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Study of Prodelphinidins: synthesis, detection, identification and reactivity with anthocyanins

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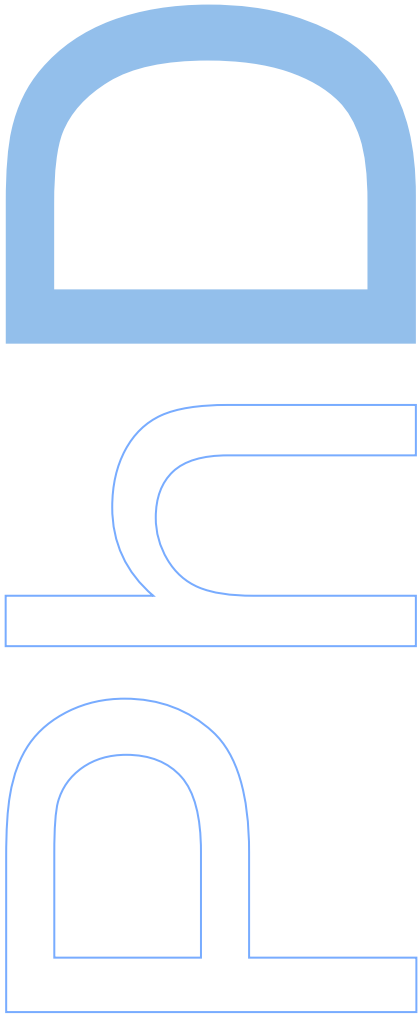

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Ao Duarte,

Aos meus Pais

"Drink wine, and you will sleep well. Sleep, and you will not sin.
Avoid sin, and you will be saved. Ergo, drink wine and be saved."
- Medieval German saying

Resumo

Este trabalho visou a obtenção de novas prodelfinidinas, o desenvolvimento de um método analítico de deteção de prodelfinidinas e a sua aplicação em matrizes alimentares.

Um dos objetivos deste trabalho prendeu-se com a síntese de novas prodelfinidinas. Para tal foi seguido um método de síntese de prodelfinidinas diméricas envolvendo a proteção das unidades monoméricas, benzilação em C4 da unidade superior, a condensação a baixa temperatura na presença de um ácido de Lewis e a hidrogenação do composto dimérico obtido. A rutura da ligação interflavânica aquando das reações de hidrogenação limitou todo o processo de síntese. Vários métodos e condições de hidrogenação foram testados, incluindo o dispositivo de H-cube® na ISM - Institut des Sciences Moléculaires, Groupe Synthèse-Molécules Bioactives na Universidade de Bordéus I, embora com baixos rendimentos.

Um segundo objetivo deste projeto foi o desenvolvimento de um novo método de LC-ESI-MS para a deteção de várias prodelfinidinas diméricas e triméricas em matrizes alimentares. Tal método não existia até então e a deteção deste tipo de compostos era apenas realizada através de outras técnicas que envolvem a rutura prévia da sua estrutura química. A partir da síntese de prodelfinidinas desenvolvida previamente, novos padrões valiosos foram obtidos e três novas prodelfinidinas diméricas podem agora ser identificadas.

Este método foi posteriormente aplicado na análise de vinhos. Um vinho tinto da Região Demarcada do Douro de 2013 mostrou ter um teor de proantocianidinas quase 2,2 vezes superior ao de um vinho verde tinto da Região Demarcada dos Vinhos Verdes (sub-região do Lima) de 2012, sendo maioritariamente composto por dímeros de procianidinas. Este vinho verde tinto é constituído por 62,5% de procianidinas triméricas, 22,9% de procianidinas diméricas, 10,2% de prodelfinidinas diméricas e 4,39% de prodelfinidinas triméricas. Ao passo que, o vinho tinto de 2013 do Douro é principalmente constituída por dímeros e trímeros de procianidinas (71,4% e 20,0%, respetivamente) e apenas 6,92% e 1,66% de dímeros e trímeros de prodelfinidinas. O conteúdo total de procianidinas no vinho verde tinto de 2012 é 6 vezes maior do que o conteúdo total de prodelfinidinas, enquanto que no vinho tinto de 2013 é quase 11 vezes maior do que o conteúdo total de prodelfinidinas. No entanto, para este estudo, todos os dímeros ou trímeros cuja unidade superior seja epicatequina ou catequina foram considerados procianidinas e não prodelfinidinas.

Uma última parte deste projeto visou o estudo do efeito de copigmentação com o propósito de analisar as interacções não covalentes de uma antocianina, a

malvidina-3-glucósido ou oenina, utilizando outros polifenóis (incluindo as prodelfinidinas) como copigmentos. A partir deste estudo pode ser concluído que a presença de um grupo hidroxilo extra no anel B da estrutura do flavan-3-ol aumenta ligeiramente o potencial de copigmentação, o que pode contribuir para as propriedades organoléticas do vinho tinto.

Abstract

This study aimed to obtain new prodelphinidins, the development of an analytical method for detection prodelphinidin and its application in food matrices.

One of the goals of this work was the synthesis of new prodelphinidins. A dimeric prodelphinidin synthetic path involving the protection of the monomeric units, benzylation at C4 of the upper unit, condensation at low temperature in the presence of a Lewis acid and the obtained dimeric compound hydrogenolysis was followed. The interflavan bond rupture when the hydrogenolysis reactions were performed limited the entire prodelphinidin synthesis. Various hydrogenolysis methods and conditions were tested, including the H-cube® device at the ISM – Institut des Sciences Moléculaires, Groupe Synthèse-Molécules Bioactives at Bordeaux University I, although with low yields.

Another goal of this work was the development of a new LC-ESI-MS method for the detection of several dimeric and trimeric prodelphinidins in food matrices. Such a method did not exist until then and the detection of this type of compounds was only performed by techniques that include prior rupture of their chemical structures. From the previously developed synthesis, new valuable standards were obtained and three new prodelphinidin dimers can now be identified.

This method was later applied on wine analysis. A 2013 red wine from the Demarcated Region of Douro showed to have an almost 2.2 times higher content of proanthocyanidins than a 2012 red vinho verde wine from the Demarcated Region of Vinho Verde (Lima's sub-region), mostly composed by PC dimers. This red vinho verde wine was constituted by 62.5% procyanidin trimers, 22.9% procyanidin dimers, 10.2% prodelphinidin dimers and 4.39% prodelphinidin trimers. In the meantime, the 2013 Douro red wine is mainly constituted by procyanidin dimers and trimers (71.4% and 20.0% respectively) and only 6.92% and 1.66% of prodelphinidin dimers and trimers. The total procyanidin content of the 2012 red vinho verde wine is only 6 times higher than the total prodelphinidin content, while in the 2013 red wine is 11 times higher than the total prodelphinidin content. However, all first unit epicatechin/catechin dimers or trimers were considered to be procyanidins and not prodelphinidins.

A final part of this project aimed the studying of the copigmentação effect for the purpose of analysing non-covalent interactions of an anthocyanin, malvidin-3-glucoside or oenin, using other polyphenols (including prodelphinidins) as copigments. From this study it could be concluded that the presence of an extra hydroxyl group in the B ring of the flavan-3-ol structure slightly increases the

copimentation potential, which may contribute to the organoleptic properties of red wine.

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C – catechin

EC – epicatechin

(E)C – catechin or epicatechin

GC - gallocatechin

EGC – epigallocatechin

CG – catechin gallate

GCG – gallocatechin gallate

ECG – epicatechin gallate

EGCG – epigallocatechin gallate

PA - proanthocyanidin

PD - prodelphinidin

PC - procyanidin

Mv3glc – malvidin-3-glucoside

C-glc – catechin-glucoside

FW – fresh weight

THF - tetrahydrofuran

DMF - dimethylformamide

MeOH - methanol

EtOAc – ethyl acetate

C4Bn (**19**) – 5,7,3',4'-*Tetra-O-benzylcatechin*

EGC5Bn (**20**) – 5,7,3',4',5'-Penta-*O*-benzylepigallocatechin

EGCG8Bn (**21**) – 5,7,3',4',5'-Penta-*O*-benzyl-3-*O*-(3,4,5-tri-*O*-benzylgalloyl)epigallocatechin

(EGC5Bn)Bn (**22**) – 4,5,7,3',4',5'-Hexa-*O*-benzylepigallocatechin

EGC-C9Bn (**23**) – 5,7,3',4',5'-Penta-*O*-benzylepigallocatechin(4→8)5,7,3',4'-Tetra-*O*-benzylcatechin

EGC-EGC10Bn (**24**) – 5,7,3',4',5'-Penta-*O*-benzylepigallocatechin(4→8)5,7,3',4'-Tetra-*O*-benzylcatechin

EGC-EGCG13Bn (**25**) – 5,7,3',4',5'-Penta-*O*-benzylepigallocatechin(4→8)5,7,3',4'-Tetra-*O*-benzylcatechin

Pd/C 10% – 10% palladium-charcoal

$Pd(OH)_2$/C 20% – 20% palladium hydroxide on carbon

TLC – thin layer chromatography

HPLC – high performance liquid chromatography

LC-ESI-MS - liquid chromatography-electrospray ionization- mass spectrometry

List of publications

The Influence of a Flavan-3-ol Substituent on the Affinity of Anthocyanins (Pigments) Toward Vinylcatechin Dimers and Procyanidins (Copigments) – Frederico Nave, Natércia Brás, Natércia Teixeira, Nuno Mateus, Maria Ramos, Florent Di Meo, Patrick Trouillas, Olivier Dangles, Víctor de Freitas, **J. Phys. Chem. B** 2012, *116*, 14089-14099.

Structural features of copigmentation of oenin with different polyphenol copigments – Natércia Teixeira, Luís Cruz, Natércia F. Brás, Nuno Mateus, Maria João Ramos, Víctor de Freitas, **J. Agric. Food Chem.** 2013, *61*, 6942-6948.

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Screening of Anthocyanins and Anthocyanin-Derived Pigments in Red Wine Grape Pomace Using LC-DAD/MS and MALDI-TOF Techniques – Joana Oliveira, Mara Alhinho da Silva, Natércia Teixeira, Victor De Freitas and Erika Salas, **J. Agric. Food Chem.** 2015, *63* (35), 7636–7644.

Proanthocyanidin screening by LC-ESI-MS of Portuguese red wines made with teinturier grapes – Natércia Teixeira, Joana Azevedo, Nuno Mateus, Victor de Freitas, **Food Chem.** 2016, *190*, 300-307.

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List of participations in science meetings

Co-pigmentation studies involving Prodelphinidins and Anthocyanins – Natércia Teixeira, Nuno Mateus, Victor de Freitas. 22 – 26 July 2012, XXVIth International Conference on Polyphenols – Florence, Italy.

Synthesis of Prodelphinidins - work in progress – Natércia Teixeira, Nuno Mateus, Victor de Freitas. 04 – 06 September 2013, 10º Encontro Nacional de Química Orgânica, 1º Simpósio Luso-Brasileiro de Química Orgânica – Lisbon, Portugal.

Structural features of copigmentation of oenin with different polyphenol copigments – Natércia Teixeira, Luís Cruz, Natércia F. Brás, Nuno Mateus, Maria João Ramos, Victor de Freitas. 09 – 11 September 2013, 7th International Workshop on Anthocyanins – Porto, Portugal.

Synthesis of Prodelphinidins – Natércia Teixeira, Nuno Mateus, Victor de Freitas. April 29 to May 1 2014, 4th Portuguese young chemists meeting – Coimbra, Portugal.

Characterization of pigments from red wine grape pomace by mass spectrometry – Joana Oliveira, Sílvia Maia, Mara Alhinho, Natércia Teixeira, Nuno Mateus, Victor de Freitas, Erika Salas. 02 – 06 September 2014, XXVIIth International Conference on Polyphenols & 8th Tannin Conference – Nagoya, Japan.

Analysis of grape seed tannins by mass spectrometry (MALDI-TOF and ORBITRAP ESI-MS)– Erika Salas, Natércia Teixeira, Sílvia Maia, Joana Oliveira, Nuno Mateus, Victor de Freitas, 14 – 17 July 2015, In vino analytica scientia – Trento, Italy.

Chapter 1

1. General introduction

Plant physiologists separate metabolism into two categories i.e. primary metabolism and secondary metabolism. All organisms possess metabolic pathways by which they synthesize and utilize certain essential chemical species: sugars, amino acids, common fatty acids, nucleotides and polymers derived from them (e.g. lipids, proteins and polysaccharides). This is known as the primary metabolism and these compounds, which are essential for the survival and well being of the organisms, are called primary metabolites. Most organisms also utilize other metabolic pathways, producing compounds for the purposes other than primary physiological functions, these are secondary metabolites also known as natural products and the pathways for their synthesis and utilization constitute the secondary metabolism. Many of these secondary metabolites found in plants, animals and microorganisms possess pharmacological properties.

1.1. Polyphenolic compounds

Polyphenols are secondary metabolites of plants and are generally involved in defence against ultraviolet radiation or aggression by pathogens (Beckman, 2000). In food, polyphenols may contribute to the bitterness, astringency, color, flavour, odour and oxidative stability. Epidemiological studies and associated meta-analyses performed in the late 20th century, strongly suggested that long term consumption of diets rich in plant polyphenols offered some protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Arts & Hollman, 2005; Graf, Milbury, & Blumberg, 2005).

Fruits like grapes, apple, pear, cherries and berries contains up to 200–300 mg polyphenols per 100 grams fresh weight. Typically, a glass of red wine or a cup of tea or coffee contains about 100 mg polyphenols. Cereals, dry legumes and chocolate also contribute to the polyphenolic intake (Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005; Spencer, Abd El Mohsen, Minihane, & Mathers, 2008). The total dietary intake is about 1 g/d. It is much higher than that of all other known dietary antioxidants, about 10 times higher than that of vitamin C and 100 times higher than those of vitamin E and carotenoids (Scalbert & Williamson, 2000).

Distribution of phenolics in plants at the tissue, cellular and sub cellular levels is not uniform. Insoluble phenolics are found in cell walls, while soluble phenolics are

present within the plant cell vacuoles (Wink, 1997). Polyphenolic compounds are commonly divided in two groups: non-flavonoids and flavonoids.

1.1.1. Non-flavonoid compounds

Non-flavonoids are divided into hydroxybenzoic and hydroxycinnamic acids, volatile phenols and stilbenes (Figure 1).

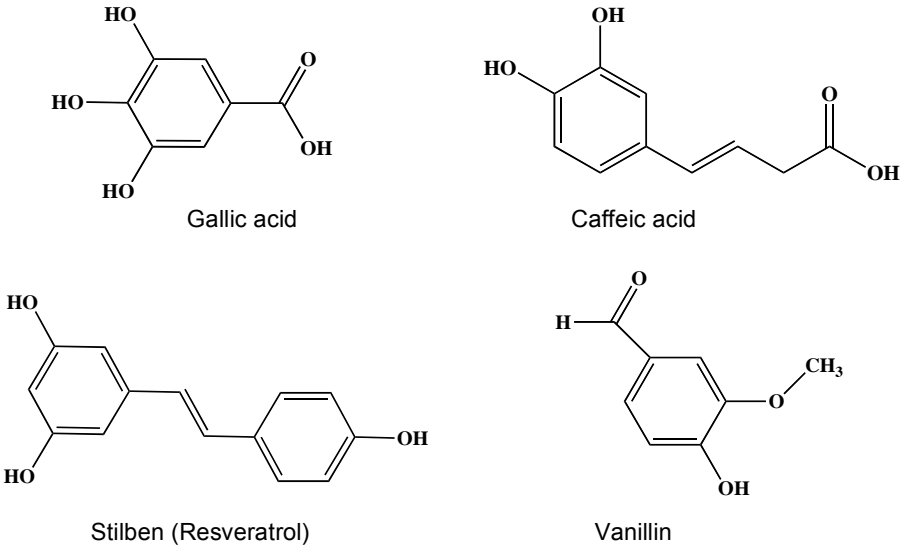


Figure 1. Non-flavonoid polyphenol structure examples

One of the most common phenolic acids is caffeic acid, present in many fruits and vegetables, most often esterified with quinic acid as in chlorogenic acid, which is the major phenolic compound in coffee. Another common phenolic acid is ferulic acid, which is present in cereals and is esterified to hemicelluloses in the cell wall. Vanillin is a phenolic aldehyde most commonly associated with the vanilla notes in wines that have been aged in oak. Trace amounts of vanillin are found naturally in grapes, but they are most prominent in the lignin structure of oak barrels.

1.1.2. Flavonoid compounds

Flavonoids are distributed among several classes: flavones, flavonols, flavanones, flavanols and anthocyanins (Figure 2).

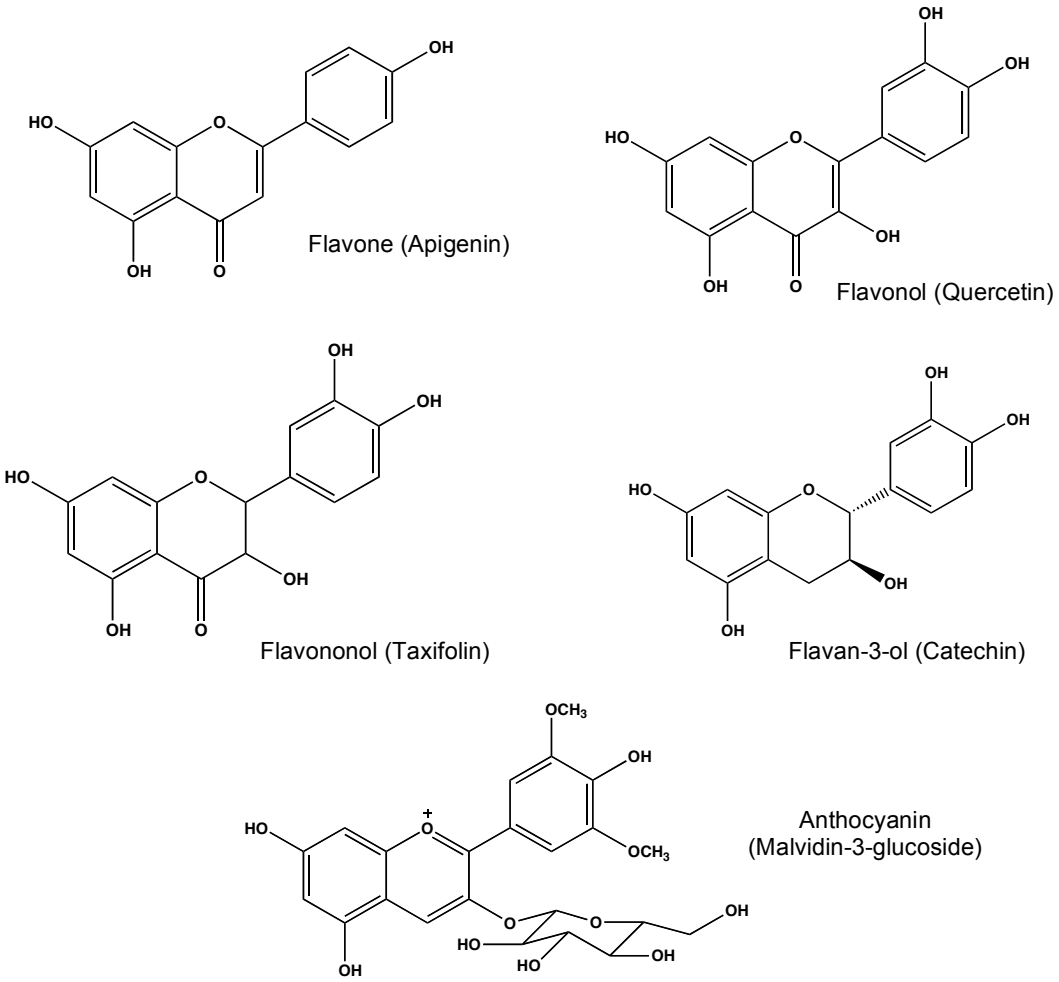


Figure 2. Flavonoid polyphenol structure examples

The flavanoid skeleton, the standard letters to identify the rings and the numbering are shown in figure 3.

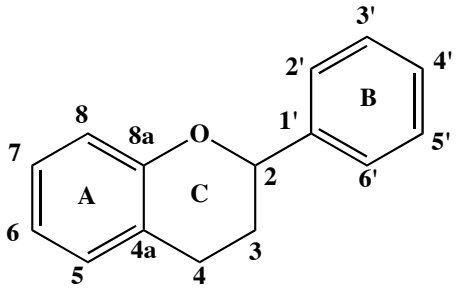


Figure 3. Flavanoid skeleton

1.1.2.1. Proanthocyanidins

Proanthocyanidin (PA) or condensed tannins are known for their astringent taste. They have the ability to complex and to form precipitates with macromolecules such as proteins and polysaccharides, with alkaloids and with heavy metals (Tarascou, Barathieu, André, Pianet, Dufourc, & Fouquet, 2006; Yoneda, Kawamoto, & Nakatsubo, 1997). The word “proanthocyanidin” refers to their ability to form red pigments in concentrated acids upon heating.

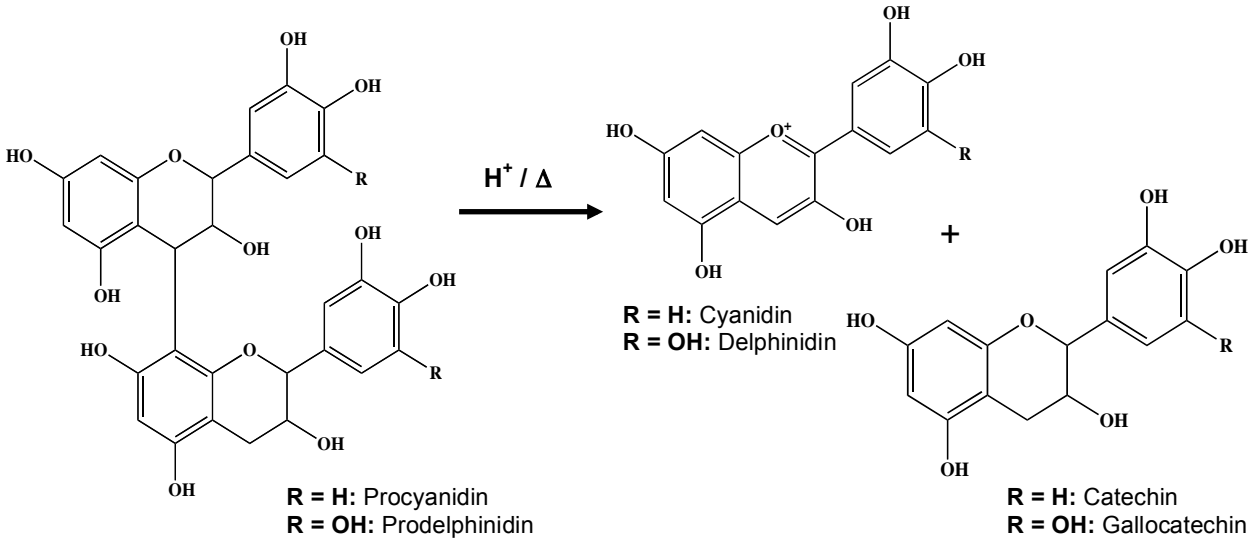


Figure 4. Bate-Smith reaction

According to their structure, tannins can be broadly divided into two classes of macromolecules, termed hydrolysable tannins and condensed tannins (Figure 5).

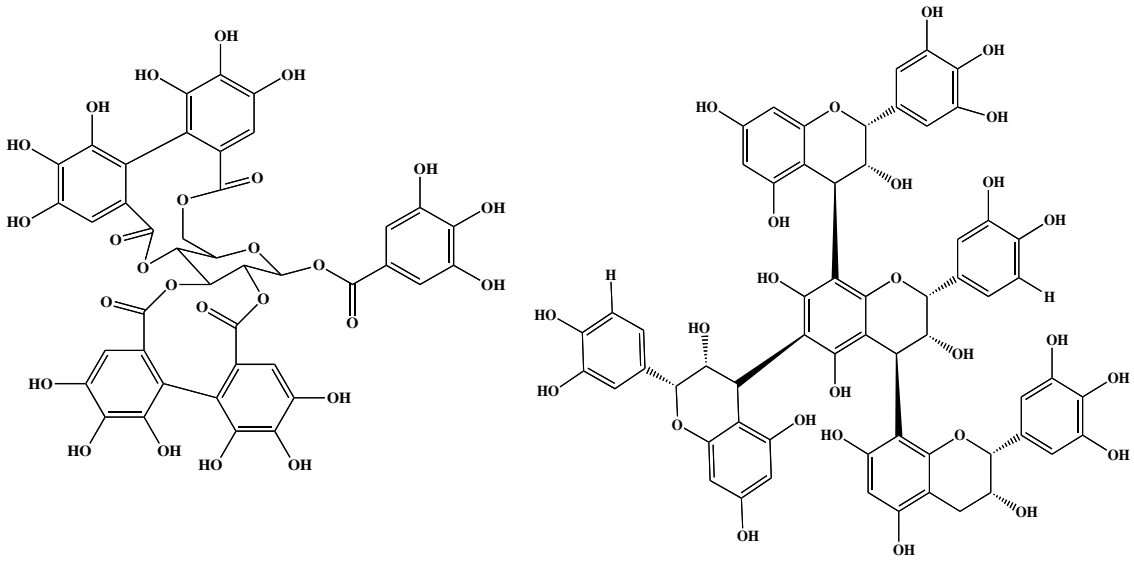
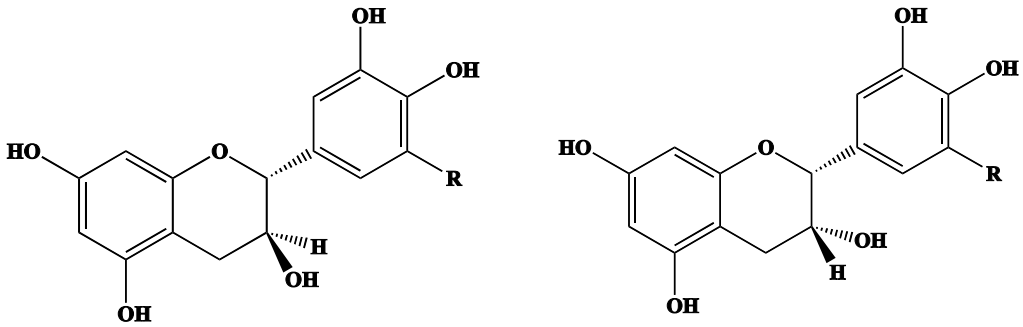


Figure 5. Examples of hydrolysable tannins (left) and condensed tannins (polymeric flavan-3-ols, right).

Hydrolysable tannins comprise numerous polygalloyl esters of glucose (so-called gallotannins), and esters of ellagic acid (ellagitannins and complex tannins). Hydrolysable gallotannins present in wine have been extracted from oak, or were added as oenotannins during winemaking (traditionally to remove undesirable proteins).

As opposed to hydrolysable tannins (such as gallo- and ellagitannins), all grape-derived tannins having enological importance are condensed tannins. Condensed tannins are large macromolecules formed by polymerisation of flavan-3-ol subunits (Figure 5). The subunit composition varies among tannins from grape skins, seeds and stems (Prieur, Rigaud, Cheynier, & Moutounet, 1994; Souquet, Cheynier, Brossaud, & Moutounet, 1996; Souquet, Labarbe, Le Guernevé, Cheynier, & Moutounet, 2000).

The most studied condensed tannins are based on the flavan-3-ols (+)-catechin (C) and (-)-epicatechin (EC). The addition of a third hydroxyl group to the B ring yields gallocatechin (GC) and epigallocatechin (EGC). Much less common are flavan-3-ols with only one hydroxyl group on the ring B (afzelechin and epiafzelechin).



R = H: Catechin
R = OH: Gallocatechin

R = H: Epicatechin
R = OH: Epigallocatechin

Figure 6. Chemical structures of (gallo)catechin and epi(gallo)catechin

These monomers can also be present in the esterified form at the ring-C O-3 position with gallic acid (galloylated) or glycosylated.

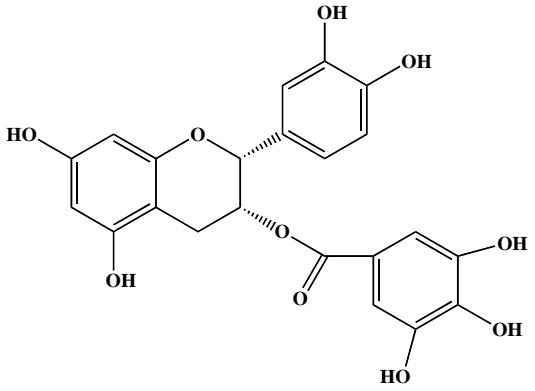
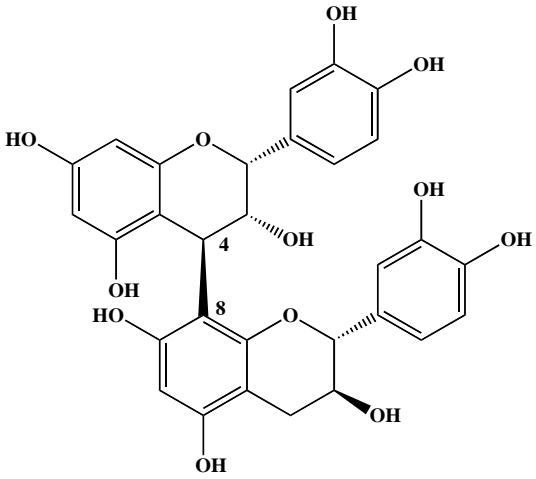


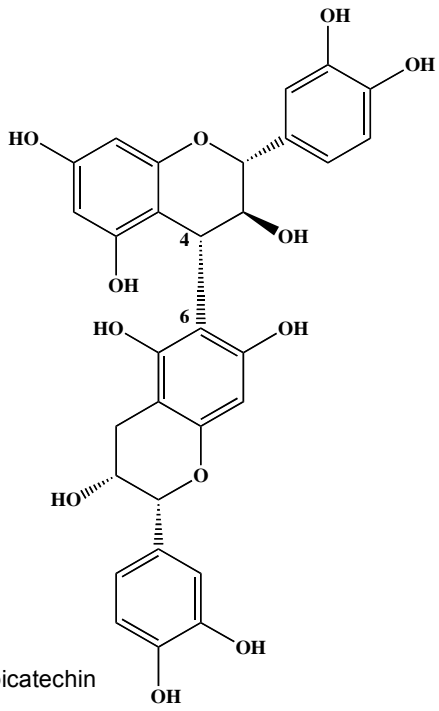
Figure 7. Chemical structure of epicatechin-3-O-gallate

Freudenberg and Weinges designated all the colourless material isolated from plants and which form anthocyanidins when heated with acid as proanthocyanidins (PAs) (Freudenberg, Böhme, & Purrmann, 1922; Freudenberg & Weinges, 1960). Later, Weinges reserved the term leucoanthocyanidin for the monomeric PAs such as flavan-3,4-diols and the name condensed PCs for the various flavan-3-ol dimers and higher oligomers (Weinges, Bhr, Ebert, Goritz, & Mark, 1969).

PAs, especially those that yield cyanidin and delphinidin upon acid treatment, were the condensed tannins characterized in first place. They are linked via a carbon-carbon bond between C4 of the extender unit and C8 (C4-C8) of the terminal unit or by C4 of the extender unit and C6 of the terminal unit (C4-C6).



PCB1: epicatechin-(4β→8)-catechin



PCB8: catechin-(4α→6)-epicatechin

Figure 8. Chemical structures of B type dimeric procyanidins

PAs exist as oligomers, containing two to five or six units, and polymers. Polymerization leads to C4-C8 polymers and less common C4-C6 and both C4-C6 and C4-C8 linkages.

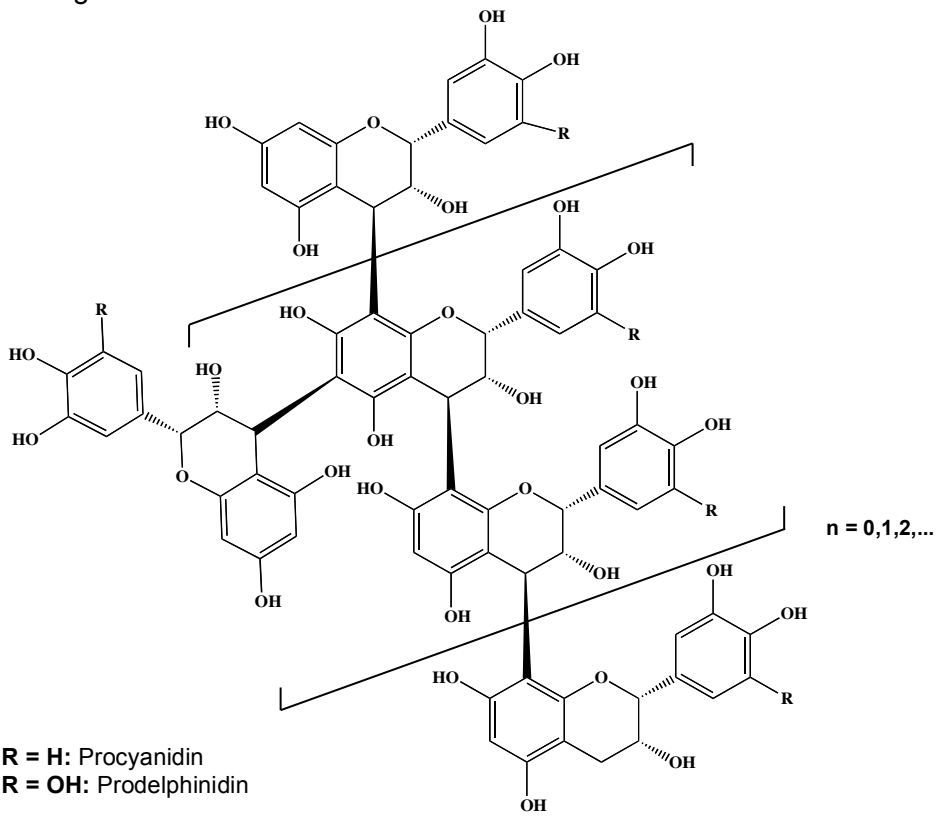


Figure 9. Chemical structures of polymeric proanthocyanidins

The PA initial studies were based on extraction and identification from fresh plant tissues. Weinges and his group were the first to identify B-type dimeric procyanidin (PC) PCB1, PCB2, PCB3 and PCB4 as their peracetates (Weinges & Perner, 1967). Later Haslam and his colleagues were able to identify their free phenolic forms (Thompson, Jacques, Haslam, & Tanner, 1972). In the late 1980's, a group led by Porter created the nomenclature still used nowadays:

PCB1: epicatechin-(4β→8)-catechin

PCB2: epicatechin-(4β→8)-epicatechin

PCB3: catechin-(4α→8)-catechin

PCB4: catechin-(4α→8)-epicatechin

PCB5: epicatechin-(4β→6)-epicatechin

PCB6: catechin-(4α→6)-catechin

PCB7: epicatechin-(4β→6)-catechin

PCB8: catechin-(4α→6)-epicatechin

PCC1: epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin

PCC2: catechin-(4α→8)-catechin-(4α→8)-catechin

Prodelphinidins (PDs) were first reported in 1929 (Romero & Bakker, 1999) and later in 1957 by Roux (Roux & Maihs, 1958) when reporting condensed tannins present in black wattle or commercial "mimosa" extracts from the bark of *Acacia mollissima*. However, only in 1978 was possible to isolate and characterize two naturally occurring dimeric PDs with stereochemistry based on GC (Foo & Porter, 1978).

PAs can also adopt a called A-type conformation when, besides a B-type interflavanolic linkage, it has a C-O linkage between C2 of the upper unit and the hydroxyl group of C7's lower unit (Figure 10).

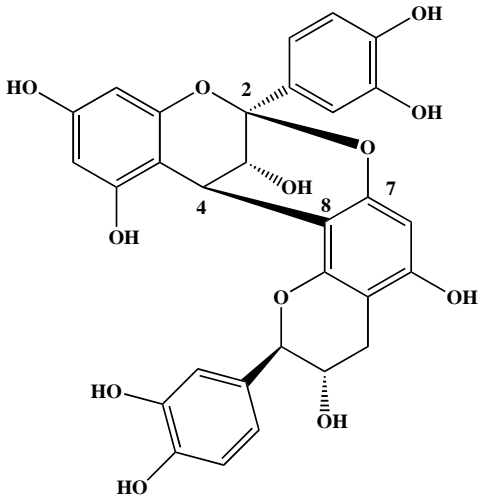


Figure 10. Chemical structure of A-type dimeric procyanidin

1.1.2.2. Anthocyanins

Anthocyanins are flavonoid compounds and are responsible for colours ranging from pale pink to red to purple and deep blue. They are most prevalent in the epidermal and hypodermal layers of the fruit skin, but may also occur throughout the fruit (many berries), in tissues surrounding seeds or pip (pomegranate and peach, respectively), or may be confined to the flesh (blood orange) (Gross, 1987).

Structurally they are flavylium cation derivatives with different methylation degrees in the B ring and different number of hydroxyl groups (Figure 11). The principal naturally occurring anthocyanidins are listed in table 1.

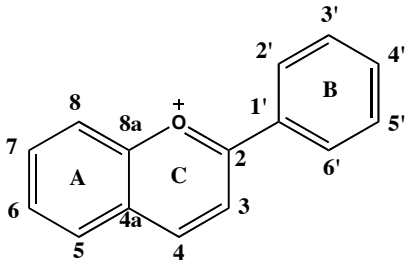


Figure 11. Chemical structure of the flavylium cation

Table 1. Principal naturally occurring anthocyanidins

Anthocyanidin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OMe	H
Petunidin	OMe	OH
Malvidin	OMe	OMe

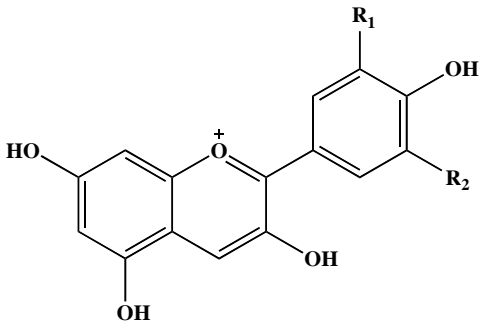


Figure 12. Anthocyanidin basic structure

Anthocyanins are anthocyanidins with at least one sugar group. The most common are 3-glucosides and 3,5-diglucosides of anthocyanidins (Andersen & Jordheim, 2001). Glycosyl groups (i.e. glucose, galactose, xylose, glucuronic acid, and arabinose) linked to anthocyanidins are frequently further glycosylated and/or acylated (Figure 13).

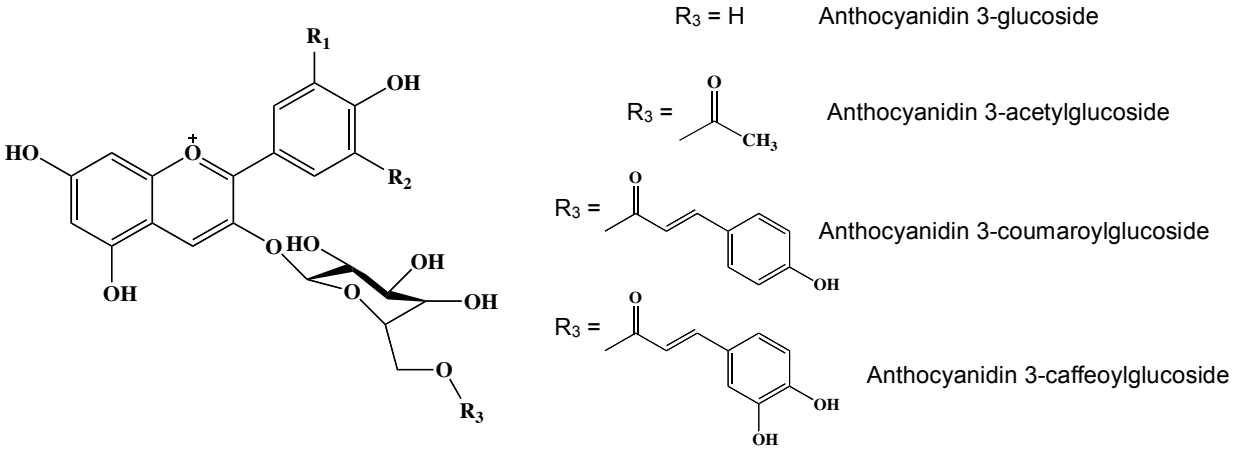


Figure 13. Chemical structure of anthocyanidins 3-monoglucoside

Glycosylation is an important modification for increasing the hydrophilicity and stability of hydrophobic flavonoids. It is also essential for color stability because the aromatic acylation that plays a key role in color stability is generally linked to glycosyl groups of anthocyanins.

Anthocyanins co-exist in aqueous solution in equilibrium between five species depending on the solution's pH: flavylium cation, carbinol base, chalcone, quinonoidal base and anionic quinonoidal base (Brouillard, 1982; Brouillard & Bernard Delaporte, 1977; Brouillard & Dubois, 1977; Brouillard & Lang, 1990; Mistry, Cai, Lilley, & Haslam, 1991) (Brouillard and Delaporte 1977; Brouillard and Dubois 1977; Brouillard and Lang 1990; Brouillard 1982; Mistry et al. 1991) (Figure 14).

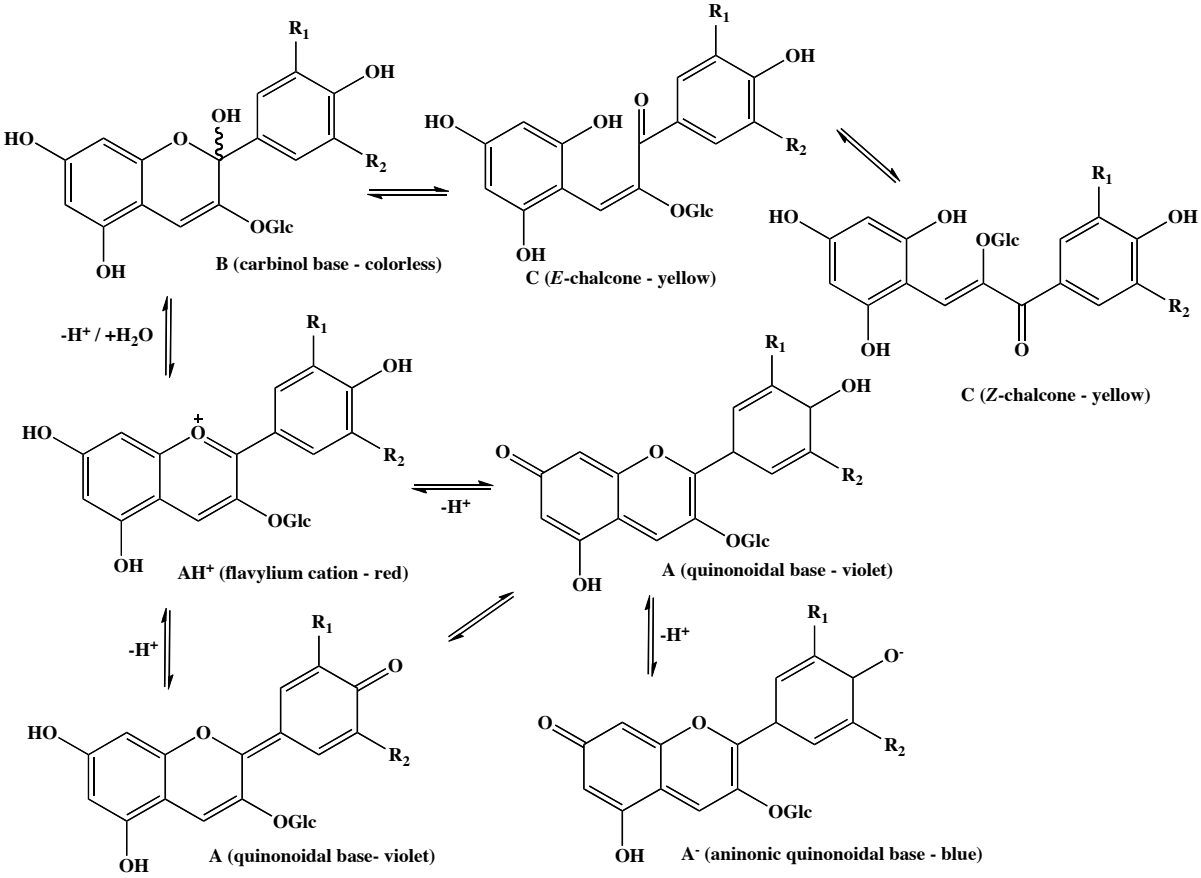


Figure 14. Structural transformations of anthocyanins in aqueous solutions depending on pH (Brouillard & Lang, 1990)

In solution at pH above 3.5, these natural compounds present little color expression and, for most plants, the optimum pH range is from 5.5 to 7.0. So how can anthocyanins be responsible for flower and fruit colour? The truth is that, in natural media at physiological pH they may display more color intensity as a result of co-pigmentation phenomena (intra- and intermolecular copigmentation) (Davies & Mazza, 1993; Mazza & Brouillard, 1990). Intramolecular copigmentation is characteristic of acylated anthocyanin structures and results from the stacking of the hydrophobic acyl moieties and the flavylium nucleus, thereby reducing anthocyanin hydrolysis (Dangles, Saito, & Brouillard, 1993). Intermolecular copigmentation involves colourless compounds such as metal ions, flavonols, etc… Concerning metals, some di- and trivalent cations may cause bathochromic shifts of the

maximum absorption wavelengths causing a “blue-ing” effect of the color through the formation of weak complexes with anthocyanins. This ability of anthocyanins to interact with colourless copigments is extremely relevant to the color stability in solution as it has been considered to be the first step in the formation of new anthocyanin-derived pigments (Brouillard & Dangles, 1994).

Concerning the natural sources of these pigments, grapes are amongst the best sources of anthocyanins. Table 2 summarizes the amount of anthocyanins in some foodstuffs.

Table 2. Average amount of anthocyanins in some foodstuffs (Clifford, 2000)

Anthocyanin source	Amount ($mg.L^{-1}$ or $mg.kg^{-1}$)
Blackberry	1150
Blueberry	825 - 4200
Boisenberry	1609
Cherry	20 – 4500
Chokeberry	5060 – 10000
Cranberry	600 - 2000
Cowberry	1000
Black currant	1300 - 4000
Elderberry	2000 – 10000
Red grapes	300 – 7500
Blood orange	2000
Plum	20 – 250
Sloe	1600
Strawberry	150 – 350
Black raspberry	1700 – 4277
Eggplant	7500
Onion	Up to 250
Rhubarb	Up to 2000
Red cabbage	250
Red wine	240 – 350
Port wine	140 – 1100

Red wine is probably the foodstuff that presents the highest structural diversity of polyphenolic pigments. Structural changes of anthocyanins in red wines have been extensively studied over the last century aiming to comprehend red wine color

evolution. The levels of anthocyanins found in young red wines and their reaction with many other chemical compounds present in wines favours the occurrence of newly formed anthocyanin-derived pigments. Indeed, anthocyanins are likely to react with other colourless polyphenols and non-polyphenolic compounds present in the wine such as ketonic compounds, metals, proteins, carbohydrates, etc…

These pigments are very reactive mainly due to the electronic deficiency of their flavylium nuclei but also by having some positions that are likely to react with different nucleophilic and electrophilic compounds (Figure 15). Anthocyanins can undergo a nucleophilic attack at the positively charge carbons C2 and C4 of the pyranic ring C (hydration in C2 gives rise to the colourless hemiacetal form). Despite its positive charge, anthocyanins were also shown to react with electrophilic compounds through its hydroxyl groups as well as carbons C6 and C8 of the phloroglucinol ring A, probably involving the uncharged hemiacetal form. The presence of a free C5-OH group is crucial for the reactivity of anthocyanins with other compounds in red wine.

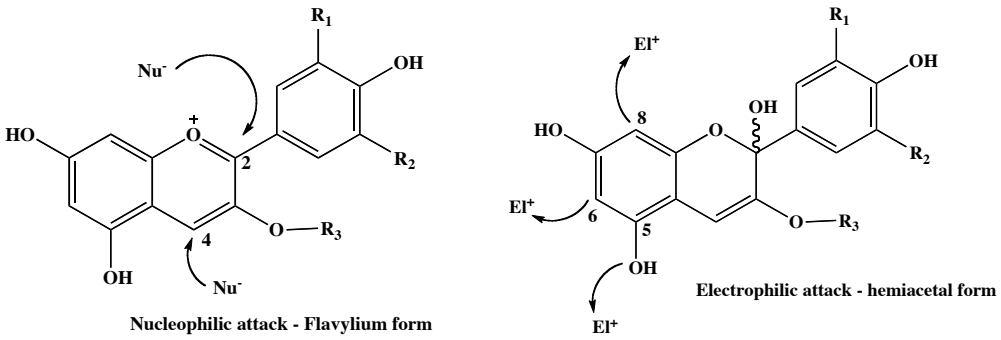


Figure 15. Schematic representation of the main reactive position of anthocyanin structures (de Freitas & Mateus, 2006)

Over the last years several anthocyanin-derived pigments occurring in red wines have been reported in the literature, especially pyranoanthocyanins (Figure 16). The major structural feature of these newly-formed pigments is the presence of an additional ring in the chromophore, which allows a higher distribution of the positive charge (Cameira-dos-Santos, Brillouet, Cheynier, & Moutounet, 1996). Overall, the major new families of anthocyanin-derived pigments detected in red wines were described to result from a:

a) Reaction between anthocyanins and small compounds (e.g. pyruvic and phenolic acids, acetaldehyde, p-vinylphenol) giving rise to new pyranoanthocyanin pigments:

a_1) Anthocyanin-pyruvic acid adducts (carboxypyranoanthocyanins) (Fulcrand, Benabdeljalil, Rigaud, Cheynier, & Moutounet, 1998);

a_2) Pyranoanthocyanins (Vitisin B) (Bakker & Timberlake, 1985);

a_3) Pyranoanthocyanin-phenol pigments (Fulcrand, dos Santos, Sarni-Manchado, Cheynier, & Favre-Bonvin, 1996; Schwarz, Wabnitz, & Winterhalter, 2003);

b) Condensation between anthocyanins and flavanols mediated by aldehydes (e.g. acetaldehyde):

b_1) Anthocyanin-ethyl-flavanol pigments (Timberlake & Bridle, 1976);

b_2) Pyranoanthocyanin-flavanol pigments (Francia-Aricha, Guerra, Rivas-Gonzalo, & Santos-Buelga, 1997; Mateus, Carvalho, Carvalho, Melo, González-Paramás, Santos-Buelga, et al., 2003; Mateus, Pascual-Teresa, Rivas-Gonzalo, Santos-Buelga, & de Freitas, 2002; Vivar-Quintana, Santos-Buelga, Francia-Aricha, & Rivas-Gonzalo, 1999);

c) Direct condensation between anthocyanins and flavanols (Remy, Fulcrand, Labarbe, Cheynier, & Moutounet, 2000).

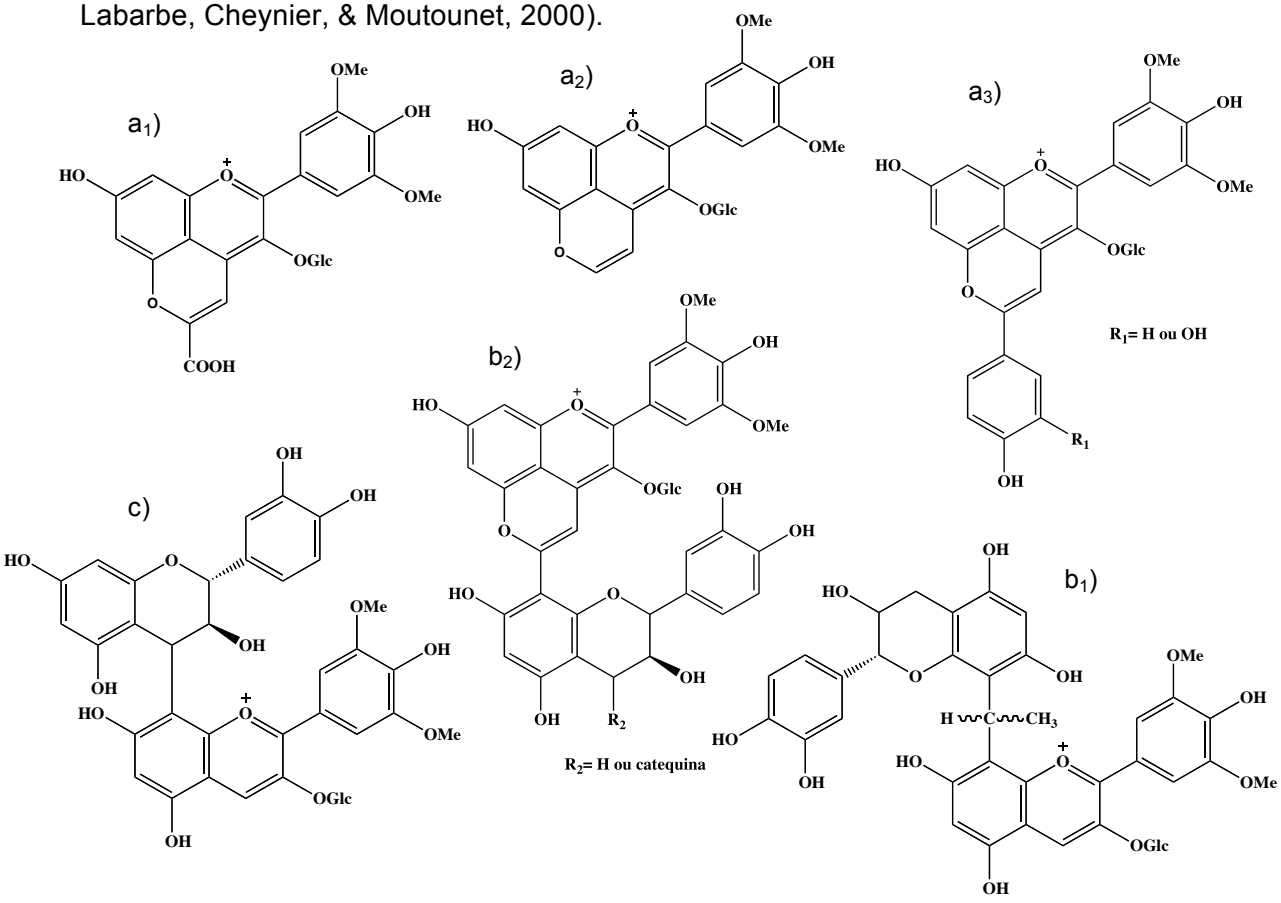


Figure 16. Structure of: a_1) malvidin-3-glucoside-pyruvic acid adducts; a_2) vitisin B; a_3) pyranomalvidin-3-glucoside-phenol pigments; b_1) malvidin-3-glucoside-ethyl-flavanol; b_2) Pyranomalvidin-3-glucoside-flavanol; c) Catechin-Mv3glc ($F\text{-}A^+$) dimer pigments (Mv3glc = malvidin-3-O-glucoside)

Later, other pigment classes were synthetized and identified in wines. Portisins (as these pigments were first and only reported in Port wines) may be synthetized through reacting anthocyanin-pyruvic acid adducts and flavanols in the presence of acetaldehyde (Figure 17) (Mateus, Oliveira, Haettich-Motta, & de Freitas, 2004).

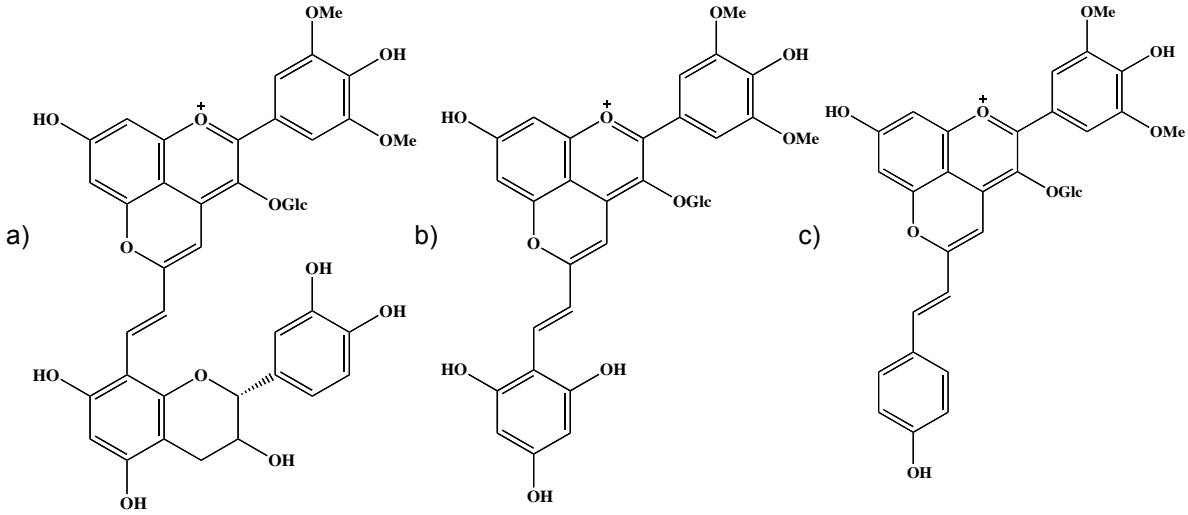


Figure 17. Structure of: a) vinylpyrano-mv3glc-p-phenol, b) vinylpyrano-mv3glc-phloroglucinol, and c) vinylpyrano-mv3glc-catechin portisins

And pyranoanthocyanin dimers, a new family of turquoise blue anthocyanin-derived pigments also found in Port Wine. These compounds can be synthetized through a reaction between a carboxypyranoanthocyanin and a methylpyranoanthocyanin (Figure 18) (Oliveira, Azevedo, Silva, Teixeira, Cruz, Mateus, et al., 2010).

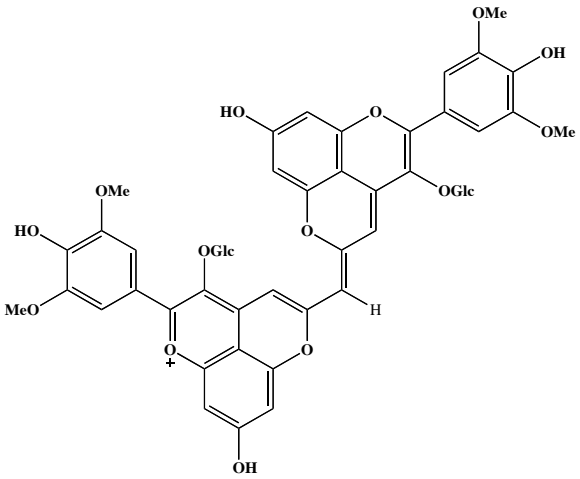


Figure 18. Structure of a pyranoanthocyanin dimer

1.2. The interest of polyphenols

The initial spark of interest into these compounds came from the discovery that they were involved in the defence mechanisms of many plants. The astringent nature of these materials caused an unpalatable taste in the mouths of predators thereby insuring the plants survival. The reason behind this was identified as being due to the complexation of plant proanthocyanidins with salivary proteins. Allied to this discovery was the realization that these compounds also demonstrated anti-microbial and anti-viral properties in plants. Because these compounds are found in the human diet this was an important discovery.

The basis of many oriental herbal remedies has been shown to be due to the presence of proanthocyanidins. For example these compounds have demonstrated a positive effect against *streptococcus mutans,* the primary causative agent of plaque and dental cavities, by limiting the adhesion of the microbe on the smooth dental surface (Kakiuchi, Hattori, Nishizawa, Yamagushi, Okuda, & Namba, 1986). As a result many commercially available mouthwashes contain proanthocyanidins.

The anti-fungal properties of these compounds have been known since the 1910's when Knudson demonstrated that very few fungi could survive in the presence of 2% (V/V) tannin solutions (Knudson, 1913). Studies into the anti-viral capacity of these compounds have also been undertaken. Cadman reasoned that polyphenols complexed with the virus thus making it non-infective (Cadman, 1960).

1.2.1. Industry applications

The Food and Beverage Industry have a major interest in the area of PAs. Since these compounds are found in numerous plant and fruit types, they form part of the human food chain and are thus important in this Industry. Polyphenols contribute to the taste and flavour of foods and their presence or absence must be controlled.

Colour appearance of food products is one of the major concerns of the food industry. Colour is also the first attribute to be perceived in foods and beverages and is usually positively correlated with standards of quality by the consumer. Within the Food Industry there has been a trend away from synthetic colorants due to possible toxic effects associated with these chemicals. Anthocyanins are compounds that are known to possess colouring capability and also may be of pharmaceutical importance. The stability of these compounds is based upon several factors: pH, temperature, partial oxygen pressure, types of co-product present, light radiation and

glycosylation, as well as the nature of the heterocyclic rings. The development of commercial processes to isolate and purify these compounds is of great importance.

1.2.2. The prevention of diseases

Polyphenols and other food phenolics are the subject of increasing scientific interest because of their possible beneficial effects on human health. Bioavailability is the proportion of the nutrient that is digested, absorbed and metabolized through normal pathways. Bioavailability of each and every polyphenol differs and there is no relation between the quantity of polyphenols in food and their bioavailability in human body.
As antioxidants, polyphenols may protect cell constituents against oxidative damage and, therefore, limit the risk of various degenerative diseases associated to oxidative stress. Antioxidant capacity of the plasma is related to dietary intake of antioxidants; it has been found that the intake of antioxidant rich diet is effective in reducing the deleterious effects of aging and behaviour. Several researches suggest that the combination of antioxidant/anti-inflammatory polyphenolic compounds found in fruits and vegetables may show efficacy as anti-aging compounds (Cao, Booth, Sadowski, & Prior, 1998). Oxidative damage to cell components, DNA, proteins, and lipids accumulates with age and contributes to the degeneration of the somatic cells and to the pathogenesis of these diseases. Antioxidants present in food can help limit this damage by acting directly on reactive oxygen species or by stimulating endogenous defence systems. The phenolic groups in polyphenols can accept an electron to form relatively stable phenoxyl radicals, thereby disrupting chain oxidation reactions in cellular components.

Many studies have demonstrated that consumption of polyphenols limits the incidence of coronary heart diseases (Dubick, Michael, Omaye, & Stanley, 2001; Nardini, Natella, & Scaccini, 2007; Renaud & de Lorgeril, 1992). Atherosclerosis is a chronic inflammatory disease that develops in lesion-prone regions of medium-sized arteries. Atherosclerotic lesions may be present and clinically silent for decades before becoming active and producing pathological conditions such as acute myocardial infarction, unstable angina or sudden cardiac death (Vita, 2005). Polyphenols are potent inhibitors of LDL oxidation and this type of oxidation is considered to be a key mechanism in development of atherosclerosis (Aviram, Dornfeld, Rosenblat, Volkova, Kaplan, Coleman, et al., 2000). Other mechanisms by which polyphenols may be protective against cardiovascular diseases are antioxidant, anti-platelet, anti-inflammatory effects as well as increasing HDL, and

improving endothelial function (García-Lafuente, Guillamón, Villares, Rostagno, & Martínez, 2009).

Polyphenols, when given to rats or mice before and/or after the administration of a carcinogenic agent or the implantation of a human cancer cell line, are most often protective and induce a reduction of the number of tumours or of their growth (Yang, Landau, Huang, & Newmark, 2001). These effects have been observed at various sites, including mouth, stomach, duodenum, colon, liver, lung, mammary, or skin. Many polyphenols, such as quercetin, catechins, isoflavones, lignans, flavanones, ellagic acid, red wine polyphenols, resveratrol, or curcumin, were tested; all of them showed protective effects in some models.

Neurodegenerative diseases represent an increasing concern to our aging societies. About 15% of the population over 65 years old are afflicted by Alzheimer's disease and 1% by Parkinson's disease, not including other type of dementia resulting from ischemic injury (Cantuti-Castelvetri, Shukitt-Hale, & Joseph, 2000). Such diseases are dependent of oxidative stress, which particularly affects brain tissues (Halliwell, 2001), and antioxidants may, therefore, contribute to their prevention (Cantuti-Castelvetri, Shukitt-Hale, & Joseph, 2000). Feeding aging rats a diet supplemented with aqueous extracts of spinach, strawberry, or blueberry rich in polyphenols improved their cognitive functions and neuronal signal transduction (James A. Joseph, Shukitt-Hale, Denisova, Bielinski, Martin, McEwen, et al., 1999; J. A. Joseph, Shukitt-Hale, Denisova, Prior, Cao, Martin, et al., 1998). Blueberries rich in anthocyanins were particularly effective. These effects were not explained by a sparing of vitamins E and C in the brain (Martin, Prior, Shukitt-Hale, Cao, & Joseph, 2000); a direct implication of polyphenols as antioxidants is, therefore, suspected.

Numerous studies report the antidiabetic effects of polyphenols. Polyphenols may affect glycemia through different mechanisms, including the inhibition of glucose absorption in the gut or of its uptake by peripheral tissues. Caffeic acid and isoferulic acid, when administered intravenously to rats, reduce the fasting glycemia and attenuate the increase of plasma glucose in an intravenous glucose tolerance test (Hsu, Chen, & Cheng, 2000). More interestingly, some hypoglycemic effects were also observed with polyphenols administered orally, shortly before consumption of the glucose source. An ill-defined leucodelphinidin (probably a mixture of PDs) reduced fasting glycemia in rats and lowered the plasma glucose peak in a glucose tolerance test (Geetha, Mathew, & Augusti, 1994). Similar effects were observed with

4-hydroxybenzoic acid (Peungvicha, Temsiririrkkul, Prasain, Tezuka, Kadota, Thirawarapan, et al., 1998).

All scientific data on the effects of polyphenols on diseases have increased the general consumer awareness about polyphenols and led to the marketing of new polyphenol dietary supplements and polyphenol-rich food products. Although no claims have been attached to these products, different polyphenols have been proposed to limit oxidative stress and aging, and isoflavones have been recommended to limit hot flushes and to improve bone health in post-menopausal women. The consumption of such products leads to increase the intake of particular polyphenols beyond common levels of exposure associated to the diet. It is often said that polyphenols consumed in high amounts could have pro-oxidant effects, so caution is recommended. The first epidemiological data on polyphenols intake were published around 10 years ago and the progress has been slow due to the lack of food composition tables an lack of validated biomarkers. A table for flavonoids based on available literature was published by the United States Department of Agriculture (Bhagwat, Haytowitz, & Holden, 2013) but more research is still needed to cover missing polyphenols and food sources. Only then the real evaluation of the consumption of polyphenols in populations can be made.

1.3. Prodelphinidin identification in natural sources

Polyphenols show highly diverse structures and over 500 different molecules are known in foods (Neveu, Perez-Jiménez, Vos, Crespy, du Chaffaut, Mennen, et al., 2010; Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010). This diversity must be taken into account when considering bioavailability, biological properties and health effects, as the latter largely depend on their specific chemical structures (Loke, Proudfoot, Stewart, McKinley, Needs, Kroon, et al., 2008; Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). The diversity of polyphenols also makes it difficult to estimate the total polyphenol content of foods, an important fact for food and nutrition researchers and the food industry.

In most cases, natural sources contain complex mixtures of polyphenols. Numerous factors affect the polyphenol content of plants including the degree of ripeness at the time of harvest, environmental factors, processing and storage. Other environmental factors like soil type, sun exposure, rainfall, etc… also can affect the polyphenol content. The next section will focus on PD (and other PAs) content of cereals, vegetables and fruits. Table 3 summarizes all published data on their PD content.

1.3.1. Barley and Hop

Outtrup *et al.* were among the first workers to characterize the individual PAs present in barley (*Hordeum vulgare*) (Outtrup, 1981a, 1981b; Outtrup & Schaumburg, 1981). They identified three types of PCs and one PD. Reverse phase HPLC was employed to isolate and purify these compounds and their characterization was carried out by 270MHz ^{1}H-NMR of their acetylated derivatives, because the compounds in their free form were observed to be sensitive to oxidation and hence were considered to be unstable.

Barley grains contain a range of flavan-3-ols from monomeric, dimeric, and trimeric PAs to higher molecular weight condensed tannins. These include (+)-C, (−)-EC, dimeric PDB3 and PCB3, as well as trimeric PCC2 and three trimeric PDs (Goupy, Hugues, Boivin, & Amiot, 1999; McMurrough, Loughrey, & Hennigan, 1983; McMurrough, Madigan, & Smyth, 1996).

Medium values show that the concentration of C is 1.23 mg/100 g FW (fresh weight), for PCB3 it is 10.90 mg/100 g FW and for PDB3 it is 23.07 mg/100 g FW (Madigan, McMurrough, & Smyth, 1994). In 2008 Dvorakova *et al.* proceeded to the characterization and quantification of flavan-3-ols and concluded that C is present at an average concentration of 3.01 mg/100 g FW, PCB3 at 3.35 mg/100 g FW, PCB2 at 0.84 mg/100 g FW, PCC2 at 1.43 mg/100 g FW and PDB3 at 12.97 mg/100 g FW (Dvorakova, Moreira, Dostalek, Skulilova, Guido, & Barros, 2008). This study also identified other PD trimers, namely: two trimers GC-C-GC, five trimers GC-C-C and C-glc (catechin-7-O-glucoside (Wolfgang Friedrich & Galensa, 2002)). Another group, in 1999, reported an average concentration of PDB3 of 4.85 mg/100 g and 0.84 for trimers (expressed as equivalents of C) (Goupy, Hugues, Boivin, & Amiot, 1999). The concentration of each compound, however, depends greatly on the barley variety. This 2008 study, for instance, shows concentration values for PDB3 from 8.7 to 19.7 mg/100 g.

Hop (*Humulus lupulus*) is used exclusively to give beer its characteristic aroma, bitterness, foam and light stability. The estimated amount of total hop PAs ranges from 0.5 to 5% on a dry weight basis, depending on the variety, geographic origin, freshness, and harvesting procedure. Previous studies have also shown that up to 30% of the PAs present in beer is derived from hops (Callemien, Jerkovic, Rozenberg, & Collin, 2004; Stevens, Miranda, Wolthers, Schimerlik, Deinzer, & Buhler, 2002). The chemical structures of hop PAs consist of C, EC, GC, PCB1,

PCB2, PCB3, PCB4, PDB3, GC-(4α→6)-C, C-(4α→8)-GC, C-(4α→6)-GC, Afzelechin-(4α→8)-C, PCC2, EC-(4β→8)-C-(4α→8)-C, EC-(4β→8)-EC-(4β→8)-C, C-(4α→8)-GC-(4α→8)-C, and GC-(4α→8)-GC-(4α→8)-C (Li & Deinzer, 2006).

1.3.2. Broad beans, Peas and Lentils

Vicia faba L. (broad, fava or faba bean) today is extensively cultivated for their edible seeds, being used green or dried, fresh or canned (Jensen, Peoples, & Hauggaard-Nielsen, 2010). Seven isomers of (E)GC–(E)GC, six isomers of (E)GC–(E)C, five PC dimers (dimers B1, B2, B3, B4, and B6) and five isomers of (E)C–(E)GC were identified by UHPLC-ESI-QTOF-MS. In a similar way, six trimers formed by two (E)GCs and a (E)C or a (E)GC and two (E)Cs and three PC dimers (including C1 and C2) were also characterized (Abu-Reidah, del Mar Contreras, Arráez-Román, Fernández-Gutiérrez, & Segura-Carretero, 2014).
According to De Pascual-Teresa *et al.*, 2000 (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000), Spanish broad bean contains 23.50 mg of PDB3, 8.17 mg of PCB3, 11.26 mg of PCB1, 18.47 mg of PCB4, 12.08 mg of PCB2 and 0.13 mg of PCC1. In terms of monomers, it was quantified 9.68 mg of GC, 17.38 mg of EGC, 16.23 mg of C and 37.55 mg of EC. All these values are expressed as mg/100g of FW. PCB6, PD GC-GC and PC C-GC were also detected but not quantified due to the lack of standards. In total, the average flavanol concentration in broad beans was 154.5 mg total flavanols/100 g FW.

Lentils (*Lens culinaris*) seem to be much less rich in flavanols. The average concentrations include 0.28 mg/100 g FW of C, 3.15 mg/100 g FW of C-3-O-glc, 0.14 mg/100 g FW GC, 0.10 mg/100 g FW of EC, 0.34 mg/100 g FW of PCB2, 0.71 mg/100 g FW of PCB3 and 0.45 mg/100 g FW of PDB3 (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000; Dueñas, Hernández, & Estrella, 2007).

Another study describes the total PA concentration in two different species of mature seeds of pea (*Pisum sativum*) being 367 and 294 mg/100 g FW, lentils 269 to 378 mg/100 g FW and faba bean 654 mg/100 g FW fresh weight. These data also shows, once more, that variation in seed PA content occurs within and among species (Jin, Ozga, Lopes-Lutz, Schieber, & Reinecke, 2012).

1.3.3. Redcurrants, Blackcurrants, Raspberries and Sea buckthorn

Black currant (*Ribes nigrum*) and red currant (*Ribes rubrum*) are widely cultivated in Europe and North America for berry production. Berries are traditionally used for producing juices, jams, jellies and syrups and for pastry and culinary purposes. Red currants contain, in average, 1.27 mg/ 100 g FW of C, 1.28 mg/ 100 g FW of GC, 0.08 mg/ 100 g FW of EC, 0.15 mg/ 100 g FW of EGC, 0.20 mg/ 100 g FW of PCB3 and 1.70 mg/ 100 g FW of PDB3 (Arts, van de Putte, & Hollman, 2000; Sonia de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000).

In 2004, Wu, X. *et al.*, showed by mass spectrometric data that black currants contained both PCs and PDs. Total PAs in six cultivars of black currant ranged from 120.6 to 165.8 mg/100 g FW. Gooseberries and red currant had profiles of PAs similar to that for black currant but lower concentrations. They contained PCs and PDs, and the predominant PAs were in the polymeric form. Chokeberry contained only PCs. Unlike other berries, elderberry contained no detectable higher oligomers and polymers. In 2011, Mattila *et al.* studied the polyphenol and vitamin C contents in European commercial blackcurrant juice products and concluded that PAs in all samples were always mixtures of PCs and PDs; thus, they were constituted of both (E)C and (E)GC subunits. In most cases, (E)GC (i.e. PDs) dominated and made up over 60% of the structural flavan-3-ols (Mattila, Hellström, McDougall, Dobson, Pihlava, Tiirikka, et al., 2011). Later, in 2014, P. Liu *et al.* identified, by HPLC-ESI-MS, one PD dimer and four PD trimers in black currant buds and leaves (Liu, Kallio, & Yang, 2014). C and GC were also detected and quantified in white currants in 0.30 and 0.70 mg/ 100 g fresh weight respectively (Arts, van de Putte, & Hollman, 2000).

Raspberries (*Rubus idaeus*) are reported containing 0.01 mg/100 g FW of GC, 0.03 mg/100 g FW of PCB3, 0.05 mg/100 g FW of C, 1.11 mg/100 g FW of EGC, 2.79 mg/100 g FW of PCB2, 1.78 mg/100 g FW of EC, 0.18 mg/100 g FW of PCB7 and 0.30 mg/100 g FW of PCC1. No PDs were detected so far.

Sea buckthorn (*Hippophae rhamnoides*) exists nowadays in various regions of Asia, Europe, and North America. It has been used as a medicinal plant in Tibet since 900 AD (Lu, 1992). Sea buckthorn seeds were extracted, fractionated by Sephadex LH20 and analyzed by HPLC–ESI-MS after thiolysis with benzyl mercaptan. Nine dimeric PAs were identified in one of the fractions: two (E)GC-(E)GC, one (E)GC-(E)C, three (E)C-(E)GC, one (E)C-(E)C and PCB3 and PCB4. The structural composition and characteristic data obtained by thiolysis degradation of each of the PA fractions

showed that PD units predominated in the extended chains and were the major components of all tannin fractions. The %PD range was between 51.4 and 88.5 (Fan, Ding, & Gu, 2007).

1.3.4. Cranberries and Blueberries

Whole cranberries (*Vaccinium oxycoccos*) contain approximately 1.7 mg/100 g of total PA, while only 2.16 to 2.23 mg/L of total PCs are found in cranberry juices (Prior, Lazarus, Cao, Muccitelli, & Hammerstone, 2001). 63% of the total PAs in cranberries are polymeric PAs (Gu, Kelm, Hammerstone, Beecher, Cunningham, Vannozzi, et al., 2002). Foo and Porter (Foo & Porter, 1980) reported that the ratio of PCs to PDs is 78:22 in European cranberries. 20 years later, Foo et al. (Foo, Lu, Howell, & Vorsa, 2000) isolated and identified three PA trimers with A-type doubly linked interflavonoid linkages in ripe American cranberry fruits. Subsequently, Prior et al. (Prior, Lazarus, Cao, Muccitelli, & Hammerstone, 2001) reported that EC and its dimers and A-type trimers (A1, A2, C1, C2) are the predominant PAs in cranberries. These authors also detected trace amounts of B-type (single linked interflavonoid linkages) and A-type tetramers. In 2002, Kandil et al. (Kandil, Smith, Rogers, Pépin, Song, Pezzuto, et al., 2002) detected EC, C, GC and EGC as well as higher molecular weight PAs in American cranberries.
Blueberries (*Vaccinium myrtillus*) are around 5 times richer in flavanols, having 5.27 mg/100 g FW of total flavanols. This value includes 0.59 mg/100 g FW of GC, 0.08 mg/100 g FW of PCB3, 0.81 mg/100 g FW of C, 0.75 mg/100 g FW of PCB1, 0.37 mg/100 g FW of EGC, 0.37 mg/100 g FW of PCB4, 0.19 mg/100 g FW of PCB2, 0.30 mg/100 g FW of EC, 0.27 mg/100 g FW of PCB7 and 0.71 mg/100 g FW of PCC1. GC-GC, C-GC and PCB6 were also detected but not quantified (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000).

1.3.5. Strawberries and Strawberry tree fruit

The strawberry tree fruit (*Arbutus unedo*) is a red berry with 1–2 cm diameter, with a rough surface, maturing 12 months at the same time as the next flowering. It is widespread in the Mediterranean region and it is has a total flavanol concentration of 20.47 mg/100 g FW. This value includes 1.60 mg/100 g FW of GC, 7.48 mg/100 g FW of C and 1.11 mg/100 g FW of EC. In terms of dimers and trimers, it includes an average concentration of 1.99 mg/100 g FW of PDB3, 1.69 mg/100 g FW of PCB3,

1.72 mg/100 g FW of PCB1, 0.43 mg/100 g FW of PCB4, 0.22 mg/100 g FW of PC trimer EC-EC-C. PD GC-GC, PCB6 and PC C-GC were also detected (Sonia de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000).
As for the more known strawberries (*Fragaria ananassa*), no PD dimers were yet detected, but they have, in average, 0.05 mg/100 g FW of GC and 0.06 mg/100 g FW of EGC. In addition to these monomers they also have 6.36 mg/100 g FW of C, 0.0075 mg/100 g FW of EC and 0.28 mg/100 g FW of ECG. In terms of PC dimers and trimers, it was detected 0.62 mg/100 g FW of PCB1, 0.03 mg/100 g FW of PCB2, 1.10 mg/100 g FW of PCB3, 0.13 mg/100 g FW of PCB4 and 0.50 mg/100 g FW of EC-EC-C (Arts, van de Putte, & Hollman, 2000; de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000; Määttä-Riihinen, Kamal-Eldin, & Törrönen, 2004).

1.3.6. Bananas, Pomegranates and Quinces

Some researchers think that the banana (*Musa*) peel has compounds and nutrients important for food and for food industry and should be utilized. The banana peel is rich in dietary fiber, protein, essential amino acids, polyunsaturated fatty acids and potassium (Happi Emaga, Andrianaivo, Wathelet, Tchango, & Paquot, 2007). It also contains antioxidant compounds including polyphenols, catecholamines and carotenoids. In average, bananas have 1.34 mg/100 g FW of C, 0.11 mg/100 g FW of EC, 0.00214 mg/100 g FW of EGC and 0.10 mg/100 g FW of PDB3 (Arts, van de Putte, & Hollman, 2000; de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000; Harnly, Doherty, Beecher, Holden, Haytowitz, Bhagwat, et al., 2006).
Pomegranates (*Punica granatum*) contain high levels of flavonoids and polyphenols, potent antioxidants offering protection against many diseases. A glass of pomegranate juice has more antioxidants than red wine, green tea, blueberries, and cranberries (Seeram, Aviram, Zhang, Henning, Feng, Dreher, et al., 2008). PD dimers were detected in pomegranates, namely: PDB3 and PD GC-GC, but not quantified. The average concentration for compounds identified and quantified are:
0.17 mg/100 g FW for GC, 0.16 mg/100 g FW for PCB3, 0.40 mg/100 g FW for C, 0.13 mg/100 g FW for PCB1, 0.16 mg/100 g FW for EGC and 0.08 mg/100 g FW for EC. It were also detected PC C-GC and PCB6 (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000).

Because quince (*Cydonia oblonga*) is a hard, acid, and astringent, it is not edible unprocessed. Nevertheless, it is often used to prepare jam, jelly, liqueur, and marmalade, as well as applied in canning and for aromatic distillation (Wojdyło,

Oszmiański, & Bielicki, 2013). Its flavan-3-ol content includes 0.08 mg/100 g FW of PDB3, 0.10 mg/100 g FW of PCB3, 0.75 mg/100 g FW of C, 0.73 mg/100 g FW of PCB1, 0.28 mg/100 g FW of EC-EC-C, 0.24 mg/100 g FW of PCB4, 1.34 mg/100 g FW of PCB2, 0.67 mg/100 g FW of EC, 0.94 mg/100 g FW of PCC1 and 0.12 mg/100 g FW of PCB5; making total flavan-3-ol concentration in quinces 5.24 mg/100 g FW (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000).

1.3.7. Persimmons, Mangosteen and Cashew apple

The persimmon (*Diospyros kaki L.*) is a widely consumed fruit that is characterized by its high PA content (Piretti, 1991) with a more potent antioxidant than apple or grape seed PA (Ahn, Jeon, Lee, Hwang, Lim, & Park, 2002). Persimmon PAs consist of C, EGCG, and myricetin terminal units and EGC, (E)GCG, C, and (E)CG as extension units. Myricetin is slightly more common than the flavan-3-ol terminals. PDs are about 58% of the total crude tannins.
In 2010, Li *et al.* identified two A-linked dimers: (E)GCG-(C2-O-C7)-(E)GCG and (E)C-(C2-O-C7)-(E)C (Li, Leverence, Trombley, Xu, Yang, Tian, et al., 2010). According to De Pascual-Teresa *et al.*, 2000 (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000), persimmons have, in average, 0.17 mg/100 g FW of GC, 0.30 mg/100 g FW of PDB3, 0.01 mg/100 g FW of PCB3, 0.63 mg/100 g FW of C, 0.13 mg/100 g FW of PCB1 and 0.04 mg/100 g FW of PC trimer EC-EC-C. The PC C-GC was also detected in this study, but not quantified.

Garcinia mangostana L., commonly known as mangosteen, is a slow-growing tropical evergreen tree with leathery, glabrous leaves. The tree can attain 6–25 m in height and is mainly distributed in India, Myanmar, Sri Lanka, and Thailand. Reversed-phase HPLC followed the thiolysis analysis revealed that epicatechin predominately occurred as both terminal and extension units. Normal-phase HPLC-ESI-MS showed the heterogeneity of mangosteen tannins in constituent units, the interflavan linkages (A-type linkage) and the presence of polymers with less than 10 units. Condensed tannins fractions from mangosteen pericarp were characterized by MALDI-TOF-MS to further determine the polymer chain length and the sequential succession of monomer units in individual chains. In each fraction the dominating mass was correspondent to (E)C, but another strongly repeated pattern within each main set of peaks was the signals separated by 16 Da difference. These masses were produced by the early detected heterogeneity of flavan-3-ol units that showing the different masses among (E)Afz, (E)C and (E)GC (Reed, Krueger, & Vestling,

2005). These findings suggested the coexistence of propelargonidin, PC and PD units, with a significant amount of propelargonidin units but much lower signals of PDs units (Zhou, Lin, Wei, & Tam, 2011).

The cashew apple (*Anacardium occidentale*) is a fruit native to northeast Brazil, whose pulp can be processed into a sweet, astringent fruit drink or distilled into liqueur. The cashew nut is served as a snack or used in recipes, like other nuts, although it is actually a seed. Depolymerization by thiolysis has been proved to be an efficient method for determining the nature of the flavan-3-ol units within condensed tannins (and for determining the mean degree of polymerization (mDP)). In the thiolysis reaction, terminal units from condensed tannins are released as free flavonoids. When applying this method to tannin extracts from cashew apples it was concluded that tannins were mainly built of EGC (and/or GC) associated with some EC (and/or C) units. In fact, HPLC analysis of depolymerization products of skin and flesh tannins in acidic methanol with the presence of $(NH_4)_2Fe(SO_4)_2$ revealed strongly dominant proportions of delphinidin along with lower amounts of cyanidin, in average ~85% and 15%, respectively (Michodjehoun-Mestres, Souquet, Fulcrand, Meudec, Reynes, & Brillouet, 2009).

1.3.8. Pecan nuts

The pecan nut (*Carya illinoinensis*) is native to Mexico and the south central and southeastern regions of the United States. The total content of phenolics in Stuart and Schley pecan varieties ranges from 7.80 to 14.20 mg/g of kernel. Total flavanol content is between 2.78 and 4.43 mg/g of kernel (Senter, Forbus, & Smit, 1978).
Tannins present in pecans may affect the color of nutmeats as well as their flavour quality and consumer acceptability. Polles et al. (Polles, Hanny, & Harvey, 1981) determined the content of condensed tannins in 31 types of pecan nut kernels. Their content of tannins ranged from 0.70 to 1.71%, thus indicating a significant difference among different cultivars. Using carbon-13 cross-polarization magic-angle spinning NMR, Preston and Sayer (Preston & Sayer, 1992) later detected only PD polymer in the packing tissue inside pecan shells.
In terms of flavan-3-ol concentrations, pecan nuts have 7.20 mg/100 g FW of C, 0.80 mg/100 g FW of GCG and EC, 5.60 mg/100 g FW of EGC and 2.30 mg/100 g FW of EGCG (Perez-Jimenez, Neveu, Vos, & Scalbert, 2010).

1.3.9. Grapes

PA content and composition in grape berries depend on different factors such as climatic and geographical conditions, cultivation practices as well as stages of ripeness (de Andrés-de Prado, Yuste-Rojas, Sort, Andrés-Lacueva, Torres, & Lamuela-Raventós, 2007; Mateus, Proença, Ribeiro, Machado, & De Freitas, 2001; Pérez-Magariño & González-San José, 2006). Moreover, grape variety has also an important contribution on grape phenolic contents and composition. The mean degree of polymerization for PAs isolated from the seeds of grapes (cv. Cabernet franc) ranges from 4.7 to 17.4 mg/kg. For those isolated from grape skin, it is between 9.3 and 73.8 and for those extracted from grape stems between 4.9 and 27.6 mg/kg (Labarbe, Cheynier, Brossaud, Souquet, & Moutounet, 1999; Souquet, Labarbe, Le Guernevé, Cheynier, & Moutounet, 2000).

So far no PD have been quantified in grape skins but, in terms of PCs, the average concentrations that have been described are: PCB1 0.43 mg/100 g, PCB2 0.36 mg/100 g, PCB3 0.12 mg/100 g, PCB4 0.33 mg/100 g and PCC1 0.38 mg/100 g (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000). In terms of monomers there have been reported 5.46 mg/100 g of C, 5.24 mg/100 g of EC, 1.68 mg/100 g of ECG and 0.03 mg/100 g of EGC (Arts, van de Putte, & Hollman, 2000; de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000). De Pascual-Teresa et al. also found PD GC-GC, PC C-GC and PCB6 in white grapes, but didn't quantified them due to the lack of standards.

1.4. Phenolic Compounds of Beverages

Popular beverages in the world include tea, coffee, cocoa, beer, wine and fruit juices; all of these beverages contain phenolic compounds. The content of phenolics in beverages depends on species, degree of maturity and processing and climatic factors of the starting materials.

1.4.1.Tea

Polyphenolic compounds constitute up to 35% of dry weight of tea. The major constituents of tea polyphenolics are flavanols, such as ECG, EGC, EGCG, EC, C (Figures 6 and 7), and their derivatives, flavonols (quercetin, kaempferol and their glycosides), flavones (vitexin, isovitexin), phenolic acids, gallic acid, chlorogenic acid

and theogallin (Balentine, Wiseman, & Bouwens, 1997; Finger, Engelhardt, & Wray, 1991; Harbowy, Balentine, Davies, & Cai, 1997; Wickremasinghe, 1978).

According to Haslam (Edwin Haslam, 1989), tea flush (the immature vegetative portions of the tea plant) may contain up to 30% polyphenols, of which the major components are flavan-3-ols. However, their composition may vary depending on the variety of tea, its geographical origin, and environmental conditions, as well as the agronomic situation. Using tannase hydrolysis and thiolytic degradation it was possible to identify the following PAs: EC-(4β→8)-EC-3-O-G, EC-3-O-G-(4β→8)-EGC-3-O-G, C-(4α→8)-EGC-3-O-G, PD B4-3'-O-G, EC-3-O-G-(4β→6)-EGC-3-O-G, EGC-3-O-G-(4β→6)-EC-3-O-G, epiafzelechin-3-O-G-(4β→6)-EGC-3-O-G and PD B2-3'-O-G (Hashimoto, Nonaka, & Nishioka, 1989).

For production of good quality tea fermentation takes place, leading to the formation of orange-yellow to red-brown pigments and volatile flavour compounds (Balentine, Wiseman, & Bouwens, 1997). During this process, the polyphenolic compounds present in tea leaves are oxidised to their corresponding *o*-quinones. This oxidation leads to the formation of theaflavins and therubigins (Figure 19), responsible for the formation of the characteristic colour and flavour of fermented tea (black tea) (Robertson, 1992). Fermentation significantly reduces the total content of flavanols in processed tea.

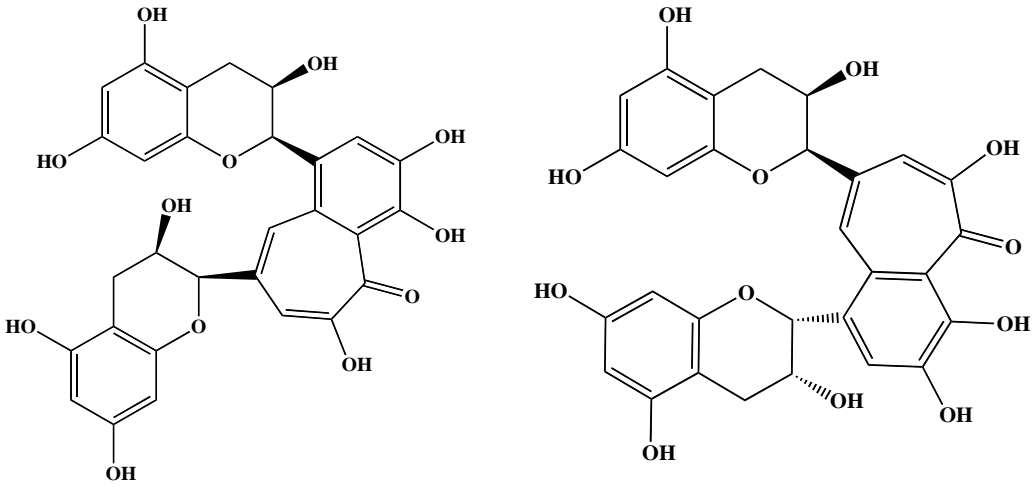


Figure 19. Structure of a theaflavin and a therubigin

Green tea has, in average, 43.83 mg total flavanols/100 g fresh weight, while black tea has only 26.80 mg (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000). Average numbers also indicates that green tea has 0.70 mg/100 mL (of infusion) of C, 2.26 mg/100 mL of GC, 0.47 mg/100 mL of GCG, 7.93 mg/100 mL of EC, 7.50 mg/100 mL of ECG, 19.68 mg/100 mL of EGC, 27.16 mg/100 mL of EGCG, 0.56

mg/100 mL of PCB1, 0.75 mg/100 mL of PCB2, 0.37 mg/100 mL of PCB3, 1.83 mg/100 mL of PCB4, 0.63 mg/100 mL of PCB7, 1.07 mg/100 mL of PCC1 and 0.27 of PDB3 (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000; Khokhar & Magnusdottir, 2002; Lin, Juan, Chen, Liang, & Lin, 1996). PD GC-GC and PC C-GC were also detected but not quantified.

For black tea the values are quite distinct: 2.45 mg/100 mL of C, 4.74 mg/100 mL of CG, 14.01 mg/100 mL of GC, 0.67 mg/100 mL of GCG, 3.94 mg/100 mL of EC, 7.34 mg/100 mL of ECG, 7.19 mg/100 mL of EGC, 9.12 mg/100 mL of EGCG, 3.70 mg/100 mL of PCB1, 2.51 mg/100 mL of PCB2, 0.49 mg/100 mL of PCB3, 1.80 mg/100 mL of PCB4, 0.01 mg/100 mL of PCB5, 0.46 mg/100 mL of PCB7, 0.76 mg/100 mL of PCC1 and 1.65 mg/100 mL of PDB3. It were also detected PD GC-GC and PC C-GC (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000; Khokhar & Magnusdottir, 2002; Liang, Lu, Zhang, Wu, & Wu, 2003; Lin, Juan, Chen, Liang, & Lin, 1996).

1.4.2. Beer

The production of beer involves malting, mashing, fermentation and storage. Yeasts are added to the wort and utilize nutrients present in it to produce ethanol, carbon dioxide, and other by-products. The characteristic flavour of beer arises from malt.

Beer contains 283 ± 3 mg/L of non-tannin and non-flavonoid phenolic compounds and 31 ± 3 mg/L of tannins (Fantozzi, Montanari, Mancini, Gasbarrini, Addolorato, Simoncini, et al., 1998). Some 67 different phenolic compounds have been identified in beer. Simple phenolics, aromatic carboxylic- and phenol carboxylic acids, hydroxycoumarins, catechins, leucoanthocyanidins, anthocyanidins, flavonols, flavanones, flavones, prenylated flavonoids and phenolic glycosides are included in this list (Bohm, 1989; Stevens, Taylor, Clawson, & Deinzer, 1999; Stevens, Taylor, & Deinzer, 1999).

Polyphenols in beer may contribute to the formation of haze (Bamforth, 1999; Siebert, 1999; Siebert, Troukhanova, & Lynn, 1996), which may contain up to 55% polyphenols. About 90% of polyphenolic compounds found in aged beer have a molecular weight of 500 to 10,000 Da; only trace quantities of phenolics have a molecular weight over 10,000 Da (Sogawa, 1972). Due to their chemical character and reactivity, polyphenols may undergo oxidative and acid-catalysed polymerization in beer upon storage (Gramshaw, 1968).

PAs from hops and malt have a tremendous influence on haze development. Among

them where identified: two monomeric flavan-3-ols (C and EC), a few B-type (with a single C4–C8 bond between successive units) PC and PD dimers (B3 and B9), and two A-type (with a single C4–C8 or C4–C6 bond and an additional ether bond between C2 and O–C7 or O–C5) PD dimers (Figure 20) (Delcour & Tuytens, 1984; Madigan, McMurrough, & Smyth, 1994; McMurrough & Baert, 1994; McMurrough, Madigan, Kelly, & Smyth, 1996).

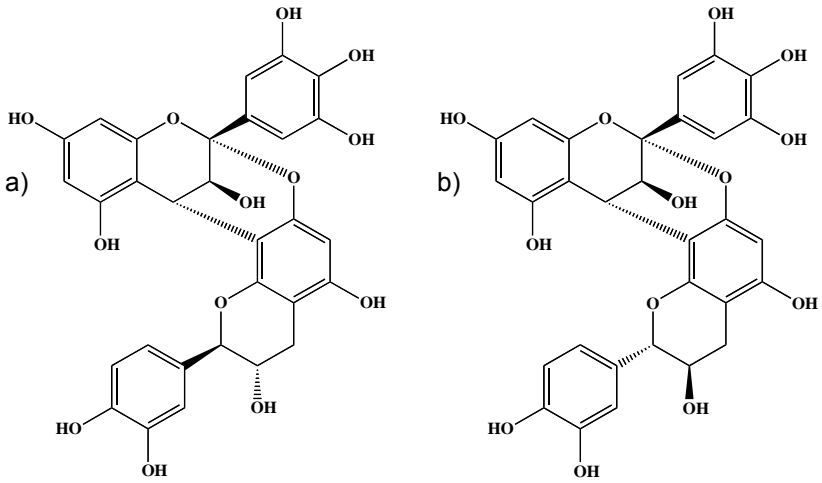


Figure 20. A-type dimer prodelphinidins: a) epigallocatechin-(4α-8, 2α-O-7)- catechin and b) epigallocatechin-(4α-6, 2α-O-7)-catechin

Quantification of beer flavan-3-ols shows that the average concentrations are: C 0.11 mg/100 mL, EC 0.06 mg/100 mL, PCB3 0.16 mg/100 mL, PCC2 0.03 mg/100 mL, PDB3 0.18 mg/100 mL, PC C-GC-C 0.02 mg/100 mL, PD GC-C-C 0.01 mg/100 mL and PD GC-GC-C 0.04 mg/100 mL (McMurrough & Baert, 1994; McMurrough, Madigan, & Smyth, 1996). Besides these compounds, de Pascual-Teresa et al. also found GC 0.10 mg/100 mL, PCB2 0.16 mg/100 mL and PCC1 0.07 mg/100 mL. It was also detected PD GC-GC and PC C-GC.

1.4.3. Wine

Phenolic compounds are important components of wine as they contribute to sensory characteristics such as color, flavour, astringency and hardness of wine directly or by interaction with proteins, polysaccharides, or other phenolic compounds (García-Viguera, Bakker, Bellworthy, Reader, Watkins, & Bridle, 1997; Lee & Jaworski, 1987). The total content of phenolics in wine depends on a number of factors, for example: grape variety, nature of crushing, possible inclusion or elimination of stems, skins and seeds prior to fermentation, vinification process, maceration time, aging.

After fermentation, wine usually is matured first in barrels or tanks. This maturation process from end of vinification to bottling typically lasts 12 to 24 months. Following this, the wine can be further aged in bottles to reduce contact with oxygen. The presence of oxygen induces the chemical transformation of phenolics and affects the flavour and color of wine (Auw, Blanco, O'Keefe, & Sims, 1996; Constantinos Dallas & Laureano, 1994; Dallas & Laureno, 1994; Gómez-Plaza, Gil-Muñoz, López-Roca, & Martínez, 2000; Somers & Evans, 1986; Somers & Pocock, 1990; Zoecklein, Jasinski, & McMahon, 1998).

Condensed tannins contribute to astringency, browning and turbidity in wines and also participate in the aging processes of wine (Haslam, Lilley, & Butler, 1988). Young wines contain mainly low to medium molecular weight phenolics while aged wines are relatively higher in polymerized phenolics. Typical Italian red and white wines contain 203 to 805 and 11 to 49 mg/L of flavanols, respectively. Furthermore, the concentration of (+)-C and (−)-EC in French wines ranges from 32.8 to 209.8 mg/L and from 22.1 to 130.7 mg/L, respectively. The level of PCs in these wines is between 7.8 and 39.1 mg/L for PCB1, 18.3 to 93 mg/L for PCB2, 21.4 to 215.6 mg/L for PCB3, 20.2 to 107.2 mg/L for PCB4, 8.6 to 36.9 mg/L for PCC1 and 26.7 to 79.3 mg/L for PCT2 (Carando, Teissedre, Pascual-Martinez, & Cabanis, 1999).

Usually the flavan-3-ol monomers (C, EC, ECG, GC) a few dimers (B1, B2, B3, B4) and one or two trimers are identified and quantified, but PA characteristics are mainly determined using other ways such as mean degree of polymerization (mDP), percentage of galloylation (%G) and percentage of PD (%PD). Red wine contains on average 6.81 mg/100 mL of C, 3.78 mg/100 mL of EC, 0.77 mg/100 mL of ECG, 0.08 mg/100 mL of GC and 0.06 mg/100 mL of EGC (Arts, van de Putte, & Hollman, 2000; de Pascual-Teresa, Rivas-Gonzalo, & Santos-Buelga, 2000; Goldberg, Karumanchiri, Tsang, & Soleas, 1998). Only 5 PC dimers were quantified: PCB1, PCB2, PCB3, PCB4 and PCB7 where detected on an average of 4.14 mg/100 mL, 4.97 mg/100 mL, 9.47 mg/100 mL, 7.29 mg/100 mL and 0.27 mg/100 mL respectively. PCC1 trimer was found at 2.56 mg/100 mL and PCT2 at 6.71 mg/100 mL. In terms of PDs, only PDB3 was found with an average concentration of 0.11 mg/100 mL (Carando, Teissedre, Pascual-Martinez, & Cabanis, 1999; de Pascual-Teresa, Rivas-Gonzalo, & Santos-Buelga, 2000; Teissedre & Landrault, 2000). de Pascual-Teresa et al. also found PD GC-GC, PC C-GC and PCB6 in red wines.

Table 3. Average concentrations of PD, PC and monomeric flavan-3-ols. The values are expressed in mg/100 g of fresh weight or mg/100mL. [a)] Neveu, Perez-Jiménez, Vos, Crespy, du Chaffaut, Mennen, et al., 2010.

Food/Beverage	C	EC	GC	EGC	ECG	GCG	EGCG	PCB1	PCB2	PCB3	PCB4	PCB5	PCB6	PCB7
Barley	1.23	--	--	--	--	--	--	--	--	10.90	--	--	--	--
Broad Beans	16.23	37.55	17.38	--	--	--	--	11.26	12.08	8.17	18.47	--	n.q.	--
Lentils	0.28	0.10	0.14	--	--	--	--	--	0.34	0.71	--	--	--	--
Red Currant	1.27	0.08	1.28	0.15	--	--	--	--	--	0.20	--	--	--	--
Raspberries	0.05	1.78	0.01	1.11	--	--	--	--	2.79	0.03	--	--	--	0.18
Cranberries	--	4.20	--	--	--	--	--	--	--	--	--	--	--	--
Blueberries	--	0.30	0.59	0.37	--	--	--	0.75	0.19	0.81	0.37	--	--	0.27
Strawberry tree fruit	7.48	1.11	1.60	--	--	--	--	1.72	--	1.69	0.43	--	n.q.	--
Strawberry	6.36	0.0075	0.05	0.06	0.28	--	--	--	0.03	1.10	0.13	--	--	--
Banana	1.34	0.11	--	0.0021	--	--	--	--	--	--	--	--	--	--
Pomegranate	0.40	0.08	0.17	0.16	--	--	--	0.13	--	0.16	--	--	--	--
Quince	0.75	0.67	--	--	--	--	--	0.73	1.34	0.10	0.24	0.12	--	--
Persimmon	0.63	--	0.17	--	--	--	--	0.13	--	0.01	--	--	--	--
Pecan nuts	7.20	0.80	--	5.60	--	0.80	2.30	--	--	--	--	--	--	--
Grapes	5.46	5.24	--	0.03	1.68	--	--	0.43	0.36	0.12	0.33	--	n.q.	--
Green Tea	0.70	7.93	2.26	19.68	7.50	0.47	27.16	0.56	0.75	0.37	1.83	--	--	0.63
Black Tea	2.45	3.94	14.01	7.19	7.34	0.67	9.12	3.70	2.51	0.49	1.80	0.01	--	0.46
Beer	0.11	0.06	0.10	--	--	--	--	--	0.16	0.16	--	--	--	--
Red Wine [a)]	6.81	3.78	0.08	0.06	0.77	--	--	4.14	4.97	9.47	7.29	--	n.q.	0.27

n.q. – not quantified

Table 3 (cont.). Average concentrations of PD, PC and monomeric flavan-3-ols. The values are expressed in mg/100 g of fresh weight or mg/100mL. [a)] Neveu, Perez-Jiménez, Vos, Crespy, du Chaffaut, Mennen, et al., 2010.

Food/Beverage	PCC1	PC C-GC	PC EC-EC-C	PDB3	PD GC-GC	PCC2	PC C-GC-C	PD GC-C-C	PD GC-GC-C
Barley	--	--	--	23.07	--	--	--	--	--
Broad Beans	0.13	n.q.	--	23.50	n.q.	--	--	--	--
Lentils	--	--	--	0.45	--	--	--	--	--
Red Currant	--	--	--	1.70	--	--	--	--	--
Raspberries	0.30	--	--	--	--	--	--	--	--
Cranberries	--	--	--	--	--	--	--	--	--
Blueberries	0.71	--	--	--	--	--	--	--	--
Strawberry tree fruit	--	n.q.	0.22	1.99	n.q.	--	--	--	--
Strawberry	0.62	--	0.50	--	--	--	--	--	--
Banana	--	--	--	0.10	--	--	--	--	--
Pomegranate	--	--	--	n.q.	n.q.	--	--	--	--
Quince	0.94	--	0.28	0.08	--	--	--	--	--
Persimmon	--	n.q.	0.04	0.30	--	--	--	--	--
Pecan nuts	--	--	--	--	--	--	--	--	--
Grapes	0.38	n.q.	--	--	n.q.	--	--	--	--
Green Tea	1.07	n.q.	--	0.27	n.q.	--	--	--	--
Black Tea	0.76	--	--	1.65	--	--	--	--	--
Beer	0.07	n.q.	--	0.18	n.q.	0.03	0.02	0.01	0.04
Red Wine [a)]	2.56	n.q.	--	0.11	n.q.	--	--	--	--

n.q. – not quantified

Chapter 2

2. Synthesis of Prodelphinidins

2.1. Introduction

Up until the 1980's much of the isolation and characterization of PAs had been carried out on compounds extracted from various plant sources, since little was known on their synthesis.

One of the most important aspects in the chemistry of polyphenols is their sensitivity. Like other phenols, they are susceptible to complex free-radical oxidation reactions by air and reactive oxygen species, especially in the presence of bases. This is the reason why this class of compounds has been so thoroughly investigated with regard to their antioxidant capabilities in biological systems. On the other hand, they are subjected to a reversible opening of their ring-C by way of a transient quinone methide at elevated temperatures or in the presence of bases. This can lead to stereochemical scrambling at C2 (Foo & Porter, 1983) or, after ring closure involving different hydroxyl groups or aromatic carbon atoms, to rearrangement products (Burger, Kolodziej, Hemingway, Steynberg, Young, & Ferreira, 1990; Laks, Hemingway, & Conner, 1987; Steynberg, Bezuidenhoudt, Burger, Young, & Ferreira, 1990). With this information in mind, it is advisable to perform all requisite synthetic steps and separations on protected precursors and to de-protect pure precursors at the very end of the synthesis under the mildest possible conditions.

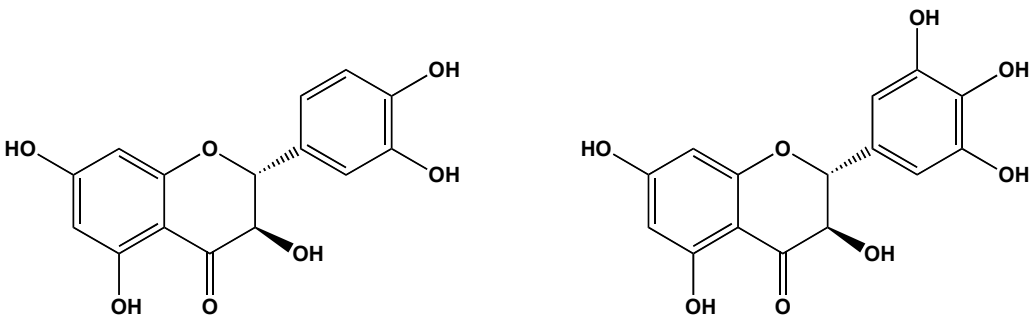
2.1.1. Earlier synthetic work

PD synthesis has always been linked to the synthesis of PCs due to chemical structure resemblance and PC widespread detection and identification in natural sources. The first synthesis of PCs was performed by Geissman in 1966 (Geissman & Yoshimura, 1966). In this work, the interflavan link was created under acidic conditions (aqueous HCl, 0.1 N) between a unit protected flavan-diol, tetra-O-methyl-3',4',5,7-tetrahydroxyflavan-3,4-diol and (+)-C. Two inseparable major products were recovered with a yield of 67%. Geissman et al. suggested that the formation of two products in equivalent amounts is induced by an epimerization at C4.

Later, PA synthesis involved complex natural PCs thiolysis giving 4-benzylthioflavan-3-ols and acid-catalysed condensation of unprotected monomers with building blocks that bear a good leaving group in C4 position. An example of the latter reaction is the

reduction of (2R,3R)-dihydroquercetin ((+)-taxifolin) with sodium borohydride in ethanol, under nitrogen, in the presence of a molar equivalent of (+)-catechin, and adjustment of the solution to pH 5 after the addition of an equal volume of water (Eastmond, 1974). Inherent characteristics of this synthetic approach are the formation of regioisomers in inter-flavan bonds from either the C6 or the C8 position of the nucleophile acceptor, as well as the re-entry of already formed oligomers into the process to generate higher oligomers, even if an excess of the nucleophile acceptor is employed (Delcour, Ferreira, & Roux, 1983). Moreover, although it is a rapid method, the resulting mixtures are sensitive to oxidation, sometimes the formed compounds are not easily isolated and there is still no better reasonably priced electrophile building block.

So far, all of the synthetic work had mainly focused on the PCs with little attention being paid to the PDs. After a work on PDs synthesis was presented at the Phytochemical Society of Europe International Symposium in 1981 (Outtrup, 1981b), Delcour *et al.* proposed a method for the direct synthesis of PDs using (+)-catechin and (+)-dihydromyricetin as precursors (Delcour & Vercruysse, 1986). The same group also identified trimers with both (C4-C8 and C4-C6) linked units which they identified as PCC2, PDC2 and a trimer comprised of (4,8:4,8)-(+)-GC-(+)-C-(+)-C, as well as (4,8:4,8)-(+)-C-(+)-GC-(+)-C.



(2R,3R)-(+)-dihydroquercetin (2R,3R)-(+)-dihydromyricetin

Figure 21. Chemical structures of dihydroquercetin and dihydromyricetin

2.1.2. Protecting groups

In 1974 Sears *et al.* (Sears, Casebier, Hergert, Stout, & McCandlish, 1974) and, later, Laks *et al.* (Laks, Hemingway, & Conner, 1987) performed studies on base reactions leading to an intra rearrangement of (+)-C yielding catechinic acid and isocatechinic acid. This acid was shown to be an enolic form of (+)-C-(+)-phloroglucinol (Figure 22 a)). Since this occurred for (+)-C it was postulated that the same could occur in higher oligomeric compounds. Polymeric PCs were reacted with phloroglucinol at pH 12.0 at 23°C and 50°C. It was found that both the interflavanyl bond and the pyrane ring underwent rapid cleavage to form a reactive quinone methide that, through an intramolecular condensation and rearrangement, leads to the 6-phloroglucinol adduct b) in figure 22. It also can undergo an epimerization reaction after C ring opening.

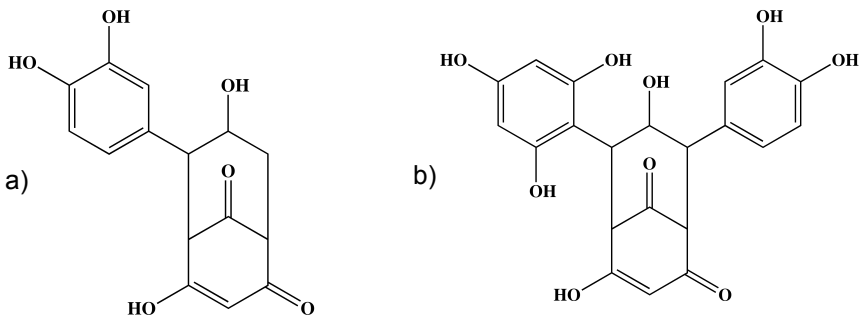


Figure 22. Chemical structure of: a) catechinic acid and
b) 6-(3,4-dihydroxyphenyl)-7-hydroxy-6-(2,4,6-trihydroxyphenyl)-bicyclo[3,3,1]-nonane-2,4,9-trione

Meanwhile, when extracted in acid solution, polymerization reactions take place, making catechins unstable compounds outside of pH 5-8. They are equally sensitive to oxidation reactions where, in the presence of oxygen, the hydroxyl groups are transformed in quinones, leading to the formation of brown compounds (Es-Safi, Le Guernevé, Cheynier, & Moutounet, 2000).

Given the disadvantages of synthesis from unprotected precursors, many efforts have been made from protected precursors. The flavanol hydroxyl group has indeed a nucleophilic character, acidic nature (pK_a 10-18) and high potential for oxidation. The difference in acidity of phenol functions with that of the alcohol in position C3 protects the four phenol functions without protecting the alcohol and thus leaves this position free for future functionalization as the galloylation (Figure 23).

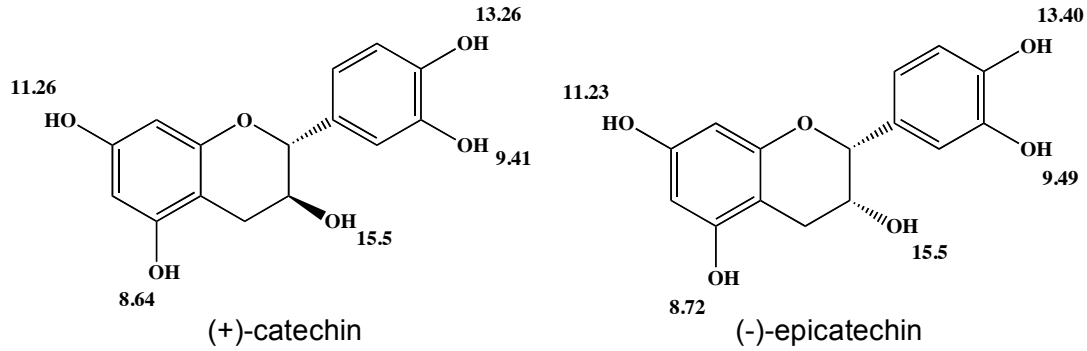


Figure 23. Protonation constants of alcohols and phenols functions (Kennedy, Munro, Powell, Porter, & Foo, 1984; Slabbert, 1977).

Methylation of the phenolic hydroxyls has commonly been employed for PC characterization purposes, and in a few cases methylated building blocks have been entered into oligomer-forming condensation reactions. However it is difficult to cleave methyl ethers without breaking the inter-flavan bond and, thereby, renders methyl unsuitable as a protecting group for PCs (Kolodziej, Ferreira, & Roux, 1984; Sweeny & Iacobucci, 1979).

Other protective groups include acetylation with acetic anhydride and pyridine (Kawamoto, Tanaka, Nakatsubo, & Murakami, 1993; Kolodziej, Ferreira, & Roux, 1984); silylation using *tert*-butyldimethylsilyl and trimethylsilyl derivatives (Corey & Venkateswarlu, 1972; Kendall, Johnson, & Cook, 1979); and benzylation (Kawamoto, Nakatsubo, & Murakami, 1991; Kawamoto, Tanaka, Nakatsubo, & Murakami, 1993).

Protection with benzyl functions remains the preferred choice because of their stability in acid/basic medium, but mainly because of the easy de-protection in neutral medium by hydrogenolysis (Deme, 1976; Haruo Kawamoto, Nakatsubo, & Murakami, 1989).

2.1.3. Inter-flavan bond formation using benzyl-protected building blocks

The first synthesis of PCs involving a coupling via an organometallic was conducted in 1967 by Weinges (Weinges & Perner, 1967). The electrophile unit **1** was obtained after methylation of taxifolin hydroxyl groups (Figure 24). The second **2** was obtained from catechin after protection of the hydroxyl groups by methoxyl groups and bromination at C8 to produce the halogen-metal exchange. After the protection of the alcohol at C3 by a benzyl group, the lithium derivative **3** was formed to react with the electrophilic unit **1**. After catalytic hydrogenolysis and acetylation of the hydroxyl in

C3, the dimer **4** was obtained with a yield of 20%.

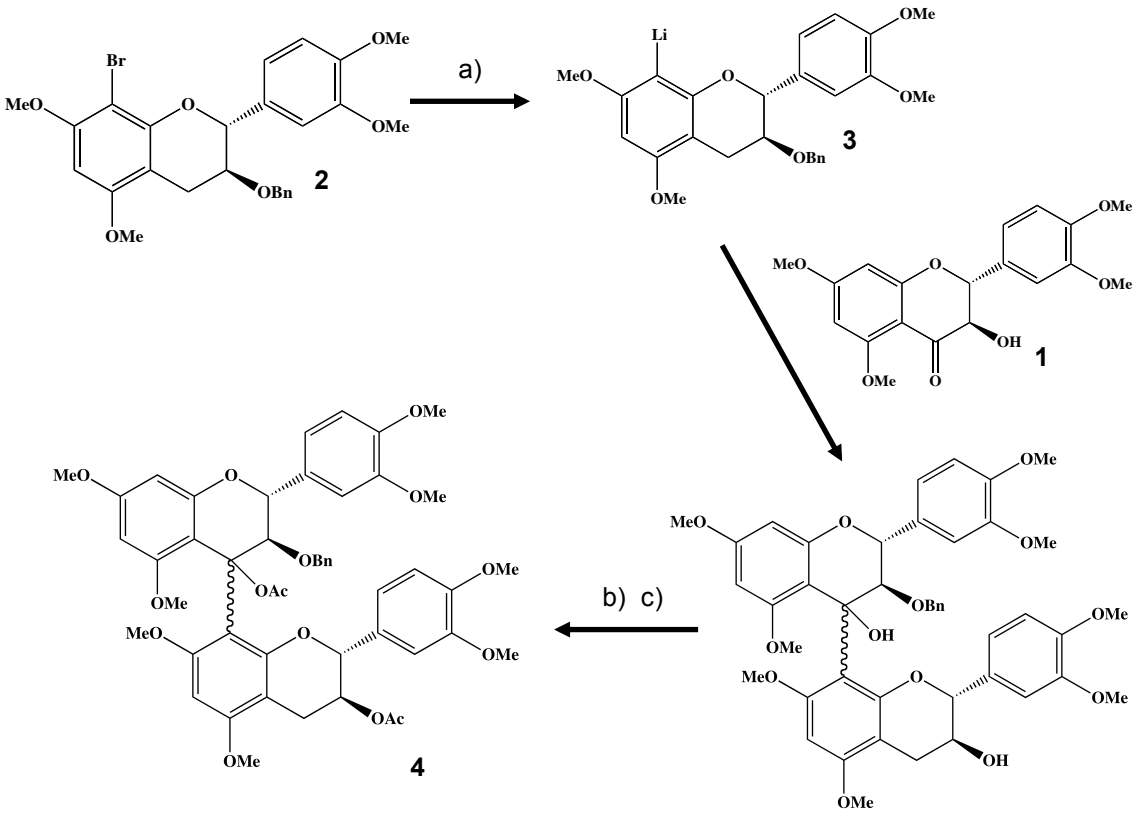


Figure 24. First synthesis of procyanidins involving a coupling via an organometallic.
a) *n*BuLi; b) H_2 – Pt; c) Ac_2O, pyridine.

Later, Kozikowski *et al.* developed another method for coupling via an organometallic (Kozikowski, Tückmantel, & Hu, 2001). After the obtainment of the electrophilic unit **5**, where all hydroxyl groups are protected with benzyl groups and the hydroxyl group at C3 either protected by benzylation or by silylation, C4 is oxidized with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and the resulting alcohol **6** oxidized to form the ketone **7** (Figure 25). The nucleophilic unit **8** is initially prepared like the electrophilic unit **5**, but then is brominated at C8 to allow the metal-halogen exchange. Treatment of the nucleophilic unit by *tert*-butyllithium (*tert*BuLi), followed by addition of the electrophilic unit leads to the formation of a dimer (**9**) as a single isomer. After reduction and deprotection the EC-(4α-8)-EC dimer was obtained.

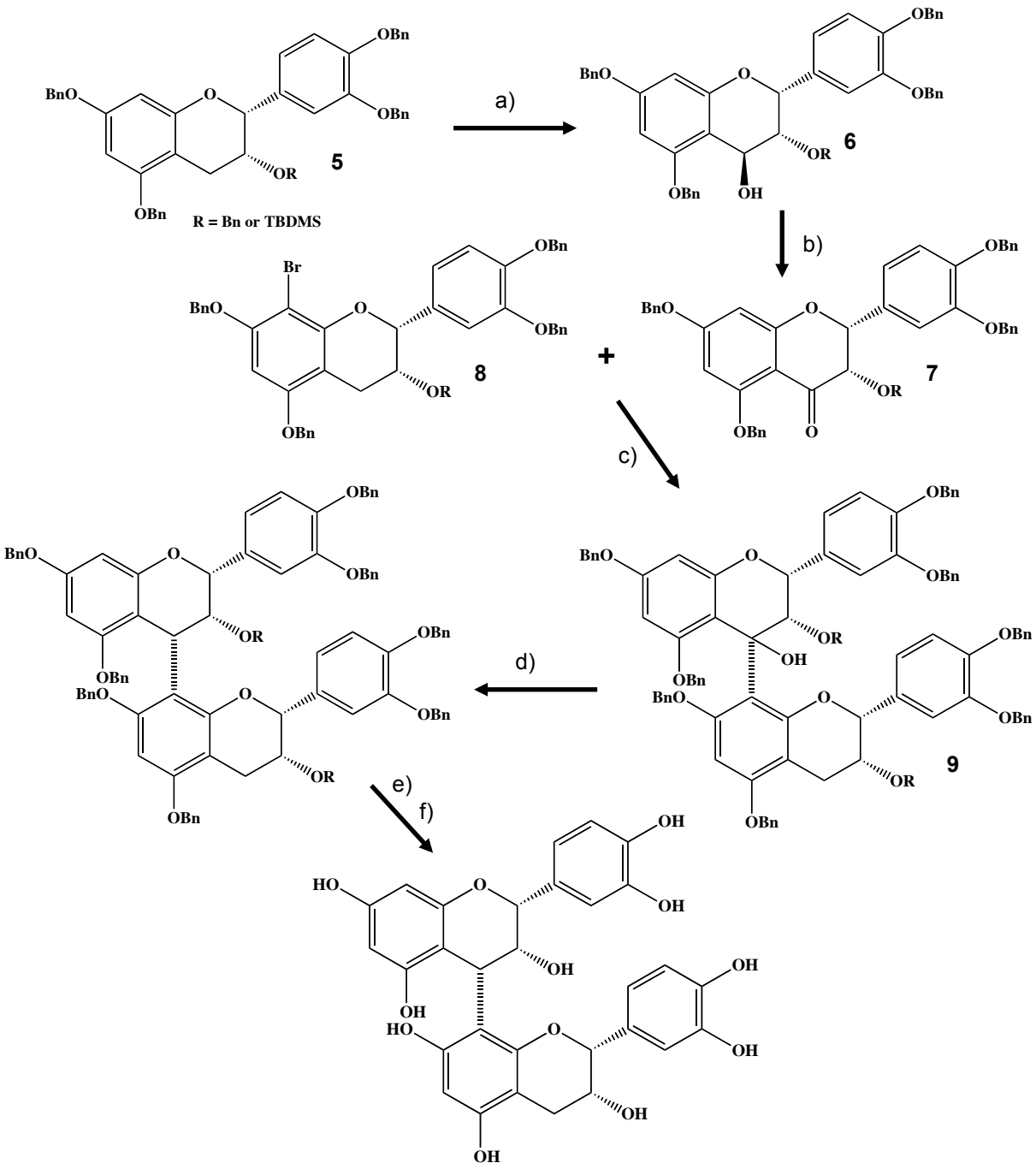


Figure 25. Synthesis of procyanidins developed by Kozikowski *et al.* a) DDQ, H_2O, THF; b) NMMO, cat. TPAP, CH_2Cl_2; c) *tert*BuLi, THF; d) $n$$Bu_3SnH$, CF_3COOH, CH_2Cl_2; e) R = TBDMS: 48% HF, CH_3CN; f) 1 bar H_2, 20% $Pd(OH)_2/C$, EtOAc/MeOH.

DDQ oxidation of the EC derivative in dichloromethane/ methanol ($CHCl_3$/MeOH) gave generally low yield, and improved result was obtained by the use of ethylene glycol instead of MeOH to give flavan-3,4-diol hydroxy ethyl ether derivatives in 52% yield. Saito *et al.* re-examined the benzylic DDQ oxidation of a C derivative (5,7,3',4'-tetrabenzylcatechin) using five alcohols as nucleophiles (Saito, Nakajima, Tanaka, & Ubukata, 2002). Among them, benzyl alcohol (BnOH) reacted in 86% yield to give

the 4-*O-benzyl* derivative. This reaction was performed at 0ºC because, at room temperature, it led to the formation of many by-products including bis-oxidized products.

The first synthesis involving cationic coupling activated by a Lewis acid was described in 1989 by Kawamoto (Kawamoto, Nakatsubo, & Murakami, 1989). The coupling between a flavan-diol (5,7,3',4'-tetrabenzylflavan-3,4-diol) and phloroglucinol was performed in the presence of titanium tetrachloride ($TiCl_4$). Since then, many researchers use this synthetic route to obtain PCs: in the presence of a Lewis acid, a nucleophilic unit is coupled with an electrophilic unit, which doesn't necessarily have to be protected at C3 and/or C8 but always activated at C4.
Until some years ago, only Tuckmantel (Tuckmantel, Kozikowski, & Romanczyk, 1999), Saito (Saito, Nakajima, Tanaka, & Ubukata, 2002; Saito, Noriyuki, Akira, & Ubukata, 2003) and Fouquet (Tarascou, Barathieu, André, Pianet, Dufourc, & Fouquet, 2006) were the only ones that had complete control of the stereoselectivity of the interflavan link upon condensation. In most cases, the control is achieved through the use of a large excess of the nucleophilic unit. Blocking the C8-position of the electrophilic unit prevents electrophilic unit respond to C8 of the formed dimer, thus blocking all access to higher order oligomers.

2.1.4. Deprotection of the benzyl groups

The deprotection step must be carried out under mild and neutral conditions to avoid both breaking the interflavan link and the degradation of the native dimer obtained by ring opening and/or epimerization. The various methods in the literature offer catalytic hydrogenolysis that differ in the nature of the catalyst, the hydrogen donor solvent or the reaction temperature. Using palladium on charcoal and a hydrogen source: H_2 (Kawamoto, Nakatsubo, & Murakami, 1989; Kawamoto, Nakatsubo, & Murakami, 1991), 1,4-cyclohexadiene (Felix, Heimer, Lambros, Tzougraki, & Meienhofer, 1978) and acetic acid (Deme, 1976); and the nature of the solvent: dioxane (Kawamoto, Tanaka, Nakatsubo, & Murakami, 1993; Tuckmantel, Kozikowski, & Romanczyk, 1999), ethanol/THF 1/5 (Yoneda, Kawamoto, & Nakatsubo, 1997) and methanol, debenzylation is not complete. Best results are obtained with another palladium catalyst: Pearlman's catalyst, $Pd(OH)_2$ on charcoal ($Pd(OH)_2/C$). This catalyst allows debenzylation in various solvent systems: THF/H_2O (Arnaudinaud, Nay, Vergé, Nuhrich, Deffieux, Mérillon, et al., 2001), THF/MeOH/H_2O (Saito, Doi, Tanaka, Matsuura, Ubukata, & Nakajima, 2004; Saito, Tanaka, Ubukata,

& Nakajima, 2004), AcOEt/MeOH (Kozikowski, Tückmantel, & Hu, 2001; Tarascou, Barathieu, André, Pianet, Dufourc, & Fouquet, 2006).

2.2. Studies involving prodelphinidins

The antioxidant properties of PD dimers from pomegranate peel were studied using two methods: inhibition of ascorbate/iron-induced peroxidation of phosphatidylcholine liposomes; and scavenging of the radical cation of 2,2-azinobis (3-ethyl-benzothiazoline-6-sulphonate, ABTS) relative to the water-soluble vitamin E analogue Trolox C (expressed as Trolox C equivalent antioxidant capacity, TEAC). The results revealed that they are potent antioxidants in the aqueous phase, being much more effective than the GC monomer. However, in the lipid phase, only one of the dimers (GC-(4-8)-C) was significantly more effective than the GC monomers in the inhibition of lipid peroxidation of phosphatidylcholine vesicles (Plumb, de Pascual-Teresa, Santos-Buelga, Rivas-Gonzalo, & Williamson, 2002).

In a chemical investigation with several flavan-3-ols isolated from the testa of faba beans, including the PDs GC-(4α→8)-C, GC-(4α→8)-EC and GC-(4α→8)-EGC. PA samples were compared for their trypsin inhibitory activity and the results suggest that the degree of polymerization, the number of phenolic hydroxyl groups and the 2,3-stereochemistry of the constituent units affect remarkably the strength of the inhibition. PDs showed trypsin inhibitor activity values of 4.7±0.5 to 9.5±0.9 for bovine trypsin and 9.5±3.1 to 13.9±2.1 for porcine trypsin against values around 2 and 4 for PCs (values expressed as mg trypsin inhibited per g test sample) (Helsper, Hoogendijk, van Norel, & Kolodziej, 1993).

PDs B3, T1, T2 and T3 isolated from a polyphenol extract from barley bran induced 26-40% nitro blue tetrazolium (NBT)-positive cells and 22-32% alpha-naphthyl butyrate esterase-positive cells in HL60 human myeloid leukemia cells (Tamagawa, Fukushima, Kobori, Shinmoto, & Tsushida, 1998).

Green tea extract is well known to reduce the risk of a variety of diseases. Studies on structure-activity relationship using five of PDs of green tea revealed that the galloyl moiety might have anti-inflammatory properties through blocking mitogen-activated protein kinase (MAPK)-mediated cyclooxygenase-2 (COX-2) expression (Hou, Masuzaki, Hashimoto, Uto, Tanigawa, Fujii, et al., 2007).

Luobuma tea, prepared from the leaves of *Apocynum venetum L.*, is a popular beverage in China. Fractionation of this extract allowed to identify seven polyphenolic

compounds: GC, EGC, C, EC, PD EGC-(4β→8)-EC, PC EC-(4β→8)-GC, and PCB2. The activity of Luobuma leaf extract and its components against the formation of advanced glycation endproducts (AGEs), which are largely involved in the pathogenesis of diabetic vascular complications, was examined using the in vitro glycation reaction. Inhibition of AGEs is considered to be one promising approach for the prevention and treatment of diabetic vascular diseases and strong inhibitory activity against the formation of AGEs was shown by Luobuma aqueous extract. These identified compounds (after purification) also exerted inhibitory activities that were more potent than the positive control, aminoguanidine (Yokozawa & Nakagawa, 2004).

PDB2-3′-*O*-G isolated from green tea leaf, was investigated for its anti-proliferative activity in human non-small cell lung cancer A549 cells. The results showed that not also inhibited the proliferation of A549 cells but also showed no detectable toxic effects on normal WI-38 cells. In addition, PDB2-3′-*O*-G effectively induced A549 cell apoptosis as determined by assessing the nucleosome level in cytoplasm (Kuo, Hsu, Lin, & Lin, 2004; Kuo, Hsu, Lin, & Lin, 2005).

Fujii and co-workers have focused their interest on examining the antitumor activities of newly synthesized PDs. The synthesis of PDB3 and PDC2 allowed obtaining sufficient quantities of purified compounds to screen against **PC-3** prostate cancer cell lines together with PCB3, PCC1 and PCC2 (Figure 26).

EGCG was used as a positive control. EGCG and all PDs exhibited significant cytotoxic activity suggesting that these were clearly associated with the presence of the pyrogallol moiety of the B ring. Treatment of **PC-3** prostate cancer cells with 50 μM of PDB3 for 48 h induced a G1/G0 phase population (cells first growth/cells resting phase) increase from 62.88% to 74.50%, blocking the **PC-3** prostate cancer cell cycle partly at the G1/ G0 phase within these 48 h; and an S phase fraction (DNA replication phase) decrease from 16.06% to 8.67%, indicating a slower cell division and growing tumor. EGCG and the rest PDs showed a similar effect. On the other hand, no effect from PCB3, PCC1 or PCC2 was observed. PDB1, PDB2, and PDB4, which have two pyrogallol moieties, seemed to have stronger activity than PDB3, which has one pyrogallol moiety. Therefore, the additional pyrogallol moieties might enhance the cytotoxic effects. EGCG has two pyrogallol moieties but one of them is esterified; this might be the reason for a weaker cytotoxic activity than PDB1, PDB2, and PDB4 (Fujii, Toda, Matsumoto, Kawaguchi, Kawahara, Hattori, et al., 2013).

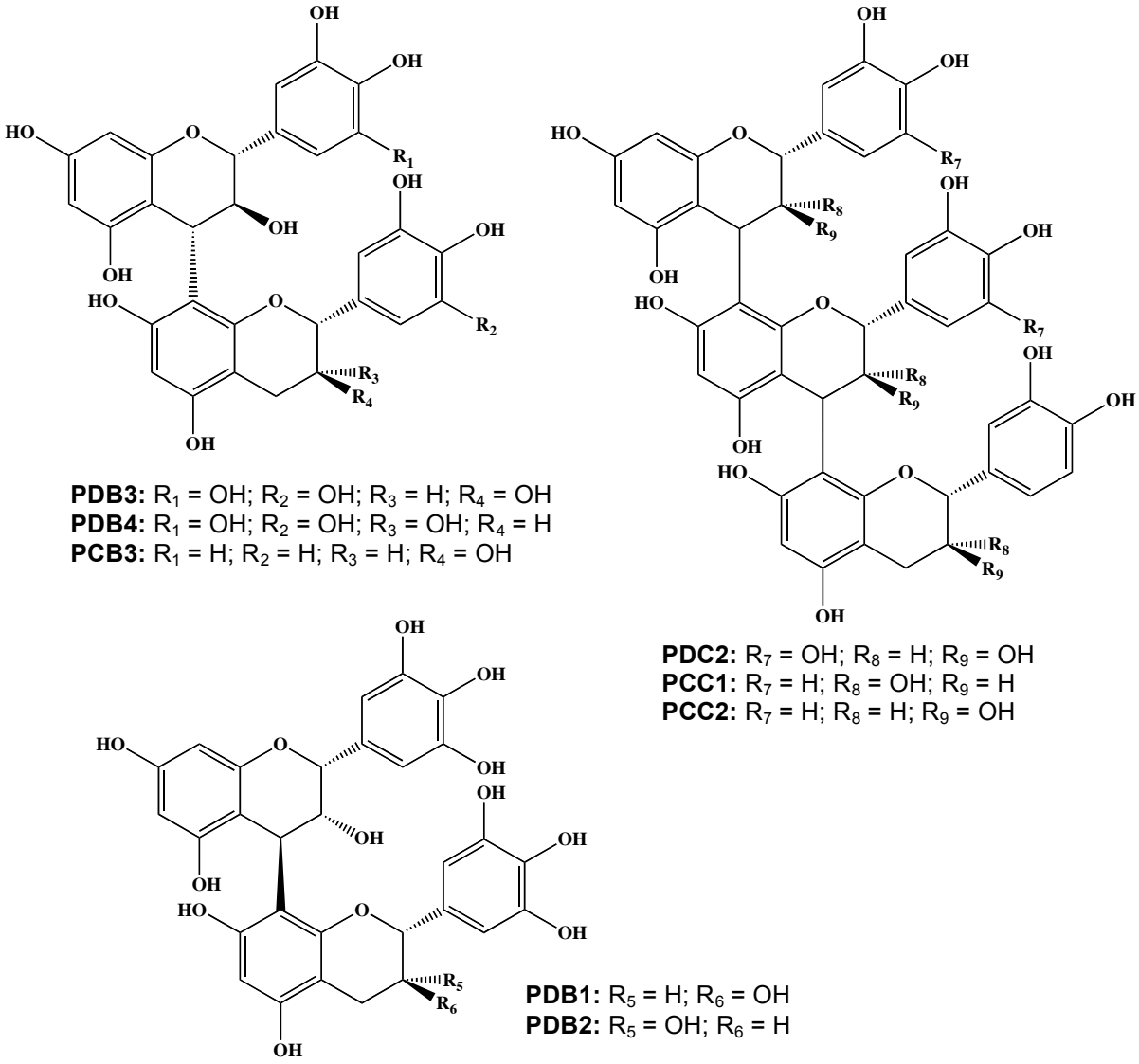


Figure 26. The chemical structures of PDB1, PDB2, PDB3, PDB4, PCB3, PDC2, PCC1 and PCC2.

Compounds which promote cell apoptosis and inhibit proliferation of cancer cells, are likely to be good candidates as antitumor agents (Zi & Agarwal, 1999). These findings suggest that PDs might be promising chemopreventive agents against prostate cancer (Fujii, Toda, Kawaguchi, Kawahara, Katoh, Hattori, et al., 2013).

2.3. Synthesis of new Prodelphinidins

Synthetic studies on PDs are quite limited. It was decided to follow a synthesis pathway proposed by Krohn and co-workers (Krohn, Ahmed, & John, 2009; Krohn, Ahmed, John, Letzel, & Kuck, 2010). Initially the idea was to follow their exact steps (Figure 27). After the obtainment of methyl-tribenzyloxybenzoate **10**, this was reduced by lithium aluminium hydride (LAH) in tetrahydrofuran (THF) for 21 hours at

room temperature and for 5 hours in reflux (Meuzelaar, van Vliet, Maat, & Sheldon, 1999; Zhao, Hao, Lu, Cai, Yu, Sevnet, et al., 2002), yielding tribenzyloxybenzylalcohol **11**. Next the tribenzyloxybenzaldehyde **12** was obtained by oxidation of the alcohol by pyridinium chlorochromate in dichloromethane at 0 °C for 6h.

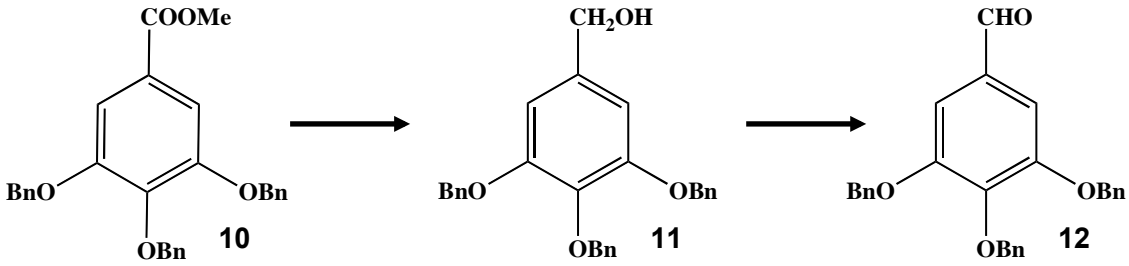


Figure 27. Synthesis of benzylated aldehyde **12**

The second reagent needed was the benzylated acetophenone **15**. Phloroglucinol (**13**) was subjected to Friedel-Crafts acetylation (Mateeva, Kode, & Redda, 2002). Trihydroxyacetophenone (**14**) was obtained by treating equimolar amount of phloroglucinol **13** and acetic anhydride with 2.5 times molar excess of boron trifluoride solution in ether at 50-60 °C for 20 minutes (Figure 28). Trihydroxyacetophenone (**14**) was protected as the benzyl ether by reaction with 4.4 eq of potassium carbonate and 2.8 eq of benzyl bromide, in DMF, at 60-70ºC.

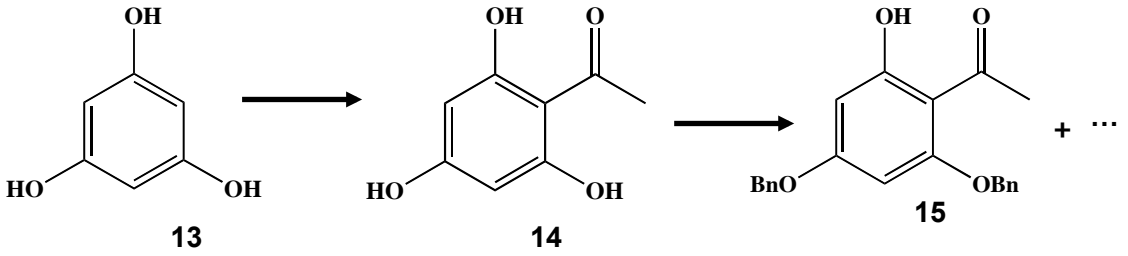


Figure 28. Synthesis of acetophenone **15**

Since this approach was consuming a lot of time and the authors of the method also experienced a few problems with the obtaining of the electrophile part (due to racemic mixtures) and, since the monomers that constitute the dimers building parts are available commercially, it was decided to continue from here.

The overall description of the synthesis of new PDs is described in figure 29

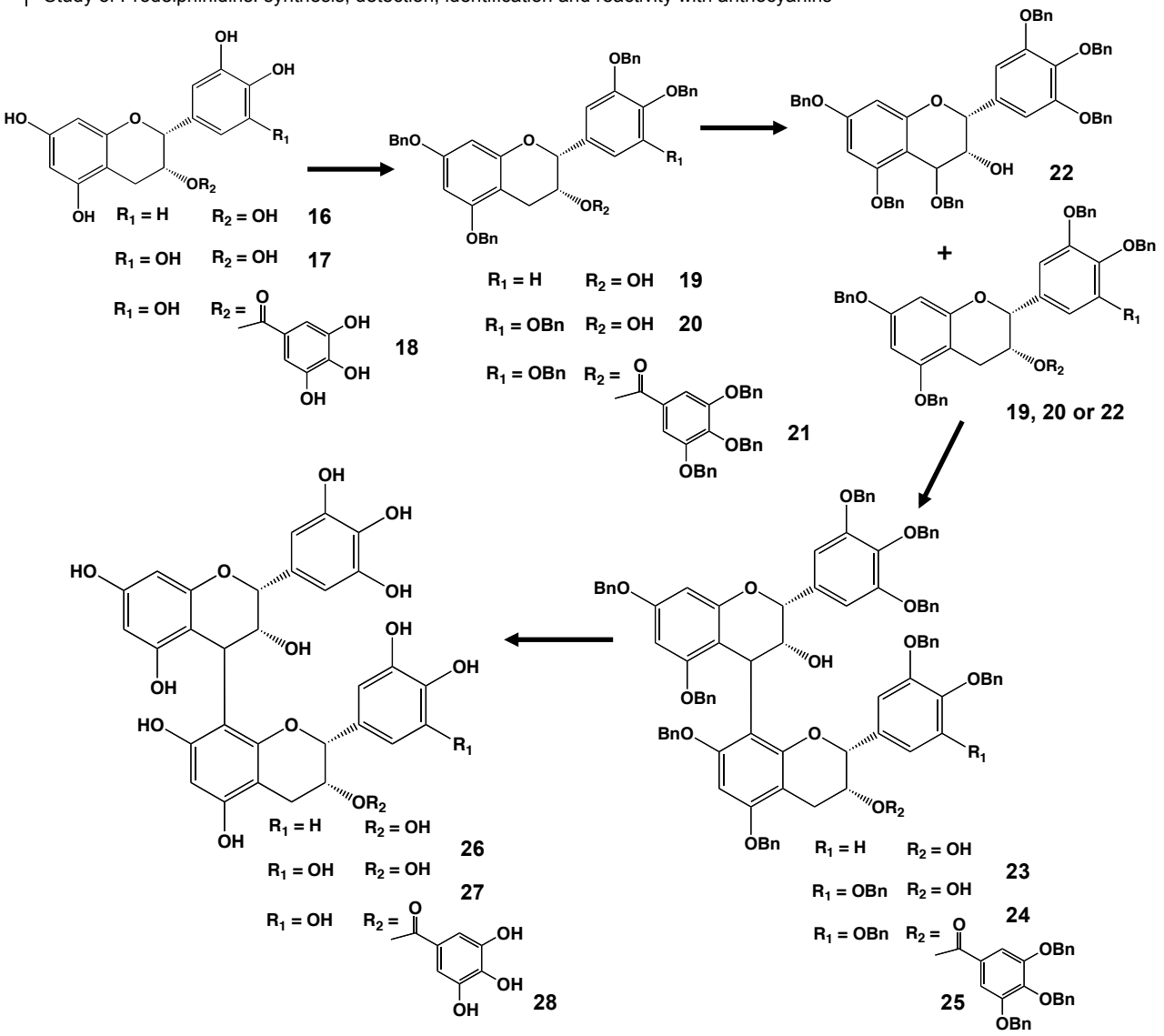


Figure 29. Synthesis of prodelphinidin epigallocatechin-(4β→8)-catechin (EGC-C) **26**, epigallocatechin-(4β→8)-epigallocatechin (EGC-EGC) **27** and epigallocatechin-(4β→8)-epigallocatechin gallate (EGC-EGCG) **28**.

2.4. Materials and Methods

(+)-Catechin (C) and (-)-epicatechin (EC), were purchased from Sigma-Aldrich® (Madrid, Spain). (-)-Epigallocatechin (EGC) and (-)-epigallocatechin gallate (EGCG) were purchased from Biopurify Phytochemicals Ltd (Sichuan, China).

The identity and purity of the compounds was achieved by LC-MS-ESI and NMR (^{1}H NMR and ^{13}C NMR spectra were measured in $CDDI_3$ or D_2O on a Bruker Avance 400 spectrometer). ^{1}H NMR spectra performed at ISM – Institut des Sciences Moléculaires, Groupe Synthèse-Molécules Bioactives at Bordeaux University, were measured in $CDDI_3$ or $CDDI_3$/MeOD 1:1 on a Bruker 200 MHz spectrometer.

All LC-ESI-MS analysis were performed in a Finnigan Surveyor Plus HPLC system fitted with a PDA Plus detector, an autosampler Plus and a LC quaternary pump plus

coupled to a Finnigan LCQ Deca XP Plus mass detector equipped with a ESI source and an ion trap quadrupole equipped with an atmospheric pressure ionization (API) source. The stationary phase was a Thermo Finnigan Hypersil Gold column (150 x 4.6 mm i.d., 5 mm). The mass spectrometer was operated in the negative-ion mode with source, with a capillary temperature of 275ºC and capillary voltages of 4.5 kV. The mass spectra were recorded between 250 and 2000 m/z. The mobile phase was composed by solvent A, 0.1% (v/v) formic acid, and solvent B, 100% (v/v) methanol. The flow rate was 0.20 mL/min and the gradient method started with a linear gradient ranging from 90% A to 60% A in 90 minutes, then reaching 100% B in 5 minutes and a final isocratic gradient of 100% B during 5 minutes.

HPLC analysis were performed on a Thermo® Scientific HPLC with a Thermo® Scientific Spectra System P4000 pump using a 250 × 4.6 mm i.d. reversed-phase C18 column (Merck®, Darmstadt) at 25 °C. The detection was carried out between 200 and 800 nm using a Thermo® Scientific Spectra System UV8000 diode array detector; 30 µl of each sample was injected using an autosampler Thermo® Scientific Spectra System AS3000. The solvents were A: water/acetic acid (2.5%), and B: 20% solvent A and 80% acetonitrile. The gradient consisted of an isocratic gradient 7% B for 5 minutes, followed by 7–20% B for 85 min and 20-100% in 5 minutes and a final isocratic gradient of 100% B for 5 minutes at a flow rate of 1.0 ml/min.

2.4.1. Benzylation of monomeric flavan-3-ols

2.4.1.1. Using benzyl chloride (BnCl)

To a stirred suspension of sodium hydride (NaH) (4.25 eq for **16** and 8.25 eq for **17**) in anhydrous dimethylformamide (DMF) under nitrogen at -78°C in a sealed reaction flask, was sequentially added a solution of (+)-C **16** and (-)-EGC **17** (1 eq) in dry DMF and BnCl (5 eq for **16** and 8 eq for **17**) in one batch *via* a syringe. The mixture was stirred at this temperature for 15 minutes, the acetone bath removed and stirring continued at room temperature for 7 hours. The reactions were quenched by adding 1N HCl (2 mL) and water (10 mL). The aqueous layer was extracted with ethyl acetate (EtOAc) and the organic layer washed with hexane to remove mineral oil and water to remove acid. The organic layer was dried over sodium sulphate (Na_2SO_4), and evaporated to dryness. The O-benzylated products were purified by silica gel column chromatography using hexane/EtOAc (2:1, v/v) as eluent.

2.4.1.2. Using benzyl bromide (BnBr)

To a stirred solution of (+)-C **16**, (-)-EGC **17** and (-)-EGCG **18** in dry DMF, under argon, was added potassium carbonate (K_2CO_3) (6 eq for **16**; 10 eq for **17**; 17.6 eq for **18**) and BnBr (4.3 eq for **16**; 7.7 eq for **17**; 13.6 eq for **18**). The solution was stirred at 0ºC for 2 hours and left at room temperature for 48 hours for **16**, 72 hours for **17** and 24 hours for **18**. The mixture was extracted with ethyl acetate and water, dried over Na_2SO_4, filtered and concentrated. The crude product was purified with silica gel column chromatography (dichloromethane (CH_2Cl_2) for **19** and **20** and hexane/ EtOAc 2:1 for **21**).

2.4.2. Benzylation at C4

To a solution of EGC5Bn **20** and benzyl alcohol (BnOH) (10.3 eq) in CH_2Cl_2 was slowly added, at 0ºC, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (2.3 eq). After reacting overnight at room temperature, it was added 4-dimethylaminopyridine (DMAP) (2,4 eq) at 0ºC and left to react for 30 minutes. Then the mixture was filtered and washed with water and brine, dried over Na_2SO_4, filtered and concentrated. The crude product was purified with silica gel column chromatography with EtOAc 2:1 as eluent to afford EGC5Bn(Bn) **22**.

2.4.3. Condensation

EGC5Bn(Bn) **22** and C4Bn **19** (4 eq), EGC5Bn **20** (4 eq) or EGCG8Bn **21** (4 eq) were dissolved in CH_2Cl_2 and trimethylsilyl trifluoromethanesulfonate ($TMSOT_f$) (0.5 M solution in CH_2Cl_2, 1.5 eq) was added dropwise at -78ºC. Therefore the proper time of reaction was tested by following each reaction by TLC. At first the solutions were left to react for 5 min, following the method described by Krohn et al. (Krohn, Ahmed, John, Letzel, & Kuck, 2010). However, after that period of time and after checking the reaction products by TLC, it was observed that the upper unit (and limiting reagent) EGC5Bn(Bn) was still present in good quantity. Thereby, the solutions were left stirring for 60, 120 and 90 min respectively (reaching -22ºC), and left to reach 0ºC for 2h 20 min, 80 min and 3h 30 min. The reactions were then quenched by addition of saturated aqueous sodium bicarbonate (Na_2HCO_3) (1 mL). The mixture was extracted with chloroform and the organic phase was washed with water and brine, dried over Na_2SO_4, filtered and concentrated. The crude product

was purified with silica gel column chromatography with hexane/ethyl acetate 1:1, hexane/EtOAc 2:1 and CH_2Cl_2, respectively, as eluent to afford EGC-C9Bn **23**, EGC-EGC10Bn **24** or EGC-EGCG13Bn **25**.

2.4.4. Hydrogenolysis

2.4.4.1. Hydrogenolysis with palladium on carbon

General procedure using a hydrogen reactor. A solution of EGC-C9Bn **23** (under argon) and 0.5 eq for each removing group of 10% palladium-charcoal catalyst (Pd/C 10%) (10-20% by weight), in THF/MeOH/H_2O 20/20/1 (V/V), were placed in a hydrogen reactor. The reaction proceeded at 1.5 bar of H_2 (g) pressure for 3 hours. When the reaction was complete, each mixture was filtered through a 0.20 mm PET Chromafil® syringe filter and the solvent was evaporated under vacuum.

General procedure at atmospheric pressure. A solution of EGC-C9Bn **23** (under argon) and 0.5 eq for each removing group of Pd/C 10%, in THF/MeOH/H_2O 20/20/1 (V/V), were placed in a schlenk flask. A balloon attached to the flask provided the hydrogen. When the reaction was complete, each mixture was filtered through a 0.20 mm PET Chromafil® syringe filter and the solvent was evaporated under vacuum.

2.4.4.2. Hydrogenolysis with palladium hydroxide on carbon

General procedure using a hydrogen reactor. A solution of EGC-C9Bn **23** (under argon) and 0.5 eq of $Pd(OH)_2$/C 20% for each removing group, in THF/MeOH/H_2O 20/20/1 (V/V), were placed in a hydrogen reactor. The reaction proceeded at 1.5 bar of H_2 (g) pressure. When the reaction was complete, each mixture was filtered through a 0.20 mm PET Chromafil® syringe filter and the solvent was evaporated under vacuum.

General procedure at atmospheric pressure. A solution of EGC-C9Bn **23** and EGC-EGC10Bn **24** (under argon) and 0.5 eq of $Pd(OH)_2$/C 20% for each removing group, in THF/MeOH/H_2O 20/20/1 (V/V), were placed in a schlenk flask. A double balloon attached to the flask provided the hydrogen. When the reaction was complete, each mixture was filtered through a 0.20 mm PET Chromafil® syringe filter

and the solvent was evaporated under vacuum.

Later this reaction was tested with 100% (m/m) of $Pd(OH)_2$/C 20% . The catalyst and the compound were placed (under argon for 10 min) in a glass balloon with a rubber septum with two needles. Then it was added 1-2 mL of MeOH/THF/AcOEt 1:1:1 and placed the double layer balloon filled with H_2 (g) attached to a long needle. The H_2 was left to bubble for 3-4 min to saturate the catalyst and then the needle was lifted to the headspace of the glass balloon. The outlet needle was then removed.

2.4.4.3. Hydrogenolysis *in situ*

To a stirred solution of EGC-C9Bn **23** and EGC-EGCG13Bn **25** under argon and Pd/C 10% in MeOH (2-3 mL) was added neat triethylsilane (TES) (10 mmol for each removing group). A few drops of THF were added to dissolve the reagents. When the reaction was complete (TLC), each mixture was filtered through a 0.20 mm PET Chromafil® syringe filter and the solvent was evaporated under vacuum.

2.4.4.1. Hydrogenolysis *in situ* using the H-cube® equipment

The following hydrogenolysiss were performed at the ISM – Institut des Sciences Moléculaires, Groupe Synthèse-Molécules Bioactives at Bordeaux University I, under the supervision of Professor Eric Fouquet and Professor Philippe Garrigues.

EGC-C9Bn **23**, EGC-EGC10Bn **24** and EGC-EGCG13Bn **25** were dissolved in THF/AcOEt/MeOH 1:1:1 and filtered through a 0.45 mm PET Chromafil® syringe filter. Then the solutions were injected in the H-cube® and the resulting product collected at the equipment outlet.

2.5. Results and Discussion

2.5.1. Benzylation of monomeric flavan-3-ols

Benzyl chloride was used to protect the starting monomers C (**16**), EGC (**17**) and EGCG (**18**) in the presence of potassium carbonate as a weak base and DMF as a solvent. Benzyl ethers are stable under basic or mildly acidic conditions.

2.5.1.1. Using BnCl

Following a method described by Mustafa et al. (Mustafa, Khan, Khan, & Ferreira, 2004), they describe a 90% yield for C **16** and a 85% yield for EGC **17**. However, ESI-MS and 1H-NMR analysis did not allow to detect the presence of the respective O-benzylated compounds **19** and **20**. In fact, TLC analysis to the reaction mixture revealed the presence of various other compounds, including unreacted BnCl.
Krohn et al. performed this catechin benzylation at 120-130°C in the presence of K_2CO_3 in DMF and it was completed within 5 hours with 80 % yield. However, it yielded 5% of pentabenzylated catechin (C5Bn) as side product (Ahmed, 2007).

2.5.1.2. Using BnBr

The compounds C4Bn **19**, EGC5Bn **20** and EGCG8Bn **21** were prepared by simple protection of the phenol functions of C **16**, EGC **17** and EGCG **18** using BnBr. It followed a method described by Fabre (Fabre, 2009) for C4Bn obtainment. The reaction time for the protection of EGC **17** and EGCG **18** were tested and 72 hours for **17** and 24 hours for **18** revealed to be the most appropriate.
This method yielded much better results then the previous one.

5,7,3',4'-Tetra-*O*-benzylcatechin (C4Bn) (19). Amorphous white solid, yield 95.3%. ESI-MS found $[M+H]^+$: 651. ^{1}H NMR ($CDCl_3$) δ/ppm: 2.64 (H4α, dd, J=5.6; 16.4 Hz), 3.09 (H4β, dd, J=9.0; 16.4 Hz), 4.62 (H2, d, J=8.1 Hz), 4.05 (H3, m), 5.02, 5.03, 5.15, 5.16 (OCH_2Bn, 4s), 6.21 (H6, d, J=2.3 Hz), 6.26 (H8, d, J=2.3 Hz), 6.94 (H5' and H6', br s), 7.02 (H2', br s), 7.27-7.44 (H-Ar, m); ^{13}C NMR ($CDCl_3$) δ/ppm: 27.58 (C4), 65.32 (C3), 68.11, 69.87, 70.10, 71.18, 71.24 (OCH_2Bn), 81.54 (C2), 93.79 (C6), 94.37 (C8), 102.26 (C4a), 113.82 (C2'), 114.85 (C5'), 120.56 (C6'), 130.93 (C1'), 126.99-128.51, 136.82-137.09 (C-Ar), 149.05 (C3'), 149.30 (C4'), 155.27 (C8a), 157.78 (C5), 158.76 (C7).

5,7,3',4',5'-Penta-*O*-benzylepigallocatechin (EGC5Bn) (20). Amorphous buff solid, yield 83.9%. ESI-MS found $[M+H]^+$: 757. ^{1}H NMR ($CDCl_3$) δ/ppm: 2.98 (H4, ABX), 4.20 (H3, br), 4.88 (H2, s), 5.01, 5.06, 5.13 (OCH_2Bn, 3s), 6.28 (H6 and H8, s), 6.81 (H2' and H6', s), 7.23-7.43 (H-Ar, m); ^{13}C NMR ($CDCl_3$) δ/ppm: 28.12 (C4), 66.30 (C3), 69.88, 70.08, 71.23 (OCH_2Bn), 78.63 (C2), 94.17 (C8), 94.75 (C6), 101.14

(C4a), 106.16 (C2' and C6'), 126.90-138.20 (C-Ar), 140.99 (C4'), 152.96 (C3' and C5'), 155.21 (C5), 158.27 (C8a), 158.78 (C7).

5,7,3',4',5'-Penta-*O*-benzyl-3-*O*-(3,4,5-tri-*O*-benzylgalloyl)epigallocatechin (EGCG8Bn) (21). Amorphous yellow solid, yield 97.2%. ESI-MS found $[M+H]^+$: 1179. ^{1}H NMR ($CDCl_3$) δ/ppm: 3.22 (H4, ABX), 4.25 (H3, br), 5.05 (H2, s) 5.10-5.16 (OCH_2Bn, m), 6.87 (H2'' and H6'', br s), 6.54 (H2' and H6', s), 7.24-7.51 (H-Ar, m); ^{13}C NMR ($CDCl_3$) δ/ppm: 26.32 (C4), 68.55 (C3), 70.16, 70.30, 71.20, 71.33, 75.16, 75.25 (OCH_2Bn), 78.03 (C2), 94.23 (C6), 94.92 (C8), 101.25 (C4a), 106.94 (C2' and C6'), 109.29 (C2'' and C6''), 127.35-128.69 (C-Ar), 130.01 (C1'), 136.57 (C1''), 136.93-138.61 (C-Ar), 142.90 (C4''), 152.58 (C3' and C5'), 153.07 (C3'' and C5''), 133.40 (C4'), 155.84 (C8a), 158.19 (C5), 159.04 (C7), 164.94 (CO).

2.5.2. Benzylation at C4

The abbreviation chosen for this compound was EGC5Bn(Bn) instead of EGC6Bn to illustrate that an extra –OBn group is added to the molecule and does not occur another benzylation of a pre-existing hydroxyl group.

The oxidation of benzylic C–H bonds by DDQ gives quinol ethers that have been reported previously (Foster & Horman, 1966; Wallace, Gibb, Cottrell, Kennedy, Brands, & Dolling, 2001). Batista *et al.* have isolated benzylic ethers resulting from the oxidative C–H activation of arylmethanes by DDQ (Batista, Crabtree, Konezny, Luca, & Praetorius, 2012). A combination of experimental and computational studies suggests that this C–H activation occurs through hydride abstraction by DDQ. It is, in fact, an oxidative nucleophilic substitution (Lemaire, Doussot, & Guy, 1988). DMAP it is a useful nucleophilic catalyst for a variety of reactions.

The activation at C4 of the upper unit with a good leaving group is essential for the cationic coupling activated with a Lewis acid that will follow next. This step can be performed by functionalization with a heteroatom such as a bromine, sulphur or oxygen (Hemingway & Foo, 1983; Steenkamp, Ferreira, & Roux, 1985; Steynberg, Nel, van Rensburg, Bezuidenhoudt, & Ferreira, 1998). According to this method, multiple nucleophilic such as water or alcohols can be used.

The electrophile **22** was prepared from his corresponding EGC5Bn **20** by treatment with DDQ for benzylic oxidation. DDQ oxidation at C4 was performed with benzyl alcohol as the nucleophile and DMAP as nucleophilic catalyst. The reaction proceeded smoothly to give EGC5Bn(Bn) **22** in 98.3% isolated yield.

4,5,7,3',4',5'-Hexa-*O*-benzylepigallocatechin ((EGC5Bn)Bn) (22). Amorphous buff solid, yield 98.3%. ESI-MS found $[M+H]^+$: 863. ^{1}H NMR ($CDCl_3$) δ/ppm: 3.93 (H3, m), 4.22 (H4, m), 4.73 (OCH_2Bn, d, J=1.8 Hz), 5.04 (OCH_2Bn, d, J=4.5 Hz), 5.13 (OCH_2Bn, s), 5.22 (H2, s), 6.28 (H8, d, J=2.3 Hz), 6.31 (H6, d, J=2.2 Hz), 6.77 (H2' and H6', s), 7.25-7.44 (H-Ar); ^{13}C NMR ($CDCl_3$) δ/ppm: 28.91 (C4), 70.18 (C3), 70.46, 70.57, 71.44, 72.17, 75.11, 75.38 (OCH_2Bn), 94.21 (C6), 94.52 (C8), 106.32 (C2' and C6'), 127.66-129.09 (C-Ar), 133.44 (C1'), 134.53, 136.46, 136.77, 137.11, 137.92, 138.34 (C-Ar), 139.04 (C4'), 153.13 (C5'), 156.04 (C3'), 159.90 (C5), 160.77 (C7).

2.5.3. Condensation

At first the solutions were left to react for 10-15 min, following the method described by Krohn et al. (Krohn, Ahmed, John, Letzel, & Kuck, 2010). However, after that period of time and after checking the reaction products by TLC, it was observed that the upper unit (and limiting reagent) EGC5Bn(Bn) **22** was still present in good quantity. Therefore, the solutions were left stirring for 60, 120 and 90 min respectively (reaching -22ºC), and left to reach 0ºC for 2h 20 min, 90 min and 3h 30min, respectively.

Couplings of monomeric flavans are usually performed in Friedel-Crafts-type reactions. Electron-rich aromatics such as the C4Bn **19**, EGC5Bn **20** and EGCG8Bn **21** represent the nucleophilic units, whereas their benzyloxylated counterparts **22**, after activation with a Lewis acid (TMSOTf), act as the electrophiles (see figure 29).

Higher oligomer formation cannot be avoided completely but it could be minimized by adding the nucleophilic unit **19**, **20** or **21** in excess (which later can be recovered). By using the nucleophilic unit in excess it also prevents the need for protection at the electrophile C8 position.

From this condensation reaction, two possible isomers may result at the C4 position of the electrophile i.e. 4α or 4β flavanyl linkage. This linkage is controlled by the neighboring group participation of the OH present at the C3. NMR studies showed two rotational isomers at C4 to C8 position. These rotamers are not separable and these are in the ratio of 2:1 in CD_3Cl. Condensation at lower temperature increased this ratio up to 55:1 (Ahmed, 2007). In fact, the use of a solution of TMSOTf in CH_2Cl_2 (0.5M) followed the procedure of Saito et al. (Saito, Tanaka, Ubukata, & Nakajima, 2004), because it has been reported that the TMSOTf-catalyzed intermolecular condensation is very specific for the formation of the natural 3,4-*trans*

isomers.

5,7,3',4',5'-Penta-*O*-benzylepigallocatechin(4→8)5,7,3',4'-Tetra-*O*-benzylcatechin (EGC-C9Bn) (23). Amorphous white solid, yield 66.9%. ESI-MS found $[M+H]^+$: 1407. ^{1}H NMR ($CDCl_3$) δ/ppm: 2.58-2.66 (H-4F, m), 3.80-3.86 (H-3F, m), 3.89-3.95 (H-3C, m), 4.06 (H-4C, d, J=7.0 Hz), 3.08 (H-2F, d, J=5.3 Hz), 4.58 (H-2C, d, J=7.6 Hz), 4.92-5.00 (H-Ar, m), 5.15 (OCH_2Bn, s), 6.20 (H-6C, d, J=2.3 Hz), 6.24 (H-6F, s), 6.25 (H-8C, d, J=2.3 Hz), 6.89 (H-2'C and H-6'C, s), 7.00 (H-6'F and H-5'F, br s), 7.03 (H-2'F, s), 7.20-7.41 (H-Ar, m); ^{13}C NMR ($CDCl_3$) δ/ppm: 27.72 (C-4F), 28.81 (C-4C), 68.14 (C-3F), 69.95, 70.08, 70.15, 70.61, 71.12, 71.25, 71.34, 77.38 (OCH_2Bn), 71.28 (C-3C), 81.41 (C-2C), 81.59 (C-2F), 91.34 (C-6F), 93.91 (C-6C), 102.42 (C-2'C and C-6'C), 102.62 (C-4aF and C-4aC), 110.38 (C-8F), 114.02 (C-2'F), 115.01 (C-5'F), 120.63 (C-6'F), 129.96 (C-1'C), 131.15 (C-1'F), 131.53 (C-4'C), 127.18-128.98, 136.98-137.43 (C-Ar), 149.09 (C-3'F), 149.10 (C-4'F), 149.33 (C-3'C), 153.11 (C-8aF), 155.36 (C-7C), 155.69 (C-8aC), 155.95 (C-7F), 157.83 (C-5F), 158.86 (C-5C).

5,7,3',4',5'-Penta-*O*-benzylepigallocatechin(4→8)5,7,3',4'-Tetra-*O*-benzylcatechin (EGC-EGC10Bn) (24). Amorphous pale yellow solid, yield 55.3%. ESI-MS found $[M+H]^+$: 1511. ^{1}H NMR ($CDCl_3$) δ/ppm: 3.37-3.45 (H-4F, m), 3.71 (OH, br), 3.89 (H-4C, d, J=4.1 Hz), 4.35 (H-3F, m), 4.67 (H-3C, tr), 4.89 (H-2C and H-2F, d, J=4.0 Hz), 4.98-5.08 (OCH_2Bn, m), 5.56 (H-6F, s), 5.72 (H-6C and H-8C, s), 6.41 (H-2'C, H-6'C, H-2'F and H-6'F, s), 7.10-7.44 (H-Ar, m); ^{13}C NMR ($CDCl_3$) δ/ppm: 14.12 (C-4F), 15.67 (C-4C), 19.56 (C-3F), 20.90, 21.34, 29.68 (OCH_2Bn), 28.90 (C-3C), 66.25 (C-2C), 69.92 (C-2F), 71.26 (C-6F and C-6C), 75.26 (C-8C), 94.12 (C-2'C and C-6'C), 94.77 (C-2'F and C-6'F), 101.07 (C-4aF and C-4aC), 105.61 (C-8F), 127.14-128.83, 137.61 (C-Ar), 134.53 (C-1'C), 136.33 (C-1'F), 136.92 (C-4'C and C-4'F), 152.91 (C-3'F, C-5'F, C-3'C and C-5'C), 155.11 (C-8aF), 158.21 (C-7C and C-5F), 158.71 (C-8aC and C-7F), 160.60 (C-5C).

5,7,3',4',5'-Penta-*O*-benzylepigallocatechin(4→8)5,7,3',4'-Tetra-*O*-benzylcatechin (EGC-EGCG13Bn) (25). Yellow oil, yield 92.1%. ESI-MS found $[M+H]^+$: 1935. ^{1}H NMR ($CDCl_3$) δ/ppm: 4.12 (H-4C, br), 4.39 (H-3C, br), 4.60 (H-2C, d, J=1.9 Hz), 4.73-5.00 (OCH_2Bn, m), 5.44 (H-3F, m), 5.81 (H-2F, d, J=2.2 Hz), 6.15 (H-6F, s), 6.23 (H-8C and H-6C, s), 6.42 (H-2'C, H-6'C, H-2'F and H-6'F, s), 6.97 (H-2G and H-6G, s), 7.04-7.24 (H-Ar, m); ^{13}C NMR ($CDCl_3$) δ/ppm: 15.51 (C-4F and C-4C), 19.72

(C-3F and C3), 20.81, 21.41 (OCH_2Bn), 28.87 (C-2C and C-2F), 71.41 (C-2'F and C-2'C), 75.00 (C-8F, C-2G and C-6G), 125.73, 126.00, 127.76, 128.06, 128.82 (C-Ar), 127.05 (C-1G), 128.21 (C-1'F, C-4'F, C-1'C and C-4'C), 129.50 (C-4G), 129.74 (C-3'C, C-5'C, C-3'F, C-5'F, C-3G and C-5G), 134.50 (C-8aF), 136.34 (C-8aC), 137.53 (C-5F, C-7F and C-7C), 144.07 (C-5C), 153.06 (CO).

2.5.4. Hydrogenolysis

2.5.4.1. Hydrogenolysis with palladium on carbon

Palladium on activated carbon 10 wt.% (Pd/C 10%) is extensively used as a heterogeneous catalyst for hydrogenolysis in synthetic organic chemistry because of its high catalyst activity, cost efficiency and easy separation from the reaction mixture.

Concerning safety and the possibility of using a thigh vessel with air and pressure control, we decided to use a hydrogen reactor. The gaseous H_2 pressure was set at 1.5 bar and EGC-C9Bn **23** was de-benzylated for 3 hours. After ESI-MS analysis by direct injection it was concluded that the de-benzylation was not completed and it yielded basically C **16**. This demonstrates that a rupture of the interflavan bond of the desired EGC-C **26** dimer occurred. This procedure was carefully repeated but the result was the same.

2.5.4.2. Hydrogenolysis with palladium hydroxide on carbon

The palladium hydroxide on carbon, $Pd(OH)_2/C$ 20 wt.%, is generally known as Pearlman's catalyst. This is probably the most used catalyst for PA debenzylation (Arnaudinaud, et al., 2001; Ferreira & Coleman, 2011; Ferreira & Slade, 2002; Mohri, Sagehashi, Yamada, Hattori, Morimura, Kamo, et al., 2007; Saito, Nakajima, Tanaka, & Ubukata, 2003). The use of $Pd(OH)_2/C$ in THF/H_2O at room temperature, instead of Pd/C 10% in dioxanne at 90°C, for deprotection, allowed Arnaudinaud *et al.* to avoid the formation of benzylated catechin by-product resulting from the cleavage of the interflavanolic linkage (Arnaudinaud, et al., 2001). Tarascou *et al.* first attempts using Pd/C 10% leaded to partial debenzylation and Pearlman's catalyst was found to be far more efficient (Tarascou, Barathieu, André, Pianet, Dufourc, & Fouquet, 2006). Various solvents were tried, including alcohols, ethyl acetate and THF, and a 1:1 mixture of MeOH/AcOEt was found to offer a good dissolution of the starting and final

compounds.

This reaction was tested for compound **23** using the hydrogen reactor, according with the procedure earlier described. After 4 hours, the reaction was stopped and analyzed by LC-ESI-MS (Figure 30).

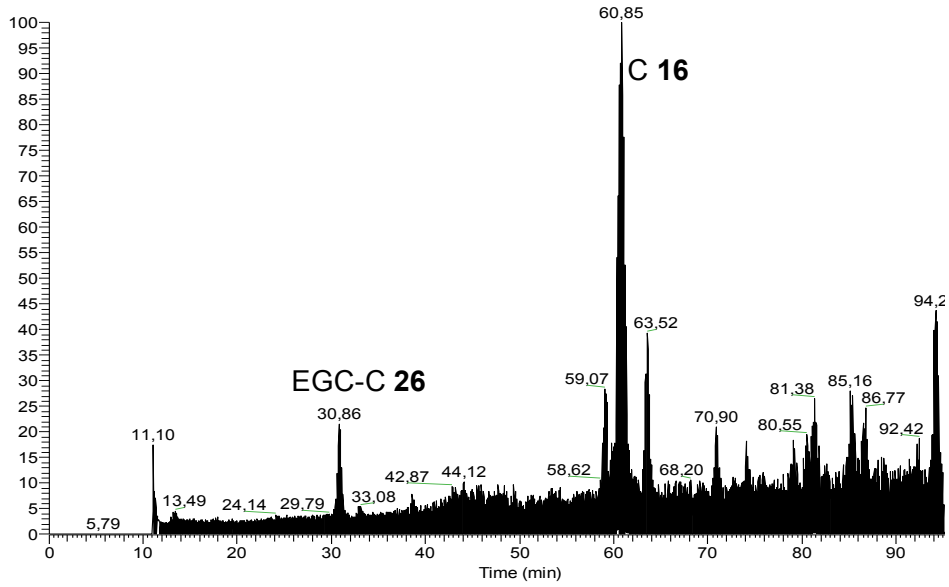


Figure 30. LC-ESI-MS chromatogram for EGC-C9Bn **23** hydrogenolysis with $Pd(OH)_2/C$ using the hydrogen reactor

The result was a mixture of C **16** (in larger amount), EGC-C **26** and other by-products. The presence of C **16** demonstrates, again, that a rupture of the interflavan bond occurred.

Next, we tested this reaction with EGC-EGC10Bn **24** but this time using a schlenk flask and gaseous H_2 provided by a double layer balloon. After 3h 30 min of reaction, and again after 21 hours, the initial compound was still detected, along with EGC **17** and EGC-EGC **27**. After 26 hours, the reaction mixture was analysed by ESI-MS by direct injection. The pseudomolecular ions with *m/z* $[M]^-$ 305 and 609 corresponding to EGC **17** and EGC-EGC **27** were again detected as well as the pseudomolecular ion with *m/z* $[M]^-$ 782, probably corresponding to EGC-EGC2Bn (since the TLC also revealed the presence of benzylated compounds), demonstrating that this was not a complete reaction.

Kozikowski *et al.* reported that it was advisable to perform this reaction in bicarbonate-washed glassware, as partial fragmentation to lower oligomers was occasionally observed without this precaution, quite probably as a consequence of an acidic reaction of the glass surface of the reaction flask (Jacques, Haslam, Bedford, & Greatbanks, 1974; Kozikowski, Tückmantel, Böttcher, & Romanczyk, 2002). Therefore we decided to rinse the hydrogen reactor with a sodium

bicarbonate solution. After 15 min an aliquot was taken for ESI-MS analysis by direct injection and it were detected pseudomolecular ion with *m/z* $[M]^-$ 305 corresponding to EGC **17**, *m/z* $[M]^-$ 607 probably corresponding to the oxidize form of EGC-EGC **27** and *m/z* $[M]^-$ 1513 corresponding to the initial compound EGC-EGC10Bn **24**. After 30 min all compounds were degradated.

Tarascou *et al.* worked with benzylated compounds bromated at the C8 of the initial monomer unit to prevent oligomerization reactions. The first attempt to de-benzylate the compounds with $Pd(OH)_2$ led to a mixture of partially debrominated procyanidin dimers. Then they optimized the reaction by using triethylamine (Tarascou, Barathieu, André, Pianet, Dufourc, & Fouquet, 2006). The amine first activates the catalyst by reducing it *in situ* to Pd^0, but also traps the generated hydrobromic acid, thus avoiding premature degradation of the native procyanidin dimers. Therefore we decided to try to de-benzylate our compounds adding 1 drop of triethylamine (Et_3N) to the mixture.
A comparative study was carried out using EGC-C9Bn **23**. Starting with equal amounts of compound **23** and adding 5.22 eq of $Pd(OH)_2$/C 20% in equal amounts of AcOEt and MeOH (with a drop of Et_3N), one reaction took place in the hydrogen reactor and the other on a schlenk flask. After 1 hour no de-benzylation was noticed and after 3 hours of reaction only C **16** was detected by ESI-MS analysis by direct injection.

Later, we decided to try to increase the amount of catalyst. Therefore we used not 0.5 eq of $Pd(OH)_2$/C for each removing group but 100% (m/m) of $Pd(OH)_2$/C.

2.5.4.3. Hydrogenolysis *in situ*

In situ generation of molecular hydrogen by addition of TES to Pd-C catalyst results in rapid and efficient reduction of multiple bonds, azides, imines, and nitro groups, as well as benzyl group and allyl group deprotection under mild, neutral conditions. The reactions are carried out at room temperature and are rapid, often complete in 10 min or less using excess TES and 10-20% Pd/C (by weight) in MeOH. The conditions are neutral, and thus acid- or base-sensitive substrates can be reduced without damage (Mandal & McMurray, 2007).

This method was first tested with C4Bn **19**. It was used 15% by weight of Pd/C 10% and 40 eq of TES. After 17 hours reacting, still not all the amount of reagent was de-benzylated. It was decided to add another 1.5 mg of Pd/C 10% and 80 eq of TES.

After 2 hours there was no sign of any benzylated C, but degradation products started to appear. This behavior was again reported when starting with 25% by weight of Pd/C 10% and 80 eq of TES.

Meanwhile it was also tested for PCB3-8Bn. It was used 10% by weight of Pd/C 10% and 80 eq of TES. After 16 hours it was added another 80 eq of TES, and repeated after 18 hours plus 2 mg of Pd/C 10%. The result was analyzed by HPLC (Figure 31) and, by comparing the chromatogram with standards, it was possible to identify PCB3 and C **16**.

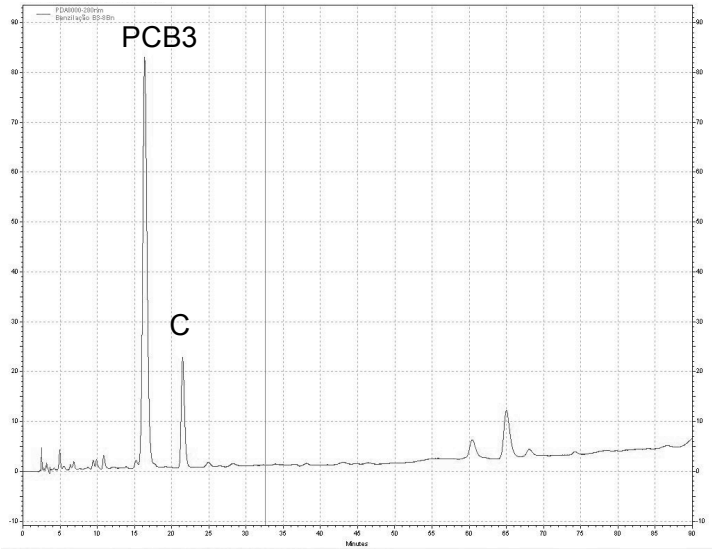


Figure 31. HPLC chromatogram for PCB3-8Bn hydrogenolysis *in situ*

When trying this method for C4Bn(Bn) de-benzylation, it was mostly obtained C **16**, but also PCB3 and C-4-OH (Figure 32).

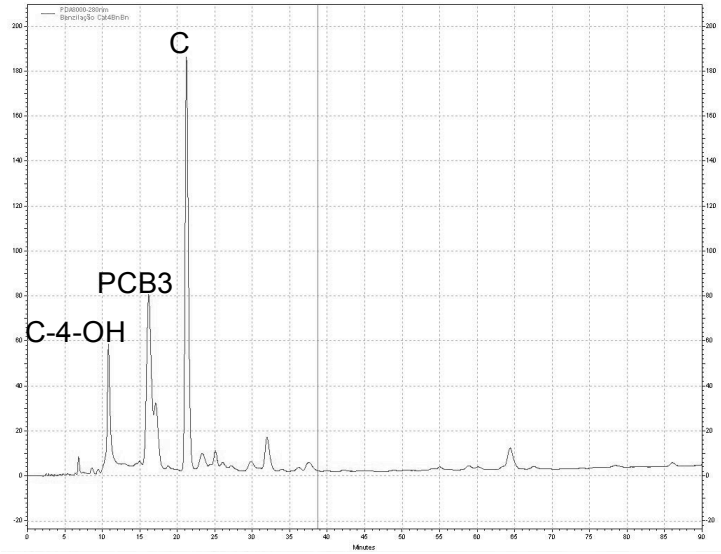


Figure 32. HPLC chromatogram for C4Bn(Bn) hydrogenolysis *in situ*

Next we tested this *in situ* method with EGC-C9Bn **23**. It was used 12% by weight of Pd/C 10% and 90 eq of TES. After reacting 18 hours, a control TLC was done to confirm if more TES was needed. Then the reaction mixture was filtered through a 0.20 mm PET Chromafil® syringe filter, the solvent evaporated under vacuum and analyzed by HPLC (Figure 33).

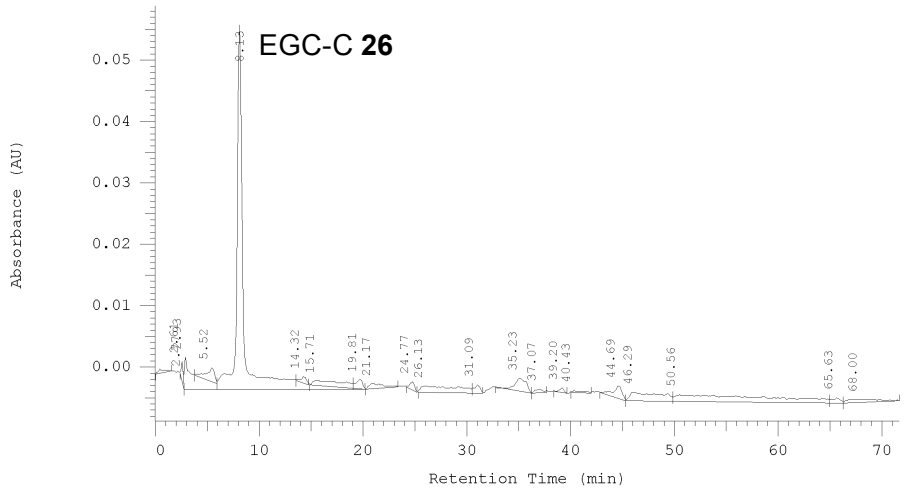


Figure 33. HPLC chromatogram for EGC-C9Bn **23** hydrogenolysis *in situ* at λ = 280 nm

This procedure was repeated in larger scale and immediately applied to a TSK Toyopearl HW40(s) gel (Tosoh, Japan) chromatography column (250 x 16 mm i.d.) connected to a ultraviolet (UV) detector. The eluent used was MeOH. The first peak was registered after 120 min and the second after 200 min.
ESI-MS analysis by direct injection revealed that the first peak corresponds to C **16** and the second peak to EGC-C **26**. This means that this procedure leaded to the breaking of the interflavan link and almost all the desired final compound was reduced to its low monomeric unit. The isolated amount was not enough for NMR analysis.

The same procedure was repeated with EGC-EGCG13Bn **25**. Again, it was noticed that the breaking of the interflavan link did occurred, as well as degradation products. However it was possible to obtain 6.4 mg of EGC-EGCG **28** and analyse it by NMR.

Epigallocatechin(4→8)epigallocatechin gallate (EGC-EGCG) (28). White powder, yield 6%. ESI-MS found $[M+H]^-$: 761. The ^1H and ^{13}C-NMR chemical shifts are reported in table 4 and COSY correlations in figure 34.

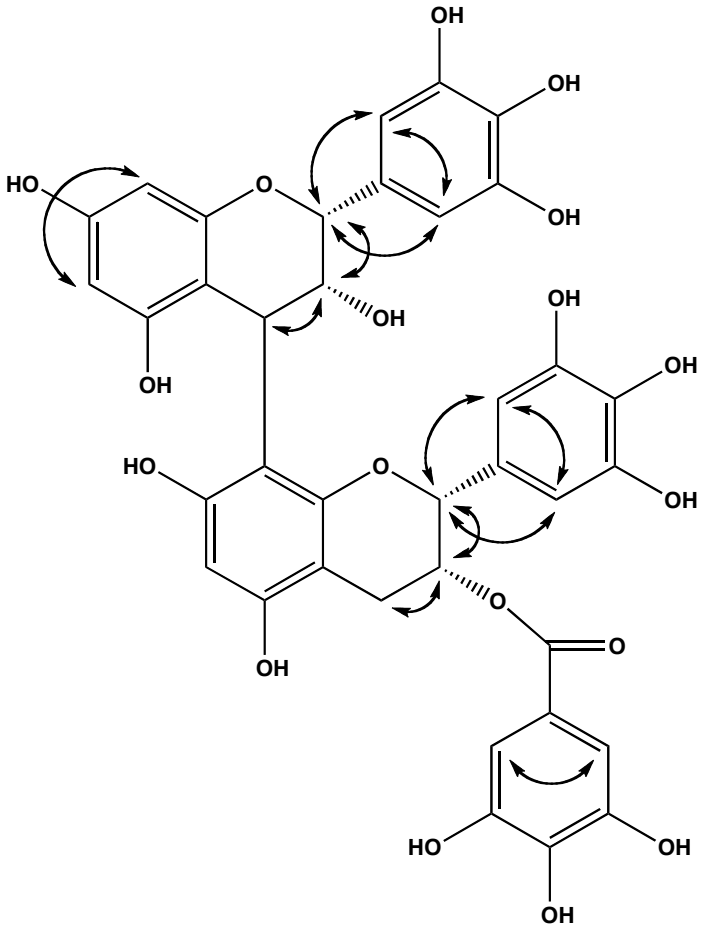


Figure 34. COSY correlations of EGC-EGCG **28**.

Table 4. ^{1}H and ^{13}C-NMR data and HMBC and HSQC correlations of EGC-EGCG **28**, determined in MeOD.

Position	δ ^{1}H (ppm) J (Hz)	δ ^{13}C (ppm)	HMBC	HSQC	NOESY
2C	5.20; br	79.1	H-2'C; H-6'C	H-2C	--
3C	4.58; tr, 3.3	69.2	H-2C	--	H-4C; H-2C; H-6F
4C	4.19; d, 3.1	29.4	H-2C	--	--
4aC	--	102.5	--	--	--
5C	--	154.9	--	--	--
6C	6.08; br d	98.8	--	H-6C	--
7C	--	150.1-152.3	--	--	--
8C	6.10; br d	98.8	--	H-8C	--
8aC	--	150.1-152.3	--	--	--
1'C	--	130.5	H-2'C	--	--
2'C	6.77; s	105.9	H-2C; H-4C; H-6'C	H-2'C	--
3'C	--	145.2	H-2'C	--	--
4'C	--	133.2	H-2'C	--	--
5'C	--	145.2	H-6'C	--	--
6'C	6.77; s	105.9	H-2C; H-4C; H-2'C	H-6'C	--
2F	5.71; br d	79.1	H-4F; H-2'F; H-6'F	--	--
3F	5.68; br	66.7	H-4F	H-3F	--
4F	2.99; dd, 17.8 3.09; dd, 17.5	22.8	--	H-4F	H-2C; H-6C
4aF	--	102.5	H-4F	--	--
5F	--	154.9	--	--	--
6F	6.21; s	98.8	--	H-6F	--
7F	--	150.1-152.3	--	--	--
8F	--	128.0	--	--	--
8aF	--	150.1-152.3	--	--	--
1'F	--	130.5	H-2'F	--	--
2'F	6.77; s	100.7	H-6'F	--	--
3'F	--	145.2	H-2'F	--	--
4'F	--	133.2	H-2'F	--	--
5'F	--	145.2	H-6'F	--	--
6'F	6.77; s	100.7	H-2'F	--	--
1G	--	119.7	--	--	--
2G	6.85; s	108.9	--	H-2G	--
3G	--	139.6	H-2G	--	--
4G	--	141.8	--	--	--
5G	--	139.6	H-6G	--	--
6G	6.85; s	108.9	--	H-6G	--
C=O	--	166.5	H-2G; H-6G	--	--
OH	4.99; br	--	--	--	--
3C-OH	4.04; br	--	--	--	--

2.5.4.4. Hydrogenolysis *in situ* using the H-cube equipment

The H-Cube® is a bench-top hydrogenolysis reactor for continuous hydrogenolysis reactions using a disposable catalyst cartridge system. The hydrogenolysis reactions are performed in a flow system where the necessary hydrogen gas for the reaction is generated in-situ from the electrolysis of water. Reactions take place on disposable proprietary CatCarts®, packed catalyst columns modelled after conventional HPLC systems (Jones, Godorhazy, Varga, Szalay, Urge, & Darvas, 2006).

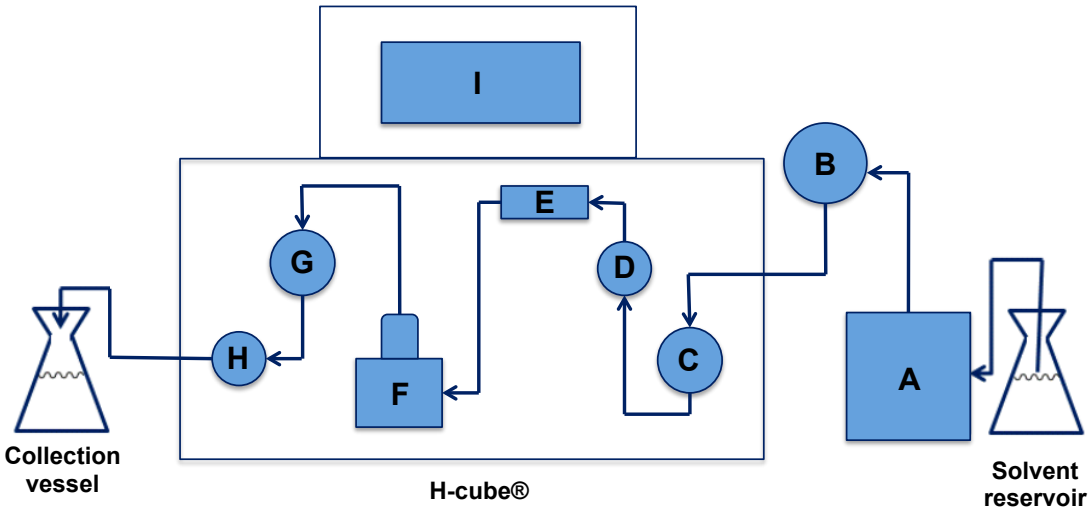


Figure 35. H-cube® scheme. A – pump; B – Manual injector; C – inlet pressure sensor; D – gas-liquid mixing chamber; E – bubble detector; F – CarCart® holder and heating unit; G – outlet pressure sensor; H – back pressure regulator; I - touch screen panel. Arrows indicate the flow direction. Adapted from (Bryan, Wernick, Hein, Petersen, Eschelbach, & Doherty, 2011)

The hydrogen/substrate mixture can be heated and pressurized up to 100°C (212°F) and 100 bar (1450 psi). The mixture is passed through a packed catalyst cartridge (CatCart®), where the reaction takes place, and the product continuously elutes out of the CatCart® and into a collection vial. No external storage of hydrogen is necessary and no catalyst filtration or direct catalyst handling. Pressure and temperature can then be changed to optimize product conversion to 100%.

According to the manufacture (and previous known studies with PCs), it allows fast and cost-efficient hydrogenolysis with superior yield when compared to conventional methods (Fabre, 2009).

The H-cube® can be used in three different modes: no H_2 (where no H_2 will be produced), full H_2 and controlled mode. In the full H_2 mode, the maximum amount of H_2 that can be produced by the electrolytic cell (25 mL/min) is delivered into the gas mixing chamber with no back pressure at the outlet of the system. When running in

full H_2 mode, the system pressure is normally working at 0-1 bar, but it can be raised manually. The flow rate of hydrogen in the controlled mode is dependent upon the liquid back pressure. The controlled mode settings (10-100 bar) are used to set the total back pressure while the system maintains a roughly constant pressure differential between the H_2 inlet pressure (internal sensor) and the liquid inlet pressure. As a consequence, setting the system to the controlled mode introduces less H_2 into the reactant stream than in the full H_2 mode setting (Bryan, Wernick, Hein, Petersen, Eschelbach, & Doherty, 2011).

All experiments were conducted using EGC-C9Bn **23** and the resulted compound analysed by TLC and ^{1}H-NMR. First, we tried to hydrogenate using the CarCart® $Pd(OH)_2$/C 20% cartridge in full H_2 mode with a pressure of 7-8 bar. The flow rate was kept at 0.5 mL/min. TLC were performed at the outlet to ensure that the entire amount of compound passed through the device. After collecting for 40 min, the result was only C **16**. This result was confirmed by LC-ESI-MS.

Next we decided to use the CarCart® Pd/C 10% cartridge with a pressure of 8-10 bar, again in full H_2 mode at 0.5 mL/min. After 20 minutes the result was a mixture of benzylated and de-benzylated compounds. Then, the collected sample was concentrated and re-passed through the H-cube® in the same conditions but in controlled mode at 20 bar of pressure. Since the result was the same, we decided to re-pass a third time in full H_2 mode (8-10 bar) with the CarCart® $Pd(OH)_2$/C 20% cartridge with a flow rate of 1 mL/min. After 25 min the benzylated compounds were still present.

Finally we used the CarCart® $Pd(OH)_2$/C 20% cartridge in controlled mode at 20 bar with a flow rate of 1 mL/min. After 10 min it resulted in a mixture of benzylated and de-benzylated compounds (especially C **16**). The collected mixture was re-passed two more times in the same conditions, but at a pressure of 30 bar. No changes were detected.

2.5.4.5. Hydrogenolysis methods comparison

Besides all these hydrogenolysis methods, it was also tested EGC-C9Bn **23** debenzylation with boron tribromide (BBr_3) (McOmie & West, 1973) and tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$). However it only resulted in the obtainment of C **16**. This later reaction was compared to a simultaneous hydrogenolysis of EGC-C9Bn **23** with Pd/C 10%, held on a schlenk flask. The result was a mixture of EGC-C **26**, C **16** and many other compounds (Figure 36).

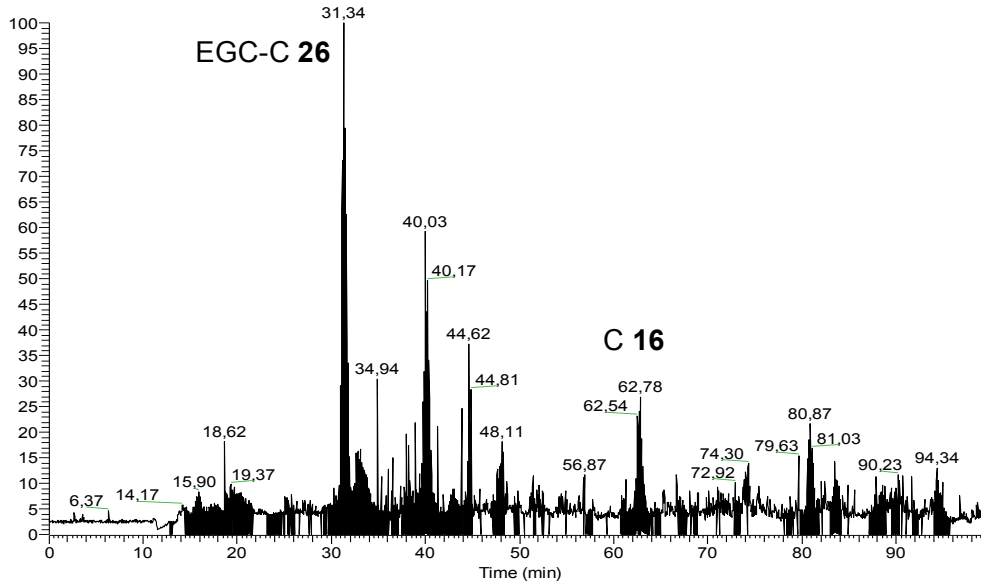


Figure 36. LC-ESI-MS chromatogram for EGC-C9Bn **23** hydrogenolysis with Pd/C 10%

Two other comparative studies to perform the deprotection of EGC-C9Bn **23** were performed simultaneously. The first study took place between hydrogenolysis *in situ* with TES and with Pd/C 10% in the hydrogen reactor. After 5h 30 min the reaction with Pd/C 10% did not show any benzylated compounds but the compound in larger amount was C **16**. On the other hand, the reaction with TES only yielded C **16**.

The second study was performed with Pd/C 10%. One of the reactions was conducted in the hydrogen reactor and the other on a schlenk flask with a drop of Et_3N. Both reactions were followed by ESI-MS by direct injection. After 3 hours, both reaction vessels showed the presence of C4Bn **19** and little amount of EGC-C **26**. After 5 h 30 min reaction mixture in the hydrogen reactor showed the presence of C **16** but no PD dimer, while the reaction with Et_3N did not show any familiar compounds. After 24 h the reaction in the hydrogen reactor showed the presence of EGC-C **26** and C **16**.

As it has been described, later we took a different and more careful approach to the hydrogenolysis using $Pd(OH)_2/C$ 20%. We used the catalyst in the same mass amount as the compound to hydrogenate and the H_2 (g) was placed to bubble in the solvent, to avoid any chances that the gas would not diffuse into the solvent. The glass balloon was covered with a rubber septum.

After the hydrogenolysis, to obtain a readily soluble polyphenol, it was necessary to dilute the filtered solution of the crude product with water, to evaporate only partially so as to remove most of the organic solvents, and to lyophilize the residual solution. If the crude polyphenol solutions are directly evaporated to dryness, partially yellow-brown insoluble materials result, indicating that some decomposition has occurred.

EGC-C9Bn **23**, EGC-EGC10Bn **24** and EGC-EGCG13Bn **25** were dissolved in THF/AcOEt/MeOH 1:1:1 and hydrogenated for 2 h for compound **23** and 1 h 30 min for compounds **24** and **25**. The solvent was filtered through a 0.20 mm PET Chromafil® syringe filter and then evaporated. The crude extract was purified by semi-preparative HPLC using the LC-MS-ESI method described forward with an Elite LaChrom L-2130 quaternary pump and an Elite LaChrom L-2420 detector.

The idea of using this catalyst quantity was to try to avoid the compound degradation by completing the reaction faster. The result was always a mixture of the desired PD dimer with the respective low monomer unit (and also gallic acid in the case of EGC-EGCG13Bn **25**) and degradation by-products.

To try to realise what was happening we decided to repeat an EGC-C9Bn **23** hydrogenolysis and follow it by ESI-MS by direct injection. After 5 min of reaction there was a mixture of the initial compound in diverse states of benzylation, C **16** and little amount of EGC-C **26**.

2.6. Conclusions

All benzylations of monomeric units, benzylations at C4 and condensation reactions proceeded without major problems with good yields. When using benzylated commercially available enantiomerically pure compounds, enantiomerically pure coupling products can reasonably be expected. The TMSOTf-catalyzed condensation proceeded smoothly in all reactions tested, supposedly to afford a mixture of 4α and 4β in a 50:1 ratio. However, all coupling products should be carefully checked by chiral HPLC.

It has been already reported that hydrogenolysis using Pd/C with H_2 (g) is quite harsh for this type of molecule because a lot of benzylic positions are available. In fact, while trying to obtain PCB3 and PCC2, a mixture of dimer, trimer along with monomeric C **16** was observed (Ahmed, 2007). This means that this procedure can also break to some extent the interflavanyl bond to yield the monomeric C (**16**).

Pearlman's catalyst i.e. $Pd(OH)_2$, is considered to be a milder reagent for hydrogenolysis and suitable for deprotection of PC dimers and trimers (Saito, Tanaka, Ubukata, & Nakajima, 2004). However that was not what was observed in this study with PDs.

The various performed hydrogenolysiss resulted, in the best cases, in a mixture of PD dimers and their respective low monomeric units. Even performing this reaction with 100% (m/m) of the most used catalyst to avoid degradation, the result, after

HPLC-preparative isolation, is a 6% yield for EGC-EGC **27** and a 2% yield for EGC-EGCG **28**. The key interflavan bond rupture limits the entire PD synthesis.

As mentioned before, the hydrogenolysis *in situ* using the H-cube® was previously used to hydrogenate PC dimers. For instance, PC B3BnBr was hydrogenated at 20ºC in MeOH/AcOEt, with 40 bar of H_2 pressure and a $Pd(OH)_2/C$ 20% cartridge at 1 mL/min. After 10 minutes, PCB3 was obtained quantitatively (Fabre, 2009). This is a rapid method (10-15 minutes Vs 18 hours) and, in the end, no filtration is necessary. The problem is that, apparently, the H-cube® device is not effective to perform hydrogenolysiss on benzylated PD for, in most cases, it results in a mixture of benzylated and de-benzylated compounds. This involves evaporating the solvent, re-dissolving and re-passing the resulting mixture through the device, leading to compound degradation. The best conditions tested were using the CarCart® $Pd(OH)_2/C$ 20% cartridge in full H_2 mode with a pressure of 7-8 bar and flow rate at 0.5 mL/min. However the result was only C **16** and no EGC-C **26**.

The results indicate that the hydrogenolysis reactions led to the breaking of the interflavan link and the desired final PD dimers were reduced to their low monomeric units.

By ESI-MS by direct injection following of the hydrogenolysis reaction, it was clear that the benzylated compounds degrade while benzyl groups are removed. In fact, not all benzyl groups were removed and the interflavan bond has already been broken, leading to the detection of the lower monomer unit of the desired PD dimer.

Chapter 3

3. Prodelphinidin analyses in grapes and wines

3.1. Introduction

As previously referred, the PA composition of wines depends on climatic and geographical conditions, cultivation practices and stages of ripeness (de Andrés-de Prado, Yuste-Rojas, Sort, Andrés-Lacueva, Torres, & Lamuela-Raventós, 2007; Mateus, Proença, Ribeiro, Machado, & De Freitas, 2001; Pérez-Magariño & González-San José, 2006). Furthermore, it depends on grape variety for the phenolic content and composition may be specific to a certain grape variety.

PAs are very difficult to extract and isolate from natural sources. The most usual technique for PAs detection is high-performance liquid chromatography (HPLC), normally coupled to an ultraviolet (UV) detector or a photodiode array detector (PDA) and reversed-phase columns. However, finding the right combination of acidic/organic solvents and gradient conditions is a hard task since these compounds usually exist with co-eluting substances present in higher amounts and/or with higher molar extinction coefficient. Moreover, their low stability, oxidation tendency and structural complexity make understanding their chemistry a challenge. Another problem is the proper compound identification of PAs that usually requires commercially non-available standards or difficult isolation from natural sources extracts. Among PAs, PDs are particularly difficult to detect because they elute earlier from reversed-phase columns, show lower UV extinction coefficient and are usually present at lower concentration than PCs (de Pascual-Teresa, Rivas-Gonzalo, & Santos-Buelga, 2000).

3.1.1. Proanthocyanidins in grapes

The annual growth cycle of the grapevine involves many processes and events in the vineyard each year. Annual growth of grapevines is frequently described using the following stages: budburst, flower cluster initiation, flowering, fruit set, berry development, harvest and dormancy.

Berry development consists of two successive sigmoidal growth periods separated by a lag phase (Figure 37) (Coombe & McCarthy, 2000). The first period of growth

lasts from bloom to approximately 60 days afterward. During this first period, the berry expands in volume as tartaric and malic acids, hydroxycinnamic acids (Romeyer, Macheix, Goiffon, Reminiac, & Sapis, 1983), tannins (including the monomeric catechins) (Kennedy, Hayasaka, Vidal, Waters, & Jones, 2001; Kennedy, Troup, Pilbrow, Hutton, Hewitt, Hunter, et al., 2000), minerals (Possner & Kliewer, 1985), amino acids (Stines, Grubb, Gockowiak, Henschke, HØJ, & van Heeswijck, 2000), micronutrients, and aroma compounds accumulate.

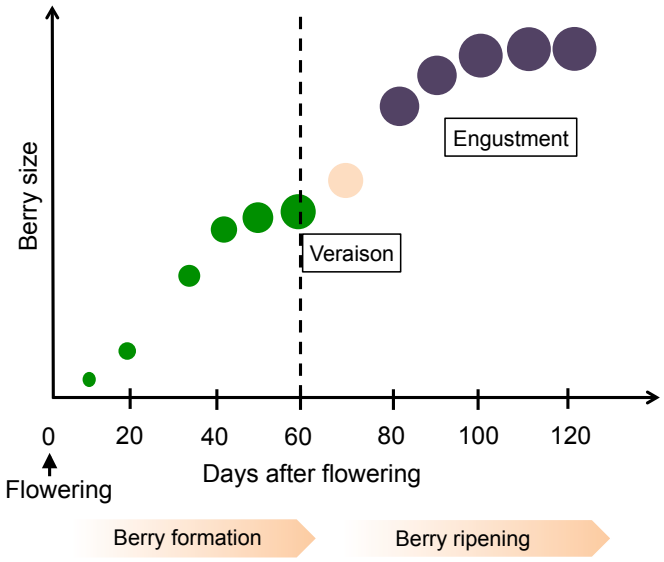


Figure 37. Diagram showing the development of grape berries (adapted from Jordan Koutroumanidins, Winetitles)

The second phase of berry growth or fruit ripening (véraison) is characterized by softening and colouring of the berry. Overall, the berry approximately doubles in size and some compounds produced during the first period of growth are reduced on a per-berry basis, mainly malic acid. Tannins also decline considerably due to oxidation as the tannins become fixed to the seed coat (Kennedy, Matthews, & Waterhouse, 2000). There is also a fructose and sucrose accumulation and, in red grape varieties, anthocyanin production.

Not all polyphenolic compounds are equally located in all grape parts. In terms of PA, grape seeds contain only the PCs (monomer, oligomers and polymers) while grape skins contain also PDs (Czochanska, Foo, & Porter, 1979; de Pascual-Teresa, Rivas-Gonzalo, & Santos-Buelga, 2000; Freitas & Glories, 1999). They both contain phenolic acids, but skins also contain anthocyanins being malvidin-3-glucoside the main anthocyanin in *Vitis vinifera* L. followed by peonidin-3-glucoside (Bakker & Timberlake, 1985; Mateus, Proença, Ribeiro, Machado, & De Freitas, 2001). PCs in grape seeds can exist in their free form or esterified with gallic acid but always preferentially in their polymerized form (Edwin, 1980).

3.1.2. Proanthocyanidins in red wine

PAs are one of the most important polyphenolic compounds in wine. They are extracted from grapes during winemaking operations (crushing, macerations and fermentation) and play a significant role in wine organoleptic evaluation. The total average PA content in red wines is around 175 mg/L (Sánchez-Moreno, Cao, Ou, & Prior, 2003) and it is approximately 20 times higher than that of white wines due to different grape material use and different enology practices.

Among PAs, PD dimers and trimers have not been widely detected in wines due to the lack of available commercial standards and the difficulty to detect and isolate them from natural sources. PA constitutive units are usually determined by acid-catalysed cleavage, in the presence of a nucleophilic agent. The more common nucleophiles include benzylhydrosulfide (*syn.* phenylmethanethiol, toluene-a-thiol), the method referred to as thiolysis (Thompson, Jacques, Haslam, & Tanner, 1972), and 1,3,5-trihydroxybenzene (*syn.* phloroglucinol) (Foo & Porter, 1978). Rupture of the interflavanoid bond in acidified methanol yields a carbocation from the upper and extension units of the molecule (initially substituted in C4) whereas the lower part (nonsubstituted in C4) is released as such. The generated carbocation the reacts with the nucleophiles to give a stable adduct (Figure 38).

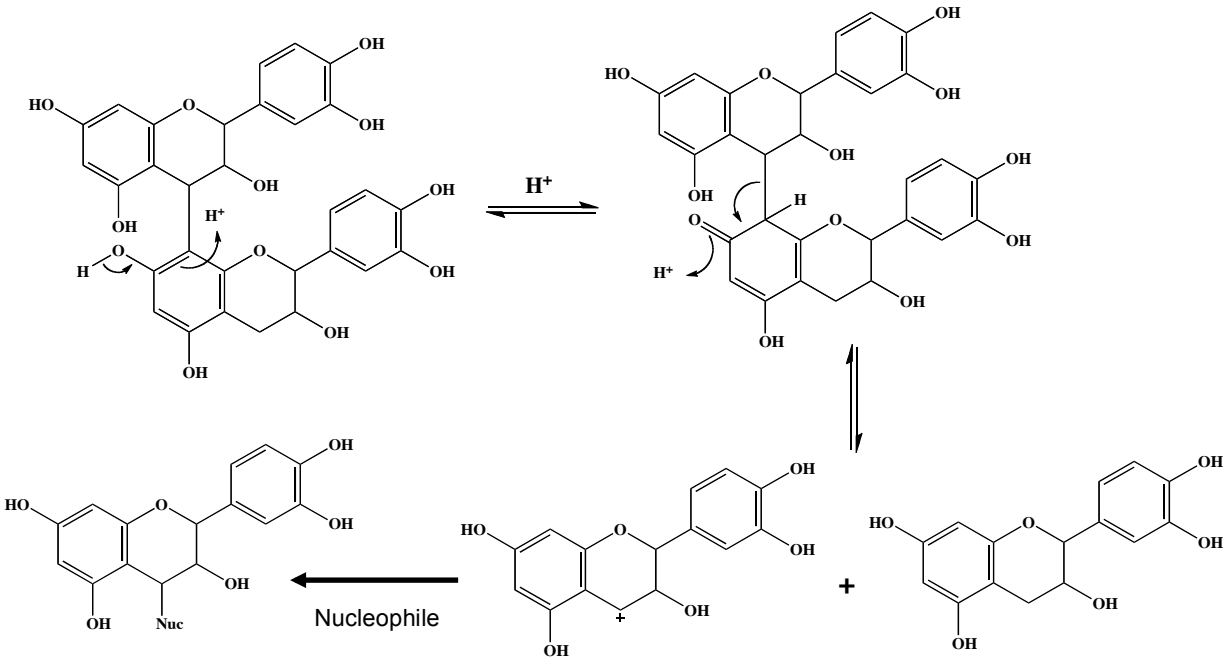


Figure 38. Reaction mechanism explaining the acid-catalyzed cleavage of proanthocyanidins.

When starting from the trimers, for instance, cleavage under mild conditions followed by HPLC analysis allows the distinction between constitutive dimers and monomers linked to the used nucleophile (Figure 39). The distinction between isomers can be achieved by comparing their retention times with standards (Ricardo da Silva, Rigaud, Cheynier, Cheminat, & Moutounet, 1991; Rigaud, Perez-Ilzarbe, Da Silva, & Cheynier, 1991; Shen, Haslam, Falshaw, & Begley, 1986).

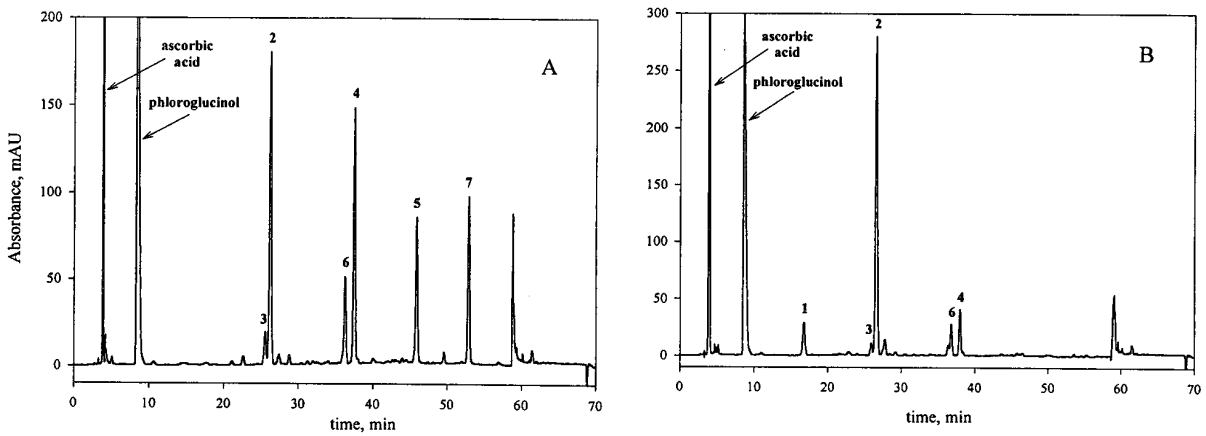


Figure 39. HPLC chromatogram of proanthocyanidin cleavage products from (A) Chardonnay grape seed and (B) Chardonnay grape skin following acid-catalysis in the presence of phloroglucinol. **1-** epigallocatechin-(4β→2)-phloroglucinol; **2-** epicatechin-(4β→2)-phloroglucinol; **3-** catechin-(4α→2)-phloroglucinol; **4-** epicatechin gallate-(4β→2)-phloroglucinol; **5-** epicatechin; **6-** catechin; **7-** epicatechin gallate (Kennedy & Jones, 2001).

However, some epimerization, especially from (-)-EC (2,3 cis) to its trans isomer, may take place when the reaction is carried out at high temperature. Besides that, no information is available on the linkage position in dimeric species. Acylation with gallic acid is maintained under the mild acidic conditions used in thiolysis and phloroglucinolysis so that galloylated units present either in upper or in terminal positions can be determined (Boukharta, Girardin, & Metche, 1988).

Application of thiolysis to grape PA polymers showed that those extracted from seeds are partly galloylated PCs whereas those of skins and stems consist of both PCs and PDs (confirming earlier results obtained by ^{13}C NMR) (Czochanska, Foo, Newman, Porter, Thomas, & Jones, 1979; Prieur, Rigaud, Cheynier, & Moutounet, 1994; Souquet, Cheynier, Brossaud, & Moutounet, 1996; Souquet, Labarbe, Le Guernevé, Cheynier, & Moutounet, 2000). The major constitutive extension units of grape skin PAs are EC and EGC. Their 3-gallates are also encountered as extension units whereas C and GC are relatively more abundant in the terminal positions. Skin PAs possess a much higher degree of polymerization around 30 units (Souquet, Cheynier, Brossaud, & Moutounet, 1996), while seeds (Prieur, Rigaud, Cheynier, & Moutounet, 1994) and stems (Souquet, Labarbe, Le Guernevé, Cheynier, &

Moutounet, 2000) only have PAs around 10 units. The proportions of galloylated units are also quite different in skins (5%), stems (15%), and seeds (30%).

Another PA constitutive units determination method is the reaction with *p*-dimethylaminocinnamaldehyde (DMACA). The use of this reagent gives coloured adducts with flavanols showing maximum absorption between 632 and 640 nm in HPLC analysis (Figure 40). At these wavelengths no interference of other coloured compounds that might be present in the same extracts (such as anthocyanins) is detected (Treutter, 1989; Treutter, Feucht, & Santos-Buelga, 1994; Treutter, Santos-Buelga, Gutmann, & Kolodziej, 1994).

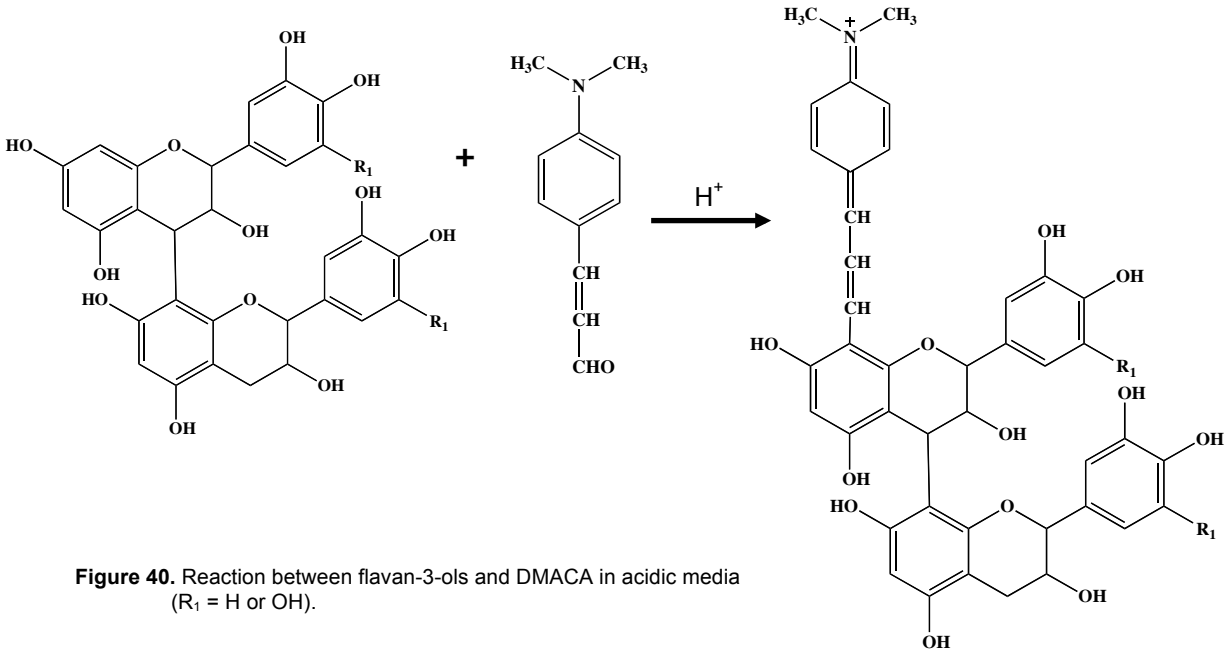


Figure 40. Reaction between flavan-3-ols and DMACA in acidic media (R_1 = H or OH).

Information about the chemical structure of the compounds can be obtained from the ratio of their peak areas at 640 and 280 nm (Santos Buelga & Treutter, 1995; Treutter, Santos-Buelga, Gutmann, & Kolodziej, 1994). This characteristic is very useful for the identification of substances in chromatograms, especially when this information is combined with their chromatographic behaviour and the UV absorbance spectra measured prior reaction with a diode array detector.

The results of individual PD identification and characterization are usually only presented as percentages of PDs (%PD). This kind of results only allows having a general idea about that same content. The aim of this work was to identify the PAs in tainturier red wine and achieve a useful PD analysis method that can prove that these compounds are not present only in residual quantities in wines.

3.2. HPLC-ESI-MS

HPLC in combination with electrospray ionization mass spectrometric detection in the negative ion mode has been used successfully to investigate PAs from many extracts. The first application of HPLC–ESI-MS to grape polyphenols was published in 1995 (Baldi, Romani, Mulinacci, Vincieri, & Casetta, 1995).

Electrospray (ESI) is a soft ionization technique that offers the possibility of generating only pseudomolecular ions without any fragmentation. Tandem mass spectrometry (MS–MS) and ion trap mass spectrometry (IT-MS, MS^n), which permit fragmentation patterns to be obtained on selected individual ions, are progressively replacing the classical ESI-quadrupole mass spectrometers in HPLC–MS coupling. Fragmentation patterns also provided insight on the sequences of flavanol units in proanthocyanidin oligomers (de Pascual-Teresa, Rivas-Gonzalo, & Santos-Buelga, 2000) and of anthocyanin and flavanol units in flavanol–anthocyanin adducts (Salas, Fulcrand, Meudec, & Cheynier, 2003). As with other mass spectrometric techniques no differentiation between stereoisomers is possible and no information about the position and stereochemistry of the interflavanoid linkage (4α→8 or 4β→6) is available.

Before ESI, thermospray (TSP) and fast atom bombardment (FAB) were the most widespread, versatile and powerful interfaces in LC-MS coupling. With a TSP interface the structure elucidation of PAs has been well established, although the high degradation in the ion source often resulted in a very small pseudomolecular ion. The electrospray ionization (ESI) and atmospheric pressure ionization (API) replaced them. This is a soft ionization technique at atmospheric pressure that produce ions even from thermally labile, non-volatile, polar compounds with high sensitivity. Normally, only the pseudomolecular ion is produced, without any fragmentation (Aramendía, García, Lafont, & Marinas, 1995; Cheynier, Doco, Fulcrand, et al., 1997; Maillard, Giampaoli, & Cuvelier, 1996).

Ions can be measured in the positive ion mode as well as in negative ion mode. Polyphenols are weakly acidic compounds, indicating that dissociation is easier than protonation. Using acetic acid as one of the HPLC eluents has advantages for chromatographic resolution and ion formation efficiency, but leads to acetate and phenolate adducts which in some cases complicate the interpretation in the negative ion mode (Whittle, Eldridge, Bartley, & Organ, 1999). This is why most users prefer measuring in the positive mode. On the other hand, better sensitivity and selectivity have been described when the mass spectrometer was operated in negative

ionization mode (Poon, 1998; Whittle, Eldridge, Bartley, & Organ, 1999). Acetic acid can be substituted by formic acid but it can form dimerization in the equipment source, especially when analyzing flavan-3-ol monomers.

A general LC-ESI-MS with ion trap equipped with an API source consists of an interface to generate ions, ion optical elements to guide the ions from the interface to the mass analyser (ion trap), an ion trap to collect the ions and then release them according to mass-to-charge ratio, ion detector (and its electronics and software) to convert the ions to a mass spectrum) and vacuum pumps to keep the system at low pressure to ensure efficient ion transmission and detection (Figure 41).

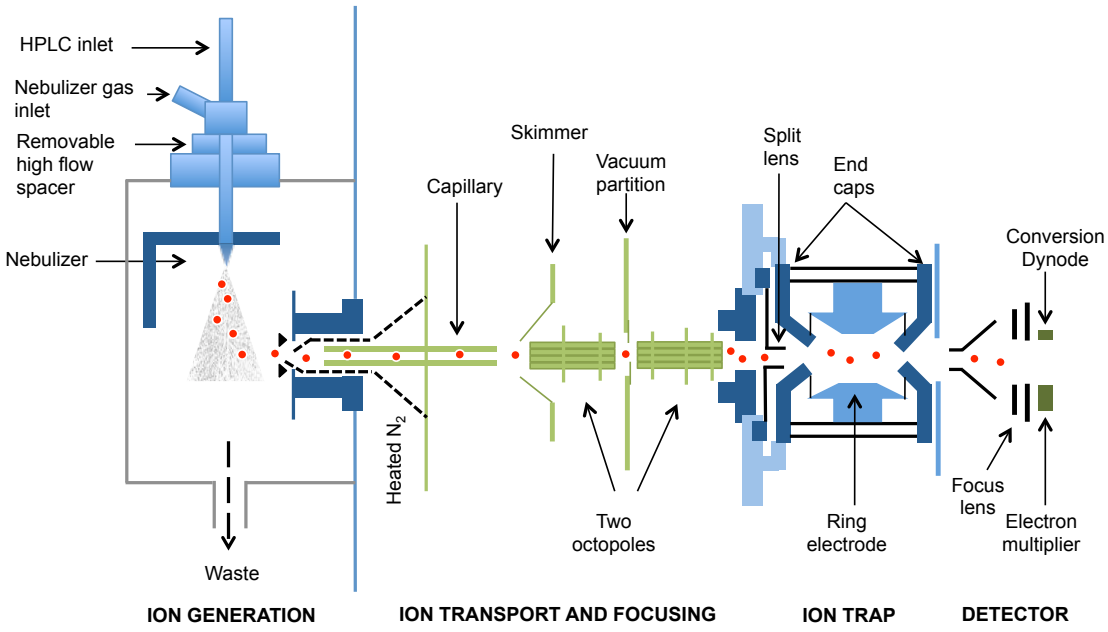


Figure 41. Trap mass spectrometer for ion transmission and detection

The electrospray interface generates ions in a spray chamber. The system then transports the ions into the ion trap mass analyser. Electrospray ionization (ESI) consists of four steps: formation of ions, nebulization, desolvation and ion evaporation.

Ion formation in API-electrospray occurs through more than one mechanism. When possible, ions can be generated in solution before nebulization. This results in high analyte ion concentration and good API-electrospray sensitivity. However, analytes that do not ionize in solution can still be analysed. The process of nebulization, desolvation, and ion evaporation creates a strong electrical charge on the surface of the spray droplets. This can induce ionization in analyte molecules at the surface of the droplets.

Nebulization (aerosol generation) starts with the sample solution entering the spray chamber through a grounded needle. For high flow electrospray, nebulizing gas enters the spray chamber concentrically through a tube that surrounds the needle. The combination of strong shear forces generated by the nebulizing gas and the strong voltage (2-6 kV) at the mesh electrode and end plate in the spray chamber draws out the sample solution and breaks it into droplets. As the droplets disperse, ions of one polarity preferentially migrate to the droplet surface due to electrostatic forces. Consequently, the sample is simultaneously charged and dispersed into a fine spray of charged droplets, hence the name electrospray.

Before the ions can be analysed, the solvent must be removed to yield bare ions. A counter current of neutral, heated drying gas (typically nitrogen) evaporates the solvent, decreasing the droplet diameter and forcing the predominantly like surface charges closer together. When the force of the Coulomb repulsion equals that of the surface tension of the droplet, the droplet explodes, producing smaller charged droplets that are subject to further evaporation.

The choice of solvents and buffers is a key to successful ionization with electrospray. Solvents like methanol that have lower heat capacity, surface tension, and dielectric constant, promote nebulization and desolvation. Because the sample solution is not heated when the aerosol is created, ESI does not thermally decompose most analytes.

3.3. Materials and methods

(+)-Catechin (C) and (-)-epicatechin (EC), were purchased from Sigma-Aldrich® (Madrid, Spain). (-)-Epigallocatechin (EGC) and (-)-epigallocatechin gallate (EGCG) were purchased from Biopurify Phytochemicals Ltd (Sichuan, China).

PCB3 and PDB3 were extracted from barley. The barley was finely ground and the ground sample extracted twice with a mixture of methanol/acetone/water 2:1.5:2 (V/V). Then it was filtered with glass wool and centrifuged at 2500 rpm for 5 minutes. The supernatant was collected and evaporated under vacuum at 35ºC for methanol and acetone removal. Then the sample was extracted with C18 gel on a G3 glass Buckner funnel and recovered with methanol. After evaporation under vacuum at 35ºC, it was resolved in water, re-dissolved in water and freeze-dried. This polyphenol extract was latter purified by column chromatography (250 x 16 mm i.d.) in a TSK Toyopearl HW40(s) gel (Tosoh, Japan) column connected to a ultraviolet (UV) detector. The required dimers were then re-purified by semi-preparative HPLC

using an Elite LaChrom L-2130 quaternary pump and an Elite LaChrom L-2420 detector. The column used was a reversed-phase C18 column (250 x 4.6 mm i.d.) and the mobile phase was composed by solvent A, 2.5% (v/v) EtOAc, and solvent B, 20% (v/v) solvent A and 80% (v/v) acetonitrile. The flow rate was 1 mL/min and the gradient method started with an isocratic gradient of 93% A during 5 minutes, followed by a linear gradient ranging from 93% A to 80% A in 90 minutes and a final isocratic gradient of 100% B during 10 minutes (Teixeira, Cruz, Brás, Mateus, Ramos, & de Freitas, 2013). The purified dimers were re-dissolved in water and freeze-dried for further use. The identity and purity of PCB3 and PDB3 was achieved by LC-MS-ESI (Finnigan Surveyor equipped with a Thermo Finnigan (Hypersil Gold) 150 mm x 4.6 mm, 5mm, C18 reversed-phase column at 25 ºC; Finnigan LCQ DECA XP MAX mass detector (Finnigan Corp., San Jose, CA, USA) quadrupole ion trap equipped with an atmospheric pressure ionization source, using an electrospray ionization interface) and NMR (^{1}H NMR spectra were measured in D_2O on a Bruker Avance 400 spectrometer) by comparing with the literature data (Dvorakova, Moreira, Dostalek, Skulilova, Guido, & Barros, 2008).

C-(4α→8)-EC (PCB4) and C-(4α→6)-C (PCB6) standards where synthetized from direct condensation with dihydroquercetin according to literature (Delcour, Ferreira, & Roux, 1983). PDs EGC-(4β→8)-C (PDB1) and EGC-(4β→8)-EGC (PDB10) were synthetized according to a procedure developed by Karsten Khron (Krohn, Ahmed, John, Letzel, & Kuck, 2010) as described in chapter 2. PCs EC-(4β→8)-EC (PCB2), and trimer EC-(4β→8)-EC-(4β→8)-EC (PCC1) standards were extracted from lyophilized grape seed with the same purification steps as PCB3 and PDB3.

The identity and purity of the compounds was achieved by LC-MS-ESI and NMR (^{1}H NMR and ^{13}C NMR spectra were measured in $CDCl_3$ or D_2O on a Bruker Avance 400 spectrometer).

3.3.1. Wine sample preparation

For this study four samples of 2012 red vinho verde from the Demarcated Region of Vinho Verde (Lima's sub-region) and three samples of 2013 red wine from the Demarcated Region of Douro. The red Vinho Verde wines analysed in this study are mainly "Vinhão" cultivar and the Douro region red wine mainly "Sousão" cultivar. These are actually the same grape variety, which originates from the northern Minho region of Portugal, and is appreciated for its colour properties.

20 mL of each wine were extracted three times with 20 mL of EtOAc and fractionated according to the procedure described elsewhere (Freitas & Glories, 1999).

3.3.2. LC-MS-ESI analysis

All samples were analysed by LC-ESI-MS performed in a Finnigan Surveyor Plus HPLC system fitted with a PDA Plus detector, an autosampler Plus and a LC quaternary pump plus coupled to a Finnigan LCQ Deca XP Plus mass detector equipped with a ESI source and an ion trap quadrupole equipped with an atmospheric pressure ionization (API) source. The stationary phase was a Thermo Finnigan Hypersil Gold column (150 x 4.6 mm i.d., 5 mm). The mass spectrometer was operated in the negative-ion mode with source, with a capillary temperature of 275ºC and capillary voltages of 4.5 kV. The mass spectra were recorded between 250 and 2000 m/z. The mobile phase was composed by solvent A, 0.1% (v/v) formic acid, and solvent B, 100% (v/v) MeOH. The flow rate was 0.20 mL/min and the gradient method started with a linear gradient ranging from 90% A to 60% A in 90 minutes, then reaching 100% B in 5 minutes and a final isocratic gradient of 100% B during 5 minutes.

3.4. Results and Discussion

3.4.1. PAs standard analysis

The synthetized and isolated compounds were analysed by LC-ESI-MS, using the analytic conditions described earlier, for standard distribution determination and retention time reference. The resulting chromatograms are shown in figure 42. Later a catechin calibration curve was calculated for compound quantification and comparison.

LC-ESI-MS with the right chromatographic conditions has proved to be a powerful tool for detection and identification of PAs in complex samples with no need to perform excessively compound isolation. Tandem mass spectrometry (MS/MS) with ion trap provided additional information about the structures of these compounds through the fragmentation patterns of the pseudomolecular ions. Analysis of dimeric PAs with constitutive units showing different molecular weights demonstrates that RDA (retro-Diels-Alder) fission takes place specifically on the upper unit (substituted only in C4) and thus can be used to determine the sequence (de Pascual-Teresa, Rivas-Gonzalo, & Santos-Buelga, 2000; Friedrich, Eberhardt, & Galensa, 2000).

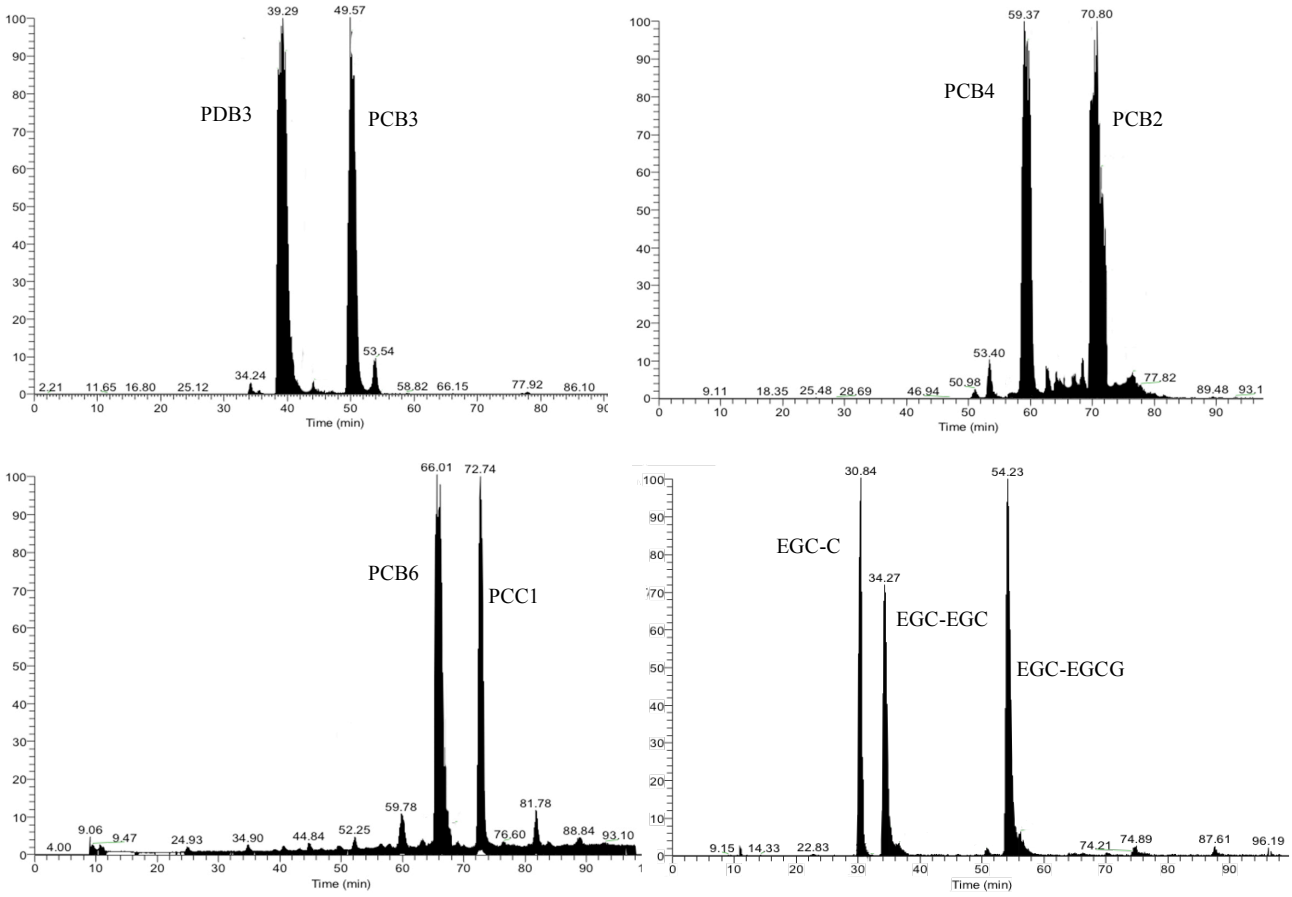


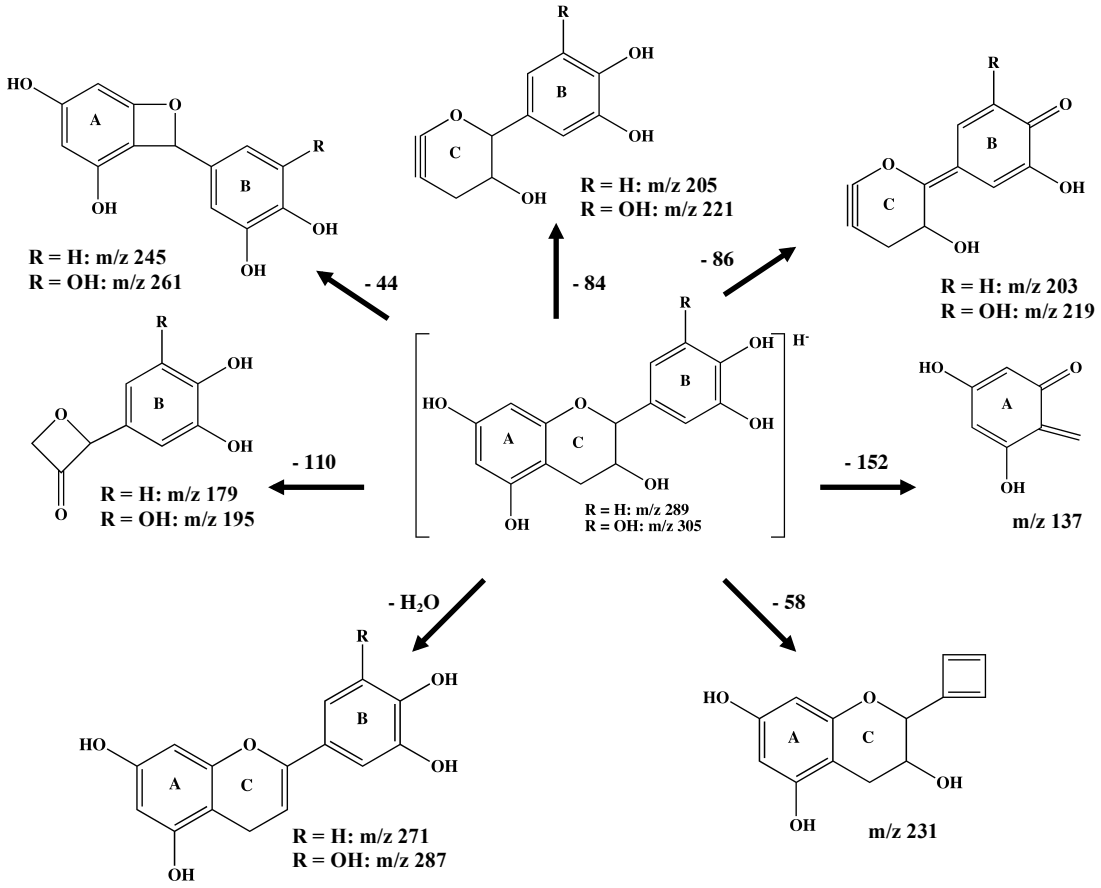
Figure 42. Proanthocyanidins PDB3, PCB3, PCB2, PCB4, PCB6, PCC1, EGC-C **11**, EGC-EGC **12** and EGC-EGCG **13** negative mass chromatograms by LC-ESI-MS.

3.4.2. Wine fractionation and analysis

Like in the grape skin extracts, the red wine samples were extracted with ethyl acetate and thereafter fractionated by Toyopearl gel column chromatography yielding two fractions: fraction A that consists in low molecular weight compounds including PAs monomers and fraction B that consists mainly in PAs dimers and trimers.

3.4.2.1. Fraction A

Table 5 shows the PA monomeric units detected in the fraction A of the analysed wines. Scheme 1 shows all the possible fragmentations this compounds can undergo.



Scheme 1. Mass fragmentation patterns of flavan-3-ol monomers

Table 5. HPLC-MS retention times, molecular ions $[M-H]^-$ and Mass Spectrometric Fragments of the fraction A of 2013 red wine from the Demarcated Region of Vinho Verde (Lima's sub-region).

Compound	R_t (min)	*m/z* $[M]^-$	Product ions *m/z* $[M-H]^-$
GC	38.41	305	221;219;261;137;287
EGC	61.35	305	219;221;261;287;137
C	63.53	289	245;205;179;203;231;271
EC	83.34	289	245;205;179;231;271;203

The analysed wines revealed similar qualitative composition and showed the presence of the four flavan-3-ol monomers in the following order: GC at 39.14 min, EGC at 61.46 min, C at 62.78 min and EC at 83.69 min (Figure 43a). This observation shows once more that, when using reversed-phase columns, ring-B trihydroxylated monomers elute earlier that the corresponding dihydroxylated ones, and 2,3-trans earlier than 2,3-cis, i.e. (G)C earlier then E(G)C. No galloyl derivatives were found suggesting that they are likely degraded during the wine-making process (de Pascual-Teresa, Rivas-Gonzalo, & Santos-Buelga, 2000) and/or they are difficult to extract from grape seeds (which contain most of the gallate forms) and grape skins.

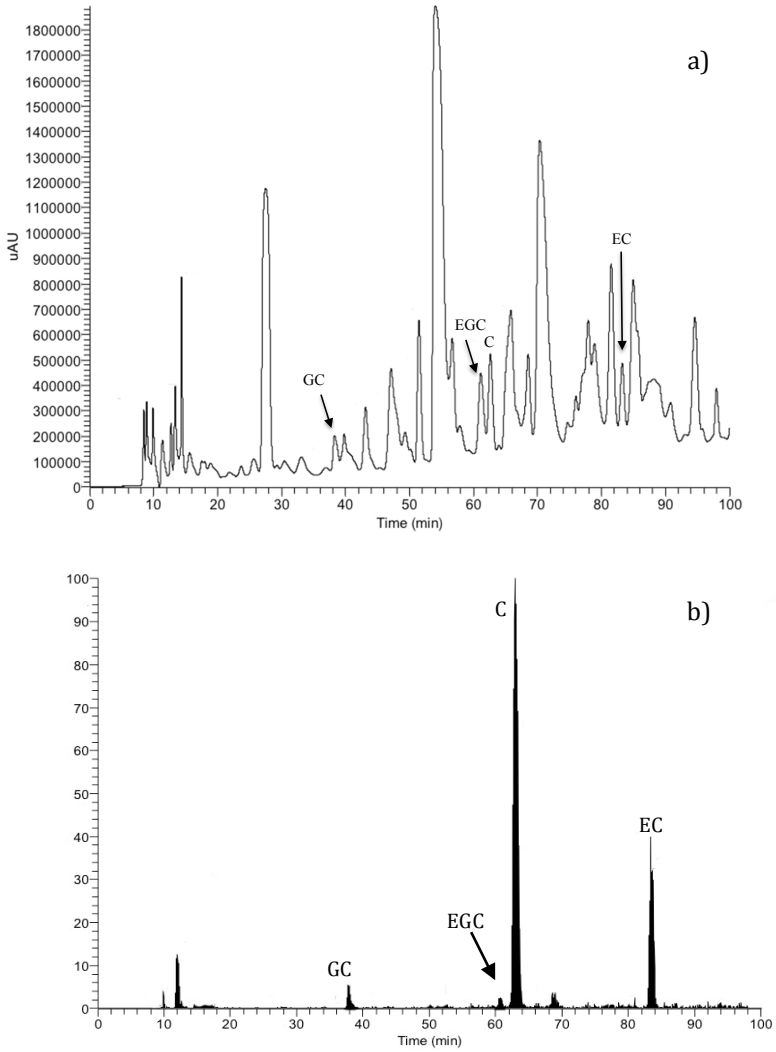


Figure 43. a) HPLC chromatogram at λ = 280 nm, b) SIM-MS (selected ion monitory – mass spectrometry) chromatogram of the fraction A of 2012 red vinho verde wine from the Demarcated Region of Vinho Verde (Lima's sub-region).

3.4.2.2. Fraction B

The LC-ESI-MS analysis of Wine fraction B allowed 3 dimeric PAs to be identified with *m/z* product ions $[M]^-$ 609, 8 dimeric PAs with *m/z* $[M]^-$ 593, 6 dimeric PAs with *m/z* $[M]^-$ 577, 3 trimeric PAs with *m/z* $[M]^-$ 897 (only in 2012 red Vinho Verde from the Demarcated Region of Vinho Verde), 10 trimeric PAs with *m/z* $[M]^-$ 881 and 11 trimeric PAs with *m/z* $[M]^-$ 865 making a total of 41 different PAs. The 2012 red Vinho Verde from the Demarcated Region of Vinho Verde (Lima's sub-region) presented 37 different PAs and the 2013 red wine from the Demarcated Region of Douro only 28 different PAs and both wines have 24 PAs in common.

Figure 44 and 45 show a LC-ESI-MS chromatogram of the B fraction for each wine type analysed where each number corresponds to a different PA. Table 6 and 7 shows the retention times (R_t), negative pseudomolecular ion (*m/z* $[M]^-$) and the product ions of the main fragments obtained for each compound.

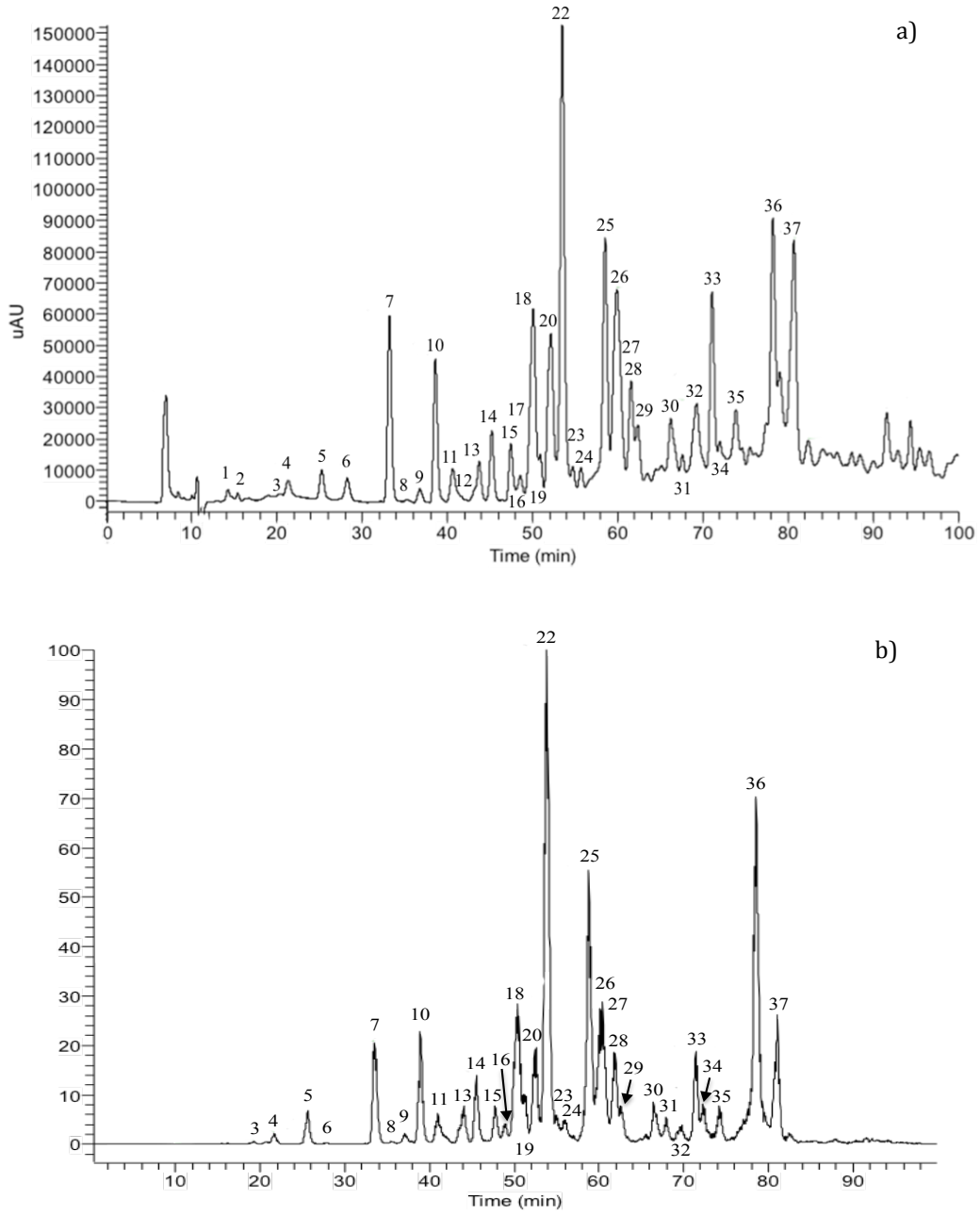


Figure 44. HPLC chromatogram at λ = 280 nm, b) SIM-MS (selected ion monitory – mass spectrometry) chromatogram of the fraction B of 2012 red vinho verde wine from the Demarcated Region of Vinho Verde (Lima's sub-region).

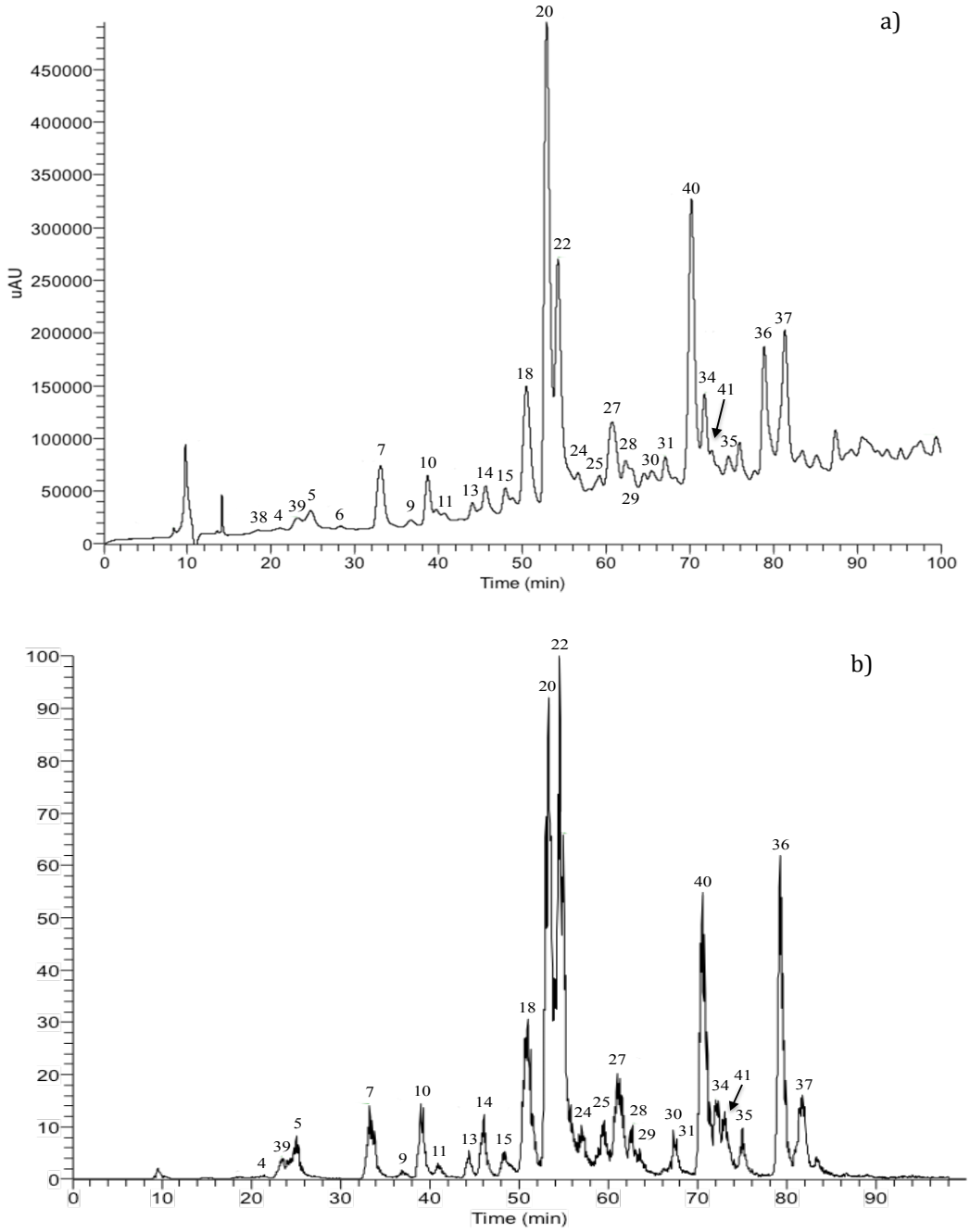


Figure 45. HPLC chromatogram at λ = 280 nm, b) SIM-MS chromatogram of the fraction B of 2013 red wine from the Demarcated Region of Douro.

Table 6. HPLC-MS retention times, pseudomolecular ions *m/z* $[M]^-$ and Mass Spectrometric Product ions of the fraction B of 2012 red vinho verde wine from the Demarcated Region of Vinho Verde (Lima's sub-region).

	Compound	R_t (min)	$[M]^-$ (*m/z*)	Product ions (*m/z*)
1	(E)C-(E)GC-(E)GC	15.70	897	609;483;745;305
2	(E)GC-(E)C-(E)C	19.23	881	577;695;755;451;289
3	(E)C-(E)C-(E)GC	20.81	881	593;711;575;467;423;755;287;863
4	(E)GC-(E)GC	21.67	609	305;441;423;483;591;453
5	(E)C-(E)C-(E)C	25.63	865	577;695;713;739;451;287
6	(E)GC-(E)GC	27.95	609	305;441;423;591;483;453
7	PDB1	33.49	593	289;467;425;407;303;575;205
8	(E)GC-(E)C-(E)GC	35.64	897	593;711;771;729;603;879
9	PDB3	37.11	593	289;467;425;245;303;437;575
10	(E)C-(E)GC	38.91	593	305;423;467;441;575;221
11	(E)C-(E)GC	40.90	593	305;423;441;467;575;453;219;287
12	(E)GC-(E)GC-(E)C	41.72	897	593;711;729;771;407;289;879
13	(E)C-(E)C-(E)GC	44.09	881	593;711;755;575;305;441;729;863
14	(E)GC-(E)C-(E)C	45.52	881	577;695;755;451;425;303
15	(E)GC-(E)C	47.41	593	289;467;425;303;575;407;245
16	(E)C-(E)C-(E)GC	48.87	881	593;711;755;575;467;423;729;441;863;305;287
17	(E)C-(E)C-(E)C	49.73	865	577;695;739;407;713;287
18	PCB3	50.22	577	289;425;407;451;559;245
19	(E)C-(E)C-(E)C	51.23	865	577;695;713;739;425;451;287
20	(E)C-(E)C	52.60	577	289;425;407;451;559;245
21	(E)GC-(E)C-(E)C	53.10	881	577;695;755;863;451;407;289
22	(E)C-(E)C-(E)C	53.82	865	577;695;739;713;425;407;451;287;847
23	(E)C-(E)C-(E)GC	55.03	881	593;755;711;575;863;467;729;423;305;441;287
24	(E)C-(E)GC	56.02	593	305;423;441;467;575;221
25	(E)C-(E)C-(E)C	58.74	865	577;695;739;713;425;407;847;289
26	(E)GC-(E)C-(E)C	59.58	881	577;695;755;407;425;289;863
27	PCB4	60.38	577	289;425;407;451;245;559
28	(E)C-(E)C-(E)C	61.91	865	577;695;739;407;425;713;847;289
29	(E)C-(E)GC	62.64	593	305;423;467;575;441;261;453
30	(E)GC-(E)C	66.45	593	289;467;425;407;245;303;575
31	PCB6	67.01	577	289;425;451;407;245;559
32	(E)C-(E)GC-(E)C	67.93	881	593;711;755;729;425;407;863
33	(E)C-(E)C-(E)C	71.48	865	577;695;739;713;425;407;451;289;847
34	PCC1	72.21	865	577;695;739;407;425;451;713;245;287;847
35	(E)GC-(E)C-(E)C	74.25	881	577;695;755;713;287;407;451
36	(E)C-(E)C-(E)C	78.48	865	577;695;739;713;451;425;413;847
37	(E)C-(E)C	81.13	577	289;425;407;451;559;245

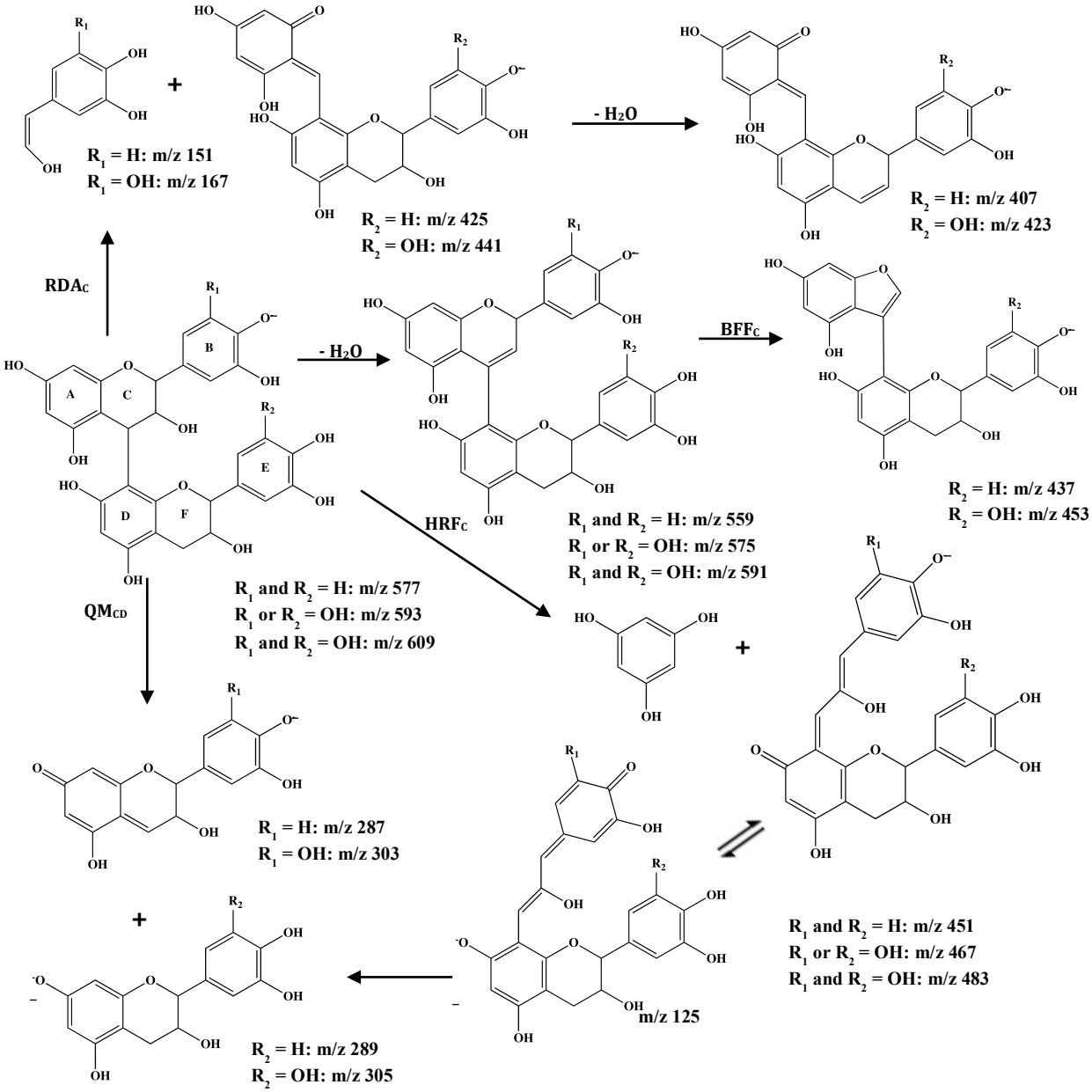
Table 7. HPLC-MS retention times, pseudomolecular ions *m/z* $[M]^-$ and Mass Spectrometric Product ions of the fraction B of 2013 red wine from the Demarcated Region of Douro.

	Compound	R_t (min)	$[M]^-$ (*m/z*)	Product ions (*m/z*)
38	GC-GC	18.66	609	305;441;423;483;591
4	(E)GC-(E)GC	21.46	609	305;441;423;483;591
39	(E)C-(E)C-(E)C	23.63	865	695;713;575;739;425;287
5	(E)C-(E)C-(E)C	25.05	865	577;695;713;739;425;287
6	(E)GC-(E)GC	27.15	609	---
7	PDB1	33.23	593	289;467;425;303;203;245;575
9	PDB3	36.87	593	289;467;425;407;303;575;245;437
10	(E)C-(E)GC	39.00	593	305;467;423;441;575;261
11	(E)C-(E)GC	40.92	593	305;467;441;423;575;221
13	(E)C-(E)C-(E)GC	44.33	881	593;711;755;729;467;423;441;287
14	(E)GC-(E)C-(E)C	46.05	881	577;695;755;425;863;407;451;303
15	(E)GC-(E)C	48.25	593	289;425;467;407;303;575;203;245;437
18	PCB3	50.82	577	289;425;407;451;559;205;245
20	(E)C-(E)C	53.17	577	289;425;407;451;559;287;245
22	(E)C-(E)C-(E)C	54.53	865	695;713;739;575;425;451;407;287;847
24	(E)C-(E)GC	57.01	593	305;467;441;423;575;221;261;421
25	(E)C-(E)C-(E)C	59.50	865	577;695;739;587;713;407;287;425;847
27	PCB4	61.11	577	289;425;407;451;559;245
28	(E)C-(E)C-(E)C	62.72	865	577;695;739;407;713;451;287;847
29	(E)C-(E)GC	63.47	593	305;467;423;441;575;219
30	(E)GC-(E)C	67.27	593	289;467;425;303;407;245;575;203
31	PCB6	67.67	577	289;425;407;451;559;245
40	PCB2	70.57	577	289;425;407;451;559;287;245;203
34	PCC1	72.18	865	577;695;739;713;425;407;287
41	(E)C-(E)C-(E)C	72.94	865	577;695;739;713;407;425;587;451
35	(E)GC-(E)C-(E)C	74.97	881	577;695;755;407;713;289;425
36	(E)C-(E)C-(E)C	79.21	865	577;695;739;713;587;407;425;847;451
37	(E)C-(E)C	81.50	577	289;425;407;451;559;245;287

3.4.2.2.1. Fraction B dimers

Compounds 4, 6 and 38 presented pseudomolecular ions with *m/z* $[M]^-$ 609. The MS^2 spectra shows product ions with *m/z* 305 indicating C and D rings quinone methide fission (QM_{CD}) (Scheme 2); *m/z* 441 indicating a retro Diels-Alder fission in the C ring (RDA_C) and also a consequent loss of a water molecule originating the pseudomolecular ion *m/z* 423; *m/z* 483 indicating a heterocyclic ring fission in ring C (HRF_C); and *m/z* 591 which corresponds to the loss of a water molecule and also a

consequent novel benzofuran-forming fission again in ring C (BFF_C) originating the pseudomolecular ion *m/z* 453. These pseudomolecular ion values and fragmentations indicate that these compounds are PDs formed with two (E)GC units. None of them could be identified as the synthetized standard EGC-(4β→8)-EGC but, according to the literature (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000) and the previous results for monomers, it can be suggested that compound 4 is GC-(4β→8)-GC, and compounds 6 and 38 GC-(4α→8)-EGC and EGC-(4β→8)-GC.



Scheme 2. Mass fragmentation patterns of flavan-3-ol dimers (adapted from (Quideau, 2006)).

Compounds 18, 20, 27, 31, 37 and 40 presented pseudomolecular ions with *m/z* $[M]^-$ 577 indicating they are B-type PAs formed with two (E)C units. The MS^2 spectra

shows with *m/z* 289 and *m/z* 287 indicating QM_{CD} fission; *m/z* 425 indicating RDA_C and also a consequent loss of a water molecule originating the pseudomolecular ion *m/z* 407; *m/z* 451 indicating a HRF_C; the loss of 18 m.u corresponding to a water molecule and originating the product ion *m/z* 559; and the product ions *m/z* 245 and *m/z* 203 corresponding to the loss of 44 and 86 m.u. from the lower unit after the QM_{CD} fission. By comparing with PC dimers standards, it can be concluded that compound 18 is in fact PCB3, while compounds 27, 31 and 40 are PCB4, PCB6 and PCB2, respectively. In Figure 45b, PCB4 peak appears co-eluted with compound 26.

Compounds 7, 9, 10, 11, 15, 24, 29 and 30 presented pseudomolecular ions with *m/z* $[M]^-$ 593 indicating they are B-type PAs formed with one (E)C unit and one (E)GC unit. The MS^2 spectra show two types of fragmentation. One type of fragmentation for compounds 7, 9, 15, and 30 with product ions with *m/z* 289 (C or EC) indicating QM_{CD} fission or a second fission after HRF_C; *m/z* 425 indicating RDA_C and *m/z* 407 for a consequent loss of a water molecule; *m/z* 437 indicating the loss of a water molecule followed by a BFF_C; *m/z* 205 and *m/z* 245 corresponding to the (E)C loss of 84 and 44 m.u.; and *m/z* 303 corresponding to the oxidized upper unit residue when the dimer is formed with an (E)GC monomer in that unit. Another MS^2 fragmentation type was identified for compounds 10, 11, 24 and 29 with product ions with *m/z* 305 (GC or EGC) indicating QM_{CD} fission; *m/z* 441 indicating RDA_C and *m/z* 423 for a consequent loss of a water molecule; *m/z* 453 indicating the loss of a water molecule followed by a BFF_C; *m/z* 221, *m/z* 219 and *m/z* 261 corresponding to the (E)GC loss of 84, 86 and 44 m.u.; and *m/z* 287 corresponding to the oxidized upper unit residue when the dimer is formed with an (E)C monomer in that unit. Both presented product ions with *m/z* 575 corresponding to the loss of a water molecule and *m/z* 467 corresponding to a HRF_C. According to the literature RDA fission can occur either in the upper or in the lower monomer subunit of the dimer. However, according to other studies (de Pascual-Teresa, Rivas-Gonzalo, & Santos-Buelga, 2000; Quideau, 2006) and with what was observed for the standards and synthetized PDs (data not shown), the RDA fission occurs fundamentally in the upper subunit of the PA dimer. Therefore, it can be concluded that compounds 7, 9, 15, and 30 are formed with (E)C in the upper subunit and (E)GC in the lower subunit, while compounds 10, 11, 24 and 29 are formed the other way around. From the comparison with the standards previously obtained it can be concluded that compound 7 is in fact EGC-C and compound 9 PDB3.

Usually, it is considered that the subunits of PCs are (+)-catechin and (-)-epicatechin, and the subunits of PDs are (-)-gallocatechin and (-)-epigallocatechin. However, if a

PC can be described as a compound that releases cyanidin in acid medium when heated (Bate-Smith, 1954) and a PD as a compound that releases delphinidin in the same conditions, then a catechin-epigallocatechin dimer can not be named a PD. Assuming this, there are more PCs in nature then the 8 already described. In fact, such compounds were detected in these wine analyses (compounds 10, 11, 24 and 29). Table 8 presents a suggestion for PC and PD identification nomenclature taking into account the already adopted one. Considering this suggested identification the three PDs synthetized for the first time in this work are: PDB1 Epigallocatechin-(4β→8)-Catechin **11**, PDB10 Epigallocatechin-(4β→8)-Epigallocatechin **12** and PDB10G Epigallocatechin-(4β→8)-Epigallocatechin Gallate **13**.

Table 8. Suggested identification nomenclature for PCs and PDs.

Compound	PC	PD
B1	EC-(4β-8)-C	EGC-(4β-8)-C
B2	EC-(4β-8)-EC	EGC-(4β-8)-EC
B3	C-(4α-8)-C	GC-(4α-8)-C
B4	C-(4α-8)-EC	GC-(4α-8)-EC
B5	EC-(4β-6)-EC	EGC-(4β-6)-EC
B6	C-(4α-6)-C	GC-(4α-6)-C
B7	EC-(4β-6)-C	EGC-(4β-6)-C
B8	C-(4α-6)-EC	GC-(4α-6)-EC
B9	EC-(4β-8)-GC	EGC-(4β-8)-GC
B10	EC-(4β-8)-EGC	EGC-(4β-8)-EGC
B11	C-(4α-8)-GC	GC-(4α-8)-GC
B12	C-(4α-8)-EGC	GC-(4α-8)-EGC
B13	EC-(4β-6)-EGC	EGC-(4β-6)-EGC
B14	C-(4α-6)-GC	GC-(4α-6)-GC
B15	EC-(4β-6)-GC	EGC-(4β-6)-GC
B16	C-(4α-6)-EGC	GC-(4α-6)-EGC

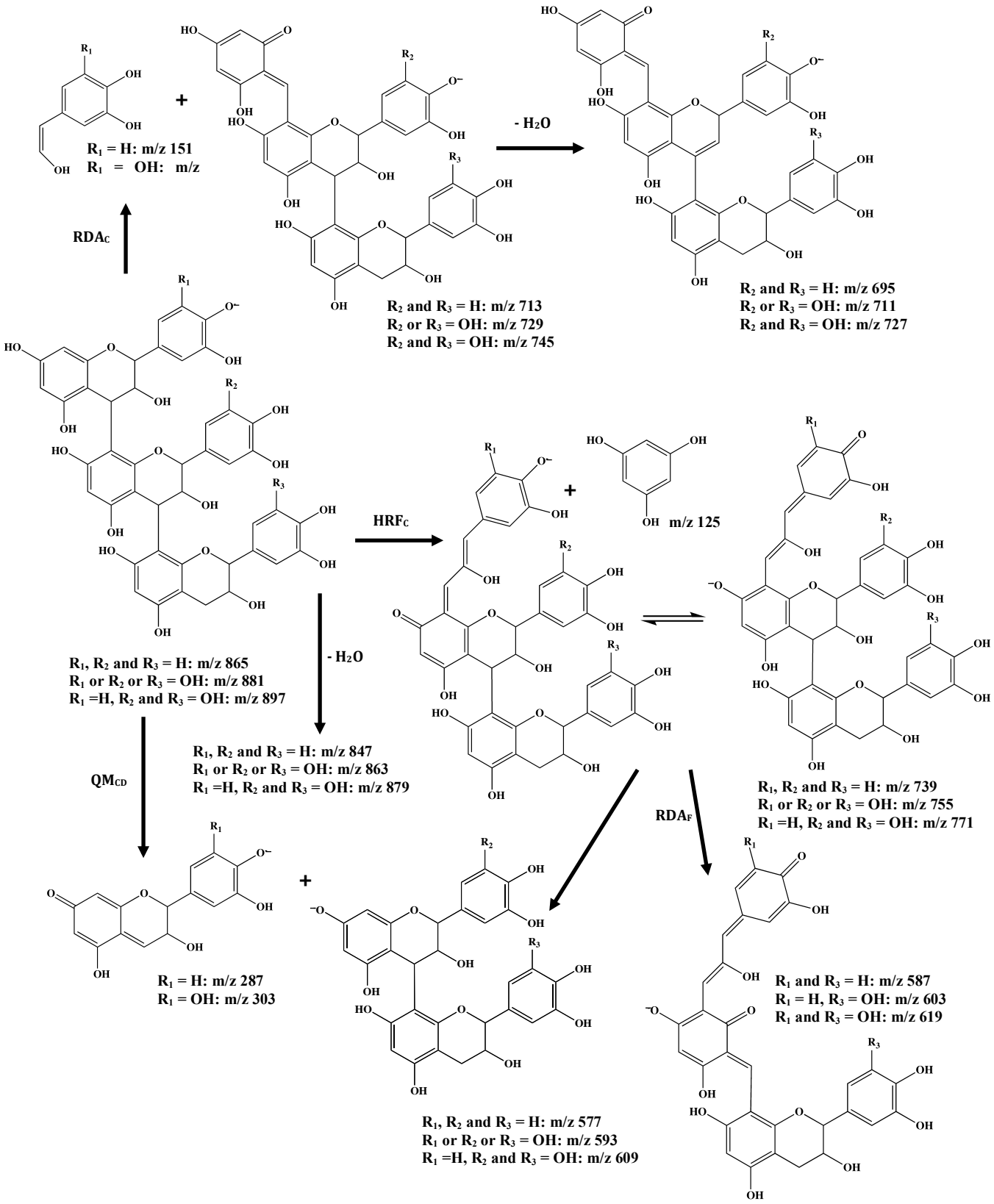
3.4.2.2.2. Fraction B trimers

Compounds 5, 17, 19, 22, 25, 28, 33, 34, 36, 39 and 41 presented pseudomolecular ions with *m/z* $[M]^-$ 865 indicating they are C-type PCs formed with three (E)C subunits (Scheme 3). The MS^2 spectra show product ions with *m/z* 577 (PC) indicating QM_{CD} fission; *m/z* 287 corresponding to the trimer upper subunit after the QM_{CD} fission; *m/z* 713 indicating RDA_C and also a consequent loss of a water molecule originating the pseudomolecular ion *m/z* 695; *m/z* 739 corresponding to a

HRF_C; *m/z* 847 corresponding to a water molecule; *m/z* 451 corresponding to a PC dimer HRF_F, meaning that the PC with *m/z* 577 undergoes further fragmentation; *m/z* 425 corresponding to a PC dimer RDA_F and *m/z* 407 due to consequent loss of a water molecule; *m/z* 289 indicates a PC dimer QM_{FG} fission and consequent loss of 44 m.u. giving pseudomolecular ion *m/z* 245. It was concluded that compound 34 corresponded to PCC1.

Compounds 2, 3, 13, 14, 16, 21, 23, 26, 32 and 35 presented pseudomolecular ions with *m/z* $[M]^-$ 881 indicating they are C-type PCs or PDs formed with two (E)C subunits and one (E)GC subunit. The MS^2 spectra show three types of fragmentation. One type of fragmentation for compounds 2, 14, 21, 26 and 35 with product ions with *m/z* 577, a dimeric PC, indicating a QM_{CD} fission where the upper subunit is the pseudomolecular ion with *m/z* 303; *m/z* 713 corresponding to a RDA_C and the consequent loss of a water molecule originating the pseudomolecular ion *m/z* 695. The rest of the pseudomolecular ions results from the dimeric PC direct fragmentation: *m/z* 451; *m/z* 425 corresponding to a RDA_F and *m/z* 407 for its consequent loss of a water molecule; *m/z* 289 corresponding to a QM_{FG} fission and *m/z* 287 its resulting upper subunit. This fragmentation pattern shows that these compounds are of the type (E)GC-(E)C-(E)C.

The second type of fragmentation for compounds 3, 13, 16 and 23 with product ions with *m/z* 593, a dimeric PC or PD, indicating QM_{CD} fission with pseudomolecular ion with *m/z* 287 as the upper released subunit; *m/z* 729 corresponding to a RDA_C and the sequent loss of a water molecule originating the pseudomolecular ion *m/z* 711. Once again the rest of the pseudomolecular ions result from the dimeric PC or PD direct fragmentation: *m/z* 575 corresponding to the loss of a water molecule; *m/z* 467 corresponding to a HRF_F; *m/z* 305 corresponding to a QM_{FG} fission and *m/z* 441 corresponding to a RDA_F and the consequent loss of a water molecule originating the pseudomolecular ion *m/z* 423. This fragmentation pattern shows that these compounds are of the type (E)C-(E)C-(E)GC.



Scheme 3. Mass fragmentation patterns of flavan-3-ol oligomers

The third type of fragmentation was only detected in one compound. Compound 32 shows product ions with *m/z* 593, again a dimeric PC or PD, indicating QM_{CD} fission; *m/z* 729 corresponding to a RDA_C and the consequent loss of a water molecule

originating the pseudomolecular ion *m/z* 711; and *m/z* 425 corresponding to a RDA_F and the consequent loss of a water molecule originating the pseudomolecular ion *m/z* 407, proving that this trimer lower subunit is a (E)C, and so this compound is of the type (E)C-(E)GC-(E)C. All compounds with *m/z* $[M]^-$ 881 presented product ions with *m/z* 863 corresponding to the loss of a water molecule and *m/z* 755 corresponding to a HRF_C.

Finally, compounds 1, 8 and 12 presented pseudomolecular ions with *m/z* $[M]^-$ 897 indicating they are C-type PCs or PDs formed with two (E)GC subunits and one (E)C subunit. The MS^2 spectra show three types of fragmentation for the three compounds. Compound 1 with product ions with *m/z* 609, a dimeric PD, indicating a QM_{CD} fission; *m/z* 483 corresponding to a HRF_C; *m/z* 745 corresponding to a RDA_C; and *m/z* 305 corresponding to a QM_{FG} fission. These fragmentations indicate that this compound is of type (E)C-(E)GC-(E)GC. Compounds 8 and 12 presented product ions with *m/z* 593 indicating a QM_{CD} fission; *m/z* 771 corresponding to a HRF_C; *m/z* 729 corresponding to a RDA_C and consequent lost of a water molecule originating *m/z* 711; *m/z* 879 indicating the initial trimer dehydration. Compound 8 also presented product ions with *m/z* 603 indicating a RDA_F after the HRF_C and compound 12 also presented product ions with *m/z* 407 corresponding to a RDA_F followed by dehydration and *m/z* 289 corresponding to a QM_{FG}. With this data, it can be concluded that compound 8 corresponds to a trimer type (E)GC-(E)C-(E)GC, while compound 12 to a trimer type (E)GC-(E)GC-(E)C.

3.4.3. PA quantification

Since we did not have standards for the most of the identified compounds, we decided to quantify them as equivalents of catechin (C-eq.). Table 9 and 10 illustrates the results for both analysed wines.

For some compounds the concentration was below the limit of quantification for the catechin calibration curve, especially compounds which presented pseudomolecular ions with *m/z* $[M]^-$ 897 and 609.

Table 9. HPLC-MS retention times, pseudomolecular ions *m/z* $[M]^-$ and concentration in catechin equivalents in mg/100mL of the fraction B of 2012 red vinho verde wine from the Demarcated Region of Vinho Verde (Lima's sub-region).

	Compound	R_t (min)	$[M]^-$ (*m/z*)	Conc. (mg/100mL)
1	(E)C-(E)GC-(E)GC	15.70	897	<LOQ
2	(E)GC-(E)C-(E)C	19.23	881	<LOQ
3	(E)C-(E)C-(E)GC	20.81	881	<LOQ
4	(E)GC-(E)GC	21.67	609	<LOQ
5	(E)C-(E)C-(E)C	25.63	865	0.00887
6	(E)GC-(E)GC	27.95	609	<LOQ
7	PDB1	33.49	593	0.0725
8	(E)GC-(E)C-(E)GC	35.64	897	<LOQ
9	PDB3	37.11	593	<LOQ
10	(E)C-(E)GC	38.91	593	0.0552
11	(E)C-(E)GC	40.90	593	0.00935
12	(E)GC-(E)GC-(E)C	41.72	897	<LOQ
13	(E)C-(E)C-(E)GC	44.09	881	0.00780
14	(E)GC-(E)C-(E)C	45.52	881	0.0260
15	(E)GC-(E)C	47.41	593	0.0114
16	(E)C-(E)C-(E)GC	48.87	881	<LOQ
17	(E)C-(E)C-(E)C	49.73	865	<LOQ
18	PCB3	50.22	577	0.0729
19	(E)C-(E)C-(E)C	51.23	865	<LOQ
20	(E)C-(E)C	52.60	577	0.0603
21	(E)GC-(E)C-(E)C	53.10	881	<LOQ
22	(E)C-(E)C-(E)C	53.82	865	0.181
23	(E)C-(E)C-(E)GC	55.03	881	<LOQ
24	(E)C-(E)GC	56.02	593	<LOQ
25	(E)C-(E)C-(E)C	58.74	865	0.0865
26	(E)GC-(E)C-(E)C	59.58	881	<LOQ
27	PCB4	60.38	577	<LOQ
28	(E)C-(E)C-(E)C	61.91	865	0.0206
29	(E)C-(E)GC	62.64	593	<LOQ
30	(E)GC-(E)C	66.45	593	0.00648
31	PCB6	67.01	577	<LOQ
32	(E)C-(E)GC-(E)C	67.93	881	<LOQ
33	(E)C-(E)C-(E)C	71.48	865	0.0563
34	PCC1	72.21	865	0.136
35	(E)GC-(E)C-(E)C	74.25	881	0.0130
36	(E)C-(E)C-(E)C	78.48	865	0.0586
37	(E)C-(E)C	81.13	577	0.00634

<LOQ – concentration below the limit of quantification for the catechin calibration curve

Table 10. HPLC-MS retention times, pseudomolecular ions *m/z* $[M]^-$ and concentration in catechin equivalents in mg/100mL of the fraction B of 2013 red wine from the Demarcated Region of Douro.

	Compound	R_t (min)	$[M]^-$ (*m/z*)	Conc. (mg/100mL)
38	GC-GC	18.66	609	<LOQ
4	(E)GC-(E)GC	21.46	609	<LOQ
39	(E)C-(E)C-(E)C	23.63	865	<LOQ
5	(E)C-(E)C-(E)C	25.05	865	0.0209
6	(E)GC-(E)GC	27.15	609	<LOQ
7	PDB1	33.23	593	0.125
9	PDB3	36.87	593	0.00996
10	(E)C-(E)GC	39.00	593	0.0601
11	(E)C-(E)GC	40.92	593	<LOQ
13	(E)C-(E)C-(E)GC	44.33	881	0.0127
14	(E)GC-(E)C-(E)C	46.05	881	0.0180
15	(E)GC-(E)C	48.25	593	<LOQ
18	PCB3	50.82	577	0.191
20	(E)C-(E)C	53.17	577	0.579
22	(E)C-(E)C-(E)C	54.53	865	0.186
24	(E)C-(E)GC	57.01	593	0.00973
25	(E)C-(E)C-(E)C	59.50	865	<LOQ
27	PCB4	61.11	577	0.0124
28	(E)C-(E)C-(E)C	62.72	865	<LOQ
29	(E)C-(E)GC	63.47	593	<LOQ
30	(E)GC-(E)C	67.27	593	<LOQ
31	PCB6	67.67	577	0.0303
40	PCB2	70.57	577	0.396
34	PCC1	72.18	865	0.0711
41	(E)C-(E)C-(E)C	72.94	865	<LOQ
35	(E)GC-(E)C-(E)C	74.97	881	0.0143
36	(E)C-(E)C-(E)C	79.21	865	0.0982
37	(E)C-(E)C	81.50	577	0.108

<LOQ – concentration below the limit of quantification for the catechin calibration curve

Fraction B of 2012 red vinho verde wine from the Demarcated Region of Vinho Verde (Lima's sub-region – Table 9) shows a total concentration of PA of 0.890 mg/100 mL of C equivalents. PDB1 and PCB3 are present in the same amount and, in this case, PDs represent 10.2% of total PA concentration and PCs represent 22.9%. It should be notice that all (E)C-(E)GC dimer were considered to PCs and not PDs. Otherwise the PD concentration would represent 17.4%.

In terms of trimer concentration, it can be notice that the concentration for compounds which presented pseudomolecular ions with *m/z* $[M]^-$ 881 was between 0.00780 – 0.0260 mg/100 mL of C equivalents and 0.00887 – 0.181 mg/100 mL of C

equivalents for PC trimers (*m/z* $[M]^-$ 865). This represents 62.5% for PC trimers and 4.39% for the other trimers.

Meanwhile, the fraction B of 2013 red wine from the Demarcated Region of Douro (Table 10) shows a total concentration of PA of 1.94 mg/100 mL of C equivalents, almost 2.2 times higher than the 2012 red vinho verde wine. In this PC concentration represents 71.4% of total PA concentration while PD represents only 6.92%.
PC trimers represent 20.0% of the total PA concentration and PD trimers only 1.66%. This wine has a much higher PA content but it is mainly due to PC dimer content.

3.5. Conclusions

The development of mild mass spectrometry techniques has led to further progress in the determination of PA size distribution. In particular, ESI-MS studies have demonstrated that PD and PC units coexist within the polymers, in an apparent random way. The aim of this work was to present a method to extract and identify PAs in wine samples, especially PDs, without the need to break interflavan bonds. This is the first time that an exhaustive wine PAs evaluation is achieved.
In this case, with this LC-ESI-MS method, it was possible to detect several dimeric and trimeric PDs for the first time in wines. The detected dimeric PDs included not only (E)GC dimers but also (E)C-(E)GC and (E)GC-(E)C dimers. Among the PD trimers, (E)C-(E)GC-(E)GC, (E)GC-(E)GC-(E)C, (E)GC-(E)C-(E)GC, (E)GC-(E)C-(E)C, (E)C-(E)C-(E)GC and (E)C-(E)GC-(E)C were detected.
2013 red wine from the Demarcated Region of Douro has an almost 2.2 times higher PA content than 2012 red vinho verde wine from the Demarcated Region of Vinho Verde (Lima's sub-region), mostly composed by PC dimers. The later wine main content of total PC content is 6 times higher than total PD content, while for the Douro wine is almost 11 times higher.
It can be concluded that red vinho verde wine may be a good source of PDs and that the PD content in wines can now be more accurately analysed.

Chapter 4

The next chapter refers to an already published paper (Teixeira, Azevedo, Mateus & de Freitas, 2016). My contribution to this specific work was all copigmentation assays and respective data processing, except the one assay using Oenin-(O)-catechin as copigment.

4. Copigmentation

4.1. Introduction

Anthocyanins are natural pigments widespread in plant kingdom and are responsible for the colors of many flowers, fruits and beverages such as red wine. It is well known that the color exhibited by anthocyanins in aqueous solution is pH-dependent of the medium. At pH < 2, the red flavylium cation is predominant and, as the pH increases, the other anthocyanin forms (hemiketal, chalcones and quinonoidal bases) occur in equilibrium (Brouillard & Delaporte, 1977).Taking into account that the pK_h of anthocyanins is between 2 and 3 in red wines (pH 3.2 - 4.0) it is expectable that anthocyanins occur largely as colorless hemiketals in equilibrium with other forms (> 70%). However, in nature, anthocyanins found some stabilizing mechanisms that allow them to exist mainly as flavylium cations and quinonoidal forms. Such mechanisms are described in the literature as resulting from non-covalent interactions of anthocyanins with themselves (self-association), with metal cations (metal complexation), with their own acylated residues (intramolecular copigmentation) and with other polyphenols (*e.g.* catechins and procyanidins) acting as copigments (intermolecular copigmentation) (Asen, Stewart, & Norris, 1972a, 1972b; Boulton, 2001; Dangles, 1997; Goto & Kondo, 1991; Haslam, 1998; Robinson & Robinson, 1931). The copigmentation phenomenon consists essentially in van der Waals interactions (vertical π-π stacking) between the planar polarizable nuclei of the anthocyanin and the copigment. The anthocyanin:copigment complexes adopt a sandwich-like structure that stabilizes the flavylium cation chromophore (benzopyrylium) and partially protects it from the nucleophilic attack of water, thus preventing color loss (Goto, 1987; Goto, Tamura, Kawai, Hoshino, Harada, & Kondo, 1986; Santos-Buelga & De Freitas, 2009). Because flavonols generally have planar polyphenolic nucleus, they are excellent copigments that can interact with anthocyanins and protect them from water addition at C2 (the first step for anthocyanin discoloration) (Cruz, Brás, Teixeira, Mateus, Ramos, Dangles, et al.,

2010; Furtado, Figueiredo, Chaves das Neves, & Pina, 1993) whereas flavan-3-ols are comparatively weak copigments. According to the literature flavan-3-ols do not have a good ability to act as copigments, probably due to their non-planar structure that does not allow a close access to the anthocyanin (Brouillard, Wigand, Dangles, & Cheminat, 1991; Gómez-Míguez, González-Manzano, Escribano-Bailón, Heredia, & Santos-Buelga, 2006). Nevertheless, EC is a better copigment than C (Brouillard, Wigand, Dangles, & Cheminat, 1991; Liao, Cai, & Haslam, 1992) due to its B ring conformation, allowing it to be approximately coplanar; PC dimers with C4-C6 interflavanic linkages seem to be better copigments that their respective C4-C8 dimers (Berké & de Freitas, 2005); the presence of more hydroxyl groups (Chen & Hrazdina, 1981) and galloylation at C3 (Berké & de Freitas, 2005) increases the copigmentation effect.

Usually copigmentation produces an increase in absorbance (hyperchromic effect) and a positive shift of the wavelength of the visible absorption maximum (bathochromic effect). Besides the pigment and copigment molecular structure and their relative concentration, copigmentation was shown to be dependent on ionic strength, pH, solvent, temperature and the presence of metal salts (Asen, Norris, & Stewart, 1972; Asen, Stewart, & Norris, 1971, 1972a; Brouillard & Dangles, 1994; Mistry, Cai, Lilley, & Haslam, 1991).

The levels of polyphenols in red wines depend on the characteristics of the grape, environmental factors and the winemaking process. In general, it is assumed that flavan-3-ols and anthocyanins are the major phenolic components in red wines. Red wine color evolution during ageing and storage is in part attributed to copigmentation phenomena. These non-covalent interactions have been reported as the first step for the formation of covalent bonds between two molecules that result into new anthocyanin-derived pigments (Brouillard & Dangles, 1994; Cai, Lilley, & Haslam, 1990; Liao, Cai, & Haslam, 1992). Indeed, during wine processing and ageing, several chemical reactions involving anthocyanins, flavan-3-ols and small molecules released by yeasts (*e.g.* pyruvic acid, acetaldehyde, acetoacetic acid, cinnamic acids) take place, yielding new families of anthocyanin-derived pigments, which will contribute to the modification of wine sensorial properties. Among them, direct and acetaldehyde-mediated condensations between anthocyanins and flavan-3-ols have been widely studied (Constantin Dallas, Ricardo-da-Silva, & Laureano, 1996; Es-Safi, Fulcrand, Cheynier, & Moutounet, 1999; Hayasaka & Kennedy, 2003; Jurd, 1969; Pissarra, Mateus, Rivas-Gonzalo, Santos Buelga, & De Freitas, 2003; Remy, Fulcrand, Labarbe, Cheynier, & Moutounet, 2000; Rivas-Gonzalo, Bravo-Haro, & Santos-Buelga, 1995; Somers, 1971; Timberlake & Bridle, 1976). Direct reactions

between anthocyanins and flavan-3-ols originate the dimeric-type Flavanol-(4,8)-Anthocyanin (F-A) and Anthocyanin-(4,8)-Flavanol (A-F) adducts. A-F adducts formation in red wines is described in the literature to arise from a nucleophilic attack of flavanols (C6/C8) to the electropositive C4 of anthocyanin yielding a colorless product (flavene structure). This adduct could further evolve to the colorless bicyclic form (supplementary ether linkage type-A, A-(O)-F) or undergo oxidation to give the red pigment A^{+}-F which could dehydrate to the orange-yellow xanthylium salt (Asen, Stewart, & Norris, 1972a; Dueñas, Salas, Cheynier, Dangles, & Fulcrand, 2005; Jurd, 1969). Recently, the oenin-(O)-catechin dimeric adduct was hemisynthesized in model solution, isolated and structurally characterized (Cruz, Mateus, & de Freitas, 2012), but its ability to act as a copigment with anthocyanins was never studied.

The aim of this work was to study the previously obtained PDs to act as copigments of oenin (the main anthocyanin of red wine) by determination of the respective copigmentation binding constants and to compare it with other polyphenols present in wine. The relationship between copigmentation ability and the structure of complexes was evaluated.

4.2. Material and methods

4.2.1. Samples

Oenin was purchased from Extrasynthèse (France). (+)-Catechin (C) and (-)-epicatechin (EC) were purchased from Sigma-Aldrich® (Madrid, Spain). (-)-epigallocatechin (EGC) and (-)-epigallocatechin gallate (EGCG) were purchased from Biopurify Phytochemicals Ltd (Sichuan, China).

PCB3 and PDB3 were extracted from barley according to the procedure described elsewhere (see chapter 3.3). The identity and purity of PCB3 and PDB3 was achieved by LC-MS-ESI (Finnigan Surveyor equipped with a Thermo Finnigan (Hypersil Gold) 150 mm x 4.6 mm, 5mm, C18 reversed-phase column at 25 ºC; Finnigan LCQ DECA XP MAX mass detector (Finnigan Corp., San Jose, CA, USA) quadrupole ion trap equipped with an atmospheric pressure ionization source, using an electrospray ionization interface) and NMR (^{1}H NMR spectra were measured in D_2O on a Bruker Avance 400 spectrometer) by comparing with the literature data (Dvorakova, Moreira, Dostalek, Skulilova, Guido, & Barros, 2008).

Oenin-(O)-catechin (Oenin-(O)-C) was obtained by hemisynthesis (Cruz, Mateus, & de Freitas, 2012). Briefly, a solution containing oenin (2.3 mM):(+)-catechin (molar

ratio of 1:20) was prepared in water (200 mL) at pH 2.5 (adjusted with dilute HCl or NaOH), protected from light and placed in the oven at 50 ºC.

4.2.2. Copigmentation

All solutions used were prepared in a citrate buffer solution with 12% ethanol (0.2 M) at pH 3.5, and the ionic strength was adjusted to 0.5 M by addition of sodium chloride. Each pigment:copigment solution was prepared by mixing a volume of pigment solution (10^{-4} M) with an aliquot of copigment solution to give the required pigment:copigment molar ratio of 1:0, 1:5, 1:10, 1:20, 1:30, 1:40. Each experiment was performed in triplicate. All the solutions were left to equilibrate for 30 min before spectroscopic measurements. The absorbance values were collected at the maximum absorption wavelength of free oenin at pH 3.5 (λ_{max} 523 nm). Parameter r, which represents the ratio between the molar absorption coefficient of the complex (oenin:copigments molar ratio = 1:40) and the free flavylium ion (10^{-4} M), was determined in strongly acidic solutions (1 M aqueous HCl, pH ≈ 0) to assure that flavylium ion is the sole anthocyanin form.

4.2.3. UV-Visible spectroscopy

UV-visible spectra were recorded on a BIO-TEK Power Wave XS spectrophotometer at a constant temperature of 25 ºC from 360 to 830 nm (1 nm sampling interval) using a 1 cm path length cell.

4.2.4. Data analysis

The curve fittings were carried out on a PC using the Scientist program (MicroMath, Salt Lake City, UT). Curve fittings were achieved through least-squares regression method. Statistical analysis reported standard deviations and correlation coefficients.

4.2.5. Molecular Dynamic Simulations

The starting geometries of the copigment (EC, EGC, PCB3 and Oenin-(O)-C) and pigment (oenin) molecules were obtained at the HF/6-31G(d) level of calculation, using the Gaussian 09 package (Frisch, 2009). Atomic charges were further

recalculated using the RESP procedure (Bayly, Cieplak, Cornell, & Kollman, 1993). MD simulations were performed with GAFF (generalized amber force field) (Wang, Wolf, Caldwell, Kollman, & Case, 2004) and the TIP3P model for the solute and water, respectively. Explicit solvation was included as a truncated octahedral box with a 12 Å distance between the box faces and any atom of the compounds. Energy minimization occurred in two stages: first, the solute was kept fixed and only the position of the water molecules and counter-ion was optimized (500 steps using the steepest descent algorithm and 1,500 steps carried out using conjugate gradient); second, the full system was minimized (1,000 steps using the steepest descent algorithm and 2,000 steps carried out using conjugate gradient). Following a 100 ps equilibration procedure at constant volume and temperature, 30 ns MD simulations were carried out. The Langevin thermostat was used to control the temperature at 303.15 K, (Izaguirre, Catarello, Wozniak, & Skeel, 2001) and all the simulations were carried out in the NPT ensemble with periodic boundary conditions. All MD simulations were carried out using the Sander module, implemented in the Amber 10.0 simulations package (Case, 2008). Bond lengths involving H-atoms were constrained using the SHAKE algorithm, and the equations of motion were integrated with a 2 fs time step using the Verlet leapfrog algorithm (Ryckaert, Ciccotti, & Berendsen, 1977). Nonbonded interactions were truncated with a 12 Å cutoff.

4.2.6. Calculation of binding free energies

The MM_PBSA script (Molecular Mechanics−Poisson−Boltzmann Surface Area) (Huo, Massova, & Kollman, 2002; Kollman, Massova, Reyes, Kuhn, Huo, Chong, et al., 2000; Massova & Kollman, 2000) as implemented in Amber 10.0 simulations package (Case, 2008) was used to calculate the binding free energies ($\Delta G_{binding}$) for all complexes. A series of 150 geometries was extracted every 100 steps of each simulation. The internal energy (bond, angle, and dihedral), the electrostatic and the van der Waals interactions were calculated using the Cornell force field (Cornell, Cieplak, Bayly, Gould, Merz, Ferguson, et al., 1995) with no cutoff. The electrostatic solvation free energy was calculated by solving the Poisson−Boltzmann equation with the PBSA program, implemented in the Amber 10.0 simulations package (Case, 2008). The nonpolar contribution to the solvation free energy due to van der Waals interactions between the solute and the solvent and cavity formation was modelled as a term that is dependent on the solvent accessible surface area of the molecule. As these compounds possess similar structures and binding modes, the relative

binding energies ($\Delta\Delta G_{binding}$) were calculated with respect to the most stable complex.

4.3. Results and discussion

In this work, oenin (malvidin-3-*O*-β-D-glucoside) was mixed with increasing concentrations of different copigments, namely: C, EC, EGC, EGCG, PDB3, PCB3 and Oenin-(O)-C (Figure 46), for a quantitative evaluation of the corresponding copigmentation complexes. No copigmentation studies had been previously performed with compounds with the Oenin-(O)-C structure nor with PDB3 due to the difficulty in obtaining them from natural sources.

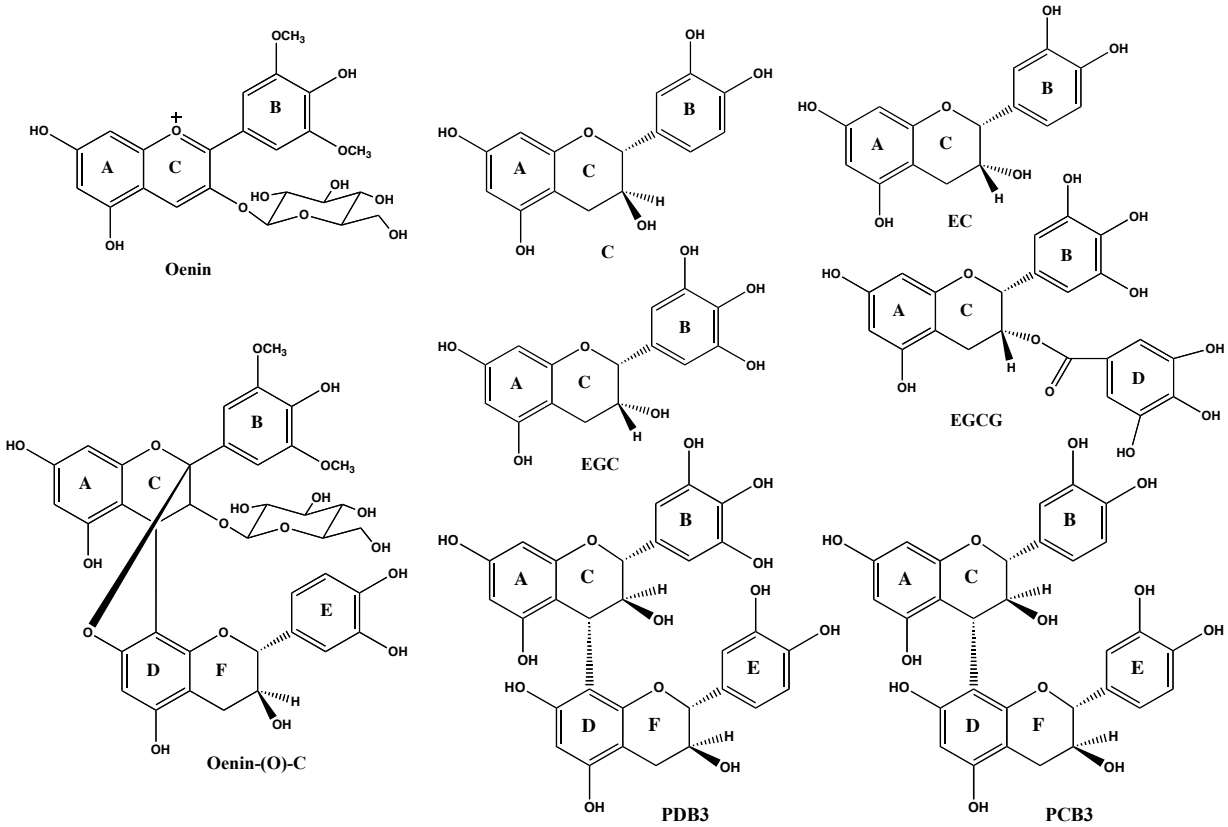


Figure 46. Chemical structures of the pigment (oenin) and copigments used.

The intermolecular copigmentation studies were performed with a concentration of pigment of $1x10^{-4}$ M to minimize any self-association effects. It is known that ethanol largely reduces the copigmentation effect (Dangles & Brouillard, 1992), however, its inclusion in the experimental conditions attempts to mimic red wine composition. Also, previous copigmentation studies were attempted using only aqueous citrate buffer but some copigments were found to be very difficult to dissolve properly and precipitated during the spectroscopic analysis. In weak acidic conditions (pH 3.5), the

flavylium and hemiketal forms are the anthocyanin predominant species (pK_h oenin = 2.70 ± 0.01) (Dangles & Elhajji, 1994; Malien-Aubert, Dangles, & Amiot, 2002). The quinonoidal bases are only present in a very small extent (Brouillard & Delaporte, 1977) and can be neglected (first pK_a ≈ 4 (Brouillard & Delaporte, 1977; Macanita, Moreira, Lima, Quina, Yihwa, & Vautier-Giongo, 2002)).

The copigmentation equilibrium is in competition with the hydration process, i.e., the copigment molecule competes with water for the flavylium ion. Bearing this, the copigmentation binding constants (K) between oenin and the referred copigments could be evaluated from a general mathematical treatment that takes into account the thermodynamics of water addition onto the flavylium ion. Assuming a 1:1 stoichiometry for the complex and no complexation between the copigment and the colourless forms, the variations of visible absorbance A as a function of the total copigment concentration CP_t can be expressed as equation (1):

$$A = \frac{A_0}{\frac{a}{r-a} + \frac{1}{(r-a).K}.\frac{1}{CP_t}} + A_0 \qquad (1)$$

where A_0 is the visible absorbance of the pigment in the absence of copigment, r is the ratio of the molar absorption coefficient of the complex to that of the free flavylium ion and $a = \frac{1}{1+K_h.10^{pH}}$ (Malien-Aubert, Dangles, & Amiot, 2002). The absorbance values obtained for the oenin-copigment complexes at the maximum absorption wavelength of free oenin at pH 3.5 (λ_{max} 523 nm) are presented in Table 11.

Table 11. Absorbance values for the oenin/copigment complexes at the maximum wavelength (λ_{max} 523 nm)

	Oenin (10^{-4} M)						
r	0.953	0.956	0.897	0.884	0.958	0.964	0.891
Pigment/copigment molar ratio	C	EC	EGC	EGCG	PDB3	PCB3	Oenin-(O)-C
1:0 (A_0)	0.528	0.520	0.521	0.540	0.507	0.537	0.635
1:5	0.564	0.538	0.569	0.586	0.525	0.549	0.715
1:10	0.598	0.555	0.603	0.625	0.540	0.563	0.767
1:20	0.648	0.605	0.649	0.679	0.560	0.576	0.922
1:30	0.696	0.642	0.703	0.732	0.573	0.604	1.009
1:40	0.737	0.680	0.784	0.757	0.603	0.616	1.150

The absorbance increase with the copigment concentration reflects the preferential binding of the copigment to the flavylium ion and the subsequent shift of the hydration equilibrium toward the coloured forms.

For each selected copigment, a plot of absorbance as a function of wavelength was performed and the typical behaviour of copigmentation phenomena was observed: increase of absorbance (hyperchromic effect) and a slight batochromatic shift of λ_{max} as the copigment concentration increased. Figure 47 shows the absorption spectra of free oenin and oenin-(O)-catechin:oenin complex obtained at different molar ratios (A) and the respective mathematical treatment to determine K according equation (1) (B).

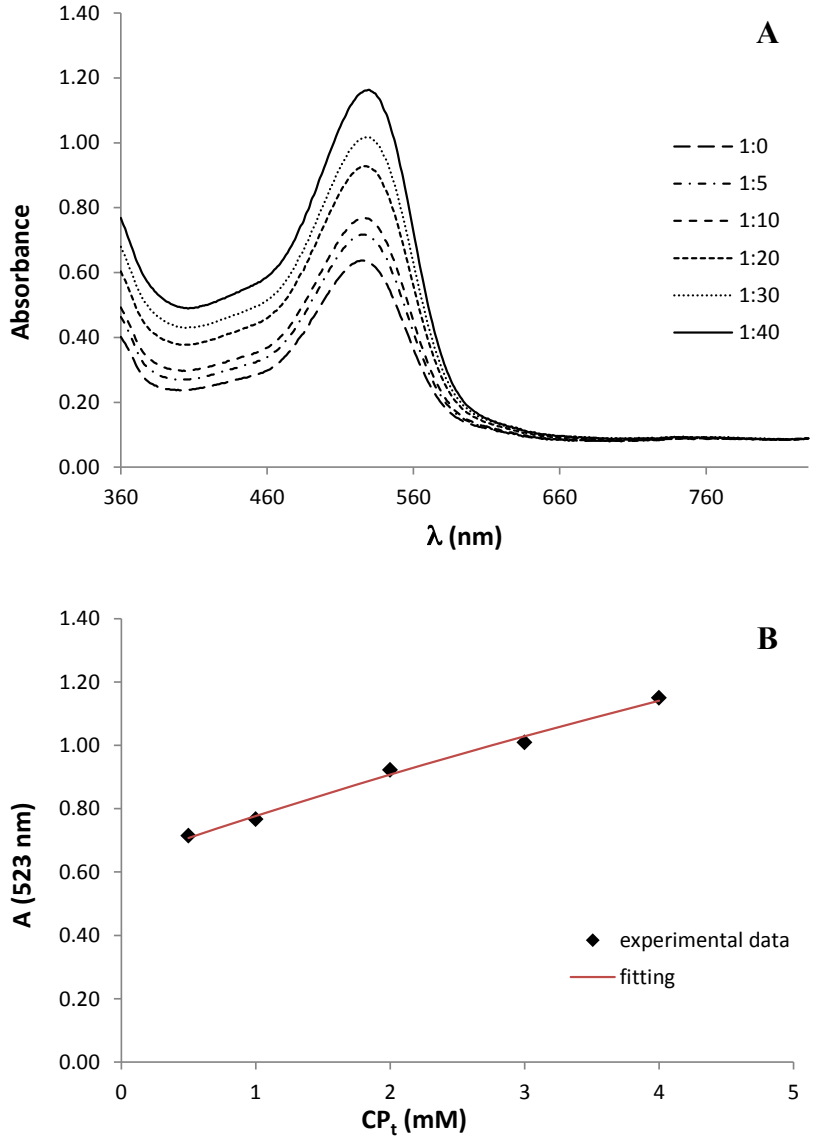


Figure 47. (A) Visible spectra of free oenin (1:0) and oenin:oenin-(O)-C solutions obtained at different molar ratios, (B) Plot of absorbance at 523 nm as a function of Oenin-(O)-C concentration.

Equation (1) was used for the curve fitting of $A = f(CP_t)$ to the experimental data to obtain the optimized values for K as the sole adjustable parameter. Statistical analysis gave good correlation coefficients and standard deviations for the copigmentation constants (*K*) (Table 12).

Table 12. Copigmentation Constants for the Oenin/Copigment Complexes[a]

Copigment	*K* (M^{-1})	R^2
C	136 (± 4)	0.998
EC	99 (± 2)	0.999
EGC	177 (± 7)	0.992
EGCG	162 (± 10)	0.991
PDB3	60 (± 3)	0.991
PCB3	48 (± 2)	0.992
Oenin-(O)-C	309 (± 7)	0.997

[a]Values in parentheses are the standard deviations of the curve-fitting procedure

The experimental results show that all copigments have a r parameter close to 1 at 523 nm with the solution of the complex copigment:oenin showing a slight discoloration at pH ≈ 0 comparatively to oenin. Oenin-(O)-C was found to be the copigment with the highest copigmentation binding constant in 12% ethanol, pH 3.5, followed by EGC, EGCG, C, EC, PDB3 and PCB3. The highest value of K obtained for oenin-(O)-C is probably due to the existence of an extra glucose unit, allowing more hydrophobic interactions between glucose residue and the pigment rings. This results in the flavylium stabilization and prevents its hydration and consequently color loss.

The results obtained with EC, EGC, PDB3 and PCB3 showed that the presence of one more hydroxyl group in the B ring of the flavan-3-ol structure increases the copimentation potential. Futhermore, the presence of an additional OH group in ring B seems to be more efficient than the presence of a galloyl group, comparing the results for EC, EGC and EGCG. Despite the fact that esterification of the C3 hydroxyl function by gallic acid adds a well exposed planar π–π stacking (Berké & de Freitas, 2005), some steric hindrance may occur. This also can explain the lower K values obtained for dimers comparatively to monomers.

Under the conditions chosen for this work, catechin seems to be more efficient to form copigment complexes with oenin than its isomer epicatechin. To confirm and better explain these experimental results, some computational studies were performed.

4.3.1. Computational Studies of four pigment−copigment complexes

Molecular dynamics simulations were carried out for four distinct complexes, EC:oenin, EGC:oenin, PCB3:oenin and Oenin-(O)-C:oenin complexes, which allowed sampling of the potential hypersurface so as to identify several conformations for each copigmentation complex studied. The relative binding energies obtained by the MM_PBSA approach (Huo, Massova, & Kollman, 2002; Kollman, et al., 2000; Massova & Kollman, 2000) are in relatively good agreement with the experimental results (Table 13), which confirms the relevance of the MD procedure to provide an accurate picture for conformational analysis. It is noticed that the theoretical $\Delta\Delta G_{binding}$ values fit only qualitatively with the experimental values, whereas the small quantitative differences could be due to some approximations within the MM_PBSA methodology. The results reveal that the $\Delta G_{binding}$ energy of the Oenin-(O)-C:oenin complex is the most negative compared to the copigmentation complexes with EC, EGC and PCB3. Therefore, Oenin-(O)-C:oenin complex displays higher stability and its formation is thermodynamically favoured when compared to the other complexes. Although the tendency obtained for the binding free energies is Oenin-(O)-C > EGC > EC > PCB3, the small differences in $\Delta\Delta G_{binding}$ between Oenin-(O)-C and EGC, as well as EC and PCB3 (0.14 and 0.16 kcal/ mol, respectively) reveal similar stability for the first two complexes and second two complexes.

Table 13. Relative Binding Free Energies of the Copigmentation Complexes

Complex	Theoretical $\Delta\Delta G_{binding}$ (kcal/mol)	Experimental $\Delta\Delta G_{binding}$ (kcal/mol)*
EC:oenin	2.73	0.85
EGC:oenin	0.14	0.51
PCB3:oenin	2.89	1.28
Oenin-(O)-C:oenin	0.00	0.00

*Experimental values calculated from Table 11 using $\Delta\Delta G = RT \ln(K_{Oenin\text{-}(O)\text{-}C}/K_{CPx})$

Figure 48 shows the closest geometries to the average structures for the four copigment:oenin complexes. It is well known that the formation of these complexes is driven by van der Waals interactions between the large planar surfaces of the pigment and copigment molecules and the concomitant release of high-energy water molecules from the solvation shells (hydrophobic effect).

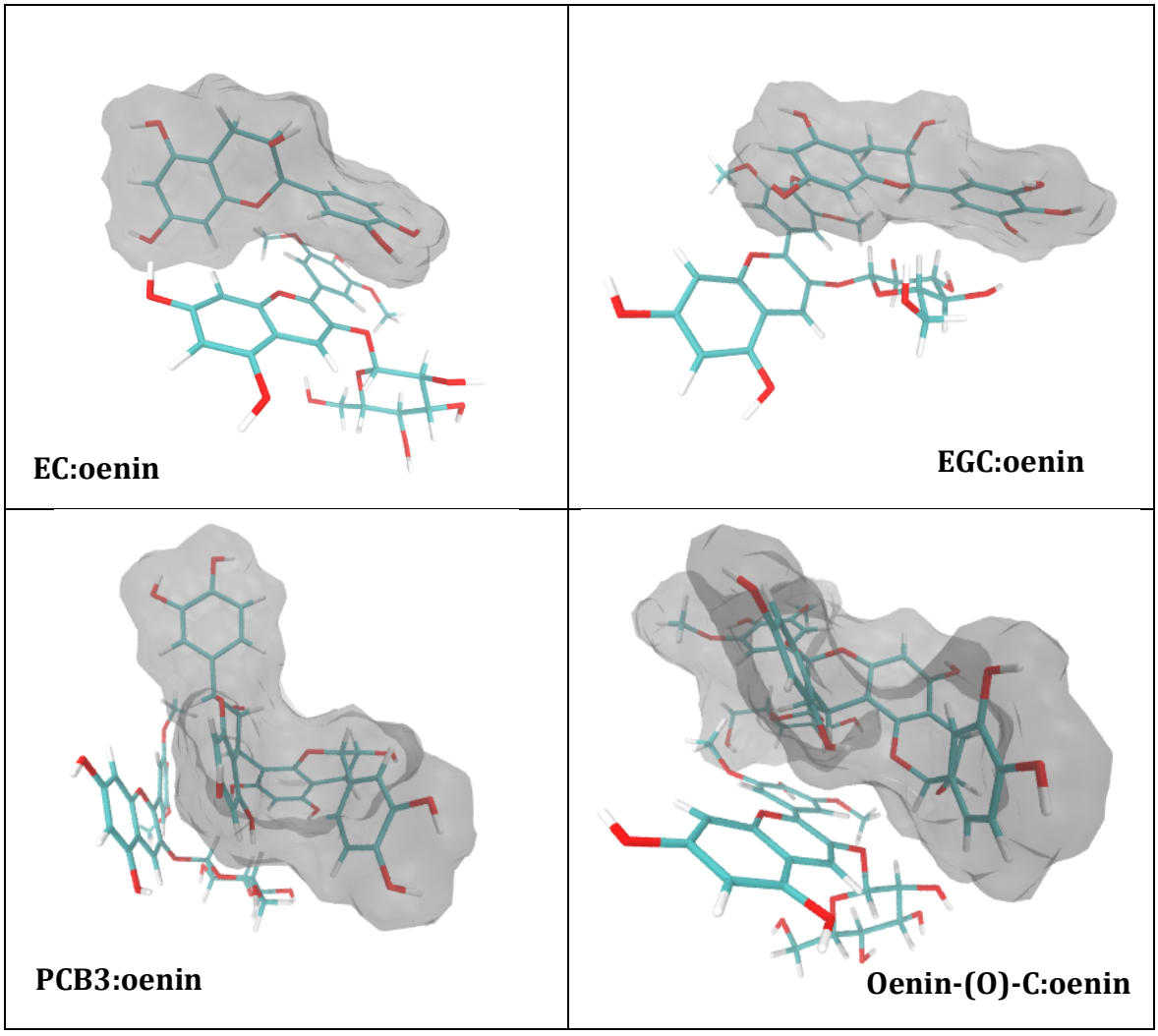


Figure 48. Closest geometries to the average structures of the EC:oenin, EGC:oenin, PCB3:oenin and Oenin-(O)-C:oenin complexes. Copigment structures are depicted with surface representation.

As this kind of copigmentation systems has a large number of degrees of freedom, the steric hindrance does not allow optimized π-stacking arrangements. However, the binding could also be strengthened by H-bonding involving the numerous OH groups from glucose and phenolic units. Hence, the proximity between planar surfaces of the pigment and copigment molecules should reflect the stability of each complex. The interplanar distances between the benzopyrylium nucleus (AC), B ring and glucose unit of the oenin and the aromatic and pyran rings of each copigment were calculated during each MD simulation. The minimal distances thus obtained are shown in table 14. The results show that the AC rings of EGC and Oenin-(O)-C are the closest planes to the benzopyrylium nucleous of oenin (4.34 Å and 4.92 Å, respectively). Furthermore, the AC plane of Oenin-(O)-C and the B ring of EGC are the nearest planes to the B ring (4.76 Å and 4.33 Å, respectively). Although all copigment planes are very distant to the glucose residue, the minimal distances were

obtained with the EGC and Oenin-(O)-C molecules (6.76 Å and 6.86 Å, respectively). As the intermolecular distances of about 4 Å are consistent with van der Waals contacts, these data reveal that the shortest copigment:oenin distances were obtained for the complexes with the Oenin-(O)-C and EGC copigments, which agrees with their highest stability constants (K).

Table 14. Average minimal distances between approximately planar surfaces of the oenin and copigment molecules in the copigmentation complexes.

	Average minimal distance (Å)		
Complex	Benzopyrylium nucleus (AC)	B ring	Glc
EC:oenin	5.06 (AC)	5.35 (AC)	6.97 (B)
EGC:oenin	4.34 (AC)	4.76 (B)	6.76 (B)
PCB3:oenin	5.01 (AC)	5.53 (AC)	7.32 (AC)
Oenin-(O)-C:oenin	4.92 (AC)	4.33 (AC)	6.86 (Glc)

Recently, the copigmentation between the 3-*O*-methycyanidin and quercetin compounds was computationally studied by Meo et al. (Di Meo, Sancho Garcia, Dangles, & Trouillas, 2012). According to their results, the contribution of copigmentation to color stability is dependent on the relative concentrations of pigment and copigment. It was verified that equal pigment and copigment concentrations results in complexes with high stability, that is the prevalent mechanism to avoid the hydration of the anthocyanin chromophore. The present results are in agreement with these data, and it was also verified that the driving force in the formation of copigmentation complexes appears to be dispersive interactions that greatly contribute to high stable complexes and prevents the hydration of the pigment.

According to the structures of the four complexes shown in figure 48, it was observed that Oenin-(O)-C offers a very large planar surface for the establishment of multiple van der Waals interactions with the pigment. The untypical covalent interaction between the anthocyanin and catechin units observed in this copigment provides a huge roughly planar polarizable surface (AC-DF nucleus) that must be prone to strong π stacking interactions with the aromatic rings of oenin. In addition, it was observed that the benzopyrylium nucleus of EGC is strategically positioned in the middle of the pigment structure, establishing an accessible face and a closer contact with all planes of oenin. This fact contributes to a great binding of EGC and oenin molecules and thus to the also higher K values obtained for this complex.

For EC:oenin, it was observed that the AC nucleus of the copigment is perpendicularly located to the flavylium plane of oenin, which difficults the establishment of nonpolar contacts between both molecules. In relation to PCB3:oenin complex, it was noticed that the EGC has its catechin unit facing opposite sides that prevents a simultaneous interaction of both moieties with the flavylium nucleus of oenin. As the hydrophobic contacts with the flavylium nucleus of oenin are great contributions to the copigmentation driving force, it is expected that EC and PCB3 have the smallest copigmentation binding constants.
All these structural data show that, within the complexes studied, Oenin-(O)-C and EGC are closer to the pigment molecule than EC and PCB3. This is in accordance with their highest copigmentation binding constants calculated experimentally.

4.4. Conclusions

The data yielded from this study allowed concluding that: a) the presence of a pyrogallol group in the B ring of the flavan-3-ol structure slightly increases the copimentation potential; and b) within all copigments tested oenin-(O)-catechin revealed to be the best. According to computational studies performed on epicatechin:oenin, epigallocatechin:oenin, procyanidin B3:oenin and oenin-(O)-catechin:oenin complexes, the $\Delta G_{binding}$ energy of the oenin-(O)-catechin:oenin complex is the most negative comparatively to the other copigmentation complexes, hence being more stable and thermodynamically favoured. All structural data show that oenin-(O)-catechin and epigallocatechin are closer to the pigment molecule, which is in accordance with these two copigments having the highest experimental copigmentation binding constants for oenin.

Chapter 5

5. General conclusions

Polyphenols offer great hope for the prevention of chronic human diseases. This work contributes to the organic synthesis of PDs, an each day passing more important class of proanthocyanidins. Some of the earliest references of PA synthesis date back as far as the turn of the 20th century, indicating the importance with which this theme was viewed. Much of the synthetic work was derived from a need to be able to assign the absolute configurations of those compounds that were isolated from natural sources. This, coupled to the fact that these compounds show countless health benefits, it would appear to be a good indicator of the importance of such studies. It has also been demonstrated the difficulty involved in the isolation and purification of such compounds. With the growing pressures involved in the use of more natural food additives, allied to the industrial applications, it is not misplaced to affirm that the analysis of these compounds should continue to flourish in the future.
A new LC-ESI-MS method was developed, allowing the detection of several dimeric and trimeric PDs in wines for the first time. This method was used to analyse and quantify the PA composition of a 2013 red wine from the Demarcated Region of Douro and a 2012 red vinho verde wine from the Demarcated Region of Vinho Verde (Lima's sub-region). The PD content in wines can now be more accurately analysed and it can be concluded that the red vinho verde wine may be a good source of PDs.
Copigmentation studies allowed concluding that the presence of a pyrogallol group in the B ring of the flavan-3-ol structure slightly increases the copimentation potential, but a A-type link between both monomeric units of a flavan-3-ol-anthocyanin dimer like oenin-(O)-catechin revealed to be the best among all tested compounds. This conclusion was supported by computational studies.

Future perspectives

Finishing this work, the most important thing still to be done is finding proper hydrogenolysis reaction conditions that do not allow the rupture of the key dimeric interflavan bond. We believe that this work does not involve finding new protection groups but perhaps finding new catalysts that work in even softer conditions and keep the regioselectivity of the debenzylation. By so, new prodelphinidins can be synthesized and also identified and quantified in food and beverages matrices. New

in vitro studies will also be possible, enlightening more the prodelphinidin potential concerning human health.

It should also be interesting to analyse red vinho verde grapes in terms of prodelphinidin nature and concentration. It is important to verify if the prodelphinidin profile found in this study is due to the skin of the grapes or contact with grape stems during vinification.

Chapter 6

6. Bibliography

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