraction from skins, cell walls are considered as one of the main factors that control their final composition in wines (Ortega-Regules *et al.,* 2006; Bindon *et al.,* 2010). Tannin extraction especially is limited by their interactions with skin insoluble cell wall constituents, i.e. polysaccharides and proteins (Ruiz-Garcia *et al.,* 2014). Interactions between tannins and cell wall polysaccharides were evidenced by means of adsorption experiments using purified cell walls and proanthocyanidins (Bindon *et al.,* 2010). Both pectins and hemicelluloses play an important

part in these interactions (Castro-López *et al.,* 2016; Watrelot *et al.,* 2013). When in solution, the degree of methylation of pectins increases their interactions with proanthocyanidins. Although they are minor components compared to polysaccharides, proteins have a much stronger affinity for tannins (Nunan *et al.,* 1997; Poncet-Legrand *et al.,* 2007). They account for about 10% of the insoluble cell wall components and are mainly structural proteins rich in hydroxyproline, proline, and glycine Carpita & Gibeaut, 1993; Vicens *et al.,* 2009). Their role in the extractability of tannins, less studied, has been mentioned by several authors (Springer & Sacks, 2014; Vicens *et al.,* 2009). Changes in skin/pulp cell wall composition occur during berry ripening, leading to a loosening of cell walls and fruit softening. Among these changes, a decrease of pectins, related to their solubilization, has been evidenced (Vicens *et al.,* 2009; Ortega-Regules *et al.,* 2008). These structural modifications have been hypothesized to induce changes in skin cell wall structure (porosity, accessibility to interaction sites) and rigidity that may modulate tannin (Bindon et al., 2012) or anthocyanin extraction (Segade *et al.,* 2011). It has also been suggested that anthocyanin may influence tannin extraction/solubility and that high anthocyanin/tannin ratios induce higher tannin concentration in wines, regardless of the initial tannin concentration in fruits (Kilmister *et al.,* 2014).

The objectives of this work were to: (i) characterize the impact of grape variety, berry size, and ripeness on the extraction and evolution of anthocyanins and tannins during skin maceration; (ii) examine links between extraction and skin composition in insoluble materials; iii) identify the main mechanisms involved. Experiments were performed in wine-like model conditions for two contrasted varieties in terms of anthocyanin/tannin ratios (Carignan and Grenache), in the absence of pulp components (to avoid the impact of adsorption/precipitation events not related to skin composition) and fermentation (to avoid chemical changes related to reactions with yeast metabolites and adsorption by yeast cells). Most of the previous studies were performed on varieties harvested through time at different degrees of ripeness (Ortega-Regules *et al.,* 2006; Hernández-Hierro *et al.,* 2014; Bindon *et al.,* 2012; Fournand *et al.,* 2006). Grape heterogeneity in terms of berry size and ripeness was considered here at technological maturity.

Materials and Methods

Chemicals

Acetonitrile, methanol, ethanol, acetic acid, and formic acid were HPLC grade from VWR. KOH Titrisol 1M was purchased from Merck. Acetone, D (+) galacturonic acid hydrated were provided by Fluka. Sodium chloride, tartaric acid, epicatechin, epigallocatechin gallate, lithium

chloride, N,N-dimethylformamide, trifluoroacetic acid, myo-inositol, allose, m-hydroxydiphenyl (MHDP), alcohol oxidase from *Pichia pastoris,* Norleucine, the 18 amino-acid standard kit, and hydrochloric acid 37 % were provided by Sigma-Aldrich, sulphuric acid by Roth. Sodium hydroxide 1M was obtained from Fisher. The lithium citrate loading buffer was obtained from Biochrom. Flavanol dimer B2, flavanol trimer C1 and Malvidin-3-O-Glucoside chloride were purchased from Extrasynthese (Genay, France). Ultra-pure water was obtained from a Milli-Q Advantage A10 system (Millipore).

Grape sampling

Two *Vitis vinifera* grape varieties (Carignan and Grenache) were harvested at an average potential alcohol of 12 % vol. in the vineyard of the Pech Rouge experimental unit (INRAE, Gruissan, France). The berries were sorted according to their natural heterogeneity in terms of volume (vol) and density (degree of maturity: deg). This heterogeneity was determined on 1000 berries the day before the harvest by measuring their diameter and estimating their density by flotation in different salt solutions, corresponding to a total soluble solid difference between two successive baths of 1 % vol potential alcohol. Berries were recovered by cutting them at the pedicel level and sorted first as a function of their size using a grading machine (vol^-, vol^+) and then as a function of their density (deg^-, deg^+) using an aqueous solution of concentrated rectified grape must at the adequate density (Figure 25).

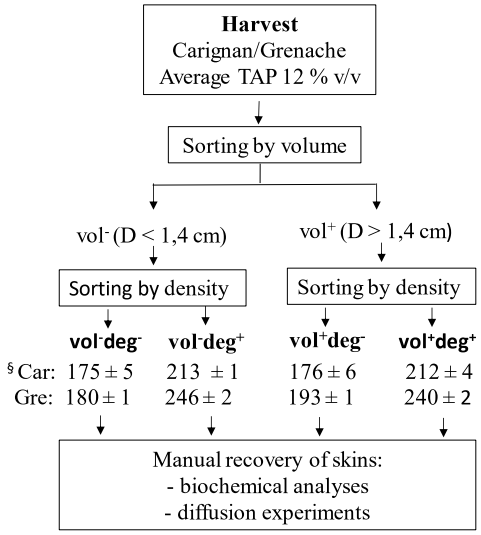


Figure 25. Preparation of the different modalities. D = berry diameter; §: sugar content expressed in $g.L^{-1}$, Car = Carignan, Gre = Grenache**.**

The oenological characteristics of the four batches (vol^-deg^-, vol^-deg^+, vol^+deg^- and vol^+deg^+) obtained for the Carignan and Grenache varieties are presented in Table 3 below. Samples

of 180 berries of each modality were recovered. Grape skins were separated from the berries with a scalpel and immediately used for diffusion experiments or frozen in liquid nitrogen and stored at -80°C for later analysis of their composition.

Table 3. Technological maturity (sugars, pH, Total acidity, PA (%vol)) of grape berries (Carignan, Grenache) sorted according to size and maturity as described in Figure 25.

Variety	Modality	Sugars ($g.L^{-1}$)	pH	Total acidity	PA (% vol)
Grenache					
0.5-1.4 cm	$vol^{-}deg^{-}$	180 ± 1	3.23 ± 0.01	4.36 ± 0.08	10.68 ± 0.04
	$vol^{-}deg^{+}$	246 ± 2	3.44 ± 0.01	3.56 ± 0.08	14.62 ± 0.09
1.4-2 cm	$vol^{+}deg^{-}$	193 ± 1	3.29± 0.02	4.03 ± 0.04	11.40 ± 0.05
	$vol^{+}deg^{+}$	240 ± 2	3.43 ± 0.01	4.61 ± 0.08	14.24 ± 0.04
Carignan					
0.8-1.4 cm	$vol^{-}deg^{-}$	173 ± 5	3.23 ± 0.01	5.21 ± 0.28	10.28 ± 0.16
	$vol^{-}deg^{+}$	213 ± 1	3.46 ±0.01	3.95 ± 0.07	12.67 ± 0.05
1.4-2 cm	$vol^{+}deg^{-}$	176 ± 6	3.30 ± 0.02	5.23 ± 0.22	10.16 ± 0.20
	$vol^{+}deg^{+}$	212 ± 4	3.54 ± 0.08	3.72 ± 0.05	12.63 0.14

Preparation of skin alcohol insoluble cell wall material (AIS)

Frozen skins of each variety and modality (from 30 berries, triplicates) were ground in liquid nitrogen. The alcohol insoluble cell wall solids (AISs) were then isolated from the powders using the procedure described in Apolinar–Valiente *et al. (*Apolinar-Valiente *et al.,* 2010), with slight modifications. AISs were prepared in triplicate and analysed separately. Skin powder (5 g) was suspended in 15 ml of boiling water for 5 min and homogenized. One part of the homogenized material was purified with two parts of 96% ethanol for 30 min at 40°C in an ultrasound bath. The alcohol insoluble solids (AIS) were separated by centrifugation and extracted again with 70% ethanol for 30 min at 40 °C. A sample from the liquid phase was taken for soluble sugar assay, done with the sulphuric phenol method. When no more sugar was detected, AISs were further washed twice with 96% ethanol and once with acetone. After being dried with an air flux overnight, they were weighed and used for the following analyses.

Carbohydrates composition of AISs

The neutral sugar composition of the AISs was determined by gas chromatography after polysaccharide hydrolysis with 72% sulphuric acid at 100°C for 3h and conversion of neutral sugars into volatile alditol acetates (Saeman *et al.,* 1954; Harris *et al.,* 1984). Inositol and allose were used as internal standards. The alditol acetates were quantified by gas chromatography with flame ionization detection (GC-FID) (GC 2010 Plus Shimadzu) using a DB225 (30 m × 0.25 mm ID, 0.25 µm film) capillary column and hydrogen 5.6 B50 as the carrier gas. Calibration was done with commercial monosaccharides. Uronic acids were determined colorimetrically in triplicates by the *m*-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973). The AISs were first submitted to pre-hydrolyse by the action of sulfuric acid, as described by Ahmed and Labavitch (Ahmed & Labavitch, 1977). A calibration curve was built using pure galacturonic acid solutions (0 to 100 mg/L). The degree of methylesterification of uronic acids (DE) was measured by the saponification of the AIS pectins in the presence of KOH, thus allowing the release of methanol. Methanol was converted to formaldehyde that was determined using the colorimetric method of Klavons and Bennet (Klavons & Bennett, 1986).

Amino acid composition of AISs

Cell wall material (5 mg) was hydrolysed in 1 mL of 6N HCl for 24 h at 120°C. Norleucine was added as an internal standard. After evaporation of the acidic aqueous solution under the air stream, samples were washed twice in water and then in 95% ethanol. Finally, samples were dissolved in a 0.2 M pH 2.2 lithium citrate loading buffer and filtered through a 0.22 µm filter (Millipore Millex-GV). Amino acids were quantified by ion-exchange chromatography with a Biochrom 30 amino acid analyzer (Biochrom, Cambridge, England), as described in Vicens *et al.* (Vicens *et al.,* 2009).

Preparation of AIS skin cell wall materials for polymers composition analysis (polysaccharides, proteins) with Comprehensive Microarray Polymer Profiling method (CoMPP)

Frozen grape skins were ground in liquid nitrogen into powder. The alcohol insoluble skin cell walls solids (AISs) were then isolated using the following procedure, as the optimal one to CoMPP technology (Nguema-Ona *et al.,* 2012; Moore *et al.,* 2014; Zietsman *et al.,* 2015). The resulting frozen powder (10 g) was incubated in 100% v/v absolute ethanol at 80°C for 15 min to deactivate endogenous enzymes. After centrifugation, the pellets were then washed sequentially by a series of solvents (ethanol, methanol, chloroform and acetone) using a stirring plate. Thereafter, the

pellet material was suspended in milliQ water and freeze-dried to yield a dry powder of grape AIS skin cell wall materials which were used for structural composition analysis.

Comprehensive Microarray Polymer Profiling (CoMPP) of AIS skin cell wall materials (see Appendix B for more details)

AIS skin cell wall samples were sequentially extracted first with 50 mM CDTA (cyclo-hexane-diamino-tetra-acetic acid pH 7.5) and then with NaOH (4M) (Moller *et al.,* 2007) to obtain the pectin and hemicellulose rich fractions. After centrifugation, the extracts from each fraction were printed onto a nitrocellulose membrane and then probed with a series of monoclonal antibodies (mAbs) and carbohydrate-binding modules (CBMs). The raw data was normalized and converted into heatmap for visualization and the relative abundance of different polymers epitopes are displayed on a scale of 0–100. The values in the heatmaps produced are mean spot signals from three biological repeats and 4 dilutions, and the highest signal measured was set to 100 with the other data adjusted accordingly. Zero in the heatmap does not represent absolutely no signal but just below the cut-off value of 5 on the raw data.

Polyphenol extraction from skins and precipitates

Frozen initial skins and frozen skins after extraction in wine-like conditions were finely ground to a fine powder in liquid nitrogen and using a mortar grinder (Pulverisette 2, Fritsch). Powders (150 mg) were treated first with methanol (750 µL) then extracted with 5.25 mL of 60/40/1 (v/v/v) acetone/water/formic acid at room temperature on an orbital shaker (Precellys 24, Bertin technologies, program 5000-3*40-20). The extracts were pooled and after centrifugation (3000 rpm, 5 min, 4°C), 1 mL aliquots were dried in a rotary evaporator under vacuum at 35°C for 2h (EZ-2 plus, Genevac SP service). Dried extracts were re-dissolved in neutral wine-like solution for UV–visible spectrophotometry and High-Performance Liquid Chromatography (HPLC) analysis, or dimethyl formamide for High-Performance Size Exclusion Chromatography (HPSEC). The same procedure except grinding was applied for the extraction and analysis of polyphenols in the precipitates recovered at the end of the wine-like maceration experiments.

Polyphenol analysis

Total polyphenols Index (TPI) and total red pigments (TRP) were determined by UV–visible spectrophotometry (spectrophotometer UV-1800, Shimadzu) at 280 and 520 nm (1 cm path length) after adequate dilution in HCl 1 M. Free anthocyanins were analysed by HPLC using a Waters chromatography system equipped with DAD detection and a C18 reversed-phase column (Atlantis

T3, Waters) as described by Fournand *et al*. (Fournand *et al.,* 2006*)*. Anthocyanins were quantified at 520 nm, in the equivalent of malvidin-3-*O*-glucoside.

The size distribution of polyphenols in the samples and the concentrations of polymeric tannins (in eq. epicatechin) were determined by HPSEC, according to the procedure described before (Vernhet *et al.,* 2020) Commercial epicatechin, B2 dimer, epigallocatechin gallate, malvidin, and home prepared and characterized tannin fractions were used to evaluate retention times corresponding to monomers, oligomers, and polymers (Figure 26).

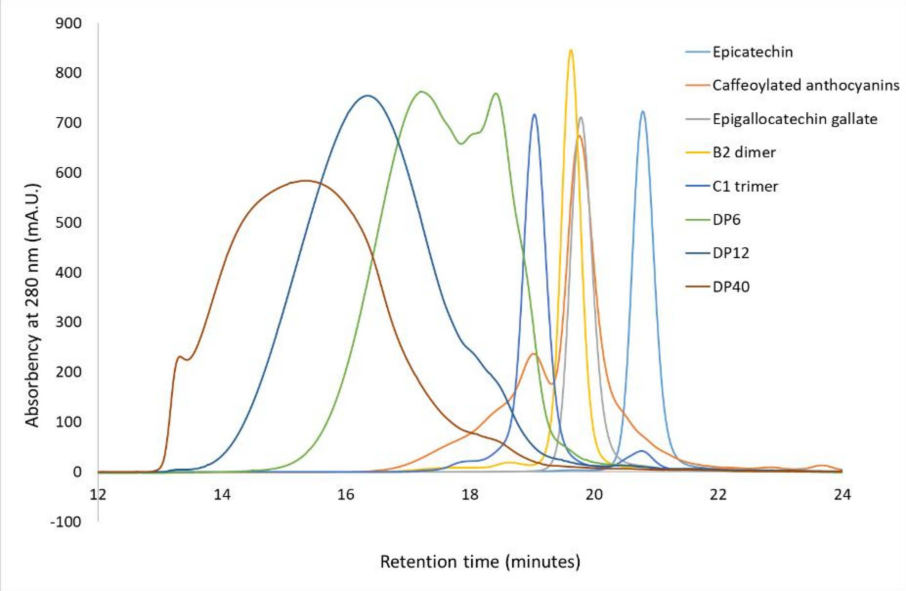


Figure 26. HPSEC profiles of anthocyanins and flavanol monomers, oligomers and polymers

Extraction of skin phenolic compounds by diffusion in model wine-like solution

Thirty berries of each modality were manually peeled and fresh skins were weighted and immediately immersed in 42 mL of a model solution containing 3 g/L tartaric acid, 50 mM NaCl, and 40 mg/L SO_2, at pH 3.5 (adjusted with NaOH 1M). Simulated maceration experiments were carried out by increasing stepwise the ethanol content from 0 to 15% (Figure 27). All experiments were performed in triplicate. Flasks were placed under argon and gently stirred in dark at 22 °C. Polyphenol diffusion was followed for 11 days by measuring the TPI and TRP daily, as well as the HPLC and HPSEC profiles of samples taken and centrifuged (15000 *x g*, 15 min, 15°C) at the end of each ethanol increase step. The dilution induced by the sampling and the addition of ethanol was considered. To

account for differences in initial fresh skin mass (dependent on the modality), results were divided by this initial mass and reduced to 1 g of fresh skin for all modalities. At the end of the diffusion, skins were recovered, and the diffusion solutions were clarified by centrifugation. Skins were then washed first with 0% (3 times for Grenache and 7 times for Carignan) and then with 15% (5 times for Grenache and 7 times for Carignan) fresh model wine-like solutions until no further extraction of skin polyphenols could be observed (Figure 27). Centrifugation pellets recovered from the diffusion solutions and "washed" skins were stored at -80 °C for further polyphenol analysis.

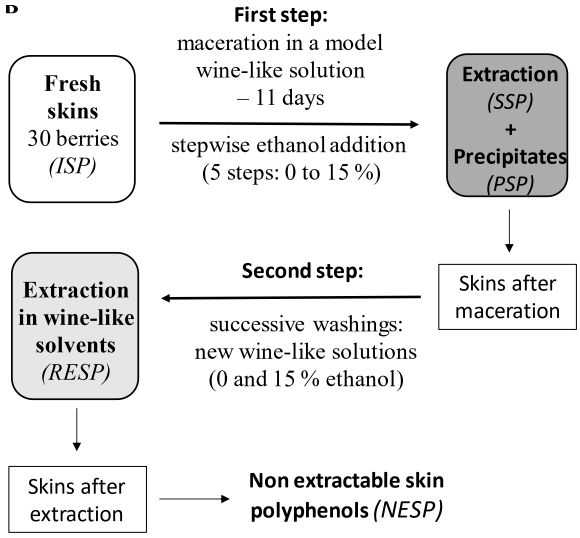


Figure 27. Experiments performed on each modality. First step: diffusion in a wine-like solvent with a gradual increase of the ethanol content. t_0, 0% ethanol – 24h; t_1 5 % ethanol – 48 h; t_2 10 % ethanol – 48 h; t_3 12% ethanol – 48 h; t_4 14 % ethanol – 48 h; t_5 15 % ethanol – 48 h. Recovery of the model wine-like solution and the skins at the end of the experiment and centrifugation of the solution to separate soluble compounds (Soluble Extracted Skin Polyphenols, *SSP*) and precipitates (Precipitated Skins Polyphenols, *PSP*). Second step: successive washings of skins with new wine-like solutions (0 and 15% ethanol) until no further extraction is observed (residual extractable polyphenols in wine-like solvents, *RESP*). Extraction in an acetonic solvent (non-extracted skin polyphenols, *NESP*).

Berry and skin firmness

The firmness of skins and berries was performed with the Penelaup robot developed by INRA and CTIFL (Abbal & Planton, 1990), see Appendix C. Specific indexes were defined and measured to quantify the firmness of berries. The type of measurement used to determine berries firmness is by compression. Penetrometry is used to determine skin hardness.

AF1(p) is the first index and corresponds to the energy required to get a deformation of p% of the berry, whereas AF2(p) equals AF1(p) normalized by the weight of the berry.

$$AF1(p) = A = \int_0^{D*p} f(x)\mathrm{d}x \qquad (1)$$

$$AF2(p) = \frac{\int_0^{D*p} f(x)dx}{M} \qquad (2)$$

where p is the percentage of the desired deformation, D the berry diameter (mm), M the fruit weight (g), x: the displacement of the tool (mm), f(x): force (Newton) applied at location x.

For skin penetrometry, the test consists of measuring the energy needed to push a needle through a berry skin for a displacement of L = 10 mm. Unlike previous tests, the penetrometry test does not take into account the diameter or the weight of the grape. We thus defined

$$AP(x=10) = \int_0^{x=10} f(x)dx \qquad (3)$$

The needle used for this experiment was 35 mm long and 2.5 mm thick and the bottom had a V shape.

Statistical analysis

Statistical analysis was performed using Statistica software. The results obtained were assessed by factorial and one-way ANOVA analysis followed by a Tukey Test. Principal Component Analysis was performed on the AIS compounds to assess the differences between varieties.

Results and Discussion

Grape skin cell walls Alcohol Insoluble Solids (AIS)

AIS amounts in skins and their composition in monosaccharides and amino acids are reported for both varieties and the four modalities (Figure 25) in Table 4 . No significant differences were observed between samples with respect to their content of AIS (mg AIS/g fresh skin) and the total content of neutral and acidic monosaccharides. For a given variety, these results are in agreement with those obtained previously on Syrah grapes (Vicens *et al.,* 2009): total amounts of skin AISs remained constant during ripening and only minor changes in their monosaccharide composition were observed.

However, factorial ANOVA followed by Tukey Test indicated (Table 5) a significant difference between varieties (Grenache vs Carignan) regarding their galactose, mannose, and glucose composition.

A centered reduced principal component analysis (PCA) was done on the obtained data set (Figure 28). Variables and individuals were represented on the first 2 principal components (PC 1-2), describing 70% of the variability in the data. Mannose, galactose, and glucose variables were

negatively correlated on axis 1 to rhamnose and fucose. Axis 2 represented arabinose and xylose. The clear separation between the two varieties confirmed the results of the factorial ANOVA. The AISs of Grenache skins were richer in mannose, galactose, and glucose compared to Carignan.

Table 4. Alcohol Insoluble Solids contents (AIS) and their monosaccharide and amino acid compositions, and polyphenol contents of the four modalities of Carignan and Grenache initial skin berries. Results are expressed in mg/g of fresh skin or AIS. Different letters indicate significant differences between samples for a given parameter Tukey's test for $p < 0.05$).

		Carignan				**Grenache**			
		vol^+deg^+	vol^-deg^+	vol^+deg^-	vol^-deg^-	vol^+deg^+	vol^-deg^+	vol^+deg^-	vol^-deg^-
AIS (mg/g fresh skin)		30.9 ± 7.1 [a]	35.7 ± 6.2 [a]	40.3 ± 6.7 [a]	42.7 ± 5.5 [a]	41.8 ± 7.2 [a]	39.0 ± 7.0 [a]	43.6 ± 3.0 [a]	39.0 ± 3.4 [a]
Amino acid/sugar ratio		0.34	0.32	0.30	0.32	0.25	0.26	0.26	0.21
Monosaccharides (mg/g AIS)	Rhamnose	7.4 ± 0.6	7.6 ± 0.5	7.6 ± 0.2	6.5 ± 0.9	6.2 ± 0.5	8.0 ± 1.5	7.2 ± 2.0	6.9 ± 0.9
	Fucose	1.8 ± 0.2	2.0 ±0.4	1.8 ± 0.0	1.8 ± 0.0	1.7 ± 0.1	1.7 ± 0.3	1.7 ± 0.2	1.7 ± 0.1
	Arabinose	22.4 ± 2.5	21.9 ± 1.7	20.8 ± 1.4	19.6 ± 1.9	18.7 ± 1.1	21.2 ±2.8	19.3 ± 2.6	19.5 ± 0.6
	Xylose	8.5 ± 1.2	7.5 ± 0.8	8.4 ±0.5	7.9 ± 1.9	8.2 ± 0.6	9.0 ±1.2	8.2 ± 0.3	9.0 ± 0.1
	Mannose	14.4 ± 1.6	14.5 ± 1.7	14.6 ± 0.7	14.8 ± 1.9	18.4 ± 1.2	16.0 ± 0.8	14.8 ± 5.9	18.0 ± 0.9
	Galactose	19.7 ± 1.0	22.2 ± 1.8	22.0 ±1.3	21.3 ± 3.5	27.3 ± 3.0	26.0 ± 1.0	24.6 ± 1.8	25.5 ± 2.5
	Glucose	138.6 ± 6.1	142.9 ±15.1	135.0 ± 6.5	134.0 ± 16.5	154.9 ± 11.8	157.7 ± 4.8	132.4 ± 26.1	159.9 ± 15.7
	Uronic acids	173.1 ± 8.4	178.1 ± 22.0	171.2 ± 13.9	163.4 ± 9.1	148.6 ± 26.0	168.2 ±33.1	162.7 ± 22.8	158.8 ± 22.2
	Total	**385.9 ± 20.3[a]**	**397.0 ± 28.7[a]**	**381.6 ± 23.9[a]**	**369.4 ± 30.8[a]**	**384.0 ± 18.5[a]**	**407.9 ± 41.7[a]**	**370.9 ± 38.3[a]**	**399.3 ± 11.5[a]**
	Ara/Gal ratio	**1.1 ± 0.1**	**1.0 ± 0.0**	**1.0 ± 0.0**	**1.0 ± 0.1**	**0.7 ± 0.0**	**0.8 ± 0.1**	**0.8 ± 0.1**	**0.8 ± 0.1**
	DE (%)	**55.2 ± 4.1[c]**	**61.4 ± 0.8[bc]**	**54.0 ±0.3[c]**	**85.2 ± 3.4[a]**	**71.9 ± 4.2[abc]**	**71.9 + 11.8[abc]**	**60.7 ± 7.6[bc]**	**78.6 ± 10.9[ab]**
Amino acids (mg/g AIS)	Hydroxypro.	3.8 ± 0.3	4.0 ± 0.4	2.6 ± 0.2	3.0 ± 0.0	2.0 ± 0.2	2.4 ± 0.1	1.8 ± 0.2	1.8 ± 0.6
	Proline	7.2 ± 0.4	7.5 ± 0.6	6.2 ± 0.3	6.5 ± 0.1	5.2 ± 0.2	6.1 ± 0.2	5.1 ± 0.5	4.8 ± 1.6
	Alanine	7.4 ± 0.3	7.5 ± 0.7	6.8 ± 0.3	7.2 ± 0.2	5.4 ± 0.2	6.0 ± 0.3	5.3 ± 0.3	4.7 ± 1.5
	Arginine	5.5 ± 0.7	4.9 ± 0.2	4.6 ±0.3	4.5 ± 0.4	4.5 ± 0.3	5.1 ± 0.2	4.6 ± 0.8	4.2 ± 1.3
	Aspartic acid	12.9 ± 0.5	13.3 ± 1.0	11.5 ± 0.5	12.2 ± 0.3	8.9 ± 0.4	10.2 ± 0.5	8.8 ± 0.7	7.9 ± 2.4
	Glutamic acid	14.6 ± 0.6	14.5 ± 1.1	12.8 ± 0.5	13.4 ± 0.7	10.9 ± 0.6	12.2 ± 0.7	10.7 ± 1.0	9.6 ± 3.3
	Glycine	8.3 ± 0.4	8.4 ± 0.6	7.7 ± 0.3	8.2 ± 0.3	6.4 ± 0.3	7.0 ± 0.4	6.2 ± 0.4	5.6 ± 1.7
	Histidine	4.8 ± 0.2	4.7 ± 0.5	4.2 ± 0.1	4.6 ± 0.2	3.7 ± 0.1	4.1 ± 0.3	3.6 ± 0.2	3.5 ± 1.1
	Isoleucine	6.0 ± 0.4	5.8 ± 0.4	5.2 ± 0.1	5.3 ± 0.3	4.7 ± 0.2	5.4 ± 0.3	4.7 ±0.5	4.2 ± 1.3
	Leucine	10.0 ± 0.6	9.7 ± 0.8	8.8 ± 0.3	9.0 ± 0.3	7.8 ± 0.3	8.8 ± 0.4	7.7 ± 0.7	6.9 ± 2.2
	Lysine	10.6 ± 0.6	10.5 ± 0.9	9.2 ± 0.4	9.8 ± 0.6	8.2 ± 0.2	9.0 ± 0.5	7.9 ± 0.4	7.1 ± 2.3
	Phenylalanine	7.2 ± 0.4	7.0 ± 0.6	6.3 ± 0.3	6.5 ± 0.2	5.1 ± 0.2	5.8 ± 0.3	5.0 ± 0.6	4.6 ± 1.4
	Serine	8.0 ± 0.4	8.1 ± 0.8	7.2 ± 0.3	7.6 ± 0.2	5.4 ± 0.2	6.1 ± 0.3	5.3 ± 0.4	4.7 ± 1.5
	Threonine	7.0 ± 0.2	7.2 ± 0.5	6.1 ± 0.3	6.4 ± 0.3	4.8 ± 0.2	5.6 ± 0.3	4.7 ± 0.4	4.3 ± 1.3
	Tyrosine	4.0 ± 0.9	3.0 ± 0.1	3.0 ± 0.8	2.5 ± 0.2	2.5 ± 0.5	3.2 ± 0.4	2.7 ±1.0	2.7 ± 0.9
	Valine	7.6 ± 0.5	7.5 ± 0.4	6.6 ± 0.1	6.9 ± 0.4	5.9 ± 0.3	6.7 ± 0.3	5.8 ± 0.5	5.2 ± 1.6
	Total	**127.4 ± 8.3[a]**	**126.2 ± 10.0[a]**	**111.4± 5.5[ab]**	**116.3 ± 5.4[a]**	**95.7 ±8.2[ab]**	**106.5 ± 5.7[ab]**	**94.9 ± 10.7[ab]**	**83.3 ±26.6[b]**

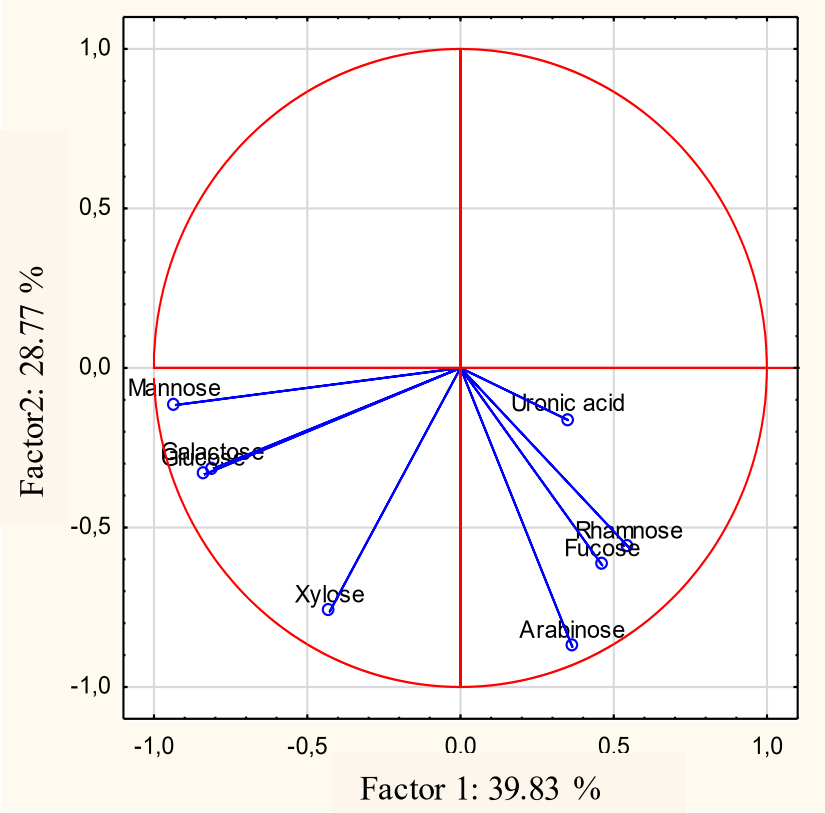
	Carignan				Grenache			
	$vol^{+}deg^{+}$	$vol^{-}deg^{+}$	$vol^{+}deg^{-}$	$vol^{-}deg^{-}$	$vol^{+}deg^{+}$	$vol^{-}deg^{+}$	$vol^{+}deg^{-}$	$vol^{-}deg^{-}$
Polyphenols								
TPI/g fresh skin	316.4 ± 34.3 ^{a}	325.9 ± 38.7 ^{a}	245.0 ±19.4^{bc}	274.0±21.7^{ab}	209.0 ± 11.4 ^{cd}	165.3 ±14.9 ^{de}	149.5±13.1 ^{de}	138.4 ± 10.2 ^{e}
TRP/g fresh skin	278.1 ± 32.5 ^{a}	286.0 ± 20.8 ^{a}	250.2±10.2^{b}	240.0±21.5 ^{b}	105.7 ± 9.7 ^{c}	97.1 ± 2.2 ^{c}	40.9 ± 3.5 ^{d}	35.1 ± 2.9 ^{d}
Anthocyanins (HPLC) (mg/g fresh skin)	5.4 ± 0.8 ^{a}	5.6 ± 0.6 ^{a}	4.9 ± 0.2 ^{b}	4.75 ± 0.4 ^{b}	2.0 ± 0.1 ^{c}	2.0 ± 0.2 ^{c}	0.9 ± 0.1 ^{d}	0.7 ± 0.1 ^{d}
Polymeric tannins (mg/g fresh skin)	8.6 ±1.7 ^{a}	9.2 ± 1.4 ^{a}	10.5 ± 1.8 ^{a}	10.5 ± 1.4 ^{a}	9.9 ± 0.8 ^{a}	9.6 ± 1.0 ^{a}	9.8 ± 0.5 ^{a}	9.5 ± 0.9 ^{a}
Anthocyanin/tannin ratio	**0.63** ^{a}	**0.61** ^{a}	**0.47** ^{a}	**0.45** ^{a}	**0.20** ^{b}	**0.21** ^{b}	**0.09** ^{c}	**0.08** ^{c}

Table 5. Factorial ANOVA assessed on the AIS compounds (monosaccharides of polysaccharides and amino acids of proteins)

	p-value (5%)											
Factors	mg AIS/g fresh skin	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acid	Total	Arab/galac	DE
Variety	1.7E-01	6.5E-01	1.2E-01	8.8E-02	1.4E-01	3.9E-02	1.0E-04	3.5E-02	1.9E-01	5.4E-01	1.2E-06	2.5E-02
Size	9.9E-01	7.5E-01	7.7E-01	7.6E-01	9.5E-01	7.7E-01	6.9E-01	1.8E-01	7.2E-01	2.9E-01	6.2E-01	1.2E-04
Maturity	7.9E-02	5.8E-01	6.7E-01	1.4E-01	8.4E-01	8.1E-01	6.1E-01	1.8E-01	7.4E-01	2.7E-01	1.4E-01	1.2E-01
var:size	1.5E-01	1.9E-01	7.6E-01	1.7E-01	3.3E-01	8.8E-01	5.5E-01	2.7E-01	6.0E-01	2.6E-01	3.8E-02	9.5E-02
vari:mat	1.5E-01	6.4E-01	6.3E-01	4.2E-01	7.6E-01	6.0E-01	2.1E-01	7.4E-01	5.5E-01	8.3E-01	2.7E-02	2.6E-02
size:maty	6.8E-01	7.2E-02	5.8E-01	3.6E-01	7.4E-01	1.7E-01	7.4E-01	4.2E-01	3.1E-01	7.0E-01	9.1E-01	1.3E-03
vari:size:maty	9.6E-01	5.9E-01	8.2E-01	6.6E-01	7.7E-01	1.9E-01	1.6E-01	2.2E-01	7.6E-01	5.6E-01	4.5E-02	5.3E-01

	p-value (5%)															
Factors	Hydroxyproline	Proline	Alanine	Arginine	Aspartique	Glutamique	Glycine	Histidine	Isoleucine	Leucine	Lysine	Phenylalanine	Serine	Threonine	Tyrosine	Valine	Total
Variety	8.0E-09	4.0E-05	1.5E-06	2.6E-01	2.5E-07	7.5E-05	8.4E-06	5.8E-04	3.2E-03	6.7E-04	1.3E-04	7.9E-06	1.8E-07	5.7E-07	2.1E-01	3.9E-04	2.7E-05
Size	4.1E-02	3.0E-01	6.3E-01	6.1E-01	4.1E-01	7.9E-01	5.7E-01	4.0E-01	9.5E-01	9.7E-01	7.6E-01	8.0E-01	5.6E-01	3.2E-01	4.4E-01	7.9E-01	6.8E-01
Maturity	2.5E-05	5.8E-03	3.5E-02	4.1E-02	1.2E-02	2.4E-02	5.2E-02	8.7E-02	1.3E-02	1.9E-02	1.6E-02	1.9E-02	2.0E-02	3.9E-03	1.2E-01	1.4E-02	1.4E-02
Var.:size	7.8E-01	9.8E-01	5.7E-01	3.7E-01	7.0E-01	8.7E-01	6.4E-01	9.5E-01	7.2E-01	9.0E-01	7.8E-01	7.8E-01	7.0E-01	7.7E-01	6.2E-02	9.2E-01	9.8E-01
Var.mat	2.1E-02	5.7E-01	7.1E-01	6.8E-01	9.7E-01	9.7E-01	5.5E-01	9.8E-01	8.8E-01	9.5E-01	9.8E-01	8.3E-01	7.6E-01	8.0E-01	3.4E-01	9.3E-01	8.8E-01
size:mat	5.9E-01	3.0E-01	3.3E-01	5.6E-01	2.6E-01	4.6E-01	4.8E-01	8.8E-01	4.4E-01	4.1E-01	5.4E-01	4.2E-01	4.1E-01	2.8E-01	8.8E-01	4.1E-01	4.3E-01
ar:size:mat	2.4E-01	2.1E-01	1.8E-01	1.8E-01	1.6E-01	1.8E-01	1.8E-01	2.0E-01	1.3E-01	1.5E-01	1.7E-01	1.8E-01	1.4E-01	1.8E-01	3.0E-01	1.5E-01	1.6E-01

A)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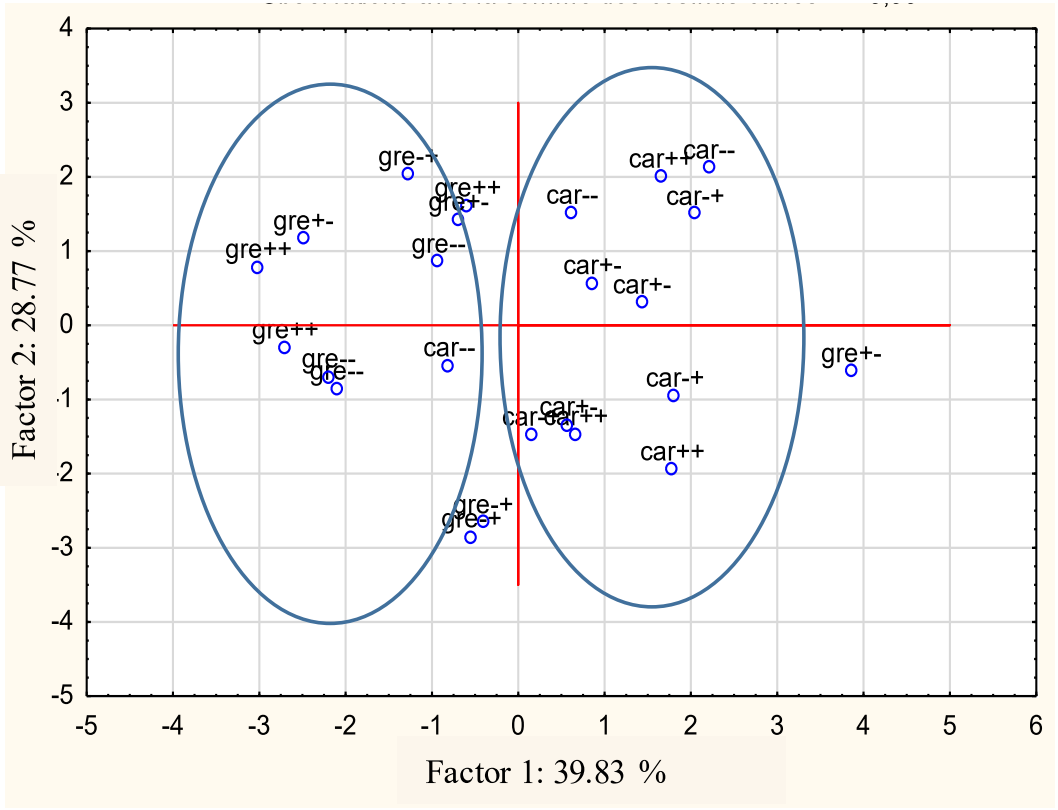
0,5
0,0
-0,5
-1,0
-1,0
-0,5
0,0
0,5
1,0
Factor 1: 39.83 %
Mannose
Galactose
Glucose
Uronic acid
Rhamnose
Fucose
Xylose
Arabinose


Factor 2: 28.77 %
Factor 1: 39.83 %
gre-+
gre++
gre+-
gre--
car++
car--
car-+
car+-
car+-
car--
car-+
car++
gre+-
gre-+
gre+-

B)

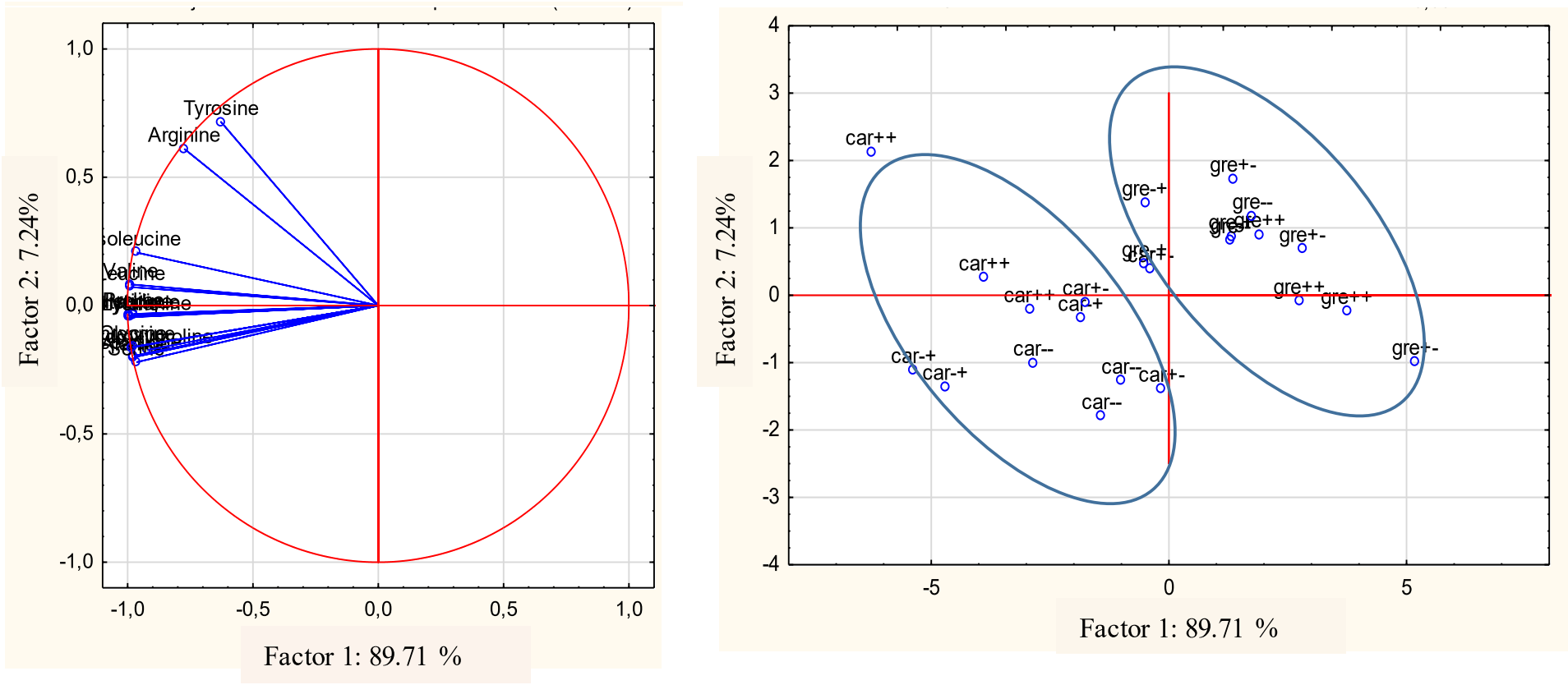


Figure 28. PCA analysis of the variables and individual distribution regarding monosaccharides constitutive of polysaccharides (A) and amino acids constitutive of proteins (B) in the skin AISs of Carignan and Grenache different modalities (vol+deg+; vol+deg-; vol-deg-; vol-deg+)

The primary cell wall of grape skins is formed by cellulose microfibrils tethered to a hemicellulosic matrix and by a more soluble domain consisting of pectic polysaccharides.

Hemicellulosic polysaccharides are mainly xyloglucans, which account for about 10% of the wall polysaccharides. Pectic polysaccharides are homogalacturonans (HG, smooth regions of pectins), rhamnogalacturonans I (RG-I) carrying side chains of arabinose and arabinogalactans (hairy regions of pectins), and rhamnogalacturonans II (RG-II). They are embedded within the primary cell wall cellulose-xyloglucan framework and in the middle lamella (Ruiz-Garcia *et al.,* 2014). As expected, galacturonic acid (from pectins, consisting mostly of homogalacturonan (HG) and rhamnogalacturonan-I (RG-I)) and glucose (from cellulose and hemicellulose) were the major sugars in skin AISs. Other neutral sugars were arabinose, galactose, and rhamnose (from pectins), along with minor contents of xylose and fucose (from hemicellulosic polysaccharides) (Ortega-Regules *et al.,* 2006). The higher glucose content found in the Grenache may then indicate higher cellulose/hemicellulose content in this variety. The calculation of different specific ratios between neutral sugars has been proposed to characterize cell wall polysaccharides (Ducasse *et al.,* 2010). Only the arabinose/galactose ratio differed between the two varieties (Table 4). This ratio is characteristic of the PRAGs-like structures (polysaccharides rich in arabinose and galactose) (Vidal *et al.,* 2003). It was higher in Carignan than in Grenache AISs, indicating different compositions in the insoluble PRAGs of the hairy regions of pectins. The degree of esterification (DE) of skin cell wall pectins ranged from 55 to 85% (Table 4), which is consistent with that found for other grape varieties harvested at maturity (Ortega-Regules *et al.,* 2008). The high deviations found for a given sample did not reveal any significant impact of variety or berry size and maturity.

Amino acids of cell wall insoluble proteins represented from 8 to 12% of skin AISs (Table 4). These amino acids belong to the network of structural proteins. In general, our results indicated higher amino acid/monosaccharide ratios for Carignan than for Grenache. A factorial ANOVA followed by the Tukey Test (Table 5) indicated an impact of both the variety and the maturity (deg^+ vs deg^- modalities). The AIS of the Carignan skins were significantly richer in amino acids than those of the Grenache and deg^+ modalities were richer than deg^- ones. Centered reduced PCA is shown in Figure 28B. Variables and individuals were represented on the first 2 axes (1-2) that described 97% of the variability. All the variables were positively correlated on the axe 1 (90% of the variability).

Characterization of the grape AIS skin cell walls with CoMPP method

The AIS skin cell wall polymer composition was analysed using the CoMPP method. The extracts resulting from the sequential extraction using CDTA (pectin rich fraction) and NaOH (hemicellulose rich fraction) were probed with 28 mAbs or CBMs.

In the heatmap table generated from the CoMPP technology (Table 6), the AIS skin cell wall materials were divided into pectin rich fraction containing homogalacturonan (HG), rhamnogalacturonan (RGI) and side chains, extensins, and AGPs. HG epitopes in samples were recognized by mAbs JIM5, JIM7, LM18, LM19, LM20/ RGI by mAbs INRA-RU1 and INRA-RU2/ and its side chains by mAbs LM6, LM13. However, RGII was not studied, as there is no monoclonal antibody for detecting this polymer in the analysis. The hemicellulose/Cellulose rich fraction contains Mannans, Glucan/Xyloglucan, Xylans, Extensine, and Cellulose, and mannans were recognized by mAb LM21, xyloglucan by mAbs LM15, LM25/ cellulose by CBM3a/ extensins by mAbs LM1, JIM11, JIM20/ and AGPs by mAbs JIM8, JIM13, LM14. These antibodies were chosen for this study as they recognize a broad range of different cell wall polymers (Appendix B: Summary of the probes used and their targets).

In the pectin rich fraction, the mAb JIM7 and LM20 showed the highest signal intensity compared to the other antibodies used for detecting the HG degree of esterification. Both mAb JIM7 and LM20 recognizes methyl-esterified HG polymers but does not bind to un-esterified HG. This confirms the previous findings that grape berry pectins are generally highly methyl esterified (Gao *et al.,* 2019). Weaker signals are observed for mAbs JIM5, LM18, and LM19. These antibodies recognize mainly partially and unesterified HG. This is also an indicator of a higher level of methyl-esterified HG present. With the CDTA fraction, the only detected signals are mainly those related to HG epitopes. The pectin-rich fraction contained low amounts of RGI, arabinans, galactans, and AGPs associated with RGI as side chains.

In the hemicellulosic rich fraction, the epitopes of samples were mainly recognized by the mAbs/CBMs for mannan (LM21), xyloglucan/glucan (BS-400-2, LM15, LM25), cellulose (CBM3a), and extensins (LM1, JIM11, JIM20). The extensins epitopes were not extracted with the CDTA but with the NaOH fraction. This may suggest that glycoproteins can be strongly associated with xyloglucan and cellulose microfibrils in the skin cell wall structure. The main hemicellulose polymers are found to be xyloglucan, which consists of a backbone of β-1,4-linked glucan where 3 out of 4 glucose units are substituted with xylose at position 6 (e.g. the XXXG motif). Other motifs are galactosylated and fucosylated such as XXFG and XLFG (Gao

et al., 2016). The epitopes of mannans and cellulose were also detected. Equally interesting is the signals observed for the glycoproteins termed extensins. Three protein groups are established based on their dominating amino acids: the hydroxyproline-rich glycoproteins (HRGPs) or extensins, the proline-rich proteins (PRPs), and the glycine-rich proteins (GRPs). In plant cell walls, the two most studied structural cell wall glycoproteins are the extensins and the arabinogalactan proteins (AGPs) rich in serine and hydroxyproline. They are known to play important role in the formation of the primary cell walls (growth, strength, elasticity, defense, environmental sensing, and signaling) (Nunan *et al.,* 1998; Carpita & Gibeaut, 1993). The raw data from the glycan microarray analysis was analysed with Factorial ANOVA (Table 7) and one-way ANOVA with a Tukey test (Table 8) for sample comparison.

Table 6. Comprehensive Microarray Polymer Profiling analysis of the CDTA (pectin-rich) and NaOH (hemicellulose-rich) fractions extracted from the AIS skin cell walls of Carignan and Grenache varieties, each sorted according to their degree of maturity and size. The values in the heatmap are the average of three biological samples and show the relative abundance of cell wall epitopes in each sample. A cut-off of 5 has been applied to the raw data.

Extraction	Samples 2018	HG with a low DE (JIM5)	HG with a high DE (JIM7)	HG partially methylesterified (LM18)	HG partially methylesterified (LM19)	HG partially methylesterified (LM20)	HG blockwise methylesterified (PAM1)	HG Ca2+ crosslinked (2F4)	Xylogalacturonan (LM8)	Backbone of rhamnogalacturonan I (INRA-RU1	Backbone of rhamnogalacturonan I (INRA-RU2	(1→4)-β-D-galactan (LM5)	(1→5)-α-L-arabinan (LM6)	Linearised (1→5)-α-L-arabinan (LM13)	(1→4)-β-D-(galacto)(gluco)mannan (LM21)	(1→3)-β-D-glucan (BS-400-2)	Xyloglucan (XXXG motif) (LM15)	Xyloglucan (LM24)	Xyloglucan / unsubstituted β-D-glucan (LM25)	(1→4)-β-D-xylan (LM10)	(1→4)-β-D-xylan/arabinoxylan (LM11)	cellulose (CBM3a)	Extensin (LM1)	Extensin (JIM11)	Extensin (JIM20)	AGP (JIM8)	AGP (JIM13)	AGP (LM14)	AGP, β-linked GlcA (LM2)
		HG								RGI		RGI and side chains			Mannans	Glucan/Xyloglucan				Xylans		Cellulose	Extensins			AGPs			
CDTA-Fraction	skin car vol+deg+	20	54	9	16	46	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	skin car vol-deg+	22	54	11	17	47	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	skin car vol-deg-	8	45	4	8	35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	skin car vol+deg-	15	59	7	14	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	skin gre vol+deg+	24	52	9	14	45	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	skin gre vol-deg+	29	56	14	18	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	skin gre vol-deg-	17	41	7	11	34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
	skin gre vol+deg-	23	58	8	12	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NaOH-Fraction	skin car vol+deg+	0	0	0	0	0	0	0	0	0	0	0	0	0	13	6	34	0	22	0	0	15	9	8	12	0	0	0
	skin car vol-deg+	0	0	0	0	0	0	0	0	0	0	0	0	0	13	11	33	0	23	0	0	14	7	9	13	0	0	0
	skin car vol-deg-	0	0	0	0	0	0	0	0	0	2	0	2	0	13	2	37	0	26	0	0	16	7	2	10	0	0	0
	skin car vol+deg-	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0	39	0	28	0	0	17	10	2	13	0	0	0
	skin gre vol+deg+	0	0	0	0	0	0	0	0	0	0	0	0	0	15	14	37	0	24	0	0	16	0	0	0	0	0	0
	skin gre vol-deg+	0	0	0	0	0	0	0	0	0	0	0	0	0	15	12	38	0	26	0	0	17	0	0	0	0	0	0
	skin gre vol-deg-	0	0	0	0	0	0	0	0	0	0	0	0	0	15	7	39	0	26	0	0	17	0	0	0	0	0	0
	skin gre vol+deg-	0	0	0	0	0	0	0	0	0	0	0	0	0	18	9	43	0	32	0	0	18	0	0	0	0	0	0

In the Factorial ANOVA of the pectin rich fraction, significant differences were mainly observed between varieties then modalities of different maturity degree (Table 7). The size had the least impact on the skin cell wall composition. Even though the signals of RGI and RGI side chains were weak, as observed in Table 7 and Table 8, significant differences between varieties were observed mainly with RGI (INRA-RU2), arabinan (LM13) higher in Carignan compared to Grenache variety. AIS skin cell walls composition of Grenache and Carignan is relatively high in HG, especially samples of high maturity degree (deg+). This was proven with the Factorial ANOVA where the signals of the HG antibodies were significantly different between maturity in the pectin rich fraction (Table 7).

In the hemicellulosic fraction, a separation based on the variety was observed. Separation according to maturity degree occurred also with Carignan variety. The high maturity degree modalities (deg+) were positively correlated with the extensin epitopes (JIM11). The signals of extensins (LM1, JIM11, JIM20) was proven to be significantly higher in the Carignan varieties according to the ANOVA tables (factorial and one-way ANOVA) (Table 7 and Table 8). Grenache variety was significantly higher with the hemicellulosic mannans, xyloglucan, and cellulose epitopes.

The CoMPP method confirmed the results of the monosaccharides and amino acid analysis of the AIS skin cell walls. It was found previously that AISs of Grenache skins were richer in mannose, galactose, and glucose compared to Carignan. On the other hand, the AIS of the Carignan skins were significantly richer in amino acids than those of the Grenache and deg+ modalities were richer than deg- ones, especially with hydroxyproline found to be associated with extensins.

Table 7. Factorial ANOVA done on the raw data of the Heatmap table. The highlighted values correspond to p<0.05.

AIS of skins	p-value (5%)	HG partially de-esterified (JIM5)	HG partially esterified (JIM7)	HG partially de-esterified (LM18)	HG partially de-esterified (LM19)	HG partially esterified (LM20)	HG blockwise de-esterified (mAb PAM1)	HG Ca2+ crosslinked (2F4)	Xylogalacturonan (LM8)	Backbone of rhamnogalacturonan I (INRA-RU1)	Backbone of rhamnogalacturonan I (INRA-RU2)	(1==> 4) beta D galactan (LM5)	(1==>5) alpha L arabinan (LM6)	linearised (1==>5) alpha L arabinan (LM13)	(1==>4) beta D galacto gluco mannan (LM21)	(1==>3) beta D glucan (BS-400-2)	Xyloglucan (XXXG motif) (LM15)	Xyloglucan (LM24)	Xyloglucan/unsubstituted beta D glucan (LM25)	(1==>4) beta D Xylan (LM10)	(1==>4) beta D xylan/arabinoxylan (LM11)	Cellulose (CBM3a)	Extensin (LM1)	Extensin (JIM11)	Extensin (JIM20)	AGP (JIM8)	AGP (JIM13)	AGP (LM14)	AGP beta linked Glc A (LM2)
	Factors	HG								RGI		RGI side chains			Mannan	Glucan/Xyloglucan				Xylans		Cellulose	Extensins			AGPs			
CDTA-Frac	Variety (V)	3.1E-06	1.4E-01	4.7E-03	1.0E-01	3.1E-01	4.2E-01	5.1E-03	8.9E-01	1.8E-06	7.5E-08	4.0E-01	2.8E-06	1.0E-06	1.7E-01	9.7E-01	9.4E-01	9.0E-01	2.8E-03	2.2E-01	1.6E-03	7.3E-02	1.9E-01	1.6E-01	1.3E-01	2.4E-07	1.3E-03	9.2E-07	8.4E-04
	Size (S)	6.5E-01	7.5E-03	1.0E-02	2.1E-01	1.6E-03	4.3E-01	7.9E-02	8.6E-02	5.8E-02	6.1E-01	1.8E-01	8.6E-01	2.9E-01	5.3E-01	5.6E-01	8.2E-02	6.5E-01	9.9E-01	9.9E-01	2.7E-04	3.7E-01	9.8E-01	4.1E-01	4.9E-01	1.8E-01	1.5E-01	6.6E-01	9.0E-01
	Maturity (M)	5.2E-07	3.6E-01	7.7E-10	1.6E-07	2.4E-02	5.8E-01	4.3E-04	8.6E-02	7.0E-01	1.1E-02	9.7E-01	1.4E-02	6.4E-02	2.7E-01	2.8E-01	9.0E-01	9.5E-01	2.2E-02	1.3E-01	6.0E-01	2.5E-01	1.7E-01	6.2E-01	3.0E-01	2.1E-01	7.9E-02	3.2E-04	1.2E-01
	V:S	8.6E-01	4.7E-01	3.5E-01	5.4E-02	1.2E-01	1.4E-01	4.2E-01	2.9E-01	4.7E-01	4.0E-01	9.3E-01	2.4E-01	3.6E-01	7.9E-01	9.3E-01	2.2E-01	9.7E-02	2.6E-01	3.9E-01	6.8E-01	1.2E-01	6.9E-01	5.5E-01	3.7E-01	2.6E-01	1.1E-01	9.7E-01	2.7E-01
	V:M	8.4E-01	1.5E-01	6.6E-01	1.5E-01	2.6E-01	4.5E-01	8.3E-01	1.7E-01	4.0E-01	5.3E-03	9.2E-01	7.0E-03	5.8E-01	5.1E-01	8.6E-01	4.7E-01	2.7E-01	4.5E-02	2.8E-01	8.8E-02	1.2E-01	8.1E-02	9.4E-01	3.9E-01	5.0E-02	9.3E-02	1.1E-01	2.1E-01
	S:M	1.0E-01	5.0E-01	2.3E-01	3.0E-01	1.5E-04	7.0E-01	6.8E-01	1.1E-01	1.4E-01	7.4E-01	1.3E-01	7.8E-01	2.4E-01	7.2E-01	7.4E-01	3.7E-01	3.3E-01	3.2E-01	6.7E-01	2.9E-01	8.0E-01	1.6E-01	4.2E-01	1.9E-01	7.3E-01	1.2E-01	3.4E-01	8.0E-01
	V:S:M	1.3E-01	6.6E-02	1.2E-01	2.4E-01	3.1E-01	7.3E-02	4.9E-01	8.9E-01	1.7E-01	3.8E-01	2.4E-01	6.7E-01	4.5E-01	1.5E-01	2.4E-01	6.5E-01	6.8E-01	7.6E-01	5.1E-01	6.4E-02	4.0E-01	9.3E-01	7.4E-01	6.9E-01	1.1E-01	6.5E-01	7.4E-01	3.0E-01
NaOH-Frac	V	6.3E-02	2.7E-01	1.9E-03	3.3E-04	6.4E-01	8.8E-01	2.1E-01	8.2E-01	6.2E-09	3.8E-08	4.1E-01	2.6E-07	9.1E-09	3.2E-02	4.2E-07	1.5E-02	3.1E-01	1.3E-02	7.7E-03	1.5E-01	2.0E-02	4.6E-09	3.7E-09	3.3E-12	1.4E-05	1.6E-06	4.7E-01	1.1E-02
	S	9.5E-01	7.3E-01	4.2E-02	8.4E-02	3.8E-01	9.3E-01	8.8E-01	5.0E-01	8.6E-01	7.9E-01	1.2E-01	9.8E-02	9.3E-02	5.9E-01	9.0E-01	4.0E-01	5.9E-01	1.9E-01	7.4E-02	8.8E-01	3.4E-01	1.1E-01	6.0E-02	4.7E-01	8.7E-01	7.1E-02	7.5E-01	1.1E-01
	M	3.1E-01	6.8E-01	6.6E-03	2.4E-02	8.5E-01	7.3E-01	1.0E+00	3.6E-01	9.2E-02	4.0E-01	2.6E-02	5.6E-01	1.7E-01	1.3E-01	1.7E-08	4.5E-04	5.6E-02	1.6E-05	1.5E-02	3.7E-01	1.6E-03	7.5E-01	9.6E-06	1.7E-01	1.4E-01	2.9E-01	3.5E-01	1.7E-02
	V:S	2.1E-01	1.7E-01	9.1E-02	8.3E-02	1.8E-01	5.0E-01	9.3E-01	7.4E-01	8.8E-01	7.0E-01	3.1E-01	3.0E-01	1.6E-01	1.9E-01	3.0E-01	6.9E-01	4.2E-01	2.9E-01	2.7E-01	2.2E-01	4.9E-01	8.0E-02	6.8E-01	1.5E-01	6.9E-01	1.1E-01	4.1E-01	5.1E-01
	V:M	9.5E-01	5.0E-01	1.6E-02	2.3E-02	5.5E-01	7.9E-01	9.3E-01	9.7E-01	2.3E-01	2.5E-01	1.2E-01	3.2E-01	2.3E-01	2.4E-01	4.6E-01	4.4E-01	4.1E-01	2.5E-01	6.8E-03	1.2E-01	1.5E-01	5.0E-01	1.4E-06	3.9E-01	8.5E-01	9.4E-01	6.7E-01	2.5E-01
	S:M	7.6E-01	1.4E-01	8.7E-02	3.1E-01	1.4E-01	5.8E-01	5.7E-01	4.1E-01	3.8E-01	7.2E-01	1.4E-01	1.9E-01	5.0E-01	5.4E-01	6.9E-02	2.5E-01	3.3E-01	1.1E-01	5.8E-01	5.0E-01	1.1E-01	6.8E-01	8.5E-01	3.2E-01	3.3E-01	5.0E-02	9.5E-01	7.5E-01
	V:S:M	7.3E-01	2.6E-01	1.7E-01	3.1E-01	3.1E-01	9.0E-01	6.0E-01	7.3E-01	5.0E-02	5.0E-01	1.2E-01	1.1E-01	6.1E-01	1.8E-01	3.3E-01	3.1E-01	8.7E-01	1.0E-01	4.3E-01	4.0E-01	2.8E-01	4.1E-01	5.5E-01	1.5E-01	5.8E-01	5.2E-01	2.4E-01	3.2E-01

AIS of skin cell wallS CDTA-Fraction	HG partially de-esterified (JIM5)	HG partially esterified (JIM7)	HG partially de-esterified (LM18)	HG partially de-esterified (LM19)	HG partially esterified (LM20)	HG blockwise de-esterified (mAb PAM1)	HG Ca2+ crosslinked (2F4)	Xylogalacturonan (LM8)	Backbone of rhamnogalacturonan I (INRA-RU1)	Backbone of rhamnogalacturonan I (INRA-RU2)	(1==> 4) beta D galactan (LM5)	(1==>5) alpha L arabinan (LM6)	linearised (1==>5) alpha L arabinan (LM13)	(1==>4) beta D galacto gluco mannan (LM21)	(1==>3) beta D glucan (BS-400-2)	Xyloglucan (XXXG motif) (LM15)	Xyloglucan (LM24)	Xyloglucan/unsubstituted beta D glucan (LM25)	(1==>4) beta D Xylan (LM10)	(1==>4) beta D xylan/arabinoxylan (LM11)	cellulose (CBM3a)	Extensin (LM1)	Extensin (JIM11)	Extensin (JIM20)	AGP (JIM8)	AGP (JIM13)	AGP (LM14)	AGP beta linked Glc A (LM2)
car vol+deg+	19.7 bcd	54.2 a	9.5 c	15.8 7ab	46.2 a	0.95 a	0.84 b	0.01 a	2.55 ab	3.80 a	0.02 a	2.91 a	0.22 b	0.04 a	0.11 a	0.16 a	0.33 a	2.40 ab	0.11 a	0.87 b	0.57 a	0.00 a	0.11 a	0.02 a	2.70 bcd	3.17 ab	0.31 bc	0.36 ab
car vol-deg+	22.0 bc	53.8 a	11.1 b	16.6 ab	46.8 a	1.55 a	0.90 a	0.00 a	2.37 bc	3.31 b	0.01 a	2.66 b	0.22 b	0.09 a	0.14 a	0.10 a	0.39 a	2.69 a	0.06 a	0.21 b	0.52 a	0.00 a	0.08 a	0.03 a	2.59 bcd	2.86 ab	0.34 abc	0.37 a
car vol-deg-	12.0 e	51.2 ab	5.7 d	12.2 c	41.7 ab	0.94 a	0.56 abc	0.00 a	1.65 cd	1.82 cd	0.02 a	1.45 c	0.21 b	0.09 a	0.06 a	0.08 a	0.55 a	2.24 ab	0.08 a	0.28 b	1.07 a	0.01 a	0.03 a	0.04 a	1.39 d	1.31 b	0.15 c	0.20 abc
car vol+deg-	14.8 de	58.a	6.8 d	13.6 bc	47.8 a	0.81 a	0.45	0.00 a	2.72 a	2.57 bc	0.06 a	1.76 bc	0.34 a	0.12 a	0.11 a	0.22 a	0.38 a	2.99 a	0.15 a	1.31 ab	0.89 a	0.01 a	0.05 a	0.01 a	2.40 cd	2.75 ab	0.15 c	0.26 abc
gre vol+deg+	24.5 ab	52.1 ab	9.4 c	14.3 bc	45.4 a	1.48 a	0.44 bc	0.01 a	1.02 d	0.71 d	0.02 a	0.70 c	0.05 c	0.11 a	0.14 a	0.16 a	0.61 a	1.10 b	0.19 a	2.47 a	0.68 a	0.00 a	0.13 a	0.05 a	4.23 a	3.40 a	0.47 ab	0.15 c
gre vol-deg+	29.4 a	55.8 a	13.6 b	18.0 a	47.8 a	1.00 a	0.75 bc	0.00 a	1.05 d	0.82 d	0.02 a	0.76 c	0.11 c	0.11 a	0.10 a	0.16 a	0.29 a	1.73 ab	0.17 a	0.85 b	1.16 a	0.00 a	0.10 a	0.04 a	3.88 abc	3.76 a	0.53 a	0.17 bc
gre vol-deg-	16.c cde	41.5 b	6.8 d	11.1 c	33.7 b	0.75 a	0.32 c	0.00 a	1.43 cd	1.17 d	0.01 a	0.98 c	0.10 c	0.14 a	0.09 a	0.11 a	0.32 a	2.27 ab	0.42 a	0.99 b	1.18 a	0.00 a	0.07 a	0.04 a	4.36 a	3.43 a	0.41 ab	0.23 abc
gre vol+deg-	22.9 bc	58.1 a	7.5 d	11.7 c	50.1 a	0.50 a	0.20 c	0.00 a	1.43 cd	0.51 d	0.08 a	0.62 c	0.11 c	0.10 a	0.09 a	0.15 a	0.39 a	2.43 ab	0.29 a	1.39 ab	0.83 a	0.00 a	0.06 a	0.02 a	4.11 ab	3.69 a	0.43 ab	0.17 bc

AIS of skins NaOH-Fraction	HG partially de-esterified (JIM5)	HG partially esterified (JIM7)	HG partially de-esterified (LM18)	HG partially de-esterified (LM19)	HG partially esterified (LM20)	HG blockwise de-esterified (mAb PAM1)	HG Ca2+ crosslinked (2F4)	Xylogalacturonan (LM8)	Backbone of rhamnogalacturonan I (INRA-RU1)	Backbone of rhamnogalacturonan I (INRA-RU2)	(1==> 4) beta D galactan (LM5)	(1==>5) alpha L arabinan (LM6)	linearised (1==>5) alpha L arabinan (LM13)	(1==>4) beta D galacto gluco mannan (LM21)	(1==>3) beta D glucan (BS-400-2)	Xyloglucan (XXXG motif) (LM15)	Xyloglucan (LM24)	Xyloglucan/unsubstituted beta D glucan (LM25)	(1==>4) beta D Xylan (LM10)	(1==>4) beta D xylan/arabinoxylan (LM11)	cellulose (CBM3a)	Extensin (LM1)	Extensin (JIM11)	Extensin (JIM20)	AGP (JIM8)	AGP (JIM13)	AGP (LM14)	AGP beta linked Glc A (LM2)
car vol+deg+	0.15 a	0.00 a	0.29 b	0.30 b	0.03 a	0.71 a	0.25 a	0.06 a	1.10 b	3.46 a	0.97 b	2.25 abc	1.19 a	13.1 b	7.06 cd	33.8 b	1.58 a	22.0 d	0.38 c	1.26 a	14.7 bc	8.60 a	8.42 a	12.4 a	1.23 a	2.00 ab	0.34 a	0.06 ab
car vol-deg+	0.13 a	0.01 a	0.19 b	0.13 b	0.10 a	0.52 a	0.21 a	0.08 a	1.69 ab	3.75 a	1.51 ab	4.10 a	1.76 a	13.1 b	10.6 ab	33.4 b	1.41 a	22.6 cd	0.78 abc	2.07 a	14.2 c	7.21 a	9.38 a	12.6 a	0.98 ab	2.48 a	0.42 a	0.05 ab
car vol-deg-	0.11 a	0.00 a	0.53 b	0.43 ab	0.05 a	0.71 a	0.26 a	0.05 a	1.51 ab	2.78 a	2.61 a	2.63 ab	1.95 a	13.9 ab	4.77 d	38.8 ab	1.40 a	27.2 abc	1.10 a	2.95 a	16.3 abc	7.06 a	5.05 b	10.3 a	0.78 abc	1.85 ab	0.43 a	0.01 b
car vol+deg-	0.13 a	0.00 a	1.48 a	1.03 a	0.06 a	0.74 a	0.21 a	0.05 a	2.10 b	3.15 a	2.02 ab	2.71 a	1.69 a	12.8 b	3.96 d	39.4 b	1.12 a	28.4 ab	0.89 ab	2.02 a	17.3 ab	9.96 a	4.57 b	12.5 3a	1.11 a	2.25 a	0.46 a	0.04 ab
gre vol+deg+	0.10 a	0.00 a	0.11 b	0.00 b	0.01 a	0.68 a	0.13 a	0.04 a	0.03 c	0.14 b	1.59 ab	0.13 c	0.23 b	14.6 ab	13.8 a	36.8 b	2.32 a	24.5 bcd	0.61 abc	1.83 a	15.7 abc	1.61 b	3.11 b	3.35 b	0.34 c	0.66 c	0.25 a	0.16 a
gre vol-deg+	0.11 a	0.00 a	0.11 b	0.00 b	0.01 a	0.70 a	0.13 a	0.09 a	0.10 c	0.25 b	2.38 ab	0.25 c	0.29 b	14.7 ab	12.2 ab	37.8 b	1.90 a	26.1 bcd	0.56 bc	1.56 a	16.9 abc	1.45 b	3.46 b	3.49 b	0.74 abc	1.47 abc	0.13 a	0.09 ab
gre vol-deg-	0.11 a	0.00 a	0.11 b	0.00 b	0.01 a	0.84 a	0.13 a	0.05 a	0.15 c	0.44 b	1.66 ab	0.48 bc	0.29 b	15.2 ab	6.6 cd	39.2 b	1.50 a	26.3 bcd	0.55 bc	0.83 a	16.6 abc	1.49 b	3.91 b	3.46 b	0.48 bc	1.07 bc	0.22 a	0.05 ab
gre vol+deg-	0.07 a	0.00 a	0.21 b	0.00 b	0.02 a	0.57 a	0.13 a	0.05 a	0.12 c	0.14 b	2.19 ab	0.17 c	0.27 b	17.9 a	9.1 bc	43.0 a	1.60 a	31.7 a	0.57 bc	2.10 a	18.0 a	1.14 b	3.31 b	2.87 b	0.35 c	0.73 c	0.22 a	0.07 ab

Table 8. One-way ANOVA and Tukey test on the raw data of the Heatmap table to determine structural differences between the AIS pulp cell wall materials of Carignan and Grenache different modalities. Different letters indicate significant differences between samples for a given parameter (Tukey's test for $p<0.05$).

Fresh skin polyphenol composition

The polyphenol compositions in the fresh skins (Initial Skin Polyphenols, **ISP)** of the different Carignan and Grenache modalities were determined using UV-visible spectrophotometry (TPI, TRP), HPLC-DAD (total free anthocyanins), and HPSEC-DAD chromatography. UV-visible spectrophotometry and HPLC-DAD data showed differences between varieties, with significantly higher total polyphenol and anthocyanin contents in Carignan skins than in Grenache ones (Table 4). They also evidenced a different composition, related to the respective proportions of non-acylated and *p*-coumaroylated anthocyanins, further discussed later. HPSEC analysis (Figure 29) was used to determine the concentrations and the size distributions of polymeric tannins (mDP > 3). HPSEC profiles evidenced three main populations, eluted between 13 and 21 min. The first one corresponds to polymeric tannins with mDP > 3, the second one (maximum A_{280} at 19 min.) to oligomeric tannins (dimers and trimer) co-eluted with anthocyanins, and the third one (maximum A_{280} at 20 min) to non-acylated anthocyanins. Polyphenols eluted later are lower molecular weight compounds. The concentrations and size distributions of polymeric tannins were similar in all cases (Table 4). Thus, the main differences between the two varieties and between the deg^+ and deg^- modalities for a given variety were their anthocyanin content and anthocyanin/polymeric tannin ratios. The size of the berries (vol^+, vol^-) had no significant impact.

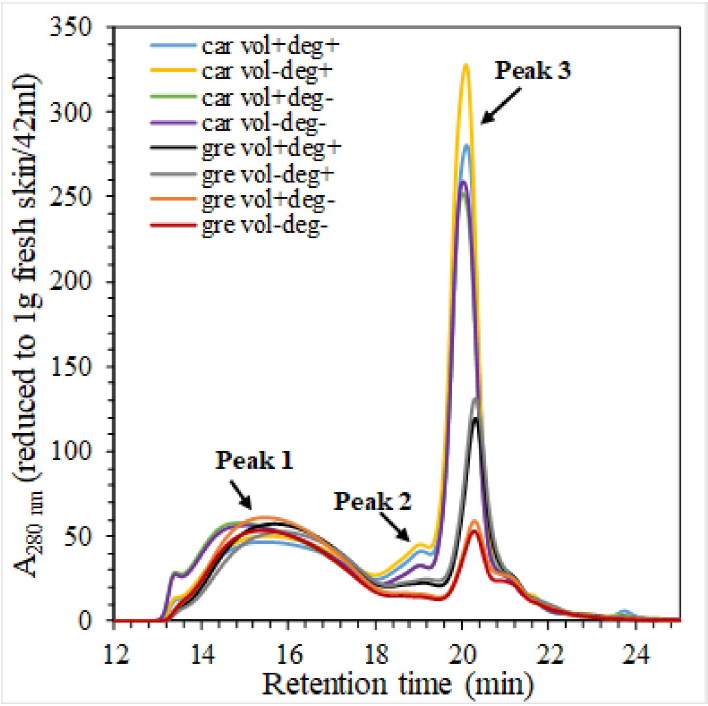


Figure 29. Initial polyphenols contents in the skins of the different modalities (vol^+ deg^+, vol^-deg^+, vol^+deg^- and vol^-deg^-) by HPSEC. Carignan: car; Grenache: gre. Peak 1: polymeric tannins with mDP > 3; peak 2: oligomeric tannins (dimers and trimer) co-eluted with acylated anthocyanins; peak 3: non-acylated anthocyanins.

Polyphenol diffusion kinetic in wine-like maceration conditions

Polyphenol diffusion in wine-like conditions was followed first by UV-visible spectrophotometry. The ethanol content in the solvent was progressively increased from 0 to 15% in 11 days to mimic solvent change during fermentation and maceration (Figure 27). However, unlike the conditions in red winemaking, samples were kept under constant stirring, i.e. under forced convective conditions in the liquid. Thus, the mass transfer is expected to be governed by the internal diffusion of polyphenols from within the skins to the solid-liquid boundary layer and to be faster than in winemaking (Setford *et al.,* 2019). TPI and TRP analysis indicated similar diffusion kinetic for both varieties and all modalities (Figure 30). A large and rapid increase in total polyphenols and red pigments was observed during the first few hours of the diffusion, up to a maximum reached after 30 hours (5% ethanol). After that, a gradual decrease in TRP continued until the end of the experiment.

This decrease was concomitant with a decrease in anthocyanins (measured by HPLC-DAD, Table 9). It may have several origins: i) degradation into low molecular weight compounds such as vanillic and syringic acids; ii) involvement in chemical reactions leading to the formation of both colorless or pigmented derived compounds with different molar extinction coefficients (Cheynier *et al.,* 2006; Fulcrand *et al.,* 2006); iii) involvement in physicochemical interactions with other skin soluble components in the medium (polysaccharides, proteins) (Bindon *et al.,* 2016; Springer *et al.,* 2016) or solubility losses, leading to precipitation; iv) re-adsorption by solid parts. In parallel, total polyphenols also decreased for all Grenache modalities, but to a much lower extent, whereas a pseudo-plateau value was observed for Carignan ones. As anthocyanins present a higher extinction coefficient than flavanols at 280 nm, their decrease may hide the extraction of other polyphenols as quantified by UV spectrophotometry (Boulet *et al.,* 2017). Thus, after 30 h extraction, TPI profiles reflected the balance between polyphenol extraction from skins and anthocyanin decrease.

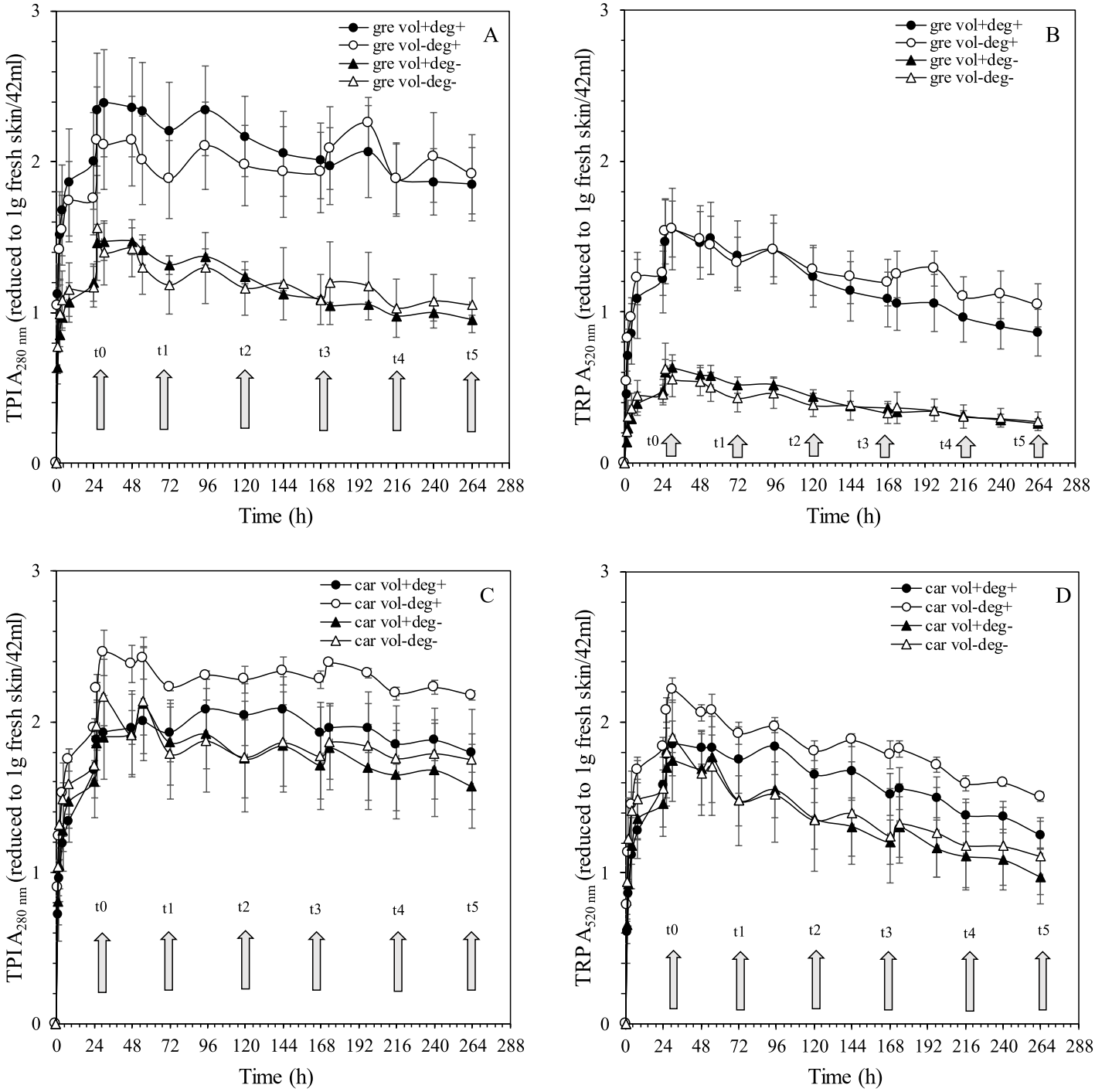


Figure 30. Polyphenols diffusion during skin maceration experiments in wine-like solvents, followed by UV-visible spectrophotometry. TPI: Total Polyphenol Index ($A_{280\ nm}$). TRP: Total Red Pigments ($A_{520\ nm}$). Arrows indicate the different ethanol additions. A) Grenache, TPI; B) Grenache TRP; C) Carignan TPI, D) Carignan TRP.

HPSEC analyses were also performed at the end of three different steps of the diffusion experiment (0, 5, and 15% ethanol) to follow the extraction of polymeric tannins. These steps were chosen based on the analyses performed at the end of all steps (t_0 to t_5) with the vol^+deg^+ modalities

of the two varieties. Since the volume had no impact, results detailed in Figure 31 are those obtained with $vol^{+}deg^{+}$ and $vol^{+}deg^{-}$ only. Polymeric tannin concentrations in the diffusion solutions increased for all Carignan modalities until the end of the 15% ethanol step (t_5), but only very slightly after the 12% one. By contrast, after a maximum reached at 5% ethanol, a slight decrease of polymeric tannins was observed for all Grenache modalities, more pronounced for the deg^{-} than the deg^{+} ones.

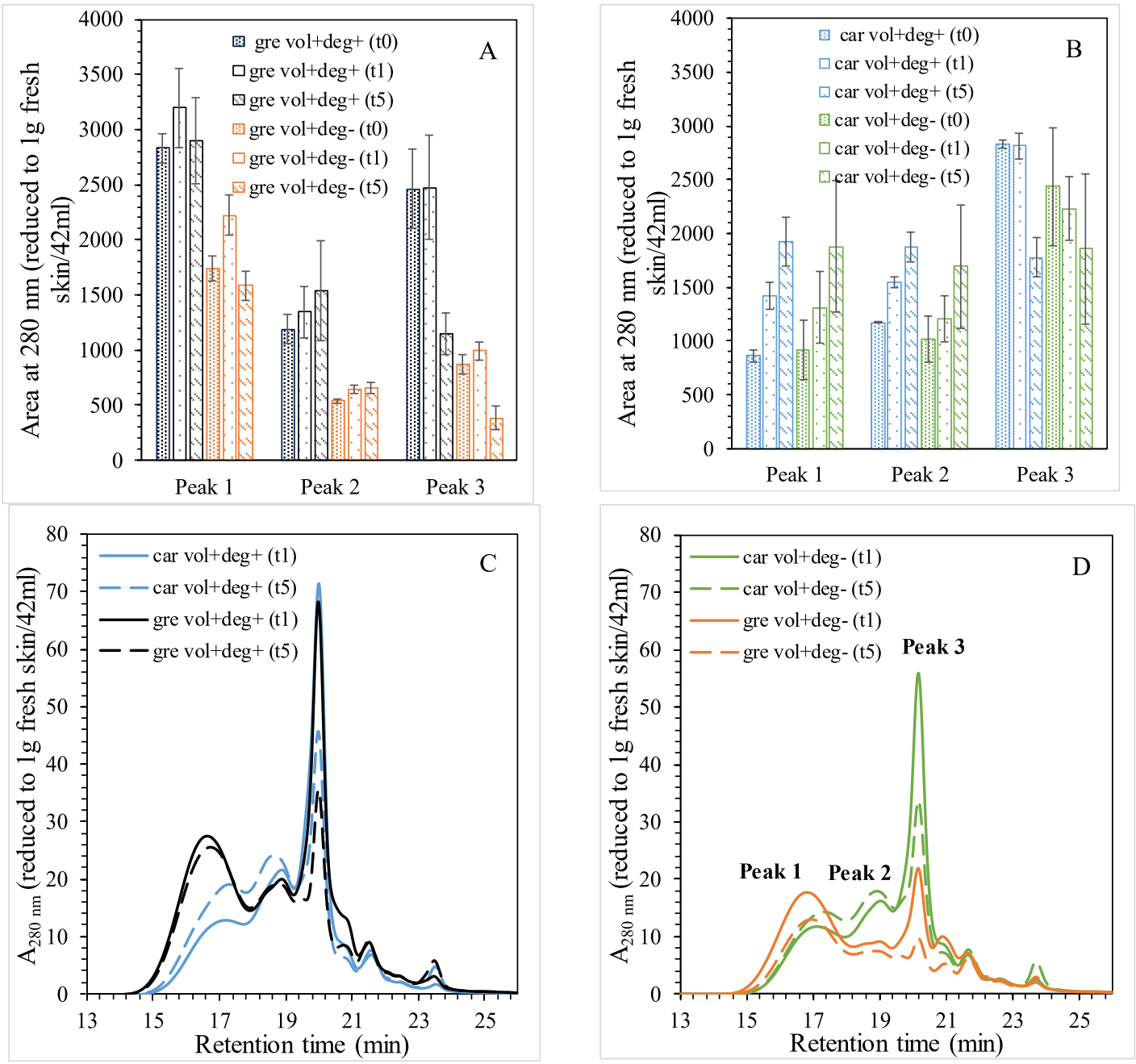


Figure 31. HPSEC analysis of polyphenols at the end of different steps of the diffusion experiments. Peak 1: polymeric tannins; Peak 2: di-trimers and anthocyanins, Peak 3: anthocyanins. Integration of the 3 main peaks of the HPSEC spectra at the end of the first (t_0, 0% ethanol), second (t_1, 5% ethanol) and last (t_5, 15% ethanol) steps of the maceration experiment for the $vol^{+}deg^{+}$ and $vol^{+}deg^{-}$ modalities of the Grenache (A) and the Carignan (B) varieties. Comparison of the HPSEC profiles of the $vol^{+}deg^{+}$ (C) and $vol^{+}deg^{-}$ (D) modalities of Grenache and Carignan at the end of t_1 and t_5.

Anthocyanin and tannin extraction: impact of variety, berry maturity and size

In wine-like maceration conditions, the concentrations of anthocyanins and tannins will be dependent on their extraction from skins but also from losses, which may be induced by chemical or physicochemical mechanisms. Maximum and final TRPs, anthocyanins, and polymeric tannins concentrations in the diffusion solutions are reported for all modalities in Table 9 and compared to their concentration in the fresh skin acetonic extracts.

Extraction of anthocyanins was not correlated to their concentrations in skins. At their maximum in the diffusion medium, the % TRP extracted was twice higher for the Grenache modalities (61-75%) than for the Carignan ones (28-32%). So, close concentrations of red pigments were obtained by diffusion for the Carignan $vol^{+}deg^{-}$ and the Grenache $vol^{+}deg^{+}$ modalities whereas anthocyanin contents in the Carignan $vol^{+}deg^{-}$ skins were twice higher. Besides, soluble anthocyanins were mainly the non-acylated ones, from the beginning to the end of the experiment (Table 9). The coumaroylated/non acylated anthocyanin ratios varied between 0.3 (deg^{+}) and 0.5 (deg^{-}) in the Carignan skins, and between 0.15 (deg^{+}) and 0.25 (deg^{-}) in the Grenache ones. In solution, these ratios fell to values between 0.05 and 0.1. These results are consistent with previous ones (Favre *et al.,* 2019; Fournand *et al.,* 2006) that showed a poor extraction of *p*-coumaroylated anthocyanins in both simulated extraction experiments and winemaking for other grape varieties. However, these structural features alone did not account for the differences observed here between Carignan and Grenache, as lower extraction was also found for non-acylated anthocyanins in Carignan. According to Ortega-Regules *et al*. (Ortega-Regules *et al.,* 2006; Ortega-Regules *et al.,* 2008), higher anthocyanin extractability could be associated with low concentrations in galactose, glucose, and mannose in the skin AISs, along with a low DE of pectins. These results, obtained from the comparison of four varieties (Cabernet-Sauvignon, Syrah, Merlot, and Monastrell), are not in total agreement with those obtained here: Carignan had lower galactose content than Grenache (and higher arabinose/galactose ratios), but also lower glucose and mannose contents (cellulose, hemicelluloses). This suggests that differences in the insoluble PRAGs of pectins could be of importance in anthocyanin extractability.

Table 9. Polyphenol extraction. Values represent concentrations reduced considering 1 g fresh skin in 42 mL solvent (in mg/L eq. malvidin-3-O-glucoside for anthocyanins and mg/L eq. epicatechin for tannins). ISP: initial skin polyphenols (acetonic solvent); SSP: soluble extracted skin polyphenols (at maximum and the end of the maceration experiment); PSP: Precipitated skin polyphenols; RESP: residual extractable polyphenols in wine-like solvents; NESP: non extracted polyphenols (acetonic solvent). Non-acyl. (non-acylated) and *p*-coum (*p*-coumaroylated) anthocyanins. Different letters indicate significant differences between samples for a given parameter (Tukey's test for $p<0.05$).

		Carignan				Grenache			
		vol^+deg^+	vol^-deg^+	vol^+deg^-	vol^-deg^-	vol^+deg^+	vol^-deg^+	vol^+deg^-	vol^-deg^-
ISP	TRP	6.9 ± 0.1^{a}	7.2 ± 0.1^{a}	6.3 ± 0.1^{b}	6.00 ± 0.03^{b}	2.5 ± 0.2 ^{c}	2.3 ± 0.1 ^{c}	1.0 ± 0.1^{d}	0.9 ± 0.1 ^{d}
	Non-acyl. anthocyanins	82.0 ± 20.3 ^{ab}	96.7 ± 8.6 ^{a}	56.0 ± 12.8^{bc}	60.5 ± 8.7 ^{bc}	38.5 ± 2.7 ^{cde}	44.6 ± 12.0 ^{cd}	15.4 ± 1.3 ^{de}	13.1 ± 1.2 ^{e}
	p-coum. anthocyanins	26.3 ± 5.6 ^{a}	29.3 ± 3.6 ^{a}	28.5 ± 7.4a	28.8 ± 2.6 ^{a}	6.0 ± 0.3 ^{b}	6.1 ± 0.9 ^{b}	4.1 ±0.5 ^{b}	3.4 ± 0.2 ^{b}
	Polymeric tannins	204.5 ± 41.0 ^{a}	220 ± 33.6^{a}	250.5 ±42.0^{a}	250.2 ±33.6 ^{a}	236.4 ±20.1^{a}	229.0 ±24.1 ^{a}	233.4 ±11.3^{a}	225.4 ±21.1^{a}
SSP at max	TRP	1.9 ± 0.1 ^{ab}	2.2 ± 0.1 ^{a}	1.8 ±0.3 ^{ab}	1.9 ±0.3 ^{ab}	1.6 ± 0.3 ^{b}	1.6 ± 0.2 ^{b}	0.6 ± 0.1 ^{c}	0.6 ±0.2 ^{c}
	% skin TRP	27.5 ± 1.9 ^{b}	31.0 ± 1.2 ^{b}	28.0 ±4.9 ^{b}	32.0 ±4.2 ^{b}	62.0 ±10.9 ^{a}	67.0 ± 8.7 ^{a}	64.0 ± 9.0 ^{a}	75.0 ± 20.4 ^{a}
	non-acyl. anthocyanins	23.3 ± 0.4 ^{ab}	26.7 ± 1.4 ^{a}	21.7 ±3.2 ^{ab}	23.1 ± 3.6 ^{ab}	20.0 ± 3.7 ^{ab}	18.8 ± 2.3 ^{b}	6.9 ± 0.7 ^{c}	5.6 ± 1.2 ^{c}
	p-coum. anthocyanins	2.4 ± 0.9 ^{a}	2.0 ± 0.2 ^{ab}	1.5 ± 0.4 ^{abc}	1.2 ± 0.2 ^{bcd}	1.2 ± 0.2 ^{bcd}	0.9 ± 0.2 ^{cd}	0.7 ± 0.04 ^{cd}	0.5 ± 0.1 ^{d}
	Polymeric tannins	39.0 ± 4.5 ^{bc}	40 ± 0.6 ^{bc}	39.0±10.5 ^{bc}	34.0 ±8.8 ^{c}	64.4 ± 7.2 ^{a}	54.0 ± 6.1 ^{ab}	45.0 ± 3.6 ^{bc}	40.0 ± 7.1 ^{bc}
	% skin polymeric tannins	19.0 ± 2.2 ^{bc}	18.0 ± 0.3 ^{bc}	16.0 ± 4.2 ^{c}	13.6 ± 3.5 ^{c}	27.0 ± 3.0 ^{a}	23.6 ± 2.6 ^{ab}	19.0 ± 1.5 ^{bc}	18.0 ± 3.1 ^{bc}
SSP at end	TRP	1.3 ± 0.1 ^{ab}	1.5 ±0.0 ^{a}	1.0 ± 0.2 ^{b}	1.1 ± 0.3 ^{b}	0.9 ± 0.2 ^{b}	1.0 ± 0.1 b	0.3 ± 0.0 ^{c}	0.3 ± 0.1 ^{c}
	% skin TRP	18.1 ±1.3 ^{d}	21.0±0.4 ^{cd}	16.0 ± 2.9 ^{d}	18.5 ± 4.2 ^{d}	34.0 ± 6.0 ^{a}	45.0 ± 6.2 ^{ab}	27.0 ± 3.0 ^{bcd}	33.0 ± 7.0 ^{abc}
	non-acyl. anthocyanins	13.3 ± 1.3 ^{ab}	15.6 ± 0.2 ^{a}	9.6 ± 1.6 ^{b}	11.3 ± 3.0 ^{ab}	9.7 ± 1.5 ^{b}	12.4 ± 1.7 ^{ab}	2.9 ± 0.2 ^{c}	3.2 ± 0.8 ^{c}
	p-coum. A	0.7 ± 0.1 ^{abc}	1.0 ± 0.0 ^{a}	0.8 ± 0.2 ^{ab}	1.1 ± 0.3 ^{a}	0.4 ± 0.07 ^{cd}	0.5 ± 0.1 ^{bc}	0.03 ± 0.01 ^{d}	0.06 ± 0.02 ^{d}
	Polymeric tannins	39.0 ± 4.5 ^{bc}	40.0 ± 0.6 ^{bc}	39.0 ± 10.5 ^{bc}	34.0 ± 8.8 ^{a}	58.0 ± 7.9 ^{a}	54.0 ± 6.1 ^{ab}	32.0 ± 2.7 ^{c}	33.0 ± 5.0 ^{c}
	% skin polymeric tannins	19.0 ± 2.2 ^{ab}	18.0 ± 0.3 ^{ab}	16.0 ± 4.2 ^{b}	13.6 ±3.5 ^{b}	25.0 ± 3.4 ^{a}	23.6 ± 2.6 ^{a}	14.0 ± 1.1 ^{b}	15.0 ± 2.2 ^{b}
PSP	mg fresh weight/g fresh skin	63 ± 2	78 ± 6	59 ± 19	101 ± 7	38 ± 11	65 ± 14	34 ± 2	67 ± 18
	TRP	0.55 ± 0.02 ^{a}	0.50 ± 0.06 ^{a}	0.45 ± 0.15 ^{a}	0.54 ± 0.06 ^{a}	0.10 ± 0.02 ^{b}	0.08 ± 0.01 ^{b}	0.05 ± 0.00 ^{b}	0.04 ± 0.01 ^{b}
	% skin TRP	7.9 ± 0.2 ^{ab}	7.0 ± 0.8 ^{abc}	7.2 ± 2.4 ^{abc}	9.0 ± 1.0 ^{a}	4.1 ± 0.6 ^{cd}	3.3 ± 0.3 ^{d}	4.8 ± 0.2 ^{bcd}	4.5 ± 1.5 ^{cd}
	non-acyl. anthoyanins	2.1 ± 0.1 ^{ab}	2.3 ± 0.2 ^{a}	1.6 ± 0.6 ^{ab}	1.5 ± 0.4 ^{b}	0.24 ± 0.2 ^{c}	0.70 ± 0.07 ^{c}	0.07 ± 0.02 ^{c}	0.17 ± 0.09 ^{c}
	p-coum. anthocyanins	8.6 ± 0.4 ^{a}	7.3 ± 0.8 ^{a}	10.0 ± 3.7 ^{a}	10.1 ± 2.6 ^{a}	0.21 ± 0.2 ^{b}	0.71 ± 0.04 ^{b}	0.07 ± 0.02 ^{b}	0.19 ± 0.08 ^{b}
	Polymeric tannins	19.0 ± 1.7 ^{abc}	20.0 ± 2.0 ^{ab}	14.0 ± 4.4 ^{bcd}	23.5 ± 3.8 ^{a}	8.6 ± 1.1 ^{de}	13.0 ± 0.8 ^{cde}	5.8 ± 0.2 ^{e}	7.4 ± 2.6 ^{de}
	% skin polymeric tannins	9.4 ± 0.8 ^{a}	9.1 ± 0.9 ^{a}	5.7 ±1.8 ^{b}	9.4 ± 1.5 ^{a}	3.6 ± 0.5 ^{bc}	5.6 ±0.4 ^{b}	2.5 ±0.1 ^{c}	3.3 ± 1.2 ^{bc}
RESP	TRP	0.64 ± 0.1 ^{a}	0.62 ± 0.0 ^{a}	0.45 ± 0.1 ^{b}	0.42 ± 0.1 ^{bc}	0.24 ± 0.0 ^{d}	0.3 ± 0.0 ^{cd}	0.07 ± 0.0 ^{c}	0.10 ± 0.0 ^{c}
	% skin TRP	9.3 ± 0.3 ^{a}	8.7 ± 0.3 ^{ab}	7.1 ± 0.8 ^{d}	7.0 ± 1.8 ^{d}	9.6 ±1.2 ^{bc}	12.6 ± 0.9 ^{a}	7.0 ± 0.0 ^{bc}	13.0 ± 1.2 ^{cd}
	Polymeric tannins	11.0 ± 0.4 ^{bc}	15.0 ± 0.3 ^{bc}	16.0 ± 2.2 ^{b}	21.4 ± 2.8 ^{a}	16.0 ± 3.0 ^{bc}	23.2 ±1.3 ^{a}	11.0 ± 0.8 ^{c}	16.0 ± 2.2 ^{bc}
	% skin polymeric tannins	5.4 ± 0.2 ^{cd}	6.9 ±0.2 ^{bc}	6.4 ± 0.9 ^{bcd}	8.6 ± 1.1 ^{ab}	6.7 ± 1.3 ^{bcd}	10.1 ± 0.6 ^{a}	4.5 ± 0.3 ^{d}	6.9 ± 1.0 ^{bc}
NESP	TRP	0.09 ±0.02 ^{a}	0.06 ± 0.0 ^{ab}	0.03 ±0.01 ^{b}	0.03 ± 0.01 ^{b}	0.08 ± 0.0 ^{a}	0.09 ± 0.0 ^{a}	0.03 ±0.0 ^{b}	0.03 ± 0.0 ^{b}
	% skin TRP	1.3 ± 0.3 ^{b}	0.8 ± 0.5 ^{b}	0.5 ± 0.2 ^{b}	0.5 ± 0.2 ^{b}	3.1 ± 0.3 ^{a}	3.9 ± 0.5 ^{a}	3.1 ± 0.3 ^{a}	3.6 ± 0.4 ^{a}
	Polymeric tannins	81.9 ± 4.9 ^{ab}	83.8 ± 0.3 ^{a}	61.1 ± 6.1 ^{c}	61.0 ± 6.4 ^{c}	58.4 ± 2.0 ^{c}	70.4 ± 6.1 ^{bc}	28.9 ±2.1 ^{d}	29.2 ± 1.5 ^{d}
	% skin polymeric tannins	40.0 ± 2.4 ^{a}	38.1 ± 0.1 ^{a}	24.4 ± 2.4 ^{c}	24.4 ± 2.6 ^{c}	24.7 ± 0.9 ^{c}	30.8 ± 2.7 ^{b}	12.4 ± 0.9 ^{d}	13.0 ± 0.7 ^{d}

Polymeric tannins extraction at maximum only represented a small proportion of skin polymeric tannins, as expected (Ortega-Regules *et al.,* 2006; Bindon *et al.,* 2010; Fournand *et al.,* 2006). It was higher for Grenache than for Carignan, especially for the deg^+ modalities (Table 9). Considering Grenache, it could be concluded from present results, and as for Syrah (Kilmister *et al.,* 2014) that higher anthocyanin/tannin ratios favour the extraction of tannins (deg^+ vs deg^- modalities). However, this was not the case for Carignan and was not verified when comparing the two varieties. Differences in the composition of AISs may have offset a positive impact of anthocyanins. Differences in tannin extractability are largely attributed to differences in the polysaccharide composition of skin AISs and/or to their protein content, higher in Carignan than in Grenache. In addition to higher maximums in solution, tannin diffusion was faster for Grenache than for Carignan and favored by increasing ethanol contents for the latter. This may be due to stronger interactions of tannins with cell walls, hydrophobic interactions, and H-bonds being weakened progressively by the ethanol concentration.

The strong affinity between tannins and the cell wall pectin layer, especially for HG and RG-I has been previously reported in apples (Watrelot *et al.,* 2013). The degree of methyl-esterification in HG, especially highly methylated ones, increases the affinity of the CWM for proanthocyanidins. In our data, a greater signal of highly methyl esterified HG was observed but no clear differences were observed between varieties (JIM7, LM20). The increase in highly-methyl-esterified HG could be also related to greater exposure of this epitope due to an increase in the skin porosity. The increase in the grape skin porosity can lead to the encapsulation of certain grape phenolics, preventing extraction into the must or wine of tannins (Bindon *et al.,* 2014).

The comparison of polymeric tannin size distribution in skins and in solutions (Figure 29 and Figure 31) indicated that the highest molecular weight tannins are not extracted in wine-like solvents (Bindon *et al.,* 2012; Fournand *et al.,* 2006).

After a maximum, a decrease in polymeric tannins was observed for all Grenache modalities that decreased differences between varieties at the end of the maceration. A much more pronounced decrease was also observed in all cases for red pigments. At the end of the diffusion, skins were recovered and the whole diffusion media clarified by centrifugation. Red-coloured pellets were present in all cases. Their visual observation showed the existence of two types of materials: one, located at the bottom of the centrifuge tube and having a fibrous-like

appearance, and another, above, having a gel-like appearance. The exact nature of these deposits was not further investigated here and will be the subject of future work. Their presence indicated that either precipitation of soluble material from skin cells or degradation of some insoluble material leading to the presence of cell fragments/fibers occurred during our maceration experiments. This material was found in higher amounts in the Carignan modalities than in the Grenache ones (Table 9).

Polyphenols in these pellets (PSP, Figure 27), were extracted and quantified. Results evidenced the presence of anthocyanins and tannins, related to precipitation or adsorption phenomena. They represented a small but non-negligible part of skin polyphenols and were in higher amounts for Carignan. Red pigments in **PSP**s accounted for 7-9% of the initial skin ones for Carignan, to be compared to 3-5% for Grenache, with no clear incidence of berry maturity or size. Polymeric tannins accounted for 6-9% of the initial skin tannins for Carignan and 3-6% for Grenache. HPLC-DAD analyses evidenced a preferential involvement of coumaroylated anthocyanins in the precipitates (Table 9), with coumaroylated/non-acylated anthocyanin ratios between 3.8 (deg^+) and 6.3 (deg^-) in Carignan **PSP**s and around 1 in Grenache ones. This indicated a lower solubility of coumaroylated anthocyanins and/or favored interactions with other constituents extracted from skins. Whatever the variety, anthocyanins in **PSPs** did not account for the losses observed during maceration, which were largely related to non-acylated anthocyanins. These losses are then likely related to chemical changes. They represented about 40 (deg^+) and 50% (deg^-) of the maximum TRP value for the Grenache modalities (to be compared to 42 and 54 % for anthocyanins) respectively, and about 30 (deg^+) and 43% (deg^-) for the and Carignan ones (42 and 53 % for anthocyanins). Thus, anthocyanin degradation and/or the formation of non-pigmented derived compounds, such as those from anthocyanin-tannin adducts, were likely the predominant phenomena in the observed losses. In the Carignan, part of anthocyanin losses could be attributed to the formation of derived red pigments such as tannin-anthocyanin adducts.

As for coumaroylated anthocyanins, it is unclear whether tannins in **PSP**s were precipitated or adsorbed very quickly after their solubilisation or whether their presence was related to their interaction inside the cells with debris/fibers released during maceration. A small but progressive decrease in tannin concentrations was observed during the experiment with the Grenache, not with the Carignan. As extraction was more gradual for this variety and analyses only carried out at the end of the various ethanol additions, extraction may have counterbalanced losses related to physico-chemical mechanisms.

Residual polyphenols in skins after maceration

Skins after diffusion experiments were successively washed with new 0 and 15% ethanol wine-like solvents until total polyphenol extraction (Figure 27). These washings led to the recovery of so-called residual extractible polyphenols in wine-like solvents (**RESP**, **Table 9**). The latter represented from 7 to 12% of the initial skin anthocyanins for both varieties and from 6 to 9% of the polymeric tannins. As solid/liquid diffusion is driven by a partition coefficient between the solid and the liquid phases, their extraction in new solvents indicated that an equilibrium between phases had been reached in our conditions at the end of the maceration. Finally, residual polyphenols in skins (non-extractable skin polyphenols **NESP**s) were extracted in an acetonic solvent, as for **ISP**s and **PSP**s. Only very small amounts of red pigments were recovered (Table 9). **NESP**s were mainly polymeric tannins (Table 9, Figure 32), along with small amounts of oligomers. Polymeric tannins recoveries in **NSEP** were higher for the Carignan than for the Grenache and, within a given variety, about twice higher for the deg^+ than for the deg^- modalities.

Similar HPSEC profiles were found for **SSP** and **RESP** (Figure 32), whatever the variety and the modality. HPSEC also evidenced a wider size distribution of polymeric tannins in **PSPs** by comparison to diffusion media, related to the presence of higher molecular polymers, and strong differences between Carignan and Grenache, related to *p*-coumaroylated anthocyanins. As expected, **NESPs** were preferentially the highest molecular weight tannins in skins. A mass balance was performed with the whole results obtained from our diffusion/extraction experiments (Table 9, Figure 33). Taking the sum of TRP in **SSP** (end of the maceration), **PSP**, **ESP,** and **NESP** resulted in the recovery of only 45 to 64% of the initial skin red pigments for Grenache (to be compared to 19-36% of the anthocyanins) and of 31 to 51% for Carignan (25-28% of the anthocyanins), with no clear impact of the maturity (deg^+ vs deg^- modalities).

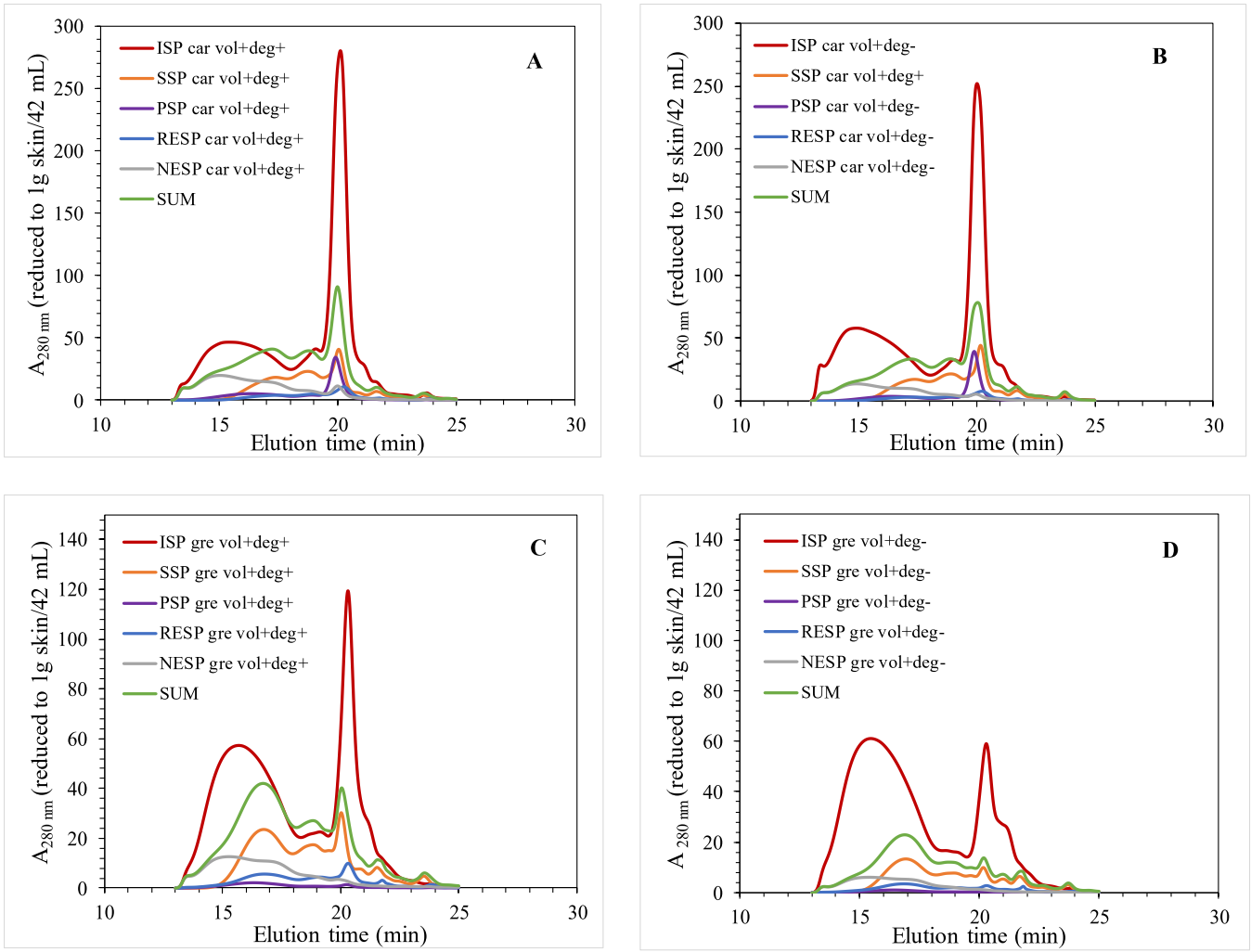


Figure 32. Comparison of the HPSEC profiles of the different phenolic extracts for the different Grenache and Carignan vol$^+$deg$^+$ and vol$^+$deg$^-$ modalities. A) Carignan vol$^+$deg$^+$ B) Carignan vol$^+$deg$^-$ C) Grenache vol$^+$deg$^+$ D) Grenache vol$^+$deg$^-$. ISP: initial skin polyphenols; SSP: soluble skin polyphenols at the end of the maceration experiment; PSP: Precipitated skin polyphenols at the end of the maceration experiment; RESP: residual extractable skin polyphenols in wine-like solvents (extraction in acetonic solvent); NESP: non-extracted skin polyphenols in wine-like solvents; SUM = SSP + PSP + RESP + NESP

Although no fermentation was carried out under our experimental conditions, our results are consistent with those of Morel-Salmi *et al.* (Morel-Salmi *et al.,* 2006), who observed a drastic loss (around 70 %) of anthocyanins during fermentation when they compared anthocyanins present in the initial skins, the wines, and the pomaces. Contrary to that observed with anthocyanins, higher tannin recoveries were found for the Carignan (72-74% for the deg$^+$ modalities and 53-56% for the deg$^-$) than for the Grenache (60-70% for deg$^+$ and 33-38% for deg$^-$), but as for anthocyanins, they were not complete. Besides, HPSEC indicated an excess of

A_{280nm} in the range of oligomers and smaller polymers for all modalities except Grenache deg^- This excess, along with the differences observed between TRPs and anthocyanins, agrees with the occurrence of chemical changes during maceration. The latter may affect anthocyanins as well as tannins alone. Indeed, even in the absence of fermentation and under conditions of protection against oxidation, cleavage and rearrangement reactions can also occur within tannins that do not imply anthocyanins (Cheynier *et al.,* 2006; Morel-Salmi *et al.,* 2006; Vidal *et al.,* 2002). These chemical reactions may also occur within skins when the integrity of the berries is broken. The quantification of polymeric tannins by HPSEC was based here on A_{280nm} measurements and was done in equivalent epicatechin. The molar absorptivity of tannins differs as a function of their degree of polymerization, even if their chemical nature is not changed (Kennedy & Jones, 2001) and can also be modified by chemical reactions. As SEC separation is based on the size, polymers with the same size but different chemical features (including different molar extinction coefficients) are coeluted, which may induce a misquantification.

Besides chemical changes, it must be considered that the same extraction procedure was performed on fresh skins, precipitates, and skins after maceration and washings, whereas these samples have different characteristics. Changes in cell-wall structures during the maceration process (11 days), leading to different permeability and solvent accessibility, as well as to stronger physico-chemical interactions than those observed in fresh skins or possibly the formation of covalent bonds, may have resulted in a lower extraction at the end (**NESP** vs **ISP**). The fact that both anthocyanins and tannins total recovery differed between the Carignan and Grenache varieties and for tannins, between the deg^+ and the deg^- modalities, is of interest and likely indicated structural differences in cells/cell walls in the initial skins and/or different changes during maceration that deserves to be further explored.

Fresh skin hardness in relation to extraction

Authors have in the past associated extractability and mechanical properties of skins during fermentation, particularly in the context of the addition of pectolytic enzymes (Rolle *et al.,* 2009; Rolle *et al.,* 2012). Indeed, they reported that during fermentation, skins seem to be characterized by increased fragility of the cell walls, which allows an easier release of colored pigments for the same variety. In our case, the varieties of Grenache and Carignan have completely different properties in terms of polyphenol extractability. Therefore, we wanted to evaluate the possibility of linking extractability, mechanical properties of the skins, and composition. To this end, the firmness of Carignan and Grenache skins was measured with

the Penelaup robot on 20 berries of each modality and average values are reported in the Figure 33.

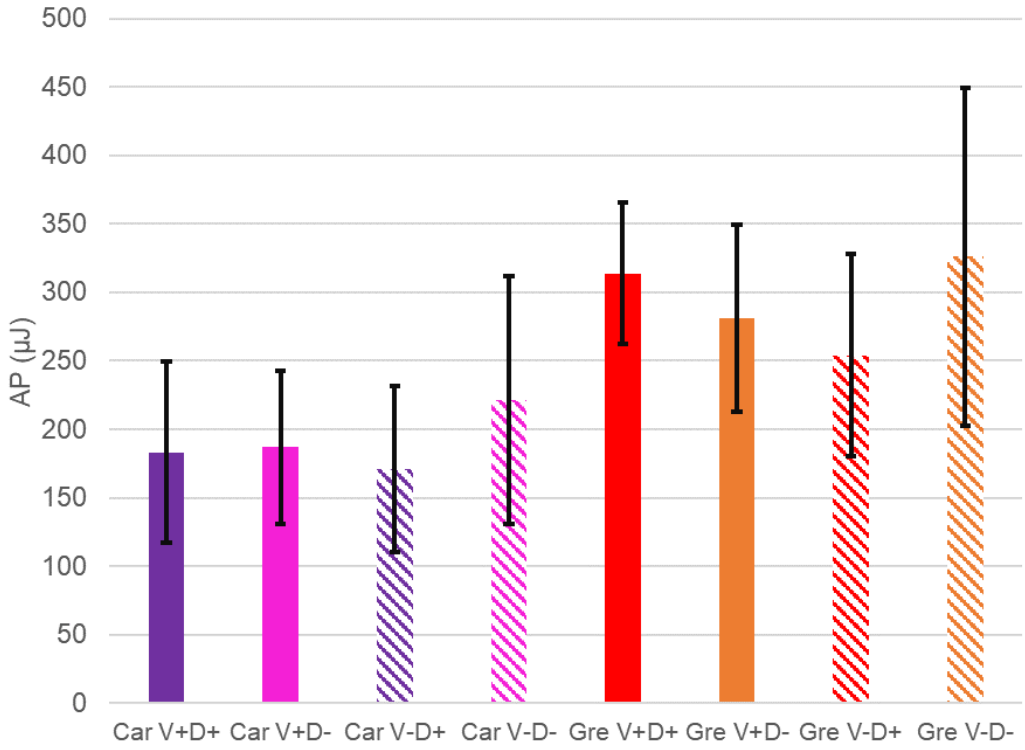


Figure 33. Skin hardness of Carignan and Grenache. Values are the average of 20 measurements

No clear relationship was observed between the concentration of sugar and skin firmness: in particular, values of firmness of deg$^-$ berries were not significantly higher than deg$^+$. The same experiments repeated in 2019 led to the same conclusions. In fact, the main factor of difference recorded in the mechanical properties of skins was related to variety, with respective values of 190 µJ for Carignan and 290 µJ for Grenache. Our results are similar to those reported by some authors (Robin *et al*., 1997), who observed a sharp decrease in firmness at ripening, with behaviour characterized by stable values during the three weeks before harvest and one week after. These results are to be linked to the fact that the cell walls of the skins differ essentially from one variety to another, in terms of extensin and hemicellulose. However, in order to be able to establish a clear correlation, it is necessary to work with a larger number of varieties.

Conclusion

The diffusion results obtained under model conditions, as well as the analyses carried out on the precipitates observed at the end of maceration and on the residual skins, made it possible to highlight differences between the two varieties studied and between berries with different levels of maturity. The impact of size was much less evident. In agreement with the literature, these differences concerned first the extraction of anthocyanins and tannins. The former was proportionally much more important in Grenache than in Carignan. This could be partly explained by the higher proportion of *p*-coumaroylated anthocyanins in Carignan skins but the extraction of non-acylated anthocyanins also was lower for this variety. The extraction of tannins was also slightly higher but above all much faster in Grenache than in Carignan, and higher for the deg^+ modalities. No decisive impact of the anthocyanin-to tannin ratio on the tannin final contents in solution was observed.

Carbohydrate and amino-acid analysis of skin AISs also evidenced differences in composition between the two varieties, and between the different modalities (especially ripeness). These differences were related to hemicellulose, cellulose, in higher content in Grenache than in Carignan, and different arabinose/galactose ratios reflecting different structures of PRAGs. Higher protein contents were found in Carignan skin AISs, especially extensins. This information, combined with a better knowledge of the polymeric skin cell wall changes during maceration and a better understanding of the part played by the chemical reactivity of tannins and anthocyanins in solution or skins, is needed to understand the impact of variety and/or berry maturity on tannin and anthocyanin extractability.

Chapter 3:

Adsorption of anthocyanins and tannins on flesh cell walls: impact of grape maturity and variety

Chapter 3: Adsorption of anthocyanins and tannins on flesh cell walls: impact of grape maturity and variety

Introduction

In the previous chapter, we studied the diffusion, in model solutions, of anthocyanins and tannins from the grape skins to a solution mimicking a grape must during fermentation. We worked with two varieties and studied the impact of berry maturity and size on the extractability of polyphenols. We highlighted a predominant impact of the variety, which was attributed to several differences in the composition of the skin:

- the anthocyanins present in Grenache and Carignan are different: there are proportionally more *p*-coumaroylated anthocyanins in Carignan, and these anthocyanins diffuse differently;

- the composition of skin cell walls is different: the cell walls of Carignan are richer in extensins, and those of Grenache are rather richer in hemicellulose (glucans/xyloglucans).

On the whole, between 15 and 25% of the tannins and between 16 to 45 % of the anthocyanins were extracted from skins (diffusion minus losses). Once extracted from skins, polyphenols may be involved in other processes that change their final concentrations in wines. Adsorption through the flesh cell walls is considered by some authors to play a major role (Bindon *et al.,* 2017; Sparrow *et al.,* 2015).

In this chapter, our objective was to investigate the effect of grape variety and maturity on the structural composition (polysaccharides, proteins) of flesh cell walls and therefore on their interactions with anthocyanins and tannins. To this end, the fresh flesh cell walls of the two different grape varieties, Carignan and Grenache, were recovered from berries at two different ripeness. The berry volume was no longer considered here and all experiments were performed with the vol+ berries. An accurate cell wall screening composition of the flesh cell walls was performed using the CoMPP method. Interactions were studied in model solutions at 0 and 15% ethanol through adsorption isotherms, using anthocyanins and tannin fractions extracted from Carignan skins in wine-like conditions. As the objective was to study the impact of flesh insoluble solids, the same polyphenol fractions were used for the two varieties. Most of the previous studies have been performed using flesh cell walls purified and freeze-dried. In

this work and to get closer to the real conditions, the choice was made to work with the fresh water-insoluble constituents of the flesh (fresh FWIM, mainly cell walls), recovered after extensive washing with aqueous buffer. Indeed, purification and drying procedures may affect the tri-dimensional structure of the network and the accessibility to interaction sites.

Materials and Methods

Grape sampling

Two *Vitis vinifera* grape varieties, of the 2018 season, (Carignan and Grenache) were harvested at an average potential alcohol of 12 % vol. in the vineyard of the Pech Rouge experimental unit (INRA, Gruissan, France). The berries were harvested at maturity and sorted according to their natural heterogeneity in terms of density (degree of maturity: deg-, deg+) using an aqueous solution of concentrated rectified grape must. This heterogeneity was determined on 1000 berries the day before the harvest by estimating their density by flotation in different salt solutions, corresponding to a total soluble solid difference between two successive baths of 1 % vol potential alcohol. Samples of berries of each modality were recovered. The flesh was separated with a scalpel and immediately frozen in liquid nitrogen and stored at - 80°C for later experiments and analysis of their composition.

Chemicals

Acetonitrile, methanol, ethanol, acetic acid, and formic acid were HPLC grade from VWR. Acetone was provided by Fluka. Sodium chloride, tartaric acid, epicatechin, epigallocatechin gallate, lithium chloride, N,N-dimethylformamide, and trifluoroacetic acid were provided by Sigma-Aldrich, sulphuric acid by Roth. Flavanol dimer B2, flavanol trimer C1 and Malvidin-3-O-Glucoside chloride were purchased from Extrasynthese (Genay, France). Ultra-pure water was obtained from a Milli-Q Advantage A10 system (Millipore).

Preparation of alcohol insoluble (AIS) flesh cell wall material for composition analysis (polysaccharides and proteins) with the CoMPP method (Comprehensive Microarray Polymer Profiling)

Frozen flesh (from 30 berries, triplicate) was ground in liquid nitrogen. The alcohol-insoluble solids (AISs) were isolated first using the same procedure as that described previously

for skins for the analysis of neutral and acidic sugars and amino acids. In the second series of experiments, the AIS (from 30 berries, triplicates) were isolated using the following procedure, as the optimal one to CoMPP technology (Nguema-Ona *et al.,* 2012; Moore *et al.,* 2014; Zietsman *et al.,* 2015). The frozen ground flesh (10 g) was incubated in 100% v/v absolute ethanol at 80°C for 15 min to deactivate endogenous enzymes. After centrifugation, the pellets were then washed sequentially by a series of solvents (ethanol, methanol, chloroform, and acetone) using a stirring plate. Thereafter, the pellet material was suspended in deionized water and freeze-dried to yield a dry powder of flesh cell wall AIS which were used for structural composition analysis. Total neutral and acidic sugars and total proteins in AIS were quantified as described in chapter 2.

Comprehensive Microarray Polymer Profiling (CoMPP) of AIS pulp cell wall materials (see Appendix B for more details)

AIS samples were sequentially extracted first with 50mM CDTA (cyclo-hexane-diamino-tetra-acetic acid pH 7.5) and then with NaOH (4M) (Moller *et al.,* 2007) to obtain the pectin and hemicellulose rich fractions. After centrifugation, the extracts from each fraction were printed onto a nitrocellulose membrane and then probed with a series of monoclonal antibodies (mAbs) and carbohydrate-binding modules (CBMs). The raw data was normalized and converted into heatmap for visualization and the relative abundance of different polymers epitopes are displayed on a scale of 0–100. The values in the heatmaps produced are mean spot signals from three biological repeats and 4 dilutions, and the highest signal measured was set to 100 with the other data adjusted accordingly. Zero in the heatmap does not represent absolutely no signal but just below the cut-off value of 5 on the raw data.

Extraction and purification of Polyphenols from grape skins

Grape berries of Carignan (deg+) were defrosted, then the skins were peeled with a scalpel and immersed in model wine-like solutions at 15% v/v ethanol containing 3 g/L tartaric acid, 50 mM NaCl, sodium azide (0.02%), and 40 mg/L SO_2 (to prevent the oxidation of phenolic compounds), the pH of which was adjusted at 3.5 with NaOH 1M. Flasks were placed under argon and gently stirred in dark at 20 °C for 3 days. The model solution was then filtered, centrifuged, and concentrated under vacuum at 40°C using a rotavapor (RII BUCHI) to eliminate the ethanol. The polyphenol extract was recovered in water and then deposited on a column filled with Fractogel toyopearl HW-50F (bed volume 226 cm^3) attached to a lab-scale chromatography system (Puriflash 430, Interchim) equipped with a UV detector. The

separation was achieved by polarity changes by applying a flow rate of 7 ml/min with an elution time of 35 min for each fraction. The column was rinsed with two bed volumes of distilled water and trifluoroacetic acid (TFA, 0.05%) to remove the water-soluble compounds (sugars, amino acids,…). The monomeric (mainly anthocyanins) fraction was then eluted with 55/45 (v/v) ethanol/water solvents acidified with TFA (0.05%). The polymeric tannin fraction was then eluted with 60/40 (v/v) water/acetone solvent acidified with TFA (0.05%). Both extracts were evaporated under vacuum at 40 °C to remove the solvents and recovered with water before freeze-drying. The two phenolic fractions (anthocyanins and tannins) were stored at −80 °C in sealed vials under argon atmosphere before further use for the adsorption experiments. 2 g of tannins and 2.7 g of anthocyanins were extracted from the skin of 1800 berries per 2.4 L of wine-like solution. A sample of each fraction was taken for UV-Vis spectrophotometry, HPLC, and SEC analysis. The mean degree of polymerization (mDP) of skin tannins was estimated to be around 10 by comparison to standards (Figure 26, Chapter 2).

Adsorption experiments

Adsorption experiments were performed in 0 and 15% ethanol model solutions composed of 3 g/L tartaric acid, 50 mM NaCl (2.16g/L), sodium azide (0.02%), and 40 mg/L SO_2 (to prevent the oxidation of phenolic compounds), the pH of which was adjusted at 3.5 with NaOH 1M.

Each experiment was performed with 1.1 g of ground frozen fresh flesh for 1 mL of polyphenol solution. This amount was chosen based on the data of the 2017 harvest where 1 kg of berries led to 700 ml of wine on average, with the pulp accounting for 80% of the berry fresh weight. The flesh weighed in a 2 mL Eppendorf was defrosted at cold temperature 4°C and washed 8 times in the 0% model solution to eliminate the must and all water-soluble components. After centrifugation (15000 *x g*, 10 min, 4 °C), a sample from the washing solution was taken to perform soluble sugars and protein assays using the sulphuric phenol and Bradford methods, respectively. After no soluble components were detected, the fresh flesh water-insoluble material (FWIM) was dried by drainage before being weighed again. Fresh FWIM represented about 10% of the initial flesh weight. It was immediately mixed with 1 mL of the model solutions at the adequate polyphenol concentrations for interaction experiments. Samples were stirred continuously on a rotator (Stuart SB3 40 rpm) in darkness and at 20 °C. A solution of polyphenols without FWIM and fresh FWIM suspended in the model solution without polyphenols served as controls. At the end of the experiment, FWIMs were removed

by centrifugation and the supernatants recovered for analysis. The adsorption was quantified from the difference in concentration between the supernatants and the controls (polyphenols solution without FWIM and FWIM suspended in model wine without polyphenols).

Kinetic studies were performed first to determine the contact time required to reach the adsorption equilibrium. These experiments were done at two different anthocyanin and tannin concentrations (0.1 g/L and 2 g/L) and in 0 and 15% ethanol solutions using a fresh FWIM of the Carignan variety. Samples were stirred for 2 days and an aliquot was taken at several time intervals (1, 3, 6, 24, 37, and 48 h). The FWIM were removed by centrifugation and the supernatant recovered for spectrophotometric measurements.

Based on adsorption kinetics, a contact time of 30 h was chosen for further adsorption experiments. The latter were performed first with the tannin fraction using concentrations ranging from 0.25 to 8 g/L (0.25, 0.5, 1, 2, 4, 6, 8 g/L) and with the anthocyanin fraction using concentrations ranging from 0.1 to 2 g/L (0.1, 0.25, 0.5, 1, 2 g/L). In another series of experiments, mixtures of anthocyanins and tannins at different concentrations (different anthocyanin/tannin ratios) were used. Two anthocyanin concentrations of 0.5 g/L and 2 g/L were selected. The former is equivalent to a frequent anthocyanin concentration in wine whereas the latter represents a very high concentration condition. Each of these anthocyanin solutions was mixed with four different tannin concentrations: 0.5, 1, 2, 4 g/L.

Reversibility of adsorption

After adsorption experiments, the FWIMs recovered by centrifugation were dispersed in 1ml of a model solution without polyphenols. Samples were stirred for 30 h and the supernatants recovered. The reversibility of the adsorption was determined through the analysis of the polyphenols recovered in solution.

Polyphenol analysis

Total polyphenols Index (TPI) and total red pigments (TRP) were determined by UV–visible spectrophotometry (spectrophotometer UV-1600, Shimadzu) at 280 and 520 nm (1 cm path length) after adequate dilution in HCl 1 M. HPLC and HPSEC analyses were however performed to distinguish between the adsorption of monomers (free anthocyanins) and that of tannins. Free anthocyanins were analyzed by HPLC using a Waters chromatography system equipped with DAD detection and a C18 reversed-phase column (Atlantis T3, Waters). The mobile phase was a gradient of solvent A (95:5, v/v, water/formic acid) and B (80/15/5, v/v/v,

acetonitrile/water/ formic acid). The flow rate was set at 0.4 ml/min and the oven temperature at 38°C. Anthocyanins were quantified at 520 nm, in equivalent of malvidin-3-O-glucoside. Tannins and their size distribution in the samples were analyzed by high-pressure size exclusion chromatography (HPSEC). After evaporation of 1 mL of the extract, it was dissolved with the mobile phase consisting of dimethylformamide with 1 % acetic acid (v/v), 5 % water (v/v), and 0.15 M lithium chloride, filtered at 0.22 µm. After solubilization in an ultrasonic bath, samples were centrifuged (15000 g for 15 min, 15 °C) and 50 µL were injected into the high-performance liquid chromatography system (Agilent HPLC 1260 Infinity II) equipped with a diode array detector. The separation was done in two Phenogel columns (Phenomenex, Le Pecq, France) (300 mm × 7.8 mm, 5 µm 50 Å and 300 mm × 7.8 mm, 5 µm 1000 Å), with an isocratic flow rate of 0.8 mL/min (run time 35 min) and a temperature of 60°C. The UV-signal was monitored at 280 nm. Commercial epicatechin, B2 dimer, epigallocatechin gallate, malvidin, and home prepared and characterized tannin fractions were used to evaluate retention times corresponding to monomers, oligomers, and polymers (Figure 26, Chapter 2).

Statistical analysis

Statistical analysis was performed using Statistica software. Samples were done in triplicate. The results obtained were assessed by factorial and one-way ANOVA analysis followed by a Tukey Test.

Results and Discussion

Characterization of flesh cell walls

The dry weight of the purified flesh cell walls, along with their global composition in neutral sugars, acidic sugars, and amino acids are given in Table 10. Statistical analyses of their sugar and amino acid composition did not evidence significant differences between the two varieties and the two maturities (results not shown).

Table 10. Alcohol Insoluble Solids contents (AIS) and their composition in total sugar (neutral and acidic) and amino acid of the four modalities of Carignan and Grenache initial flesh.

	AIS mg/g fresh flesh	Total neutral sugars mg/g AIS	Total acidic sugars mg/g AIS	Total amino acids mg/g AIS
Car deg+	3.7 ± 0.3	181.1 ± 14.6	147.6 ± 18.9	218.9 ± 10.8
Car deg-	4.1 ± 0.7	199.4 ± 8.4	159.5 ± 38.4	255.9 ± 8.0
Gre deg+	3.4 ± 0.2	161.2 ± 28.9	138.9 ± 25.8	238.4 ± 8.5
Gre deg-	3.7 ± 0.6	218.9 ± 27.4	132.2 ± 20.4	263.8 ± 27.0

The composition of the flesh cell walls was analyzed using the CoMPP method. To this end, the extracts resulting from a sequential extraction using CDTA (pectin rich fraction) and NaOH (hemicellulose rich fraction) were probed with 28 mAbs or CBMs (Table 11). These antibodies were chosen as they recognize a broad range of different cell wall polymers (see Table 1 in Appendix B for a summary of the probes used and their targets). The CDTA fraction includes pectic polysaccharides (homogalacturonans HG, rhamnogalacturonans I RGI, and arabinans), Arabinogalactan-Proteins, and extensins. The NaOH fraction is rich in mannans, glucan/xyloglucan, and cellulose. RGII was not studied, as there is no monoclonal antibody for detecting this polymer in the analysis. HG epitopes in samples were recognized by mAbs JIM5, JIM7, LM18, LM19, LM20/ RGI by mAbs INRA-RU1 and INRA-RU2/ and its side chains by mAbs LM6, LM13/ mannans by mAb LM21, xyloglucan by mAbs LM15, LM25/ cellulose by CBM3a/ extensins by mAbs LM1, JIM11, JIM20/ and AGPs by mAbs JIM8, JIM13, LM14.

In the pectin rich fraction, the mAb JIM7 and LM20 showed the highest signal intensity compared with the other HG antibodies used, confirming the previous findings that grape berry pectins are highly methyl esterified (Gao *et al.,* 2019). Both MAb JIM7 and LM20 recognizes methyl-esterified HG polymers but does not bind to un-esterified HG. Weaker signals were observed for mAbs JIM5, LM18, and LM19. This indicated the presence of low or demethyl-esterified HG zones, but in lower amounts than esterified HG. Identical signals were found for the mAbs JIM5 and JIM7in the CDTA fractions of the two varieties. The JIM5/JIM7 ratio (~ 0.26 for Grenache and ~ 0.24 for Carignan) showed that there are about 4 times less de-esterified HG than esterified HG in their flesh cell walls. The arabinan epitopes, recognized by LM6 and LM13 antibodies, were present in the flesh cell walls contrary to the galactan epitope.

The signal of arabinan (attached as side chains of RGI) was found to be higher in Carignan varieties compared to Grenache (significantly higher according to the factorial and one-way ANOVA). The RGI and RGI side-chain epitopes were extracted not only with CDTA but also with NaOH, and in higher proportions for the Carignan than for the Grenache flesh cell walls. This suggests that an RGI coating layer is strongly associated with xyloglucan and cellulose microfibrils in the cell wall structure.

Differences were also observed with the signals of extensins (hydroxy-proline-rich glycoproteins) and arabinogalactan proteins (AGPs, rich in serine and hydroxyproline). Higher extensin contents were found in the Carignan flesh cell wall CDTA fractions than in the Grenache ones. The Factorial ANOVA showed significant differences between varieties with the JIM11 and the JIM20 extensin antibodies. Extensins play an important structuring role in the assembly of plant cell walls, (Cannon *et al.,* 2008; Lamport *et al.,* 2011; Chormova & Fry, 2016). This would result, among other things, in ionic interaction with the pectic network (MacDougall et *al.,* 2001; Cannon *et al.,* 2008). More surprisingly, extensins were also detected in the NaOH fraction, but only with the Grenache variety, indicating a different localization for the two varieties and may have a more important structuring role for the Grenache flesh cell walls, with higher interactions with xyloglucans. AGPs were found in higher amounts in Grenache.

In the hemicellulosic rich fraction, the samples were mainly recognized by the mAbs/CBMs for RGI side chains (INRA-RUI, INRA-RUII, LM6, and LM13), mannan (LM21) xyloglucan/glucan (LM15, LM25), and cellulose (CBM3a). The main hemicellulose polymers were xyloglucans, which consist of a backbone of β-1,4-linked glucan where 3 out of 4 glucose units are substituted with xylose at position 6 (e.g. the XXXG motif). Other motifs are galactosylated and fucosylated such as XXFG and XLFG (Gao *et al.,* 2016).

The factorial ANOVA results also indicated an impact of the maturity (deg+ vs deg- modalities). Although both varieties were harvested at the same average maturity (12% potential alcohol), the densimetric sorting showed a greater heterogeneity for the Grenache berries than for the Carignan ones. Sugar analyses carried out after separation of the berries into 2 batches indicated a lower maturity (vol+deg- 176 ± 3 and vol+deg + 212 ± 2 g/L) for the Carignan than for the Grenache (vol+deg- 193 ± 1 and vol+deg+ 240 ± 2). This may explain the observed differences in the ANOVA analysis of the pectin-rich extracts between the two varieties: pectins in the carignan flesh cell walls are not yet impacted by the maturation.

Extraction	Samples	HG with a low DE (JIM5)	HG with a high DE (JIM7)	HG partially methylesterified (LM18)	HG partially methylesterified (LM19)	HG partially methylesterified (LM20)	HG blockwise methylesterified (PAM1)	HG Ca2+ crosslinked (2F4)	Xylogalacturonan (LM8)	Backbone of rhamnogalacturonan I (INRA-RU1)	Backbone of rhamnogalacturonan I (INRA-RU2)	(1→4)-β-D-galactan (LM5)	(1→5)-α-L-arabinan (LM6)	Linearised (1→5)-α-L-arabinan (LM13)	(1→4)-β-D-(galacto)(gluco)mannan (LM21)	(1→3)-β-D-glucan (BS-400-2)	Xyloglucan (XXXG motif) (LM15)	Xyloglucan (LM24)	Xyloglucan / unsubstituted β-D-glucan (LM25)	(1→4)-β-D-xylan (LM10)	(1→4)-β-D-xylan/arabinoxylan (LM11)	cellulose (CBM3a)	Extensin (LM1)	Extensin (JIM11)	Extensin (JIM20)	AGP (JIM8)	AGP (JIM13)	AGP (LM14)	AGP, β-linked GlcA (LM2)
		HG								RGI		RGI and side chains			Mannans	Glucan/Xyloglucan				Xylans		Cellulose	Extensins			AGPs			
CDTA-Fraction	pulp car vol+deg+	19	79	10	18	76	0	0	0	28	44	0	33	20	0	0	0	0	7	0	0	0	20	18	32	14	14	6	0
	pulp car vol+deg-	19	80	12	18	74	0	0	0	27	41	0	32	20	0	0	0	0	9	0	0	0	21	16	32	12	12	0	0
	pulp gre vol+deg+	23	86	13	25	92	0	0	0	25	44	0	27	10	0	0	0	0	0	0	0	0	15	11	19	18	15	4	0
	pulp gre vol+deg-	23	87	13	24	84	0	0	0	28	44	2	29	14	0	0	0	0	6	0	0	0	15	12	19	24	19	9	0
NaOH-Fraction	pulp car vol+deg+	0	0	0	0	0	0	0	0	10	20	0	19	12	11	0	26	0	17	0	0	9	0	0	0	0	0	0	0
	pulp car vol+deg-	0	0	0	0	0	0	0	0	11	16	0	16	11	12	0	26	0	19	0	0	9	0	0	5	0	3	0	0
	pulp gre vol+deg+	0	0	0	0	0	0	0	0	4	12	0	6	0	12	0	22	0	16	0	0	8	6	4	8	0	0	0	0
	pulp gre vol+deg-	0	0	0	0	0	0	0	0	6	10	0	4	0	14	6	23	0	18	0	0	8	6	7	9	0	4	0	0

Table 11. Comprehensive Microarray Polymer Profiling analysis of the CDTA (pectin-rich) and NaOH (hemicellulose-rich) fractions extracted from the AIS pulp cell walls of Carignan and Grenache varieties, each sorted according to their degree of maturity. The values in the heatmap are the average of three biological samples and show the relative abundance of cell wall epitopes in each sample. A cut-off of 5 was applied to the raw data.

Table 12. Factorial ANOVA assessed on the raw data of the heatmap table from the AIS samples of the flesh cell walls of Carignan and Grenache

	p-value (5%)	HG partially de-esterified (JIM5)	HG partially esterified (JIM7)	G partially de-esterified (LM18)	G partially de-esterified (LM19)	G partially esterified (LM20)	HG blockwise de-esterified (mAb PAM1	HG Ca2+ crosslinked (2F4)	xylogalacturonan (LM8)	ackbone of rhamnogalacturonan I (INRA-RU1)	Backbone of rhamnogalacturonan I (INRA-RU2)	(1→4)-β-D-galactan (LM5)	(1→5)-α-L-arabinan (LM6)	Linearised (1→5)-α-L-arabinan (LM13)	(1→4)-β-D-(galacto)(gluco)mannan (LM21)	(1→3)-β-D-glucan (BS-400-2)	Xyloglucan (XXXG motif) (LM15)	Xyloglucan (LM24)	Xyloglucan / unsubstituted β-D-glucan (LM25)	(1→4)-β-D-xylan (LM10)	(1→4)-β-D-xylan/arabinoxylan (LM11)	cellulose (CBM3a)	Extensin (LM1)	Extensin (JIM11)	Extensin (JIM20)	AGP (JIM8)	AGP (JIM13)	AGP (LM14)	AGP, β-linked GlcA (LM2)
	Factors																												
CDTA-Fraction	Variety	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.2	0.7	0.4	0.0	0.0	0.0	0.5	0.0	0.4	0.7	0.0	0.2	0.3	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.1
	Maturity	1.0	0.8	0.2	0.6	0.1	0.5	0.1	0.1	0.3	0.3	0.6	0.8	0.0	0.3	0.1	0.2	0.3	0.0	0.4	0.9	0.6	0.8	0.4	1.0	0.1	0.2	0.1	0.9
	Variety*Maturity	0.8	0.9	0.5	0.5	0.3	0.7	0.0	0.2	0.3	0.4	0.6	0.2	0.1	0.9	0.0	0.1	0.7	0.6	0.9	0.7	0.2	0.9	0.2	0.9	0.0	0.0	0.0	0.8
NaOH-Fraction	Variety	0.5	0.9	0.4	0.1	0.4	1.0	0.4	0.2	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.5	0.6	0.0	0.3	0.2	0.0	0.0	0.0	0.6	0.6	0.0	0.8
	Maturity	0.3	0.2	0.3	0.9	0.8	0.2	0.2	0.3	0.2	0.1	0.6	0.1	0.2	0.3	0.0	0.7	0.2	0.1	0.7	0.2	0.6	0.7	0.1	0.1	0.0	0.0	0.0	0.2
	Variety*Maturity	0.2	0.9	0.2	0.5	0.6	0.3	0.4	0.2	0.7	0.5	0.3	0.3	0.3	0.5	0.1	0.5	1.0	1.0	0.2	0.5	0.6	0.1	0.4	0.4	0.8	0.1	0.0	0.1

		HG partially de-esterified (JIM5)	HG partially esterified (JIM7)	G partially de-esterified (LM18)	G partially de-esterified (LM19)	G partially esterified (LM20)	HG blockwise de-esterified (mAb PAM1	HG Ca2+ crosslinked (2F4)	xylogalacturonan (LM8)	ackbone of rhamnogalacturonan I (INRA-RU1)	Backbone of rhamnogalacturonan I (INRA-RU2)	(1→4)-β-D-galactan (LM5)	(1→5)-α-L-arabinan (LM6)	Linearised (1→5)-α-L-arabinan (LM13)	(1→4)-β-D-(galacto)(gluco)mannan (LM21)	(1→3)-β-D-glucan (BS-400-2)	Xyloglucan (XXXG motif) (LM15)	Xyloglucan (LM24)	Xyloglucan / unsubstituted β-D-glucan (LM25)	(1→4)-β-D-xylan (LM10)	(1→4)-β-D-xylan/arabinoxylan (LM11)	cellulose (CBM3a)	Extensin (LM1)	Extensin (JIM11)	Extensin (JIM20)	AGP (JIM8)	AGP (JIM13)	AGP (LM14)	AGP, β-linked GlcA (LM2)
CDTA-Fraction	car vol+deg+	19 a	79 a	10 b	18 b	76b	1 a	0.3 b	0.03 a	28 a	44 a	3ab	33 a	20a	0.2 a	0.3 b	0.4 a	0.5 a	7 b	0.4 a	1 a	1 a	20 a	18 a	32 a	14bc	14 bc	6 b	19a
	car vol+deg-	19 a	80 a	12 ab	18 b	74b	1 a	0.5 b	0.02 a	27 a	41 a	2b	32ab	20a	0.3 a	0.2 b	0.4 a	0.5 a	9 a	0.6 a	1 a	1 a	21 a	16 a	32 a	12c	12 c	4 b	19a
	gre vol+deg+	23 a	86 a	13 ab	25 a	92 a	1 a	1 a	0.04 a	25 a	44 a	4ab	27 b	10b	0.2 a	0.3 b	0.5 a	0.5 a	4 c	0.2 a	2 a	0.5 a	15 a	11 b	19 b	18b	15 b	6 b	23a
	gre vol+deg-	23 a	87a	13 a	24 a	84ab	1 a	0.5 b	0.03a	28 a	44 a	4a	29ab	14b	0.3 a	0.8 a	0.2a	0.4 a	6 b	0.3 a	2 a	1 a	15 a	12 b	19 b	24a	19 a	9 a	23a
NaOH-Fraction	**car vol+deg+**	**0.1 a**	**0.00 a**	**0.1 a**	**0.2 a**	**0.1 a**	**1 a**	**0.02 a**	**0.1 a**	**10 a**	**20a**	**1a**	**19 a**	**12 a**	**11 a**	**2 b**	**26 a**	**1 a**	**17 a**	**0.4 a**	**3 a**	**9 a**	**3 b**	**2 b**	**4 b**	**2b**	**4 ab**	**0.2 b**	**0.1 a**
	car vol+deg-	**0.2 a**	**0.01 a**	**0.1 a**	**0.3 a**	**0.1 a**	**1 a**	**0.2 a**	**0.1 a**	**11 a**	**16ab**	**2a**	**16 a**	**11 a**	**12 a**	**3 b**	**26 a**	**1 a**	**19 a**	**1 a**	**3 a**	**9 a**	**4 b**	**3 b**	**6 ab**	**4a**	**5 a**	**1 a**	**0.1 a**
	gre vol+deg+	**0.1 a**	**0.00 a**	**0.1 a**	**0.2 a**	**0.1 a**	**1 a**	**0.03 a**	**0.04 a**	**5 b**	**12bc**	**1a**	**6 b**	**3 b**	**12 a**	**2 b**	**22 a**	**1 a**	**16 a**	**1 a**	**1 a**	**8 a**	**6 a**	**6 a**	**8 a**	**2b**	**4 b**	**0.1 b**	**0.1 a**
	gre vol+deg-	**0.1 a**	**0.01 a**	**0.1a**	**0.1 a**	**0.1 a**	**0.4 a**	**0.1 a**	**0.1 a**	**6 b**	**10c**	**1a**	**6 b**	**3 b**	**14 a**	**7 a**	**23 a**	**1 a**	**18 a**	**1 a**	**3 a**	**8 a**	**6 a**	**7 a**	**9 a**	**4ab**	**5 a**	**0.1 b**	**0.04 a**

Table 13. One-way ANOVA followed by the Tukey test assessed on the raw data of the heatmap table to determine structural differences between the AIS of the flesh cell walls of Carignan and Grenache deg+ and deg- berries. Different letters indicate significant differences between samples for a given parameter (Tukey's test for $p<0.05$).

Kinetic of PA and anthocyanin adsorption by FWIM

Kinetic studies were performed first to determine the time needed to reach equilibrium. Experiments were performed with the fresh FWIM of Carignan with the tannins and anthocyanin fractions, using two different concentrations (0.1 and 2 g/L). Polyphenol adsorption was followed during 48 h using absorbency measurements (Figure 34) and was the difference between the initial concentration in solution and the concentration at time t. After an increase during the first hours, a plateau value was obtained after 24 h at the higher concentration (2 g/L), indicating that the equilibrium was reached. At low concentration, the plateau was reached from the first hours. From these results, the contact time for adsorption isotherm experiments was set at 30 h.

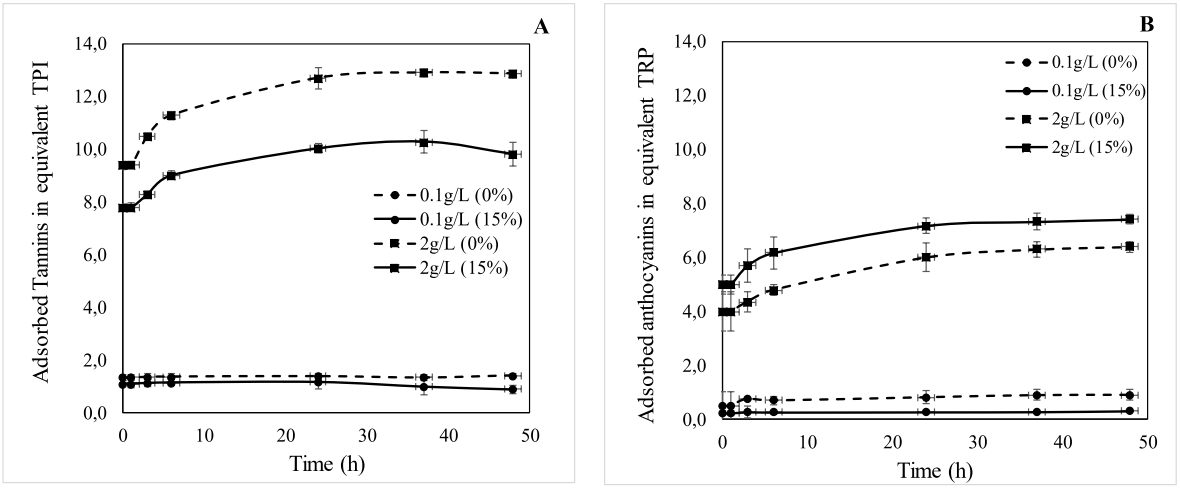


Figure 34. Adsorption kinetics at 0.1 and 2 g/L of A) tannins and B) Anthocyanins on the fresh flesh water-insoluble material (FWIM) of Carignan. Experiments were performed in 0 and 15% model solutions, using the FWIM of 1.1 g of fresh flesh per mL of solution. Results are expressed in equivalent TPI for tannins and equivalent TRP for anthocyanins.

Tannin adsorption by FWIM

Adsorption isotherms were established using tannin concentrations varying between 0.25 and 8 g/L for a fresh FWIM concentration of 10 g/L (Figure 35A). Up to initial concentrations of 0.5 g/L (initial part of the isotherm), adsorption represented 80 and 76-77% of the initial tannin contents in solution for Carignan and Grenache, respectively (Table 14). No significant differences were observed between the two varieties and modalities and the ethanol content had no impact. At higher concentrations in solution, a change in slope was observed: adsorbed amounts increased more progressively and represented between 45 and 60

% of the initial tannin concentration in solution, depending on the variety and the ethanol concentration. No plateau value could be observed within the tested concentration range.

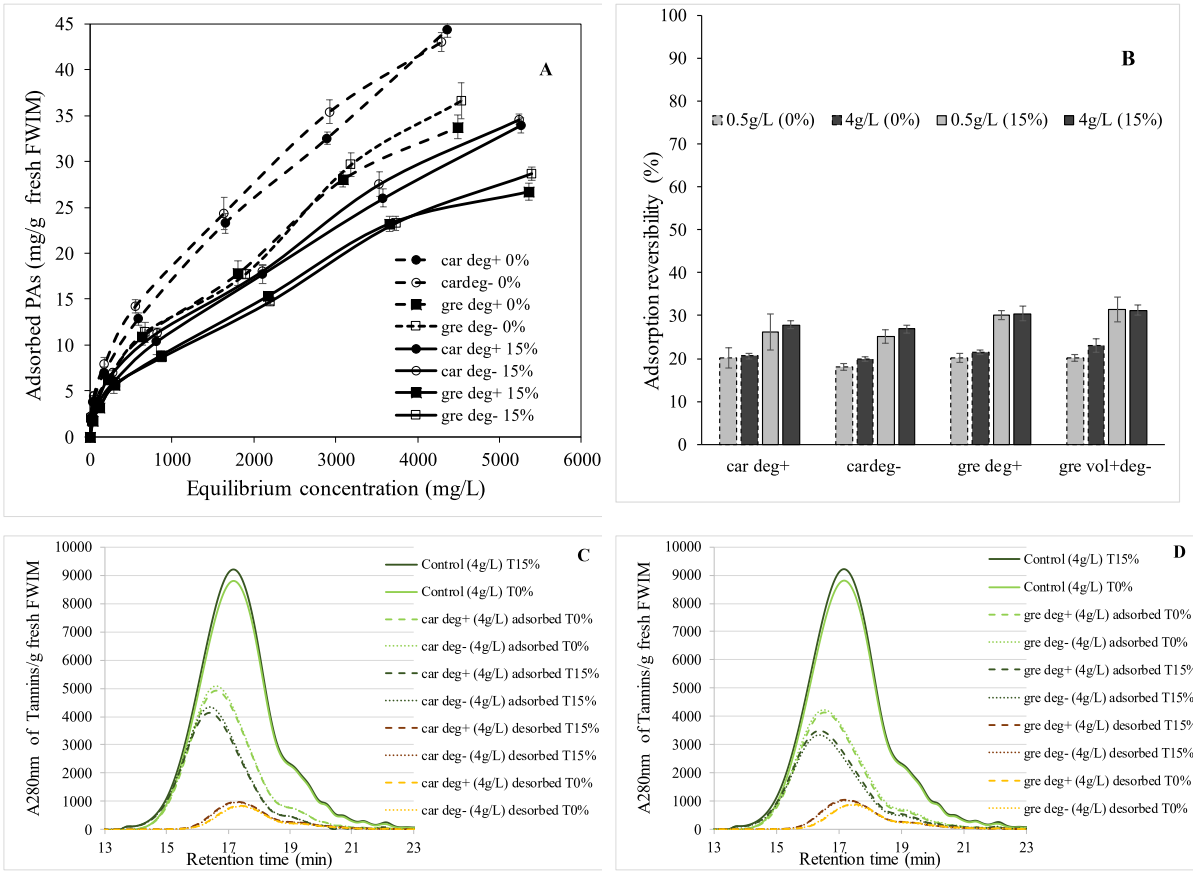


Figure 35. Adsorption isotherms of grape skin tannins (A) on the fresh FWIM of Carignan and Grenache, at two different maturity degrees (deg+; deg-) and in 0 and 15% ethanol model solutions. Results are expressed in mg adsorbed tannins/g of fresh FWIM. (B) Reversibility of the adsorption. HPSEC analysis performed for a 4 g/ initial tannin concentration in the solution for the two Carignan (C) and Grenache (D) deg+ and deg- modalities and the two ethanol concentrations showing the HPSEC chromatograms of the initial tannin solution; the adsorbed tannins, obtained by subtracting the chromatogram of the supernatant after interaction from that of the initial solution; the desorbed tannins.

	0% ethanol						15% ethanol					
	Tannins											
Initial concentration (mg/L)	500		4000		8000		500		4000		8000	
Adsorbed amount	C (mg/L)	**Q (mg/g)**	C (mg/L)	**Q (mg/g)**	C (mg/L)	**Q (mg/g)**	C (mg/L)	**Q (mg/g)**	C (mg/L)	**Q (mg/g)**	C (mg/L)	**Q (mg/g)**
car vol+deg+	436.8 ± 5.3	**3.8±0.4x**	2384.6±47.0	**23.3 ±1.1 x**	3670.0±75.0	**44.4±0.8x**	382.6±17.0	**3.7±0.6x**	1793.6±88.7	**17.7 ±1.0 x**	2838.0±58.2	**34.0±0.8x**
car vol+deg-	432.6±31.4	**4.37±0.6x**	2400.2±70.5	**24.4 ±1.7 x**	3743.7±89.2	**43.0±1.0x**	382.6±11.2	**4.0±0.1x**	1791.1±43.6	**18.1 ±0.6 x**	2861.0±65.1	**34.6±0.6x**
gre vol+deg+	436.0±6.2	**3.5±0.3x**	2219.0±22.8	**17.8 ±1.4 y**	3537.9±35.5	**33.8±1.3y**	367.9±11.5	**3.2±0.2x**	1738.8±61.7	**15.4 ±0.3 y**	2733.6±51.0	**26.7±0.9y**
gre vol+deg-	446.8±9.5	**4.0±0.2x**	2129.4±29.7	**17.7 ±0.6 y**	3495.9±128.5	**36.6±2.0y**	365.3±11.4	**3.2±0.1x**	1719.6±84.1	**14.7 ±0.3 y**	2703.1±89.2	**28.7±0.7y**
	Anthocyanins											
Initial concentration (mg/L)	500		2000				500		2000			
Adsorbed amount	C (mg/L)	**Q (mg/g)**	C (mg/L)	**Q (mg/g)**			C (mg/L)	**Q (mg/g)**	C (mg/L)	**Q (mg/g)**		
car vol+deg+	78.1±28.2	**0.8±0.2x**	425.8±54.9	**4.2 ±0.4 x**			106.6±14.0	**0.9±0.03x**	537.7±33.2	**4.5 ±0.2 x**		
car vol+deg-	83.4±3.5	**0.8±0.03x**	403.2±47.3	**3.9 ±0.5 x**			105.5±2.6	**0.9±0.03x**	476.4±13.3	**4.2 ±0.4 x**		
gre vol+deg+	91.8±8.3	**1.0±0.01x**	426.1±9.8	**4.7 ±0.1 x**			111.0±5.4	**0.8±0.02x**	557.6±11.1	**3.7 ±0.1 x**		
gre vol+deg-	73.3±20.6	**0.9±0.1x**	401.2±35.6	**4.6 ±0.1 x**			122.8±11.6	**0.8±0.00x**	566.7±25.2	**3.8 ±0.1 x**		

Table 14. Adsorbed tannin and anthocyanin amounts expressed in Concentration (mg/L) and Quantity (mg /g fresh flesh water-insoluble material cell walls) for Carignan and Grenache, in 0 and 15% model solutions. Different letters indicate significant differences (One-way ANOVA) between samples for a given parameter (Tukey's test for $p<0.05$).

At low concentrations, lateral interactions between adsorbed species are low and the initial part of the isotherm represents the polymer affinity for the sorbent. As the polymer concentration increases, adsorbed amounts depend on the number of binding sites and of their affinity for the polymer, on the accessibility of these binding sites, and possible conformational rearrangements and lateral interactions between adsorbed polymers. FWIMs are essentially cell walls, that is complex tri-dimensional networks of different polysaccharides and proteins, with interaction sites having different affinities for polyphenols (Le Bourvellec *et al.,* 2005; Le bourvellec *et al.,* 2012; Poncet-Legrand *et al.,* 2007; McRae *et al.,* 2010, Frazier *et al.,* 2010). The change in slope observed here in the isotherms may then reflect the different nature of these binding sites. The lack of plateau value even at tannin concentrations as high as 8 g/L indicates the existence of numerous interaction sites, still accessible at these concentrations, and for which tannins do not have a high affinity. Contrary to what was observed at low concentrations, adsorbed amounts were significantly higher with Carignan FWIM than with Grenache ones, but the maturity of the berry had no impact. A higher binding was always observed at 0% ethanol in comparison to 15%, as already observed (Medina-Plaza *et al.,* 2019), Le Bourvellec *et al.,* 2004) and in agreement with the impact of ethanol on polar interactions (hydrophobic interactions and H-bond formation) and the role played by the latter in polyphenol solubility and adsorption (Poncet-Legrand *et al.,* 2003; Cartalade & Vernhet, 2006)

Present results can be compared to those obtained previously by Bindon *et al.,* (2012 and 2014) using skin tannins and flesh and skin cell walls purified from Cabernet Sauvignon berries. With an mDP 33 tannin fraction and an initial tannin concentration of 8 g/L in solution, they found adsorbed amounts of the order of 280 mg/g and 170 mg/g for flesh and skin cell walls, respectively. In the present work, we have used the flesh water-insoluble materials, extensively washed with water but not with organic solvents and not dried. The dried AIS from these FWIM represents between 3.4 and 4.1 mg/g fresh flesh, depending on the variety and the modality. Considering the initial mass of flesh used for each interaction experiment (1.1g) and the corresponding AIS values, adsorption represented here about 900 mg/g and 700 mg/g dry cell walls in 0% and 15% ethanol, respectively. Besides, comparing an mDP11 (as in the present work) and mDP 33 tannin fractions for their adsorption by skin cell walls, it was found that adsorbed amounts were 1/3 lower for the mDP 11 tannins (Bindon *et al.,* 2012). Thus, it can be concluded that adsorbed amounts by fresh FWIM are about three times higher than that found with the corresponding AISs. These differences indicate that the cell wall purification, which

involves several washing steps with organic solvents, and/or the drying affect the binding capacity of flesh cell walls.

The reversibility of tannin adsorption was measured at initial concentrations of 0.5 and 4 g/L (Figure 35B). This reversibility was not influenced by the adsorbed amount and remained rather low, which suggests multiple bonding between tannins and FWIM. It was higher in the presence of ethanol and slightly higher for Grenache than for Carignan. HPSEC analyses were performed for a tannin concentration of 4 g/L in the initial solution, the corresponding supernatants after adsorption, and the supernatants recovered from reversibility experiments (Figure 35C and D). The highest molecular weight tannins were preferentially adsorbed, as in previous data, but adsorption also concerned small oligomers (di and trimers). In agreement with the irreversibility conferred by multiple bonding, desorption only concerned small tannins.

The best way to explain these results is to analyze the differences between fleshes since the polyphenols used are identical. The analysis of the CoMPPs showed us that the parameter with the greatest impact was variety, particularly on de-esterified HG, hemicellulose, extensin, and arabinan compositions. This is in agreement with adsorption data: the effect of variety is higher than the effect of maturity. Higher composition in extensins and RGI for Carignan are consistent with higher retention of tannins (Gao *et al.,* 2016), and thus higher adsorption in our experiments. Differences in composition were also observed with the partially methylesterifeid homogalacturonan (LM19 and LM20). Watrelot *et al.,* 2013 observed a positive impact of the degree of methylation of HG on their interactions with tannins in solution, but only with very different HG (0, 30, and 70% of methylation) and with relatively high molecular weight tannins (mDP30). They could not detect interactions between mDP9 tannins and homogalacturonans. The tannins used in our experiments were extracted in wine-like conditions and have a mDP around 10. This suggests that differences in homogalacturonans are not responsible for the differences observed here in adsorption.

Anthocyanin adsorption by FWIM

Results of adsorption experiments performed with anthocyanins at concentrations between 0.1 and 2 g/L are shown in Figure 36. Anthocyanins exhibited only a very low affinity for FWIM (Figure 36A): the percentage of adsorbed anthocyanin from solution varied between 16 to 28%, depending on their initial concentration in solution (Table 14). No differences were observed between the two varieties and the deg+ and deg- modalities, and ethanol content of 15% had no significant impact. Much higher reversibility was evidenced for anthocyanins

compared to tannins, in both 0 and 15% ethanol (Figure 36B), likely in relation to their monomeric nature. This reversibility was influenced by the variety (higher for Grenache than for Carignan) and was strongly decreased in the presence of 15% ethanol.

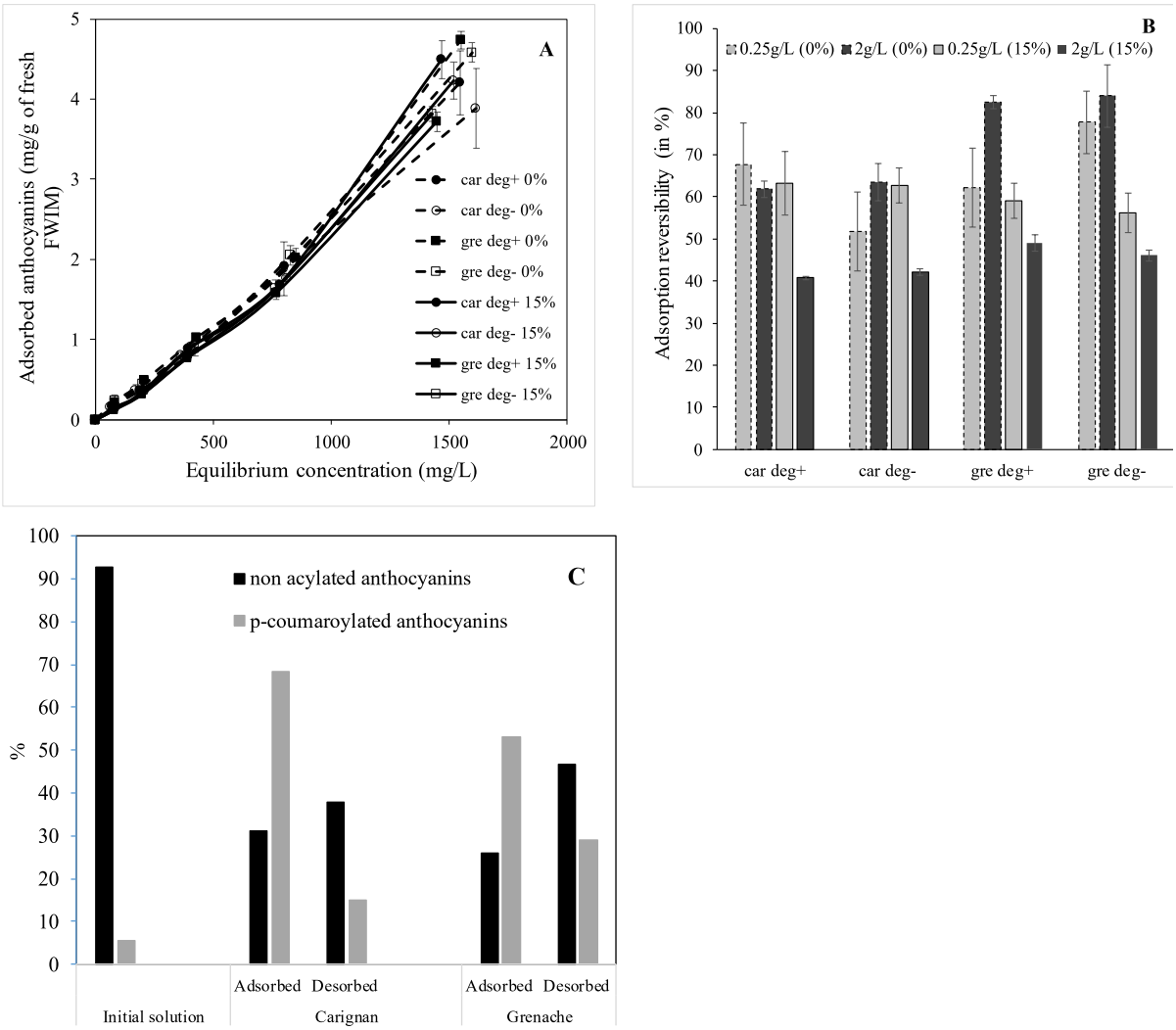


Figure 36. Adsorption isotherms of Anthocyanins (A) on the fresh pulp cell walls of Carignan and Grenache, at two different maturity degree (deg+; deg-) and in 0 and 15% ethanol model solutions. Results are expressed in mg adsorbed anthocyanins/g of fresh pulp cell walls. (B) Reversibility of the adsorption. (C) Proportions of *p*-coumaroylated and non-acylated anthocyanins in the initial anthocyanin fraction and of the adsorbed and desorbed (reversibility) anthocyanins (initial concentration in solution: 2 g/L, 15% ethanol).

Anthocyanins in the fraction extracted from skins were mainly glycosylated anthocyanins (92.7%) and the second most abundant population were *p*-coumaroylated anthocyanins (5.2%) (Figure 36C). Other acylated anthocyanins only represented a very minor proportion of the fraction. Analysis evidenced a marked selectivity for the adsorption of *p*-coumaroylated derivatives, and lower reversibility of their adsorption by comparison to non-

acylated anthocyanins. This preferential adsorption of *p*-coumaroylated anthocyanins by FWIM is in line with the previous observations that they are only very little extracted by diffusion or during winemaking compared to their initial content in skins (Fournand *et al.,* 2006; Chapter 2 of this thesis). Present results suggest that their preferential adsorption on both skin and flesh has the same cause. Although no significant impact of ethanol was observed here on anthocyanin adsorption, its impact on reversibility may be related to lower solubility of *p*-coumaroylated anthocyanins in 15% than in 0% ethanol.

A positive correlation has been observed between the cellulose content and the degree of methylation of pectins and anthocyanin extraction (Ortega-Regules *et al.,* 2006). In our study, it was difficult to link the adsorption of anthocyanins to the structural composition of the flesh cell walls since this adsorption was very low and that only minor differences were observed between the two varieties and maturity.

Impact of Anthocyanins on Tannins interaction with FWIM

In this set of experiments, adsorption was studied for mixtures of anthocyanins and tannins at different concentrations and ratios. The HPSEC chromatograms of the initial solution, non-adsorbed, adsorbed and desorbed polyphenols are represented in Figure 37 for a tannin concentration of 4 g/L in the presence of 0.5 and 2 g/L anthocyanins. All the other chromatograms, obtained with the different anthocyanins/tannin ratios are summarized in supplementary data S1 (adsorption) and S2 (desorption) at the end of this chapter. In these conditions and in agreement with previous work (Bindon *et al.,* 2014), anthocyanins did not impact tannin adsorption. This does not necessarily reflect a lack of competition between anthocyanins and tannins for potential binding sites. Indeed, monomers are easily desorbed when they do not present a very high affinity for a binding site whereas, with polymers like tannins, which usually possess several binding sites, desorption is unlikely to occur simultaneously for all sites.

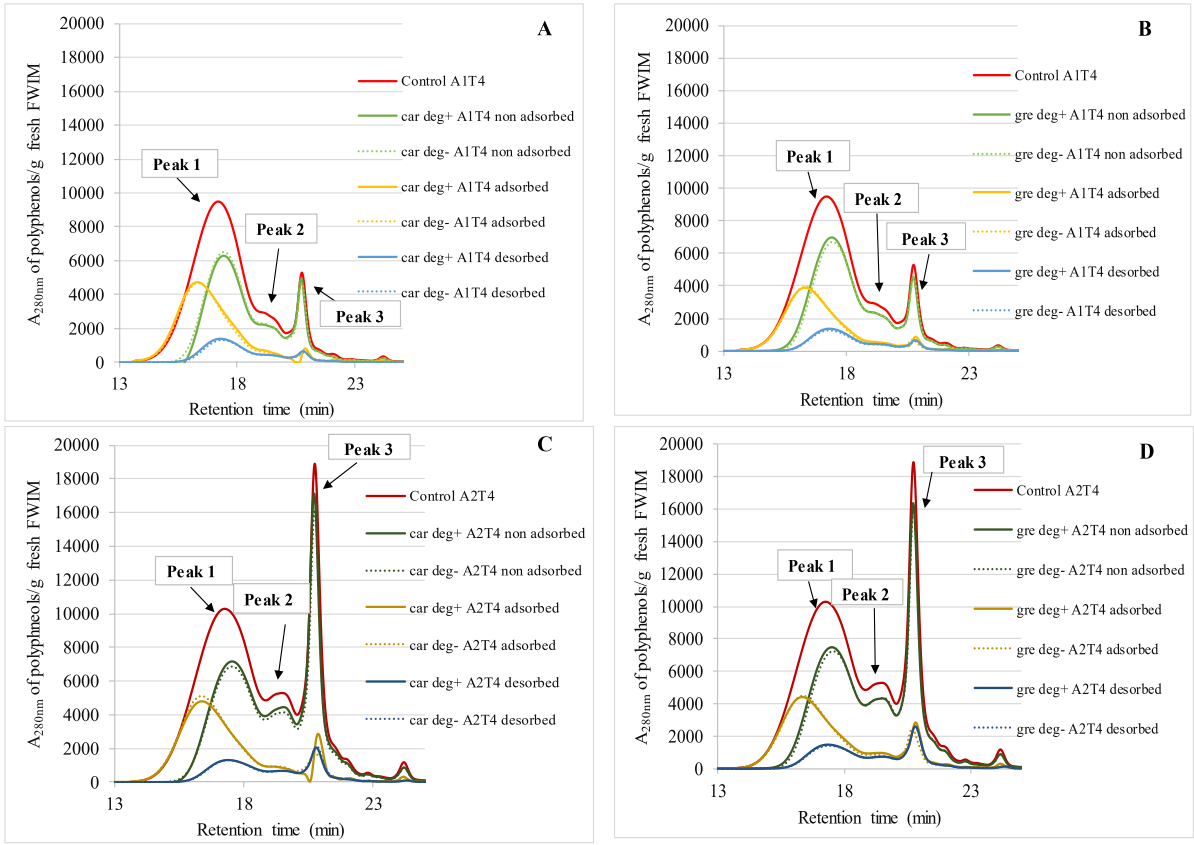


Figure 37. HPSEC analysis of the initial anthocyanin and tannin solutions and the non-adsorbed, adsorbed, and desorbed polyphenols after interactions with Carignan and Grenache pulp cell walls in a 15% ethanol model solution. Peak 1: polymeric tannins; Peak 2: di-trimers tannins coeluted with anthocyanins, Peak 3: anthocyanins. (A) Carignan A1T4 ; B) Grenache A1T4 ; (C) Carignan A2T4 ; D) Grenache A2T4. Control are samples of polyphenols without insoluble pulp cell walls served as control. Results are expressed per g of fresh insoluble pulp cell wall materials.

In our experimental conditions, the presence of anthocyanins also had a minor impact on the reversibility of the adsorption of tannins. By calculating the area of the polymeric tannin peak from the HPSEC chromatograms (Figure 38), it was found that for a 4 g/L tannin initial concentration and in 15% ethanol, desorption represented 20% (A1T4) and 28% (A2T4) of the adsorbed tannins for Carignan and was slightly higher for Grenache with 25% (A1T4) and 33% (A2T4) desorption.

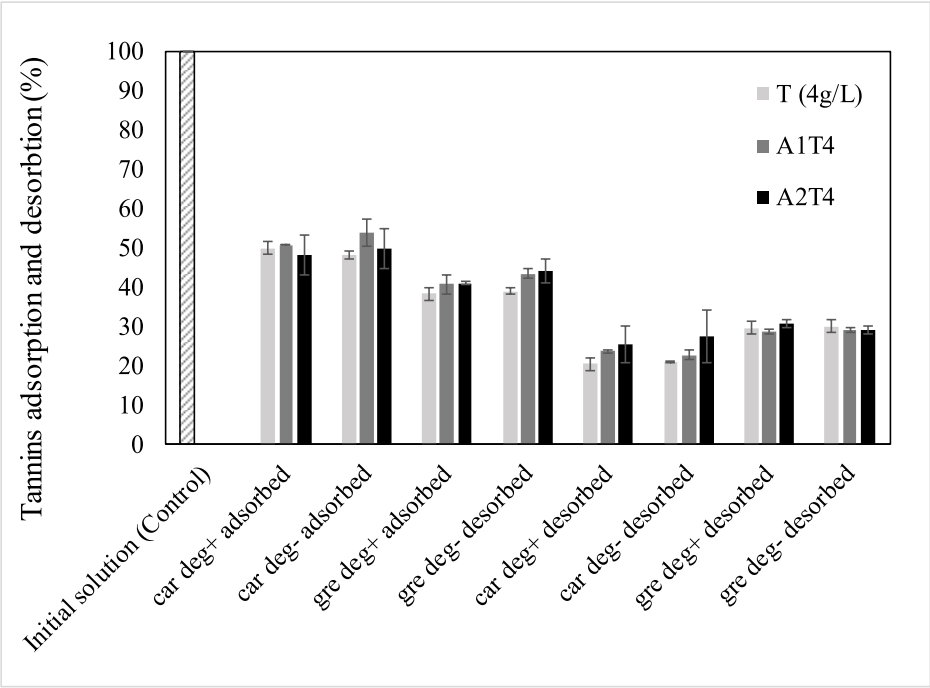


Figure 38. Comparison of the SEC results of the polymeric peak area of Tannins at 4g/L (control, adsorbed, and desorbed polyphenols) with and without the addition of anthocyanins (0.5 and 2g/L) expressed in % of Tannins adsorption and desorption for the Carignan and Grenache modalities in 15% model wine-like medium.

Impact of tannins on anthocyanin adsorption

The impact of tannins on anthocyanin adsorption was studied in 15% ethanol for 0.5 and 2 g/L concentration of anthocyanins and four different tannin concentrations (Figure 39). At usual anthocyanin concentrations in wines (0.5 g/L), the presence of tannins at low concentration (0.5 g/L) induced a decrease in anthocyanin but this impact diminished as the tannin concentration increased and was no more visible at 4 g/L. At high anthocyanin concentrations (2 g/L), a decrease of anthocyanin adsorption and enhanced reversibility were observed whatever the tannin concentration. The adsorption of *p*-coumaroylated anthocyanins was especially affected (Figure 40), but as stated before, the latter only represented a minor part (5%) of total anthocyanins in the initial solution. This decrease varied between 27 and 33% for the Carignan FWIM and between 13 and 15% for the Grenache ones, with no impact of the maturity of the berries. Results obtained at 2 g/L anthocyanins thus tends to indicate competition between anthocyanins and tannins for interaction sites on the FWIM. However, this does not explain the results obtained at low anthocyanin contents and the decreasing impact of tannins as their concentration increases. A hypothesis could be anthocyanin-tannin and anthocyanin-anthocyanin interactions. At low anthocyanin concentration, their adsorption by FWIM when the tannin concentration increases could be related to interactions between anthocyanins and

adsorbed tannins. At high anthocyanin concentrations, competition between anthocyanin-anthocyanin interactions (co-pigmentation) and anthocyanin-adsorbed tannins interactions may perhaps reduce the latter.

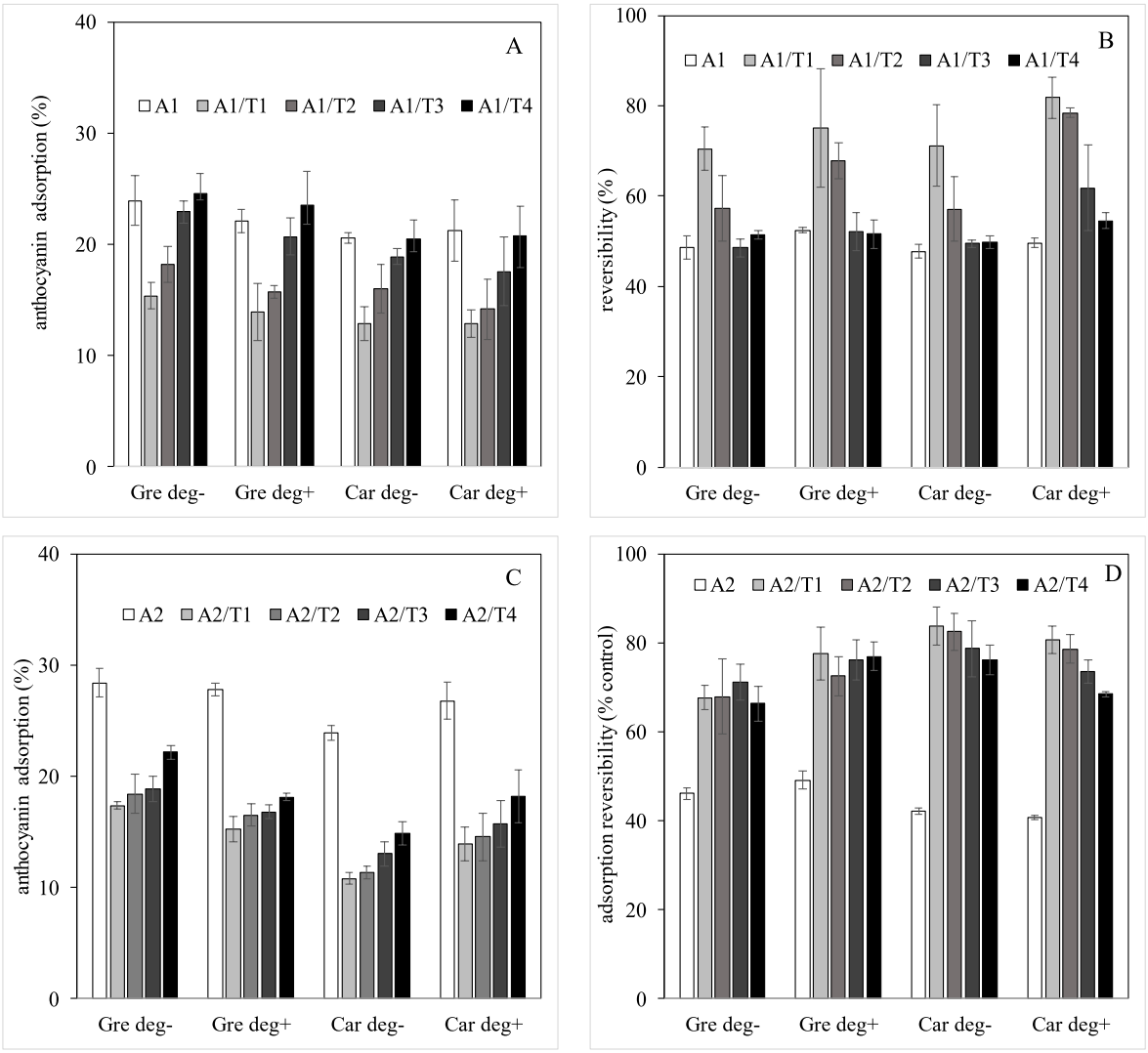


Figure 39. Adsorption and desorption of anthocyanins in the presence of different tannin concentrations in solution: T1: 0.5 g/L, T2: 1 g/L, T3: 2 g/L and T4: 4 g/L. A_1 corresponds to a concentration in anthocyanins of 0.5 g/L, A_2 corresponds to 2 g/L. Graphs A) and C): percentage of adsorbed anthocyanins. B) and D): percentage of anthocyanins that are desorbed. Values are obtained from TRP measurements. Experiments were performed in the 15% ethanol model solution.

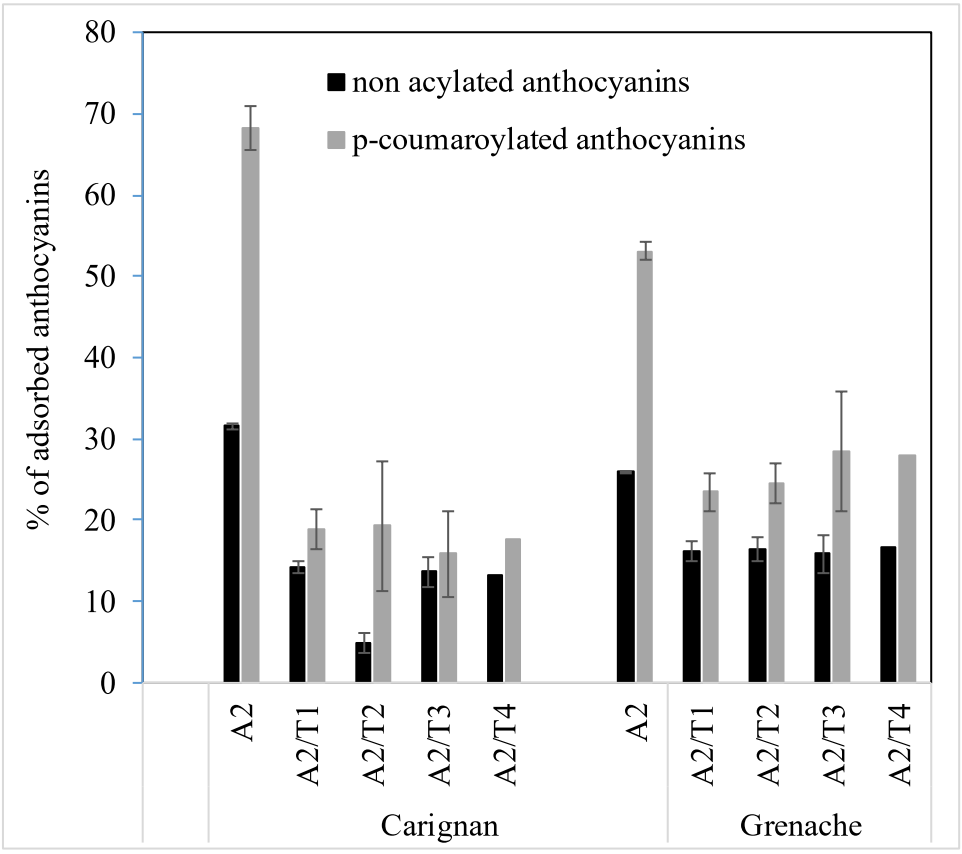


Figure 40. Percentage of adsorbed non-acylated and *p*-coumaroylated anthocyanins on FWIM at different tannin concentrations (0, T1: 0.5 g/L, T2: 1 g/L, T3: 2 g/L and T4: 4 g/L). Experiments were performed in the 15% ethanol model solution and for an anthocyanin concentration of 2 g/L.

In previous experiments, Bindon *et al.*, 2014 observed that the adsorption of anthocyanins by cell walls increased in the presence of tannins (1.25 g/L tannins) for initial concentrations of anthocyanins between 0 and 5 g/L, and then decreased at higher initial anthocyanin contents. These results can be compared to those obtained in the present study for tannin concentrations of 1 and 2 g/L and anthocyanin concentration of 0.5 and 2 g/L. In all cases, we observed a decrease and not an increase of anthocyanin adsorption. Measurements in this previous work were done after 1 hour of contact, whereas we waited 30 hours. When we performed kinetics experiments, we pointed out that tannin adsorption takes time. The exchange between anthocyanins and tannin at the interaction sites is probably a time-dependent phenomenon and probably explains the differences in results.

Conclusion

The insoluble flesh cell wall materials have the potential to significantly alter the composition of tannins in solution through the adsorption of tannins and anthocyanins. In the present study and in agreement with previous works, we found that at their usual concentration in wines, more than half of the tannins, especially those with the highest mDP, were adsorbed by FWIM and eliminated from the solution. The adsorption isotherms did not show a high affinity of the tannins for the interaction sites present in the FWIMs but underlined the high adsorption capacity of the latter: no plateau could be reached even for concentrations as high as 8 g/L. It was also found that the presence of tannins at their usual contents in wines decreases anthocyanin adsorption and that the latter only have a very low affinity for FWIMs. At a concentration of 0.5 g/L (usual concentration in wines), this adsorption can however induce a 15% decrease in their content in wine. Concerning anthocyanins, an important observation in the present work is the preferential adsorption of *p*-coumaroylated anthocyanins, which is in agreement with their lower extraction from skins during maceration. The latter only represented a minor proportion of the initial anthocyanins in the polyphenolic extract used. This extract was obtained from the maceration of Carignan skins in a 15% ethanol solution, which may have further limited their extraction (chapter 2). It would have been interesting to study interactions between FWIMs and several anthocyanin extracts with different proportions in *p*-coumaroylated anthocyanins to assess the impact of anthocyanin composition in the wine on their adsorption level.

An impact of the variety on the interactions was evidenced, whereas the modalities of different degrees of maturity did not affect the adsorption. According to the CoMPP results, this higher interaction observed with Carignan variety can be related to the high interactions between tannins and the extensin and arabinan part of its flesh cell walls. The flesh of Carignan berries was able to adsorb roughly 20% more tannins than the flesh of Grenache. The reversibility of tannin adsorption was also different, slightly higher with Grenache compared to Carignan, indicating weaker interactions This may not seem much, but if we consider that the diffusion of skin polyphenols was already less than with Grenache (about 15 % versus 25%), the two effects are cumulative. This finding, along with the preferential involvement of *p*-coumaroylated anthocyanins in interactions, has significant implications for winemaking and has the potential to contribute to the divergences often observed between total tannin concentration in grape tissues and that in wine.

To summarise, chapter 2 showed a significant impact of cell walls composition and the proportion of *p*-coumaroylated anthocyanins on the percentage of polyphenols that diffuse from the grape skins to the liquid medium. Similarly, chapter 3 showed that more polyphenols were adsorbed on Carignan fresh FWIM, and analysis of the pulp cell walls suggest that it is mainly due to the flesh cell walls composition.

During vinification, these two stages are simultaneous and it seems important to us to see if the two effects observed are cumulative, possibly with synergy, or on the contrary antagonistic. Moreover, the seeds are also a potential source of tannins and were not taken into account. In chapter 4, we will study the diffusion from these three compartments and compare the results with microvinifications.

Chapter 4:

Polyphenol diffusion and interactions: comparison between model systems and winemaking

Chapter 4: Polyphenol diffusion and interactions: comparison between model systems and winemaking

Introduction

During winemaking, polyphenol diffusion and interaction phenomena occur simultaneously. To understand their respective impact and the potential impact of variety and maturity, it was important to study diffusion and interactions with flesh insoluble material separately. This is why in the previous chapters of this manuscript, different experiments were done in model conditions to:

- study the diffusion of polyphenols from the skins of Carignan and Grenache berries sorted according to their size and maturity (vol+deg+; vol+deg-; vol-deg-; vol-deg+);
- study the interaction of flesh water insoluble materials (mainly cell walls) with anthocyanins and tannins, considering the two varieties and the two different berry maturity (deg+, deg-).

Our objective in this chapter was to study diffusion (mass transfer) and interactions together to understand their respective impact and to compare results obtained in model solutions and a real winemaking experiment (microvinification). To this end, new diffusions experiments were performed in model wine-like systems, including seeds and with or without flesh water insoluble material (FWIM). Only the vol+deg+ modalities of Carignan and Grenache were considered.

Materials and Methods

Grape sampling

The sampling of grape berries has been described in Chapter 2. The total sugar, pH, and total acidity of the musts corresponding to the vol+deg+ modality are reported in Table 15. Berries (30 berries per modality, triplicate) of each modality were used to separate the different compartments for polyphenol analysis. Skins, seeds, and pulp were immediately frozen in liquid nitrogen and stored at -80 °C before use. The average weight of berries and each of their compartments was determined (Table 15). 3 kg of berries were immediately crushed and used for the microvinification (900 g per "vat", triplicates). The sugar content, total acidity, and pH

were determined on the must of each essay before the beginning of the fermentation (Table 15). 500 g of berries were also immediately frozen at - 80°C for experiments in model solutions.

Table 15. Sugar concentration, acidity, weight, and repartition between the different compartments of Carignan and Grenache berries.

	sugars (g/L)	**Total acidity (g H_2SO_4/L)**	**pH**	**Berry weight**	**Skin weight %**	**Seeds %**
Carignan deg+	212 ± 2	3.72 ± 0.03	3.54 ± 0.08	2.41 ± 0.00	24.5 ± 6.45	2.95 ± 0.8
Grenache deg+	240 ± 2	4.61 ± 0.08	3.43 ± 0.01	2.41 ± 0.00	18.07 ± 2.07	3.03 ± 0.5

Macerations in model wine-like conditions

This experiment was designed to follow polyphenol diffusion from skins and seeds during a wine-like maceration experiment and study the impact of their interactions with flesh water-insoluble material on their diffusion and their final composition in the solution. To this end, five different maceration experiments were performed, each being made in triplicate: maceration of A) skins alone, B) seeds alone, C) skins + seeds, D) skins + FWIM, and finally E) skins + seeds + FWIM. Twenty berries of Carignan and Grenache vol+deg+ modality (stored at - 80°C) were used for each experiment. They were manually peeled to separate skins, seeds, and mesocarp. FWIM was prepared from the mesocarp as described in Chapter 3. The materials (of 20 grape berries) were immediately immersed in 28 mL of a model aqueous solution containing 3 g/L tartaric acid, 50 mM NaCl, and 40 mg/L SO_2, at pH 3.5 (adjusted with NaOH 1M). This volume was chosen to obtain a solid/liquid ratio similar to that found in winemaking. Simulated maceration experiments were carried out by increasing stepwise the ethanol content from 0 to 15% (similar as in Chapter 2). All experiments were performed in triplicate. Flasks were placed under argon and gently stirred on a stirring plate in dark at 22 °C for 11 days. Samples were taken and centrifuged (15000 g, 15min, 15°C) for phenolic analysis at the end of each ethanol increase step. The dilution induced by the sampling and the addition of ethanol was considered.

Winemaking experiment

Fermentation and maceration were performed in low volume tanks (<1 kg) using “French Press” coffee plungers at 22°C. 900g of berries of each modality were crushed.

Reactivated Lalvin ICV OKAY yeast (20 g/hL) and SO_2 (250 µL of an 8% solution) were added. During the alcoholic fermentation (AF), manual punching down of the pomace cap was carried out daily to homogenize the medium. It is important to note that on such small volumes, the cap of pomace does not form: the solids rise to the surface but do not compact. The decrease in sugar concentration was followed daily, along with polyphenol extraction (TPI and TRP measurements).After 8 days, at the end of the fermentation, the solid parts were manually pressed and the "free-run" and "press" wines gathered.

Polyphenol extraction from skins, pulps, and seeds

Frozen skins, pulps, and seeds were finely ground to a fine powder in liquid nitrogen and using a mortar grinder (Pulverisette 2, Fritsch). Powders (150 mg) were treated first with methanol (750 µL) then extracted with 5.25 mL of 60/40/1 (v/v/v) acetone/water/formic acid at room temperature on an orbital shaker (Precellys 24, Bertin technologies, program 5000-3*40-20). The extracts were pooled and after centrifugation (3000 *x g*, 5 min, 4°C), 1 mL aliquots were dried in a rotary evaporator under vacuum at 35°C for 2h (EZ-2 plus, Genevac SP service). Dried extracts were used for polyphenol analysis.

Polyphenol analysis

Polyphenols in grape berries extracts (skins, pulps, seeds) and the wines were analysed, by direct injection or after depolymerization for tannins, using Ultra High-Performance Liquid Chromatography coupled to triple-quadrupole Mass Spectrometry (UHPLC-QqQ-MS) in the Multiple Reaction Monitoring (MRM) mode, according to the methods described by Lambert *et al.,* 2015 and Pinasseau *et al.,* 2016 and using the same standards. These analyses were made by the polyphenol platform of the SPO joint research unit. UHPLC-QqQ-MS analyses allowed the detection and quantification of about 94 phenolics including hydroxybenzoic acids, hydroxycinnamic acids, and their derivatives, stilbenes, anthocyanins and their acylated derivatives, flavonols, and flavan-3-ols monomers and oligomers (dimers B1, B2, B3, and B4 and trimers). This method also allowed the quantification of pyranoanthocyanins, carboxypyranoanthocyanins, and phenylpyranoanthocyanins (catechyl, hydroxyphenyl, and guaiacylpyranoanthocyanins), of F-A and A-F dimers and F-ethyl-A and F-ethyl-F dimers. Constitutive units of PAs were analyzed by UHPLC-QqQ-MS after acid-catalyzed depolymerization in the presence of phloroglucinol.

The size distribution of polyphenols and the concentration of polymeric tannins (in eq. epicatechin) were also determined by HPSEC in the skin and seed extracts, in the wines, and at

the end of the maceration experiments in model conditions. Commercial epicatechin, B2 dimer, epigallocatechin gallate, malvidin, and home prepared and characterized tannin fractions were used to determine the retention times of different monomers, oligomers, and polymers.

Polyphenols diffusion during micro-vinifications and experiments in model solutions was followed by measuring the total polyphenol Index (TPI) and total red pigments (TRP), determined by UV–visible spectrophotometry (spectrophotometer UV-1800, Shimadzu) at 280 and 520 nm (1 cm path length) after adequate dilution in HCl 1 M. In model maceration experiments and initial skin extracts, free anthocyanins were also analysed by HPLC using a Waters chromatography system equipped with DAD detection and a C18 reversed-phase column (Atlantis T3, Waters). Anthocyanins were quantified at 520 nm, in equivalent of malvidin-3-*O*-glucoside.

Statistical analysis

Statistical analysis was performed using Minitab software. The results obtained were assessed by one-way ANOVA analysis followed by a Tukey Test.

Results and discussion

Analyses of grape polyphenols

The amounts of anthocyanins and tannins quantified in fleshes were negligible compared to the amount of polyphenols found in other grape compartments, even in considering their weight in the whole berries. Thus they have not been further considered. Anthocyanins and tannins in skins, seeds, and fleshes were analyzed by UPLC-QqQ-MS in the MRM mode (Table 16). The two varieties differed greatly in their anthocyanin content and composition (Table 16), as discussed previously (Chapter 2). However, quite higher proportions in *p*-coumaroylated anthocyanins were found in comparison to what was indicated by HPLC-DAD. This can be attributed to the quantification method, which differs between the two analytical methods. With the two methods, results are given in eq. malvidin-glucoside. However, they were not calculated in the same way with HPLC-DAD (the extinction coefficient of malvidine was used for all anthocyanins) and UPLC-QqQ-MS (quantification of the ion intensity). Calibration of the two methods with *p*-coumaroylated malvidine would be needed to accurately determine its concentration. In any case, both methods indicated much higher proportions in Carignan than in Grenache.

Unlike anthocyanins, total tannin contents in skins and seeds did not differ significantly between the two varieties. The only differences observed for skin tannins concerned their proportions of epigallocatechin (EgC) and epicatechin-gallate (Ec-G) units, in higher proportion in the Grenache skin tannins. Especially, a factor 2 was observed in Ec-G % between the two varieties. This higher proportion of Ec-G units in the Grenache variety was also found for seed tannins. As expected, the latter only had very small EgC contents in both varieties. The mDP of seed tannins was slightly higher for the Carignan than for the Grenache.

Table 16. UPLC-QqQ-MS analysis of phenolic compounds in the skins (µg/g of fresh skin), seeds (µg/g of fresh seeds) of the various raw materials. mDP: mean degree of polymerization; % EgC: % in Epigallocatechin units; % Ec-G: % in Epicatechin-Gallate units. Different letters indicate significant differences (One-way ANOVA) between varieties for a given parameter (Tukey's test for $p<0.05$).

Samples / **Phenolic compounds**	Unit: µg/g fresh skin or seed	**car vol+deg+**	**gre vol+deg+**
Total anthocyanins Non-acylated antho *p*-coum antho	Skins	6490.8±1413.7a 3345.5±773.1a 2879.1±583.1a	1980.9±220.9b 1409±153.3b 475.9±59.2 b
Total Flavanols mDP % EgC % Ec-G	Skins	9179.1±2146.1a 11.2±0.5a 9.3±0.3b 1.7±0.2b	9399.0±900.3a 10.3±1.1a 12.3±0.2a 4.1±0.6a
Total Flavanols mDP % EgC % Ec-G	Seeds	46085.9±6025.4a 6.7±0.1a 0.2±0.02a 15.8±0.8b	55371.1±4998.0a 5.6±0.1b 0.2±0.01a 20.5±0.7a

HPSEC analyses with DAD detection were also performed on skins and seeds phenolic extracts to determine the size distribution of polyphenols (Figure 41). As discussed in chapter 2, HPSEC chromatograms of skin polyphenols show three different peaks: one related to polymeric tannins (mDP > 3), one related to oligomers, and one related to anthocyanins. The size distributions of polyphenols only slightly differed between Carignan and Grenache. One can note the presence of a small exclusion peak, corresponding to polymers of large size, more pronounced in the Carignan. The anthocyanin peak is wider and shifted for Carignan compared to Grenache, in agreement with the higher content of total anthocyanins in this variety. Quite

different chromatograms were found for seed polyphenols. These chromatograms evidenced different populations: catechin/epicatechin, epicatechin-gallate, oligomeric, and polymeric (mDP>3) tannins, and a much more important exclusion peak.

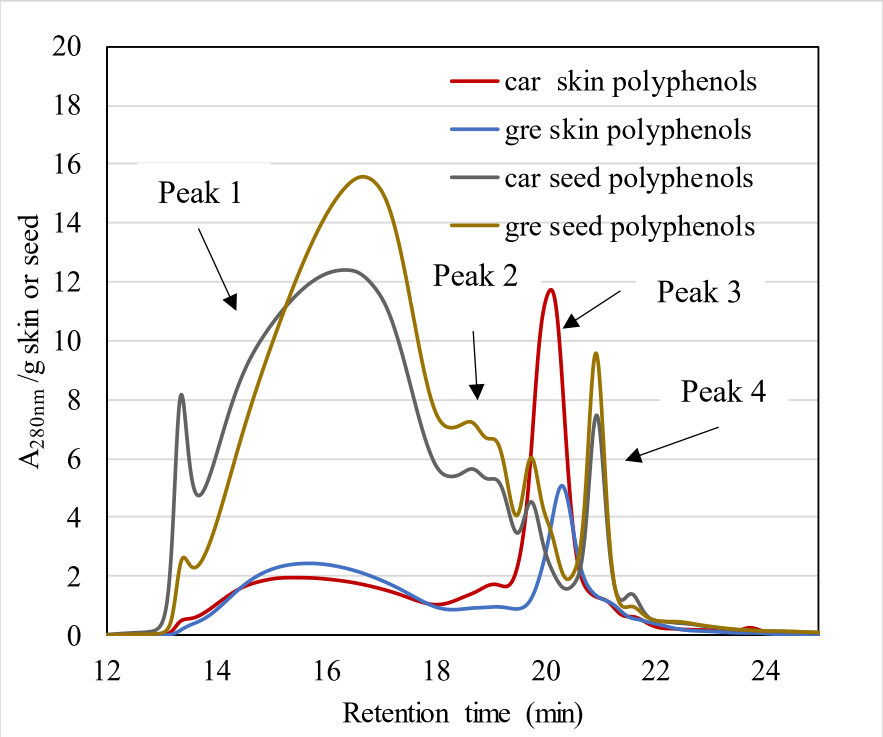


Figure 41. Analysis of the skin and seed polyphenol size distribution of the different modalities (vol+ deg+, vol-deg+, vol+deg- and vol-deg-) of Carignan (car) and Grenache (gre) grape berries by HPSEC. Peak 1: polymers; Peak 2: oligomers; Peak 3: monomers (anthocyanins, epicatechin-gallate); Peak 4: monomers (catechin/epicatechin).

These analyses showed differences in composition between the two varieties (Table 17): higher contents in flavanol polymers and oligomers in Grenache seeds than in Carignan ones, and larger size distribution of polymeric tannins in Carignan seeds (Figure 41). They also evidenced that high molecular weight tannins are in higher contents in seeds than in skins. This information is not accessible through phloroglucinolysis, which provides an average DP in number, and is important when dealing with tannin interactions.

Table 17. HPSEC analyses of phenolic compounds (polymer and oligomer peak) in the skins (mg equivalent epicatechin/g fresh skin) and seeds (mg equivalent epicatechin/g fresh seed) of Carignan and Grenache (vol+deg+ berries).

	Polymeric Tannins		Oligomers	
Samples	**car vol+deg+**	**gre vol+deg+**	**car vol+deg+**	**gre vol+deg+**
Skins (mg eq epicatechin/g fresh skin)	9 ± 2	10 ± 1	2 ± 1	1 ± 0.1
Seeds (mg eq epicatechin/g fresh seed)	59 ± 1	76 ± 1	7 ± 0.2	11 ±1

Polyphenol diffusion from skins and seeds

Tannins can diffuse from skins and seeds, anthocyanins from skins only. In Chapter 2 we studied the diffusion of polyphenols from skins alone. To study the impact of seeds, diffusion experiments were repeated on skins and new ones performed with seeds alone and then skins + seeds. The diffusion of polyphenols from skins (experiment A) and seeds (experiment B) during maceration was followed using absorbency measurements (TPI and TRP values) (Figure 42). As previously observed (Chapter 2), a higher total polyphenol extraction was observed for the skins of the Carignan variety, in relation to higher extraction of anthocyanins (red pigments). By contrast, extraction from seeds was higher with the Grenache. This extraction was observed even at low ethanol concentration and increased with ethanol concentration as reported by Canals *et al.,* (Canals *et al.,* 2005). SEC analysis evidenced different size distribution of tannins in seeds between Carignan and Grenache. This size distribution also differed for extracted seed tannins (Figure 42). The highest molecular weight tannins were not extracted, as already observed with skins, and the distribution profiles differed from what was observed with skins. Lower amounts of high molecular weight tannins diffused from skins than from seeds, likely because there are more interactions with the skin cell walls. The percentage of extraction was estimated from the area of the peaks in SEC analysis. We found a high percentage of extraction of seed polymeric tannins by comparison to skins (Table 18 and Table 19), and we also found a complete extraction of oligomers and monomers. These extractions led to final flavanol concentrations in the maceration solutions of 3.2 ± 1 and 3.6 ± 0.3 g/L in equivalent epicatechin for the Carignan and the Grenache, respectively, polymeric tannins representing 2.0 ± 0.1and 2.5 ± 0.2 g/L.

Canals *et al.,* (Canals *et al.,* 2005) studied the diffusion of seed tannins (variety Tempranillo) and reached a TPI value of roughly 18 after 7 days with 13% of ethanol, but

without agitation. Bindon *et al.,* (2017) reached a final concentration of tannins of 1.23 g/L in 15% of ethanol, but after only 48 h. After 48 h, our values were at least 30% lower than what we reached at the end of our experiments. In another work (Zouid, PhD thesis, 2011), the author studied the extraction of tannins from the seeds of Cabernet Franc berries harvested on different plots and at different stages of maturity after mid-veraison. This study was carried out on two different vintages and extraction experiments were performed in a 12% ethanol solution at pH 3.5 for 3 days, without agitation, and using the seeds of 50 berries for a 150 mL solution. This author found extraction % ranging from 25 to 55 % depending on berry maturity, plot, and vintage.

All these values are below ours, which may be explained by the fact that we worked with frozen seeds, under continuous agitation for 11 days.

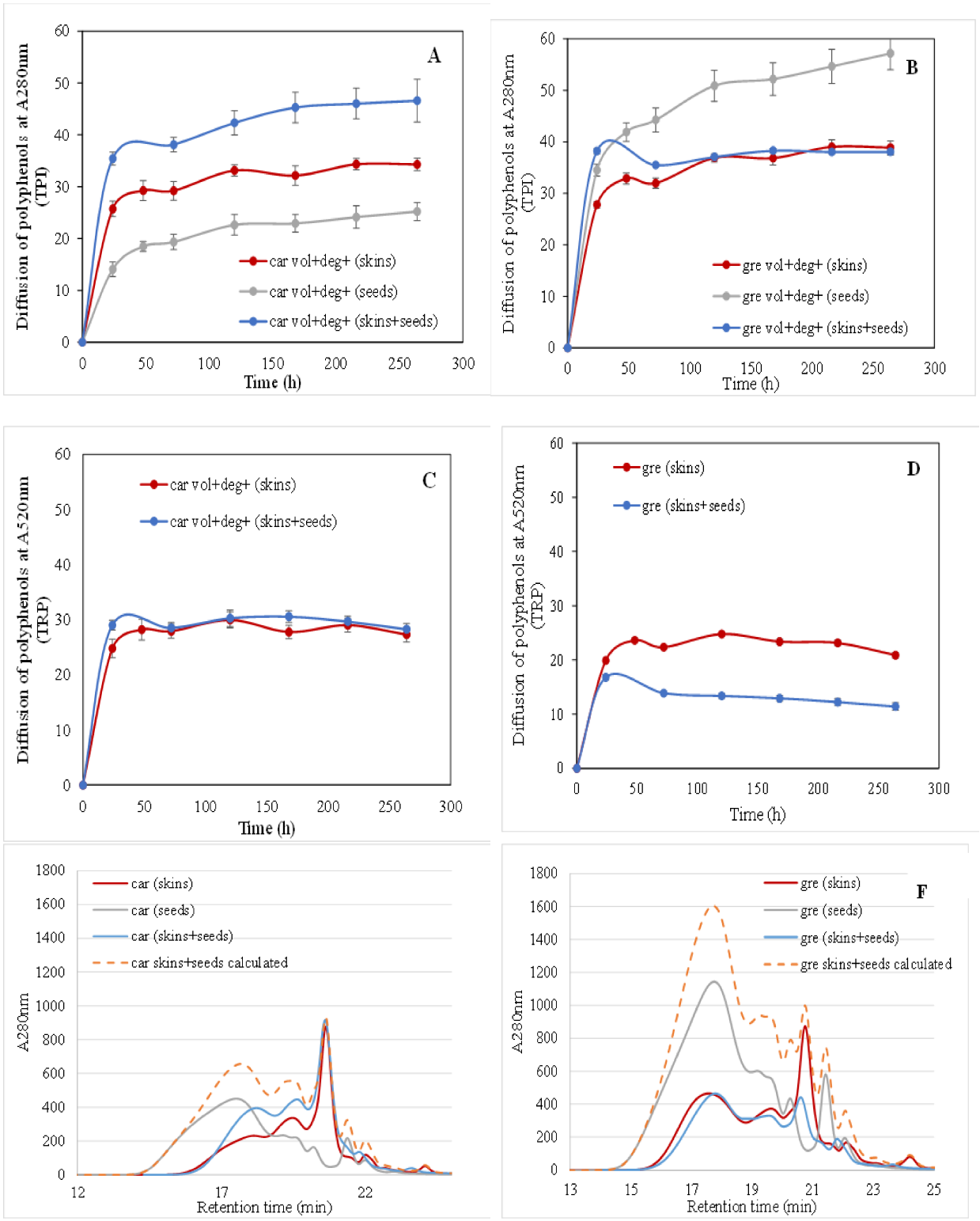


Figure 42. Polyphenols diffusion from berries different compartments (skins; seeds; skins+seeds) during wine-like model maceration experiments of Carignan and Grenache vol+deg+ modality. A) car deg+ TPI, B) gre deg+ TPI, C) car vol+ TRP, D) gre deg+ TRP. E) and F) HPSEC chromatograms at t5 (15%EtOH, 264h): E) car deg+, F) gre deg+

Table 18. Proportion (%) of polymers, oligomers extracted from skins at the end of the maceration experiment.

(%)	polymers	oligomers	total polyphenols
Carignan	25 ± 1	116 ± 12	35 ± 1
Grenache	37 ± 9	126 ± 14	50 ± 9

Table 19. Proportion (%) of polymers, oligomers extracted from seeds at the end of the maceration experiment.

(%)	polymers	oligomers	total polyphenols
Carignan	89 ± 1	129 ± 1	97 ± 1
Grenache	85 ± 5	114 ± 16	90 ± 5

The concentrations of polymeric tannins and anthocyanins in solution at the end of the maceration experiments are reported in Table 20.

Table 20. Concentrations of polyphenols at the end of the different maceration experiments.. Polymeric tannin and oligomer concentrations were determined from the HPSEC chromatograms, anthocyanin concentrations were determined by HPLC-DAD exepriments.

	Total Anthocyanins mg eq malvidin/L		Oligomers (mg eq epicat/L)		Polymeric tannins (mg eq epicat/L)	
	car	gre	car	gre	car	gre
skins	349±38	283±9	522±245	530±26	519±208	1187±131
seeds	-	-	289±25	699±73	1605±137	3441±152
skins + seeds	388±40	164±20	576±142	396.1±50	865±108	1069±82
skins + seeds theoretical	349±30.3	283±13	811±100	1229.0±115	2123±290	4628± 200

In a third maceration (experiment C), seeds and skins were mixed. The total polyphenol extraction was much lower than expected: TPI values were much lower than those obtained by summing the values of experiments A and B (Figure 42 A and B). For the Carignan variety, the presence of seeds did not impact the diffusion/extraction of anthocyanins (red pigments). The deficit observed in TPI or $A_{280\ nm}$ in SEC was mainly related to flavanols, the most important impact being observed with the high molecular weight tannins (Figure 42E). The deficit in flavanols was much more important with the Grenache than with the Carignan and important losses in TRP (45 %) were also observed for this variety. Once again, the highest molecular weight tannins extracted from seeds were the most affected (Figure 42F).

The deficit in flavan-3-ols observed when skins and seeds are present together in the diffusion medium may have different origins: 1) high concentrations in the solution that reduces the mass transfer form skins or seeds; 2) re-adsorption of seed tannins by skin insoluble material and/or interactions and precipitation with skin soluble polymers (proteins, polysaccharides); 3) reactions/interactions between polyphenols in solution leading to precipitation. Point 1 could explain the results obtained with the Carignan as the diffusion from skins + seeds is different than that observed with skins or seeds separately. However, this is not the case with Grenache. If the mass transfer may be limited by the tannin concentration in the medium, the main mechanism leading to tannin deficiency when skins and seeds are present together is likely related to physico-chemical interactions. Tannins in Grenache skins and seeds have a higher content of Ec-G. This likely enhances their adsorption/interactions with skin insoluble/soluble materials (Fournand *et al.* 2006; Bautista-Ortín *et al.,* 2014; Le Bourvellec *et al.,* 2004; Poncet-Legrand *et al.,* 2007)

Anthocyanin losses in the presence of seed tannins were only observed with the Grenache variety and anthocyanins interact very little with insoluble materials and macromolecules. Anthocyanin losses, in this case, are therefore more likely caused by chemical reactions. Grenache and Carignan differ by the anthocyanin/tannin ratio, much higher in Carignan, and by the percentage of Ec-G of seed tannins. The different characteristics of seed tannins may favor reactions between anthocyanin and tannins leading to the formation of less soluble species or non-pigmented derived compounds. It has been recently shown that derived pigments formed by anthocyanin and seeds tannin reactions in the presence of acetaldehyde are much less stable than those formed with skin tannins and tend to aggregate and precipitate (Teng *et al.,* 2019). This resulted in a loss of polymeric pigments formed from seed tannins, which increased with tannin molecular mass. Although there was no acetaldehyde in the model solutions used here, other chemical reactions cannot be excluded. Beside, anthocyanin concentrations in Carignan skins are much higher than in Grenache ones. Another suggestion for the Carignan, anthocyanin losses in the presence of seed tannins may have occurred and induced an enhanced diffusion from skins that compensate these losses.

Impact of FWIM

The presence of FWIM with skins in the diffusion medium induced a decrease of the total polyphenol concentrations (10% for Carignan, 27% for Grenache) and red pigments (14%

for Carignan, 18% for Grenache), as shown in Figure 43. This decrease was then less important (Table 21) than that expected from adsorption experiments for tannin and anthocyanin concentrations in the diffusion medium (expected losses around 45% for skin tannins and 20% for anthocyanins in 15% ethanol). It was also less important for Carignan than for Grenache, contrary to that expected from previous results (Chapter 3). HPSEC data (Figure 43 E and F) also evidenced other differences between the two varieties. With the Carignan, reduced TPI values resulted from slightly lower concentrations in polymeric tannins (7% decrease, that mainly concerned the highest molecular weight species) and in anthocyanins. With the Grenache, reduced TPI values resulted from a decrease in the concentrations of both polymeric tannins (36%), oligomers (22%), and anthocyanins.

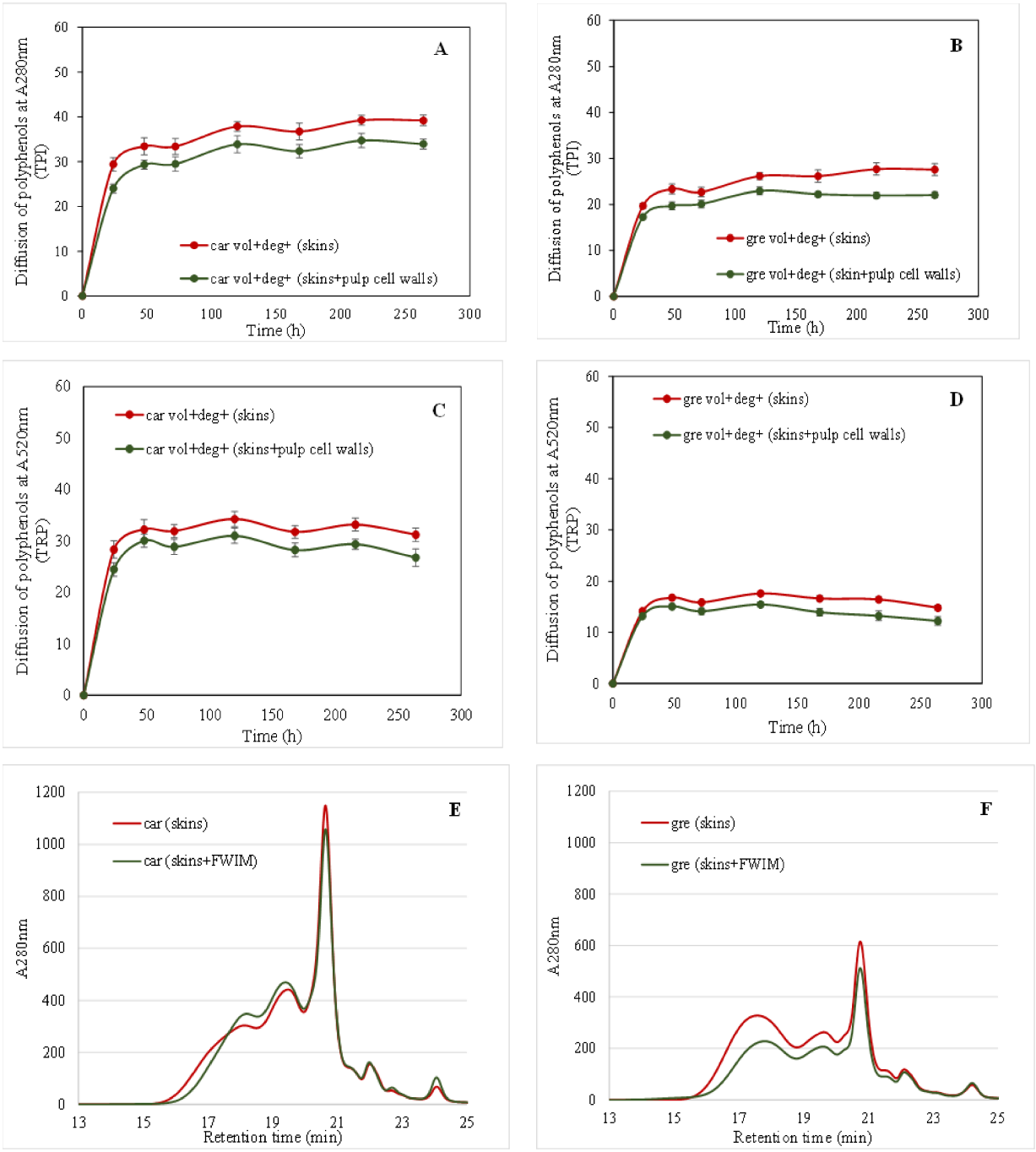


Figure 43. Comparison of polyphenols diffusion from the berries different compartments (skin, skin + FWIM) during wine-like model maceration experiments of Carignan and Grenache vol+deg+ modality. A) and B) TPI, C) and D) TRP, E) and F) SEC Chromatograms.

Table 21. Concentrations of polyphenols at the end of the different maceration experiments. Polymeric tannin and oligomer concentrations were determined from the HPSEC chromatograms, anthocyanin concentrations were determined by HPLC-DAD exepriments.

	Total Anthocyanins mg eq malvidin/L		Oligomers (mg eq epicat/L)		Polymeric tannins (mg eq epicat/L)	
	car	gre	car	gre	car	gre
skins	398±30	201±11	678±250	374±30	681±210	836±134
skins + FWIM	336±40	149±20	697±142	286±50	632±290	527±82

These results indicate first that interactions between FWIMs and extracted polyphenols during maceration shift the solid/liquid equilibrium by decreasing the concentration in solution, resulting in increased diffusion from the skins. This phenomenon is clear for tannins, not for anthocyanins as there is an impact of tannins on their interactions with FWIMs. The differences observed between Carignan and Grenache, which are in contradiction with adsorption experiment results (Chapter 3), may have two origins:

- the differences in galloylation of skin tannins between the two varieties (twice higher in Grenache than in Carignan), leading to higher adsorption for this variety that counterbalance the 20% higher adsorption capacity evidenced for Carignan with the same polyphenol pool;
- different mass transfer equilibria between polyphenols in skins and polyphenol in the diffusion medium between the two varieties, also related to a different composition of tannins and anthocyanins.

The diffusion/maceration experiments were repeated but this time by mixing skins, seeds, and FWIM (Figure 44 and Table 22). In comparison to skins+seeds experiment, a decrease in TPI and TRP concentrations was only observed for the Carignan variety (8% in TPI and 16% in TRP). TPI and TRP were slightly higher (16% and 20%, respectively) in the presence of FWIM for Grenache. HPSEC analysis confirmed the results obtained with TPI. In our experimental conditions, the whole results indicated that adsorption by FWIM modulates the polyphenol composition and impact their final concentrations but less than expected from adsorption results.

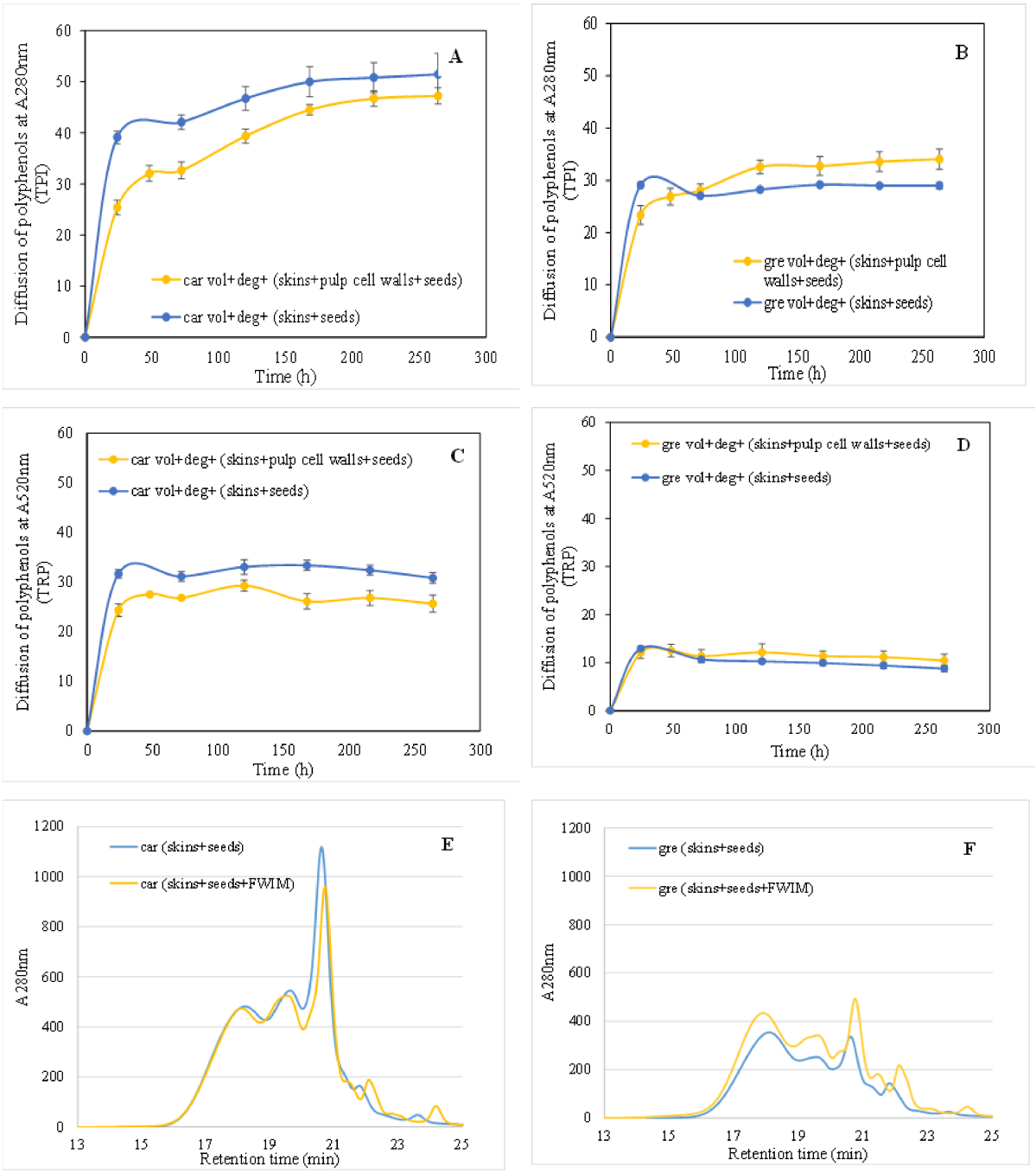


Figure 44. Comparison of polyphenols diffusion from the berries different compartments (skin + seeds, skin +seeds + FWIM) during wine-like model maceration experiments of Carignan and Grenache vol+deg+ modality. A) and B) TPI, C) and D) TRP, E) and F) SEC Chromatograms.

Table 22. Concentrations of polyphenols at the end of the different maceration experiments. Polymeric tannin and oligomer concentrations were determined from the HPSEC chromatograms, anthocyanin concentrations were determined by HPLC-DAD exepriments.

	Total Anthocyanins mg eq malvidin/L		Oligomers (mg eq epicat/L)		Polymeric tannins (mg eq epicat/L)	
	car	gre	car	gre	car	gre
skins+seeds	388±35	126±14	702±247	301±29	1055±207	814±82
skins+seeds+FWIM	315±52	121±27	764±15	464±1	947±40	1060±4

Comparison of diffusion in model systems and microvinifications

The diffusion of polyphenols from the "skins + seeds + FWIM" model system was compared with that observed in micro-vinification (Figure 45). Differences in extraction rates were observed, especially within the first days of the maceration, but overall and at the end, the extractions were close in terms of TPI and TRP. These differences in extraction rates can be attributed to differences in the stirring conditions (constant stirring in the model system versus homogeneization related to CO_2 release and to punching in microvinification) and a more progressive change in the ethanol content during the real fermentation.

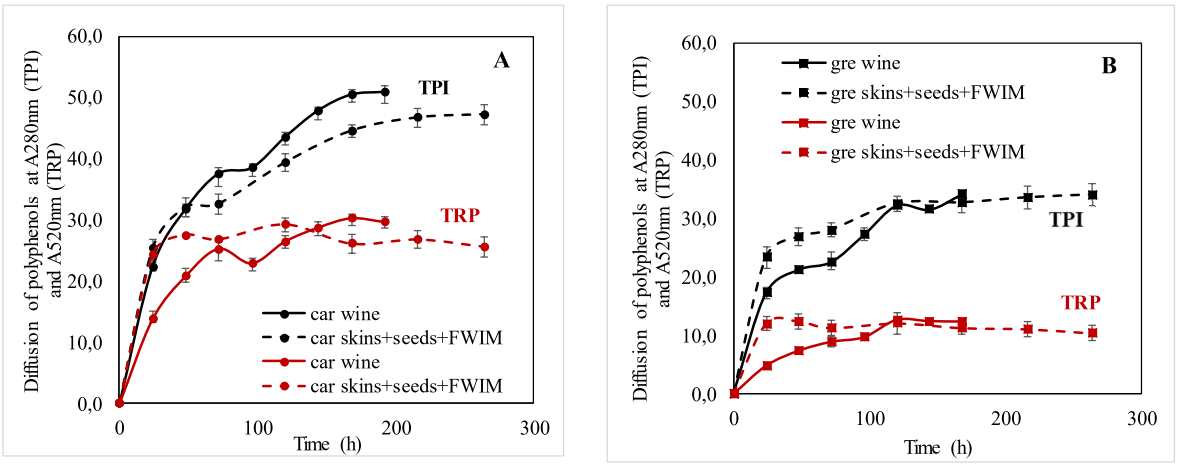


Figure 45. Comparison between the phenolic diffusion (TPI and TRP) from whole grape berries in winemaking and wine-like model maceration experiment in A) Carignan vol+deg+ and B) Grenache vol+deg+.

The wines were analyzed by UPLC-MS (MRM) and HPSEC at the end of the alcoholic fermentation. The tannin mDP, %EgC, and %Ec-G were determined by pholoroglucinolysis.

Tannins in the Grenache wine had slightly but significantly higher mDP, EgC%, and Ec-G % than those of the Carignan ones (Table 23). HPSEC analyses (Figure 46) showed close profiles, with slightly higher contents in polymeric tannins in the model maceration experiments than in wines and similar contents in oligmers (Table 22 and Table 23). Despite close TRP values, quite different anthocyanin concentrations were found by UPLC-MS compared to those determined in model maceration experiments by HPLC-DAD. This highlights once again a problem related to two different calibrations. The comparison between Carignan and Grenache at the end of winemaking indicated much higher anthocyanin extraction for the Grenache (50% versus 22%) and a limited extraction of *p*-coumaroylated anthocyanins, in accordance with the results found in Chapter 2. This however differs from what observed in model maceration experiments (Table 22): 19% for the Carignan versus 26% for the Grenache. This point will need further investigation.

Samples / **Phenolic compounds**	**car vol+deg+**	**gre vol+deg+**
Polymeric tannins (mg equivalent epicat/L)	878.0±18.5a	877.8±25.3a
Oligomers (mg equivalent epicat/L)	834.0±19.9a	421.0±5.3b
mDP	4.2±0.1b	4.7±0.1a
% EgC	4.8±0.3b	8.2±0.3a
% Ec-G	3.4±0.0b	5.4±0.1a
TRP	29.6 ± 0.9a	11.5 ± 0.1b
Total anthocyanins (mg eq. malvidin/L)	545.1 ± 30.5a	336.3 ± 4.9b
p-coumaroylated anthocyanins (mg eq. malvidin/L)	43.7 ± 0.9b	54.9 ± 1.2a

Table 23. Tannin contents in Wines from HPSEC analysis oligomer and polymer peak (mg equivalent epicatechin/L). mDP: mean degree of polymerization; % EgC: % in Epigallocatechin units; % Ec-G: % in Epicatechin-Gallate units. Different letters indicate significant differences (One-way ANOVA) between samples for a given parameter (Tukey's test for $p<0.05$).

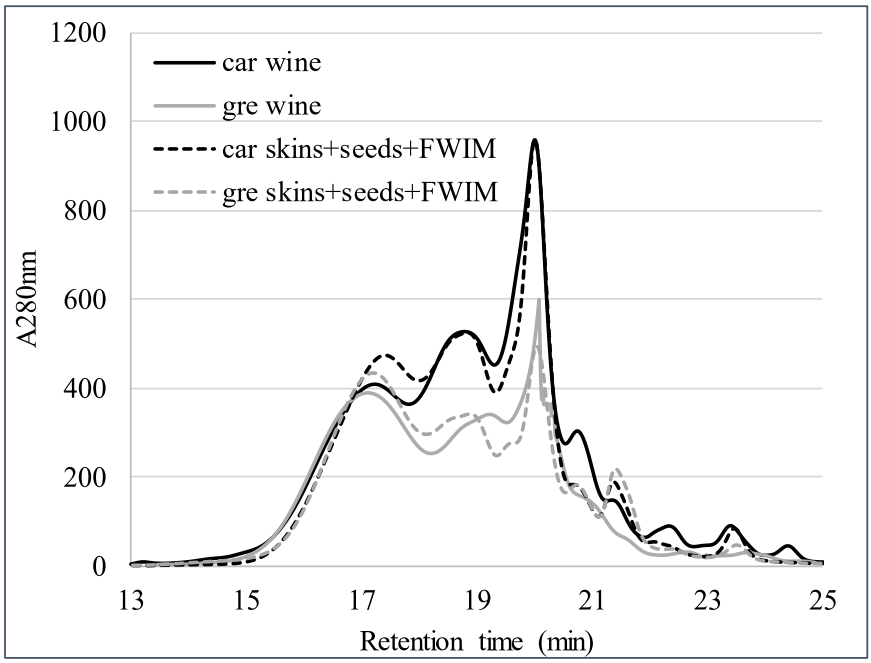


Figure 46. Comparison of the HPSEC chromatograms of wines and model maceration experiments (Skins + Seeds + FWIM).

The percentage of galloylation of wine tannins, between 3 and 5%, is much lower than that of seed tannins (between 15 and 20%). However, it is higher than that of skin tannins (between 2 and 4%), which leads us to conclude that the tannins found in wine are mostly those of skins. Similarly, the percentage of epigallocatechin units, which come almost exclusively from the tannins of skins, decreases slightly in the wine. Both observations lead to the conclusion that higher levels of skin tannins are found in wine, despite seeds having much higher initial tannin content than the skin. These low contents of seed tannins in wines were attributed to the low and slow extraction of seed tannins requiring longer maceration time (Bautista-Ortin *et al.,* 2016). However, this is not what we observed. Previous results (Zouid, 2011), from extraction experiments in a 12 % ethanol solution, suggest that extraction rates are highly variable, depending on the vintage and not on the variety. In our experiments, seed tannins were extracted rapidly and easily from seeds, even at low ethanol contents (Figure 42 A and B). Our results suggest then that the low % of Ec-G units in wine can be related to : i) a selective adsorption of the most galloylated tannins (seed tannins) by skin or flesh insoluble materials; ii) a selective precipitation of galloylated tannins with skin soluble polymers or related to chemical reactions. Analyses by phloroglucinolysis of tannins at the end of the wine-like maceration experiments, as performed on wines, are missing here to be in position to clearly conclude.

During winemaking, yeasts may be involved in changes in the polyphenol composition by means of two different mechanisms: 1) chemical reactions involving polyphenols

(anthocyanins and tannins) by the release of metabolites such as acetaldehyde or pyruvic acid and also some enzymatic activities (minor); 2) adsorption by cell walls and/or whole cells (Mekoue *et al.,* 2015 ; Vernhet *et al.,* 2020). The impact of chemical reactions related to yeast has not been studied in depth in this work. Pyranoanthocyanins and dimeric ethyl adducts were analyzed by UPLC-MS but represented less than 3% of the total analyzed pigments at the end of the fermentation. These chemical reactions may also involve however polymeric and oligomeric tannins, not detected by the UPLC-MS method used in the present study. When dealing with red wines, adsorption by yeast cell walls only has a minor impact the total red wine polyphenolic content (Mekoue *et al.,* 2015) and specifically affect high molecular weight polymers. Adsorption by whole yeasts have a higher impact (15-20% of TPI losses, 5% of TRP losses) but occurs within the days after the end of the fermentation, essentially after their death and mainly concern oligomers and polymers (Mekoue et al., 2015 ; Vernhet *et al.,* 2020). Analysis on wines were performed immediately at the end of fermentation, so that significant decreases in TPI and TRP related to adsorption are not expected.

Conclusion

Macerations in model solutions do not reflect the initial conditions of the winemaking process, but they gave however results close to what is observed during microvinifications and led to several conclusions:

- seed tannins diffuse very well on their own and in our experimental conditions (variety, ethanol content, duration), much more than expected from literature; there may have been a concern in our experiments due to freezing, but the final concentrations usually observed using fresh seeds are of the order of 1-1.5 g/L, which is far from negligible; in terms of selectivity, the highest molecular weight tannins are not extracted;

- as soon as they are in the presence of cell walls of skin/flesh, and/or anthocyanins, the concentration of seed tannins in solution drops abruptly, due to a combined effect of adsorption and/or precipitation and/or chemical reactions;

- the flesh water-insoluble materials certainly absorb tannins, but they also tend to shift the extraction balance and it seems that more tannins can be extracted from skins and seeds when they are added;

- the impact of the yeasts is weak in terms of TPI and TRP if the maceration is stopped at the end of fermentation, because adsorption occurs mainly when yeasts are dead, a few days after the end of fermentation, and mainly involve oligomers/polymers.

Discussion et conclusion générale

Discussion et Conclusion générale

Rappel des objectifs de la thèse

La qualité et la typicité des vins rouges est étroitement liée à leur composition initiale en anthocyanes et en tannins, et aux évolutions de ces composés lors des opérations de vinification et d'élevage des vins. Ces évolutions peuvent être liées à des mécanismes réactionnels et/ou physico-chimiques (précipitations, traitements de stabilisation). Ce travail de thèse s'est focalisé sur la composition initiale, autrement dit la composition telle qu'obtenue à l'issue de la fermentation alcoolique et de la macération. En vinification en rouge, la macération est l'opération technologique permettant l'extraction des composés phénoliques des parties solides de la baie de raisin, pellicules et pépins, dans lesquels ils sont essentiellement localisés. Dans la pratique, le vinificateur peut moduler cette extraction en mettant en œuvre différents moyens physiques (température et durée de macération, homogénéisation du milieu, …) ou biochimiques (utilisation de glycohydrolases pour favoriser la déstructuration des parois cellulaires et la diffusion des polyphénols).

Outre les conditions dans lesquelles la macération est effectuée, la composition initiale des vins est dépendante des caractéristiques de la baie de raisin, dont sa composition en polyphénols. Cependant, la seule composition en polyphénols des pellicules et des pépins n'est pas un critère suffisant pour prévoir la composition des vins à l'issue de la macération. Cette dernière est fonction de l'« extractibilité » de ces composés. A procédé constant, il existe une variabilité entre cépages, et pour un même cépage une variabilité en fonction de l'origine ou du millésime, qui ne sont pas directement et simplement liées à la composition en polyphénols des baies. Ces différences sont attribuées aux constituants structuraux de la matière première, qui limitent la diffusion des composés phénoliques de la phase solide vers la phase liquide dans le cas des pellicules et des pépins, et qui sont à l'origine de phénomènes d'adsorption (constituants insolubles de pulpe et de pellicules) ou de précipitation (extraction de protéines et de polysaccharides solubles) une fois ces composés sont en phase liquide. Parmi les facteurs liés à la matière première, une attention particulière a été portée dans la littérature à la composition des parois cellulaires de pulpe et de pellicules et à leur capacité à adsorber les anthocyanes et les tanins. S'il est admis que cette adsorption joue un rôle prépondérant dans la composition finale des polyphénols dans les vins, son impact exact comme celui des caractéristiques structurales et de la composition de ces parois n'est pas clairement établi. De même la composition des baies en polyphénols, la structure et la composition des parois cellulaires varient en fonction du cépage et de la maturité et est susceptible d'être influencée par les

conditions environnementales. Dans un contexte d'évolution des conditions climatiques et des pratiques culturales d'une part, et de création variétale d'autre part, une meilleure connaissance des mécanismes jouant un rôle clé dans l'extractabilité des composés phénoliques (composition initiale des vins vs composition des baies) et l'identification de marqueurs permettrait d'orienter les choix faits au vignoble et/ou d'adapter les procédés de transformation.

L'objectif de ce travail de thèse était de contribuer à une meilleure compréhension de l'impact de la matière première et de sa variabilité sur la composition des vins en anthocyanes et en tanins. Pour cela, nous avons sélectionné deux variétés très contrastées en termes de composition en polyphénols (rapport anthocyanes / tanins). Les raisins ont été récoltés à un degré de maturité moyen donné et les baies triées ensuite en quatre lots, en fonction de leur volume (vol+/vol-) puis de leur degré de maturité (deg+/deg-). Le volume des baies peut avoir un impact sur les proportions de pellicule/pépins/pulpe. Le degré de maturité impacte la composition polyphénols (en particulier anthocyanes) et celle des parois cellulaires. Ce choix ne permet pas d'obtenir des différences très importantes en termes de maturité mais limite l'impact potentiel des facteurs environnementaux pour un millésime donné.

Les différents lots ainsi obtenus ont été caractérisés de la façon suivante :

- poids moyen des baies, teneur en sucres, pH et acidité totale des moûts, proportions en pellicule, pépins et pulpe ;
- composition en polyphénols des différents compartiments (pellicules, pépins et pulpe)
- analyse des constituants pariétaux des pellicules et des pulpes.

Des expériences de macération en conditions modèles ont été réalisées en utilisant ces différents compartiments pour évaluer les impacts respectifs :

- De la diffusion des polyphénols des pellicules et des pépins vers la phase liquide ;
- Des interactions avec des parois cellulaires de pulpe et pellicule.

Elles ont été faites à l'abri de l'oxygène et en absence de levures pour pouvoir obtenir des conditions de milieu identiques entre les différentes matières premières et s'affranchir autant que possible et dans un premier temps des réactions chimiques.

En parallèle, 900 g de baies de chaque lot ont été utilisés pour réaliser des micro-vinifications en conditions standardisées et en triplicat (Figure 47), afin de relier les résultats obtenus en conditions modèles à l'extraction des composés phénoliques en conditions réelles.

Figure 47. Dispositif de microvinification dans une cafetière à piston. A gauche, en cours de fermentation alcoolique avec le marc immergé, à droite avec le piston baissé pour simuler un pigeage une fois par jour.

Impacts respectifs de la diffusion et des interactions avec les parties solides

Anthocyanes et tannins dans les cellules végétales sont majoritairement localisés dans des vacuoles, sous forme d'inclusions vacuolaires pour les anthocyanes, et d'«accressions » ou d' « agrégats vacuolaires », plus récemment appelés tannosomes pour les tanins. Une petite partie des tanins est retrouvée associée à maturité aux parois cellulaires. Pour être extraits, ils doivent donc être solubles/solubilisés et diffuser à partir de leur vacuoles et traverser vers la phase liquide, la barrière physique représentée par la structure de la cellule : membranes, cytoplasme et enfin parois. Cette solubilisation et cette diffusion au travers des structures cellulaires sont dépendantes du solvant et de la structure chimique des composés phénoliques, de leurs interactions avec les constituants cellulaires, et du « gradient de concentration » entre phase solide et solution. Depuis les années 2000, différents auteurs ont souligné l'impact prépondérant des interactions entre polyphénols et parois cellulaires sur l'extractibilité des

tanins et des anthocyanes. Ces interactions sont elles-mêmes modulées par la structure des parois cellulaires, la structure des polyphénols et les paramètres physiques de la matrice (teneur en éthanol, température, permeabilité et non accessibilité des sites…) (Le Bourvellec *et al.,* 2004 ; Bindon *et al.,* 2010, 2011, 2012 ; Fournand *et al.,* 2006; Bautista Ortin *et al.,* 2014 ; Canal *et al.,* 2005). Une fois extraits, les polyphénols peuvent également être adsorbés sur les débris solides des cellules de la pulpe ou interagir avec des macromolécules solubles des constituants cellulaires (protéines, polysaccharides) et être impliqués éventuellement dans des précipitations. La structure (réseau tridimensionnel) des parois cellulaires des pellicules et de la pulpe peuvent varier en fonction du cépage et de son degré de maturité, influençant ainsi l'extraction et la composition finale en polyphénol dans le vin. L'objectif ici a été d'étudier les phénomènes séparément pour mieux évaluer leur impact.

Extraction des polyphénols à partir des pellicules – diffusion et interactions

Lors d'expériences de maceration en condition modèle (des pellicules en milieu hydro-alcoolique, Chapitre 2), nous avons mis en évidence des différences marquées entre Carignan et Grenache, en termes de diffusion des anthocyanes et des tanins, ainsi qu'un impact de la maturité. Les tanins diffusent proportionnellement plus, et plus vite à partir des pellicules de Grenache (18-19% pour deg-, 24-27% pour deg+ au maximum de la concentration) que des pellicules de Carignan (14-16% pour deg-, 18-19% pour deg+), et ce pour des teneurs en tanins dans les pellicules très proches. Ces différences dans les proportions extraites sont encore plus marquées dans le cas des anthocyanes : 40-47% pour le Grenache, 25-27% pour le Carignan, sans impact clair de la maturité ni du volume des baies dans ce cas-là. En accord avec les données de la littérature l'extraction ne concerne que les tanins de faible DP. Le fait de renouveler le milieu de diffusion (milieu hydroalcoolique) permet d'extraire une proportion résiduelle de composés phénoliques, mais qui reste très faible (5 à 10% pour les tanins, 7 à 12% pour les anthocyanes).

Une analyse plus fine de la composition en polyphénols et de la composition des parois a mis en évidence trois différences majeures entre les deux variétés :

i) des concentrations en anthocyanes totales beaucoup plus élevées et une plus grande proportion d'anthocyanes *p*-coumaroylées dans le Carignan ;

ii) des tanins légèrement plus riches en épicatéchine gallate dans le Grenache. Dans un travail antérieur, Fournand *et al.,* (2006) ont montré un % en unités galloylées plus important dans les tanins non extraits que dans ceux extraits en milieu hydroalcoolique, et des études en solutions modèles ont mis en évidence des interactions plus fortes avec les protéines, les polysaccharides ou des parois cellulaires pour les tanins galloylés (Bautista-Ortín *et al.,* 2014; Le Bourvellec *et al.,* 2004; Poncet-Legrand *et al.,* 2007).

iii) d'un point de vue de la composition pariétale, des parois de pellicules plus riches en extensines et arabinanes pour le Carignan, et plus riches en hemicelluloses pour le Grenache.

Les teneurs en tanins dans les pellicules et la répartition de leur taille moléculaire étaient similaires pour les deux variétés et les différentes modalités. Les différences liées à la composition des parois peuvent expliquer les différences observées dans l'extraction de ces composés : les extensines sont des protéines riches en proline susceptibles de développer des interactions fortes avec les tanins alors que ces derniers ne développent que peu d'interactions avec les hémicelluloses. Avec les polysaccharides pectiques, il y a des affinités moins marquées que pour les protéines. Ceci pourrait expliquer une rétention des tanins moins importante dans le cas du Grenache, en dépit d'une teneur en unités galloylées plus forte des tanins de pellicules qui devrait favoriser ces interactions. Cette teneur, même si elle est supérieure à celle du Carignan, reste faible.

Les analyses CoMPP ont également montré des différences au niveau des chaines homogalacturoniques partiellement estérifiées des pectines et des β-glucanes et xyloglucanes des parois des pellicules en fonction du degré de maturité, qui pourraient rendre compte des différences d'extractions observées (deg+ > deg-).

Dans le cas des anthocyanes et du Carignan, et par comparaison avec le Grenache, les faibles proportions extraites durant la macération ne peuvent pas être attribuées à une limitation de la diffusion par les équilibres solide/liquide. Il est clairement apparu dès les études de diffusion et dans la suite de ce travail de thèse que les anthocyanes *p*-coumaroylées ont un comportement très différent des anthocyanes non acylées. Leur taux d'extraction à partir des pellicules est très faible : 4 à 9% dans le cas du Carignan et 15 à 20% dans le cas du Grenache. Ceci peut être lié à une solubilisation/solubilité différente de ces dérivés et à des interactions plus fortes avec le milieu environnant. La plus grande richesse du Carignan en anthocyanes *p*-

coumaroylées ne suffit cependant pas à rendre compte des différences d'extractabilité observées entre les deux variétés, l'extraction étant également plus faible pour les anthocyanes non acylées dans le cas de ce cépage.

Les teneurs moyennes en composés insolubles (AISs) des pellicules (parois essentiellement) ne diffèrent que légèrement entre les modalités des 2 cépages, sans lien avec la maturité des baies. Elles sont de l'ordre de 40 mg/g de pellicule fraiche. Les teneurs en tanins sont quant à elles de l'ordre de 10 mg/g, soit de l'ordre de 250 mg de tanins pour 1 g d'AIS. Par le biais d'isothermes d'adsorption, il a été montré que les capacités d'adsorption des parois de cellules de pellicule sont de l'ordre de quelques centaines de mg/g en milieu hydroalcoolique (2 à 300 mg/g) (Bindon *et al*., 2012). Ces parois ont une forte capacité d'adsorption des tanins et sont donc susceptibles d'être le principal élément limitant dans le cas de leur extraction à partir des pellicules. Il n'en est cependant pas de même pour les anthocyanes, qui n'ont qu'une affinité faible avec les parois cellulaires (Bindon *et al.,* 2014 ; Mekoue *et al.,* 2015 ; chapitre 3). Outre des interactions avec les parois, des différences de solubilisation des composés phénoliques en fonction de leurs structures (*p*-coumaroylation pour les anthocyanes, DPm et % de galloylation pour les tanins) et de leur localisation/état d'agrégation dans les compartiments sont également possibles, et pourraient être à l'origine des différences d'extraction observées entre Grenache et Carignan pour les polyphénols.

Extraction à partir des pépins et interactions pépins/pellicules

En l'absence des parois cellulaires de pellicule et de pulpes, le % de tanins extraits par diffusion à partir des pépins était de l'ordre de 80-90%. Même si ce % élevé peut être lié à nos conditions opératoires, cela démontre des interactions avec leur environnement cellulaire moins important qu'observé dans le cas des pellicules. Lorsque ces diffusions ont été effectuées en présence de pellicules, nous avons observé un déficit important : les concentrations en tanins dans la solution en fin de macération sont beaucoup plus faibles que ce que l'on aurait pu attendre en considérant leur diffusion à partir des pellicules et des pépins séparément. Ce déficit est plus marqué dans le cas du Grenache et s'accompagne d'une forte diminution des pigments totaux. Dans le cas des tanins, il est attribuable à des interactions avec les parois des pellicules et/ou à des interactions avec des constituants solubles extraits de ces pellicules (protéines, polysaccharides) conduisant à des précipitations. Ce déficit montre que les parois de pellicules ne sont pas saturées par les tanins de pellicules et il correspond à une élimination préférentielle des tanins de plus haut DPm. Une diminution de la concentration en anthocyanes n'a été observée que dans le cas du Grenache. Cette diminution suggère des réactions chimiques

impliquant la formation de pigments dérivés éventuellement moins solubles ou moins colorés, ou la formation d'adduits incolores. Le fait que ceci soit observé uniquement avec cette variété pourrait être lié à un rapport anthocyanes/tanins plus faible et/ou à une teneur plus importante en unité galloylées. Des analyses plus fines des anthocyanes et des pigments dérivés par la plateforme polyphénols pourraient apporter des éléments de réponse.

Impact des constituants insolubles de la pulpe : interactions et diffusion

Une fois extraits, les polyphénols peuvent être adsorbés par les constituants insolubles de la pulpe, ce qui modifie leur concentration finale dans les vins. Les expériences suivantes ont consisté à comparer l'adsorption des anthocyanes et des tannins par les insolubles de pulpes des deux cépages, et en considérant les modalités deg+ et deg-. Pour pouvoir effectuer cette comparaison les mêmes fractions de polyphénols ont été utilisées pour les deux variétés. Elles ont été extraites à partir des pellicules de Carignan avec un milieu hydroalcolique à 15% d'éthanol de façon à avoir des tanins représentatifs de ceux retrouvés dans les vins en termes de DPm.

Ces expériences, réalisées dans les chapitres 3 et 4, ont montré que :

i) l'adsorption des anthocyanes est faible par rapport à celle des tanins et essentiellement réversible et il existe une adsorption sélective des anthocyanes para-coumaroylées ;

ii) l'adsorption des tanins est quantitativement plus importante mais aucun plateau n'a pu être mis en évidence dans les gammes de concentration testées et avec la fraction considérée; cette adsorption est peu réversible et concerne environ 50 % des tanins en solution pour des concentrations initiales supérieurs à 0,5 g/L;

iii) les constituants insolubles des pulpes des baies de Carignan adsorbent environ 20% de tanins en plus que ceux des pulpes des baies de Grenache, ceci pouvant être lié à leur teneur plus élevée en extensines ;

iv) la présence d'anthocyanes dans le milieu n'a pas d'impact notable sur l'adsorption des tannins.

v) cette adsorption n'a pas d'impact notable sur la concentration finale globale en composés phénoliques dans le milieu (TPI, TRP) mais affecte par contre leur composition.

Le fait que les interactions entre composés phénoliques et constituants insolubles de la pulpe n'affectent pas les teneurs finales en tanins en fin de macération, et ce que ce soit dans le cas des pellicules ou des pellicules en présence de pépins, était inattendu. Cela indique que cette adsorption déplace les équilibres solide/liquide en faveur d'une diffusion accrue à partir des pellicules et des pépins. Cela conduit par ailleurs à masquer les différences de concentrations en tanins attendues à partir des études de diffusion et d'interactions. Une analyse plus poussée des caractéristiques (DPm, % de galloylation) des tannins dans les milieux de diffusion permettra de mieux caractériser les mécanismes impliqués.

Composition de la baie vs composition des vins

L'extraction des composés phénoliques a été suivie tout au long des microvinifications par des mesures d'IPT (Indice de polyphénols totaux = Absorbance à 280 nm) et de PT (Pigments Totaux, absorbance à 520 nm. La Figure 48 montre ces extractions pour les quatre modalités des deux cépages (vol+deg+, vol+deg-, vol-deg+, vol-deg-). Comme nous l'avons observé dans le Chapitre 4 dans le cas de la modalité vol+deg+, nos modèles de diffusion incluant pellicules, pépins et pulpe conduisent à des teneurs en IPT et PT semblables à celles obtenues en microvinification. L'impact des levures en fermentation est resté relativement faible : ceci est probablement dû au fait que les vins ont été analysés dès la fin de la fermentation, avant que les levures ne meurent (elles fixent les polyphénols essentiellement une fois mortes), et avant que les réactions chimiques impliquant les polyphénols et les métabolites de la levure n'aient eu un impact trop important.

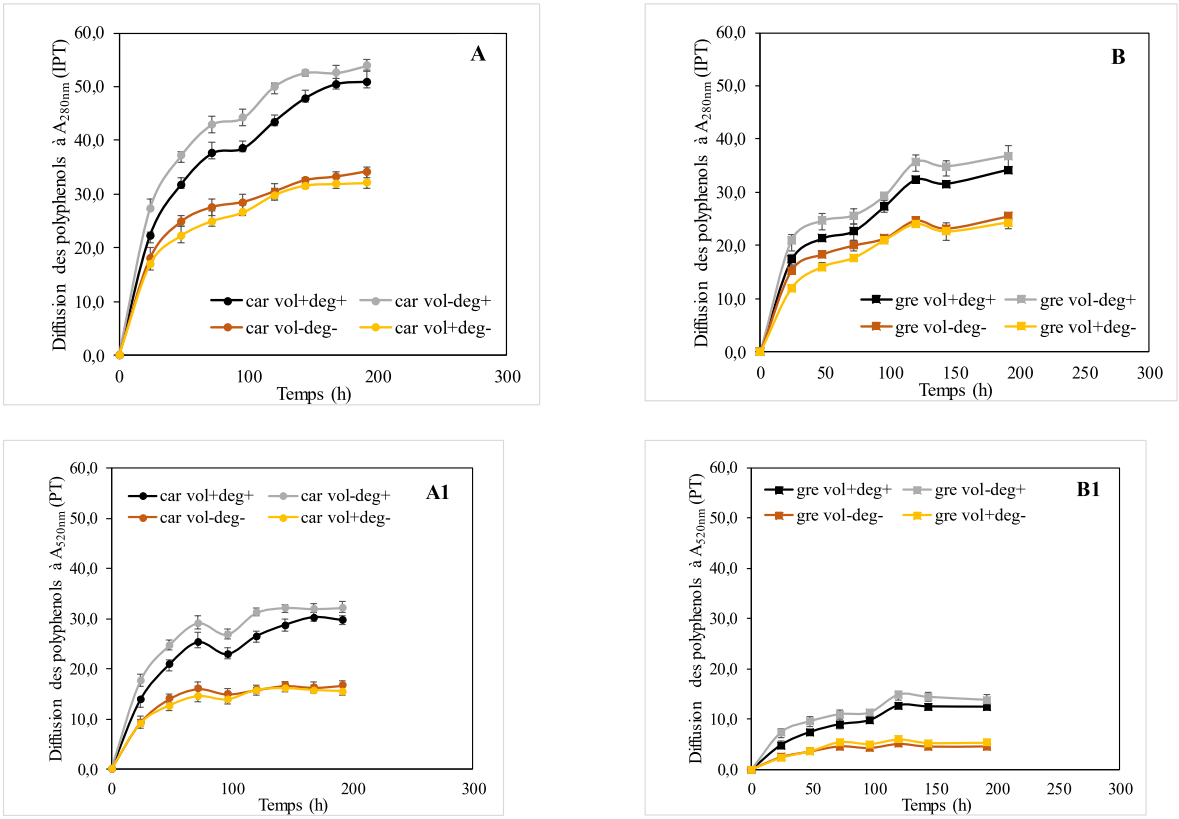


Figure 48. Suivi des vinifications par spectrophotométrie UV-visible : IPT (Indice de Polyphénols Totaux, A280nm) and PT (Pigments Totaux, A520nm). A) Carignan (vol+deg+ ; vol-deg+ ; vol-deg- ; vol+deg), B) Grenache (vol+deg+ ; vol-deg+ ; vol-deg- ; vol+deg-), A1) PT Carignan (vol+deg+ ; vol-deg+ ; vol-deg- ; vol+deg-), B1) PT Grenache (vol+deg+ ; vol-deg+ ; vol-deg- ; vol+deg-)

Les polyphénols des différents compartiments de la baie de raisin et des vins ont été analysés par la Plate-Forme Polyphénols de l'UMR SPO comme décrit dans le Chapitre 4. Le Tableau 24 regroupe les différentes valeurs trouvées pour les 4 modalités de Carignan et de Grenache en anthocyanes (anthocyanes non acylées ; anthocyanes para-coumaroylées) et flavanols (degré de polymérisation moyen DPm ; pourcentage de gallate d'épicatéchine %Ec-G ; pourcentage d'épigallocatéchine %EgC déterminés par phloroglucinolyse).

Comme discuté précédemment, la composition initiale des pellicules en anthocyanes varie en premier lieu selon le cépage, puis en fonction de la maturité : le Carignan est plus riche que le Grenache, les baies mures plus riches que les baies moins mures. La proportion d'anthocyanes *p*-coumaroylées est également très différente pour les deux cépages : un peu moins de 50% des anthocyanes totales pour le Carignan, entre 20 et 35 % pour le Grenache selon les modalités. Les teneurs totales en flavan-3-ols sont similaires dans les pellicules pour toutes les modalités, mais les degrés de polymérisation, les pourcentages de gallolylation et

d'épigallocatéchine peuvent différer : les pépins et pellicules de Grenache sont plus riches en épicatéchine gallate que ceux de Carignan, les pellicules de Grenache plus riche en épigallocatéchine que celles de Carignan. Enfin, le rapport anthocyanes/ tanins est beaucoup plus faible dans le cas du Grenache. Les vins issus des microvinifications ont été analysés à la fin de la fermentation alcoolique, après pressurage. Les vins ont des teneurs en anthocyanes très contrastées, tout comme les matières premières (variété et maturité). Cependant, alors que les pellicules de Carignan sont trois à sept fois plus riches en anthocyanes que celles de Grenache, les vins de Carignan sont entre 1,5 et 3 fois plus riches que ceux de Grenache : on a extrait proportionnellement plus d'anthocyanes totales avec le Grenache. Une analyse plus fine des résultats montre un déficit dans l'extraction des dérivés *para*-coumaroylés pour les deux cépages. Ces observations sont en accord avec les résultats issus des études en solutions modèles et les proportions retrouvées dans le vin Carignan vol+deg+ (22% des anthocyanes extraites) sont en accord avec les proportions retrouvées dans le cas d'une diffusion en milieu modèle avec pellicules, pépins et constituants insolubles de pulpe (25%). Ceci n'est pas le cas pour le Grenache : on retrouve 43% des anthocyanes de la matière première dans le vin vol+deg+ alors que l'on n'en dose que 25% dans le cas de la diffusion en milieu modèle. Pourtant les teneurs en pigments (TRP) étaient voisines dans les deux cas. Cela tend à indiquer des réactions plus importantes en milieu modèle pour le grenache, réactions conduisant à la formation de composés dérivés colorés (adduits tanin-anthocyane T-A). Ceci serait à vérifier par l'analyse de ces adduits dans les milieux modèles de diffusion.

Les concentrations finales en flavanols dans les vins sont en général un peu plus importantes pour le Grenache (de 580 à 692 mg/L) que pour le Carignan (425 à 600 mg/L) et plus importantes dans les deg+ que les deg-. les teneurs en polymères de DP >3 telles que dosées en SEC (et eq. epicatéchine), sont toujours supérieures aux teneurs en flavanols totaux dosées par phloroglucinolyse. Cela peut provenir d'un rendement incomplet de la réaction de dépolymérisation. Si l'on considère uniquement ces polymères de DP > 3, il n'existe plus de différences entre Grenache et Carignan pour les modalités deg+. On retrouve des concentrations en solution plus faibles pour les modalités deg- et plus faibles pour le Carignan que pour le Grenache. Seulement 10% des flavanols totaux présents dans la matière première sont extraits dans les vins, ce qui est très peu. Ceci contribue, avec l'extraction importante observée à partir des tanins de pépins, à rendre difficile la mise en évidence d'un impact de la composition des constituants structuraux (parois) de la matière première.

A partir de l'analyse de composition en polyphénols dans les baies et les vins, nous avons réalisé une analyse en composantes principales, dont les axes 1 et 2 expliquent environ 80 % de la variance observée (Figure 49). La séparation le long de l'axe 1 se fait selon le cépage (Carignan à gauche, Grenache à droite), la séparation selon l'axe 2 se fait selon la maturité : degrés – en haut, degrés + en bas. La taille des baies ne joue pas un rôle majeur, comme observé dans nos expériences en milieux modèles.

Si l'on regarde de plus près la contribution des différentes variables, on peut noter que :

- Les degrés de polymérisation moyens des tanins des pellicules et pepins sont négativement corrélés aux degrés moyens de polymérisation DPm des tanins du vin. Cela s'explique par le fait que seuls les tanins de DP les plus faibles sont extraits ;
- Les concentrations en flavanols totaux des vins, des pépins et des pellicules ne sont pas corrélées ;
- Les pourcentages de galloylation des tanins des vins et des pépins sont corrélés, tout comme les pourcentages d'épigallocatéchine des tanins des vins et des pellicules ;
- Les concentrations en anthocyanes non-acylées dans les vins sont très faiblement corrélées à celles dans les pellicules, les concentrations en anthocyanes *p*-coumaroylées dans les vins et dans les pellicules ne sont pas corrélées. Cela est due à une extraction des anthocyanes *p*-coumaroylées plus faible.

Ces résultats sont cohérents avec ce que nous avons observé dans les systèmes modèles pour les anthocyanes. Dans le cas des tanins, les taux de galloylation des tanins des vins confirment une extraction à partir des pépins, plus importante dans le cas du Grenache que dans celui du Carignan. Il faudrait analyser ces proportions dans les systèmes modèles pour voir si les proportions finales en unités galloylées dans les vins résultent d'une extraction moins importante en vinification par rapport à nos systèmes modèles ou d'une adsorption/précipitation préférentielle des tanins de pépins par rapport à ceux des pellicules quand ils sont présents ensemble dans le milieu.

Tableau 24. Composés phénoliques dans la matière première et les vins. Anthocyanes totales, non-acylés et para-coumaroylées déterminés par injection directe. Flavanols totaux, %Ec-G (epicatéchine gallate) et %EgC (épigallocatéchine), DPm (degré moyen de polymérisation) déterminés par phloroglucinolyse. Polmère de tannins déterminés par HPSEC.

		Car vol+deg+	Car vol-deg+	Car vol-deg-	Car vol+deg-	Gre vol+deg+	Gre vol-deg+	Gre vol-deg-	Gre vol+deg-
Pellicules (µg/g de pellicules)	Tot antho Non-acyl *p*-coum	6490,8±1413,7a 3345±773ab 2879±583,1a	6657,7±756,2a 3581,0±370,7a 2799,4±343,4a	5624,4±913,1a 2367,5±416,6bc 3048,8±481,0a	5786,8±1191,1a 2425,3±460,6bc 3142,3±689.6a	1980,9±220,9b 1409±153,3cd 475,9±59,2 b	1900,6±494,0b 1391,9±374,1cd 421,7±95,4b	794,0±36,4c 464,7±45,2d 295,0±16,5b	970,0±74,6c 600,1±37,7d 326,0±33,6b
	Tot Flav DPm %EgC %Ec-G	9179,1±2146.1a 11,2±0.5abc 9,3±0.3bc 1,7±0.2b	8272,1±1500,7a 11,9±0,4abc 9,7±0,8bc 2,3±0,1b	10444,0±1344,5a 12,8±0,5a 9,1±0,4bc 2,4±0,1b	11993,5±2149,9a 12,5±0,1ab 8,9±0,7c 1,7±0,1b	9399,0±900,3a 10,3±1,1c 12,3±0,2a 4,1±0,6a	7879,8±1508,8a 10,4±0,3bc 10,7±0,9ab 4,5±0,4a	10864,8±279,1a 11,8±1,4abc 9,8±0,5bc 4,1±0,6a	10470,1±1711,2a 11,4±0,5abc 10,6±0,5b 4,5±0,8a
Pépins (µg/g de pépins)	Tot Flav mDP %EgC %Ec-G	46085,9±6025,4a 6,7±0,1ab 0,2±0,02bcd 15,8±0,8b	53241,7±5004,5a 6,1±0,1bc 0,2±0,03b 15,7±0,9b	55045,6±5377,3a 7,1±0,5a 0,3±0,02a 16,9±0,5b	53371,7±2576,0a 6,8±0,2a 0,2±0,03b 17,2±0,4b	55371,1±4998,0a 5,6±0,1cd 0,2±0,01bc 20,5±0,7a	51141,0±3165,5a 5,3±0,1d 0,2±0,03bcd 21,4±0,3a	54877,7±4195,6a 5,7±0,2cd 0,1±0,00cd 22,4±0,5a	58524,8±5286,8a 5,4±0,1d 0,1±0,01d 22,4±0,1a
mg/L vin	Tot antho ***%tot antho*** Non-acyl ***%non acyl*** *p*-coum ***%p-coum***	545,1±30,5a ***22%*** 460,7±31,0a ***27%*** 43,7±0,9d ***14%***	564,9±2,6a ***26%*** 342,6±3,2b ***33%*** 174,5±4,6a ***15%***	331,1±12,0c ***19%*** 283,1±10,9cd ***29%*** 23,7±0,9ef ***9%***	291,1±20,9c ***20%*** 251,7±18,3d ***30%*** 17,9±2,1f ***11%***	336,3±4,9c ***43%*** 269,2±4,6d ***47%*** 54,9±1,2c ***29%***	402,1± 23,22b ***38%*** 322,5±19,4bc ***40%*** 64,3±3,2b ***26%***	111,0±3,6d ***47%*** 84,6±3,8e ***62%*** 21,8±0,4ef ***25%***	137,4±2,2d ***48%*** 104,0±0,6e ***57%*** 28,0±1,7e ***26%***
	Tot Flav ***%tot flav*** mDP %EgC %Ec-G	521,6±22,2cd ***9%*** 4,2±0,1c 4,8±0,3c 3,4±0,02c	600,2±7,0b ***10%*** 4,0±0,03d 5,4±0,1c 3,7±0,5c	484,0±19,4de ***10%*** 3,9±0,04d 4,0±0,03d 3,7±0,1c	426,5±34,8e ***10%*** 4,1±0,03c 3,4±0,4d 3,9±0,2bc	691,8±23,3a ***10%*** 4,7±0,1ab 8,2±0,4a 5,4±0,2ab	647,8±27,6ab ***12%*** 4,8±0,03a 7,2±0,2b 5,7±0,8a	614,9±44,4b ***9%*** 4,8±0,1a 5,0±0,2c 6,2±1,2a	578,9±11,9bc ***10%*** 4,6±0,02b 6,8±0,3b 5,7±0,2a
mg eq epicat/L vin	Polymère de tannins (HPSEC)	878± 18	931±32	690±39	625±22	878±25	931±38	829±32	720±1

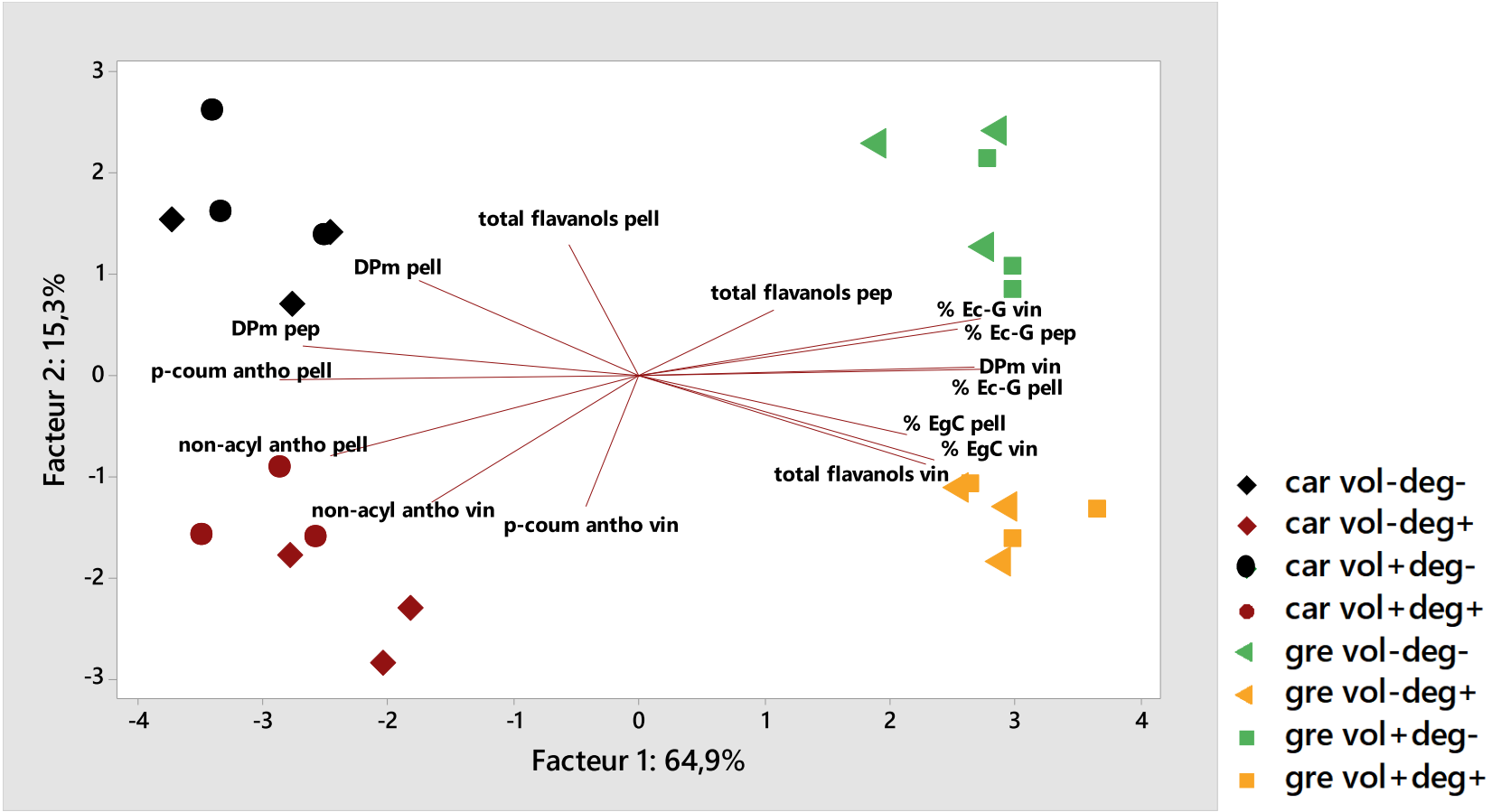


Figure 49. Analyse en composantes principales de la composition phénolique des baies et vins de Carignan et Grenache 2018.

Perspectives

Pour conclure, il reste encore des verrous techniques et scientifiques à lever concernant l'étude et la compréhension des phénomènes impliqués dans la diffusion des composés phénoliques du raisin vers la phase liquide. Au cours de ce travail de thèse, nous avons mis en évidence quelques caractéristiques de la matière première qui peuvent jouer un rôle sur la composition en anthocyanes et en tanins des vins :

1. la proportion d'anthocyanes para-coumaroylées, qui seront très peu extraites au final ;
2. la galloylation des tanins et l'extraction des tanins galloylés à partir des pépins et leur compétition avec les tanins issus des pellicules pour les phénomènes d'adsorption et d'interaction comme en termes de réactivité, notamment avec les anthocyanes;
3. la structure des parois cellulaire et notamment leur composition en extensines, AGPs, hemicellulose et les caractéristiques de structure des polysaccharides pectiques (homogalacturonanes et chaines latérales d'arabinane).

Afin de mieux exploiter les résultats trouvés, nous proposons certaines perspectives qui peuvent être des eventuelles questions pour mieux comprendre cette thématique:

La localisation et l'état d'association des composés phénoliques dans les compartiments cellulaires jouent un rôle clé dans la diffusion de ces composés. Progresser dans la comprehension de ces mecanismes nous permettra de cibler les analyses.

Les pépins constituent un élément important, même si on ne retrouve que peu de tanins de pépin dans les vins par rapport à leur concentration dans la matière première. Ils modifient la composition en polyphénols totaux par des mécanismes à approfondir.

Nous nous sommes focalisés sur les parois cellulaires, et notamment leur fraction insoluble. Or, lors de la macération, des macromolécules solubles (polysaccharides, protéines) sont extraites. Il serait intéressant de les identifier, de les quantifier et de caractériser leur impact sur la composition en polyphénols des vins. Est-il négligeable par rapport à l'impact des parois ou pas ?

Les facteurs identifiés dans cette thèse sont essentiellement impactés par le cépage et à la marge par la maturité. Nous les avons observés sur deux cépages. Il serait intéressant aussi de généraliser nos analyses et travailler sur d'autres variétés ou bien sur d'autres millésimes pour une même variété, notamment pour confirmer les résultats et l'impact de la composition des parois cellulaires de pulpe et de pellicules.

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Appendix

Appendix A: Abstract of communications in conferences

F&V Processing 2020-Third Symposium on Fruit and Vegetable Processing
Topic: Processing and reactivity of F&V (Oral communication)

Impact of grape variety, berry maturity and size on the extractability of skin polyphenols during model wine-like maceration experiments

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Anthocyanins and tannins, mainly present in the skin of grape berries and extracted during winemaking by maceration, play a decisive role in the sensory properties of red wines. Although it provides essential information, their analysis in skins is not sufficient to predict wine composition. Their extraction is partial and not proportional to initial skin contents in given maceration conditions, which is mainly attributed to interactions with cell wall material[1]. Beside, anthocyanins and tannins are reactive compounds that undergo chemical changes during winemaking, which impact their structure and properties[2]. In the present context (environmental constraints that affect grape composition, new varieties) it is needed to better identify the links between grape and wine composition and the main factors involved. This work focused on the impact of grape variety, berry size and density on the extraction of skin polyphenols during maceration.

Two contrasted varieties (Grenache, Carignan) were selected and harvested at technological maturity. The berries were sorted according to their size and density. Polyphenol extraction was followed during wine-like maceration experiments, performed on fresh skins in model systems (22°C, under argon, without yeast, stepwise ethanol addition from 0 to 15%). After diffusion, skins were successively washed with new wine like solvents until total polyphenol extraction before analysis of non-extractable polyphenols. Polyphenols in skins and solutions were analyzed by spectrophotometry and chromatography. Alcohol insoluble solids (AISs) in skins, as well as their composition in polysaccharides and proteins, were also determined.

Skin AISs differed between the two varieties by their carbohydrate composition and protein content. Fresh skins had similar contents in polymeric tannins, but strongly differed by their anthocyanin contents (higher in Carignan and in the ripest berries) and composition (higher proportions in coumaroylated anthocyanins in Carignan). In accordance with literature, tannin extraction was partial (from 14 to 25%) and selective due to interactions with skin insolubles. It was strongly impacted by the maturity for the Grenache, much less for the Carignan. Neither anthocyanin contents nor the AIS compositions could explain these differences. Anthocyanin extraction strongly differed between the two varieties and with the berry density, whereas berry size had no impact. Both a selective extraction/precipitation of anthocyanins (coumaroylated vs non-coumaroylated) and chemical changes strongly modulated final concentrations so that higher contents in skins did not lead to higher contents in wine-like solvents. Structural analyses of AIS components are needed to better identify the differences observed for tannins and link wine and grape compositions.

Keywords: grape skin polyphenols, extraction, model solution, alcohol insoluble solids.

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OenoIvas 2019, Bordeaux France
Topic: Analysis and composition of grapes, wines, wine spirits (Poster)

Skin polyphenol extraction during maceration: a quite complex problem?

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According to literature, the extraction of skin polyphenols in winemaking is mostly related to their solubility and to their interactions with cell wall material, which forms a barrier against their diffusion. In the present work, the impact of the grape variety and maturity on the extraction of skin polyphenols was studied in model systems.

Two grape varieties with contrasted polyphenol compositions (Carignan, Grenache) were sorted according to their degree of maturity and their size. Berries were manually peeled and skins recovered for biochemical analyses and extraction experiments. Analyses included polyphenol constituents, insoluble cell material (ICM) polysaccharides and proteins in ICM. Extraction experiments were performed on fresh skins, by increasing ethanol percentages from 0 to 15% to mimic maceration during fermentation. Polyphenol concentration was measured by UV-Vis spectrophotometry, HPLC and SEC. After extraction in wine-like conditions, skins were transferred several times in fresh solvents (with 0 then 15% ethanol), until no further diffusion was observed. Extractable polyphenols in wine-like solvents were determined from these experiments and compared to (i) polyphenols extracted at the end of the maceration and ii) polyphenols extracted with a good solvent (acetone/methanol/water) on both fresh and extracted skins.

Extraction profiles during maceration strongly differed between the two varieties and with the berry ripeness. Analyses indicated that these differences were first linked to the initial polyphenol composition in skins. In fact, polyphenol extraction coefficients at the end of the maceration (amount extracted/amount in skins) depended on their amount in skins but also on the anthocyanin to tannin ratios (A/T). Maximum 50 % of skin phenolics were extracted in Grenache berries in comparison to 30% in Carignan, whereas phenolic concentration and A/T ratios were higher in Carignan. However, other factors than the phenolic composition could participate in these differences, such ICM global composition. No significant differences between the different varieties and modalities, but structural differences cannot be ruled out.

Finally, after successive washings with fresh solvents, up to 90-92% of skin polyphenols were extracted in wine like conditions. This showed that interactions between skin insoluble and polyphenols modulate their extraction but do not have a major impact on the total amount of extractible skin polyphenols.

Keywords: grape skin polyphenols , extraction coefficients, diffusion, model solution

Appendix B: Comprehensive Microarray Polymer Profiling method (CoMPP method)

The analysis of the components of cell walls is challenging because of the complexity and heterogeneity of this latter. New tools are needed to better explore the grape cell wall architecture. The grape cell wall is composed of complex polysaccharides and structural proteins interlinked together in a complex network. Primary cell walls are networks of cellulose microfibrils connected by cross-linking hemicellulose. This connection is embedded in matrix of complex pectic polymers.

The classical tool for cell wall analysis is gas chromatography after extraction, acidic hydrolysis and derivatization. This latter has been used to identify and quantify the sugar monomers. However, this method has some limitations as it cannot fully answer questions at the polymer level. In response to the lack of suitable technologies for cell wall polymer analysis, an immunochemistry procedure, called Comprehensive Microarray polymer profiling (CoMPP) technology, was developed.

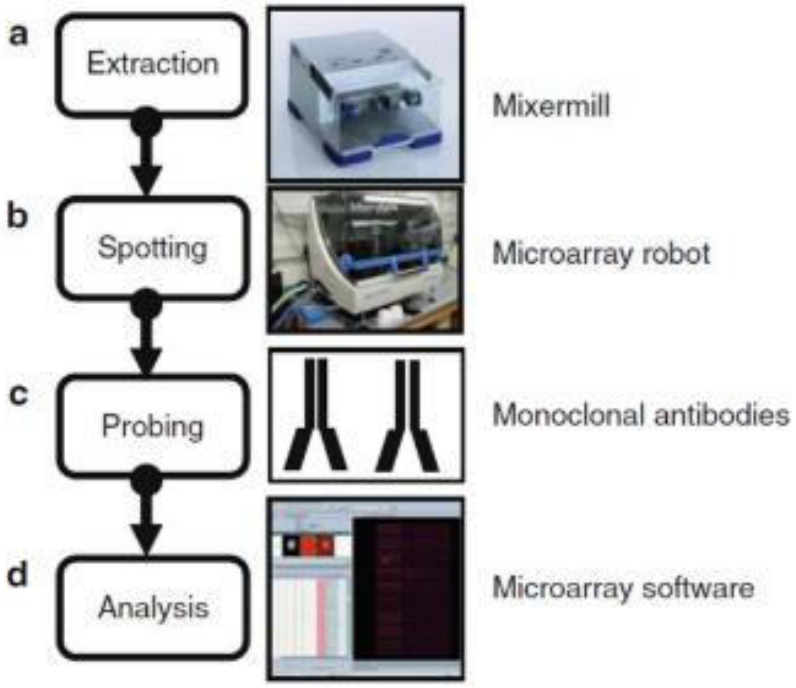
This technique consists of using highly specific molecular probes (monoclonal antibodies (mAbs) and carbohydrate binding modules (CBMs)), combined with the generation of glycan microarrays, that can cover a large range of plant cell wall polymers through epitopes detection (Moller *et al.,* 2007). This method provides a fast and effective way to profile and compare cell wall samples. It was validated on grapevine related studies, such as grape leaves, berry skins during winemaking process (Gao et al., 2016; Gao *et al.,* 2019).

The first step before analysis starts with the preparation of the AIS structural cell wall polymers using an optimized series of organic solvent. A sequential extraction is performed on the extract using CDTA (cyclohexanediamine-tetra-acetic acid) and NaOH. CDTA is used to extract the pectin rich fraction and NaOH for the hemicellulose rich fraction. The two different fractions are then printed individually on nitrocellulose membranes and probed with the antibodies (mAbs and CBMs). These fractions are then quantified using image analysis software generating a heatmap. A mean spot signal was calculated and the highest mean signal in the dataset was set to 100.

All antibodies (or probes) are rat monoclonal antibodies. Different reagent can be used for the detection such as: Peroxidase-linked (Anti-rat IgG (whole molecule)-peroxidase

produced in rabbit), Fluorochromes (Anti-rat IgG (whole molecule)-FITC produced in rabbit), Gold-linked (Anti-rat IgG (whole molecule)-Gold produced in goat). Analyses of sections of plant materials are often done with fluorescence and gold detection in conjunction with light and electron microscopies, respectively.

The procedure takes 2-3 days; it can quickly generate detailed polymeric profile over a large sample set. It has the specificity to give information on structural polysaccharides, glycoproteins, and linkages as well as methylation patterns.



The main steps of the comprehensive microarray polymer profiling (CoMPP) as illustrated in (Sorensen & Willats, 2011)

The mAbs and CBMs commonly used in our analyses are provided and better described in the table below. The table was followed by schemas illustrating the action of some antibodies on their epitopes. (Paul Knox lab website : http://www.plantprobes.net/index.php).

Table showing the antibodies used in CoMPP method and their targets

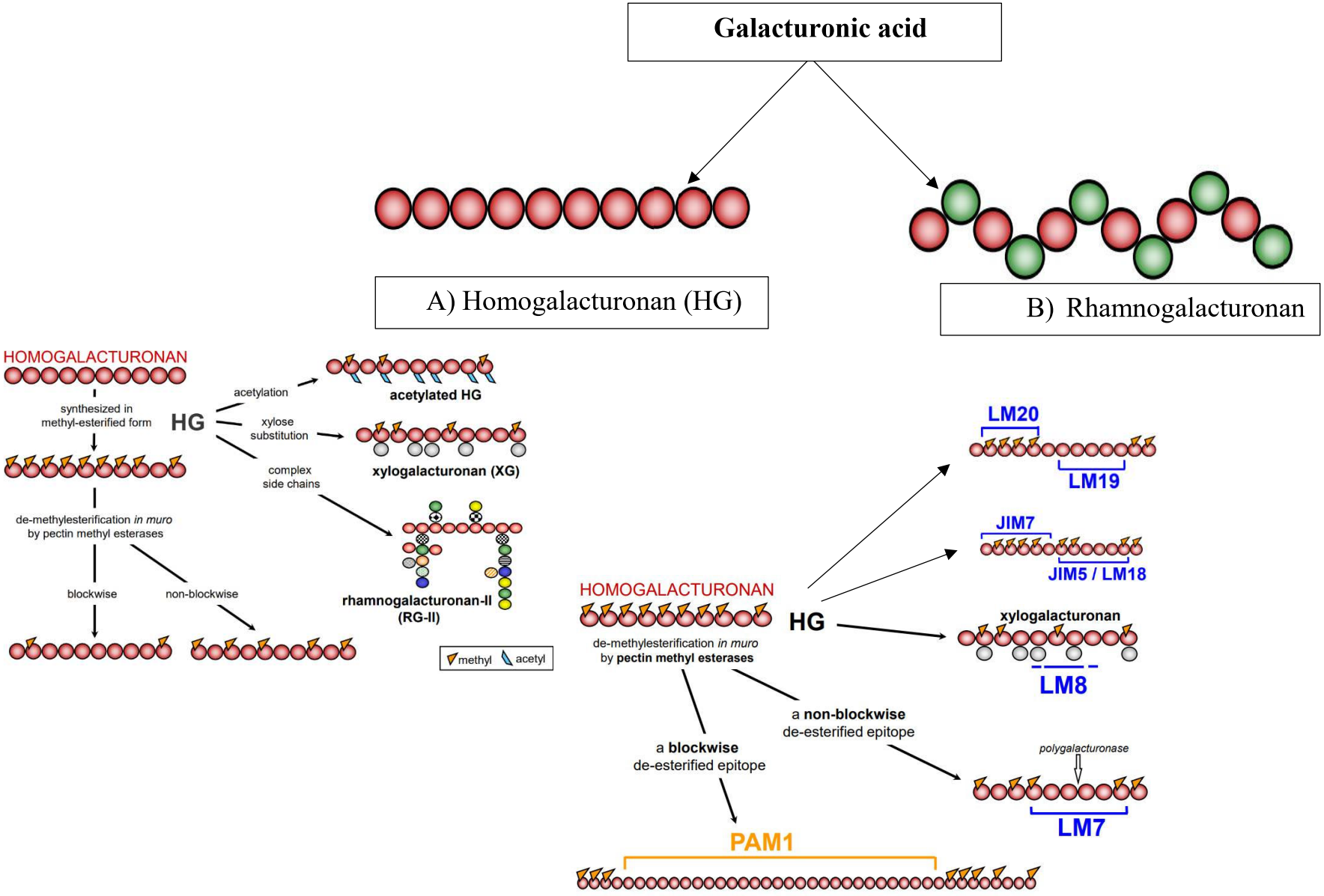
Category	mAbs/CBMs	Epitope recognition	Specificity	References
HG	JIM5	HG with a low DE	-Rat monoclonal antibody to homogalacturonan ----Recognize the α-1,4-linked homogalacturonan domain of pectic polysaccharides -Recognizes the partially methyl-esterified epitopes of homogalacturonan and can also bind to un-esterified homogalacturonan -Has no known cross-reactivity with other polymers.	Knox *et al.,* 1990 Clausen *et al.,* 2003
	JIM7	HG with a high DE	-Rat monoclonal antibody to homogalacturonan -Recognizes the α-1,4-linked homogalacturonan domain of pectic polysaccharides -Recognizes partially methyl-esterified epitopes of homogalacturonan but does not bind to un-esterified homogalacturonan. ➔It is recommended the use of LM19 and LM20 along with JIM7. The LM20 having a wide recognition of methylester epitopes and strong recognition of HG.	Knox *et al.,* 1990; Clausen *et al.,* 2003
	LM18	HG partially methylesterified	-Rat monoclonal antibody to homogalacturonan -Has a similar recognition profile to JIM5 -Binds to de-esterified HG, higher affinity to shorter chain (DP <4)	Verhertbrugger *et al.,* 2009a
	LM19	HG partially methylesterified	-Rat monoclonal antibody to homogalacturonan -Recognizes the α-1,4-linked homogalacturonan domain of pectic polysaccharides -Recognizes a range of homogalacturonan samples but appears to have a preference for and binds strongly to un-esterified homogalacturonan. Higher affinity to longer chain (DP >4) -Has no known cross-reactivity with other polymers ➔LM19 is more recommended to be use in the place of JIM5 as LM19 binds more effectively to unesterified HG	Verhertbrugger *et al.,* 2009a
	LM20	HG partially methylesterified	-Rat monoclonal antibody to homogalacturonan -Recognizes the α-1,4-linked homogalacturonan domain of pectic polysaccharides -Binds to methyl esterified HG and does not bind to un-esterified HG -Has no known cross-reactivity with other polymers. ➔LM20 binds to a higher density esterified homogalacturonan epitope than JIM7	Verhertbrugger *et al.,* 2009a

	PAM1	HG blockwise methylesterified	-Phage display monoclonal antibody to homogalacturonan (HIS-tagged recombinant proteins) -Binds to long stretches of unesterified homogalacturonan and recognises long un-esterified blocks of GalA residues	
	2F4	HG Ca2+ crosslinked	-Mouse monoclonal antibody to homogalacturonan -Recognizes the homogalacturonan domain of pectic polysaccharides. -Binds specifically to Ca2+ crosslinked HG with degrees of methyl-esterification (DM) up to 40%	Liners *et al.,* 1989
	LM8	Xylogalacturonan	-Rat monoclonal antibody to xylogalacturonan -Recognizes a specific epitope of a xylogalacturonan pectic polysaccharide that is associated with cell detachment and separation in a wide range of species -Has no known cross-reactivity with other polymers	Willats *et al.,* 2004
RGI	INRA-RU1	Backbone of rhamnogalacturonan I	-Binds to unbranched region of RGI -Needs >6 disaccharide backbone repeats -Maximum binding to DP=14	Ralet *et al.,* 2010 Jones
	INRA-RU2	Backbone of rhamnogalacturonan I	-Binds to unbranched region of RGI -Significant binding to 2 disaccharide backbone repeats -Need at least DP=4	Ralet *et al.,* 2010 Jones
RGI side chains	LM5	(1→4)-β-D-galactan	-Rat monoclonal antibody -High affinity to (1-4)-β-D-galactosyl residues found in the galactan components of certain pectic polymers such as rhamnogalacturonan-I.	Jones *et al.,* 1997
	LM6	(1→5)-α-L-arabinan	-Rat monoclonal antibody -High affinity to (1-5)-α-L-arabinosyl residues found in the arabinan components of certain pectic polymers such as rhamnogalacturonan-I	Willats *et al.,* 1998
	LM13	Linearised (1→5)-α-L-arabinan	-Rat monoclonal antibody to (1-5)-α-L-arabinan linear -Recognizes a longer linear epitope in (1-5)-α-L-arabinosyl residues that are likely to be more abundant in unbranched arabinans	Verhertbrugger *et al.,* 2009b

Mannans	LM21	(1→4)-β-D-(galacto)(gluco)mannan	-Rat monoclonal antibody to mannan -Binds to β-(1→4)-manno-oligosaccharides from DP2 to DP5 -Displays a wide recognition of mannan, glucomannan and galactomannan polysaccharides.	Marcus *et al.,* 2010
Glucan/Xyloglucan	BS-400-2	(1→3)-β-D-glucan	-Rat monoclonal antibody to mannan -Binds to (1-3)-β-D-glucan	Meikle *et al.,* 1991
	LM15	Xyloglucan (XXXG motif)	-Rat monoclonal antibody -High affinity to xylosyl residues in the XXXG motif of xyloglucan ➔ Xyloglucans have a backbone of (1→4)-β-D-glucan and some glucosyl residues are substituted with short side chains. Xyloglucans are classified as XXXG or XXGG type based on the number of backbone residues that carry side chains with the XXXG type having three consecutive glucosyl residues with xylose attached and a fourth unbranched residue. For example, an unbranched glucosyl residue is designated G, a glucosyl residue bearing a single xylose is designated X and one bearing a disaccharide of β-Gal-(1,2)-α-Xyl is designated L.	Marcus *et al.,* 2008
	LM24	Xyloglucan	-Rat monoclonal antibody to Xyloglucan -Binds preferentially to the galactosyl residues XLLG motif of xyloglucan	
	LM25	Xyloglucan / unsubstituted β-D-glucan	-Rat monoclonal antibody to Xyloglucan -Binds to xyloglucan/unsubstituted glucan -Binds to the XLLG, XXLG and XXXG oligosaccharides of xyloglucan	Pedersen *et al.,* 2012
Xylans	LM10	(1→4)-β-D-xylan	- Rat monoclonal antibody to Xylan -High affinity to (1-4)-β-D-xylosyl residues that constitute the backbone of xylans -Recognizes unsubstituted and relatively low-substituted xylans	McCartney *et al.,* 2005
	LM11	(1→4)-β-D-xylan/arabinoxylan	-Rat monoclonal antibody -High affinity to (1,4)-β-D-xylosyl residues that constitute the backbone of xylans -Recognizes unsubstituted xylans and arabinoxylans carrying a low degree of arabinose substitutions	McCartney *et al.,* 2005
Cellulose	CBM3a	cellulose	-HIS-tagged recombinant proteins -Binds to crystalline cellulose	Tormo *et al.,* 1996

Extensins	LM1	Extensin	-Recognizes an epitope that is carried by a range of hydroxyproline-rich glycoproteins (HRGPs) of the extensin class	Smallwood *et al.,* 1995
	JIM11	Extensin	-Recognizes an epitope that is carried by a range of hydroxyproline-rich glycoproteins (HRGPs) of the extensin class	Smallwood *et al.,* 1994
	JIM20	Extensin	-Recognizes an epitope that is carried by a range of hydroxyproline-rich glycoproteins (HRGPs) of the extensin class	Smallwood *et al.,* 1994
AGPs	JIM8	AGP	Arabinogalactan protein	McCabe *et al.,* 1997
	JIM13	AGP	Arabinogalactan protein	Knox *et al.,* 1991; Yates *et al.,* 1996
	LM14	AGP	Arabinogalactan protein	Moller *et al.,* 2008
	LM2	AGP, β-linked GlcA	Recognizes a carbohydrate epitope containing b-linked glucuronic acid	Smallwood *et al.,* 1996

Illustration of the pectin antibodies recognizing the structural polysaccharides epitopes (Paul knox lab website)



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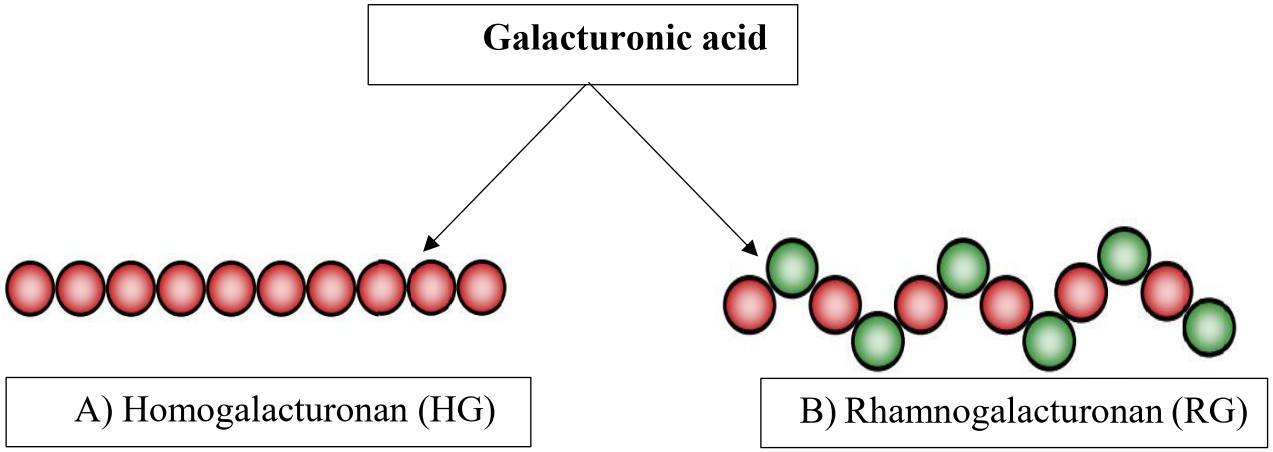

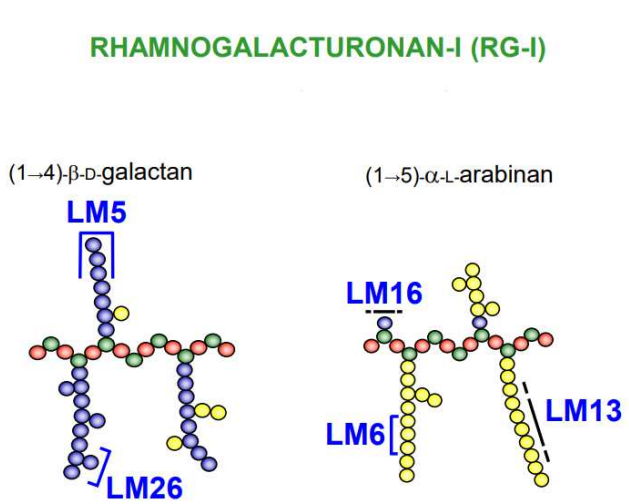

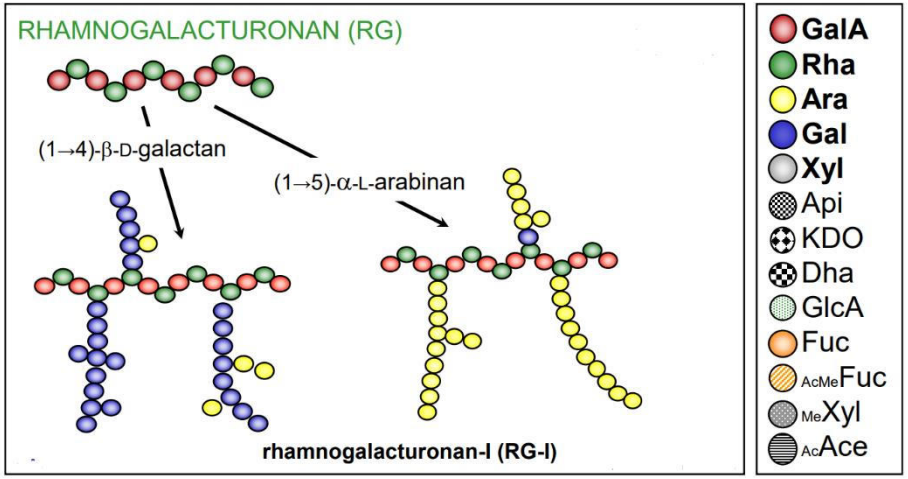

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Appendix C: Rheology of grape berries and skins

The device used to collect the measurements for this trial, the Penelaup robot, was developed by INRA and CTIFL (Abbal and Planton, 1990). This device makes it possible to automatically determine the physical characteristics such as the dimensions and / or the firmness of a product. It consists of a support, a measuring rod whose end is designed to support a part intended to come into contact with the sample (Figure 1). This tool is either a flat tool able to measure the berry firmness or a needle-like tool able to measure the berry skin hardness (Penelaup II).

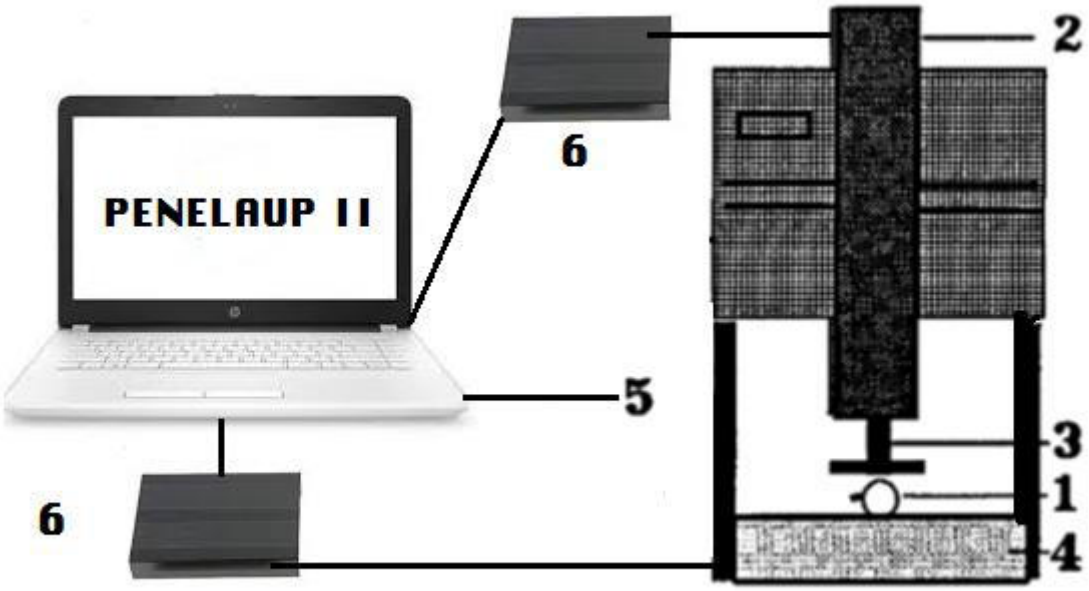

Figure 1. Scheme of the Penelaup rheometer. 1.berry; 2. column and servo screw moving system; 3. mandrel and crushing tool; 4. precision sensor; 5. computer; 6. electronic control device.

The measuring rod is mounted in translation relative to a worm extending parallel to the measuring rod, which is driven in rotation by a high-precision stepper motor. The sample is placed on a measuring sensor support and set up perpendicular to the measuring rod. The system control consists of a computer (PC Windows 10tm) connected to several microcontroller modules specifically developed for executing commands and processing analog signals.

For each measurement the robot supplies the mass, the diameter as well as the stress curve (expressed in Newton) of the product as a function of the user-selected % of crushing. Given the very high accuracy of this device, the user can work in the superficial areas of the

berry (a few %) or otherwise seek to the bursting of the berry. The recorded measurement curve can be directly interfaced to Microsoft Excel (Figure 2). The new version of the robot has a second specific device to study the skin of the berry, and measures its hardness by penetrometry.

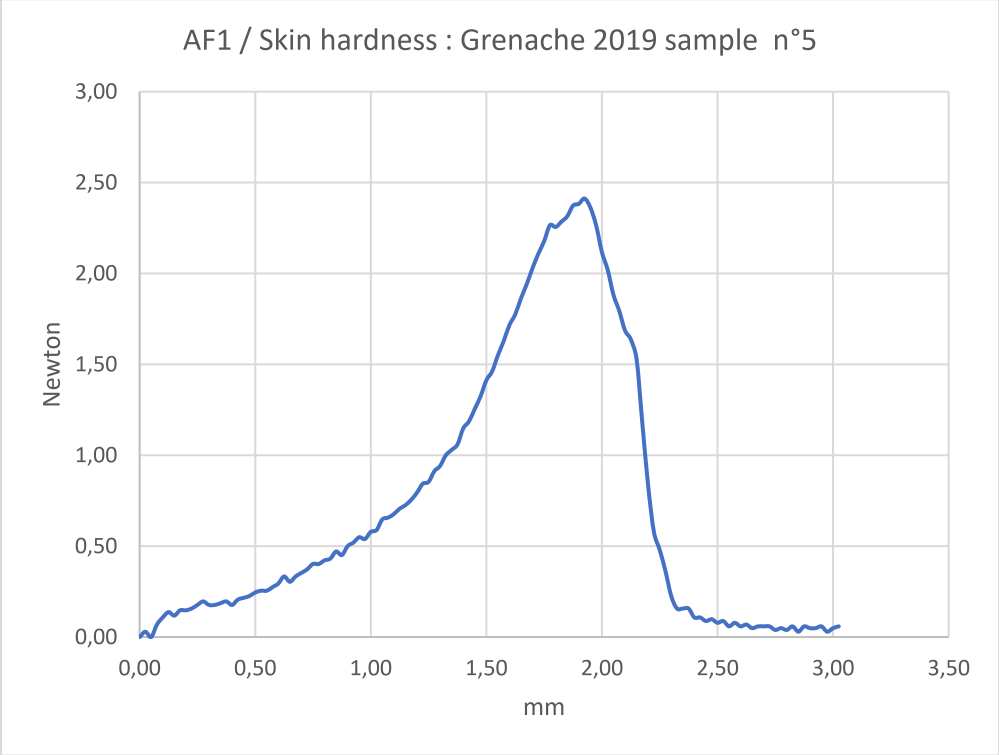

Figure 2. Stress curve recorded by Penelaup

In the present research we were able to define and calculate specific indexes to quantify the firmness of berries and hardness of their skin. These indexes could be used for other fruits like apples, cherries, or tomatoes to study firmness and skin hardness with the same methodology as for grapes.

Consider the following variables:

p: percentage of desired deformation
D: berry diameter (mm)
M: fruit weight (g)
x: the displacement of the tool (mm)
f(x): force (Newton) applied at location x

Processing the test, the deformation curve of the grape follows the equation y = f (x), x going from the contact of the tool with the grape up to the location programmed by the operator.

The integration of f(x) gives a graphical representation of the total energy A (joule) of the process. A is the sum of the force f (x) multiplied by the displacement x of the tool for every position x:

$A = \int_a^b f(x)dx$

A is the energy, represented by the area under the curve f(x).

if a = 0 (initial contact of the tool with the grape)

and b = D * p. (b is the final location of the tool: p is the percentage of the grape diameter D, programmed by the operator)

$AF1(p) = A = \int_0^{D*p} f(x)dx$ (1)

AF1(p) is called the first Abbal index (joule).

It is an index of absolute firmness for p % deformation of the initial diameter D of the fruit.

Using equation (1) if we calculate :

$$A/M = \frac{\int_a^b f(x)dx}{M}$$

with a = 0 and b= D * p we can define

$$AF2(p) = \frac{\int_0^{D*p} f(x)dx}{M} \quad (2)$$

AF2(p) is called the second Abbal index (joule/kg) and takes into account fruit weight for a p % deformation of the initial berry diameter (D).

For penetrometry, the test consists of measuring the energy needed to push a needle through a berry skin for a displacement of L = 10 mm.

Unlike previous tests, penetrometry test does not take into account the diameter or the weight of the grape. So we defined $AP(x=10) = \int_0^{x=10} f(x)dx$ (3)

AP(x=10) is called the third Abbal index (joule).

The needle is 35 mm long and 2,5 mm thick and the bottom has a V shape (Figure 3).

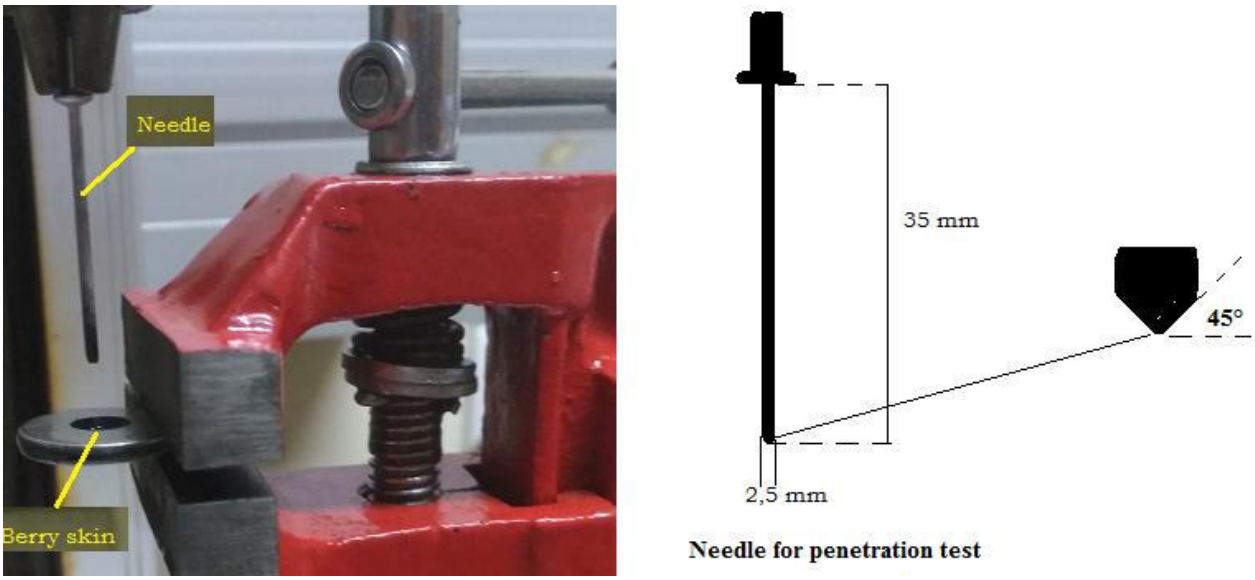

Figure 3. Details of the device and of the needle used

Extraction des anthocyanes et des tanins en vinifications en rouge : étude de certains mécanismes et impact de la matière première

Résumé. Les polyphénols du raisin, principalement localisés dans la pellicule et les pépins, jouent un rôle clé dans le goût et la couleur des vins rouges. Leur extraction par diffusion a lieu pendant la macération. Elle est partielle, et modulée par plusieurs facteurs pouvant la favoriser ou au contraire la limiter. Après diffusion, les polyphénols subissent d'autres modifications liées à leur réactivité chimique. Il est par conséquent difficile de prévoir la composition finale d'un vin à partir de celle du raisin. Les objectifs de ce travail étaient d'identifier les facteurs impactant l'extraction des polyphénols. Dans ce but, deux variétés contrastées ont été étudiées : le Grenache et le Carignan. Les compositions en polyphénols des différents compartiments ont été déterminées, et l'analyse du matériel pariétal des pulpes et pellicules réalisées. Des études en milieux modèles ont été réalisées : diffusion des polyphénols à partir des pellicules et des pépins, pris ensemble ou séparément et en présence ou non des constituants insolubles de pulpe (CIPs), adsorption d'anthocyanes et de tanins purifiés sur les CIPs. Elles ont été comparées à des microvinifications. Les résultats obtenus ont permis de montrer que : i) l'extraction des anthocyanes est dépendante de la proportion d'anthocyanes *p*-coumaroylées ; ii) la diffusion des tanins à partir des pellicules et leur adsorption sur les CIPs sont influencées par la composition des parois cellulaires, notamment en termes d'extensines et d'AGP ; iii) cette adsorption est essentiellement irreversible, sélective (tanins de plus haut DPs et galloylés) et peu influencée par la présence des anthocyanes ; iv) les diffusions de tanins observées à partir des pépins seuls sont importantes mais dès qu'ils sont en présence de parois cellulaires de pellicules/des CIPs et/ou d'anthocyanes, leur concentration chute fortement, en lien avec de l'adsorption et/ou des précipitation et/ ou des réactions chimiques; v) si les CIPs peuvent adsorber des quantités importantes de tanins, cette adsorption déplace les équilibres solide/liquide en faveur de la diffusion. Ces résultats ont permis de rendre compte des différences observées en microvinification entre ces deux cépages et de progresser dans la compréhension de l'impact de la composition de la matière première.
Mots-clès : raisin, anthocyanes, tanins, extraction, polysaccharides et protéines pariétaux.

Anthocyanins and tannins extraction in red winemaking : study of certain mechanisms and impact of the grape composition

Abstract. Grape polyphenols, mainly located in skins and seeds, play a key role in the taste and color of red wines. Their extraction occurs by diffusion during maceration. It is partial and modulated by several factors that can favour it or on the contrary limit it. After diffusion, they undergo other modifications linked to their chemical reactivity. It is therefore difficult to predict the final composition of a wine from that of grapes. The objectives of this work were to identify the factors impacting polyphenol extraction. To this end, two contrasted varieties were studied: Grenache and Carignan. The polyphenol compositions of the different compartments were determined and the analysis of the cell walls of the fleshes and skins was carried out.Studies in model solutions were performed: diffusion of polyphenols from skins and seeds, taken separately or together and in the presence or not of flesh water-insoluble materials (FWIM); adsorption of anthocyanins and tannins on FWIM. They were compared to microvinifications. Results showed that: i) the extraction of anthocyanins is dependent on the proportion of p-coumaroylated anthocyanins; ii) the diffusion of tannins from the skins and their adsorption on FWIM are influenced by the composition of the cell walls, in particular in terms of extensins and AGP ; iii) this adsorption is essentially irreversible, selective (tannins of higher DPs and galloylated) and little influenced by the presence of anthocyanins ; iv) the tannin diffusions from the seeds alone are important but their concentration drops sharply as soon as they are in the presence of skins/fleshes cell walls and/or anthocyanins, in relation to adsorption and/or precipitation and/or chemical reactions; if the pulp insolubles can adsorb large amounts of tannins, this adsorption shifts the solid/liquid equilibrium in favour of diffusion. These results made it possible to account for the differences observed in microvinification between the two grape varieties studied and to progress in the comprehension of the impact of grape composition.
Key-words: grape, anthocyanins, tannins, extraction, cell wall polysaccharides and proteins.

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