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The Evolution of Polyphenols from Grapes to Wines

Violeta-Carolina Niculescu, Nadia Paun and
Roxana-Elena Ionete

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<http://dx.doi.org/10.5772/intechopen.72800>

Abstract

Polyphenols play an important role in the quality of wines, due to their contribution to the wine sensory properties: color, astringency and bitterness. They act as antioxidants, having positive role in human health. They can be divided into non-flavonoid (hydroxybenzoic and hydroxycinnamic acids and stilbenes) and flavonoid compounds (anthocyanins, flavan-3-ols and flavonols). Anthocyanins are responsible for the color of red grapes and wines, hydroxycinnamic and hydroxybenzoic acids act as copigments, stilbenes as antioxidants and the flavan-3-ols are mainly responsible for the astringency, bitterness and structure of wines, being involved also in the color stabilization during aging. This chapter will focus on the chemical structures of the main polyphenols, their identification and quantification in grapes and wines by advanced analytical techniques, highlighting also the maceration and aging impact on the polyphenols evolution. The factors influencing the phenolic accumulation in grapes are also reviewed, emphasizing as well the relationship between phenolic content in grapes versus wine. Polyphenolic changes during the wine making process are highlighted along with the main polyphenol extraction methods and analysis techniques. This research will contribute to the improvement in the knowledge of polyphenols: their presence in grapes, the relationship with wine quality and the influence of the external factors on their evolution.

Keywords: grapes, liquid chromatography, polyphenols, chemistry, wines

1. Introduction

First, a simple question can be addressed: what are phenolic compounds? For a simple question, a simple answer is that they are compounds that have one or more hydroxyl groups attached directly to an aromatic ring. Phenol (**Figure 1**) is the basic structure, the aromatic ring being benzene.

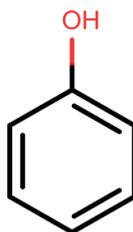


Figure 1. Phenol chemical structure.

Due to the aromatic ring, the hydrogen of the hydroxyl group is labile, which makes phenols weak acids. Polyphenols are compounds that have more than one phenolic hydroxyl group attached to one or more aromatic rings. The term is somewhat misleading since it tends to make people think of polymers of individual phenol molecules.

The general term ‘phenolic’ covers a very large and diverse group of chemical compounds, and they can be classified in many ways. Phenolic compounds are well connected with several functions in plants like protection against invading pathogens or from UV radiation, pigmentation, or attraction of pollinators and seed dispersers. These compounds were classified into groups based on the number of carbons in the molecule (**Table 1**) [1].

An alternative classification has been used by Swain and Bate-Smith [2]. They grouped the phenols in ‘common’ and ‘less common’ categories. Also, phenols were grouped into three families [3]:

1. Widely distributed phenols—ubiquitous to all plants, or of importance in a specific plant
2. Phenols that are less widely distributed—limited number of compounds known
3. Phenolic constituents present as polymers

Structure	Class
C6	Simple phenolics
C6-C1	Phenolics acids and related compounds
C6-C2	Acetophenones and phenylacetic acids
C6-C3	Cinnamic acids, cinnamyl aldehydes, cinnamyl alcohols
C6-C3	Coumarins, isocoumarins, chromones
C15	Chalcones, aurones, dihydrochalcones
C15	Flavans
C15	Flavones
C15	Flavanones
C15	Flavanonols
C15	Anthocyanidins
C15	Anthocyanins
C30	Biflavonyls

Structure	Class
C6-C1-C6, C6-C2-C6	Benzophenones, xanthones, stilbenes
C6, C10, C14	Quinines
C18	Betacyanins
Lignans, neolignans	Dimmers or oligomers
Lignins	Polymers
Tannins	Oligomers or polymers
Phlobaphenes	Polymers

Table 1. Classification of phenolic compounds.

Phenolic compounds play an important role on the sensory characteristics of both grapes and wine because they are responsible for some sensory properties: aroma, color, flavor, bitterness and astringency [4]. The chemical structure enables them to act as antioxidants, scavenging and neutralizing free radicals, and wine polyphenols have been extensively studied in relation to their positive role in human health [5].

2. Grape and wine polyphenols: a chemical perspective

During the grape ripening phase, the physiological and biochemical changes determine the quality of the fruit, the relative proportions of the grape components and wines, being influenced considerably by site (altitude, geological features, soil type, sunlight exposure), climate (cool climate or warm climate), time of harvesting (fungi and bacteria present in grapes), and viticultural practice. The studies about the formation and development of grapes showed that environmental stress factors like heat, drought and light intensity drive to changes in the development of grapes, their chemical composition and phenolic metabolism. The first period of grapes' growth consists mostly of cell division and expansion, followed by a rapid growth phase during which the berry is formed and the seed embryos are produced. In this period, several compounds accumulate in the berry, especially the tartaric and malic acids, conferring the acidity of the future wine. During the first growth period, other compounds accumulate: hydroxycinnamic acids—in the flesh and skin of the grapes; tannins and catechins—in the skin and seed tissues of the grapes; minerals, amino acids and amino acids. The most important changes in grapes' composition occur during the second growth phase (the ripening phase). Grapes switch from small, hard and acidic berries, to larger, softer, sweeter, less acidic, flavored and colored one. The majority of the solutes accumulated during the first growing period remain at harvest. During the second period, the malic acid is metabolized and used as an energy source, its proportion decreasing toward the tartaric acid concentration, which remains almost unchanged. Tannins also decrease considerably after *véraison*.

Winemaking techniques involve the extraction of juice from ripe grapes and fermentation with yeast, changes in polyphenolic composition occurring due to the participation of these compounds in various reactions such as copigmentation, cycloaddition, polymerization and oxidation. The reactions begin after grapes crushing, followed by fermentation and aging, contributing to the sensory properties of wines, mainly color and astringency.

The understanding of the relationship between the quality of a particular wine and its phenolic composition is, at the moment, one of the major challenges in oenology research. For example, the anthocyanin fingerprints of varietal wines are proposed as an analytical tool for authenticity certification [6].

Polyphenolics must be also considered from the taxonomical point of view, knowing that the patterns of some classes of flavonoids are under strict genetic control and that their distribution varies considerably among different grape cultivars [7]. There are several factors with impact on the nonvolatile wine phenolic compounds, including the '*terroir*,' the grape variety and its degree of maturation before harvesting, or the winemaking process with its specific conditions of fermentation or aging [8]. Certain technological procedures such as addition of sulfur dioxide (SO_2) and/or ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$) prior to crushing the grapes, maceration, yeast strain utilization and alcoholic fermentation, oxidation or adsorption, can also influence the levels of phenolic during the winemaking process [9].

The investigation of phenolic composition in grapes and wine may provide some specific biomarkers that allow us to better assess the chemical evolution of grapes during growth and maturation periods but also to improve the knowledge on wine authentication by developing and implementing new/improved control methods. Phenolic compound identification and quantification fit authentication purpose, protecting consumers against fraud [10].

There is a great chemical diversity in the nonvolatile phenolic composition of grapes and wines, due not only to the varieties of grapes, but also to the fact that they exist in both the free and conjugated forms, as they may be bound to one or more sugar molecules (glucose, galactose, sucrose and mannose) [11]. From the flavonoid compounds, the main phenolics involved in the red color of grapes and wines are the anthocyanins, while the flavan-3-ols are most linked with the astringency, bitterness and structure of wines, but also with the stabilization of color during aging. The nonflavonoid compounds act as copigments (hydroxycinnamic and hydroxybenzoic acids) and antioxidants (stilbenes).

2.1. Phenolic acids

They include two main groups, the benzoic acids, containing seven carbon atoms (C₆-C₁), and the cinnamic acids, with nine carbon atoms (C₆-C₃), and they exist mainly as hydroxybenzoic and hydroxycinnamic acids, in either the free or the conjugated form. Various types of hydroxybenzoic acids (HBA) have been identified in both grapes and wines, among them parahydroxybenzoic, protocatechuic, vanillic, gallic and syringic acids (**Figure 2**) [12]. Gallic acid is considered the most important phenolic acid, being the precursor of all hydrolyzable

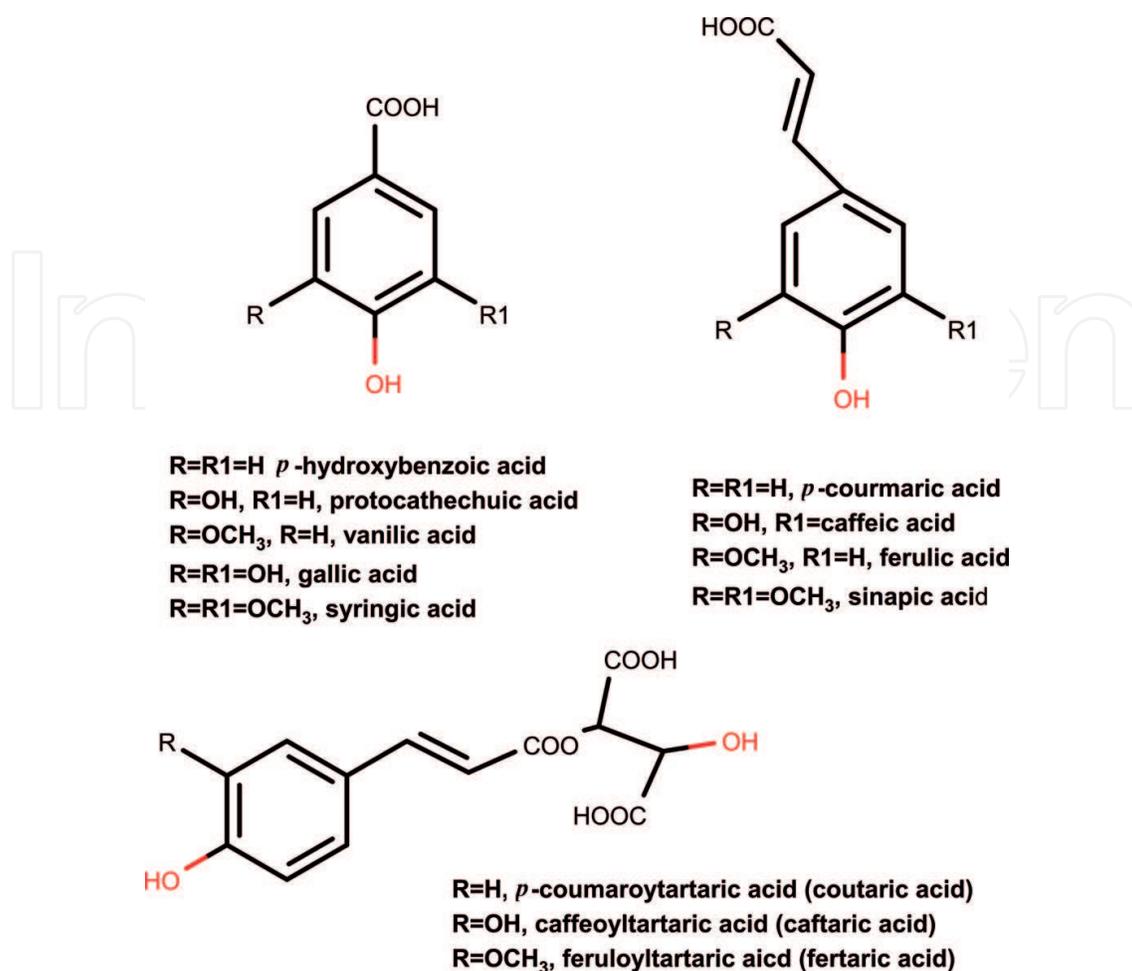


Figure 2. Chemical structures of hydroxybenzoic, hydroxycinnamic and hydroxycinnamoyltartaric acids.

tannins. Hydroxycinnamic acids (HCA) are found in both grapes and wine, the most referenced compounds among them being *p*-coumaric, caffeic, ferulic and sinapic acids (**Figure 2**), which are associated with the browning of wine [13].

Cinnamic acids exist as *cis* or *trans* isomeric forms, which are convertible either enzymatically or through the action of light. Other hydroxycinnamic acids detected in grapes and wines are *p*-coutaric acid, fertaric acid and caftaric acid (**Figure 2**). Caftaric and fertaric acids exist in their *trans* form, mainly localized in the grape pulp and, during the grape pressing, being quickly released into the juice, while a negligible fraction of the *cis* isomer has been found for *p*-coutaric acid. Contrary to this, the *trans* and *cis* isomers of *p*-coutaric acid are less extractable since they are mostly localized in the grape skin, being partially responsible for the astringent properties of both grapes and wines [14]. Although the white wines have a lower concentration of phenolic compounds compared to the red ones, they contain, in turn, a high quantity of caftaric acid. Hydroxycinnamic acids and their tartaric esters constitute the main class of nonflavonoid phenolics in red wines and the main class of phenolic compounds in white wines [14]. During the fermentation process, these esters are partially hydrolyzed, resulting free hydroxycinnamic acids, transformed then into ethyl coumarate and ethyl caffate [15].

2.2. Flavonoids

They are a chemical class with a basic structure of 15 carbon atoms, including two aromatic rings bound through a three-carbon chain (C6-C3-C6), which is responsible for the chemical. Flavonoids are grouped into several classes, differing from the oxidation degree of the central pyran ring, except of chalcones, including flavanols, flavonols, flavononols, flavones, flavanones, flavanes, anthocyanidins and anthocyanins, chalcones and dihydrochalcones (**Figure 3I**) [12].

In grapes, the highest flavonol concentrations were found at flowering, decreasing as the grapes mature. Some of the flavonoids present in both grapes and wine are represented in **Figure 3II**. In the flavonoid class, the chemical diversity *versus* complexity is related to the high variety of aglycones and glycosides, as well as to the occurrence of condensation reactions, three distinct categories being usually accepted: glycosylated flavonoids, flavonoid aglycones and anthocyanidin glycosides (anthocyanins) [16].

Flavanols (**Figure 3I**) are benzopyrans with a saturated carbon chain between C2 and C3, a hydroxyl function in C3, and no carbonyl group in C4. The most abundant flavan-3-ols in nature are catechin and its enantiomer epicatechin (**Figure 3II**), and they can be found in the grape skin, seeds and stems, as well as in wine. Some catechin derivatives, such as gallic catechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate, were identified in grapes and wine [17].

Flavonols (**Figure 3I**) have a double bond between C2 and C3 atoms and a hydroxyl group in C3, being often named 3-hydroxyflavones. Approximately 90% of the flavonols are hydroxylated in C3, C5 and C7, being designated as 3,5,7-trihydroxylated derivatives [18]. In grapes, there were identified: quercetin (3',4'-diOH), kaempferol (4'-OH), myricetin (3',4',5'-triOH), isorhamnetin (3'-MeO analog of quercetin), laricitrin (3'-MeO analog of myricetin) and syringetin (3',5'-MeO analog of myricetin) (**Figure 3II**). In *Vitis vinifera* grapes, there was detected the simultaneous presence of these aglycones [19]. The most abundant condensed flavonoids are the *O*-glucosides, the *O*-sulfates and the derivatives containing acylated sugars and aliphatic or aromatic acid groups in their structure [20]. White wine contains only quercetin, kaempferol and isorhamnetin [20]. Depending on the nature of sugar moiety bound at position C-3, there are three different complete series for flavonol 3-*O*-glycosides in red grapes. The 3-*O*-glucosides are the main derivative of the flavonol aglycones, namely kaempferol, quercetin, isorhamnetin, myricetin, laricitrin and syringetin, while the minor ones are the 3-*O*-galactoside derivatives.

Flavononols (**Figure 3I**) are characterized by the presence of a hydroxyl group in the C3 position and the absence of a double bond in the heterocyclic ring, being also named 3-hydroxyflavonones or dihydroflavonols (e.g., taxifolin, astilbin and dihydromyricetin 3-*O*-rhamnoside) [16].

Flavones (**Figure 3I**) have a double bond between carbons C2 and C3, and the hydroxyl group is absent in the C3 position. Although they are widely encountered in plants, as aglycones or glycosides, they are not present in grapes in significant amounts, except for luteolin (**Figure 3II**) [8].

Flavanones (**Figure 3I**) have a saturated carbon chain between atoms C2 and C3, being often named dihydroflavones (e.g., eriodictyol, a flavanone that has been extracted from grapes) [21].

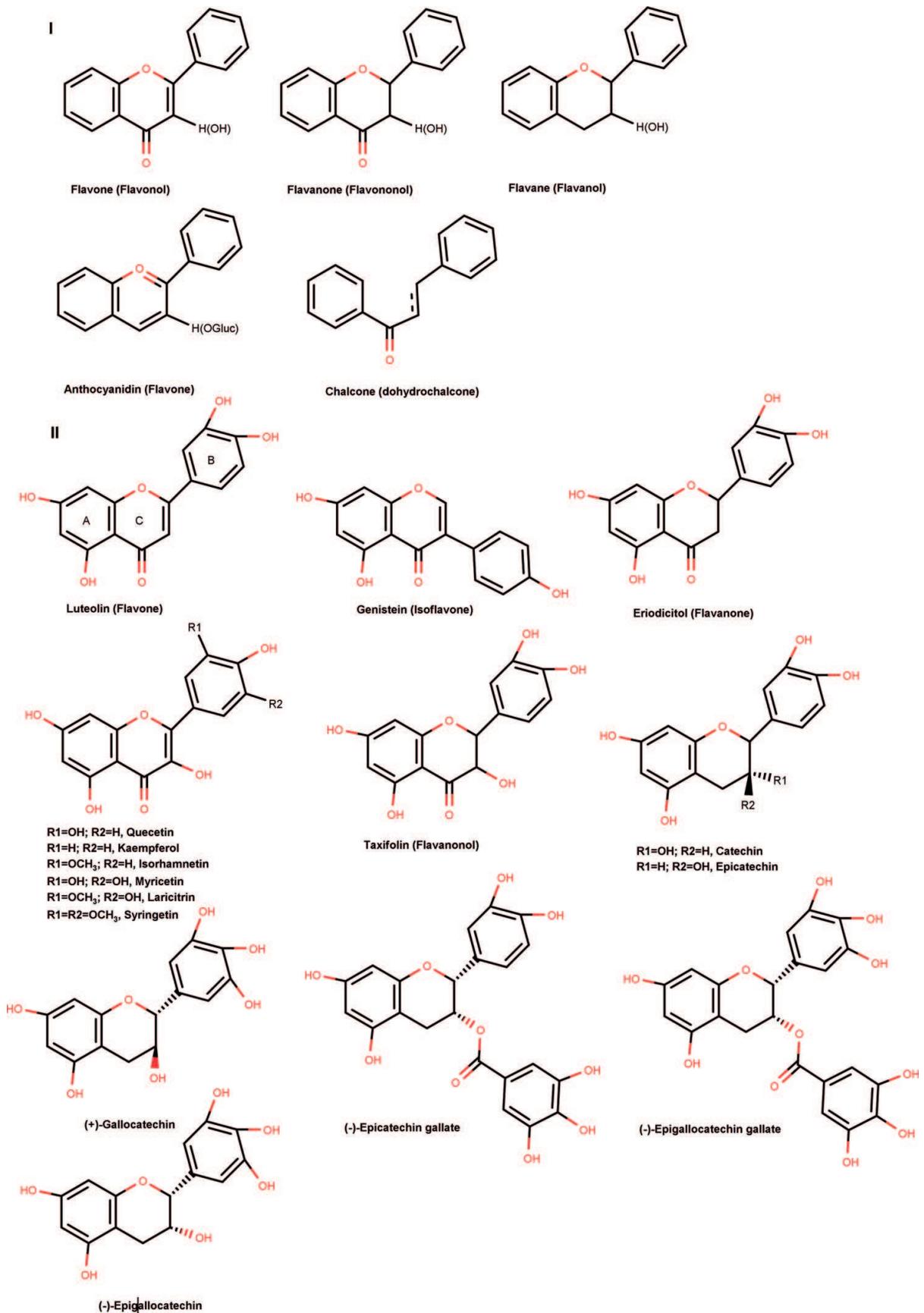


Figure 3. General chemical structure of flavonoid subgroups.

Flavanes (**Figure 3I**) contain a saturated carbon chain between C2 and C3, and no carbonyl group in C4 [22].

Chalcones have two aromatic rings linked by a carbonylic α,β -insaturated system (**Figure 3I**). Dihydrochalcones are obtained from chalcones through a reduction process [23].

Different families of anthocyanin derivatives have been reported in grapes, wine and wine-like solutions. Anthocyanidins and anthocyanins (**Figure 3I**) have as nucleus the 2-phenylbenzopyrylium (the flavylum) cation. Anthocyanins are anthocyanidin glycosides. They are responsible for the color of grapes and wines. In red grapes and wines, six anthocyanidins have been detected: cyanidin (orange red), peonidin (red), delphinidin (bluish red), pelargonidin (orange), petunidin and malvidin (bluish red) [24], the last one being the most representative compound in *V. vinifera* [25, 26]. Also, the 3-O-monoglucosides of the mentioned six anthocyanidins have already been detected in grapes [24, 25, 27]. The anthocyanins are mainly found in the skin of grapes, while the other flavonoids occur in both skin and seeds. The amount of anthocyanins and other flavonoids extracted during vinification depends on the duration of the process, the temperature or the extent of disruption of the grapes [16]. In the grape vines, anthocyanins accumulate in the leaves during senescence and they determine the coloration of the grape skin in red and rose cultivars. The anthocyanidin composition of grapes is affected by factors such as the grape-growing origin (*terroir*) and varieties, the degree of maturity and the weather conditions. The profiles of anthocyanins for each grape variety are relatively stable, while absolute concentrations can vary widely between different vintages due to environmental and agronomical factors [11].

Stilbenes have two aromatic rings linked by an ethene bridge, resveratrol (3,5,4'-trihydroxystilbene, **Figure 4**) being the most important in grapes and wine. Also, it was identified in vine leaf and in the grapes skin, and its concentration is known to decrease significantly upon grape maturation [28]. Due to the presence of resveratrol in the grapes skin, the wine-processing methods determine its concentration in the final product. Wines from grapes with longer maturation periods have an increased content of resveratrol, its concentration being higher in red wines compared to white wines [28]. The stilbenes in wines can contribute to the correlation of their profiles with winemaking procedures and with the grape wine varieties.

Tannins are polyphenols with astringent properties, able to cause protein precipitation. They are usually divided in two classes: the hydrolyzable and the nonhydrolyzable or condensed tannins [22]. Most of the natural tannins present in grapes and wine are the condensed type. In young wines, tannins occur mainly in the form of dimers or trimers. However, their concentration decreases with aging due to oxidation and precipitation processes [29]. Hydrolyzable tannins can be degraded through pH changes as well as by enzymatic or nonenzymatic hydrolysis into smaller fragments, mainly sugars and phenolic acids. Their basic unit is gallic acid (**Figure 2**) and its derivatives (e.g., ellagic acid). These acids are usually esterified with D-glucose, resulting 500–2800 molecular weight species. Aging in oak barrels promotes the extraction of low molecular weight phenolic compounds, mainly from ellagitannins, into wine. For example, ellagic acid and myricetin are probably the major phenolic compounds present in muscadine grapes (*Vitis rotundifolia*) [30]. Condensed tannins

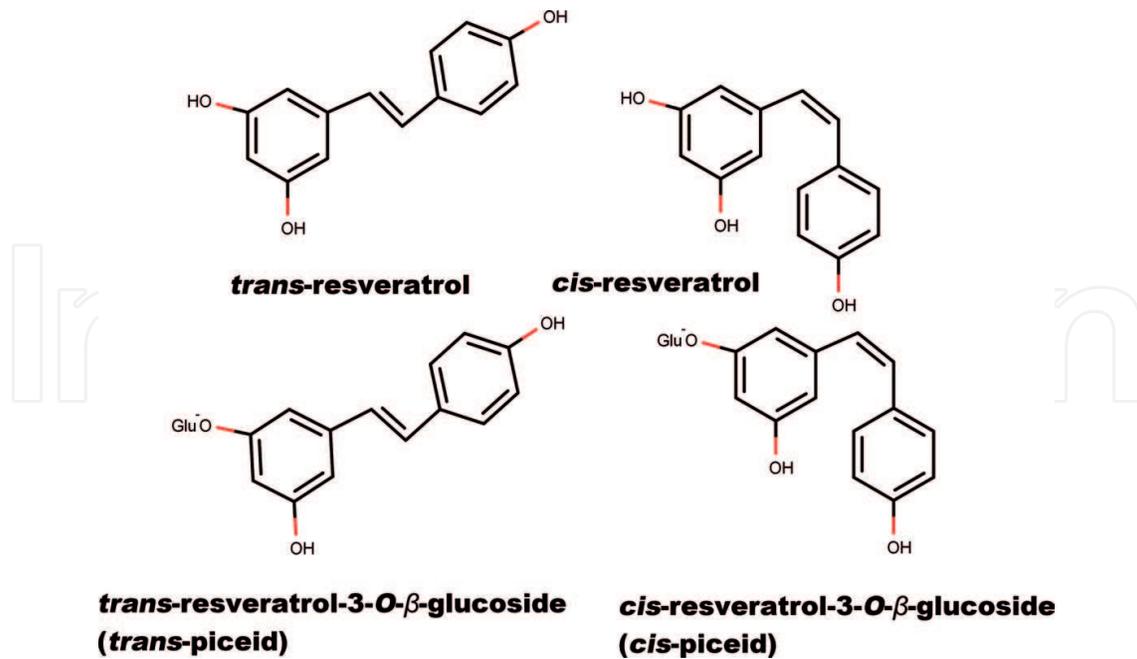


Figure 4. Chemical structures of resveratrol and resveratrol glycoside.

(proanthocyanidins) are polymeric compounds that are transformed into anthocyanidins, and they can be found in residual amounts of the solid grape components (e.g., seeds, skin) or in the pulp [27]. They are transferred into the must during winemaking operations such as crushing, maceration and fermentation [27]. Proanthocyanidins generally occur as oligomers and polymers of flavan-3-ols. The proportion of the polymer units and the average number of proanthocyanidins have been highlighted by LC-MS/MS and thiolytic degradation [31]. *V. vinifera* grapes contain mainly proanthocyanidins as oligomers and polymers of (+)-catechin and (-)-epicatechin linked through C4/C8 bonds [32]. The procyanidins and prodelphinidins, leading to cyanidin and delphinidin, are the most abundant condensed tannins in grapes and wine [32]. The relationship between monomeric, oligomeric and flavan-3-ol composition has been intensively studied as a prerequisite for improving wine quality, being showed that flavan-3-ols and their monomers ((+)-catechin and (-)-epicatechin) as predominant phenolic compounds extracted from grape seeds have a highly positive correlation with antioxidant activity. In *V. vinifera*, oligomers with a maximum degree of polymerization of 16 have been identified. The identification and quantification of proanthocyanidins is an important subject, since these compounds are mainly responsible for the sensory characteristics of wine and they play an important role in the wine aging process.

3. Polyphenolic changes in the winemaking and polyphenol's extraction techniques

The total extractable phenolic content in grapes is encountered in the pulp (about 10% or less), in the seeds (60–70%) and in the skin (28–35%). In the seeds, the phenolic content may range

between 5% and 8%, by weight [33]. There are a great number of methods for polyphenol extraction from grapes. **Table 2** summarizes the most applied extraction techniques.

The main phenolic compounds in musts from white grapes are hydroxycinnamic tartaric acid esters (HCTA). Catechins and proanthocyanidins are found mainly in the skins of white grapes. They can sustain oxidation; as a consequence, the grapes must often be protected by limiting the contact with oxygen and inhibiting polyphenoloxidase (PPO) by SO₂ addition. Due to the oxidation, the phenolic profile of the musts is characterized from lower levels of HCTA and flavanols, and high 2-S-glutathionyl caffeoyl tartaric acid (GRP). Red wines are produced by the must fermentation in the presence of the grape skins and seeds. During the process, phenolic compounds such as anthocyanins are subjected to various reactions, such as enzymatic oxidation, electrophilic substitution, degradation of anthocyanins, cyclo-accession of the carbonyl compounds to anthocyanins and formation of vitisins (A, B and C), hydrolysis of proanthocyanidins and formation of carbocation in C4 position which attacks the positions C6 and C8 of the proanthocyanidins and anthocyanins, attack of C4 carbocation of anthocyanin to C6 or C8 of proanthocyanidins, attack of acetaldehyde to C6 and C8 of proanthocyanidins and formation of structures with flavanol linked to anthocyanin by ethyl bridge [33].

Polyphenol extraction from grapes can be achieved by using alcoholic or hydroalcoholic solutions either containing, or not containing, mineral acids, or using acetone/water solutions. The use of a mineral acid (usually methanol containing HCl 1% by volume) allows extraction of all phenolic compounds. The use of nonalcoholic acid containing solutions does not guarantee satisfying extraction of HCTA. Grapes' freezing after the harvest, followed by heating at room temperature before performing extraction, allows PPO to be in touch with and to oxidize the substrates [34].

A satisfying extraction method for all polyphenols is the use of a model wine solution containing: tartrate buffer pH 3.2 (with 12% ethanol, v/v) and sodium metabisulfite 2 g/L. This can be prepared by adding 5 g tartaric acid, 22.2 mL 1 M NaOH solution and 2 g of Na₂S₂O₅ to 500 mL

Sample preparation	Extraction	Clean-up isolation	Instrumental analysis
Freeze-drying	Supercritical fluid extraction (SFE)/solid phase extraction (SPE)/solid phase micro-extraction (SPME)	Column chromatography/acidity-based fragmentation: SPE	Liquid Chromatography (LC): UV, FLUD, ECD, NMR, MS, MS ⁿ
Air drying		Thin-layer chromatography (TLC), MSPD	CE
Centrifugation	Soxhlet	High-speed countercurrent chromatography (HSCCC)	Gas Chromatography (GC): FID, ECD, MS, MS ⁿ
Filtration	Solid-liquid		
	Liquid-liquid		
	Accelerated solvent extraction/pressurized liquid extraction (ASE/PLE)		
	Microwave		
	Matrix solid phase dispersion (MSPD)		
	Sonication		

Table 2. Polyphenol extraction methods.

ultrapure water, stirring continuously, then adding 125 mL ethanol and adjusting the final volume to 1 L with ultrapure water. Often, extracts have to be concentrated by solid phase extraction (SPE) before performing analysis by high-performance liquid chromatography (HPLC) or spectrophotometry. As soon as the grapes were harvested, the seeds and skins of 50 berries must be separated from the others parts of the berry, immersed in two solutions of 50 mL tartrate buffer solutions containing 1 g/L SO₂ and immediately frozen. Before analysis, the samples must be heated at room temperature and kept for 4 h; the extracts must be then homogenized and centrifuged; the supernatant transferred in a 100-mL volumetric flask, pellets being resuspended twice in 15–20 mL tartrate buffer; after centrifugation, the liquid phases can be collected in the same volumetric flask; the final volume must be adjusted to 100 mL with tartrate buffer [33].

When using acetone/water solutions for the extraction, skins from 300 berries are rinsed with ultrapure water, after separation from the flashes, immersed in 100 mL of aqueous solution containing 100 mg/L of SO₂ and stored at 4°C. In order to perform the extraction, the sample must be laid into a 500-mL Erlenmeyer flask, over which 200 mL of acetone is added. Next, the flask is covered and placed for 24 h on a shaker, at 20°C and 100 rpm. After this procedure, the acetone is removed at 40°C, under vacuum; the solution is centrifuged, and the supernatant is recovered and diluted with ultrapure water, at 100 mL, and finally stored at –20°C until analysis [35].

Another method for extracting polyphenols from seeds and skins is by immersion of the fine powder (previously obtained by grinding the grape skins and seeds in liquid nitrogen) in an acetone/water 7:3 (v/v) solution containing 0.1% ascorbic acid added to prevent oxidation [36].

Other methods use methanol for extraction: 250 mg skins must be stored at –80°C, then grounded with mortar and pestle in liquid nitrogen and soaked in 30 mL methanol at 4°C; the extract is then concentrated to almost dryness and dissolved in 1 mL methanol [37].

Ethanol/water/chloroform solutions can be also used for phenolic compound extraction; the skins and seeds are freeze-dried until obtaining 1 g (dry weight) and 0.5 g (dry weight), respectively, and subjected to an extraction procedure using 20 mL ethanol/water/chloroform 1:1:2 (v/v/v) for 2 min. The 50% upper layer of ethanol containing the phenolic compounds is separated by the chloroform lower layer which contains undesirable compounds like chlorophylls and lipids; the ethanol is removed under vacuum; the resulted aqueous solution is then filtered through a glass membrane, diluted with water up to 100 mL and stored at –20°C until analysis [38].

Another method proposes that 1 g of freeze-dried grape seeds and skins is to be extracted, using a blender, with 5 × 20 mL with ethanol/water 1:1 (v/v). Subsequently, the obtained extracts are adjusted to a final volume of 100 mL and pH 4.0 and then purified and fractionated on a 250 × 5 mm column, type Toyopearl Gel HW-40(s), using as eluent the methanol [39].

4. Advanced techniques for polyphenol analysis

Monomer phenols in grapes and wines are usually analyzed by high-performance liquid chromatography (HPLC) using a reverse phase C18 column (usually 250 × 4 mm, 5 μm) operating close to or at room temperature.

For example, in order to obtain the anthocyanin monomer profile of grapes, a Cabernet Sauvignon grape skin extract was used, the chromatogram being displayed in **Figure 5**. A column type C18 (250 × 4 mm, 5 μm), at 45°C, using a binary solvent mixture of formic acid/water 10:90 (v/v) and formic acid/methanol/water 10:50:40 (v/v/v) and detection at 520 nm wavelength were used for the analysis. For compound elution from the column, a gradient program was applied. The sample volume injected is 20 μL [33].

Peaks of some typical anthocyanins for *V. vinifera* varieties, namely 3-glucosides, 3-glucoside acetates, 3-glucoside para-coumarates and malvidin 3-glucoside caffeate, are highlighted in the **Figure 5**. Even if different separation column is used, the sequence for compound elution from a reverse phase column is due to their polarity and is always the same. The values of single anthocyanin concentration can be expressed as area percentage on the total area amount of the peaks of all identified anthocyanins or in mg/kg grapes of malvidin-3-glucoside (compound used as external standard).

HPLC with UV-Vis detection makes possible the analysis of some anthocyanin derivatives in wines, such as vitisins, several pyranoanthocyanins and flavanol-ethyl-anthocyanin derivatives. Due to the lack of commercially available standards, identification and quantification of these compounds is difficult. Usually, the column is a reverse-phase polystyrene divinylbenzene (250 × 4.6 mm, 5 μm) operating at 30°C, elution being performed by a binary mobile phase mixture composed of aqueous H₃PO₄ 1.5% (w/w) and aqueous H₃PO₄/acetonitrile 20:80 (v/v) with a gradient program, at 520 nm, the compounds being quantified as malvidin-3-glucoside. Analysis can be performed by direct injection of the sample or, to increase the sensibility, after concentration on a C18 cartridge [40].

HCTA and flavonols can be simultaneously detected in a single run using a C18 (250 × 4 mm, 5 μm) column operating at 40°C, performing elution with a binary solvent mixture composed of H₃PO₄ 10⁻³ M and methanol, and an elution gradient program. **Figure 6** shows a typical chromatogram for flavonols, respectively, recorded in the analysis of a Cabernet Sauvignon grape skin extract. Detection wavelength was 360 nm for flavonols (sample volume injected 25 μL). Identification of compounds is performed on the elution order from the column and by recording UV-Vis spectra on the basis of maximum wavelengths [33].

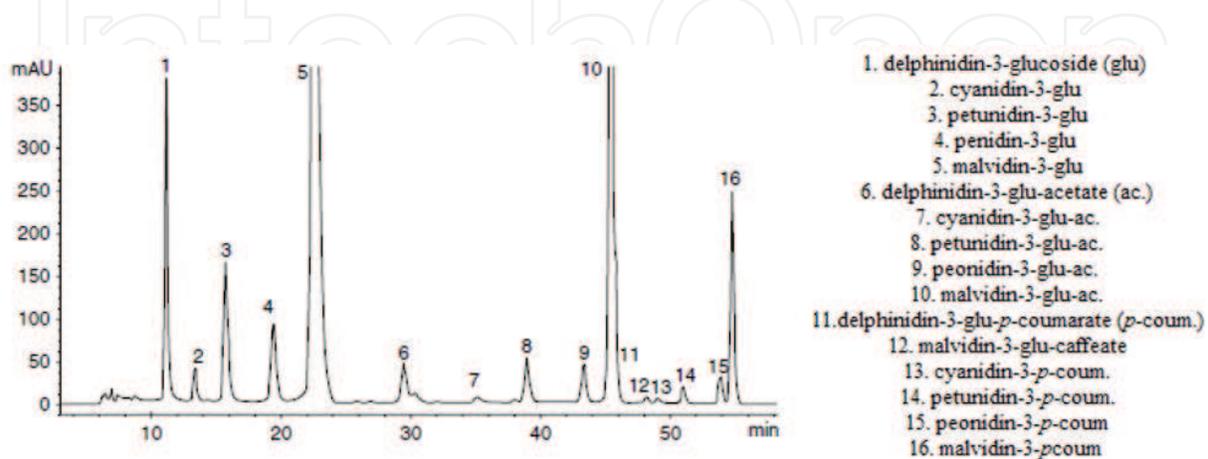


Figure 5. HPLC anthocyanin profile of Cabernet Sauvignon grape skin extract.

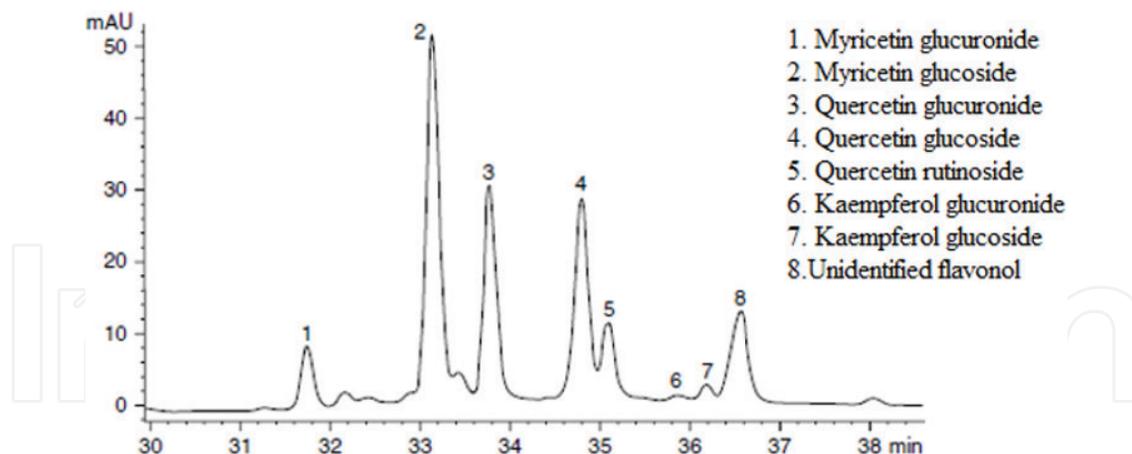


Figure 6. HPLC profile of flavonols of a Cabernet Sauvignon grape skin extract recorded at 360 nm [33].

Due to the lack of commercially available standards for HCTA, chlorogenic acid or free hydroxycinnamic acids are used as external standards for quantitative analysis. Quercetin and myricetin glucoside and other flavonols glycoside (e.g., rutin) are standards of flavonols commercially available.

For the HCTA and flavonols in wines, analysis can be performed on the acidified sample prepared by the addition of 0.5 mL 1 M H_3PO_4 to 4.5 mL of wine and filtration on 0.45 μm membrane. The HPLC conditions used are the same reported for skin extract.

The typical HPLC chromatograms of grape skins and red wines for the identification of *trans*-resveratrol in grape skins and red wines are presented in Figures 7 and 8, respectively. *Trans*-resveratrol was identified by the comparison with a commercial standard of *trans*-resveratrol. An Aquasil C18 (250 \times 4.6 mm, 5 μm) analytical column was used for separation. Chromatographic conditions included a mixed mobile phase of water:acetonitrile:acetic acid

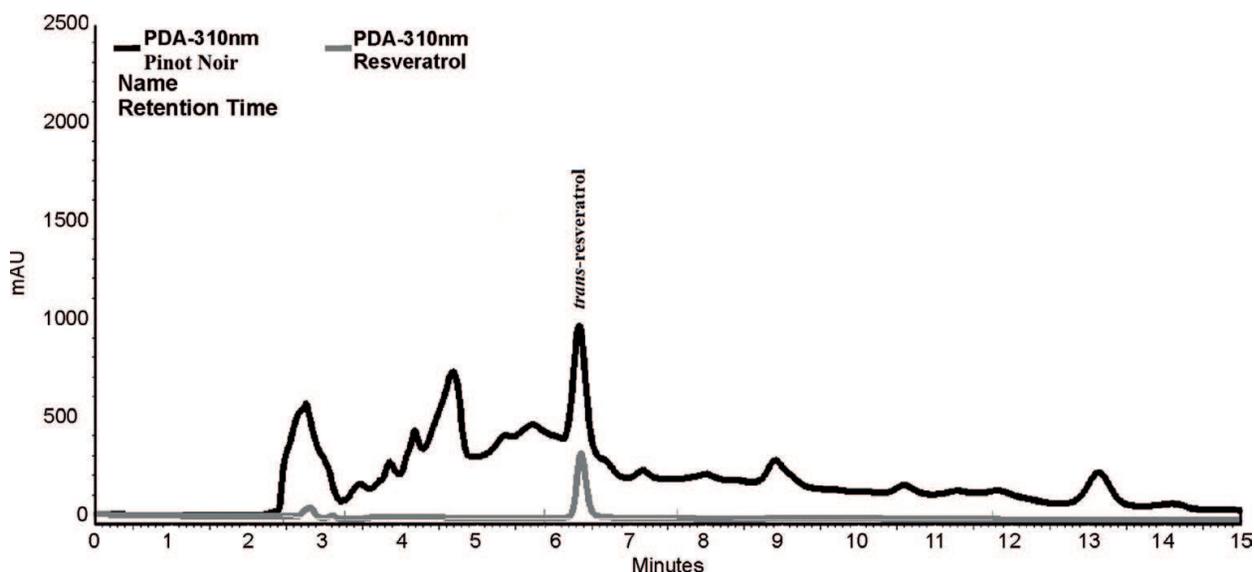


Figure 7. HPLC chromatogram of grape skin (*V. vinifera*, Pinot Noir) extract and standard [28].

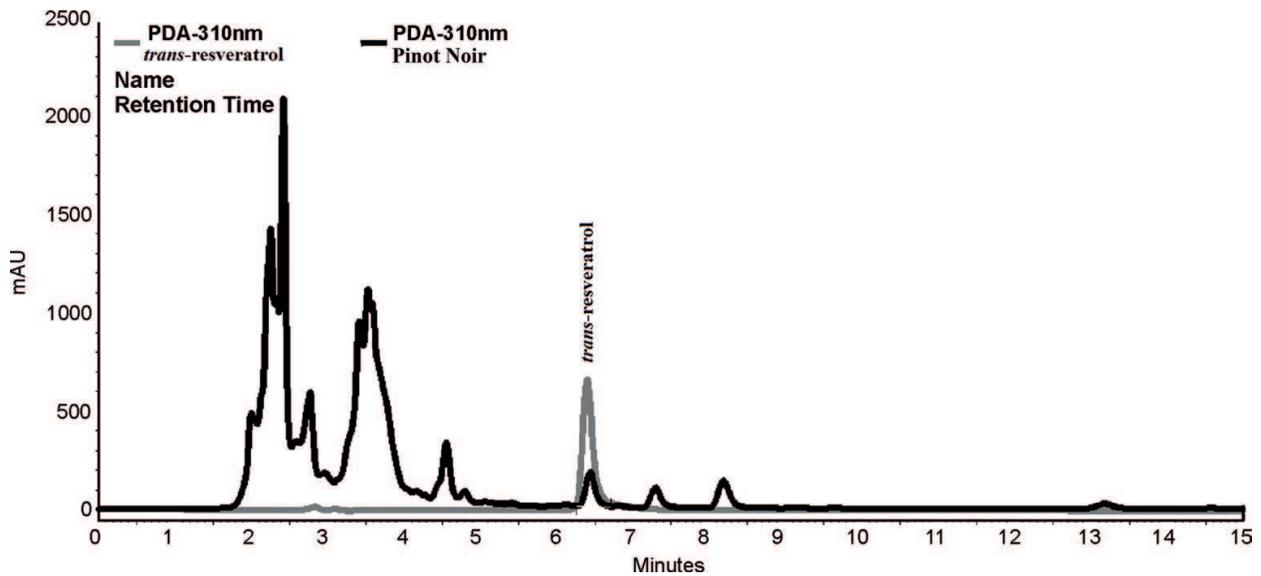


Figure 8. HPLC chromatogram of red wine (*V. vinifera*, Pinot Noir) and standard [28].

of 70:29.9:0.1, flowing through the system at the rate of 1 mL/min; the injection volume was 20 μ L; the detection was set at 310 nm [28].

In order to evaluate the difference in phenolic composition of grape skins, 35 samples of five red grape varieties (*V. vinifera* L.), namely Pinot Noir, Merlot, Cabernet Sauvignon, Feteasca Neagra and Mamaia cultivated in Southeastern Romania, were investigated by reversed-phase high-performance liquid chromatography (RP-HPLC) using a system with: a diode array detector set at 280 nm; the mobile phase—water with 0.1% formic acid and acetonitrile with 0.1% formic acid; and an Accuacore PFP (100 \times 2.1 mm, 2.6 μ m) column, operated at 30°C. The concentrations of phenolic compounds in the extracts were calculated as mg/L using external calibration curves generated from standards, which were obtained for each phenolic compound (Figure 9 and Table 3) [41].

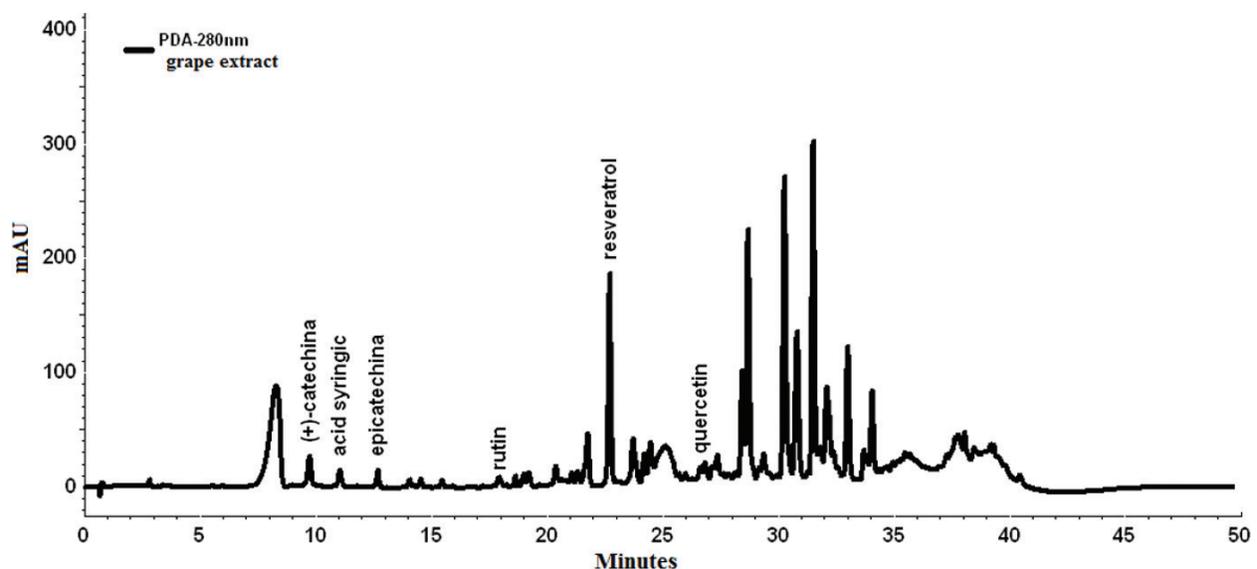


Figure 9. HPLC chromatogram of Cabernet Sauvignon grape skin extract [41].

Grape cultivar		Phenolic compound in grape skins				
		Syringic acid	Catechin	Epicatechin	Rutin	Trans-resveratrol
Cabernet Sauvignon	Average	6.1	44.2	20.3	30.0	70.6
Merlot	Average	11.5	52.6	41.5	63.5	151.6
Feteasca Neagra	Average	1.1	24.4	18.3	15.2	112.7
Mamaia	Average	3.4	14.4	13.6	18.3	18.1
Pinot Noir	Average	Not detected	28.1	38.1	88.2	203.6

Table 3. Phenolic compound in grape skins.

The phenolic profile in grape skins of red grape varieties grown in Southeastern Romania in 2013 season, under the same agronomic conditions and microclimate, showed significant differences. Each variety had a different ripening trend. The ripening influenced the phenolic composition of red grape skins, higher amounts of phenolic compounds being found on the last sampling week for Cabernet Sauvignon, Feteasca Neagra and Merlot grape cultivars, while phenolic maturity was reached for Mamaia and Pinot Noir. This observation must be taken into consideration in winemaking process, in order to obtain wines with high phenolic content [41].

Low molecular weight and volatile phenols are usually identified and quantified by Gas Chromatography coupled with Mass Spectrometry (GC-MS). For analysis and structural characterization of more polar compounds such as polyphenols, Liquid Chromatography Mass Spectrometry (LC-MS) and Multiple Mass Spectrometry (MS/MS and MSⁿ) techniques are used [42]. These methods are the most effective techniques to characterize individual polyphenols in grape extracts and wine, due to the soft ionization conditions and minor sample purification required. Opposed to the LC methods coupled with spectrophotometric detection which require hydrolysis or thiolysis for compounds identification, the LC-MS is a more reliable and advanced technique to be applied in the study of compound structure linked with the color-changing of red wines during aging formed by reactions of anthocyanins with other compounds. This technique also allows the characterization of proanthocyanidins (procyanidins, prodelfinidins) also called condensed tannins. Moreover, for the particular case of studying compounds, the MS/MS approach is a very powerful tool that allows characterization of aglycone and sugar moiety.

In order to analyze the grape flavonols, the extraction of berries with acidified methanol (0.01% 12 N HCl) must be performed. The extract is then filtered, the solvent removed under vacuum and the residue dissolved in 0.1 M citric acid buffer at pH 3.5. Polyphenolics are fractionated on the basis of their affinity to a C18 cartridge and then on a Sephadex LH-20 cartridge, the fractions being then eluted by ethyl acetate and methanol. To perform analysis of the isolate, ethyl acetate is evaporated and the residue is redissolved in the pH 3.5 buffer. The isolates can be analyzed by Liquid-Chromatography-Electrospray-Ionization-Mass Spectrometry. By connecting the LC/ESI-MS system to the probe of the mass spectrometer via the UV cell outlet, LC-UV chromatograms and spectra can also be recorded. Compounds such as vanillin and phenolic acid present in wine can be detected by LC/ESI-MS analysis [43].

To achieve structural and semiquantitative information on the anthocyanins from grapes, ESI-MS/MS direct-injection analysis of extract can be performed. An example of an ESI-MS spectrum is reported in **Figure 10**, the peaks being identified and quantified in **Table 4**.

Quantification by ESI/MS of anthocyanins present in grape extract can be done by constructing a calibration curve using two commercially available standard compounds, the Mv-3-O-glucoside (M^+ species at m/z 493) for monoglucosides and the Mv-3,5-O-diglucoside (M^+ species at m/z 655) for diglucosides [44].

Operating in the positive ionization mode, the ESI is also effective for analysis of flavan-3-ols. But since most phenolic acids in wine are not detectable in this mode, the negative ionization mode is preferable to work with (see an example in **Figure 11**) [43].

By operating with a cone voltage of 60 V, these compounds show high formation of the $[M-H]^-$ ion. The sample under investigation can be prepared by the liquid-liquid extraction of 50 mL wine, using diethyl ether (3×5 mL) and ethyl acetate (3×15 mL), after previous concentrating to 15 mL, at 30°C , under vacuum, to eliminate the ethanol. Next, the organic phases are combined and the resulting solution dried over Na_2SO_4 . The solvent is removed under vacuum, then the residue is dissolved in 2 mL of methanol/water (1:1) and the solution is filtrated prior to analysis [45]. The compounds identified by LC-ESI-MS are reported in **Table 5** [43].

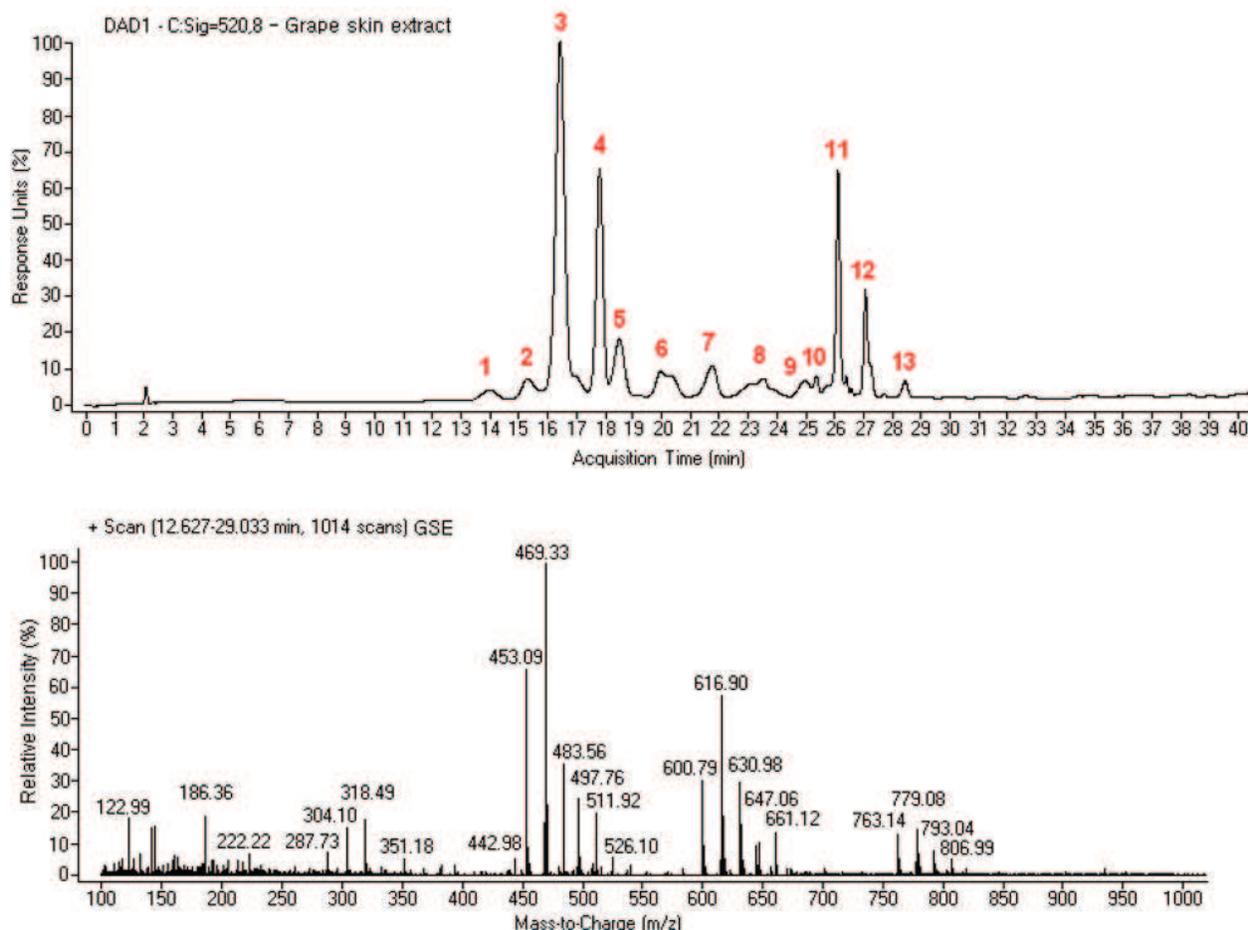


Figure 10. LC-ESI-MS anthocyanin profile of Othello grape skin extract.

Peak number	RT (min)	m/z	Mass assignment (M ⁺ sau [M + H] ⁺)	Anthocyanins content (%)
1	13.9	633	Malvidin-Delphinidin (oligomeric form)	1.87
2	15.1	617	Malvidin-Cyanidin (oligomeric form)	1.87
3	16.2	469/303	Delphinidin-3-O-glucoside	40.64
4	17.6	453/287	Cyanidin-3-O-glucoside	17.82
5	18.2	453/287	Cyanidin-3-O-galactoside	5.75
6	19.7	467/303	Delphinidin-3-O-glucoside	5.44
7	21.5	511/303	Delphinidin-3-(6-O-acetylglucoside)	4.25
8	23.2	779/287	Malvidin-Cyanidin + glucoside	4.75
		495	Malvidin-3-O-glucoside	
9	24.7	763	Cyanidin-3-glucoside-ethyl-catechin	1.16
10	25.1	763	Cyanidin-3-glucoside-ethyl-catechin	0.53
		617	Malvidin-Cyanidin (oligomeric form)	
11	25.8	807	Malvidin-3-glucoside-8-vinyl(epi)catechin	9.71
		617	Malvidin-Cyanidin (oligomeric form)	
12	26.8	601	Malvidin-3-(6-O-acetylglucoside) pyruvate	6.01
13	28.1	645	Malvidin-Petunidin (oligomeric form)	0.16

Table 4. Peak assignments, retention time, mass special data, and percentage of grape skin anthocyanins detected by LC and electrospray ionization-MS.

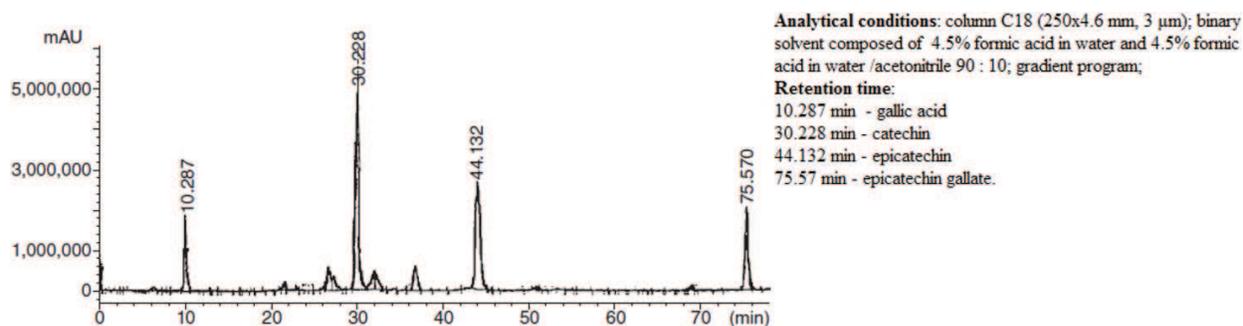


Figure 11. LC-ESI-MS of flavan-3-ols from a wine sample analysis performed in negative ionization mode.

MW	Compound	Main ions observed (m/z)	
		Fragm. 60 V	Fragm. 120 V
154	Protocatechuic acid	153 (109)	109 (153)
138	Protocatechuic aldehyde	137	137 (108)
138	<i>p</i> -Hydroxibenzoic acid	137 (93)	93 (137)
122	<i>p</i> -Hydroxibenzoic aldehyde	121	121 (92)
168	Vanillic acid	167	108 (167, 123, 91)
152	Vanillin	151 (136)	136 (151)

MW	Compound	Main ions observed (m/z)	
		Fragm. 60 V	Fragm. 120 V
194	Ferulic acid	193 (134, 149)	134 (193)
182	Syringic aldehyde	181 (166)	181 (166, 151)
164	<i>p</i> -Coumaric acid	147 (103)	147 (103)
180	Caffeic acid	179 (135)	135 (179)
178	Esculetin	177	177 (133)

Table 5. Some wine polyphenols determined by negative ionization mode.

5. Factors influencing phenolic content and composition of wine

Wine phenolic profile depends on the grapes and on the vinification technique, although other variables such as cultivar, viticulture practices can also affect it. The variety, growing season, environmental and climatic conditions, soil type and even maturity influence the concentration of phenolic compounds within grapes. The qualitative changes in the phenolic composition of the final wine, compared to the composition of the corresponding grapes, are mainly due to the production of new derivatives (such as flavenes, tyrosol and free phenolic acids—gallic, paracoumaric, caffeic, and so on). The environment in which both the must and the wine are produced can contribute to the generation of new phenolics [21]. Since wine composition is in constant evolution, the aging in bottles also seems to contribute to changes in the flavonol content, through the interaction of flavonols with other constituents [3]. It was found that the effect of vintage was significantly more pronounced on the anthocyanins than on the flavonols [46].

The total phenolic content in grapes is clearly affected by several factors: the cultivar, the year of production, the geographic origin of grapes, soil chemistry and the degree of maturation, many studies focusing on defining the effects of growing conditions on grape and wine phenolic composition and of the impact of light and temperature conditions on berry flavonoid composition [35]. The results revealed a combined effect of solar radiation and temperature on the compositional profile of flavonoids. It was observed that moderate exposure and temperature were favorable for the accumulation of anthocyanins, while an enhancement of skin tannins was noted in the grape berries from bunches exposed to sunlight versus those from shaded bunches or dense canopies. In fact, maturation is associated with polymerization of phenols, which leads to a marked decrease of astringency [16].

The phenolic composition, the relative proportions of anthocyanins and flavonols, changes mainly in the first steps of vinification and continues during storage, the applied techniques (maceration, fermentation, clarification, aging, etc.) influencing significantly both the concentration and the composition of phenolics and, therefore, also the color intensity and hue of red wines. Extraction of the beneficial components from the grape skins can occur before fermentation, during it, or after it. Fermentation can be delayed, if the must

and the skins are kept cool enough, and maceration will occur in an aqueous medium. A key decision is when to separate wine and skins after fermentation has finished. After the wine has been separated from the skins, a pulp containing a mix of juice, skins and pips remains. This mixture is introduced in a press to squeeze out the remaining juice. The applied force and the type of the press determine the quality of the wine. This juice may be finished off separately from the rest of the wine or blended back into it. By pressing too hard, high concentrations of bitter compounds from the skins and seeds can be extracted, affecting the wine quality.

The phenolic composition of the wine also changes along the wine aging process, reflecting in the color and astringency degree of the final product. The relative anthocyanin content decreases upon aging although this chemical modification is associated with a very clear change in color, this characteristic being often used as a quality standard for aged wines. One of the main factors responsible for anthocyanin loss is the storage temperature [24].

The stability of the phenolics present in the wine is different from the one in situ (nonharvested grapes), several chemical changes, beginning in the grapes, and reaching completion only after the processing period. In general, the chemical composition of the final product is much more complex than the one of the raw material, due to the formation of a variety of new compounds.

Although procyanidins are related to grape composition, in wine, they were found to evolve during wine aging. In fact, they show a remarkable stability and resistance to several winemaking processes, such as sulfite bleaching. Oxygenated wines display characteristic color changes, along with a significant increase in the concentration of pyranoanthocyanins and related pigments [29]. In summary, one can say that a diversity of products can be obtained from condensation reactions between anthocyanins and tannins.

6. Conclusions

A main conclusion can be displayed: the resulted wine is a very complex medium influenced by winemaking techniques applied to different batches of grapes. The anthocyanin content of grapes at harvest is of vital importance in achieving quality wines. Tannins are the largest contributor, their content and composition in grapes probably determining the final wine quality. Presently, tannins are not routinely measured, the total phenolic content being determined and linked to the tannin content of grapes and wine, although it is not a specific assay. The wine phenolics can be used as a fingerprint for their differentiation, according to the geographical origin, vine variety and vintage. Phenolics compounds can be used as markers to discriminate the origin of wine (e.g., geographical, vine variety and vintage). For selection of winemaking practices aimed to improve the wine quality, the knowledge of intrinsic physicochemical characteristics of the grape cultivar employed in the vinification is required, but also the influence of different technological procedure to the final product, the wine. Since there is an increasing demand for producing and protecting high-quality wines, extensive studies on the factors influencing the chemical composition and biological effects of wine are still a necessity.

The use of advanced analytical techniques leads to the identification of some structures derived from tannins and anthocyanins in wine and determined how they are formed, the diversity of methods and experimental procedures reflecting the complexity of phenolics in grapes and wine. Improvement is still necessary, since each species is present in very small amounts and too many unidentified compounds still remain, especially with the polymeric fraction. Also, various extraction and analysis techniques have been developed as an alternative to conventional procedures, offering advantages with respect to analysis time, solvent consumption, extraction yields and reproducibility, each method providing specific advantages. Currently, the best approach to properly characterize the phenolic composition of the grapes and wines is using some assay combinations.

Author details

Violeta-Carolina Niculescu*, Nadia Paun and Roxana-Elena Ionete

*Address all correspondence to: violeta.niculescu@icsi.ro

National Research and Development Institute for Cryogenics and Isotopic Technologies—ICSI Ramnicu Valcea, Ramnicu Valcea, Romania

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