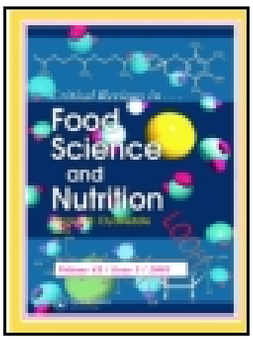

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Grape and wine polymeric polyphenols: Their importance in enology

Lingxi Li[a,b] and Baoshan Sun[b,c]

[a]School of Pharmacy, Shenyang Pharmaceutical University, Shenyang, P. R. China; [b]School of Functional Food and Wine, Shenyang Pharmaceutical University, Shenyang, P. R. China; [c]Pólo Dois Portos, Instituto Nacional de Investigação Agrária e Veterinária, I.P., Quinta da Almoinha, Dois Portos, Portugal

ABSTRACT

Phenolic compounds are important constituents of red wine, contributing to its sensory properties and antioxidant activity. Owing to the diversity and structural complexity, study of these compounds was mainly limited, during the last three decades, on their low-molecular-mass compounds or simple phenolic compounds. Only in recent years, much attention has been paid to highly polymerized polyphenols in grape and red wines. The reason for this is largely due to the development of analytical techniques, especially those of HPLC-ESI-MS, permitting the structural characterization of highly polymerized polyphenols. Furthermore, the knowledge on the biological properties of polymeric polyphenols of red wine is very limited. Grape polyphenols mainly consist of proanthocyanidins (oligomers and polymers) and anthocyanins, and low amount of other phenolics. Red wine polyphenols include both grape polyphenols and new phenolic products formed from them during winemaking process. This leads to a great diversity of new polyphenols and makes wine polyphenol composition more complex. The present paper summarizes the advances in the research of polymeric polyphenols in grape and red wine and their important role in Enology. Scientific results indicate that polymeric polyphenols, as the major polyphenols in grape and red wine, play a major role in red wine sensory properties, color stability and antioxidant activities.

KEYWORDS

Polymeric polyphenols; grape; wine; separation; sensory property; color stability; antioxidant activity

Introduction

Grape and wine phenolic compounds have attracted considerable attention of the international scientific community during the last three decades, not only for their contribution to the quality of wines, including sensory properties (color, flavor, astringency and bitterness) (Ribéreau-Gayon 1964; Somers 1966, 1968; Ribéreau-Gayon 1970; Somers 1971; Timberlake and Bridle 1976; Somers 1978; Arnold et al. 1980; Haslam 1980; Ribéreau-Gayon 1982; Lee and Jaworski 1988; Lea 1992; Bakker and Timberlake 1997; Mirabel et al. 1999; Saucier et al. 2004; Spranger et al. 2004; Sun et al. 2008; Jone et al. 2008; Mercurio and Smith 2008; Blazquez et al. 2012; McRae et al. 2010, 2013), and ageing behavior (Somers 1966; Ribéreau-Gayon 1973; Somers and Michael 1979; Ribéreau-Gayon et al. 1983; Somers and Evans 1986; Somers and Wescombe 1987; Bakker et al. 1993; Mirabel et al. 1999; Mateus et al. 2003; Sun and Spranger 2005; Sun et al. 2011; Barrio-Galán et al. 2016), but especially for their potential beneficial health effects, related to their protective action towards coronary heart disease and various biological activities (Masquellier 1988; Ricardo-da-Silva et al. 1991; Nigdikar et al. 1998; Carando et al. 1999; Santos-Buelga and Scalbert 2000; Corder et al. 2001; Diebolt et al. 2001; Leikert et al. 2002; Dell'Agli et al. 2004; Rasmussen et al. 2005; Baur and Sinclair 2006; Guo et al. 2007; Spranger et al. 2008; Covas et al. 2010; Das et al. 2010; Leopoldini et al. 2011; Rodrigo et al. 2011; Sun et al. 2011; Wang et al. 2011; Jordão et al. 2012; Mulero et al. 2011, 2015; Sun et al. 2017).

The phenolic compounds are widely distributed in the plant kingdom. They are secondary plant metabolites and consist of several groups of substances (Ribéreau-Gayon 1968; Jaganath and Grozier 2010). Phenolic compounds can be divided into flavonoids and non-flavonoid compounds. The non-flavonoid compounds include essentially the phenolic acids, which may be divided into benzoic acids (C6-C1) and cinnamic acids (C6-C3), but also other phenolic derivatives as stilbenes (including resveratrol). The flavonoids are characterized by their C6-C3-C6 skeleton. In large sense, flavonoids include anthocyanins, flavan-3-ols (catechins and proanthocyanidins), flavonols, flavanonols and flavones (Ribéreau-Gayon 1968; Jaganath and Grozier 2010).

Owing to the diversity and structural complexity of plant polyphenols, study of these compounds was limited, during a long period, on their low-molecular-mass compounds or simple phenolic compounds (Czochanska et al. 1979; Salagoïty-Auguste and Bertrand 1984; Romeyer et al. 1985; Bourzeix et al. 1986; Cheynier and Rigaud 1986; Jaworski and Lee 1987; Oszmianski et al. 1988; Cheynier et al. 1989; Foo and Karchesy 1989; Oszmianski and Lee 1990; Ricardo-da-Silva et al. 1990; Ricardo-da-Silva et al. 1991a; Ricardo-da-Silva et al. 1991b; Ricardo-da-Silva et al. 1991c; Cheynier et al. 1992; Ricardo-da-Silva et al. 1992; Kermasha et al. 1995; Santos-Buelga et al. 1995; Fuleki and Ricardo-da-Silva 1997; Carando et al. 1999). Separation and identification of phenolic compounds were performed in the past by paper chromatography (PC) (Thompson

CONTACT Baoshan Sun sun.baoshan@iniav.pt School of Functional Food and Wine, Shenyang Pharmaceutical University, 110016 Shenyang, P. R. China.

et al. 1972; Lea and Timberlake 1974; Malan and Roux 1975; Gupta and Haslam 1978; Lea 1978; Lea et al. 1979; Oh and Hoff 1979; Delcour et al. 1983), thin-layer chromatography (TLC) (Michaud et al. 1971; Michaud et al. 1973; Lea and Timberlake 1974; Piretti et al. 1976; Lea 1978; Lea et al. 1979; Kaluza et al. 1980; McMurrough 1981; Nonaka et al. 1981; Wilson 1981; Hemingway et al. 1982; Delcour et al. 1983; Hemingway et al. 1983; Nonaka et al. 1983; Delcour et al. 1985; Gujer et al. 1986; Foo and Karchesy 1989; Lee and Jaworski 1990) or column chromatography (CC) (Somers 1966; Lea and Timberlake 1974; Michaud and Margail 1977; McMurrough and McDowell 1978; Oh and Hoff 1979; Kantz and Singleton 1990). And more recently HPLC techniques which have revolutionized the study of plant phenolic compounds because of its high resolution, sensibility, speed and quantitative nature (Haslam 1980; McMurrough 1981; Foo 1984; Bakker 1986; Bakker et al. 1986; Bourzeix et al. 1986; Cheynier and Rigaud 1986; Jaworski and Lee 1987; Boukharta et al. 1988; Cheynier et al. 1988; Cheynier et al. 1989a; Cheynier et al. 1989b; Bailey et al. 1994; Adrian et al. 2000). However, although reversed-phase HPLC is almost universally used for analysis of plant phenolic compounds, this technique only permits separation and quantitative determination of low-molecular-mass polyphenols or simple phenolic compounds, while higher oligomeric and polymeric forms could not be further separated and eluted towards the end of the chromatogram as a broad, unresolved peak (Putman and Buttler 1989; Rigaud et al. 1993). Furthermore, only from the middle of 1990s, great attention has been paid to their higher molecular-mass (oligomeric and polymeric) phenolic compounds. The reason for this is largely due to the development of analytical techniques, especially that of thioacidolysis (Prieur et al. 1994) and HPLC-ESI-MS (Cheynier et al. 1997; Spranger et al. 2008), permitting the determination of the structural composition and molecular mass of highly polymerized polyphenols. On the basis of these analytical techniques, structural features of grape polymerized polyphenols have, to a great extent, been characterized. It is confirmed that grape seed tannins consist of partly galloylated procyanidins (Prieur et al. 1994; Sun et al. 1998), with chain length ranging from 2 to 34 (Sun et al. 1999), and that grape skin tannins also contain prodelphinidins (Souquet et al. 1996; Sun et al. 1998), with degree of polymerization up to 83 (Souquet et al. 1996). In our laboratory, we have succeeded in isolating, from grape seed, catechin fraction, oligomeric procyanidin fraction (DP = 2 to 12–15) and polymeric procyanidin fraction (mDP = 25) (Sun et al. 1998; Sun et al. 1999), each of which possessed potent antioxidant activities (Spranger et al. 2008). Furthermore, using ESI-MS^n analysis, polymeric procyanidins were detected to have DP up to 34 (Spranger et al. 2008).

However, until now little is known about the chemical and biological properties of polymeric polyphenols of red wine. Grape polyphenols mainly consist of proanthocyanidins (monomers, oligomers and polymers), and anthocyanins, and low amount of other phenolics such as phenolic acids, resveratrol and its derivatives, flavonols, flavanonols and flavones (Cheynier 2002; Cheynier 2012). Red wine polyphenols include both grape polyphenols and new phenolic products formed from them during winemaking process.

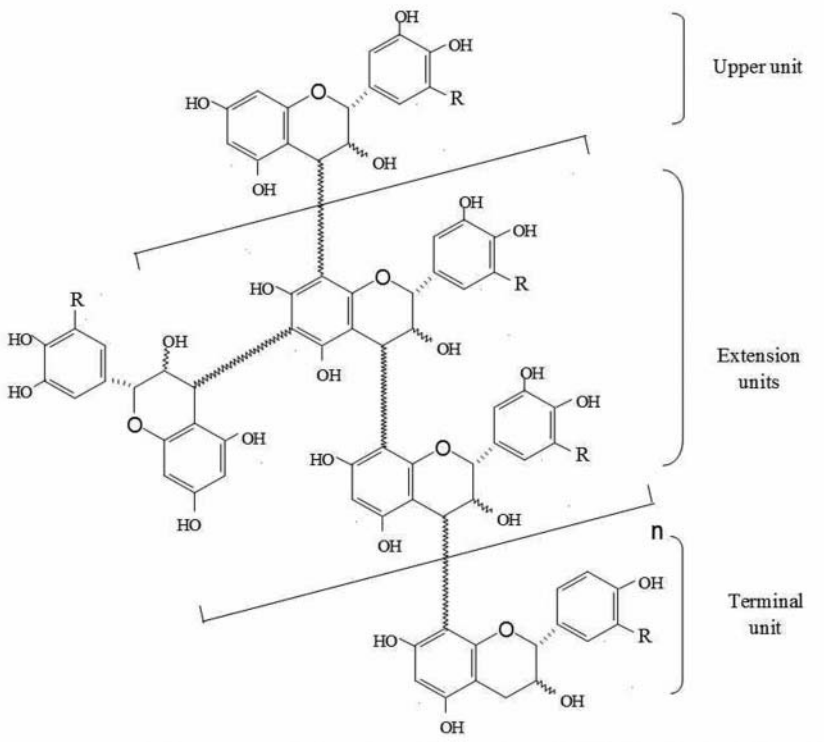

Figure 1. Structure of polymeric polyphenols presented in grape tissues.

The enzymatic and non-enzymatic reactions start as soon as the beginning of wine making (crushing) and continue throughout fermentation and ageing. This leads to a great diversity of new polyphenols and makes wine polyphenol composition more complex (Cheynier 2002). Furthermore, red wine polyphenols mainly consist of free proanthocyanidins (monomers, oligomers and polymers), free anthocyanins, anthocyanin-proanthocyanidin complexes (either direct or indirect), pyranoanthocyanins, as well as low amount of other phenolic compounds. One of the major problems encountered was the difficulty to separate oligomeric proanthocyanidins from anthocyanins (Vidal et al. 2002) and polymeric proanthocyanidins from pigmented complexes. The present paper summarizes the advances in the research of polymeric polyphenols in grape and red wine polymeric polyphenols and their important role in Enology.

Polymeric polyphenols in grapes and red wine

From the quantitative point of view, polymeric polyphenols are major group of polyphenols in grape and red wine. For example, in a one-year-old red wine, catechins occupy about 5 to 8 percent of total polyphenols, dimer procyanidins 5 to 10 percent, anthocyanidin 10 to 15%, phenolic acids 3–6%, flavonol, less than 1% and resveratrol, less than 0.3%, while polymeric polyphenols occupy 60–80%. This would indicate that polymeric polyphenols would play a major role in Enology (Sun et al. 2001).

In grapes, polyphenols are present essentially in solid part of grapes, that is to say, seed, skin and stems (Bourzeix et al. 1986; Sun et al. 1998). The seed polymeric polyphenols are exclusively polymeric procyanidins (Prieur et al. 1994; Sun et al. 1998), with mean degree of polymerization around 30 (Sun et al. 1998; Spranger et al. 2008). The skin and stems polymeric

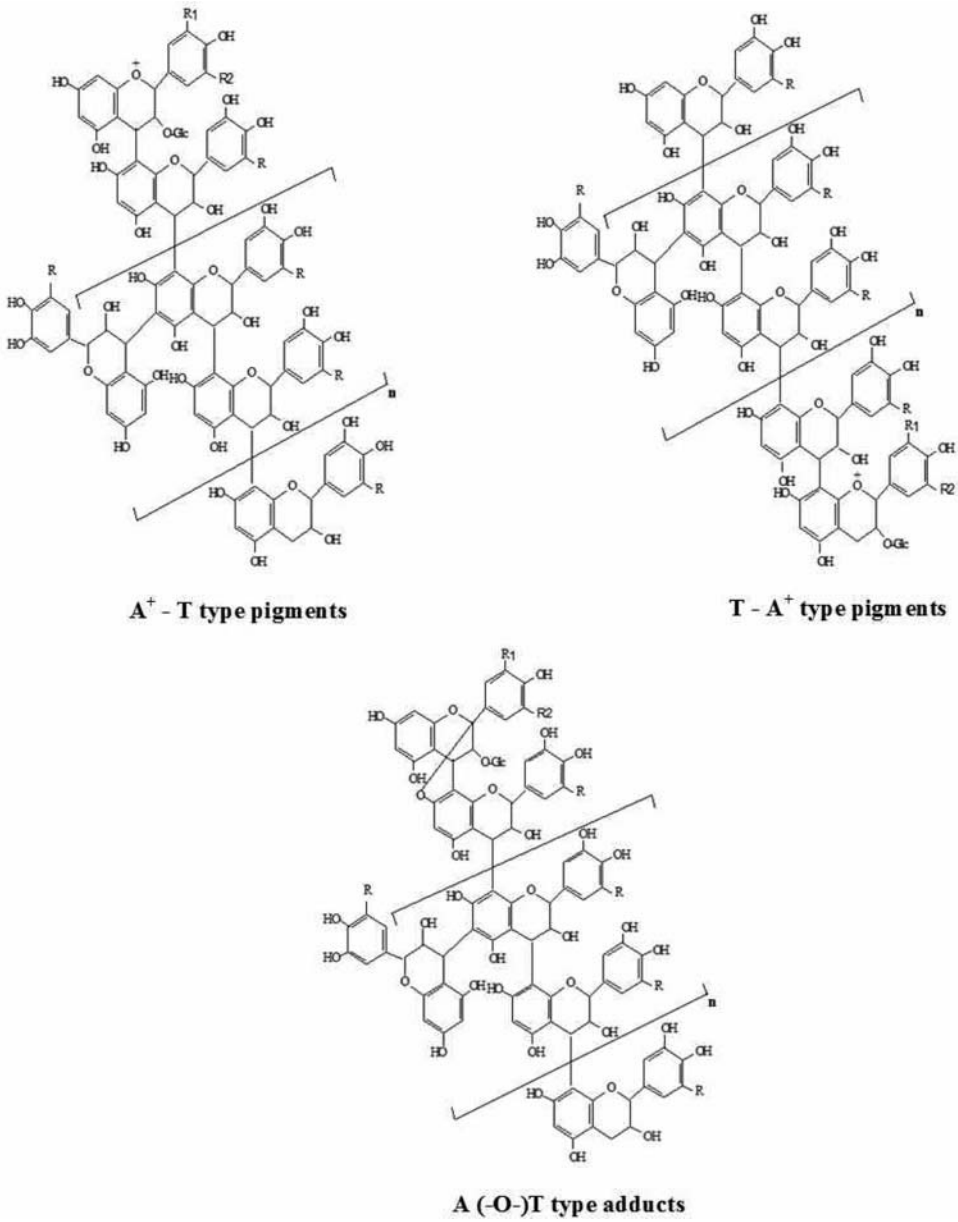

Figure 2. Direct anthocyanin-proanthocyanin condensation products in red wine.

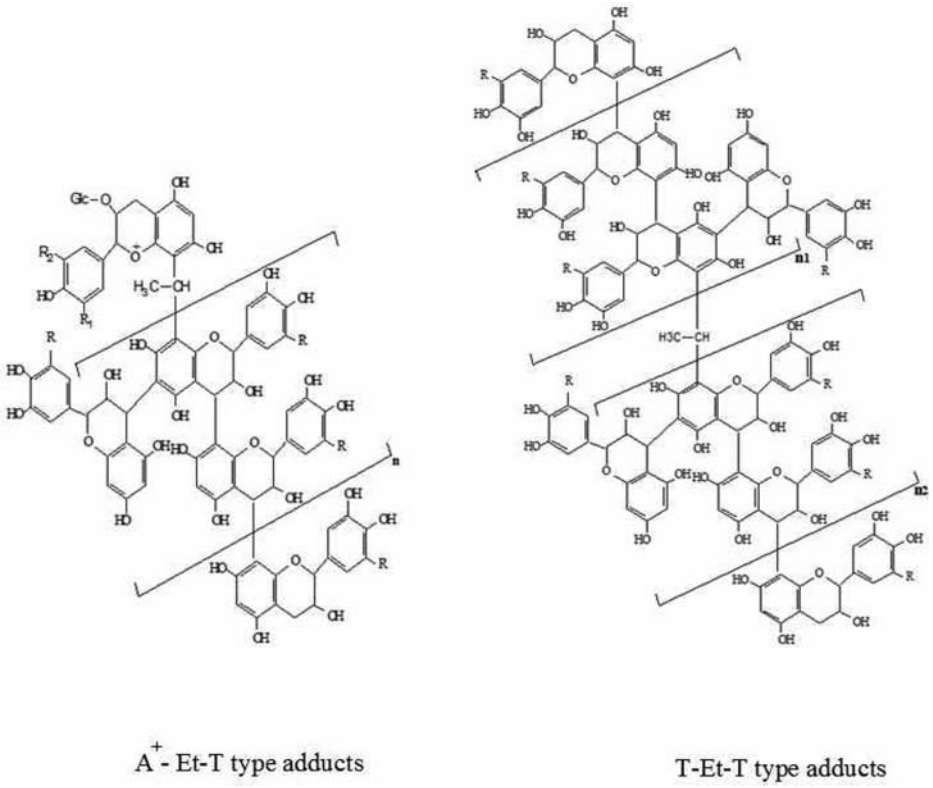

Figure 3. Indirect ethyl-linked anthocyanin-proanthocyanin and proanthocyanidin-proanthocyanidin condensation products in red wine.

polyphenols include polymeric procyanidins and polymeric prodelphinidins, with mean degree of polymerization over 80 (Souquet et al. 1996). Figure 1 presents the structures of polymeric procyanidins and polymeric prodelphinidins.

Red wine polyphenols are more complexes than the grapes. They include both grape polyphenols owing to the extraction during alcoholic fermentation, and new phenolic products formed from them during winemaking and storage (Sun et al. 2008). As a consequence, red wine polymeric polyphenols mainly consist of polymeric procyanidins, polymeric prodelphinidins (Figure 1), direct anthocyanin-proanthocyanin condensation products (Figure 2), indirect ethyl-linked anthocyanin-proanthocyanin condensation products (Figure 3), indirect hydroxylethyle-anthocyanin-ethyl-proanthocyanidin condensation products (Figure 4) and other pigmented complexes (Figure 5).

Separation of grape and wine polymeric polyphenols

Polyphenols are mainly presented in the solid parts of grape cluster (skins, seeds and stems) but only proanthocyanidins (condensed tannins) are presented in the form of oligomers and polymers, while other classes of polyphenols in grape are presented in monomer or low-molecular-weight forms. Thus, grape polymeric polyphenols are grape polymeric proanthocyanidins. The seeds account for a largest proportion of total proanthocyanidins in the entire grape cluster, then the stem and skin; the pulp is free or lack of these compounds (Bourzeix et al. 1986; Sun et al. 2001). Proanthocyanidins in seeds are procyanidins composed by catechin, epicatechin and epicatechin-3-O-gallate. In stems, proanthocyanidins are predominantly in form of procyanidins together with low amount of prodelphinidins (Souquet et al. 2000), while grape skin contained important amount of both procyanidins and prodelphinidins (Souquet et al. 1996; Sun et al. 1998).

The first research work concerning polymeric polyphenols in our previous work was that we have presented in 17th

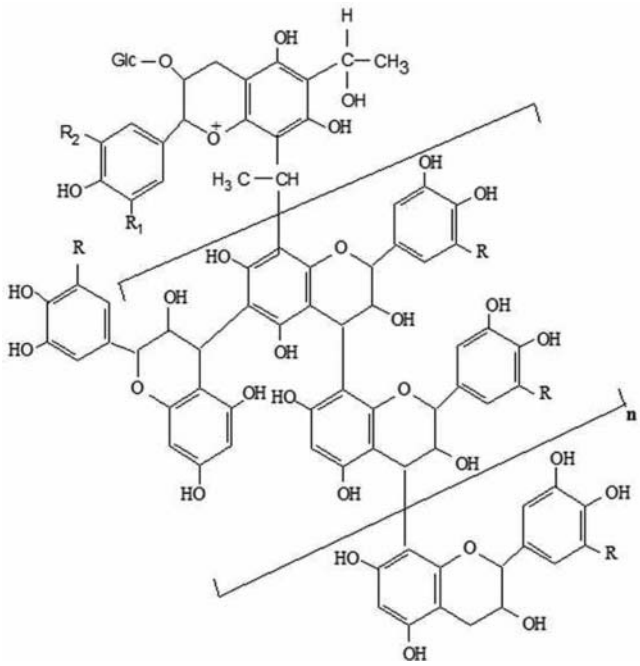

Figure 4. Indirect hydroxylethyl anthocyanin-ethyl-proanthocyanin condensation products in red wine.

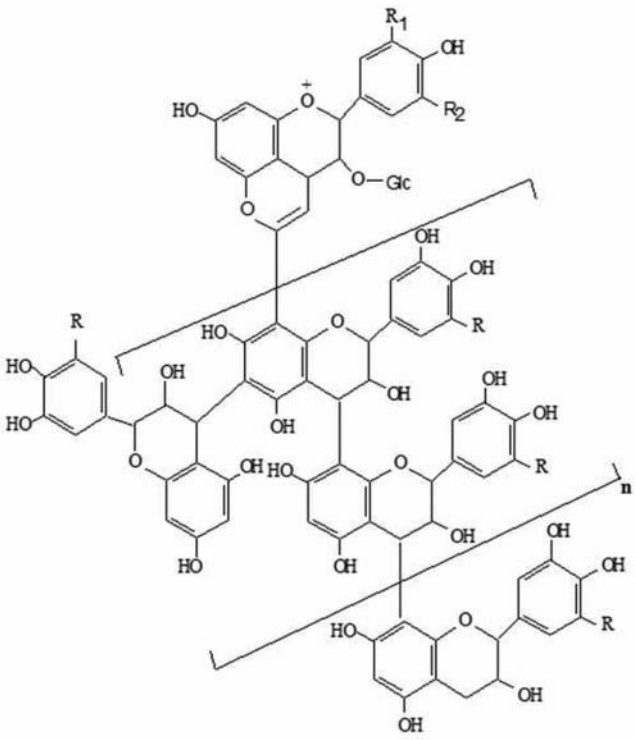

Figure 5. Flavanyl-pyranoanthocyanins.

International Conference on Polyphenols (Sun et al. 1994), in which grape and red wine polymeric proanthocyanidin fraction was firstly separated from oligomeric proanthocyanidins and other monomeric phenolic compounds, by solid phase extraction using C_{18} Sep-Pak cartridges (Figure 6); each proanthocyanidin fraction was quantified by an improved vanillin assay established (Sun et al. 1998). Thus, for the first time, oligomeric and polymeric proanthocyanidins in several Portuguese grapevine varieties and red wines were quantified (Sun et al. 2001), which provided us some important information as follows: (1) catechins, oligomeric proanthocyanidins and polymeric proanthocyanidins were located essentially in the seeds, then in the skins and very little in the pulp; (2) in wines and in each solid part of grape berry, proanthocyanidins were mainly present in polymeric forms, to a much less extent in oligomeric forms and very little in monomeric flavan-3-ols (catechins); (3) the percentages of polymeric proanthocyanidins in the skins were generally higher than those in the grape seeds.

Due to the difficulty to separate oligomeric proanthocyanidins from anthocyanins (Vidal et al. 2002) and polymeric proanthocyanidins from pigmented complexes, a combined fractionation techniques to separate red wine polyphenols into various sub-fractions has been firstly developed (Sun et al. 2006) and thus in separating oligomeric proanthocyanidins from anthocyanins and polymeric proanthocyanidins from pigmented complexes has been achieved (Figure 7). Furthermore, the phenolic composition of each fraction was verified by HPLC-DAD, ESI-MS^n and/or thiolysis-HPLC.

The traditional methods for separation of grape and wine polyphenols have some disadvantages including complex process, long separation period, low-yield, high-cost and often present secondary pollution. In recent years, the technique of high speed counter-current chromatography (HSCCC) has been widely used in separation of natural products. HSCCC is a unique liquid-liquid partition chromatography technique for separation solely based on the partition of compounds between a pair of mutually immiscible liquid phases (Ito, 2005) which avoids disadvantages such as irreversible adsorption from the solid support, secondary pollution, complex process, time

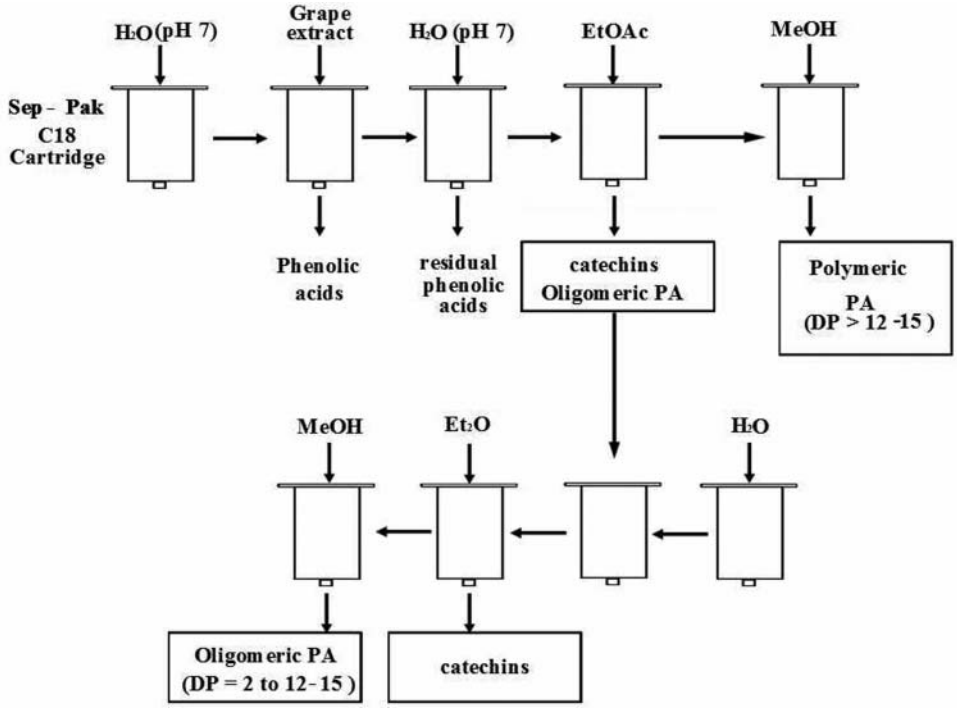

Figure 6. Separation of grape oligomeric polymeric polyphenols (Sun et al., 1994; Sun et al., 1998).

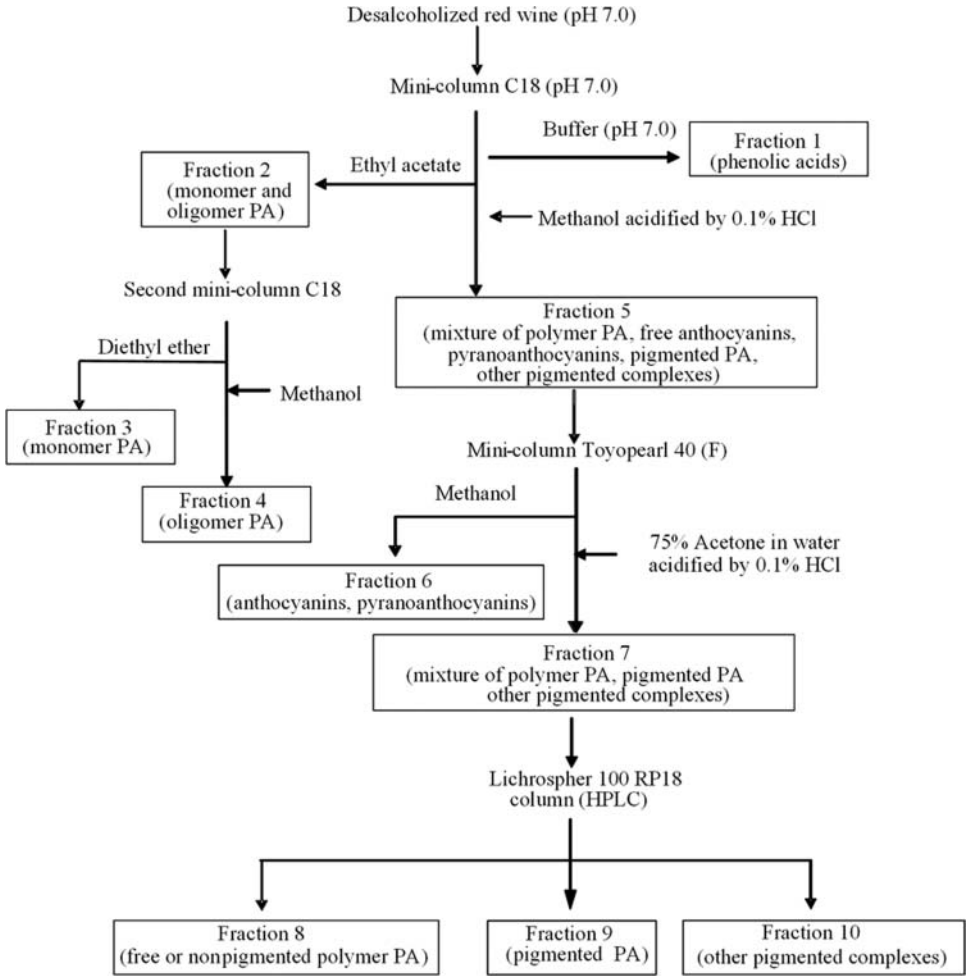

Figure 7. Fractionation of red wine polyphenols using combined column chromatography based on the method of Sun et al., (2006).

consuming, low-yield and high-cost, compared to conventional column chromatography and thin-layer chromatography. Thus, in our laboratory, we have developed a series of methods for large separation of various individual polyphenols from grape skins, grape seeds, red wine extracts even cacao beans by preparative HSCCC combined with preparative-HPLC (Zhang et al. 2015; Luo et al. 2016; Li et al 2016; Zhang et al. 2017; Li et al. 2017). These methods make possible to prepare polymeric polyphenols in large scale, as well as to obtain high-yield and high-purity of various DP fractions or individual phenolics by only one-step of HSCCC or by HSCCC coupled with preparative-HPLC (Zhang et al. 2017). Moreover, the isolated polymeric proanthocyanidins could be further degraded into monomer catechins and their nuclephile derivatives in the presence of phloroglucinol, and the nuclephile derivatives were verified to possess stronger antioxidant activities than free monomer catechins (Zhang et al. 2015), which indicated that polymeric proanthocyanidins would be important source of bioactive compounds.

Structural characterization of grape and wine polymeric polyphenols

Structural characterization of grape oligomeric and polymeric polyphenols was initiated firstly by a French research group who developed an improved thiolysis-HPLC method permitting determination of the compositional data of oligomeric and polymeric proanthocyanidins of grapes (Prieur et al. 1994; Souquet et al. 1996; Souquet et al. 2000). This method allows determining the nature and concentration of terminal and extension units and consequently calculating the mean DP (mDP) and the percentage of galloylation (%G) of proanthocyanidins. In our laboratory, we have succeeded in application of this thioacidolysis method, with slight modification, for determination of the structural characteristics of oligomeric and polymeric proanthocyanidin fractions of grape and red wines (Sun et al. 1998; Sun et al. 2006).

Electrospray ionization mass spectrometry (ESI-MS) has been proved to be a very powerful tool for characterization of proanthocyanidins, in particular for detection of individual oligomeric and polymeric proanthocyanidins in a mixture or in heterogeneous solutions, providing the molecular mass, number of constitutive moieties and substituents (Guyot et al. 1997; Roux et al. 1998; Gu et al. 2002; Hayasaka et al. 2003; Sun et al. 2010). The advantage of eletrospray ionization is that it permits the detection of the molecular ion but does not cause the fragmentation of the molecule as occurs other types of ionization such as atmospheric pressure chemical ionization (APCI) (De Pascual-Teresa and Rivas-Gonzalo 2003). Earlier works (Cheynier et al. 1997; Fulcrand et al. 1999) showed that ESI-MS analysis of grape seed extract detected various procyanidins from dimer (PC2) to pentamer (PC5) as mono-charged ion species

([M-H]$^-$) and from pentamer to nonamer (PC9) as doubly charged ion species ([M-2H]$^{2-}$), with galloylation degree up to 3. Lately, tridecamer (PC13) and its monogallate were detected in an oligomeric procyanidin fraction from grape seeds by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) (Pianet et al. 2002). More recently, Hayasaka et al., (2003) detected various single ([M-H]$^-$), double ([M-2H]$^{2-}$)and triple ([M-3H]$^{3-}$) charged ions in grape seed fractions corresponding the molecular sizes of procyanidins up to 28 units and with a galloylation degree ranging from 0 to 8, using ESI-MS under enhanced resolution. Wollmann and Hofmann (Wollmann and Hofmann 2013) analyzed the composition of the high-molecular weight polymers by HPLC-MS/MS, HPLC-UV/Vis and ion chromatography, and the key structural features of the red wine polymers were proposed. The structural backbone of the polymers seems to be comprised of a procyanidin chain with (-)-epicatechin, (+)-catechin, (-)-epicatechin-3-O-gallate units as extension and terminal units as well as (-)-epigallocatechin as extension units. Acetaldehyde was shown to link different procyanidins at the A-ring via an 1,1-ethylene bridge and anthocyanins and pyranoanthocyanins were found to be linked to the procyanidin backbone via a C-C-linkage at position C(6) or C(8), respectively.

In our previous work, ESI-MS was used for detection of highly polymerized proanthocyanidins in grape seed and in red wine (Spranger et al. 2008; Sun et al. 2010). For this purpose, both grape seed extract and red wine were fractionated, in order to obtain purified polymeric proanthocyanidin fractions. The fractions were lyophilized and powder obtained was dissolved in pure methanol prior to MS analysis. Each solution was infused directly into ESI source. Mass spectra were recorded in a negative mode, Mass range mode was selected as Standard/Normal, Standard/Enhanced and Standard/Maximum, respectively. Multiply charged ions (from [M-2H]$^{2-}$ to [M-6H]$^{6-}$) were identified by deconvoluting high charge states up to 6. Multiple fragmentation experiments MS^n were performed for getting more detailed structural information of target compounds (procyanidins). Various pseudomolecular ions corresponding grape seed polymeric proanthocyanidins were thus detected (Spranger et al. 2008). The DP ranging from 11 to 18 were detected by ESI-MS and their structures were successfully confirmed by multi-stages fragmentation analysis. Furthermore, there were also many other ions detected in polymeric fraction which would be interpreted as proanthocyanidins with DP ranging from 19 to 32, though their intensities were too low to perform further MS^n analysis.

In order to detect polymeric polyphenols in red wine, direct injection of wine sample to HPLC or ESI-MS is impossible because of the complexity of red wine composition. In other words, it is necessary firstly to fractionate red wine polyphenols into various fractions. As described above, we have firstly separated red wine polyphenols into various fractions on the basis of a fractionation method established in our laboratory (Sun et al. 2006) and the polymeric fractions were subject to thiolysis followed by HPLC analysis, or injected directly to ESI-MS. Thiolysis-HPLC analysis permits determination of mDP of proanthocyanidins while ESI-MS and multiple fragmentation allows to detect individual polymeric polyphenols in the fraction. As a consequence, the mDP values determined in two polymeric fractions from one-year-old red wine were respectively 11.1 $\pm$ 0.1 and 12.4 $\pm$ 0.2 (Sun et al. 2006). Furthermore, various polymeric proanthocyanidins, direct condensation products and indirect anthocyanin-proanthocyanidin condensation products were successfully detected (Sun et al. 2010).

Reactivity of polymeric proanthocyanidins towards human salivary proteins—contribution to red wine astringency

Astringency is defined as "the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as tannins (Clifford et al. 1996). It is generally accepted that polyphenols, particularly proanthocyanidins (or condensed tannins), are responsible for astringency of plant-derived foods owing to their complexation with salivary proteins. Astringency is an essential characteristic of red wine. Chemically, this sensation is related to the ability of wine proanthocyanidins to precipitate human salivary proteins (Arnold et al. 1980; Thorngate 1997). The interactions between salivary proteins and tannins have been intensively studied (McMurrough 1981; Baxter et al. 1997; Kallithraka et al. 1998; Sarni-Manchado et al. 1999; Prinz and Lucas 2000; De Freitas and Mateus 2001). Protein-tannin reactions depend on the pH and the composition of the proteins and the tannins. The proline-rich proteins, as found in saliva, were confirmed to be the essential agent for these reactions (Baxter et al. 1997). On the other hand, it was reported that the relative astringency of proanthocyanidins was related to their degree of polymerization (DP) (Lea 1978; Arnold et al. 1980). According to these authors, the intensity of astringency of proanthocyanidins increases with molecular size at least up to DP = 6–7 then decreases because higher proanthocyanidins became, or no longer soluble (Lea 1978) or too bulky to bind to the protein (Ribéreau-Gayon et al. 2006). However, our more recent studies have shown that proanthocyanidins presented in red wine were essentially in higher polymerized form (mDP > 15) (Sun et al. 1998; Sun et al. 1999; Sun et al. 2001; Spranger et al. 2004). Furthermore, such water-soluble and highly polymerized proanthocyanidins could be selectively precipitated by fining proteins (Sarni-Manchado et al. 1999; Maury et al. 2001), indicating that they can readily interact with proteins and thus suggesting that they could be particularly astringent (Vidal et al. 2002).

In our previous works, the reactivity of oligomeric and polymeric proanthocyanidin fractions towards to human salivary proteins was investigated (Spranger et al. 2000; Sun et al. 2013). A precipitation reaction between human salivary protein and procyanidin solution was performed. Protein composition in both supernatant and dissolved residue was verified by polyacrylamide gel electrophoresis (Figure 8), and their procyanidin composition was determined by thioacidolysis-HPLC (Table 1). The results indicated that human salivary proteins are essentially composed of low molecular proteins (6.5–66 kDa). The migrations of supernatant and residue showed that procyanidins react nearly all salivary proteins, particularly those of lower molecule weights (proximately 6.5–36 kDa), suggesting that lower-molecular-weight proteins have higher reactivity

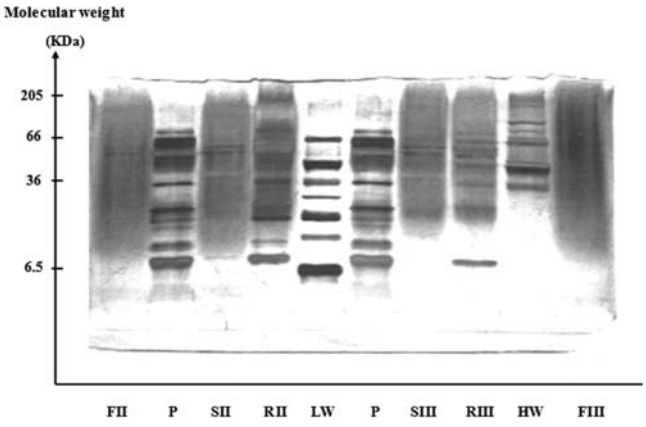

Figure 8. Separation of human salivary proteins before and after their reaction with procyanidins by gel polyacrylamide electrophoresis. (FII–Oligomeric procyanidins; P–Human salivary proteins; SII–Supernatant after reaction between oligomeric procyanidins and salivary proteins; RII–residue after reaction between oligomeric procyanidins and salivary proteins; LW–low-molecular-weight marker (MW = 6.5–66 KDa); SIII–Supernatant after reaction between polymeric procyanidins and salivary proteins; RIII–residue after reaction between polymeric procyanidins and salivary proteins; HW–high-molecular-weight marker (MW = 36–205 KDa); FIII–polymeric procyanidins.)

than higher-molecular-weight proteins. Sarni-Manchado et al., (Sarni-Manchado et al. 1999) reported that by SDS-PAGE of human salivary proteins, the bands ranging from 16 to 67 kDa corresponded to proline-rich proteins (PRP), which were considered to be the major target for the reaction with procyanidins. Furthermore, the results obtained by SDS-PAGE electrophoresis do not permit distinguishing clearly the reactivity of oligomeric and polymeric proanthocyanidin fractions towards salivary proteins.

Analysis of procyanidin composition before and after procyanidin-salivary protein reaction revealed that 82.9% of polymeric procyanidins were precipitated by salivary proteins; as compared, only 39.4% of oligomeric procyanidins were precipitated by salivary proteins (Table 1). This would indicate that polymeric procyanidins had much higher reactivity with salivary proteins than oligomeric procyanidins.

On the other hand, sensory analysis of grape seed oligomeric procyanidins fraction and polymeric procyanidins fraction, each of them with different concentrations (150, 300, 600, 900, 1200 mg/L in water), have been carried out by a sensory panel composed of 10 official judges who participated, at least once a week, in the wine sensory sessions. The judges were asked to rank the aqueous polyphenol solutions according to their astringency. The results showed that the intensity of astringency (in water solution) is positively correlated to their concentration (correlation coefficient > 0.95). Furthermore, on equivalent concentration in the range from 150–1200 mg/L, polymeric procyanidins have more astringency intensity than oligomeric procyanidins (Figure 9). Thus the results obtained by panel sensory analysis are in good agreement with those obtained by chemical reaction between polymeric proanthocyanidins-human salivary proteins. These results indicate that red wine astringency would be contributed essentially by polymeric proanthocyanidins because significant amount of highly polymerized and soluble proanthocyanidins are presented in red wine.

Reactivity of polymeric proanthocyanidins towards anthocyanins—effect on red wine color stability

It is well known that the reaction of anthocyanins and flavanols is one of the most important of reactions during ageing and storage of red wine. The newly formed pigments are more stable than their anthocyanins precursors, and present distinct sensory properties. Two major ways of anthocyanins and flavanols reactions have been reported: direct reaction and indirect reaction (Timberlake and Bridle 1976). Both of these two types of reactions have been intensively studied during the last years (Rivas-Gonzalo et al. 1995; Es-Safi et al. 2000; Cheynier 2002; Pissarra et al. 2003; Salas et al. 2003; Dueñas et al. 2006).

In our previous work (Sun et al. 2008), we have detected, in a model wine solution containing malvidin 3-glucoside, epicatechin and acetaldehyde, four isomers of a new indirect condensation product 1′′-hydroxylethyl-malvidin-3-glucoside-ethyl-epicatechin formed during the reaction. Interestingly, these newly formed condensation products were very stable and became the major pigments of reaction medium at the later stage of the reaction. This fact let us to think the possibility of the presence of the same compound in red wine. Moreover, in addition to malvidin 3-glucoside and epicatechin, other anthocyanins and flavanols (catechins, oligomer and polymer proanthocyanidins) are also present in red wine. This would suggest that red wine might contain a new class of anthocyanin-proanthocyanidin condensation products with the structures similar to that identified in model wine solution. As a consequence, by fractionation of red wine polyphenols into various fractions

Table 1. Structural composition of procyanidins before and after their reaction with human salivary proteins.

		Relative percentage of structural units (%)						
		Terminal unit			Extension unit			
Fraction		cat	epi	epiG	*cis*-cat	*trans*-cat	epicat	epiG
Oligomeric procyanidins	Before reaction	5.5b ± 0.0	4.1b ± 0.3	4.3b ± 0.1	3.1a ± 0.1	11.4b ± 0.3	47.1a ± 0.5	24.5b ± 0.4
	supernatant	6.9c ± 0.3	5.8c ± 0.1	3.5a ± 0.2	3.8b ± 0.1	13.1c ± 0.5	50.4b ± 1.3	16.5a ± 0.0
	residue	3.5a ± 0.3	1.9a ± 0.0	4.4b ± 0.1	3.1a ± 0.2	9.9a ± 0.3	47.4a ± 0.5	29.5c ± 0.6
Polymeric procyanidins	Before reaction	1.6a ± 0.1	0.8a ± 0.0	1.5b ± 0.0	2.3a ± 0.3	8.1a ± 0.4	52.2a ± 1.6	33.5b ± 0.9
	supernatant	2.7c ± 0.3	1.4b ± 0.1	1.6b ± 0.1	2.3a ± 0.5	8.5a ± 0.6	57.5b ± 2.3	26.0a ± 1.9
	residue	1.7b ± 0.0	0.8a ± 0.1	1.2a ± 0.2	2.6b ± 0.1	9.0b ± 0.9	58.1b ± 2.6	26.7a ± 2.3

Abbreviation: cat = catechin; epicat = epicatechin; epiG = epicatechin 3-O-gallate; for the same procyanidins fraction, means (n ± 3) followed by the same letter in a column are not significantly different (LSD, 5%).

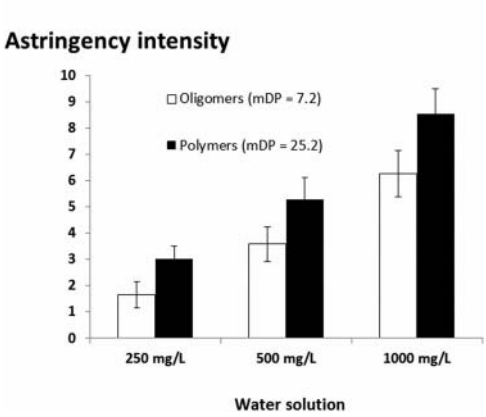

Figure 9. Astringency intensity of oligomeric and polymeric procyanidins fractions.

followed by ESI-MS^n analysis, a new class of anthocyanin-proanthocyanidin condensation products, i.e., 1''-hydroxylethyl-anthocyanin-ethyl-flavanol have been identified (Sun et al. 2010). Actually, only hydroxylethyl-anthocyanin-ethyl-dimer procyanidin were detected in red wine by ESI-MS^n analysis. Identification of higher oligomeric and polymeric proanthocyanidins involving such new class of condensation products have still been achieved due to structural complexity and very low ion intensity. In order to verify the reactivity of polymeric proanthocyanidins towards anthocyanins, we have firstly isolated oligomeric and polymeric procyanidin fractions from grape seeds and then experimented the interaction of each of them with malvidin 3-glucoside in the presence of acetaldehyde under the conditions described previously (Sun et al. 2008). These results are presented in Table 2. It can be seen that on the basis of molar concentration, the reactivity of procyanidins towards malvidin 3-glucoside was positively correlated to their degree of polymerization. In other words, polymeric procyanidins have higher reactivity towards anthocyanins than oligomeric ones. These results would indicate that polymeric proanthocyanidins may play a major role in red wine color stability during ageing. On one hand, anthocyanins would become more stable after interaction with polymeric proanthocyanidins than their free form. On the other hand, such high-molecular-weight condensation products may not be very stable in red wine medium and thus may gradually be precipitated during wine storage. In fact, in model wine solution containing polymeric procyanidins and malvidin 3-glucoside, more precipitates were formed during the reaction period than in model wine solution containing oligomeric procyanidins and malvidin 3-glucoside.

Table 2. Reactivity of oligomeric and polymeric PA towards malvidin-3-glucoside

	pH = 1.7		pH = 3.2	
Procyanidins	$K(h^{-1})$	R^2	$K(h^{-1})$	R^2
Dimer (DP = 2)	0.712 ± 0.010	0.999	0.194 ± 0.010	0.995
Oligomer (mDP = 4.81)	0.826 ± 0.030	0.996	0.234 ± 0.030	0.998
Oligomer (mDP = 7.22)	0.944 ± 0.050	0.992	0.255 ± 0.050	0.991
Polymer (mDP = 25.22)	1.117 ± 0.016	0.999	0.355 ± 0.016	0.991

K = Rate constant K; R^2 = correlation coefficient

Antioxidant activity of polymeric polyphenols from grape and red wine

During the last three decades, a great number of works have reported the biological activities of grape and red wine phenolic compounds. These include procyanidin dimmers and trimers (Masquelier 1988; Ricardo-da-Silva et al. 1991; Yun et al. 2004; Verstraeten et al. 2005; Serra et al. 2010; Ge et al. 2015), catechin and epicatechin (Carando et al. 1999; Sutherland et al. 2006; Yu et al. 2010; Noll et al. 2013; Angel et al. 2016), resveratrol and its derivatives (Blond et al. 1995; Clement et al. 1998; Burkitt and Duncan 2000; Burkhardt et al. 2001; Wolter and Stein 2002; Losa 2003; Sun et al. 2006; Yan et al. 2013; Witte et al. 2014; Kuršvietienė et al. 2016), flavonols and flavones (Herrmann 1976; Chu et al. 2000; Zapata-Torres et al. 2004; Chirumbolo 2010; Hossain et al. 2016), phenolic acids (Yeh and Yen 2003; Mudnic et al. 2010; Ozcan et al. 2014; Alim et al. 2017) and anthocyanins (Wang and Mazza 2002; Lila 2004; Einbond et al. 2004; Hassellund et al. 2012; Sancho and Pastore 2012; Hossain et al. 2016).

As compared with low-mass-polyphenols, the data about the study of biological activities of polymeric polyphenols were very limited. For *in vitro* study, Krishnan and Maru (2004) investigated the inhibitory effect(s) of polymeric black tea polyphenol fractions on the formation of [(3)H]-B(a)P-derived DNA adducts. They found that polymeric black tea polyphenol fractions retain one of the chemopreventive effects exhibited by the monomeric green tea polyphenol EGCG *in vitro*. Schoene et al., (2005) verified the anti-cancer properties of water-soluble, polymeric polyphenols from cinnamon and they confirmed that these polymers inhibited the proliferation and altered cell cycle distribution patterns of hematologic tumor cell lines. More recently, the chemopreventive efficacy and possible mechanism of polymeric black tea polyphenols were evaluated in experimental lung carcinogenesis model (Hudlikar et al. 2017). It was verified that polymeric black tea polyphenols inhibited benzo(a)pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung carcinogenesis potentially through down-regulation of p38 and Akt phosphorylation in A/J mice, indicating that the polymeric polyphenols have chemopreventive effect through inhibition of inflammation, cellular proliferation, and induction of apoptosis possibly via modulation of signaling kinases.

In our previous works, grape seed procyanidins were separated into catechins, oligomeric procyanins and polymeric procyanidins using the method described previously (Sun et al. 1998). The *in vitro* antioxidant activities of these fractions were measured using several methods as Reducing Power, $DPPH^{\bullet}$ Scavenging Activity, $O_2^{\bullet -}$ Scavenging Capacity, and the Fenton System for generating hydroxyl radical ($HO^{\bullet}$) (Spranger et al. 2008).

Reducing Power of the tested compounds is presented in Table 3. Polymeric procyanidins fraction presents the highest reducing capacity, followed by oligomeric procyanidins fraction and catechin. Ascorbic acid and trolox showed low reducing capacity. The reducing capacity of a sample is an important parameter reflecting one aspect of its antioxidation property. However, it might be oversimplified to refer to the result as "total antioxidant capacity" (Huang et al. 2005).

Table 3. FCR reducing capacity.

Antioxidant compound		FCR reducing capacity (mM catechin equiv.)
Gallic acid	$\bar{x}$	0.53 a
	SD	*0.022*
Ascorbic Acid	$\bar{x}$	0.42 a
	SD	*0.016*
Trolox	$\bar{x}$	0.20 a
	SD	*0.012*
(+)-Catechin	$\bar{x}$	1.00 b
	SD	*0.002*
Oligomeric procyanidin fraction	$\bar{x}$	4.99 c
	SD	*0.024*
Polymeric procyanidin fraction	$\bar{x}$	17.73 d
	SD	*0.269*

$\bar{x}$= mean value, SD = standard deviation. F_{olig} = Total oligomeric procyanidin fraction; F_{poly} = Total polymeric procyanidin fraction. Different letter in the same column means very significant differences, $p < 0.001$.

DPPH$^{\bullet}$ is a useful reagent for studying the free radical-scavenging activities of compounds. Since DPPH radical is not biologically relevant, the DPPH assay was performed as a preliminary study to estimate the direct free radical scavenging abilities of different tested compounds. The kinetics of the reaction was dependent on the concentration and structural type of the compound. For each tested compound, the percentage of reducing DPPH$^{\bullet}$ increased dose-dependently at a given concentration range. According to the plots of percentage of inhibiting DPPH$^{\bullet}$ (% inhibition) of each tested compound as a function of the molar ratio of the antioxidant to DPPH$^{\bullet}$, the relative concentration of each tested compound (μmol per μmol DPPH$^{\bullet}$) necessary to reduce 50% of DPPH$^{\bullet}$ (EC_{50}) can be determined. EC_{50} is a parameter widely used for the antioxidant capacity of one compound (Vinson et al. 1995), sometimes expressed as Antiradical power (ARP = $1/EC_{50}$) (Brand-Williams et al. 1995). The time to reach a steady state of the reaction at the concentration corresponding to EC_{50} (T_{EC50}) was also used by several authors for antioxidant classification (Brand-Williams et al. 1995; Sánchez-Moreno et al. 1999). A new parameter considering, both EC_{50} and T_{EC50}, was then proposed to discriminate the different antioxidant compounds: Antiradical efficiency (AE) or $1/EC_{50} \times T_{EC50}$ (Sánchez-Moreno et al. 1999). In this work, all these parameters (EC_{50}, T_{EC50}, ARP and AE) for the tested compounds were determined or calculated and the results are presented in Table 4.

According to parameter EC_{50} or ARP, polymeric procyanidins present the highest scavenging activity on DPPH$^{\bullet}$, followed by oligomeric procyanidins, whereas natural antioxidant ascorbic acid and other simple phenolics (including catechin) present very low scavenging activity on DPPH$^{\bullet}$. The scavenging activity of grape seed procyanidins on DPPH$^{\bullet}$ is positively related to their degree of polymerization, *i.e.*, polymer > oligomer > monomer (catechin). When Antiradical efficiency (AE) was used to discriminate the different antioxidant compounds, polymeric procyanidins present also the highest values, followed by ascorbic acid and oligomeric procyanidins, while other phenolics present low antiradical efficiency. It is worth mentioning that T_{EC50} is also another important parameter reflecting the antiradical efficiency of an antioxidant compound. Lower T_{EC50} signifies higher antiradical efficiency of an antioxidant compound. Table 4 shows that (+)-catechin has a little lower EC_{50} but much higher T_{EC50} than ascorbic acid, so the AE of (+)-catechin is much lower than that of ascorbic acid, indicating the latter may have more antiradical efficiency. From this sense, the parameter AE which considers both antiradical power ($1/EC_{50}$) and time to reach a steady state of the reaction (T_{EC50}) may be more efficient to discriminate the different tested phenolic compounds than ARP alone.

$O_2^{\bullet-}$ Scavenging Capacity of each tested compound can be expressed by the percentage of inhibition (% inhibition) of NBT reduction induced by $O_2^{\bullet-}$ generated by xanthine-xanthine oxidase system and also the initial rate (V_0) of the kinetic reaction. For all tested compounds, the maximum absorbance is reached at 20 minutes of reaction. Thus, the % inhibition and the V_0 can be determined. These results are presented in Table 5.

It is worth noting that the $O_2^{\bullet-}$ scavenging activity of each tested compound increased dose-dependently at a certain molar concentration range. When the concentration of a tested compound was lower than this range, no apparent inhibition reaction could be observed and if its concentration was higher than this range, the percentage of inhibiting NBT reduction reached 100% immediately (data not shown). Table 5 shows that all tested compounds decreased the reduction of NBT by

Table 4. Scavenging activity of various antioxidant compounds on DPPH radical.

Antioxidant compound		EC_{50} (moles AO/moles DPPH)	T_{EC50} (min)	ARP ($1/EC_{50}$)	AE ($1/EC_{50} \times T_{EC50}$)
Caffeic acid	$\bar{x}$	0.194 de	11.1 b	5.17 ab	0.47 a
	SD	*0.002*	*1.56*	*0.06*	*0.06*
Gallic acid	$\bar{x}$	0.091 b	17.2 bc	11.05 c	0.64 a
	SD	*0.001*	*0.50*	*0.09*	*0.02*
Quercetin	$\bar{x}$	0.124 c	25.7 de	8.07 bc	0.32 a
	SD	0.001	4.46	0.09	0.05
Trolox	$\bar{x}$	0.199 e	4.1 a	5.05 ab	1.24 b
	SD	0.020	0.62	0.50	0.31
Ascorbic acid	$\bar{x}$	0.203 e	0.9 a	4.93 a	5.27 c
	SD	*0.001*	*0.01*	*0.03*	*0.01*
(+)-Catechin	$\bar{x}$	0.174 d	32.4 f	5.76 ab	0.18 a
	SD	*0.002*	*2.26*	*0.07*	*0.01*
F_{olig}	$\bar{x}$	0.018 a	30.9 ef	55.25 d	1.79 b
	SD	*0.001*	*1.27*	*0.43*	*0.06*
F_{poly}	$\bar{x}$	0.005 a	23.5 cd	186.93 e	7.97 d
	SD	*0.001*	*1.41*	*2.47*	*0.37*

$\bar{x}$= mean value, SD = standard deviation. F_{olig} = oligomeric procyanidins fraction; F_{poly} = polymeric procyanidins fraction. Different letter in the same column means very significant differences, $p < 0.001$.

Table 5. Initial rate (V_0) of the kinetic reaction and the percentage of inhibition of NBT reduction (% inhibition) of the tested antioxidant compounds.

Antioxidant compounds	Concentration	V_o ($\Delta A, s^{-1}$)	% inhibition
Control	—	$(1.53 \pm 0.114) \times 10^{-3}$	0
SOD	400 u/mL	$(2.95 \pm 0.212) \times 10^{-4}$ *	36 ± 0.85 †
Trolox	0.20 mM	$(1.03 \pm 0.123) \times 10^{-3}$	26 ± 0.64 †
Gallic acid	0.20 mM	$(2.07 \pm 0.106) \times 10^{-4}$ *	7 ± 0.42 †
Ascorbic acid	0.20 mM	$(3.16 \pm 0.229) \times 10^{-4}$ *	15 ± 2.19 †
(+)-Catechin	0.20 mM	$(1.67 \pm 0.120) \times 10^{-4}$ *	13 ± 0.49 †
F_{olig}	0.05 mM	$(2.68 \pm 0.120) \times 10^{-4}$ *	52 ± 0.85 †
F_{poly}	0.01 mM	$(1.91 \pm 0.311) \times 10^{-4}$ *	60 ± 7.50 †

The symbols
*and † indicate very significant difference $p < 0.01$ with respect to control. F_{olig} = Total oligomeric procyanidin fraction; F_{poly} = Total polymeric procyanidin fraction.

$O_2^{\bullet-}$. The measured rates of reduction were significantly lesser than the control rate for all tested compounds except for trolox. On an equimolar basis and considering both the % inhibition and the V_0, catechin appeared higher $O_2^{\bullet-}$ scavenging activity than gallic acid; ascorbic acid (vitamin C) presents similar capacity of inhibiting NBT reduction but its V_0 value is significantly higher than that of catechin, indicating the latter is more efficient as $O_2^{\bullet-}$ scavenger. The $O_2^{\bullet-}$ scavenging activity of trolox (a water-soluble synthetic analogue of α-tocopherol) is arguable. Although trolox presents much higher capacity of inhibiting NBT reduction than simple phenolics catechin and gallic acid and also ascorbic acid, its V_0 was not significantly different from that of control. In other words, this compound only appears its strong $O_2^{\bullet-}$ scavenging activity after a prolonged reaction time.

Concerning oligomeric and polymeric procyanidins, both of them inhibited 100% NBT reduction immediately at the same molar concentration as other compounds, so it was impossible to assay the $O_2^{\bullet-}$ scavenging activity of these procyanidin fractions with other tested compounds in an equal molar concentration. However, even though much lower concentrations of procyanidin fractions were used, much higher values of % inhibition were obtained, indicating that procyanidins have much higher $O_2^{\bullet-}$ scavenging activity than other antioxidants. Moreover, polymeric procyanidins presented higher $O_2^{\bullet-}$ scavenging activity than oligomeric procyanidins even though the concentration used for the former was five-fold less than that of the latter. These results show that the grape seed procyanidins may be considered as potent antioxidants and their $O_2^{\bullet-}$ scavenging capacities are positively related to their degree of polymerization. Similar effect was also observed by Yamaguchi et al., (2000) who reported that the higher the polymerization degree of flavanols is, the stronger the superoxide-scavenging activity is.

Figure 10 presents plots of 1/A against the concentration (mM) of various tested compounds. It can be seen that the linearity of all plots obtained was good ($R^2 > 0.96$). It is evident that for all tested compounds, its scavenging activity on hydroxyl radical increased as its concentration increased in a certain concentration range. According to these results, the rate constant of the reaction (RC) of each tested compound with $HO^{\bullet}$ can be calculated (Figure 10).

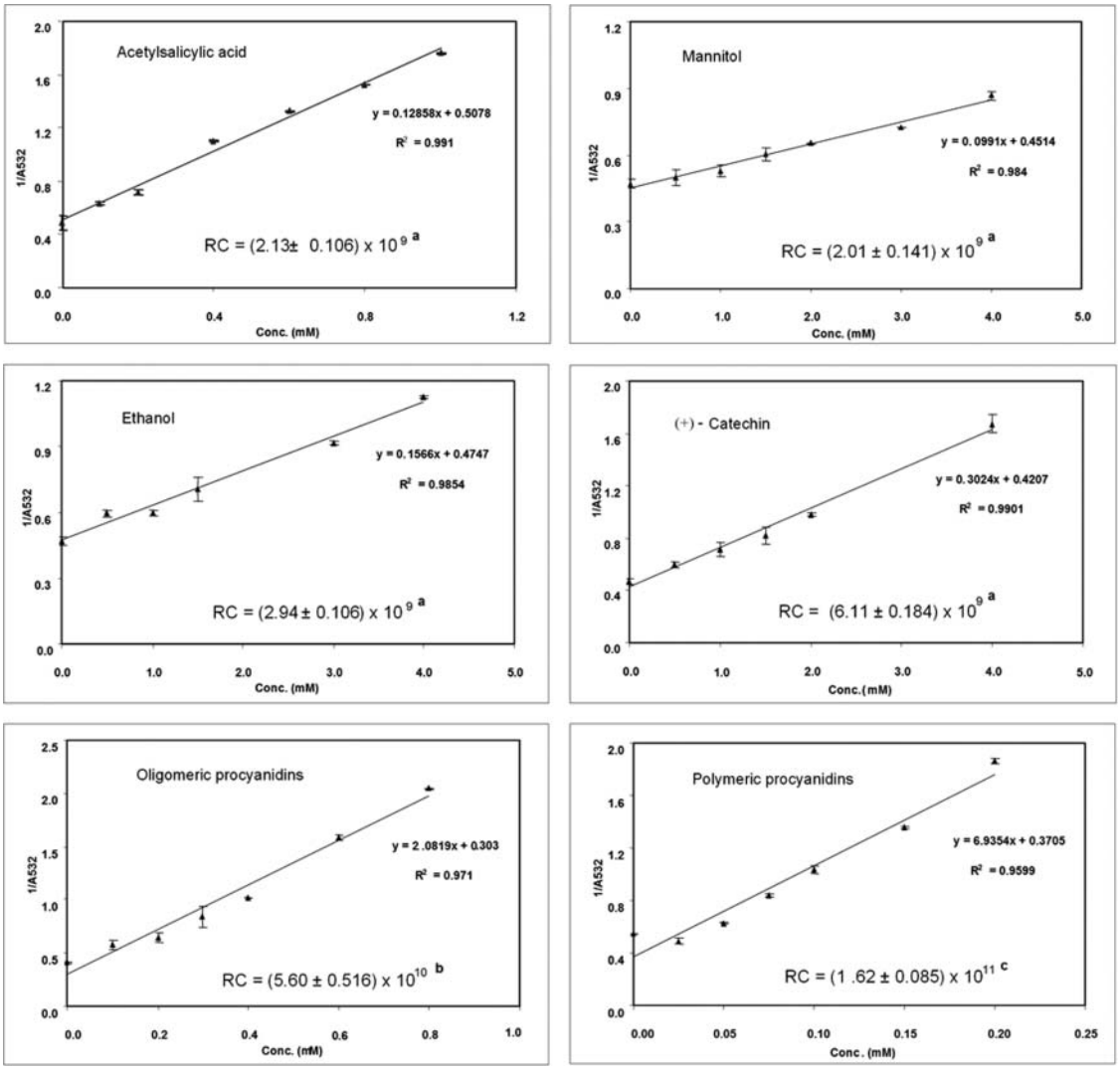

Figure 10. Hydroxyl radical ($HO^{\bullet}$) scavenging capacity of various antioxidant compounds. (RC = rate constant (M^{-1} s^{-1}). Different superscript letter after the RC values are significantly different ($p < 0.001$).)

The RC value of each tested compound is directly related with its scavenging activity on HO•. Thus, oligomeric and polymeric procyanidins are potent hydroxyl radical scavengers, as compared with some well-known antioxidants–ethanol, mannitol (a selective hydroxy radical scavenger), and acetylsalicylic acid. Furthermore, the HO• scavenging activity of procyanidins appeared positively related with their degree of polymerization (polymeric procyanidins > oligomeric procyanidins > catechin).

The results showed that on the basis of molar concentration, polymeric procyanidins appeared the highest antioxidant activities, followed by oligomeric procyanidins, whereas catechins presented a lower antioxidant activity than its oligomers and polymers. Moreover, grape seed procyanidins presented higher antioxidant activities than other well-known antioxidants such as vitamin C, suggesting that grape seed procyanidins might be of interest to be used as alternative antioxidants.

The *in vitro* antioxidant activities of red wine polymeric polyphenols were also studied in our previous works. Thus, one-year-old red wine polyphenols were fractionated into various distinct fractions, including polymeric proanthocyanidins fraction and phenolic complex fraction, by solid phase extraction and liquid chromatography as described previously (Sun et al. 2006). The results showed that both polymeric proanthocyanidin fraction and phenolic complex fraction possessed strong antioxidant activities as their compositional phenolics (Sun et al. 2009). This work provides, for the first time, the direct evidence about the *in vitro* antioxidant activities of red wine polymeric proanthocyanidins and phenolic complexes.

For *in vivo* study on biological activities of polymeric polyphenols, very few data were available in literature. Earlier work (Déprez et al. 2000) revealed that polymeric proanthocyanidins are catabolized by human colonic microflora into small phenolic acids, providing thus the first evidence of degradation of dietary phenolic polymers into low-molecular-weight aromatic compounds. These results indicate that for elucidating the nutritional properties of proanthocyanidins, it is essential to consider the biological properties of these metabolites. Later, Kimura et al., (2009) reported, by oral fat tolerance test in mice, that highly polymeric proanthocyanidins from seed shells of Japanese horse chestnut suppressed fat digestion, indicating that these polymeric polyphenols could be used as nutraceutical substances with anti-obesity effects. Very recently, Masumoto et al., (2016) performed an experiment by the administration of apple polymeric polyphenols for the alleviated obesity and regulate expression of genes related to lipid metabolism in C57BL/6J mice fed a high-fat/high-sucrose diet. They found that polymeric polyphenols from apple influence the gut microbiota and the intestinal metabolome to produce beneficial effects on metabolic homeostasis.

In our previous works, oligomeric and polymeric procyanidin fractions were verified to have potent inhibition activity against ethanol-induced hydroxyl radical production in the striatum (Huang et al 2002). More recently, the protective effects of grape seed oligomer and polymer procyanidin fractions against ethanol-induced toxicity were confirmed in mouse brain cells (Guo et al. 2007). The results indicated that the oligomer and polymer procyanidins could protect the brain against DNA damages in the mouse cerebellum and hippocampus induced by acute ethanol administration. Moreover, the oligomer and polymer procyanidins could protect DNA damage in the cerebellum, hippocampus, cerebral cortex, and hypothalamus induced by chronic ethanol. Therefore, it is reasonable to assume that scavenging of the ethanol-induced formation of oxidative species mainly contributes to the protective activities of procyanidins for the DNA damage induced by acute and chronic ethanol administration.

Conclusion

From the quantitative point of view, the major polyphenols in grape and in red wine are polymeric polyphenols, while other well-known simple phenolics, such as flavonol, catechin, resveratrol, represent only a very small portion. Polymeric polyphenols play an important role in red wine sensory properties (color, astringency). On one hand, polymeric polyphenols have higher reactivity towards human salivary proteins and thus are more astringent than oligomeric ones. On the other hand, polymeric polyphenols possess strong reactivity towards anthocyanins and thus affect significantly anthocyanins stability during wine ageing and storage. Furthermore, polymeric polyphenols showed potent *in vitro* and *in vivo* antioxidant activity as other phenolics presented in grape and red wine. This would indicate that it may be just these polymeric compounds that are mainly responsible for health-beneficial effect of red wine, due to their quantitative importance (1000–5000 mg/L in red wine), rather than other very little amount of phenolic compounds presented in red wine, such as resveratrol (generally less than 5 mg/L in red wine).

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