PHYTOCHEMISTRY AND HEALTH BENEFITS OF GRAPES AND WINES RELEVANT TO THE STATE OF TEXAS

A Dissertation

by

ARMANDO DEL FOLLO MARTINEZ

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2011

Major Subject: Food Science and Technology

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Approved by:

Chair of Committee,	Susanne Mertens-Talcott
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ABSTRACT

Phytochemistry and Health Benefits of Grapes and Wines

Relevant to the State of Texas. (August 2011)

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Chair of Advisory Committee: Dr. Susanne Mertens-Talcott

The overall objective of this work was to increase the knowledge regarding American hybrid grapes and wine-making techniques relevant to the State of Texas, specifically to investigate grape chemistry of hybrid grapes, to evaluate the effects of micro-oxygenation on wine chemistry, and to elucidate anti-cancer effects of wine compounds and extract in colon cancer cells *in vitro*. The methods used include HPLC-PDA-EIS-MS^n and molecular bioassays.

The American hybrid grapes, Black Spanish (*Vitis aestivalis hybrid)* and Blanc Du Bois (*Vitis aestivalis hybrid*), were compared to Cabernet Sauvignon and Merlot (*Vitis vinifera*) in their phytochemical composition. Total phenolics were similar in red grape varieties, but lower in white grapes. In Black Spanish grapes, anthocyanins and antioxidant capacity (ORAC) exhibited the highest values. Non-anthocyanin polyphenolics did not show qualitative differences in the four grape varieties. The presence of anthocyanins diglucosides was unique to Black Spanish grapes.

The second experiment involved application of micro-oxygenation with oak inner staves to evaluate the effect of this new vinification technology on the stability of anthocyanins. Overall, anthocyanins exhibited significant decreases over time in the following order: control, wine with oak pieces, oak barrel, and micro-oxygenation.

The anti-cancer effect of a combination of wine compounds, resveratrol/quercetin (RQ), and a polyphenolic extract from Black Spanish wine were investigated in colon cancer cells HT-29. RQ reduced the generation of reactive oxygen species (ROS), whereas the ORAC increased. RQ reduced cancer cell viability and proliferation, induced caspase-3-cleavage, and increased PARP-cleavage. Additionally, Sp1, Sp3, Sp4, and survivin were down-regulated at mRNA and protein levels. Furthermore, RQ decreased microRNA-27a (miR-27a) and induced ZBTB10, suggesting that RQ interactions with the miR-27a-ZBTB10-axis play a role in Sp down-regulation. Similar results were obtained for the wine extract.

This work will provide valuable information regarding grape varieties, potential health benefits of wine, and wine production techniques to the wine industry in Texas and beyond.

To my family

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

In recent years, the consumption of red wine has increased and now is accepted that moderate drinking, combined with a healthy diet, may have potential health benefits against chronic-degenerative pathologies. According to the American Cancer Society, after heart and coronary diseases, cancer is the second cause of death in the United States; and colorectal cancer for both, men and women, is the second most common form. The chemopreventive properties of red wine have largely been attributed to polyphenolic compounds present in grapes and wines; however, how polyphenolics exert their anti-cancer properties remains unclear, and new mechanisms are being proposed.

In order to improve the color, flavor, and reduce aging time during the winemaking process, new technologies have been developed. One of these technologies is micro-oxygenation, which consists of controlled addition of small amounts of oxygen into the wine. However, most of the available information on the usage and outcomes of this procedure comes from empirical results or winemakers' experience. Micro-oxygenation can be also used as a tool to accelerate aging with the addition of oak products, which is an attractive technology to reduce costs with the same final quality in wines.

This dissertation follows the style of *Journal of Agricultural and Food Chemistry*.

The development of the Texas wine industry differs from regions such as California, New York, Virginia, and Oregon; namely due to characteristic environmental conditions such as late freeze, hail, and plant diseases. Weather in the east and southeast regions of Texas does not allow the growth of high quality grapes for winemaking; therefore, there exists a need to import fruit from other regions such as the western part of the state of Texas. Furthermore, where wineries and tourism flourish, quality wine grapes often do not. In addition, due to the relatively large number of new wineries and winemakers, the skill and knowledge required to produce high quality wines is rapidly increasing.

The overall aim of this study is to investigate potential molecular mechanisms by which red wine and wine polyphenolics exert anti-cancer effects in colon cancer cells and determine the effect of micro-oxygenation on the chemical profile of Texas red wines. Ideally, the information obtained in this research will avail the Texas wine industry in promoting the health benefits of its wines, as well as provide technical knowledge relevant to the use of new technologies to achieve higher quality products and enhance efficiency and effectiveness in production.

CHAPTER II

LITERATURE REVIEW

WINE AND TEXAS WINE INDUSTRY

Grapes sold as fresh fruit or for processing are major sources of economic income in many countries. Currently, around 87% of harvested grapes are used in wine production, and the other 13% are sold as tables grapes or in grape-derived products (*1*). According to statistics from the World Food and Agriculture Organization wine production in 2009 was 2.71 x 10^7 tons (*2*).

Wine production and consumption has shown a steady increase worldwide over the last decade, and United States is no the exception. Moreover, while French wines are still popular options in the US, market share has moved to lower-priced wines from other regions such as Italy, Chile, Spain, Argentina, South Africa, and Australia (*3, 4*).

Currently, the United States ranks fourth among wine producing countries in terms of volume; it follows Italy, France and Spain (*5*). Within the US, the major wine producing states are California, Washington, Oregon, New York, Virginia, Pennsylvania, Ohio, and Texas (*6*).

The wine industry in Texas is one of the oldest in the US. Texas was the first state to establish vineyards in North America; as European settlers immigrated, they brought grapevine cuttings with them, providing the means for development of the wine industry in the 1800's (*7*).

In recent years, the Texas wine industry has experienced significant growth. In 2001, there were approximately 41 commercial wineries, in 2009, approximately 181, and, by 2010, they exceeded 200 (*8*). Even though over 80% of the wineries are small, producing less than 5,000 gallons per year (*6, 9*), in the last seven years, overall production has grown by more than 30% (*9*).

Although Texas is the fifth largest wine producer, the state has more land to offer growers for maintaining vineyards. The production of grapes has been inconsistent due to environmental conditions such as late freezes and hail. According to the Alcohol, Tobacco, Tax and Trade Bureau data, wine produced from local grapes totaled 462,739 gallons in 2008, and total wine production was estimated to be 2 million gallons (*6*). Preliminary data from 2010 shows that grape production in Texas was the highest ever.

In regions with warmer climates (southeast and central Texas) grapes are harvested in early summer, usually July or August, whereas regions with cooler climates (western areas) harvest during the months of September and October. This is a notable difference when compared with the other large grape producing states like California, Oregon, New York, where harvest generally occurs in September and October. In addition, due to specific growing conditions in certain parts of Texas, the wine industry uses European grape varieties; however, specifically in the east and southeast, these varieties are prone to disease pressure which has led to a need for hybrid grapevines which seem to be more disease resistant/tolerant (*4, 7, 9*).

NORTH AMERICAN GRAPES

The Vitaceae family, the *Vitis* genus, from the agronomic and economic point of view, is by far one of the most important (*10*). Currently, there are nearly 60 different inter-fertile species that exist in the Northern Hemisphere. *Vitis vinifera* is the most widely used grape in the wine industry (*10*).

Cultivation of grapes, historically, is linked to the consumption of wine. Selective cultivation and breeding increased the production of a bigger fruit size with higher sugar content (*10*). Vinifera grape varieties have thin skin, sweet flesh, and high sugar content, making them suitable for the production of high quality wines (*11*).

On the North American continent, there are several native varieties of grapes. The domestication of these has played an important role in the development of the eastern American wine industry. In fact, widespread wine production may not be possible in places where *Vitis vinifera* L. cannot grow due to environmental conditions such as severe weather or endemic diseases (*12*).

Vitis vinifera is indigenous to Europe and Asia, but was introduced in America by missionaries in colonial times (*11*). Since then, there has been extensive crossbreeding with local species. Grapes share a common ancestor, but followed different patterns of adaptation depending on species; this makes American grapes remarkably well adapted to different regions. Even though there are several indigenous American varieties, few are suitable to make wines. The fruit that they produce is often high in acid and low in sugar and contains flavors that may not be desirable. Also, the

wine from these grapes is unstable, thin, and not comparable to those made with *Vitis vinifera* (*11*).

Some North American grape varieties include *Vitis labrusca*, *Vitis rotundifolia*, *Vitis riparia*, and *Vitis aestivalis*, which have economic relevance for the Texas wine industry. Unfortunately, extensive scientific information is not available for most American grape varieties, since the majority is not suitable for making good wines.

Vitis labrusca

This grape is the most representative of the North American grapes and has large berries that come in white, red or black. It is the only variety that exhibits this range of colors. The best representative of this grape variety is the Concord grape that is widely used by the food manufacturing industry for juices and jellies (*11, 13*). This grape is characterized by a unique "foxy" aroma that is caused by the volatile compound methyl anthranilate (*11, 14*). This grape is not widely used in wine production.

Vitis rotundifolia

V. rotundifolia is the predominant grape variety grown in the southern United States with excellent potential for commercial expansion and value-added development (*15, 16*). This variety has thick, tough skin, round fruit, and sweet flavor. It grows on riverbanks, in swamps, and in bottomlands. As with most of the American grape varieties, it is necessary to add sugar to this grape for production of good wine (*11*). Muscadine grapes are the only grapes of this variety that contain ellagic acid and anthocyanins 3,5-diglucosides. These compounds make juices or wines unstable under storage conditions *(16). V. rotundifolia* is not widely used in wine production.

Vitis riparia

This grape variety is better known as riverbank grape. The principal characteristics are small size and a harsh taste. It is the most widely distributed of all American grapes. In addition, due to its characteristics, it is able to grow under a great variety of weather conditions. Recently, it has been used as a basis for hybridizing wine grapes for the cold climates in the United States (*11*).

Vitis aestivalis

Although this variety has excellent features for optimal vinification, it is not the most widely used wine grape. Unlike other grape varieties, such as *V. rotundifolia* and *V. labrusca,* it has an adequate sugar content. The most representative cultivars are Norton and Cynthiana, which are grown mainly in Missouri and Arkansas (*11, 17*). Even though these cultivars are considered as two separate ones, it is thought that they stem from the same vine, and have acquired two names throughout the course of propagation and distribution (*17*). In recent years, the Norton grape has increased in popularity in the Midwestern and Southern states, due to its adaptability and resistance to fungal diseases (*18*).

In the state of Texas the white species cultivated include Blanc Du Bois, Chardonnay, Chenin Blanc, Sauvignon Blanc, Muscat Blanc, Pinot Blanc, and Riesling. Whereas, the red varieties include Cabernet Sauvignon, Merlot, Black Spanish, Norton, Sangiovese, Syrah, and Cabernet Franc (*7*).

WINE CHEMISTRY

Wine Polyphenolics

Polyphenolics play an important role in wine chemistry and are responsible for the differences among wines. Polyphenolic compounds can affect the taste, mouth-feel, color, and aroma of wines (*19*).

Chemically, phenols are cyclic benzene derivates possessing one or more hydroxyl groups directly associated with the ring structure. Based on their carbon skeleton, polyphenolics are classified in non-flavonoid and flavonoid compounds (*19-21*). Polyphenolics present in wine stem from various sources including grapes, vine stems, products of yeast metabolism, and extractions from wood cooperage (*20, 22*). In general, grapes contain non-flavonoid compounds mainly in the pulp, while flavonoid compounds principally are located in skins, seeds, and stems (*19*).

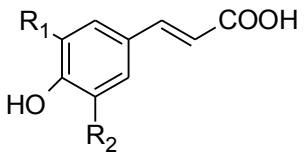
Non-Flavonoid Phenolic Compounds

The main non-flavonoid compounds present in grapes and wines are phenolic acids in the form of hydroxycinnamic acids (i.e. *p*-coumaric, caffeic, ferulic, sinapic, caftaric, etc.), hydroxybenzoic acids (i.e. vanillic, gallic, protocatechuic, syringic, etc.), and other phenolic derivatives such as stilbenes (resveratrol, piceid, piceatannol, etc.) (*19, 23*).

Hydroxycinnamic Acids

Compared to hydroxybenzoic derivatives, hydroxycinnamate derivatives are more abundant and variable in composition. Hydroxycinnamic acids can be found esterified to sugars, alcohols, or organic acids such as tartaric acid (*20, 22*), and are

located in the vacuoles of grape skin and pulp (*19*). The main hydroxycinnamic acids present in wines (Figure 1) are mainly derived from caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid (*23*). Among the hydroxycinnamic acids caftaric acid is the most predominant with up to 50% of the total hydroxycinnamic acid content in grapes and wines (*23*).

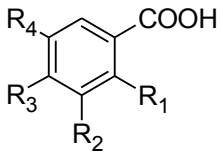


Hydroxycinnamic acids	R_1	R_2
Caffeic	OH	H
Ferulic	OCH_3	H
Sinapic	OCH_3	OCH_3
p-coumaric	H	H

Figure 1. Grape-derived hydroxycinnamic acids (*19*).

Hydroxybenzoic Acids

Of hydroxybenzoic acids (Figure 2), gallic acid is one of the most prevalent in wines. Gallic acid also occurs as hydrolysis-product with hydrolysable and condensed tannins (i.e. the gallic acid esters of flavan-3-ols) (*23*). It is found in the solid part of the grape, either in the free form or in the form of flavanol ester such as epicatechin-3-*O*-gallate (*19*).



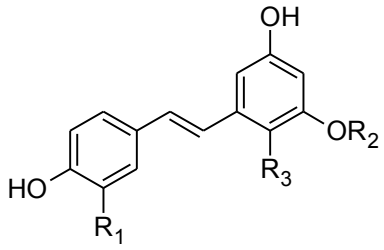
Hydroxybenzoic acids	R_1	R_2	R_3	R_4
p-Hydroxybenzoic	H	H	OH	H
Vanillic	H	OCH_3	OH	H
Gallic	H	OH	OH	OH
Syringic	H	OCH_3	OH	OCH_3

Figure 2. Grape-derived hydroxybenzoic acids (*19*).

Concentrations of cinnamic and benzoic acids vary significantly across wines, in white wines, concentrations are between 10 to 20 mg/L, whereas in red wines, these range from 100 - 200 mg/L (*22*). Furthermore, when comparing both hydroxycinnamic and hydroxybenzoic acids, the overall concentrations of the latter are relatively low in wines (*23*).

Stilbenes

Stilbenes, a non-flavonoid family of more complex polyphenolics, are also present in grapes and wines. This group is characterized by two benzene rings bonded by an ethane chain as shown in Figure 3 (*22*). Stilbenes are phytoalexins synthesized in leaves, skins and roots as a defense mechanism against fungal infections or abiotic stressors like UV radiation (*19, 22, 23*). The content of stilbenes in wines varies considerably and depends on several factors such as grape variety, environmental conditions, microbial diseases, and enological methods (*23, 24*).



Stilbenes	R_1	R_2	R_3
Trans-resveratrol	H	H	H
Trans-reveratrol-3-*O*-glucoside (Piceid)	H	OCH_3	OH
Trans-reveratrol-2-C-glucoside	H	OH	OH
Trans-astringin	H	OCH_3	OH

Figure 3. Stilbenes and stilbene derivatives (*19*).

Resveratrol is the stilbene most studied, and is mainly extracted during maceration and fermentation of wines with concentrations ranging from 0.2 to 13 mg/L for red wines and 0.1 to 0.8 mg/L in white wines (*22, 23*). In nature, resveratrol exists in two isomeric forms (*cis*- and *trans*-) in the free as well as in ß-glucoconjugated forms (i.e. 3-*O*-ß-D-glucosides) called piceids (*23*).

Flavonoid Compounds

Flavonoids are important secondary metabolites that are responsible for normal growth, development, and defense mechanisms against infections and stress in plants (*20*). These compounds are characterized by two phenol groups joined by an oxygen-containing ring structure (*20, 25*).

Flavonoids are synthesized in the endoplasmic reticulum before being stored in the vacuole (*20*). In plants, flavonoids do not impart significant color, although in some

cases, some of them possess yellow shades that contribute to the color of flowers, leaves and fruits (*20, 26*). To date, more than 400 flavonoids have been identified in plants; consequently, they have been subdivided into different categories based on the number and location of the hydroxyl and methoxyl groups located in the B ring. These categories include flavones, isoflavones, flavonols, flavanones, flavones, tannins, flavan-3-ols, and anthocyanins (*20, 25*).

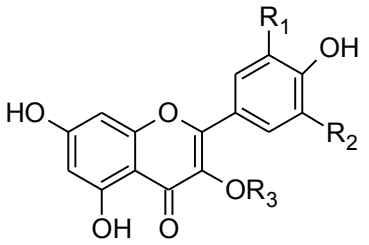
In grapes and wines, flavonoids make up a significant portion of the phenolic content, where flavonols, flavan-3-ols and anthocyanins are the most predominant; some flavanonols and flavones are also present in smaller amounts (*19, 27*).

Flavonols

Flavonols, members of the flavonoid family, range from white to yellow in color (*19, 28*), and are relatively abundant in beverages, grapes, and some others fruits and vegetables (*28, 29*). Flavonoids are found as glycosides with glucose, although some other moieties may be found such as rhamnose, galactose, and xylose (*22, 25*). In wines, flavonols maybe present as aglycon, due to fermentation and aging (*19, 22*). The most representative flavonols present in wines are quercetin, myricetin and kaempferol, either as glycosides or aglycon as shown in Figure 4 (*19, 25*). In *Vitis vinifera*, flavanols exist in the 3-*O*-gycoside forms of quercetin, myricetin, isorhamnetin, and kaempferol (*19*).

The concentration of flavonols in wine depends on the type of wine as well as the winemaking practices; thus the pre- and post-fermentation treatments, as well as the procedures enhancing cell wall disruption are important in controlling flavonol concentrations (*23*). In red wines, flavonol concentrations are within the 1000 mg/L

range, whereas in white wines values are between 0.5 to 3 mg/L (*22, 28*).



Flavonols	R_1	R_2	R_3
Quercetin	OH	H	H
Kaempferol	H	H	H
Myricetin	OH	OH	H
Quercetin-3-*O*-glucoside	OH	H	gluc
Kaempferol-3-*O*-glucoside	H	H	gluc
Myricetin-3-*O*-glucoside	OH	OH	gluc

Figure 4. Flavonols present in wine (*19*).

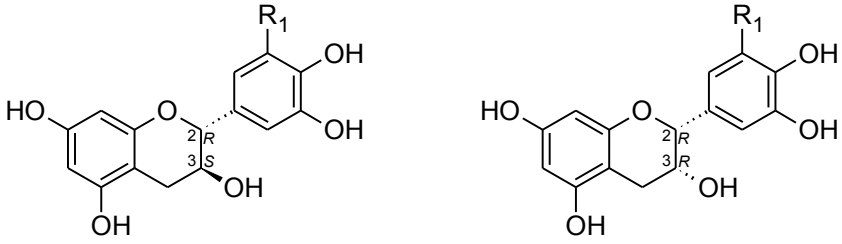
Flavan-3-ols, Condensed and Hydrolyzable Tannins

Flavan-3-ols or flavanols are responsible in part for the astringency and bitterness in wines (*27*), they can be present in monomeric, oligomeric, and polymeric forms (*23, 25*). The major flavan-3-ol monomers present in *Vitis vinifera* grapes include (+)-catechin, (-)-epicatechin, (+)-gallocatechin, and (-)-epigallocatechin as shown in Figure 5 (*19, 25*). In red wines, catechins may be present in concentrations up to 300 mg/L (*25*).

(+)-Catechins and (-)-epicatechins are orthohydroxylated in C-3' and C-4' position of the B ring, while (+)-gallocatechin and (-)-epigallocatechin posses a third hydroxyl group in the C-5' position. In addition, in the dihydroxylated form, (-)-epigallocatechin can be esterified with gallic acid at the C-3 position resulting in (-)-

epigallocatechin-3-*O*-gallate (*19*). The trihydroxylated forms (+)-gallocatechin and (-)-epigallocatechin, and (-)-epigallocatechin-3-*O*-gallate have been found in free form in grapes and wines, but are absent in grape seeds (*19, 30-32*).

The presence of (+)-catechin, (-)-epicatechin, and (-)-epigallocatechin-3-*O*-gallate has been reported in both grape skins and seeds, whereas only (+)-catechin and (-)-epicatechin have been found to be present in stems (*33, 34*). Thus, it is thought that winemaking practices influence the extraction and content of flavanol monomers in wines (*27*).



Flavan-3-ols	R_1	C-2	C-3
(+)-Catechin	H	*R*	*S*
(+)-Gallocatechin	OH	*R*	*S*
(-)-Epicatechin	H	*R*	*R*
(-)-Epigallocatechin	OH	*R*	*R*

Figure 5. Catechins and epicatechins (*19*).

Proanthocyanidins or condensed tannins are flavanol (catechins) oligomers and polymers structures. The name “tannin” comes from the capacity to interact or react with proteins (*22, 23, 25*) giving an astringent sensation in the mouth. Since they are

polymers of flavan-3-ol subunits, a wide variety of molecular weights and configurations are possible (*23, 27*).

Proanthocyanidins have the characteristic to release anthocyanins under heated acidic conditions as a result of the interflavanic bond cleavage (*22, 23*). In *Vitis vinifera*, two proanthocyanidins have been identified: prodelphinidins (composed of (+)-gallocatechin and (-)-epigallocatechin) and procyanidins (composed of (+)-catechin and (-)-epicatechin) (*19*). The distribution of proanthocyanindins in grapes varies. In seeds, only procyanidins are found, whereas, in skins, a mixture of both prodelphinidins and procyanidins may be present (*19, 23*).

In condensed tannins, the chain length plays an important role; the oligomers, which are proanthocyanidins between 2 and 5 units, are the largest structures that have been individually identified (*22, 23*). Individual proanthocyanidin polymers, which are molecules larger than 5 units, have not been isolated due to the large number of isomers (*23*).

Condensed tannins are flavanol subunits usually linked by carbon-carbon bonds in the C_4–C_6 or C_4–C_8 positions (B-type proanthocyanidins). When an additional ether bond is linked between the positions C_2-C_5 or C_2-C_7 positions, A-type proanthocyanindins are formed (*35, 36*). In grapes and wines dimmers, trimers, and tretramers of B-type proanthocyanidins of (+)-catechin, (-)-epicatechin, and (-)-epicatechin-3-*O*-gallate have been found as shown in Figure 6 (*19*).

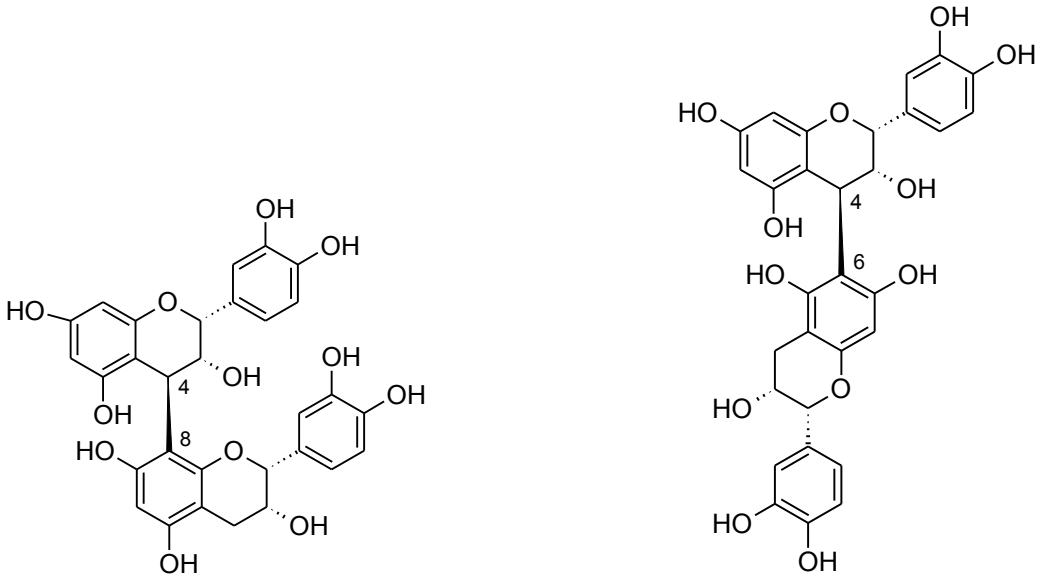


Figure 6. Structure of simple dimeric proanthocyanidins in condensed tannins.

Hydrolyzable tannins are not naturally present in grapes, but they are found in wines. The presence in wine is due to the aging process when wines are stored in oak barrels (*22, 23*). In the wine industry, these tannins are the only ones legally authorized as wine additives (*22*). Hydrolyzable tannins include gallotannins and ellagitannins (Figure 7) which are able to undergo hydrolytic cleavage of the sugar moiety (most common sugar) and gallic or ellagic acid after acid hydrolysis (*22, 37*). The presence of ellagic acid in *Vitis vinifera* wine may be due to ellagitannin cleavage from oak barrels or from the addition of tannins during the winemaking process (*22*).

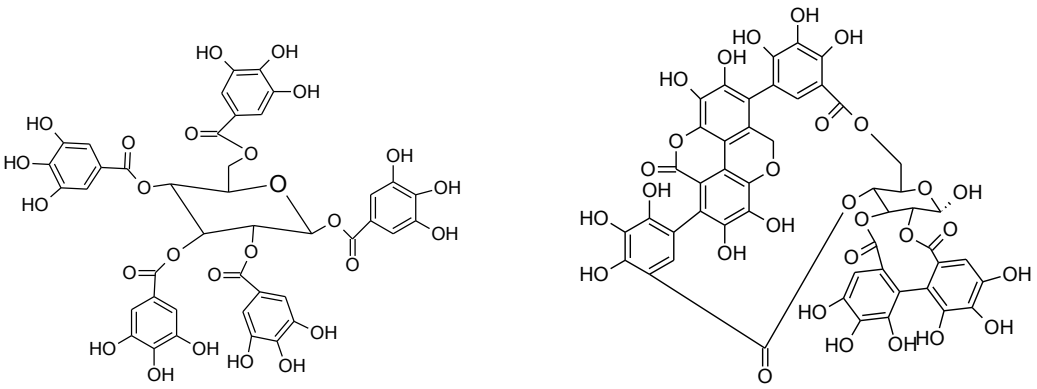


Figure 7. Gallotannin and ellagitannin (*37*).

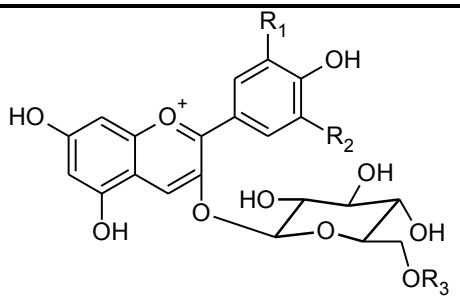
Anthocyanins

Anthocyanins are one of the most important plant pigments. They belong to the flavonoid group and are mainly located in the cell vacuole of fruits and flowers (*38*). In grapes, anthocyanins are located in the grape skins, with the exception of some varieties that also contain anthocyanins in the pulp (i.e. 'teinturier' grape varieties) (*22, 23*). Anthocyanidins are unconjugated and are composed of an aromatic ring, A, bonded to a heterocyclic ring, C, that contains oxygen (flavylium cation), which is also bonded by a carbon-carbon bond to a third aromatic ring, B (*39*).

Currently, there are more than 23 naturally occurring anthocyanidins identified, along with more than 600 anthocyanins (*23, 39, 40*). However, only six of them (Figure 8) are commonly found in higher plants: delphinidin, cyanidin, pelargonidin, petunidin, peonidin, and malvidin. They differ from each other by the number and position of hydroxyl and methoxyl groups located in the B-ring of the molecule (*39, 41*).

In grapes five anthocyanidins have been identified: delphinidin, cyanidin,

petunidin, peonidin, and malvidin. These aglycones exist in different glycosidic forms such as 3-monoglucosides, 3,5-diglucosides, and acylated forms (*42, 43*). In acylated anthocyanins, *p*-coumaric, caffeic, and acetic acids are esterified with the hydroxyl group in the six position of a glucose molecule (*22, 42, 43*).



Name	R_1	R_2
Delphinidin	OH	OH
Cyanidin	OH	H
Petunidin	OCH_3	OH
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH_3
R_3		
p-coumaroyl	acetyl	caffeoyl

Figure 8. Anthocyanins occurring in red wines (*19*).

Although some anthocyanins may be present in grapes and wines, the concentration of each depends on the climatic conditions, region of production, cultivar, maturity, and processing conditions, among other factors (*20, 23*). From the five anthocyanidins present, malvidin is by far the most abundant in red grape varieties; it represents between 50 to 90% in some grape varieties. Acylated anthocyanins are mostly

influenced by grape variety and may be absent from some varieties such as Pinot noir (*22, 23*).

Volatile Compounds

Wine is one of the most aroma-complex alcoholic beverages. Volatile compounds in wine originate from grapes, fermentation, and oak used in the winemaking process. To date, there have been more that 800 aroma compounds identified in wines displaying a wide range of polarity, solubility, and volatility (*44*).

Wine volatiles include a variety of compounds such as alcohols, phenol-derived compounds, terpenes, esters, organic acids, aldehydes, and others, with each one imparting specific characteristic aroma to the wine. Volatiles may contribute to pleasant aroma notes or unpleasant ones, which are considered flaws.

Organic Acids

Organic acids may come from grapes or may be produced during wine fermentation; the type of yeast used during the fermentation process will impart a characteristic acid profile. The major organic acids present in wines such as malic, succinic, lactic, and tartaric are non-volatile and do not impart significant aroma to the final product, but help to define its structure and balance (*23, 26*). Acids such as acetic, propionic, butyric, and formic have characteristic aromas and their presence in wines are associated with off-odors (*20, 26*). In some cases, acetic acid in concentration below its threshold levels (0.4 to 1.1 g/L) may impart complexity to wines (*23, 26*).

Alcohols

Long chain alcohols or fusel alcohols, which have more than two carbon atoms, are the major group of volatile compounds produced by yeast during alcoholic fermentation, and are responsible for some characteristic wine aromas (*20, 23*). Examples of these long chain alcohols include 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol. They tend to have fusel odors, whereas hexanols possess an herbaceous scent (*20, 23, 26*).

Winery practices that may increase production of fusel alcohols include selection of yeast, oxygen exposure, and high temperature used during fermentation. Addition of sulfites, clarification of must before fermentation, and other practices may reduce their production (*20, 23*). Generally, a concentration of these alcohols below 300 mg/L contributes desirable complexity to wine, but higher concentrations may be perceived as faults in the wine depending on the type and style of wine (*23, 26*).

Phenolics

Wine aromas may also come from phenol-derived compounds; most of these are able to withstand yeast fermentation. Phenol-derived compounds such as acetovanillone may impart a vanilla-like odor; some others like methylanthranilate are an important aroma component in some grape varieties for instance, *Vitis labrusca,* giving it the "foxy" aroma. This may also be present in some other grape varieties in much lower concentrations (*20, 45*). Perhaps the most characteristic phenol-derived aroma is 2-phenylethanol, which imparts a rose-like fragrance especially in *Vitis rotundifolia* (*20, 23, 26*).

Phenolic compounds seem to be an important source of aroma in the form of hydroxycinnamic acid esters, which can be transformed during fermentation. They come from oak cooperage or are produced by microorganisms (*20, 26*). Derivatives of hydroxycinnamic esters such as vinylphenols (4-vinylguaiacol and 4-vinylphenol) and ethylphenols (4-ethylphenol and 4-ethylguaiacol) can donate spicy, pharmaceutical, and clove-like odors and smoky, phenolic, and animal stable-like notes, respectively. Off-odors are frequently detected when ethylphenol contents exceed 400 μg/L or 725 μg/L for vinylphenols (*20, 26*). Red wines have been shown to have a greater proportion and higher concentration of ethyl- to vinylphenols when compared to white wines (*20*).

A considerable proportion of these phenol-derived volatiles come from oak cooperage during the wood seasoning and toasting from the breakdown of lignins, which are the source of several volatile phenolic acids and aldehydes such as benzaldehyde (almond-like odor), syringaldehyde and vanillin (vanilla-like scent), furfural and furfural-derivatives (*26*).

Nitrogen Compounds

The most representative of this group are the pyrazines, which are nitrogen-containing cyclic compounds. Among these, methoxypyrazines (3-isobutyl-2-methoxypyrazine, 3-*sec*-butyl-2-methoxypyrazine, 3-isopropyl-2-methoxypyrazine, and 3-ethyl-2-methoxypyrazine) represent an important group in wines. They are associated with vegetative, herbaceous, and green pepper aromas in some grape varieties especially in Sauvignon Blanc and Cabernet Sauvignon (*23, 26, 46*).

These compounds located in the grape skins are water soluble, and, therefore, are

rapidly extracted into the must (*23*). Other sources of pyrazines include stems and leaves from grapevines, which are incorporated into the vinification process due to an incomplete destemming, especially when harvesting is mechanically performed (*47, 48*).

The concentrations in grapes may be influence by variety, season, climate, solar exposure, and fruit maturity (*46*). Moreover, pyrazines can be derived from insects, including *Harmoniaaxyridis*, better known as Multicoloured Asian Lady Beetles, which are inadvertently incorporated in with the grapes during the harvest and later into the must (*49, 50*).

In general methoxypyrazines have low sensory thresholds, in the range of parts per billion (ng/L) and parts per trillion (pg/L) depending on the wine type and style (*49*). At concentrations of about 8 to 20 ng/L, some methoxypyrazines such as methoxybutylpyrazine may impart a desirable aroma, although above these values they give a strong herbaceous aroma (*26*).

Lactones

Lactones are cyclic esters formed by internal esterification between carboxyl and hydroxyl groups. Even though some lactones may come directly from grapes such as 2-vinyl-2-methyltetrahydrofuran-5-one (*26, 51*), the majority come from yeast fermentation and aging in oak barrels. The most common are the ones extracted from oak which have a coconut and oak-like scent (*26*). Oak lactones, which are often referred to as whisky lactones (*cis* and *trans* isomers of β-methyl-γ-octalactone) (*23, 26*), are the main volatile components present in the extractable fraction of oak wood (*23*). Furthermore, these lactones have been identified, not only in wines, but also in brandies

and whiskies (*52*) which are aged in oak barrels as well. Previous studies have determined concentrations for these two isomers in the rage of parts per billion depending on the time of contact, oak type, and shape of wood (*53*).

Ketones

Ketones represent a small amount of the spectrum of volatile compounds in wine. The few present are the ones that survive fermentation. Ketones like β-damascenone and the isomers α-ionone and β-ionone, are known to have a rose-like aroma (*20, 26*). This may be associated with some grape varieties such as Riesling and Chardonnay (*20*).

Several ketones are produced during wine fermentation, many of them without having any organoleptic effect in wines. One of the most important is diacetyl due to its adverse sensory characteristics when concentrations are above the threshold limit. At high concentrations, it is considered a flaw imparting a buttery and lactic off-odor, associated with bacterial spoilage and yeast that is common when fermentation occurs at high temperatures. At low concentrations, it gives a nutty, buttery and/or toasty flavor (*20, 26*).

Aldehydes

Grapes produce small amounts of aldehydes that may impart aromas in wine as a result of their reduction to alcohol during yeast fermentation. Those able to withstand fermentation typically are C_6 aldehydes such as hexanals and hexenals, which impart green, grassy aromas to wines mainly attributed to use of unripe grapes, but also associated with certain grape varieties (*20*). However, most of the aldehydes found in wines are produced during fermentation, winemaking practices, and oak cooperage (*20*).

Acetaldehyde, the most common aldehyde, represents nearly 90% of the total content in wines; small amounts impart complexity, but above threshold levels, it results in an off-odor (*20, 26*). Acetaldehyde is the one of the first metabolic by-products of fermentation, but is also produced by the oxidation of ethanol and the by autooxidation of *o*-diphenols (*26*). Since acetaldehyde plays an important role in several chemical reactions such as polymerization with anthocyanins and with sulfur dioxide reactions, the final concentration in wine is generally below its threshold detection limit (*26*).

There are some other phenol-derived aldehydes, like cinnamaldehyde and vanillin that are extracted from oak wood during aging (*20, 26*), which are products of lignin degradation during the oak wood toasting process.

Esters

In young red wines and white wines, esters derived from fermentation are responsible for the characteristic fruity aroma. Although at wine pH many esters hydrolyze during aging, some may still be present in aged wine above the threshold detection limit, suggesting its participation in wine aroma (*23*). Currently, there are more than 160 esters that have been identified in wines, but the two main groups include acetate esters (ethyl acetate, isobutyl acetate, amyl and isoamyl acetate, 2-phenylethyl acetate, etc.) and ethyl fatty acid esters (ethyl C_3-ethyl C_{12}) (*23, 26*). The most significant esters found in wine are formed between ethanol and short chain fatty acids, acetic acid and various short chain alcohols, and nonvolatile acids and ethanol (*26*).

Ethyl acetate is the most significant among this group. At low concentrations, (< 50 mg/L) it may add complexity to wines. Higher concentrations (> 150 mg/L) generate

an acetone-like odor, which is considered undesirable (*26*). In addition, some particular esters give the characteristic aroma to certain grapes or wines like methyl anthranilate, which is a phenolic ester present in *Vitis labrusca* varieties (*26*).

The low molecular weight esters formed from higher alcohols (isoamyl and isobutyl) are considered to have fruit-like aromas. However, as the length of the hydrocarbon chain increases, the odor shifts from fruity to soap-like, and finally to lard-like with C_{16} and C_{18} fatty acids (*26*).

NEW TECHNOLOGIES FOR WINE PRODUCTION

Wine technology and production techniques have changed in the last century, and in the last three decades there has been a great interest in developing new technologies and equipment to improve quality, increase production, and reduce operation costs.

Wine, as a product, needs to meet consumer preferences and those may change throughout time, and new technologies play an important role in satisfying those preferences. Among these new technologies, micro-oxygenation and accelerated aging are of great interest in the wine industry due to the high impact that may have on the final product.

The use of micro-oxygenation and acceleration of aging, alone or in combination, have shown to be a feasible option for wineries to modify the chemical profile and sensory characteristics of wines in a short period of time and even improve the quality of wines that otherwise would not reach the market (*54-58*). In the following sections, these

two technologies, micro-oxygenation and artificial aging, will be discussed in further detail.

Micro-oxygenation

During the winemaking process, oxygen is required as it plays an important role in wine maturation. At early stages, oxygen aids in fermentation, and later helps to stabilize color and develop aromas during aging. Despite these beneficial effects of oxygen, an excess may lead to wine oxidation which has negative effects (*59*) including phenol oxidation, higher astringency, and adverse microbial activity (*60*).

The micro-oxygenation technology and research started in the mid-1980's and was commercially available in the mid-1990's (*61*). To date, European countries such as France and Italy are leaders in the use of micro-oxygenation, followed by South Africa, Chile, USA, and Australia (*58*). This new technology is based on the dosage of very small amounts of oxygen to the wine which attempts to mimic the oxygen transport that takes place in oak barrels (*56, 58, 60*).

Micro-oxygenation can be applied at any stage of the winemaking process, but research has shown that the best results are obtained after alcoholic fermentation and before malolactic fermentation (*57, 60*) to enhance color stability, as well as reduce the herbaceous aromas and improve wine structure (*58, 60*).

The most significant reason for considering micro-oxygenation use in the winemaking process is the opportunity to avoid barrel aging, which involves high costs due to intensive labor operations, long periods of time due to slow production, and extensive cellar space that few wineries can afford (*58*).

When considering the application of this process, it is important to understand the reactions that take place during the incorporation of oxygen into the wine throughout winemaking. These are not limited to micro-oxygenation treatments. Oxygen has an impact on the phenolic composition of wines, which establishes an indirect influence on astringency and color stability (*60*). The reactions in which oxygen can participate include oxidation, condensation, and polymerization of different compounds. As a result, new compounds and pigments are formed giving the wine its characteristic structure and color stability (*60, 62*).

Oxygen may also participate in the oxidation of ethanol to acetaldehyde, which can react with flavonols to produce very reactive carbocation species that react with other flavonols or anthocyanins to produce new pigments and polymeric compounds via the formation of ethyl-bridged adducts (*60, 63, 64*). Furthermore, researches have demonstrated the formation of these polymeric compounds and more stable anthocyanin pigments in wine model solutions and in wines after micro-oxygenation treatments (*57, 62, 64-68*).

Accelerated Aging

Traditionally, after alcoholic and malolactic fermentation, wines are placed in wooden barrels to mature. This process, commonly known as aging, gives the wine its organoleptic structure and color characteristics. These vary depending on several factors such as geographical origin, oak species, degree of toasting, and aging time (*53, 69*). During the aging process, wine polyphenolics react with oxygen and compounds extracted from oak wood such as tannins, lactones, vanillin derivates, and phenolic

acids. These increase wine complexity and improve color stability (*53, 69*). Studies on the chemical compounds that come from oak that contribute to the organoleptic characteristics in wine, have identified furfural, whisky lactones, guaiacol, eugenol, vanillin and syringaldehyde, among others which are produced during the toasting process (*55*).

Aging in an oak barrel is a common practice in wineries, however, it requires long periods of time, is labor intensive, demands a considerable amount of space, and results in high costs. These may be limiting factors for some small to mid-size wineries (*58*). Hence, the necessity to find new technologies to accelerate this process and make them affordable for even small wineries has been presented.

In recent years, new techniques have been introduced in winemaking to accelerate the aging process to mimic the desirable characteristics in wines. The use of oak chips, inner staves, oak powder, oak beans, and even oak extracts are the most common in the market (*53, 55, 58*). While these practices have been used in countries such as Chile, Australia, and South Africa for several years, it has been recently approved and legislated by the European Community (*58*). Furthermore, the characteristics of the final product will depend on the chip size, oak origin, degree of toasting, time of application, and doses (*53*). The aging time is considerably reduced since the oak is being put into the wine where the whole surface is available to interact with the wine, as compared to the traditional oak barrel where the contact surface is no more than 40% (*55*).

Techniques for accelerating aging may be use in combination with micro-oxygenation treatments to mimic the oxidation process that naturally occurs in wood barrels, a process called industrial aging (*58, 70*). Several studies address the effects of the addition of oak along or in combination with micro-oxygenation on the chemical composition, sensory properties, and volatile profile of wines when compared to traditional aging barrels (*53, 55, 56, 58, 69*).

HEALTH BENEFITS OF WINE AGAINST CHRONO-DEGENERATIVE DISEASES

The consumption of red wine in European countries such as France has been discussed frequently in the news. The so-called "French Paradox" was described in a study that compared dietary intake and disease incidence in several countries including the United States. The study showed that consumption of red wine might provide protection in the French population. Despite the high consumption of saturated fat in the French diet, the incidence of cardiovascular-related deaths when compared to other countries in the study was lower (*71-73*).

The health benefits of red wine later on were attributed to the same polyphenolic compounds present in fruits and vegetables (*71*). Red wine is an excellent source of polyphenolics, which mainly come from the grape skin (*74*) and have demonstrated protective effects against cardiovascular disease, inflammation, and cancer (*72, 74-77*). How these polyphenolic compounds act against chronic-degenerative diseases is still debated, but several mechanisms have been proposed including anti-oxidant and

scavenger properties, quench reactive oxygen species, metal chelators, induction of apoptosis, modulators of cell signaling pathways, and anti-inflammatory properties (*72, 75, 76, 78, 79*). These compounds are thought to exert these properties in such complex ways that even more than one mechanism may be acting at the same time.

Inflammation is an immune response to cellular or tissue insult or infection by pathogens initiated by cytokines and interleukins (*80, 81*). Acute inflammation may be considered as a protective attempt by the organism to stop the injurious stimuli, as well as initiate the healing process, thus it is considered beneficial to the organism. Chronic inflammation has been demonstrated to be responsible for the triggering of chronic-degenerative diseases such as arteriosclerosis, platelet aggregation, obesity, diabetes, and cancer (*82, 83*).

The inflammation response triggers the activation of mitogen-activated protein kinases; as a result, transcription factors such as NF-kB are activated (*80*); these transcription factors trigger the expression of pro-inflammatory genes (cytokines, cycloxygenase-2, chemokines, etc.), which amplify the inflammatory response (*80, 82*).

Recent studies have demonstrated that dietary polyphenolics, as well as red wine polyphenolics, at least in part, reduce the inflammatory response and confer cardiovascular protection in *in vitro* and *in vivo* models (*78, 79, 84*). Walter and others demonstrated *in vivo* that red wine polyphenolics were able to reduce the effects of angiotensin II and the effectors VGEF and matrix metalloproteinases (*79*). Moreover, a study *in vivo* with anthocyanins from red wine, showed a decrease in MCP-1 levels in plasma, as well as an increase in antioxidant capacity (*84*). In concordance with previous

studies, Lelfert and others demonstrated that grape seed and red wine extracts were effective in reducing the proinflammatory 5-LOX, as well as cholesterol uptake *in vitro* (*78*).

It is recognized that chronic inflammation, as well as an imbalance between anti-inflammatory and proinflammatory mediators, in some cases are responsible for the initiation of signal cascades that are able to promote a carcinogenesis process (*80, 83, 85*). Furthermore, malignant cells are able to produce different types of cell signals (cytokines) and growth factors, which facilitate cell proliferation and survival (*83*). Recent studies have demonstrated the potential link between chronic inflammation and cancer (*78, 86, 87*).

Research has also shown the protective effects of wine polyphenolics and dietary sources of polyphenolics against several types of cancer cell lines, as well as in animal models (*76, 88-97*). In general, results have demonstrated that polyphenolics are effective to reduce cell proliferation, induce apoptosis, modulate cell signals through different pathways, and arrest cells in specific stages of the cell cycle.

MOLECULAR ASPECTS OF CANCER

Cancer is recognized as a genetic disease characterized by abnormal cell growth and development of normal cells beyond their natural boundaries (*98*). Despite the efforts to control and limit the incidence and mortality rate, cancer is still one of the leading causes of death among men and women (*75, 98, 99*). According to the American Cancer Society, cancer is the second leading cause of death after heart diseases in the

United States. Furthermore, a report from the World Cancer Research Fund, showed that between 30-40% of cancers can be avoided just by changing life style habits such as increasing physical activity, consuming an appropriate diet, and maintaining a healthy weight (*85*).

Carcinogenesis is a multi-step process in which molecular and cellular alterations occur. The current accepted process of carcinogenesis consists of three stages: initiation, promotion, and progression (Figure 9).

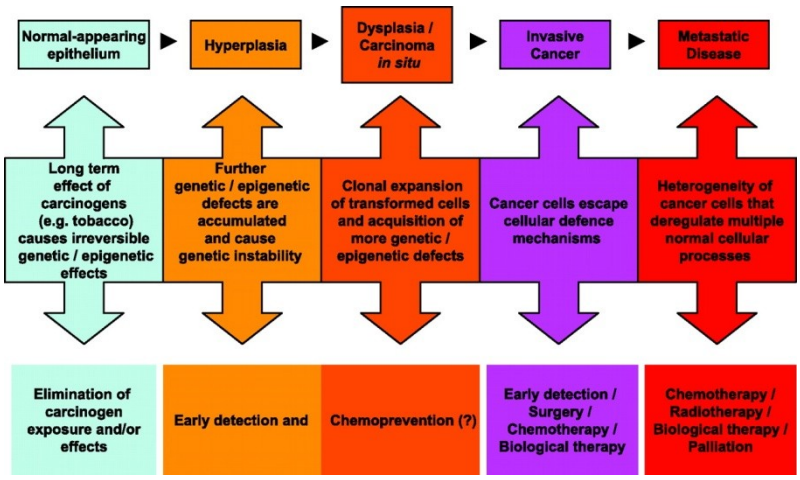


Figure 9. General carcinogenesis model (*100*).

Colon Cancer

Colon cancer is the second most common type of cancer in men and women in the United States (*101, 102*) and the third most common type worldwide (*103*). Despite an increase incidence from 2004 to 2007 by 6%, the mortality rate decreased by 5% during the same period of time (*102*). According to the National Cancer Institute, in

2009 there were an estimated 106,100 incidents of colon cancer, 40,870 incidents of rectal cancer, and 49,920 deaths from colon and rectal cancers combined.

Dietary Risk Factors

Epidemiological and data from migrant studies have shown that when low-risk populations such as Japanese move to high-risk areas like the USA, the incidence of developing colorectal cancer increases within the first generation of migrants, and the second generation has an even higher risk than the white population in the USA (*104*). Thus, there is evidence that diet is an important exogenous factor in colorectal cancer.

Colorectal cancer may be influenced by nutritional risk factors such as diets high in saturated fat, high in red meat, high in processed meats, high in caloric intake, low in fiber, and low in fruits and vegetables, as well as consumption of alcoholic beverages which provide more than 30 g per day of ethanol (*86, 88, 103, 105, 106*). Furthermore, physical activity has shown greater protective effects against colon cancer than for rectum cancer (*103*).

Research has shown that polyphenolics from dietary sources have chemopreventive and chemotherapeutic properties including activity as antioxidants, free radical scavenging, inducing apoptosis, inducing detoxifying enzymes, inhibiting transcription factors, and reducing inflammation (*81, 107*). In a study with delphinidin and colon cancer HCT116 cells, results showed a reduction in cell proliferation, induction of apoptosis, and reduction of the inflammatory response of NFκB (*108*). Furthermore, with grape seed proanthocyanidins and several colon cancer cells, results showed inhibition of cell proliferation in some cell lines which was associated with the

induction of apoptosis through loss of membrane potential and caspase-3 activation (*109*).

In accordance with previous studies on red wine and grape seed polyphenolics, Lelfert and others demonstrated that in colon cancer cells these compounds reduced cell proliferation, induced apoptosis, as well as inhibited cholesterol uptake and the pro-inflammatory 5-LOX activity (*78*). Furthermore, with isolated extracts from muscadine grapes, Talcott and others showed induction of apoptosis in Caco-2 colon cells, as well as reduction in cell proliferation in a concentration-dependent manner (*95*).

To date, there is an increased interest in the study of the effects of dietary polyphenolics alone or in combination as chemopreventive and chemotherapeutic agents against colon cancer (*91, 107, 110, 111*).

Non-Dietary Risk Factors

Non-dietary risk factors for colon cancer include chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) and aspirin, smoking, genetic predisposition, and some colorectal diseases (*103*).

The effects of NSAID drugs have been reviewed and evidence shows that aspirin reduced the recurrence of sporadic adenomatous polyps. In addition, in a short-term trial, evidence showed a regression, but not prevention or elimination, of colorectal polyps in familial adenomatous polyposis (*112*).

Smoking has been associated with the formation of large colorectal adenomas, which are accepted as being precursor lesions for colorectal cancer. Furthermore, the

genotoxic exposure to tobacco compounds may have a temporal effect on the induction period of cancer between three to four decades and cancer diagnosis (*103*).

Additionally, inflammatory pathologies like Crohn's disease have been associated with the risk of developing colorectal cancer (*103*). Similarly, those patients that have previously been diagnosed and treated with colorectal cancer have a higher risk of reoccurrence (*113, 114*).

Genetic Risk Factors

As a genetic risk factor, colon cancer has been associated with either polyposis or nonpolyposis syndromes (*103*). Furthermore, colorectal cancer may be caused by two known pathways: chromosomal instability in tumor suppressors and oncogenes such as p53, K-ras, DCC, and APC, or by microsatellite instability, which is characterized by deficiency in DNA mismatch repair (often caused by promoter methylation) and alterations in small stretches of short repetitive DNA sequences (*86, 115, 116*).

Colorectal Criteria for Stage Classification

The criteria for stage classification of colorectal cancer is defined according to the TNM classification as shown in Table 1, which is a dual system that includes clinical and pathological classification. There is a stage grouping classification, as well, shown in Table 2 and Figure 10.

Table 1. TNM classification.

Primary tumor (T)	
TX:	Clinical evidence of primary tumor cannot be assessed
T0:	No evidence of primary tumor
Tis:	Carcinoma in situ
T1:	Tumor invades and is confined to mucosa or submucosa
T2:	Tumor invades the muscular wall or serosa; but no extension beyond
T3:	Tumor invades through the muscularispropia into the subserosa, or into the nonperitonealized pericolic or perirectal tissues; no fistula present
T4:	Fistula present; tumor invades other organs or structures and/or perforates the visceral peritoneum
Regional lymph nodes (N)	
NX:	Nodes cannot be assessed
N0:	No regional lymph nodes metastasis; nodes not involved
N1:	Metastasis in 1 to 3 regional lymph nodes
N2:	Metastasis in 4 or more regional lymph nodes
Distant metastasis (M)	
MX:	Metastasis cannot be assessed
M0:	No known distant metastasis
M1:	Distant metastasis present

Modified from Labianca and others, 2010; and Zinkin, 1981.

Table 2. Stage classification and equivalence with TNM classification.

Stage 0	Cancer is just found in the innermost lining of the colon or rectum	Tis, N0, M0
Stage I	Tumor has grown into the inner walls of the colon or rectum, but has not grown trough the walls	T1, N0, M0 T2, N0, M0
Stage IIA	Tumor extends more deeply into of through the wall	T3, N0, M0
Stage IIB	of the colon and rectum; and may be invade nearby tissues. No evidence of spread to lymph nodes.	T4, N0, M0
Stage IIIA	Tumor extends more deeply into of through the wall	T1-2, N1, M0
Stage IIIB	of the colon and rectum; and may be invade nearby	T3-4, N1, M0
Stage IIIC	tissues. No evidence of spread to lymph nodes.	Any T, N2, M0
Stage IV	Metastasis to other parts of the body (liver and/or lungs)	Any T, any N, M1

Modified from Labianca and others, 2010; and Zinkin, 1981.

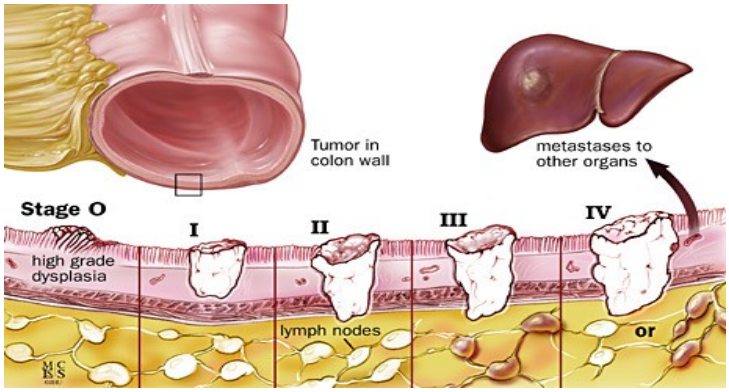


Figure 10. Colon cancer stages (*117*).

Apoptosis

Apoptosis is a type of cell death that is highly regulated and requires energy to be completed (*118*). Necrosis, is also a cell death process, but it occurs in an uncontrolled manner, is energy independent and un-orchestrated, and the main characteristic is the release of intracellular content onto neighboring cells leading to inflammation (*102*).

Apoptosis is a process that plays an important role during organism development, morphogenesis, and tissue remodeling (*119*). The main function of apoptosis is to maintain cellular homeostasis, thus it plays an important role in cancer research. The classic hallmarks of apoptosis include plasma and nuclear membrane blebbling, cytosolic condensation, organelle relocalization, chromatin condensation, protein cross-linking condensation, DNA fragmentation, cell shrinkage, and the formation of apoptotic bodies (*119-122*) which later are removed by phagocytes (*118, 122*). One characteristic of apoptosis is that cell death is associated with a lack of inflammatory response contrary to necrosis (*121*).

Researchers have found different pathways that can lead to apoptosis. The classic pathways are caspase-dependent. Caspases are cystein proteases that exist in inactive form in cells (zymogens) (*122*). The term caspase reflects that these proteases cleave substrate proteins after aspartate residues (*122-124*). To date, caspase activity has been classified in effector caspases (caspase 8 or 9) and executioner caspases (caspase 3, 6 and 7) (*118, 122, 124*); although, other caspases are currently being studied (*118*).

Caspase-dependent programmed cell death can be triggered by extracellular cell signaling or by intrinsic pathways which promote mitochondrial membrane permeabilization which releases caspase-activating factors (*121, 123*) as shown in Figure 11.

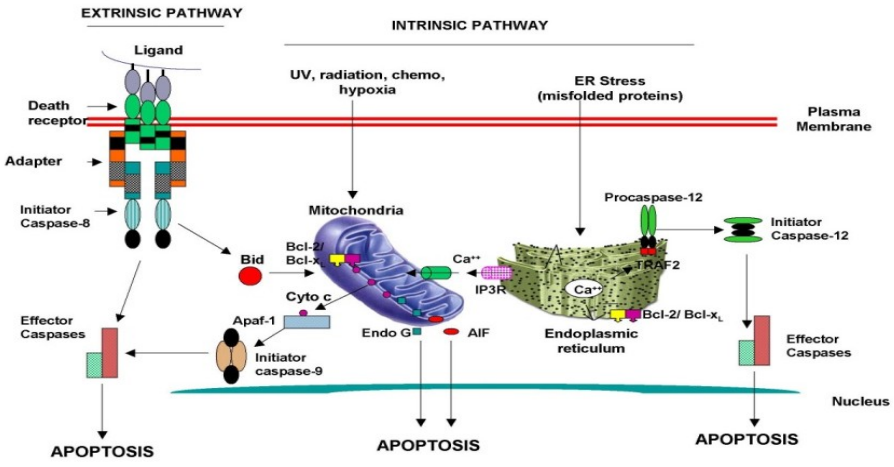


Figure 11. Caspase-dependent apoptosis pathways (*121*).

Programmed cell death through caspase-independent pathways often depends on proteolytic enzymes and mitochondrial membrane permeabilization (*123*). Some of these

proteases include cathepsin, calpains, and granzymes. Even though these enzymes may participate in caspase-dependent programmed cell death, recent studies have shown that they are able to produce apoptotic morphological changes in a caspase-independent manner (*123, 125, 126*) as shown in Figure 12.

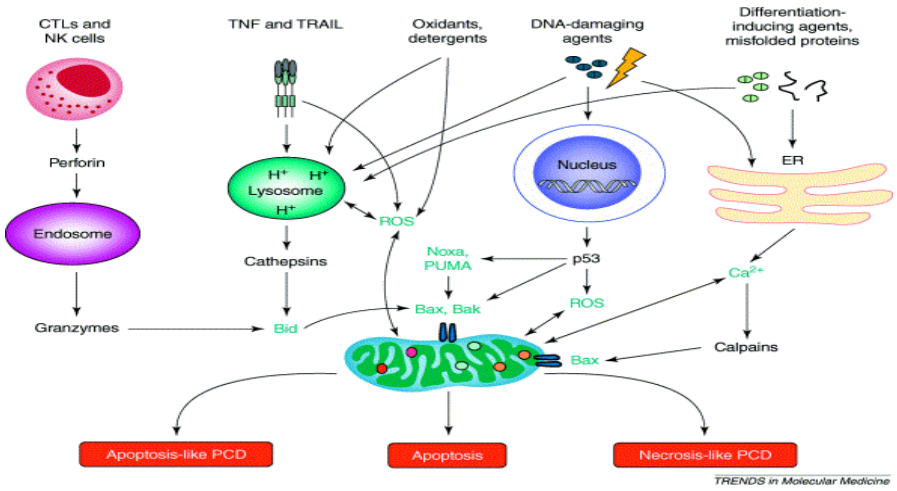


Figure 12. Caspase-independent apoptosis pathways (*123*).

MicroRNAs and Phytochemicals

Since the discovery of microRNAs (miRNAs) in the early 1990's (*127*), these small non-coding RNA molecules, no longer than 18-25 nucleotides in length (*128*), have played an important role in providing a better understanding of the molecular basis of cancer. MiRNAs regulate gene expression through interactions with 3'-untranslated regions (3'-UTR) of target genes causing mRNA cleavage and repressed translation

(*127, 129-131*). MiRNA-mediated oncogenic activity may result from either down-regulation of tumor suppressor genes or up-regulation of oncogenic genes (*131, 132*).

The first studies on miRNAs were focused on the biogenesis and function of these molecules and their relationship with chronic-degenerative diseases (*133-135*). Data from those studies demonstrated that one miRNA could target not only one mRNA, but hundreds (*128*). So far, oncogenic miRNAs such as miR-21, miR-27a, miR155, and miR-106a have been found in different tumors and cancer cell lines, as well as some tumor suppressor miRNAs such as miR-15, miR-16, miR-127, and miR-181, that may be potential target for cancer therapies. Recent evidence also suggests that miRNAs may play an important role in the development and progression of cancer.

Despite the advances in this research, there is still little focus on the effects of phytochemicals and miRNAs. Data, so far, has shown promising results for protection against several diseases *in vitro* and *in vivo*. Furthermore, results indicate that phytochemicals may inhibit or over-express some miRNAs, and as a result, could induce drug sensitivity, inhibit cell proliferation, and potentially reduce or block cancer invasion and metastasis (*136*). More importantly, phytochemicals could be used to increase malignant cells sensitivity to conventional agents facilitating inhibition or cell death.

In a study with epigallocatechin gallate and HepG2 cells, results showed that when cells were treated with this polyphenol, miR-16 was up-regulated, whereas the anti-apoptotic protein Blc-2 exhibited significant down-regulation. This may evidence that miR-16 in conjunction with polyphenolic compounds induce apoptosis through down-regulation of Bcl-2 (*137*).

In addition, a study with curcumin and pancreatic cell treatments showed down-regulation of miR199a* and up-regulation of miR-22 which suppressed the Sp1 protein and estrogen receptor 1 suggesting that modulation of miRNAs may be important to understanding the biological properties of curcumin (*138*).

The effect of miRNAs and colon cancer cells has also been studied with synthetic drugs and dietary supplements (*139-141*). A study with methyl 2-cyano-3,11-dioxo-18b-olean-1,12-dien-30-oate showed anticancer properties on SW480 and RKO colon cancer cells suggesting inhibition of miR-27a as a potential mechanism (*139*). Furthermore, with 5-fluorouracil and oxaliplatin, Zhou and others (*140*) evaluated the expression profiles of miRNAs in HCT-8 and HCT-116 colon cancer cells to determine whether the pharmacodynamic mechanisms of the chemotherapeutics could rely in part on their influence on miRNA expression. Similarly, Tili and others(*141*) studied the effect of resveratrol on miRNAs in colon cancer cells SW480. Results showed that resveratrol treatments decrease the levels of several oncogenic miRNAs while increasing the levels of miR-663, a tumor suppressor miRNA targeting TGF beta 1 transcripts.

CHAPTER III

CHARACTERIZATION AND COMPARISON OF AMERICAN HYBRID GRAPES RESISTANT TO PIERCE'S DISEASE WITH TRADITIONAL *VITIS VINIFERA* GRAPE VARIETIES GROWN IN TEXAS

SUMMARY

Environmental conditions play an important role in grape quality and survival. Where humidity and hot weather are predominant, some grape varieties are under disease pressure. The physicochemical composition and polyphenolic profiles of Cabernet Sauvignon, Merlot, Black Spanish and Blanc Du Bois were studied. Total phenolics in Black Spanish, Cabernet Sauvignon, and Merlot were similar with values of 2.53, 2.51, 2.53, respectively; whereas Blanc Du Bois was lower with 0.43 mg GAE/gr FW. The skinned-red grapes showed a difference in anthocyanins with values of 1.47, 0.40, and 0.29 mg/gr FW, respectively. Likewise, Black Spanish had the highest ORAC followed by Merlot, Cabernet Sauvignon, and Blanc Du Bois with values of 15.72, 8.63, 7.61, and 1.95 μmol TE/gr FW, respectively. Non-anthocyanin polyphenols didn't show qualitative differences, the four grape varieties contained similar hydroxycinnamic acids, hydroxybenzoic acids, flavan-3-ols, and flavonols. Moreover, possible genetic differences between *V. vinifera* and *V. aestivalis* had strong influences on anthocyanin profiles. The presence of diglucoside forms of anthocyanins and its derivatives of acetyl and *p*-coumaric were relevant in Black Spanish.

INTRODUCTION

In regions with humid and warmer climates, grapes are highly susceptible to diseases, particularly to Pierce's disease (*142*). Pierce's disease is a bacterial infection caused by *Xylella fastidiosa* in which the "glassy-winged sharpshooter" serves as a vector (*142, 143*). This insect's natural habitat is in the southeastern region of the country in states including Texas, Louisiana, Mississippi, and Florida (*143*). Therefore, these areas are considered "hot zones" for Pierce's disease propagation, which is considered to be endemic (*142, 144*).

Since the late 1800's, there have been attempts to grow *Vitis vinifera* in regions where Pierce's disease is prevalent without success (*145*). Therefore, grape growers are looking for American hybrids or grapes native to America, which have shown resistance or tolerance to diseases such as Pierce's disease, black rot, and powdery mildew (*16, 142*). In the state of Texas, due to specific growing conditions in certain regions, grape growers are able to produce *Vitis vinifera* varieties (Merlot, Cabernet Sauvignon, Sangiovese, Shiraz, etc.) of good quality, whereas in the east and southeast, near the gulf coast, hybrid grapes (Black Spanish and Blanc Du Bois) or native grapevines like *Vitis aestivalis* (Norton or Cynthiana) are suitable for propagation (*4, 7, 9*).

Information on the chemical characterization of *V. vinifera* is available and current (*146-148*). In contrast, information about American natives or hybrid grapes such as Black Spanish, Blanc Du Bois, Norton, and other varieties with the potential to benefit the winemaking industry is scarce and outdated.

Black Spanish grapes, also known as Jaques or Lenoir, are considered natural

hybrids of *Vitis aestivalis*, and scientists agree that this grape variety contains characteristics from three species *V. aestivalis*, *V. cinerea,* and *V. vinifera*(*142, 149, 150*). This variety has a long history and tradition in the state of Texas, yet, in recent years, the Black Spanish grape has incited the interest of winemakers due to its unique aroma, taste, and the potential for satisfactory growth in warmer regions susceptible to Pierce's disease.

Blanc Du Bois was developed with the intention to obtain a high yield plant able to produce quality grapes for premium white wines (*151*). Blanc Du Bois was the result of crossing between Florida D6-148, a hybrid resistant to Pierce's disease, and Cardinal in 1968 (*151*). Although is a hybrid, its origin can be traced back as a distant descendent of *Vitis aestivalis* ssp. *smalliana*, Pixiola and Golden muscat (*151*). Principal advantages and characteristics of Blanc Du Bois include its resistance to fungal and bacterial infections and the ability to grow in warmer climates (*151*), which makes it attractive where *V. vinifera* varietals can't survive.

The objective of this study is to investigate the chemistry of American hybrid grapes resistant to endemic diseases as compared with traditional *Vitis vinifera* varieties of economic interest for the winemaking industry in Texas.

MATERIALS AND METHODS

Grape Sources

Commercial grape varieties were collected at different locations in the state of Texas during the harvest season of 2010. Black Spanish grapes were collected from the

experimental vineyard at Texas A&M University (College Station, TX); Merlot grapes from Shofner (southeast of Lamesa, TX) were donated by Messina Hof winery (College Station, TX), Cabernet Sauvignon from the Texas AgriLife experimental vineyard station (Lubbock, TX), and Blanc Du Bois donated by Austin County Vineyards (Cat Spring, TX). After harvest, grapes were stored at -20 ^{o}C and shipped overnight to the Department of Food Science (Texas A&M University, College Station, TX). Upon arrival, grapes were stored at -20 ^{o}C until needed for chemical analysis.

Extraction and Fractionation of Grape Polyphenols

Grapes were thawed at room temperature and the seeds were removed. Skins and pulp were collected and homogenized with a hand blender and 25 gr of sample was taken in triplicate. An extraction solvent consisting of methanol:acetic acid (95:5) was used to extract polyphenolic compounds. Samples where homogenized with a homogenizer PowerGen 500 (Fisher Scientific, Pittsburg, PA) for 1 min with 50 mL of the solvent, and clarified by centrifugation (Eppendorf centrifuge 5810R, Hauppauge, NY) at 2500 rpm for 5 min. The supernatant was collected and the process was repeated two more times. The final extract was evaporated by rotary evaporation in a rotavapor (Büchi Labortechnik AG, Flawil, Switzerland) at 40 ^{o}C to remove the excess of solvent.

Grape polyphenols were isolated using C_{18} solid phase extraction as follows: grape extract was loaded onto the C_{18} Sep-Pak Vac cartridges (Waters Corporation, MA, USA) washed with water, and eluted, with ethyl acetate to remove non-anthocyanin compounds and to enrich the isolate in phenolic acids, flavan-3-ols, and flavonols, followed by acidified methanol to remove the anthocyanin-rich fraction. Both solvent-

fractions were evaporated by rotary evaporation in a rotavapor (Büchi Labortechnik AG, Flawil, Switzerland) at 40 ^{o}C to dryness. The final samples were reconstituted with methanol and stored at -80 ^{o}C until further use. Prior to analysis of anthocyanins, methanol was removed in a speedvac concentrator (Savant ISS110, Thermo Scientific, Waltham, MA) and the residue was dissolved in an equivalent amount of 5% acetic acid (v/v) in water.

Determination of Total Soluble Phenolics

Total soluble phenolics were determined by Folin-Ciocalteu assay (*152*) using a microplate reader FLUOstar (BMG Labtech Inc., Durham, NC) with absorbance read at 726 nm, and quantified by linear regression using a gallic acid standard and expressed as gallic acid equivalents (GAE).

Determination of Monomeric Anthocyanins

Total monomeric anthocyanins were determined spectrophotometrically by pH differential method using a Helios Gamma spectrophotometer (Thermo Electron, Waltham, MA) at 520 nm, and quantified as equivalents of malvidin-3-*O*-glucoside with a molar extinction coefficient of 28,000 (*153*).

Determination of Oxygen Radical Absorbance Capacity

The antioxidant capacity was determined using the oxygen radical absorbance capacity assay (ORAC) (*154*), using fluorescein as the fluorescent probe with a FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission, BMG Labtech Inc., Durham, NC). Results were reported in μmol of Trolox equivalents (TE) /gr of sample fresh weight (FW).

HPLC-PDA-ESI-MS^n Analysis of Grape Polyphenols

Mass spectrometric analysis of the non-anthocyanin fraction was performed on a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source (ThermoFisher, San Jose, CA). The non-anthocyanin fraction was analyzed using a Sunfire C_{18} column (Waters Inc., Milford, MA) (250 x 4.6 mm, 5 μm) at a temperature of 25 ^oC. The chromatographic separation was performed with a mobile phase consisting of solvent A and B in gradient mode, where mobile phase A was 0.1% acetic acid (v/v) in water, and mobile phase B was 0.1% formic acid (v/v) in methanol. The gradient program with 0.4 mL/min was run as follows: 0 min 100%, A; 1 min 95%, A; 15 min 70%, A; 40 min 35%, A; 50 min 5%, A; 55 min 5%, A; 55.5min 100%, A; 60 min 100%, A. Ionization was conducted in negative mode as follows: sheath gas (N_2), 40 units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 4.5 kV; capillary temperature, 300 °C; capillary voltage, 7 V; tube lens offset, 40 V. The detection wavelengths were set at 280 nm and 360 nm. The identification of wine polyphenols was carried out based on mass-fragmentation pattern and wavelengths.

For the anthocyanin fraction, mass spectrometric analysis was performed using a Symmetry C_{18} column (Waters Inc., Milford, MA) (250 x 4.6 mm, 5 μm) at a temperature of 25 ^oC. The chromatographic conditions consisted of mobile phase A water/acetic acid/methanol (85:10:5), and mobile phase B of 0.1% formic acid (v/v) in methanol. A gradient program with 0.25 mL/min was run as follows: 0 min 95%, A; 10 min 80%, A; 30 min 50%, A; 35 min 25%, A; 55 min 25%, A; 56 min 95%, A; 60 min 95%, A. Ionization was conducted in positive mode as follows: sheath gas (N_2), 40

units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 4.5 kV; capillary temperature, 300 °C; capillary voltage, 3.3 V; tube lens offset, -60 V. The detection wavelength was set at 520 nm, and the identification was carried out based on fragmentation pattern and wavelengths.

Statistical Analyses

Data from each chemical analysis were analyzed by one-way-analysis of variance (ANOVA) using JMP version 8.0 (SAS Institute Inc., Cary, NC). Mean separations were deemed significant at $p \leq 0.05$ using a Tukey-Cramer HSD comparison for all pairs.

RESULTS

Chemical Analyses

The chemical properties of two *V. vinifera* and two American hybrids varieties grown in Texas were determined using data for total phenolics, total monomeric anthocyanins, and ORAC as shown in Table 3. The total phenolics content in Black Spanish, Cabernet Sauvignon, and Merlot did not show significant differences; with values of 2.53, 2.51, 2.53, respectively; whereas, Blanc Du Bois was significantly lower with 0.43 mg GAE/gr FW.

The three red grapes; Black Spanish, Merlot and Cabernet Sauvignon, showed significant differences in monomeric anthocyanins with concentrations of 1.47, 0.40, and 0.29 mg/gr FW, respectively. Likewise, the antioxidant capacity assessed by ORAC, showed similar trends with significant differences among grape varieties, in which Black

Spanish had the highest ORAC followed by Merlot, Cabernet Sauvignon, and Blanc Du Bois with values of 15.72, 8.63, 7.61, and 1.95 μmol TE/gr FW, respectively.

Table 3. Chemical characterization of Texas grape varieties.

Analysis	Black Spanish	Cabernet Sauvignon	Merlot	Blanc Du Bois
Total phenolics (mg GAE/gr FW)[1]	2.53±0.01a	2.51±0.07a	2.53±0.00a	0.43±0.01b
Anthocyanins (mg/gr FW)[2]	1.47±0.03a	0.29±0.01c	0.40±0.00b	n.d.
ORAC (μmol TE/gr FW)[3]	15.72±0.14a	7.61±0.05c	8.43±0.03b	1.95±0.03d

[1]Quantified as equivalents of gallic acid per gram of fresh weight. [2]Monomeric anthocyanins quantified as equivalents of malvidin-3-*O*-glucoside per gram of fresh weight. [3]Reported in μmol Trolox equivalents per gram of fresh weight. Data marked with different letters within the same row are significantly different ($p < 0.05$).

Chemical Profile of Grape Non-Anthocyanin Polyphenolics

The non-anthocyanin polyphenolics found in all the wine grape varieties studied were from the hydroxycinnamic acids, hydroxybenzoic acids, flavan-3-ols, and flavonols families. The chromatographic profiles of *V. vinifera* varieties, Cabernet Sauvignon and Merlot, showed common qualitative patterns as shown in Figures 13A-B and Figures 14A-B.

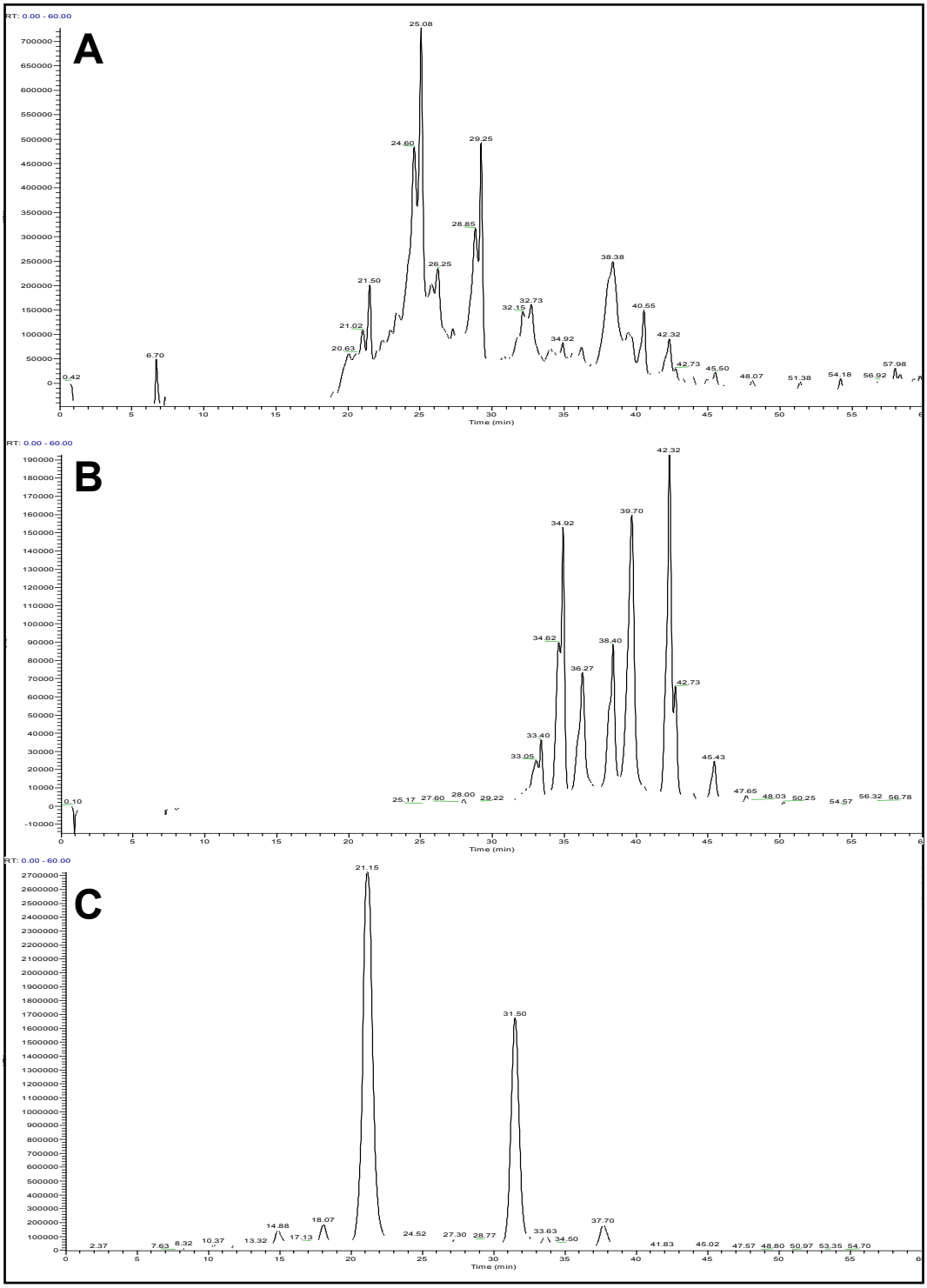


Figure 13. Chromatograms of a Cabernet Sauvignon grape extract. ***A,*** phenolic acids, flavan-3-ols, and procyanidins at 280 nm; ***B,*** flavonols at 360 nm; and ***C,*** anthocyanins at 520 nm.

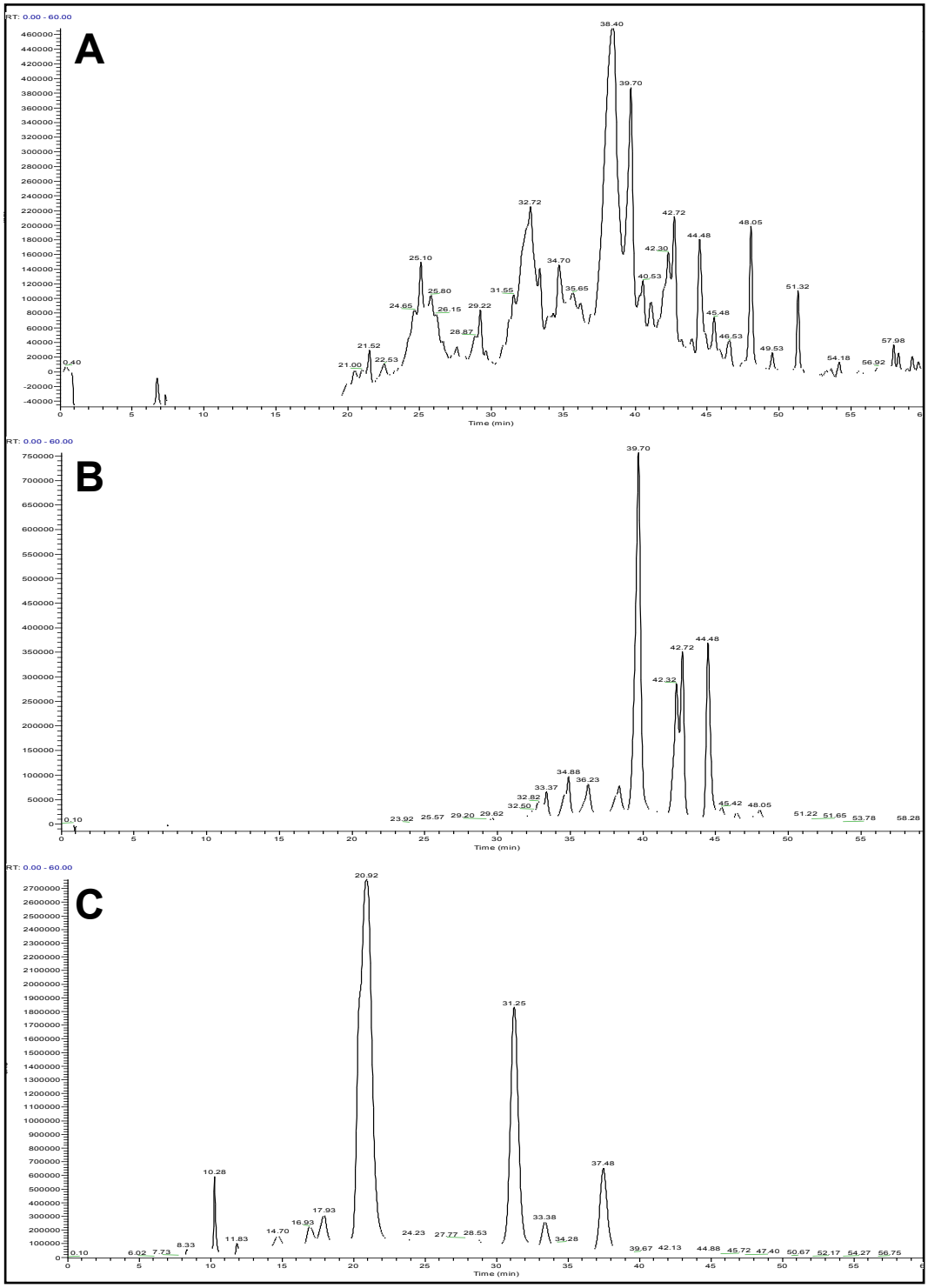


Figure 14. Chromatograms of a Merlot grape extract. ***A,*** phenolic acids, flavan-3-ols, and procyanidins at 280 nm; ***B,*** flavonols at 360 nm; and ***C*,** anthocyanins at 520 nm.

For American hybrids grapes, Blanc Du Bois and Black Spanish similar trends similar chemical profiles were present compared to *V. vinifera* varieties as shown in Figures 15A-B and Figures 16A-B.

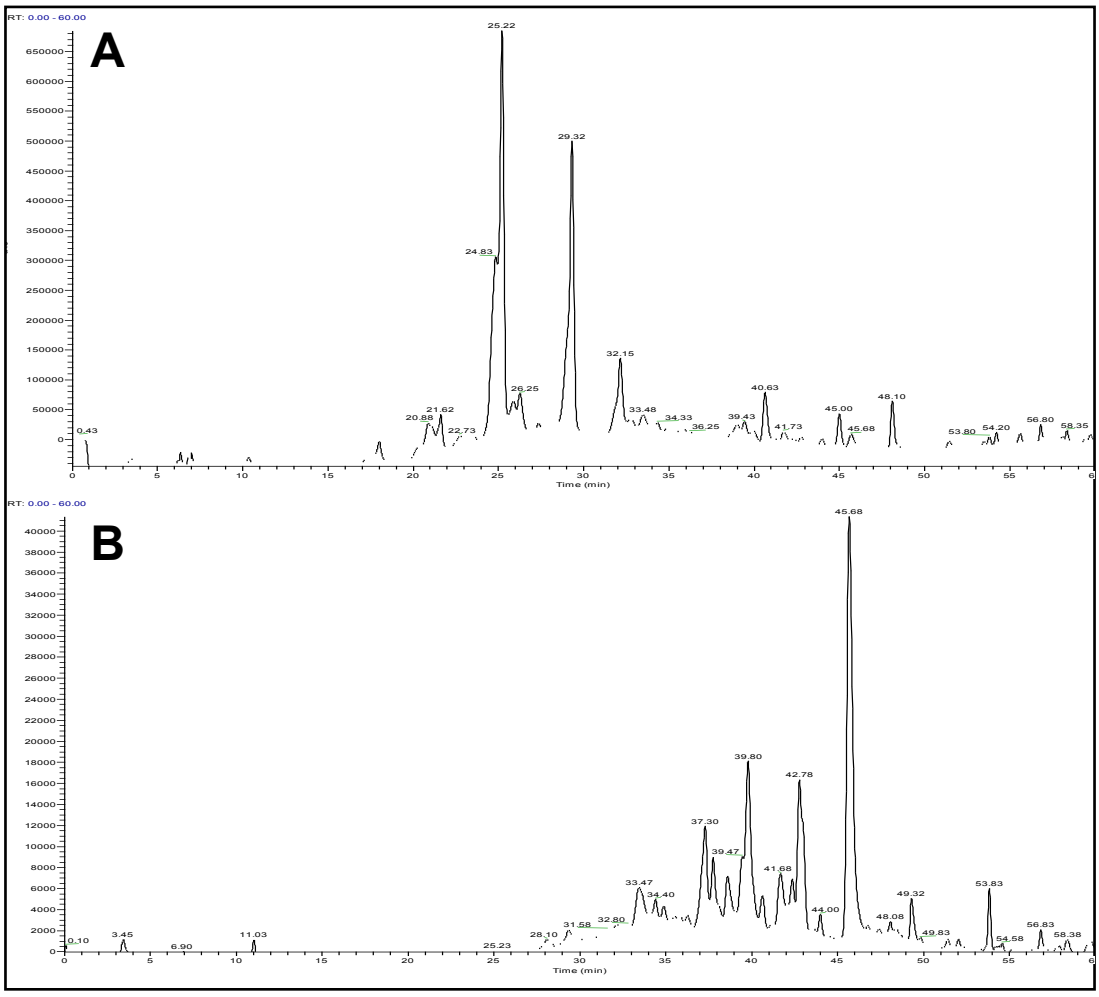


Figure 15. Chromatograms of a Blanc Du Bois grape extract. ***A,*** phenolic acids, flavan-3-ols, and procyanidins at 280 nm; and ***B,*** flavonols at 360 nm.

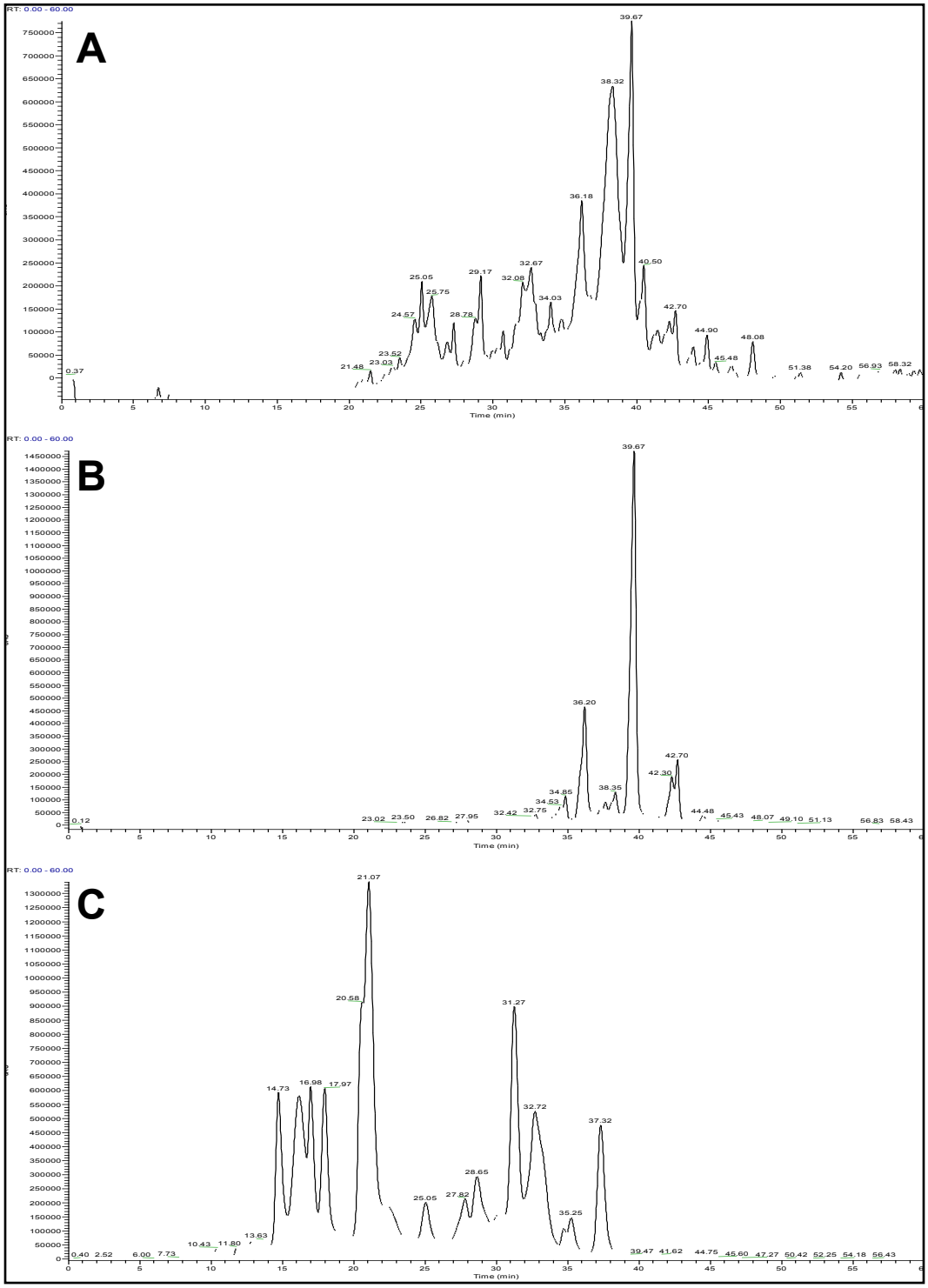


Figure 16. Chromatograms of a Black Spanish extract. ***A,*** phenolic acids, flavan-3-ols, and procyanidins at 280 nm; ***B,*** flavonols at 360 nm, and ***C*,** anthocyanins at 520 nm.

The major non-anthocyanin polyphenols identified in all four grape varieties studied are shown in Table 4. From the hydroxybenzoic acid group, only gallic acid was identified in Blanc Du Bois. Likewise, two hydroxycinnamic acids were identified with the same *m/z* ratio at different retention times for Black Spanish, and based on their fragmentation pattern could be associated to a glucosides form of *p*-coumaric acid.

Flavan-3-ols, both (+)-catechin and (-)-epicatechin were detected at 280 nm and identified in all grape varieties. Several forms of procyanidins were detected in all grape varieties. Polymeric procyanidins included dimers, trimers, and tetramers forms of catechin and/or epicatechin.

Five compounds exhibiting typical flavonoid spectral properties were detected in all grape varieties in glycoside forms of myricetin, laricitrin, isorhamnetin, and quercetin. Only for quercetin, a glucoronic form was identified in Merlot and Blanc Du Bois grapes.

Matrix complexity, coelution and relative low abundance of certain non-anthocyanin compounds made it difficult to identify these grape varieties. By direct infusion onto the mass spectrometer, it was possible to detect their fragmentation patterns and determine the presence of more polyphenolic compounds in the samples.

Table 4. Retention times, mass spectra by HPLC-MS-ESI-(-)-MS^n, and UV-data of polyphenolics present in Cabernet Sauvignon, Merlot, Blanc Du Bois and Black Spanish grapes.

T_R (min)	Compound	Grape Variety[1]	λ_{max}	MS $[M - H]^-$ (*m/z*)[2]	MS^2 (*m/z*)
18.0	Gallic acid	BDB	n.d.	169.2	125.3
21.6	Polymeric procyanidins	CS, M, BDB, BSp	280	1153.0, 865.0, **577.0**	451.0, 425.0, 407.1, 289.1
23.2	Polymeric procyanidins	BDB	281	1153.0, 865.0, **577.0**	451.0, 425.0, 407.1, 289.1
25.0	Polymeric procyanidins	BSp	279	1153.0, 865.0, **577.0**	451.0, 425.0, 407.1, 289.1
25.2	Unknown	CS, M, BDB	n.d.	324.8	
25.2	Polymeric procyanidins	BDB	279, 238	577.0	
25.2	(+)-Catechin	CS, M, BDB, BSp	279, 238	289.0	
26.4	Polymeric procyanidin	CS, M, BDB	279	1153.2, **865.0**	694.9, 577.0, 448.9
27.3	*p*-Coumaroyl hexose	BSp	315	325.0	187.1, 163.1, 145.3
29.4	(-)-Epicatechin	CS, M, BDB, BSp	279, 237	289.0	
29.4	Unknown	CS, M, BDB, BSp	n.d.	324.9	
30.7	Polymeric procyanidin	CS	n.d.	865.0	694.9, 577.1, 289.0
30.7	*p*-Coumaroyl hexose	BSp	311	325.0	162.9
36.2	Myricetin-3-*O*-hexoside	CS, M, BSp	351, 300sh, 252	479.1	316.1
39.5	Laricitrin-3-*O*-hexoside	CS, M, BSp	356, 300, 256	493.1	330.9
39.8	Qercetein-3-*O*-hexoside	CS, M, BDB, BSp	354, 295sh, 256	463.1	301.1
39.8	Polymeric procyanidin	CS, M, BSp	n.d.	576.8	
42.8	Unknown	CS, M, BSp	347, 300sh, 254	447.1	
42.8	Isorhamnethin-3-*O*-glucoside	CS, M, BDB, BSp	352, 295sh, 265sh, 255	477.2	314.0
45.7	Quercetin-3-*O*-glucoronide	M, BDB	356, 295sh, 256	476.9	301.1

[1]CS: Cabernet Sauvignon, M: Merlot, BDB: Blanc Du Bois, BSp: Black Spanish. [2]Ions in bold indicate the most intense product ion on which further MS analyses were performed.

For hydroxybenzoic acids, identification of compounds in the grape varieties was based on their characteristic *m/z* signals as follows: 4-hydroxybenzoic acid at *m/z* 137.2 ([M - H]$^-$) with fragmentation to the *m/z* 93.1, protocatechuic acid at *m/z* 153.2 ([M - H]$^-$) with fragmentation to the *m/z* 109.1, vanillic acid at *m/z* 167.2 ([M - H]$^-$) with fragmentation at *m/z* 123.1, gallic acid at *m/z* 169.2 ([M - H]$^-$) with fragmentation to the *m/z* 125.3, syringic acid at *m/z* 197.1 ([M - H]$^-$), vanillic acid hexoside at *m/z* 329.1.2 ([M - H]$^-$) with fragmentation at the *m/z* 167.1; for vanillin only in Cabernet Sauvignon, Merlot and Black Spanish was detected at *m/z* 151.1 ([M - H]$^-$) with fragmentation at the *m/z* 136.1 and 106.8. Furthermore, only in Cabernet Sauvignon and Merlot ethyl gallate was detected at *m/z* 197.0 ([M - H]$^-$) with fragmentation to the *m/z* 182.1, 165.1 and 151.0.

Hydroxycinnamic acids were detected in all grape varieties including: cinnamic acid at *m/z* 147.0 ([M - H]$^-$), *p*-coumaric acid at *m/z* 163.1 ([M - H]$^-$) with fragmentation to the *m/z* 119.2, caffeic acid at *m/z* 178.8 ([M - H]$^-$) with fragmentation to the *m/z* 135.2, ferulic acid at *m/z* 192.9 ([M - H]$^-$) with fragmentation to the *m/z* 178.1, 149.3 and 134.4, caffeoyl tartaric acid at *m/z* 311.1 ([M - H]$^-$) with fragmentation to the *m/z* 178.9 and 149.3,feruryltartaric acid at *m/z* 324.9 ([M - H]$^-$) with fragmentation to the *m/z* 193.2. In addition, coumaroyl tartaric acid at *m/z* 295.1 ([M - H]$^-$) with fragmentation to the *m/z* 163.1 and dicaffeoyl tartaric acid at *m/z* 311.0 ([M - H]$^-$) with fragmentation to the *m/z* 293.0 and 183.0 were detected in all grapes except Blanc Du Bois. Likewise, in Cabernet Sauvignon and Black Spanish, a *p*-coumaroyl hexose was detected at *m/z* 325.0 ([M - H]$^-$) with fragmentation to the *m/z* 163.1 and 145.3.

Flavan-3-ols in all grape varieties, it was possible to identify either gallocatechin or epigallocatechin at *m/z* 305.0 ([M - H]$^-$), catechin gallate or epigallocatechin gallate at *m/z* 441.0 ([M - H]$^-$) with fragmentation to the *m/z* 289.1 and 169.2, and gallocatechin gallate or epigallocatechin gallate at *m/z* 457.2 ([M - H]$^-$) with fragmentation to the *m/z* 289.2. Similarly, in all grapes varieties except Blanc Du Bois, a dimer of epigallocatechin-epicatechin was identified at *m/z* 593.0 ([M - H]$^-$) with fragmentation to the *m/z* 424.9, as well as a procyanidin dimer gallate at *m/z* 729.0 ([M - H]$^-$) with fragmentation to the *m/z* 576.8.

For flavonols, several compounds were common for all grape varieties including: quercetin at *m/z* 301.1 ([M - H]$^-$) with fragmentation to the *m/z* 273.2, 179.1 and 151.0, myricetin at *m/z* 317.1 ([M - H]$^-$), kaempferol-3-*O*-hexoside at *m/z* 447.1 ([M - H]$^-$) with fragmentation to the *m/z* 285.2, quercetin3-*O*-glucoronide at *m/z* 477.0 ([M - H]$^-$) with fragmentation to the *m/z* 301.1, myricetin-3-*O*-hexoside at *m/z* 479.1 ([M - H]$^-$) with fragmentation to the *m/z* 316.2, laricitrin-3-*O*-hexoside at *m/z* 493.1 ([M - H]$^-$) with fragmentation to the *m/z* 331.1. Moreover, the following flavonols were absent in Blanc Du Bois: quercetin-3-*O*-rhamnoside at *m/z* 447.1 ([M - H]$^-$) with fragmentation to the *m/z* 301.1, myricetin-3-*O*-glucoronide at *m/z* 493.0 ([M - H]$^-$) with fragmentation to the *m/z* 317.3, and quercetin-3-*O*-rutinoside at *m/z* 609.0 ([M - H]$^-$) with fragmentation to the *m/z* 463.0 and 301.1.

Chemical Profile of Grape Anthocyanins

Using HPLC-PDA-ESI-MS analysis in positive mode, anthocyanins were identified in different grape varieties. Cabernet Sauvignon and Merlot grapes, both *V.*

vinifera, presented similar chromatographic profiles (Figures 13C and 14C); Black Spanish, a *V. aestivalis* hybrid, displayed significant differences (Figure 16C) when compared to *V. vinifera*.

Cabernet Sauvignon and Merlot grapes showed similar anthocyanin compositions (Tables 5 and 6). In Black Spanish grapes, the anthocyanin profile was quite different (Table 7). For individual anthocyanins, five conjugated forms of 3-*O*-glucoside of delphinidin, cyanidin, petunidin, peonidin and malvidin were identified in both *V. vinifera* and *V. aestivalis* hybrid varieties. In addition to the 3-*O*-glucoside moieties, in Black Spanish grapes, the 3,5-*O*-diglucosides were also identified for the five anthocyanins previously mentioned.

For the three grape varieties studied, acetyl derivates with 3-*O*-glucoside moieties were identified for petunidin and malvidin, in which the latter was detected at two different retention times with the same *m/z* signal; this could be associated to the presence of different hexoses. Furthermore, in Black Spanish grapes, acetyl derivates with the 3,5-*O*-diglucoside moieties were also found for petunidin and malvidin.

Merlot grapes contained delphinidin, cyanidin, petunidin, and malvidin in two isomer forms *cis* and *trans*; Cabernet Sauvignon grapes only contained petunidin and malvidin, in isomer forms as well. Black Spanish had not only the monoglucoside forms of cyanidin, petunidin and malvidin, but also the 3,5-*O*-diglucoside forms of the five anthocyanins present in wine grapes.

Table 5. HPLC-MS-ESI-(+)-MS^n of anthocyanins present in Cabernet Sauvignon grapes.

T_R (min)	Compound	λ_{max}	MS $[M + H]^+$ (*m/z*)	MS^2 (*m/z*)
14.9	Delphinidin-3-*O*-glucoside	526, 278	465.0	303.2
17.3	Cyanidin-3-*O*-glucoside	523, 278	449.0	287.0
18.1	Petunidin-3-*O*-glucoside	528, 278	478.9	317.1
20.7	Peonidin-3-*O*-glucoside	522, 279	463.0	301.1
21.2	Malvidin-3-*O*-glucoside	528, 346, 274	493.1	331.1
24.5	Malvidin-3-*O*-(6-*O*-acetyl)-glucoside	530, 278	535.0	331.1
25.3	Delphinidin-3-*O*-(6-*O*-acetyl)-glucoside	531, 278	507.0	464.4, 303.3
27.1	Malvidin derivate	531, 333, 279	510.9	348.8, 330.7
27.4	Unknown	n.d.	531.1	369.2
28.6	Unknown	533, 279	573.0	369.2
28.7	Petunidin-3-*O*-(6-*O*-acetyl)-glucoside	532, 278	521.0	317.2, 302.2
29.6	Malvidin derivate	524, 279	552.8	348.8, 331.0
31.5	Malvidin-3-*O*-(6-*O*-acetyl)-glucoside	528, 347, 272	535.1	331.1
31.8	Malvidin derivate	n.d.	756.8	331.1
32.5	A-type vitisin of malvidin-3-*O*-(6-*O*-acetyl)-glucoside	n.d.	603.0	399.0
33.7	Malvidin-3-O-(6-caffeoyl)-glucoside	534, 279	655.0	331.1
33.8	Petunidin-3-(6-*O*-*p*-coumaroyl)-glucoside *cis*	536, 280	625.0	317.2
34.6	Malvidin-3-(6-*O*-*p*-coumaroyl)-glucoside *cis*	536, 280	639.0	331.1
35.6	Petunidin-3-(6-*O*-*p*-coumaroyl)-glucoside *trans*	534, 280	625.0	317.2
35.6	Peonidin derivate	n.d.	611.1	449.1, 301.2
37.7	Malvidin-3-(6-*O*-*p*-coumaroyl)-glucoside *trans*	534, 282	639.0	331.1
38.2	A-type vitisin of Malvidin-3-(6-*O*-*p*-coumaroyl)-glucoside	n.d.	707.0	399.1

Table 6. HPLC-MS-ESI-(+)-MS^n of anthocyanins present in Merlot grapes.

T_R (min)	Compound	λ_{max}	MS $[M + H]^+$ (*m/z*)	MS^2 (*m/z*)
14.7	Delphinidin-3-*O*-glucoside	527, 278	465.0	303.2
16.9	Cyanidin-3-*O*-glucoside	520, 279	448.9	287.2
17.9	Petunidin-3-*O*-glucoside	527,278	479.0	317.1
20.5	Peonidin-3-*O*-glucoside	518, 279	463.0	301.2
20.9	Malvidin-3-*O*-glucoside	528, 345, 273	492.9	331.1
24.1	Malvidin-3-*O*-(6-*O*-acetyl)-glucoside	526, 279	535.1	331.2
26.3	Malvidin derivate	533, 277	653.2	635.1, 447.2, 311.4
26.9	Malvidin derivate	533, 277	510.9	493.0, 348.9, 331.0
27.2	Unknown	526, 279	531.1	369.1
27.8	Cyanidin-3-*O*-(6-*O*-acetyl)-glucoside	529, 277	491.2	287.2
28.5	Petunidin-3-*O*-(6-*O*-acetyl)-glucoside	532, 279	521.0	317.2
29.5	Malvidin derivate	533, 279	552.9	348.9, 330.9
31.3	Malvidin-3-*O*-(6-*O*-acetyl)-glucoside	528, 277	535.0	331.1
32.3	A-type vitisin of malvidin-3-*O*-(6-*O*-acetyl)-glucoside	528, 280	603.1	399.3
32.8	Delphinidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside	532, 280	611.1	303.2
33.4	Peonidin-3-*O*-(6-*O*-caffeoyl)-glucoside	532, 280	625.0	301.2
33.4	Malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside	532, 280	655.0	331.1
34.6	Malvidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside *cis*	534, 280	639.0	331.1
34.9	Cyanidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside	527, 280	595.0	287.3
35.4	Petunidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside *trans*	531, 274	625.0	317.0
37.5	Malvidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside *trans*	532, 283	639.1	331.1

Table 7. HPLC-MS-ESI-(+)-MS^n of anthocyanins present in Black Spanish grapes.

T_R (min)	Compound	λ_{max}	MS $[M + H]^+$ (*m/z*)	MS^2 (*m/z*)
9.7	Delphinidin-3-*O*-glucoside-5-*O*-glucoside	528, 275	627.1	464.8, 303.2
13.0	Cyanidin-3-*O*-glucoside-5-*O*-glucoside	521	611.0	448.8, 287.2
13.5	Petunidin-3-*O*-glucoside-5-*O*-glucoside	526	641.0	478.8, 317.1
14.7	Delphinidin-3-*O*-glucoside	525, 340, 277	465.0	303.2
16.1	Peonidin-3-*O*-glucoside-5-*O*-glucoside	518, 277	625.0	462.9, 301.1
16.5	Malvidin-3-*O*-glucoside-5-*O*-glucoside	522, 277	655.1	492.9, 331.1
17.0	Cyanidin-3-*O*-glucoside	519, 279	449.1	287.3
17.9	Petunidin-3-*O*-glucoside	527, 340, 277	479.0	317.1, 302.1
19.2	Petunidin3-*O*-(6-*O*-acetyl)-glucoside-5-*O*-glucoside	n.d.	683.1	520.9, 478.7, 317.0
20.7	Peonidin-3-*O*-glucoside	519, 279	463.0	301.2
21.1	Malvidin-3-*O*-glucoside	527, 340, 277	493.0	331.1
22.3	Malvidin-3-*O*-(6-*O*-acetyl)-glucoside-5-*O*-glucoside	526, 279	697.1	534.9, 492.8, 331.1
22.6	Peonidin derivate	526, 279	667.0	505.0, 462.9, 301.1
24.5	Malvidin-3-*O*-(6-*O*-acetyl)-glucoside	531, 283	535.1	331.2
25.1	Delphinidin-3-*O*-(6-*O*-acetyl)-glucoside	530, 280	507.0	303.2
26.8	Petunidin derivate	534, 282	803.2	640.9, 317.2
27.8	Delphinidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside-5-*O*-glucoside	528, 281	773.0	611.0, 464.8, 303.2
28.0	Cyanidin-3-*O*-(6-*O*-acetyl)-glucoside	527, 281	491.0	287.2
28.7	Petunidin-3-*O*-(6-*O*-acetyl)-glucoside	531, 281	521.0	317.2
29.2	Cyanidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside-5-*O*-glucoside	531, 282	757.4	595.0, 449.0, 287.0
29.2	Malvidin derivate	532, 284	817.0	654.9, 492.8, 331.1
30.0	Petunidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside-5-*O*-glucoside	531, 282	787.2	624.9, 478.9, 317.1
31.3	Malvidin-3-*O*-(6-O-acetyl)-glucoside	528, 279	535.2	331.1
32.7	Malvidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside-5-*O*-glucoside	534, 283	801.2	638.9, 493.0, 331.2
33.9	Peonidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside-5-*O*-glucoside	n.d.	771.1	608.9, 462.9, 301.2
34.7	Cyanidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside	527, 310, 284	595.0	287.2
35.3	Petunidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside	534, 283	625.1	317.1
37.3	Malvidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside	532, 283	639.0	331.1

Moreover, in *V. vinifera* varietals, it was possible to identify some caffeic glycoside derivates for peonidin and malvidin in Merlot and malvidin in Cabernet Sauvignon. In Black Spanish, no caffeic glycoside derivates were identified. In addition, some pyranoanthocyanins were found in *V. vinifera*, but not in *V. aestivalis* grapes. A-type vitisin of malvidin-3-*O*-(6-*O*-acetyl)-glucoside was identified in both Cabernet Sauvignon and Merlot varieties; whereas A-type vitisin of malvidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside was found in Cabernet Sauvignon.

Some additional anthocyanin-type derivatives were found in both *V. vinifera*, and *V. aestivalis,* as shown in Tables 5-7, for which it was not possible to obtain total confirmation based on spectral properties and *m/z* signals.

DISCUSSION

Chemical Analyses

Total phenolics are an important parameter in determination of grape and wine quality. Differences in total phenolics were evident between red-skinned and white varietals as previously reported (*155*). These differences are attributed to the presence of anthocyanins in red-skinned grapes, which significantly contribute to the phenolic content. Although Black Spanish grapes displayed the highest anthocyanin concentration, they presented similar amounts of total phenolics when compared to Cabernet Sauvignon and Merlot. This suggests that the phenolic profile or concentrations of these grape varietals may be different. Furthermore, concentrations obtained in this study were within an expected range (*155, 156*).

Possible genetic differences between *V. vinifera* and *V. aestivalis* hybrids seemed to have a great impact on anthocyanin content. Previous studies have shown similar tendencies in anthocyanins content in *V. aestivalis* when compared to *V. vinifera* varietals (*157*). In addition, in Black Spanish grapes, since fresh grapes contained also anthocyanins, this may contribute to the total amount. In general, the anthocyanin content found in both *V. vinifera* and *V. aestivalis*, were comparable to the range reported in previous studies (*155, 157, 158*).

In grapes and other fruits rich in anthocyanins, studies have shown a relationship with high ORAC values (*159*). The ORAC values for red-skinned grapes were significantly higher than for Blanc Du Bois, and were in accordance with previous studies (*154, 155*). In other red-skinned varieties, antioxidant capacities have been reported in the range of 46.8 to 22.9 μmol TE/gr FW (*157, 160*) comparable to the range found in this study. The differences in total phenolics, monomeric anthocyanins, and ORAC values may be due to the extraction procedure, grape variety, vineyard location, growing conditions, and use of whole grapes or only parts of them in analysis.

Chemical Profile of Grape Non-Anthocyanin Polyphenolics

In grapes, most of the non-flavonoid compounds are present in the pulp, whereas flavonoids are located in the stems, seeds and skins (*19*). Under the analytical conditions used in this study, polyphenolics, which have been previously identified in grape varieties, were detected (*161-163*) for both *V. vinifera* and American hybrid grapes as shown in Tables 5-7.

The relative abundance and extraction method made it difficult to identify hydroxybenzoic and hydroxycinnamic acids. In addition, under the chromatographic conditions used, only gallic acid and *p*-coumaroyl hexose were confirmed for Blanc Du Bois and Black Spanish, respectively.

Flavan-3-ols are present in both, skins and seeds, but higher concentrations can be found in seeds (*19*); chromatographic coelution and relative low abundance made it difficult to obtain detailed information about procyanidin identities. The UV properties and the characteristic signals at *m/z* 577.0 ($[M - H]^-$) indicated the presence of procyanidin dimers, and previous studies on proanthocyanidins agreed that the *m/z* 577.0 ion is indicative of B type procyanidin dimers (*164, 165*), whereas signals at *m/z* 865.1 ($[M - H]^-$) were attributed to procyanidin trimers and signals at *m/z* 1153.0 ($[M - H]^-$) were attributed to procyanidin tetramers (*166-168*).

Flavonols are mainly present in glycosidic, glucoronic, or acylated forms which may, due to enzymatic processes during wine fermentation, hydrolysis, etc., be found as aglycones (*19*). No significant qualitative differences were observed between *V. vinifera* and American hybrid grapes for the flavonols investigated. Previous results have shown myricetin and quercetin derivates being the most common flavonols in grape skins (*169*).

In a study of white grape varieties from a warm climate, the main flavonol glycoside was quercetin glucoronide, whereas isorhamnetin glucoside was found in very low concentrations (*169*), which was similar to the results obtained for Blanc Du Bois. Furthermore, some other authors have shown that flavonols like isorhamnetin and myricetin glycosides are specific to red grapes (*170*) but not white; the singularity of

myricetin glycosides was supported by this study.

Moreover, by means of direct infusion onto the mass spectrometer, other compounds were tentatively identified in all grape varieties, which are in accordance with previous reports done with *V. vinifera* and non-vinifera varieties (*161, 163, 169*).

Lastly, although there is a remarkable difference between grapes with regard to their origin, including genetic background, this does not seem to be the most important factor in determining which polyphenols are present. Environmental factors such as light, soil content, temperature, degree of ripeness, etc (*156, 169*), may be more influential in determining the differences observed between *V. vinifera* and American hybrid grapes as evidenced by this study.

Chemical Profile of Grape Anthocyanins

Anthocyanins are responsible for the color of grape skins and an important determinant of red wine hues as well. Grapes from *V. vinifera* varieties like Cabernet Sauvignon, Merlot, Sangiovese, etc. contain only 3-*O*-glucoside forms of the five anthocyanins: delphinidin, cyanidin, petunidin, peonidin and malvidin according to reports (*43, 171-173*). In addition, they can be found as monoglucoside derivates of acetic acid, coumaric acid, and caffeic acid, which have been reported for these grape varieties as well (*173*). In this study, Cabernet Sauvignon and Merlot grapes, showed anthocyanin profiles similar to profiles previously reported (*161, 163*) from other regions; because the to grapes have a similar genetic background (*43*), this trend was expected. In consideration of relative anthocyanin abundance, malvidin-3-*O*-glucoside and its derivative forms of acetyl and coumaroyl were the most predominant, along with

the monoglucoside of peonidin, delphinidin, and petunidin. This tendency in anthocyanins relative abundance has been reported previously for wine grapes of *V. vinifera* varieties and wines (*43, 161*).

For Black Spanish grapes, a *V. aestivalis* hybrid, in addition to the 3-*O*-glucoside anthocyanins, they also contain 3,5-*O*-diglucosides, and additional sugar-moiety is attached at the 5 position of the A ring in the anthocyanidin structure (*174*). Likewise, they are known to contain acylated diglucoside derivatives of the five anthocyanins (*175*). These characteristics are mainly present in non-vinifera species, and have been reported previously for other grapes varieties like *Vitis rotundifolia* (*15, 176, 177*) and *Vitis labrusca* (*178*). The anthocyanin fractions of Black Spanish grapes contained these characteristic anthocyanins and were identified as delphinidin, cyanidin, petunidin, peonidin and malvidin 3,5-*O*-dilgucosides with parent ions at *m/z* 627.1 ($[M + H]^+$), *m/z* 611.0 ($[M + H]^+$), *m/z* 641.0 ($[M + H]^+$), *m/z* 625.0 ($[M + H]^+$),and *m/z* 655.2 ($[M + H]^+$), respectively. This was also found for 3,5-*O*-diglucosides of acetyl and coumaroyl derivatives (Table 7). Malvidin in its different moieties was the most abundant anthocyanin, which is in accordance with previous results in wine grapes varieties and in non-vinifera varieties (*171, 179*).

Different authors have suggested that flavonoid biosynthesis, in this case anthocyanins, is affected by a myriad of factors such as light, altitude, temperature, soil, and water (*161, 163, 169*).

CONCLUSION

Non-anthocyanin polyphenols didn't show qualitative differences. Both *V. vinifera* and American hybrids Black Spanish and Blanc Du Bois contained similar hydroxycinnamic acids, hydroxybenzoic acids, flavan-3-ols, and flavonols. It may be concluded that white grape Blanc Du Bois had similar qualitative composition to that of red grapes varieties in terms of non-anthocyanin polyphenols. Moreover, possible genetic differences and background between *V. vinifera* and *V. aestivalis* seems to strongly have influenced the anthocyanins profile. The most important difference was the presence of diglucoside forms of anthocyanins and anthocyanin-derivatives of acetyl and *p*-coumaric acid in *V. aestivalis*, which were not present in *V. vinifera* varieties.

Further studies need to be considered in order to fully characterize non-anthocyanin polyphenolics and quantify individual polyphenolics in American hybrid grapes. The selected American hybrid grapes hold potential for use in Texas viticulture and similar climates.

CHAPTER IV

EFFECT OF INDUSTRY-SCALE MICRO-OXYGENATION ON THE STABILITY OF ANTHOCYANINS IN TEXAS RED WINE

SUMMARY

Oxygen plays an important role throughout the vinification process and micro-oxygenation (MOX) is a technology dosing very small amounts of oxygen into the wine mimicking the oxygen transfer into wine through oak barrels. This study evaluated wine chemistry after industry-scale micro-oxygenation (MOX) was performed in one of the largest Texas wineries. MOX with oak inner staves, oak pieces in a stainless steel drum and American oak barrel were applied for 96 days in a red wine blend. Wine samples were characterized by means of HPLC-PDA-MS, and results showed the typical polyphenolic profile for non-anthocyanins and anthocyanins compounds. Malvidin-3-*O*-glucoside and its derivates were the most predominant anthocyanins with an average abundance of 90.1% in all wine treatments. In general, the anthocyanins content decreased in concentration for all treatments including the control. The control was the lowest (65.5%), followed by the treatment with the addition of oak pieces (53.2%), oak barrel (43.9%), and finally MOX (27.1%). Likewise, for some anthocyanins starting at day 24, concentrations decreased compared to the control. These changes were more evident at day 52.Moreover, at days 66, 94, and 96 of the MOX, anthocyanin concentrations were higher in all treatments compared to the control, potentially due to

difference in uncontrolled oxygen-uptake due to vessel-opening. The total phenolic content did not show significant differences over time in any of the treatments; hence oxygen did not seem to affect the total phenolic content directly, a finding which is consistent with previous studies.

INTRODUCTION

Oxygen is required and plays an important role throughout the vinification process (*180*). At early stages oxygen aids in the fermentation process and stabilizes color and aids in developing aromas during aging (*181*). Despite the beneficial effects of oxygen, an excess may lead to wine oxidation having negative effects such as higher astringency, color degradation, and adverse microbial activity (*59, 60*).

Micro-oxygenation (MOX) is a technology based on the dosage of small amounts of oxygen into wine within the same range as oxygen uptake that takes place in oak barrels (*56, 58, 60*). The objective is to accelerate wine-aging and improve color, aroma and texture of wines (*182*). MOX can be applied at any stage during winemaking, however best results are obtained after yeast fermentation and before malolactic fermentation (*57, 60, 61, 182*). MOX is being considered as a feasible option for some wineries to avoid the use of oak barrels for aging, and to reduce costs involved with raw materials, labor, and cellar space (*58*). MOX may have an impact on phenolic compounds, which in red wines are among the constituents responsible for color and taste (*60, 182*). Furthermore, the addition of oxygen during MOX, may contribute to the formation of new compounds, especially with anthocyanins and flavan-3-ols to form

more stable colored compounds through condensation, oxidation and polymerization reactions (*60, 62*). Oxygen may also participate in the oxidation of ethanol to acetaldehyde, which can react with flavonols to produce very reactive carbocation species that react with other flavonols or anthocyanins to produce new pigments and polymeric compounds via the formation of ethyl-linked adducts (*60, 63, 64*).

Traditionally, wines are placed in oak barrels to mature; this process gives the wine its color and organoleptic characteristics, which are influenced by several factors like geographical origin, oak species, toasting levels, aging time, etc (*53, 69*). During this time, wine polyphenolics react with oxygen and compounds extracted from oak wood to increase wine complexity and improve color stability (*53, 69*). However, in recent years new technologies have been introduced to accelerate the aging process. The uses of oak products (chips, inner staves, oak powder, etc) are becoming popular options in the market (*53, 55, 58*). The aging time is considerably reduced since the surface area of oak is increased compared to the oak barrel where the contact surface is no more than 40% (*55*). Accelerating aging techniques may be use in combination with MOX treatments to mimic the oxidation process that naturally occurs in oak barrels (*58, 70*). Several studies have addressed the effects of the addition of oak alone or in combination with MOX on chemical composition, sensory properties, and volatile profile of wines compared to traditional aging barrels (*53, 55, 56, 58, 69*).

The objective of this study was to evaluate the effects of MOX and the use of inner staves and oak shavings on the chemical profile of anthocyanins in an industry-scale operation at a commercial winery in Texas.

MATERIALS AND METHODS

Winemaking

A red wine blend consisted of Cabernet Franc 45%, Malbec 45%, and Merlot 10% was made following traditional winemaking practices and bought it by Llano Estacado Winery (Lubbock, TX). At the winery, malolactic fermentation was allowed to completion before MOX.

Experimental Design

The experimental design consisted of four treatments: a 55 gallons stainless steel drum (Control), a 55 gallon stainless steel drum with the addition of inner staves, the same type used in the micro-oxygenation tank (SSD+oak), a standard 59 gallons two years old American oak barrel medium toast (Demptos, Napa, CA) (Oak Barrel), and 3142 gallons tank with 30% oak portion in the inner stave (Innerstave, Sonoma, CA) (MOX), which was used previously one time. Winery laboratory staff was in charge of the sampling during the experiment that lasted approximately three months. Wine samples were taken in triplicate in a 750 mL bottle, then were immediately flushed with nitrogen, and stored at 4 ^{o}C until the need for chemical analysis.

Micro-oxygenation Procedure

A proprietary MOX procedure developed by Llano Estacado Winery (Lubbock, TX) was used during the experiment. In brief, sulfur dioxide was adjusted to 0.5 molecular before start wine MOX. The initial oxygen level was set to 10 mg/L/month using a micro-oxygenation unit model SAEN 400x/5 (PARSEC Srl, American Tartaric Products Inc. Windsor, CA).

The wine was monitored on a weekly basis for pH, SO_2 (free, molecular, and total), volatile acidity and sensory; and depending on the degradation of free SO_2 and sensory, oxygen concentration was kept or adjusted as follows: if SO_2 drops the first time below 0.3 molecular, then is adjusted again to 0.5 and the oxygen level is maintained to 10mg/L/month; when the SO_2 drops for the second time below 0.3 molecular, the oxygen level is reduced to 5 mg/L/month and the SO_2 is adjusted to 0.5 once again; the third time that SO_2 goes below 0.3 the oxygen level is reduced to 2 mg/L/month and molecular is adjusted once again to 0.5; finally when the SO_2 reaches 0.3 or below, the micro-oxygenation is stopped. During the micro-oxygenation process, tasting was guiding the procedure of MOX.

Extraction of Wine Polyphenols for HPLC-PDA-ESI-MS^n Analysis

A wine sample (100 mL) was diluted with acidified water (5X) prior fractionation. Wine polyphenols were isolated using solid phase extraction following manufacture's recommendations. Diluted samples were loaded onto the C_{18} Sep-Pak Vac cartridges (Waters Corporation, MA, USA) washed with water, and eluted first with ethyl acetate to remove non-anthocyanin compounds; then a second wash with acidified methanol was applied to remove the anthocyanin fraction. Both solvents were evaporated by rotary evaporation in a rotavapor (Büchi Labortechnik AG, Flawil, Switzerland) at 40 ^oC to dryness. The final samples were reconstituted with methanol and stored at -80 ^oC until further use. Prior analysis of anthocyanins, methanol was removed in a vacuum concentrator (Thermo Scientific, Savant ISS110 Speedvac concentrator, Waltham, MA) and the residue dissolved in an equivalent amount of acidified water.

HPLC-PDA-ESI-MS^n Analysis of Wine Polyphenols

Mass spectrometric analysis of the non-anthocyanin fraction was performed on a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source (ThermoFisher, San Jose, CA). The non-anthocyanin fraction was analyzed using a Sunfire C_{18} column (Waters Inc., Milford, MA) (250 x 4.6 mm, 5 μm) at a temperature of 25 ^oC. The chromatographic separation was performed with a mobile phase consisting of solvent A and B in gradient mode, where mobile phase A was 0.1% acetic acid (v/v) in water, and mobile phase B was 0.1% formic acid (v/v) in methanol. The gradient program with 0.4 mL/min was run as follows: 0 min 100%, A; 1 min 95%, A; 15 min 70%, A; 40 min 35%, A; 50 min 5%, A; 55 min 5%, A; 55.5 min 100%, A; 60 min 100%, A. Ionization was conducted in negative mode as follows: sheath gas (N_2), 40 units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 4.5 kV; capillary temperature, 300 °C; capillary voltage, 7 V; tube lens offset, 40 V. The detection wavelengths were set at 280 nm and 360 nm. The identification of wine polyphenols was carried out based on fragmentation pattern and wavelengths.

For the anthocyanin rich fraction, mass spectrometric analysis was performed using a Symmetry C_{18} column (Waters Inc., Milford, MA) (250 x 4.6 mm, 5 μm) at a temperature of 25 ^oC. The chromatographic conditions consisted of mobile phase A water/acetic acid/methanol (85:10:5), and mobile phase B of 0.1% formic acid (v/v) in methanol. A gradient program with 0.25 mL/min was run as follows: 0 min 95%, A; 10 min 80%, A; 30 min 50%, A; 35 min 25%, A; 55 min 25%, A; 56 min 95%, A; 59 min 95%, A. Ionization was conducted in positive mode as follows: sheath gas (N_2), 40

units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 4.5 kV; capillary temperature, 300 °C; capillary voltage, 3.3 V; tube lens offset, -60 V. The detection wavelength was set at 520 nm, and the identification was carried out based on fragmentation pattern and wavelengths.

Quantification of Anthocyanins by HPLC-PDA

A wine sample (25 mL) was diluted with acidified water (2X) prior to fractionation. Wine polyphenols were isolated using solid phase extraction (SPE) following manufacture's recommendations. Diluted samples were loaded onto the C_{18} Sep-Pak Vac cartridges (Waters Corporation, MA, USA) washed with water, and eluted with ethyl acetate to remove non-anthocyanin compounds; then acidified methanol was applied to remove the anthocyanin fraction. Both solvents were evaporated by evaporation in a rotavapor (Büchi Labortechnik AG, Flawil, Switzerland) at 40 ^{o}C to dryness. The final samples were reconstituted with methanol for the non-anthocyanin fraction and acidified water for the anthocyanin fraction and stored at 4 ^{o}C until further use.

Monomeric anthocyanins were quantified by HPLC-PDA using a standard of cyanidin-3-*O*-glucoside (ChromaDex, Irvine, CA), and reported as equivalents of cyanidin-3-*O*-glucoside. The chromatographic separation was performed in an Alliance 2695 system (Waters Inc., Milford, MA), and carried out in a using a Symmetry C_{18} column (Waters Inc., Milford, MA) (250 x 4.6 mm, 5 μm) at a temperature of 25 ^{o}C. The chromatographic conditions were of mobile phase A water/acetic acid/methanol (85:10:5), mobile phase B 0.1% formic acid (v/v) in methanol. A gradient program with

0.25 mL/min was run as follows: 0 min 95%, A; 15 min, 80% A; 30 min, 50% A; 35 min, 25% A; 55 min, 25% A; 55.5 min, 0% A; 64 min, 0% A; 64.5 min, 95% A; 70 min, 95% A. The detection wavelength was set at 520 nm.

Determination of Total Soluble Phenolics

Total soluble phenolics were determined by Folin-Ciocalteu assay (*152*) using a microplate reader FLUOstar (BMG Labtech Inc., Durham, NC) with absorbance read at 726 nm, and quantified by linear regression using a gallic acid standard and expressed as gallic acid equivalents (GAE).

Statistical Analyses

Data from each chemical analysis were analyzed by one-way-analysis of variance (ANOVA) using JMP version 8.0 (SAS Institute Inc., Cary, NC). Mean separations were deemed significant at $p \leq 0.05$ using a Tukey-Cramer HSD comparison for all pairs.

RESULTS

Chemical Profile of Non-Anthocyanin Polyphenolics

The chemical profile of the ethyl acetate fraction of a red wine containing non-anthocyanin polyphenolics was investigated by spectral characteristics and mass spectrometry analyses. The polyphenolics present in the ethyl acetate fraction are shown in Figures 17 A and B. Compounds in this fraction, were monitored at two wavelengths 280 nm and 360 nm, which corresponded namely to hydroxycinnamic and hydroxybenzoic acids, flavan-3-ols, and flavonols.

The red wine contained hydroxybenzoic acids, including gallic acid, protocatechuic acid, and syringic acid (Table 8). Gallic acid was the most abundant, followed by syringic acid. In addition of hydroxybenzoic acids, hydroxycinnamic acids were identified, which included *p*-coumaric acid hexoside, caffeic acid, and esters of tartaric acid such as caffeoyl tartaric acid, and *p*-coumaroyl tartaric acid, and ferouyl tartaric acid. The most abundant were caffeic, syringic, and *p*-coumaroyl tartaric acid.

Flavan-3-ols were found in the wine extract as well. (+)-Catechin and (-)-epicatechin were monomers identified, whereas a mixture of procyanidin polymers dimers, trimers, and tetramers were also detected. Specific identification of polymeric forms of procyanidins was no possible due to coelution of compounds and chromatographic conditions.

Five flavonols were identified in the red wine (Table 8). The main flavonols were laricitrin-*O*-hexoside, quercetin and myricetin derivatives of glucoronic acid, as well as the aglycon form of myricetin. A quercetin derivative, myricetin, and quercetin-3-*O*-glucoronide occurred in higher quantities.

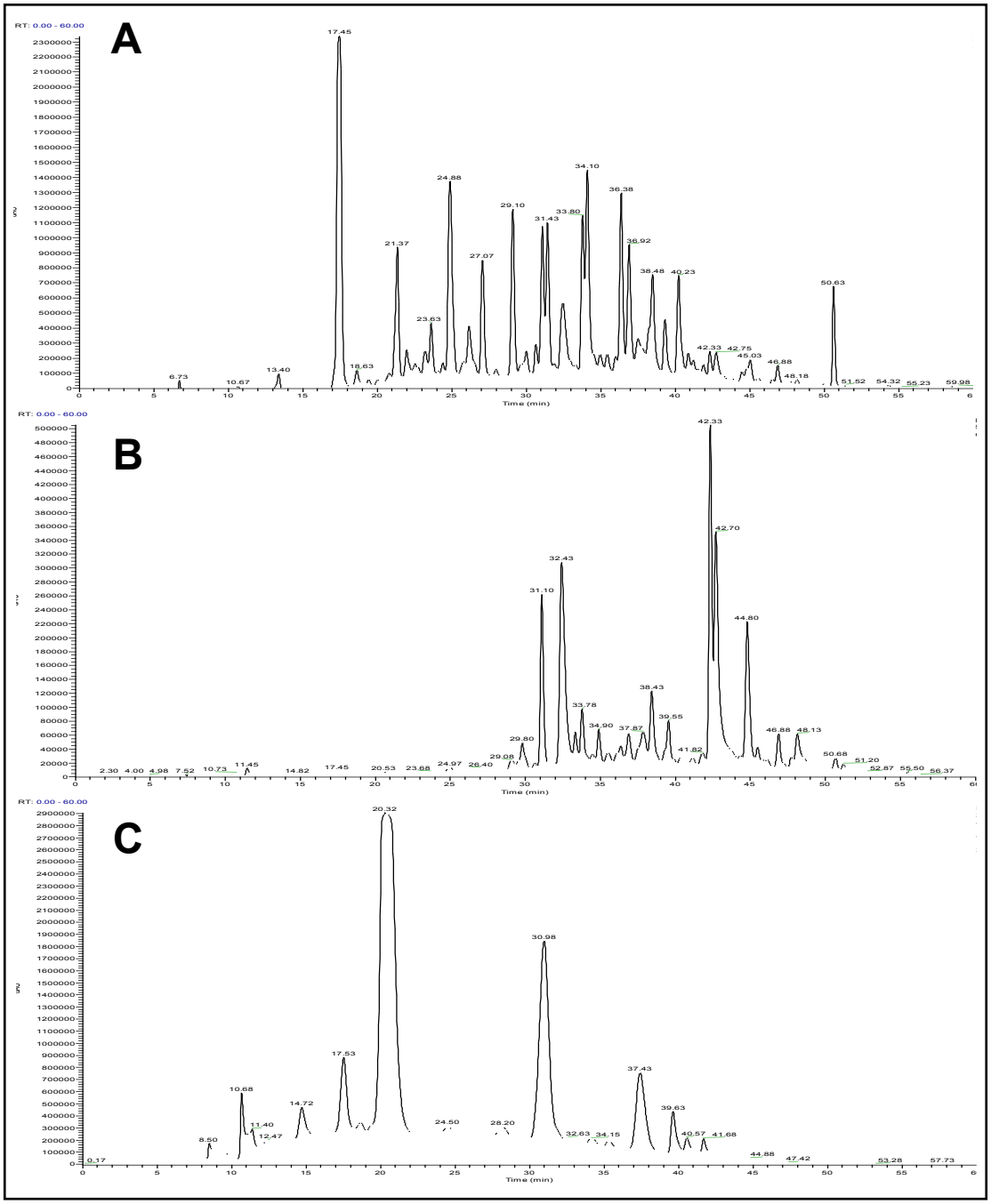


Figure 17. HPLC chromatograms of polyphenolic compounds present in a red wine. ***A,*** phenolic acids, flavan-3-ols, and procyanidins at 280 nm; ***B,*** flavonols at 360 nm, and ***C*****,** anthocyanins at 520 nm.

Table 8. Retention times, mass spectra by HPLC-MS-ESI-(-)-MS^n, and UV data of polyphenolics present in a red wine.

T_R (min)	Compound	λ_{max}	MS $[M - H]^-$ (*m/z*)[1]	MS^2 (*m/z*)
17.2	Prodelphinidin B3	278	593.1	466.9, 425.0, 407.1, 302.8, 289.0
17.5	Gallic acid	271	169.1	125.3
18.6	Catechin-gallocatechin	281	593.1	467.1, 440.8, 423.2, 305.0
21.5	Polymeric procyanidins	279	1153.0, **865.0,** 577.1	739.0, 694.9, 577.0
23.1	Polymeric procyanidins	279	1153.0, 865.1, **577.1**	450.9, 424.9, 407.1, 289.1
24.8	Polymeric procyanidins	279	1153.1, **577.1**	450.9, 424.9, 407.1, 289.1
24.9	(+)-Catechin	289	289.1	
26.2	Polymeric procyanidins	278	1153.0, **865.0**	695.0, 739.1, 577.0, 424.9
29.1	(-)-Epicatechin	279	289.1	
30.7	*p*-Coumaroyl hexose	311, 28sh	324.9	265.0, 243.9
31.1	Caffeic acid	323, 297, 247	179.0	135.3
31.4	Unknown	275	507.2	492.1, 327.0
32.5	Caffeoyl tartaric acid	329, 302sh, 249	310.8	179.1, 149.1
33.8	Unknown	291	510.8	467.2, 474.0, 331.1
34.1	Syringic acid	273	197.2	197.1, 169.1
36.9	*p*-Coumaroyl tartaric acid	310	294.9	163.1, 149.2
37.9	Ferouyl tartaric acid	311	324.9	192.9
39.3	Vanillic acid	289	167.3	123.2
39.6	Laricitrin-3-*O*-hexoside	286	493.0	331.0
41.5	Syringic aldehyde	294, 259	181.3	153.2, 151.9, 166.1
42.1	Myricetin-3-*O*-glucoronide	292, 379sh	493.0	317.0
42.4	Unknown	358, 254	507.2	387.1, 344.0
42.8	Myricetin	372, 305, 255	317.1	179.0, 151.1
45.0	Quercetin-3-*O*-glucoronide	357, 295sh, 257	477.0	301.0

[1]Ions in bold represent the most abundant ion on which further mass spectrometry analyses were conducted.

Chemical Profile of Anthocyanins

A large number of anthocyanins have been identified in red wines; in this study anthocyanins in the methanolic fraction were identified using HPLC-PDA-ESI-MS in positive mode. The chromatogram of the anthocyanin fraction is shown in Figure 17C, whereas detailed information about individual anthocyanins are shown in Table 9. The wine contained 4 conjugated forms of 3-*O*-glucoisde of delphinidin, petunidin, peonidin, and malvidin were identified. Likewise, only one conjugated form of 3,5-*O*-diglucoside was found for malvidin. The presence of acetyl glycoside derivates for delphinidin and malvidin were detected, in which the latter, was identified at two different retention times with same *m/z* ratios. Furthermore, five types of coumaroyl glycoside derivates were present for delphinidin, petunidin, peonidin, and malvidin; the latter showed two different retention times with same *m/z* ratio, which may be an indication of the isomer forms *cis* and *trans*. Finally, a caffeoyl glucoside derivate was identified for malvidin.

At least seven different pyranoanthocyanins were identified, which included vitisin B, vitisin B derivate of malvidin-3-*O*-(6-*O*-acetyl)-glucoside. In addition, vitisin A-type derivates of malvidin malvidin-3-*O*-(6-*O*-acetyl)-glucoside and malvidin-3-*O*-(6-*O*-*p*-coumaric)-glucoside were detected. Similarly, one vinylcatechol derivate of malvidin-3-*O*-(6-*O*-acetyl)-glucoside, and two vinylphenol pyranoanthocyanins of malvidin-3-*O*-(6-*O*-acetyl)-glucoside and malvidin-3-*O*-(6-*O*-*p*-coumaric)-glucoside were detected.

Table 9. Retention times, mass spectra by HPLC-MS-ESI-(+)-MS^n, and UV-vis data of anthocyanins present in a red wine.

T_R (min)	Compound	λ_{max}	MS $[M + H]^+$ (*m/z*)	MS^2 (*m/z*)
14.7	Delphinidin-3-*O*-glucoside	526, 340sh, 277	465.0	303.3
17.4	Petunidin-3-*O*-glucoside	527, 345sh, 276	479.3	317.1
18.7	Malvidin-3-*O*-glucoside-5-*O*-glucoside	529, 340sh, 276	655.0	331.2
19.3	Malvidin derivate	531, 277	657.0	495.0, 331.3
19.9	Peonidin-3-*O*-glucoside	516, 275	462.9	301.2
20.4	Malvidin-3-*O*-glucoside	527, 350sh, 272	493.1	331.1
23.6	Vitisin B	529, 280	517.1	355.2
24.1	Malvidin-3-*O*-(6-*O*-acetyl)-hexoside	530, 310sh, 280	535.0	331.1
24.5	Delphinidin-3-*O*-(6-*O*-acetyl)-glucoside	530, 310sh, 280	507.1	303.3
24.9	B-type vitisin of malvidin-3-*O*-(6-*O*-acetyl)-glucoside	530, 310sh, 280	559.1	355.1
26.8	Malvidin derivate	529, 305sh, 279	510.9	492.9, 348.9, 331.1
27.4	Malvidin derivate	529, 305sh, 279	985.0	823.0, 331.1
27.9	Malvidin-3-O-glucoside-8-ethyl-derivative catechin/epicatechin	530, 310sh, 279	809.0	646.9, 518.8, 357.2
28.2	Petunidin-3-*O*-(6-*O*-acetyl)-glucoside	530, 310sh, 279	521.1	317.1
29.4	Malvidin derivate	527, 280	553.0	348.8, 331.1
30.9	Malvidin-3-*O*-(6-*O*-acetyl)-hexoside	530, 345sh, 276	535.1	331.1
32.0	Malvidin-3-*O*-(6-*O*-acetyl)-glucoside pyruvate	520,279	603.0	399.1
32.6	Delphinidin derivate	528, 320sh, 281	611.2	303.3
33.3	Malvidin derivate	529, 320sh, 281	685.1	523.0, 477.1, 331.2
33.4	Malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside	529, 320sh, 281	655.2	493.0, 331.1
34.2	Malvidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside *cis*	533, 280	639.1	331.1
35.3	Petunidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside	529, 315sh, 281	625.1	317.1
35.5	Malvidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside-8-ethyl derivative catechin/epicatechin	526, 315sh, 281	955.0	664.2, 357.1
37.5	Malvidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside *trans*	532, 310sh, 282	639.1	331.1
38.0	A-type vitisin of malvidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside	520, 305sh, 281	707.0	399.1
38.9	Malvidin-3-*O*-(6-*O*-acetyl)-glucoside-4-vinylcatechol	520, 320sh, 280	667.1	463.2
39.7	Peonidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside	505, 271	609.0	447.0, 301.0
40.6	Malvidin-3-*O*-(6-*O*-acetyl)-4-vinylphenol	508, 277	651.0	447.1
41.7	Malvidin-3-*O*-(6-*p*-coumaroyl)-4-vinylphenol	508, 278	755.0	447.1

Possible acetaldehyde-mediated condensation products were detected for malvidin and identified as possibly malvidin-3-*O*-glucoside-8-ethyl-epicatechin and malvidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside-8-ethyl-epicatechin.

Other anthocyanins were detected at different retention times, but not fully identified. Based on the presence of the major fragment ion in the MS^2 at *m/z* 303.0 and *m/z* 331.0 indicated that these compounds might be delphinidin and malvidin derivatives, respectively (*183*).

Quantification of Monomeric Anthocyanins and Total Soluble Phenolics

Individual and total monomeric anthocyanins were quantified throughout the MOX duration of 96 days. However, some of the aforementioned anthocyanins were minor compounds that could not be quantified, and some others co-eluted, and were quantified combined. As expected, malvidin-3-*O*-glucoside and malvidin derivates were the most predominant anthocyanins with an average abundance of 90.1%, followed by petunidin derivates with 6.2%, dephinidin-3-*O*-glucoside with 1.98%, and penoidin-3-*O*-(6-*p*-coumaroyl)-glucoside with 1.62%.

The control wine stored in a stainless steel drum showed a significant decrease in most of the individual anthocyanins as shown in Table 10. Malvidin-3-*O*-glucoside and malvidin-3-*O*-(6-*O*-acetyl)-hexoside, were the most abundant with initial concentrations of 91.37 and 14.31 μg/mL, respectively.

Table 10. Individual and total monomeric anthocyanins in red wine stored in a stainless steel drum (control).

Anthocyanins (μg/mL)	Time						
	0 days	**13 days**	**24 days**	**52 days**	**66 days**	**94 days**	**96 days**
Dp-3-gluc	2.60±0.05a[1]	1.96±0.12bc	2.29±0.12ab	2.33±0.09ab	1.66±0.14c	0.83±0.00d	0.79±0.04d
Pt-3-gluc	6.37±0.08a	4.79±0.13b	4.81±0.22b	5.03±0.08b	3.97±0.14c	2.53±0.02d	2.33±0.05d
Mv-3,5-digluc	0.59±0.03a	0.51±0.02ab	0.50±0.03ab	0.52±0.02ab	0.43±0.03b	0.29±0.00c	0.25±0.01c
Pn/Mv-3-gluc	91.37±1.66a	68.75±1.37b	66.71±2.51b	70.59±0.61b	54.02±2.50c	30.24±0.24d	28.48±0.31d
Dp/Mv-3-acet-hex	0.51±0.02a	0.34±0.01bcd	0.40±0.02abc	0.38±0.01abc	0.20±0.05d	0.28±0.00cd	0.45±0.05ab
Pt-3-acet-gluc	1.24±0.04a	0.84±0.01bc	0.82±0.05bc	0.89±0.02b	0.72±0.03cd	0.61±0.00d	0.64±0.01d
Mv-3-acet-hex	14.31±0.52a	10.03±0.32b	9.27±0.49bc	10.18±0.24b	7.83±0.54c	4.65±0.01d	4.72±0.06d
Pt-3-coum-gluc	0.47±0.02a	0.33±0.02b	0.33±0.04b	0.30±0.01b	0.30±0.04b	0.27±0.01b	0.27±0.01b
Mv-3-coum-gluc *trans*	7.25±0.23a	4.67±0.33b	4.30±0.53bc	5.18±0.13b	3.56±0.48bcd	2.26±0.98d	2.85±0.26cd
Pn-3-coum-gluc	2.21±0.02a	1.98±0.09a	2.10±0.17a	2.28±0.02a	2.17±0.20a	1.85±0.05a	2.27±0.02a
Mv-3-acet-4-vp	0.61±0.01a	0.49±0.04a	0.53±0.06a	0.59±0.01a	0.55±0.07a	0.44±0.01a	0.61±0.01a
Mv-3-coum-4-vp	0.39±0.01a	0.27±0.03a	0.30±0.06a	0.40±0.01a	0.29±0.07a	0.23±0.02a	0.37±0.01a
Total anthocyanins	127.93±2.55a	94.93±2.32b	92.33±4.26b	98.71±1.12b	75.67±4.27c	44.47±0.57d	44.09±0.63d

[1]Data represent average values ± standard error from triplicate measurements; values with different letters between columns represent a significant difference (Tukey-Kramer HSD, $p < 0.05$). Dp, delphinidin; Pt, petunidin; Pn, peonidin; Mv, malvidin; digluc, diglucoside; hex, hexoside; gluc, glucoside; acet, acetyl; coum, *p*-coumaroyl; vp, vinylphenol.

Whereas a form of malvidin-3-*O*-(6-*O*-acetyl)-hexoside together with delphinidin-3-*O*-(6-*O*-acetyl)-hexoside along with petunidin-3-*O*-(6-*p*-coumaroyl)-glucoside and malvidin-3-*O*-(6-*p*-coumaroyl)-4-vinylphenol, were the lowest with initial concentrations of 0.51, 0.47, and 0.39 μg/mL, respectively.

Anthocyanins with major significant decreases over time were delphinidin-3-*O*-glucoside, peonidin/malvidin-3-*O*-glucoside, and malvidin-3-*O*-(6-*O*-acetyl)-hexoside with up to 69.5%, 68.8% and 67.0% decrease, respectively. However, anthocyanins such as peonidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside, malvidin-3-*O*-acetyl-4-vinylphenol, and malvidin-3-*p*-coumaroyl-4-vinylphenol did not show significant losses over time. For monomeric anthocyanins, two major ones were identified, one at early stages (day 13) with 25.8% of loss compared to the initial time, and on day 94 with 41.2% reduction compared to the previous date. Overall there was a 65.5% reduction in anthocyanins.

The wine stored in an American oak barrel showed similar abundance of major anthocyanins and tendencies in anthocyanin losses over time as the control wine (Table 11). Losses were below 50% for the major anthocyanins like delphinidin-3-*O*-glucoside (44.0%), petunidin-3-*O*-glucoside (39.6%), peonidin/malvidin-3-*O*-glucoside (44.0%), malvidin-3-*O*-(6-*O*-acetyl)-hexoside (46.6%), and *trans*-malvidin-3-*O*-(6-*p*-coumaroyl)-glucoside (49.8%). Moreover, as in the control wine, anthocyanins like peonidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside, malvidin-3-*O*-acetyl-glucoside-4-vinylphenol, and malvidin-3-*O*-*p*-coumaroyl-4-vinylphenol, did not show significant differences over time.

Table 11. Individual and total monomeric anthocyanins in a red wine stored in an American oak barrel.

Anthocyanins (μg/mL)	Time						
	0 days	13 days	24 days	52 days	66 days	94 days	96 days
Dp-3-gluc	2.52±0.04a[1]	1.78±0.10c	2.18±0.02b	2.09±0.09bc	1.79±0.02c	1.41±0.07d*	1.41±0.04d*
Pt-3-gluc	6.03±0.10a	4.53±0.18bc	4.64±0.07b	4.41±0.10bc*	4.09±0.06cd	3.69±0.14d*	3.64±0.03d*
Mv-3,5-digluc	0.57±0.03a	0.49±0.01ab	0.47±0.00b	0.43±0.02bc*	0.42±0.00bc	0.41±0.03bc*	0.37±0.02c*
Pn/Mv-3-gluc	92.00±0.72a	66.76±3.23b	65.73±0.80bc	61.36±1.62bc*	58.62±0.99cd	51.90±2.01d*	50.95±0.39d*
Dp/Mv-3-acet-hex	0.52±0.02a	0.37±0.02bc	0.37±0.01b	0.35±0.01bc	0.32±0.00bcd	0.31±0.03cd	0.29±0.01d*
Pt-3-acet-gluc	1.21±0.05a	0.80±0.04b	0.82±0.02b	0.79±0.01b*	0.74±0.03b	0.75±0.05b	0.72±0.02b*
Mv-3-acet-hex	13.87±0.16a	9.07±0.46bc	9.54±0.13b	8.85±0.24bc*	8.29±0.18bcd	7.86±0.44cd	7.41±0.09d*
Pt-3-coum-gluc	0.41±0.01a*	0.26±0.03b	0.27±0.01b	0.27±0.01b*	0.23±0.01b	0.23±0.03b	0.20±0.00b*
Mv-3-coum-gluc *trans*	6.24±0.06a*	4.25±0.41b	4.49±0.22b	4.19±0.08bc*	3.71±0.10bc	3.71±0.31bc	3.13±0.14c
Pn-3-coum-gluc	1.97±0.02a*	1.97±0.14a	2.03±0.07a	2.03±0.04a*	1.97±0.05a	2.13±0.14a	1.86±0.07a*
Mv-3-acet-4-vp	0.53±0.01a*	0.47±0.05a	0.50±0.03a	0.50±0.02a*	0.47±0.01a	0.55±0.05a	0.44±0.02a*
Mv-3-coum-4-vp	0.29±0.01a*	0.26±0.05a	0.28±0.03a	0.28±0.03a*	0.26±0.01a	0.35±0.05a	0.23±0.02a*
Total anthocyanins	126.15±0.84a	91.00±4.69b	91.32±1.15b	85.54±2.11b*	80.90±1.44bc	73.31±3.20c*	70.68±0.71c*

[1]Data represent average values ± standard error from triplicate measurements; values with different letters between columns represent a significant difference (Tukey-Kramer HSD, $p< 0.05$). Dp, delphinidin; Pt, petunidin; Pn, peonidin; Mv, malvidin; digluc, diglucoside; hex, hexoside; gluc, glucoside; acet, acetyl; coum, *p*-coumaroyl; vp, vinylphenol. Values with an asterisk (*) mean significant difference when compared to control (Tukey-Kramer HSD, $p< 0.05$).

When comparing the American oak barrel with the control, no significant differences were observed up to 24 days after experiment begin. However, on day 52, a significant trend was observed towards a decrease in anthocyanins in the oak barrel. In contrast, on days 94 and 96, there was significant retention of the major and more abundant anthocyanins in the oak barrel when compared to the control.

Significant differences in concentrations of monomeric anthocyanins were noted after day 13 with 27.8% decrease in anthocyanin content compared to the initial concentration, followed by a stable period with no significant decrement, to reach 43.9% in loss after 96 days.

In order to analyze the effects of oak on red wine anthocyanins, oak pieces made from staves were added into a stainless steel drum, keeping the oak/wine proportion similar as in the MOX tank. Similar tendencies were detected for the most abundant anthocyanins as well as the ones in lower concentrations (Table 12) when compared to previous treatments (control and oak barrel). In this case, for the most abundant anthocyanins like peonidin/malvidin-3-*O*-glucoside, malvidin-3-*O*-(6-*O*-acetyl)-hexoside, *trans*-malvidin-3-*O*-(6-*p*-coumaroyl)-glucoside, and delphinidin-3-*O*-glucoside the reduction in concentrations went up to 55.1%, 52.6%, 50.1%, and 42.3% respectively. Likewise, as in previous treatments, vinylphenol anthocyanins, such as malvidin-3-*O*-acetyl-glucoside-4-vinylphenol, and malvidin-3-*O*-*p*-coumaroyl-4-vinylphenol, reductions were below 10%, when compared to the other anthocyanins with reductions up to 69% for delphinidin/malvidin-3-*O*-(6-*O*-acetyl)-hexoside.

Table 12. Individual and total monomeric anthocyanins in red wine stored in a stainless steel drum with oak pieces.

Anthocyanins (μg/mL)	Time						
	0 days	13 days	24 days	52 days	66 days	94 days	96 days
Dp-3-gluc	2.56±0.14a[1]	1.35±0.03d*	1.55±0.03bcd*	1.77±0.07bc*	1.75±0.04bc	1.85±0.04b*	1.48±0.03cd*
Pt-3-gluc	6.31±0.13a	4.24±0.09bcd*	4.38±0.09bc*	4.53±0.07b*	3.84±0.06d	4.04±0.06cd*	3.27±0.7e*
Mv-3,5-digluc	0.59±0.01a	0.45±0.03b	0.47±0.05b	0.41±0.02b*	0.25±0.01c*	0.28±0.00c	0.23±0.01c*
Pn/Mv-3-gluc	93.52±2.88a	63.69±1.33b	65.32±1.63b	65.71±1.43b*	51.51±0.61c	53.81±1.08c*	41.90±1.03d*
Dp/Mv-3-acet-hex	0.48±0.02a	0.25±0.02c	0.29±0.02c*	0.38±0.01b	0.22±0.01cd	0.16±0.00d*	0.15±0.01d*
Pt-3-acet-gluc	1.20±0.03a	0.76±0.03b	0.79±0.03b	0.85±0.04b	0.40±0.01c	0.43±0.01c*	0.39±0.01c*
Mv-3-acet-hex	13.71±0.28a	9.54±0.27bc	9.54±0.43bc	9.72±0.35b	8.05±0.15d	8.20±0.16cd*	6.50±0.20e*
Pt-3-coum-gluc	0.44±0.02a	0.25±0.01bc	0.26±0.02bc	0.27±0.02b*	0.21±0.01bc	0.22±0.00bc	0.20±0.00c*
Mv-3-coum-gluc *trans*	6.36±0.54a	4.55±0.06bc	4.49±0.57bc	5.09±0.26ab	3.74±0.17bc	3.95±0.03bc	3.17±0.13c
Pn-3-coum-gluc	2.08±0.10ab*	1.96±0.05ab	2.11±0.12ab	2.12±0.06a	1.76±0.05b	1.79±0.03ab	1.88±0.03ab*
Mv-3-acet-4-vp	0.56±0.04a	0.47±0.02abcd	0.53±0.05abc	0.54±0.02ab	0.40±0.02cd	0.39±0.02d	0.42±0.02bcd*
Mv-3-coum-4-vp	0.31±0.04a	0.23±0.02a	0.32±0.05a	0.33±0.02a	0.25±0.03a	0.25±0.01a	0.28±0.01a*
Total anthocyanins	128.12±3.35a	87.73±1.70b	90.03±3.00b	91.72±2.36b	72.39±1.01c	75.37±1.26c*	59.86±1.55d*

[1]Data represent average values ± standard error from triplicate measurements; values with different letters between columns represent a significant difference (Tukey-Kramer HSD, $p< 0.05$). Dp, delphinidin; Pt, petunidin; Pn, peonidin; Mv, malvidin; digluc, diglucoside; hex, hexoside; gluc, glucoside; acet, acetyl; coum, *p*-coumaroyl; vp, vinylphenol. Values with an asterisk (*) mean significant difference when compared to control (Tukey-Kramer HSD, $p< 0.05$).

A comparison to the control wine demonstrated some significant differences starting at day 24, in which concentrations for delphinidin-3-*O*-glucoside and petunidin-3-*O*-glucoside were higher in the stainless drum with oak, whereas the most abundant peonidin/malvidin-3-*O*-glucoside was present in lower concentration compared to the control. Similar trends were observed at days 94 and 96. Total monomeric anthocyanins showed at least three significant decreases in concentrations; during the early stage (day 13) for the control and oak barrel with 31.5% when compared to the initial, the second at day 66 with 21% when compared to previous date, and finally a third one at day 96 with 20.5% when compared to previous date. Overall total anthocyanins decreased 53.2%.

In the treatment of MOX along with oak inner staves malvidin-3-*O*-glucoside and all its conjugated forms were the most predominant with an average abundance of 95.5% (Table 13).

In general, major individual anthocyanins like delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin/malvidin-3-*O*-glucoside, and malvidin-3-*O*-(6-*O*-acetyl)-hexoside, showed losses of 6.6%, 25.4%, 31.5%, 22.4%, respectively. Furthermore, when compared with the other treatments, MOX caused the least reduction in anthocyanin concentrations in this treatment.

Table 13. Individual and total monomeric anthocyanins in red wine during micro-oxygenation (MOX) and oak inner staves.

Anthocyanins (μg/mL)	Time						
	0 days	13 days	24 days	52 days	66 days	94 days	96 days
Dp-3-gluc	2.33±0.16a[1]	2.14±0.05a	2.32±0.05a	2.39±0.05a	2.12±0.07a*	2.27±0.05a*	2.17±0.03a*
Pt-3-gluc	6.00±0.05a*	4.75±0.15bc	4.93±0.05b	4.75±0.05bc*	4.21±0.05d	4.61±0.06bc*	4.48±0.07cd*
Mv-3,5-digluc	0.58±0.00a	0.38±0.01bc*	0.38±0.01bcd*	0.39±0.01b*	0.31±0.03d	0.37±0.02bcd*	0.32±0.01cd*
Pn/Mv-3-gluc	89.11±0.48a	66.40±2.61bc	67.97±0.55b	65.14±1.49bc*	57.28±0.56d	62.51±0.81bcd*	61.07±0.75cd*
Dp/Mv-3-acet-hex	0.48±0.01a	0.23±0.02a*	0.28±0.02a*	0.24±0.02a*	0.25±0.01a	0.27±0.03a	0.27±0.01a*
Pt-3-acet-gluc	1.14±0.02a	0.60±0.04b*	0.61±0.01b*	0.60±0.04b*	0.55±0.01b*	0.60±0.01b	0.61±0.02b
Mv-3-acet-hex	12.18±0.14a*	10.06±0.45b	10.26±0.17b	10.05±0.29b	8.78±0.10c	9.51±0.15bc*	9.46±0.20bc*
Pt-3-coum-gluc	0.35±0.01a*	0.28±0.03a	0.30±0.03a	0.29±0.04a	0.24±0.04a	0.29±0.01a	0.28±0.02a
Mv-3-coum-gluc *trans*	5.06±0.16a*	5.59±0.48a	5.85±0.27a	5.70±0.49a	4.76±0.11a	5.27±0.10a	5.34±0.39a*
Pn-3-coum-gluc	1.93±0.02b*	2.23±0.14ab	2.28±0.07a	2.28±0.13ab	2.18±0.03ab	2.23±0.04ab	2.27±0.08ab
Mv-3-acet-4-vp	0.51±0.00a*	0.57±0.05a	0.63±0.03a	0.57±0.04a	0.55±0.01a	0.57±0.01a	0.60±0.04a
Mv-3-coum-4-vp	0.26±0.01b*	0.43±0.08ab	0.54±0.05a*	0.49±0.08ab	0.43±0.02ab	0.45±0.03ab	0.53±0.07a*
Total anthocyanins	119.94±0.90a*	93.67±4.03b	96.45±1.12b	92.79±2.59b	81.65±0.87c	88.94±1.23bc*	87.40±1.60bc*

[1]Data represent average values ± standard error from triplicate measurements; values with different letters between columns represent a significant difference (Tukey-Kramer HSD, $p< 0.05$). Dp, delphinidin; Pt, petunidin; Pn, peonidin; Mv, malvidin; digluc, diglucoside; hex, hexoside; gluc, glucoside; acet, acetyl; coum, *p*-coumaroyl; vp, vinylphenol. Values with an asterisk (*) mean significant difference when compared to control (Tukey-Kramer HSD, $p< 0.05$).

When comparing MOX and control treatment, some differences become evident after day 52, MOX wine exhibited significant lower anthocyanins concentrations specifically for peonidin/malvidin-3-*O*-glucoside. Moreover, by days 94 and 96 this tendency was reversed, wine treated with MOX had significantly higher concentrations for most of the anthocyanins.

Likewise, during the MOX treatment, total monomeric anthocyanins were significantly decreased after 13 days with nearly 22% reduction when compared to day zero; during the rest of the time anthocyanins concentrations were kept at similar concentration levels reaching at the end losses of 27.1%.

The total anthocyanin content in wines evaluated during the MOX study is summarized in Figure 18. In all treatments, monomeric anthocyanins decreased where wine control was the lowest followed by the treatment with the addition of oak pieces, oak barrel, and finally MOX.

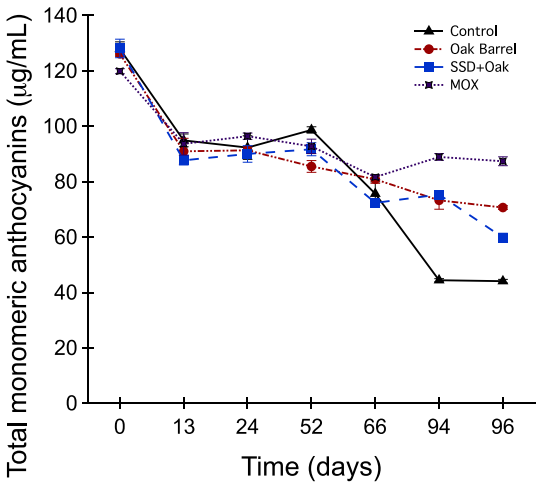


Figure 18. Total monomeric anthocyanins in red wines through time.

Total soluble phenolics were determined for all wine treatments as shown in Table 14. The concentration of total phenolics did not show significant differences over time for any of the treatments evaluated and the concentrations were approximately ~2200 μg GAE/mL. Likewise, when treatments were compared to the control wine, no significant differences were present.

Table 14. Total soluble phenolics in red wines in μg GAE/mL[1].

Time (days)	Control	Oak Barrel	SSD+Oak[2]	MOX[3]
0	2266.77±53.57a[4]	2224.54±23.69a	2276.99±1.03a	2287.26±27.71a
13	2165.82±82.41a	2256.47±4.12a	2274.93±48.29a	2267.74±14.38a
24	2260.59±2.06a	2219.38±51.51a	2253.35±20.55a	2328.36±13.36a*
52	2339.91±7.21a	2267.80±19.57a	2280.07±39.04a	2270.82±40.07a
66	2331.67±56.66a	2181.27±151.43a	2256.44±33.91a	2188.62±5.14a
94	2289.43±10.30a	2332.70±30.90a	2279.04±11.20a	2309.86±54.46a
96	2271.92±21.63a	2333.73±13.39a	2249.24±51.37a	2249.24±53.43a

[1]Quantified as equivalents of gallic acid. [2]SSD+Oak: Stainless steel drum with oak pieces. [3]MOX: Micro-oxygenation treatment with oak inner staves. [4]Data represent average values ± standard error from triplicate measurements; values with different letters between rows in the same column represent a significant difference (Tukey-Kramer HSD, $p < 0.05$). Values with an asterisk (*) mean significant difference when compared to control (Tukey-Kramer HSD, $p < 0.05$).

DISCUSSION

Characterization of Wine Non-Anthocyanin Polyphenolics

In this study, structural information and compound identification were obtained by means of mass spectrometric analyses. Non-anthocyanin polyphenolics that have been previously identified in wine grapes and red wines were present as well (Table 8) (*76, 161-163, 183-185*). The relative low abundance, extraction method, and the wine made it difficult to identify hydroxybenzoic and hydroxycinnamic acids. Of the ones

identified, gallic acid was most predominant, confirmed by previous studies (*161, 184*), along with caffeic, coumaric, and cinnamic acids and its derivates (*184*).

Flavan-3-ols are located in both, skins and seeds, but higher concentrations are found in seeds (*19*). During the winemaking process, time of contact with the must, temperature, pressing the skin grapes, etc; affects the extraction of flavan-3-ols and the final concentration in the wines (*185*). In this study, trace amounts of prodelphinidin B3 were detected with its characteristic *m/z* signal at 593.1 ($[M - H]^-$) and MS^2 fragmentation ions at *m/z* 466.9, 425.0, 407.1, 302.8 and 289.0. Likewise with same *m/z* signal at 593.1 ($[M - H]^-$) but a different retention time, a possible dimer of catechin-gallocatechin was detected with MS^2 ions at 467.1, 440.8, 423.2 and 350.0, which are accordance with previous wine reports (*183*). In addition, coelution made it difficult to obtain detailed information about procyanidins identities. The UV properties and the characteristic signals at *m/z* 577.0 ($[M - H]^-$) indicated the presence of procyanidin dimers, and previous studies on proanthocyanidins agreed that the *m/z* 577.0 ion is an indicative of B type procyanidin dimers (*164, 165*), whereas signals at *m/z* 865.1 ($[M - H]^-$) were attributed to procyanidin trimers and signals at *m/z* 1153.0 ($[M - H]^-$) were attributed to procyanidin tetramers (*166-168*).

Flavonols may be present in glycosidic, glucoronic, or acylated forms, which may be cleaved enzymatically during fermentation, hydrolysis, and aging (*19, 22*). Previous studies in wines have shown myricetin and quercetin derivates are the most common flavonols (*169, 184*), which is in accordance with the results obtained in this study, where myricetin glucoronide was identified at *m/z* 493.0 ($[M - H]^-$), myricetin at

m/z 317.1 ([M - H]$^-$), and quercetin glucoronide at *m/z* 477.0 ([M - H]$^-$). Differences in the non-anthocyanin polyphenolics found in this study, compared to previous reports may be also be attributed to grape origin, environmental conditions like soil type, temperature, water, etc (*156, 169*).

Characterization of Wine Anthocyanins

In red wines, anthocyanins are key elements for color development and play an important role for quality aspects especially in young wines (*61*). The red wine blend analyzed in this study, made from *V. vinifera* varieties (Cabernet Franc, Malbec, and Merlot) contained the characteristic 3-*O*-monoglucosides and the 3-*O*-acetylated monoglucosides (acetyl, *p*-coumaroyl, and caffeoyl) of the main anthocyanins delphinidin, petunidin, peonidin, and malvidin; where UV-vis spectra, molecular and fragments ions *m/z* have been widely reported in red wines (*19, 43, 61, 161, 172, 186*). Moreover, malvidin-3-*O*-glucoside, and its acylated forms (acetyl and coumaroyl), which represent more than 88% of the total anthocyanin content in the wine, was found as previously determined in wines made of *V. vinifera* (*43, 161*).

Malvidin-3-*O*-glucoside acylated with *p*-coumaric acid, was found in isomers forms *cis* and *trans*, and based on previous work, since hydroxycinnamic acids may exist in both cis and trans conformations, the only tentative form to identify in between them was base on retention times, *cis* isomer eluted before the *trans* isomer (*187*).

As common in *V. aestivalis* derived grapes, the wine contained small amounts of malvidin-3,5-*O*-diglucoside. These anthocyanins have been found in other wine varieties at less than 0.5% (*186*), which was within the same range found in this study. Different

authors have suggested that flavonoid biosynthesis, in this case anthocyanins, is affected by many factors such as environmental conditions including light, altitude, temperature, soil, water (*161, 163, 169*).

In red wine, pyranoanthocyanins were identified, which are formed when a pyrano-ring is introduced between the C4 and the hydroxyl group attached to the C5 in the anthocyanin molecule (*61, 62*). In this study, two types of pyranoanthocyanins were detected, the ones that are classified as A-type vitisins, which are formed by the addition of pyruvic acid, and the B-type vitisins or vinyl adducts resulting by the cycloaddition of acetaldehyde to malvidin-3-*O*-glucoside (*61, 62, 65*). The pyranoanthocyanins found in this wine have also been identified in previous work (*61, 62, 65, 186*). According to some authors, these compounds are important to color stability in red wine, since the cycloaddition increases anthocyanin-stability to changes in pH and sulfites (*61, 188*).

Other types of anthocyanins, the acetaldehyde-mediated, were detected in all treatments but were not quantified in red wine due to low concentrations. These compounds, some authors suggest, are formed by direct condensation of acetaldehyde with malvidin-3-*O*-glucoside and/or catechin and epicatechin, which yields an ethyl bridge-linked compound (*61*). The acetaldehyde-mediated anthocyanins found in this study, have previously been identified in wines and are also resistant to changes in pH and sulfites (*61, 66, 189*).

Quantification of Monomeric Anthocyanins and Total Soluble Phenolics

During winemaking, oxygen takes part in reactions in every step of the vinification process; involving the early stages in yeast fermentation through wine aging (*182*). In this study, the reaction of oxygen in reaction with anthocyanins was observed, since individual and total anthocyanins decreased over time, although it was not the objective of this project to identify types of reactions. Based on these results, the first decrease in anthocyanins concentration at day 13, for all treatments, may be due to oxygen uptake during the process of initiating treatments, since wines needed to be transfer to their respective vessels and the MOX tank. In addition, the oxygen uptake may have initiated reactions of anthocyanin reactions with other phenolics (*60, 61, 190*).

The control wine, exhibited slightly higher anthocyanin concentrations compared to the other treatments including MOX, as previously reported (*60-62, 190*). In addition, at the last two dates sample days, the possibility of a higher oxygen concentration inside the tank due to increasing headspace may have caused increased decrease in anthocyanin concentrations. Previous studies have shown that control wines often exhibited higher anthocyanins concentration when compared to other treatments evaluated such as MOX (*60, 182, 191*), in which this case was the contrary, excess in oxygen on the system lead to potential wine oxidation.

In the case of American oak barrel, the major source of oxygen occurred during the filling and storage time, where the oxygen that passed through the wood porous and timbers, as well as every time the barrel was opened to take wine samples. As shown in Table 11, anthocyanins decreases were progressive without any drastic changes over

time. Studies have reported that in oak barrels, type of oaks used is a key factor on oxygen transport (*63*); and that the age of the barrel also affects oxygen permeation since wine deposits can plug the wood porous (*65*). The changes in anthocyanins concentrations exhibited over time may be attributed more to the opening rather than the oxygen permeation, since the permeation rate is quite low and it has been reported for French oak barrels in the range of 2.5 to 20 mL of O_2/L of wine per year (*63*). Furthermore, one possible beneficial aspect at the latest stages of the experiment was the potential interaction of polyphenolic compounds extracted from the oak barrel; which may help to retain anthocyanins through copigmentation phenomena (*192, 193*). This phenomenon could be associated with anthocyanins retention was higher at day 94 and 96 when compared to the control, which did not contain compounds to protect anthocyanins.

In the stainless steel drum with oak chips, the only possible source of oxygen assumed during the experiment was during the opening to take the wine samples and the oxygen that was dissolved already in the wine. Previous studies have demonstrated that artificial systems for wine aging, the type of oak and selection is fundamental for wine evolution (*54, 194, 195*). In this study, the treatment with oak chips displayed similar but slightly higher anthocyanin reduction compared to the American oak barrel treatment. Some authors suggest that the evolution of anthocyanins and low molecular polyphenols aged in different wine systems, such as the addition of oak pieces experienced much rapid aging and greater rate of polymerization than the wine aged in barrels (*54, 194, 195*). This phenomenon was also observed in this study mainly after day 66, in which the

stainless steel drum with oak showed lower anthocyanin content and it was evident at the end of the treatment that this may be due to polymerization reactions of anthocyanins (*54*). It may be assumed that an excess of oxygen due to the headspace in the tank, contributed to have a more reduced anthocyanins concentration. As in the oak barrel, possible extraction of polyphenolics from the oak pieces, may contribute to promote molecular association with through copigmentation helping to retain or stabilize anthocyanins (*192, 193*).

Studies have shown that MOX wines tend to have an overall lower anthocyanins content over time when compared with their controls (*60, 190*), but in this work this was not always the case. Only some individual anthocyanin monoglucosides exhibited significant lower concentrations when compared to the no MOX counterpart, which was more evident at day 56. Even though oxygen was administrated and controlled in a regular basis, it was expected to have greater impact on anthocyanins as previously reported (*60, 61, 69, 190*). One of the main factors attributed to these results, may be the vessel size and the better control of headspace in the tank than in the barrels.

In previous studies, MOX treatments with or without the addition of oak products have shown the formation of new anthocyanin compounds due to the reaction of oxygen with anthocyanins, in which vitisins compounds and anthocyanin-ethyl-flavan-3-ols showed lower concentrations in control wines, whereas wines treated with MOX there was an increase in concentrations (*61, 190*). In this study, these observations were not made in general, however pyranoanthocyanin malvidin-3-*O*-(6-*p*-coumaroyl)-4-vinylphenol experienced a slight increase over time in the MOX wine and compared to

the control. Some authors suggest that oxygen or reactive oxygen species are required to promote de cycloaddition of pyruvic acid, acetaldehyde, vinylphenols, and vinylflavanols to malvidin-3-*O*-glucoside, as an oxidation step to recover the flavylium moiety of the anthocyanin (*61, 182, 196*). Although anthocyanin concentration decreased in MOX wine, quality, sensory evaluation is a valid evaluation method of wine quality (*60*).

Previous studies on total phenolics content in wines from different varieties have reported concentrations from ~1000 to 3000 μg GAE/mL (*57, 76, 197-199*), which are comparable to this study. Differences in phenolic content are influenced by geographical regions, grape varieties, environmental, and winemaking conditions (*161*). In addition, total phenolics in this study did not significantly change over time and were similar to all wine samples, which are in accordance with previous results and showed that MOX had little influence in the evolution of the phenolic content (*57, 60, 200*).

CONCLUSION

In summary, results indicate that oxygen influenced anthocyanin stability over time. At early stages anthocyanin stability exhibited common degradation patterns in all treatments. At day 52 it was evident the oxygen involvement in anthocyanin degradation was present in all treatments including MOX, compared to the control. Furthermore, at the end of experiments, vessel size for wine storage seemed to play a role for oxygen uptake when compared to the MOX tank. Significant losses of anthocyanins were observed in the small containers, which were likely due to an uncontrolled oxygen

exposure than the MOX tank. Moreover, MOX seemed to be an effective and controlled way to incorporate oxygen into the wine. Other factors such as grape quality, initial wine conditions, aroma and flavor play an important role in winemaking and choice of aging process.

CHAPTER V

RESVERATROL AND QUERCETIN IN COMBINATION HAVE ANTI-CANCER ACTIVITY IN COLON CANCER CELLS AND REPRESS ONCOGENIC MICRORNA-27a

SUMMARY

Resveratrol and quercetin (RQ) previously have been shown to inhibit growth in human leukemia cells. This study investigated the anticancer activity of these compounds in HT-29 colon cancer cells. RQ decreased the generation of reactive oxygen species (ROS) by up to 2.25-fold, and antioxidant capacity was increased up to 3-fold in HT-29 cells within a concentration range of 3.8 – 60 μg/mL. Cell viability also was decreased after 48, 72 or 96 h, and IC_{50} values for viability were 24, 21 and 14 μg/mL, respectively. RQ also induced caspase-3-cleavage (2-fold) and increased PARP cleavage, indicating apoptosis. Specificity protein (Sp) transcription factors, Sp1, Sp3, and Sp4 are over-expressed in cancers and regulate genes required for cell proliferation survival and angiogenesis. RQ (5 - 30 μg/mL) decreased the expression of Sp1, Sp3, and Sp4 mRNA and protein. Moreover, mRNA expression and protein level of the Sp-dependent survival-gene survivin were decreased. RQ decreased microRNA-27a and induced zinc finger protein ZBTB10, a Sp repressor, suggesting that RQ interactions with the microR-27a-ZBTB10-axis play a role in Sp down-regulation. This was confirmed by transfection of cells with the specific microRNA-27a mimic, which

partially reversed the effects of RQ. This is consistent with previous studies using botanical anticancer drugs in colon cancer cells.

INTRODUCTION

Colon cancer is the fifth most common form of cancer in the United States. Diets high in red meat, saturated fat and low in fiber-rich fruits and vegetables have been identified as important risk factors (*88, 107*). The beneficial role of a diet rich in fruits and vegetables in colon cancer prevention has been, in part, attributed to plant-derived botanicals including polyphenolics, which may protect against various illnesses including cancer and chronic-degenerative diseases (*201*). Quercetin is a dietary flavonoid identified in tea, onions, grapes, wines and apples (*202*) and the anti-cancer activities of quercetin have been show in several cancer cell lines, as well as *in vivo* (*107, 203, 204*). Resveratrol, a phytoalexin, is produced by plants as a defense mechanism in response to fungal diseases, stress and UV radiation. The primary dietary sources of resveratrol are grapes and peanuts (*94*). Resveratrol exhibited anticancer properties by inhibiting cell proliferation, inducing apoptosis, decreasing angiogenesis and causing cell cycle arrest in several cancer cell lines (*94, 201, 203, 205*). The combination of resveratrol and quercetin previously exhibited synergistic interactions in their anti-cancer effects in MOLT-4 leukemia cells (*203*).

The aim of this study was to investigate the effects of the mixture on Sp1, Sp3 and Sp4 expression and the potential involvement of miR-27a in the down-regulation of Sp transcription factors in HT-29 colon carcinoma cells.

MATERIALS AND METHODS

Botanical Extract

Polyphenolics were extracted from a standardized *trans*-resveratrol and quercetin dehydrate supplement (ratio 1:1) in capsule form which was provided by Designs for Health (East Windsor, CT). Polyphenolics were extracted with methanol (50 mg/50 mL), followed by centrifugation at room temperature for 10 min at 3000 rpm to remove inactive and insoluble components. Methanol was evaporated in a rotavapor (Büchi Labortechnik AG, Flawil, Switzerland) at 40 ^{o}C. Residual moisture was evaporated in a speedvac concentrator (Thermo Scientific, Waltham, MA) at 43 °C. The final resveratrol-quercetin mixture (RQ) was stored at -80 ^{o}C and dissolved in dimethyl sulfoxide (DMSO) prior to use.

HPLC-PDA Analysis

The polyphenolic mixture was analyzed and quantified by retention time and PDA spectra by HPLC-PDA. The chromatographic separation was performed in an Alliance 2695 system (Waters Inc., Milford, MA), and carried out in a Discovery C_{18} column (Supelco Inc. Bellefonte, PA) (250 x 4.6 mm, 5 μm) at room temperature. The chromatographic conditions used were: mobile phase A water/acetic acid (98:2), mobile phase B acetonitrile/water/acetic acid (68:30:2). A gradient program with 1 mL/min was used as follow 0 min 100%, A; 20 min, 60% A; 30 min 30%, A; 32 min 0%, A; 35 min 100%, A. The detection wavelengths were set at 306 nm and 360 nm for *trans*-resveratrol and quercetin, respectively. Standard compounds for the identification and

quantitative analysis of quercetin and resveratrol were obtained from Acros Organics (Morris Plains, NJ.) and ChromaDex (Irvine, CA), respectively.

Determination of Oxygen Radical Absorbance Capacity

The antioxidant capacity was determined using the oxygen radical absorbance capacity assay (ORAC) (*154*), with fluorescein as a fluorescent probe in a FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission, BMG Labtech Inc., Durham, NC). Results were reported in μmol of Trolox equivalents/mL.

Cell Culture

Human adenocarcinoma cells HT-29 (American Type Culture Collection (ATCC, Manassas, VA), were cultured in McCoy's medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (100,000 U/L penicillin and 100 mg/L streptomycin). Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. For experiments, cells were seeded in DMEM/F-12 medium with 2.5% FBS (stripped with activated charcoal as previously described (*203*)) and 1% antibiotic (100,000 U/L penicillin and 100 mg/L streptomycin).

Generation of Reactive Oxygen Species

Generation of reactive oxygen species (ROS) was determined using the 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay (*206*). In brief, cells ($1x10^4$ cells/well) were seeded in a clear bottom 96-well plate and incubated for 24 h, then treated with different concentrations of RQ (0 - 60 μg/mL). After 24 h, cells were twice washed with phosphate buffer (PBS) and incubated with 200 μM hydrogen peroxide for 2 h at 37 °C. Hydrogen peroxide was removed with a PBS-wash and 10 μM DCFH-DA diluted in

PBS was added to cells and incubated for 15 min at 37 °C. After removing DCFH-DA, the fluorescence intensity was measured after 15 min at 37 °C using a FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission, BMG Labtech Inc., Durham, NC).

Cell Viability

Cells were seeded ($3x10^3$ cells/well) in a 96-well plate for 24 h, after which the growth medium was removed and experimental media containing different extract concentrations of RQ (from 0 to 60 μg/mL), was added and incubated for up to 96 h. Cell viability was assessed at 48, 72 and 96 h with the Cell Titer 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI) according to manufacturer's protocol using a FLUOstar microplate reader at 490 nm (BMG Labtech Inc., Durham, NC). The concentration at which cell viability was inhibited by 50% (IC_{50}) was calculated by linear regression analyses on percentage cell inhibition as a ratio to the control.

Cell Proliferation

HT-29 cells ($2x10^4$ cells/well) were grown in a 24-well plate for 24 h, and then the growth medium was replaced with the experimental medium containing different extract concentrations (from 0 - 60 μg/mL). After 72 h cell proliferation was determined using a cell counter (Beckman Coulter, Fullerton, CA). Cell counts were expressed as a percentage of control cells. The concentration at which cell proliferation was inhibited by 50% (IC_{50}) was calculated by linear regression analyses.

Cleaved Caspase-3 Activation

Cells were grown ($6x10^5$ cells/well) for 24 h, and then incubated with different RQ concentrations (0 - 30 μg/mL) for 24 h. Cleaved caspase-3 activation was determined using an ELISA kit (Cell Signaling Technology Inc. Danvers, MA) according to the manufacturer's protocol using a microplate reader FLUOstar (BMG Labtech Inc., Durham, NC) at 450 nm.

Real-Time PCR Analysis of miRNA and mRNA

HT-29 cells were grown ($2x10^5$ cells/well) in a 6-well plate for 24 h and then incubated with different concentrations of RQ (0 - 30 μg /mL). Total RNA, which contains both mRNA and miRNA, were isolated using the mirVana™ miRNA Isolation Kit (Applied Biosystems, Foster City, CA) following the manufacturer's recommended protocol. Nucleic acid was evaluated for quality and quantity using the NanoDrop®*ND-1000 Spectrophotometer* (NanoDrop Technologies, Wilmington, DE) at 260 nm and 280 nm.

SuperScript™ III First-Strand (Invitrogen, Carlsbad, CA) was used to reverse transcribe mRNA. TATA binding protein (TPB) was used as mRNA endogenous control. For real time PCR (RT-PCR), proprietary primers for Sp3 and Sp4 (Qiagen, Valencia, CA) were used. The following primers were purchased from Integrated DNA Technologies (Coralville, IA) and used for amplification: TBP (sense 5'-TGC ACA GGA GCC AAG AGT GAA-3'; antisense 5'-CAC ATC ACA GCT CCC CAC CA-3'), caspase-3 (sense 5'-CTG GAC TGT GGC ATT GAG ACA-3'; antisense 5'-CGG CCT CCA CTG GTA TTT TAT G-3'), ZBTB10 (sense 5'-GCT GGA TAG TAG TTA TGT

TGC-3'; antisense 5'-CTG AGT GGT TTG ATG GAC AGA G-3'), Sp1 (sense 5'-TCA CCA ATG CCA ATA GCT ACT CA-3'; antisense 5'-GAG TTG GTC CCT GAT GAT CCA-3'), survivin (sense 5'-CCA TGC AAA GGA AAC CAA CAA T-3'; antisense 5'-ATG GCA CGG CGC ACT T-3'). RT-PCR for mRNA was performed using the SYBR GreenER qPCR SuperMix (Invitrogen, Carlsbad, CA) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

The TaqMan® MicroRNA Assay for miR-27a and RNU6B (used as control) (Applied Biosystems, Foster City, CA) was used to reverse transcribe mature miRNA following the manufacturer's protocol in a MasterCycler (Eppendorf, Westbury, NY). RT-PCR for miRNA was carried out with TaqMan® assay, which contained the forward and reverse primers as well as the TaqMan® probe and TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Foster City, CA). After completion of RT-PCR, relative quantification for both mRNA and miRNA of gene expression was evaluated by utilizing the comparative critical threshold (C_T) method as previously performed (*129*).

Transfection with miR-27a Mimic

Transfections with 50 nM and 100 nM miR-27a mimic (Dharmacon, Lafayette, CO) were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 6 h as previously described (*129*). After transfection for 6 h, cells were incubated with 20 μg/mL of RQ for 24 h.

Cell Cycle Kinetics

Cells were seeded ($5x10^5$ cells/plate) and the cell cycle was synchronized with medium containing 2.5% FBS for 24 h. Then cells were treated with RQ (0 - 20 μg/mL) for 24 h. Cells were fixed with 90% ethanol and stored at -20 ^oC as previously described (*203*). DNA was stained with propidium iodide containing RNAse (0.2 mg/mL) solution and analysis was carried out at 488 nm excitation and 620 nm emission wavelengths on a FACScan flow cytometer (Becton-Dickinson Immonunocytometry Systems, San Jose, CA). The percentage of cells in each cell cycle phase was analyzed using the ModFit LT version 3.2 for Macintosh by Verity Software House (Topsham, ME).

Western Blotting

Cells were grown ($2x10^6$ cells/plate) for 24 h and treated with RQ (0 - 30 μg/mL) for 24 h. Cells were harvested and cell lysates were obtained using a high-salt buffer [1.5 mmol/L $MgCl_2$, 500 mmol/L NaCl, 1 mmol/L EGTA, 50 mmol/L HEPES, 10% glycerol, 1% Triton X-100; adjusted to pH 7.5] supplemented with protease inhibitors (Sigma-Aldrich, St. Louis, MO) (*129*). Samples were incubated at 100^oC for 5 min in 1X Laemmli buffer (0.1% bromophenol blue, 175 mM ß-mercaptoethanol, 50 mM Tris-HCl, 2% SDS). Proteins were separated on a 10% (Sp-proteins) or 12% (survivin) SDS-PAGE at 100 V and transferred to PVDF membrane (Bio-Rad, Hercules, CA) as previously performed (*129*). Membranes were blocked in non-fat milk in PBS-Tween and incubated with primary antibodies overnight at 4 ^oC. After washing-steps, membranes were incubated with secondary antibodies. Membranes were washed and incubated with chemiluminescence substrate (Santa Cruz Biotechnology Inc., Santa

Cruz, CA), and proteins were visualized with a Kodak Molecular Imaging System (Carestream Health, Rochester, NY).

Statistical Analyses

Data from *in vitro* experiments were analyzed by one-way-analysis of variance (ANOVA) with JMP 8.0 (SAS Institute Inc., Cary, NC). Differences were deemed significant at $p \leq 0.05$ using a Tukey-Cramer HSD comparison for all pairs. For transfections with miR-27a mimic, differences were deemed significant at $p \leq 0.05$ using a *t*-student comparison for all pairs. The analysis of linear (pairwise) correlations was performed where correlations with a *p*-value less than 0.05 were deemed significant.

RESULTS

Chemical Composition

The chromatographic profiles (Figures 19A and 19B) of RQ demonstrate the presence of two major polyphenols, *trans*-resveratrol (peak 2) and quercetin (peak 3), respectively, as major ingredients of this botanical supplement. A small amount of *cis*-resveratrol, an isomer less abundant than *trans*-resveratrol, was also detected (peak 1). The presence of small amounts of an isomer of resveratrol, *cis*-resveratrol, may be produced by photo isomerization and UV-radiation (*207*). *Cis*-resveratrol might also form during the extraction and concentration process when exposed to light. However, *trans*-resveratrol is the isomer with more bioactive properties and it also represents the major form of resveratrol in grapes, wines and in the dried roots of *Polygonum cuspidatum* (*201, 207*).

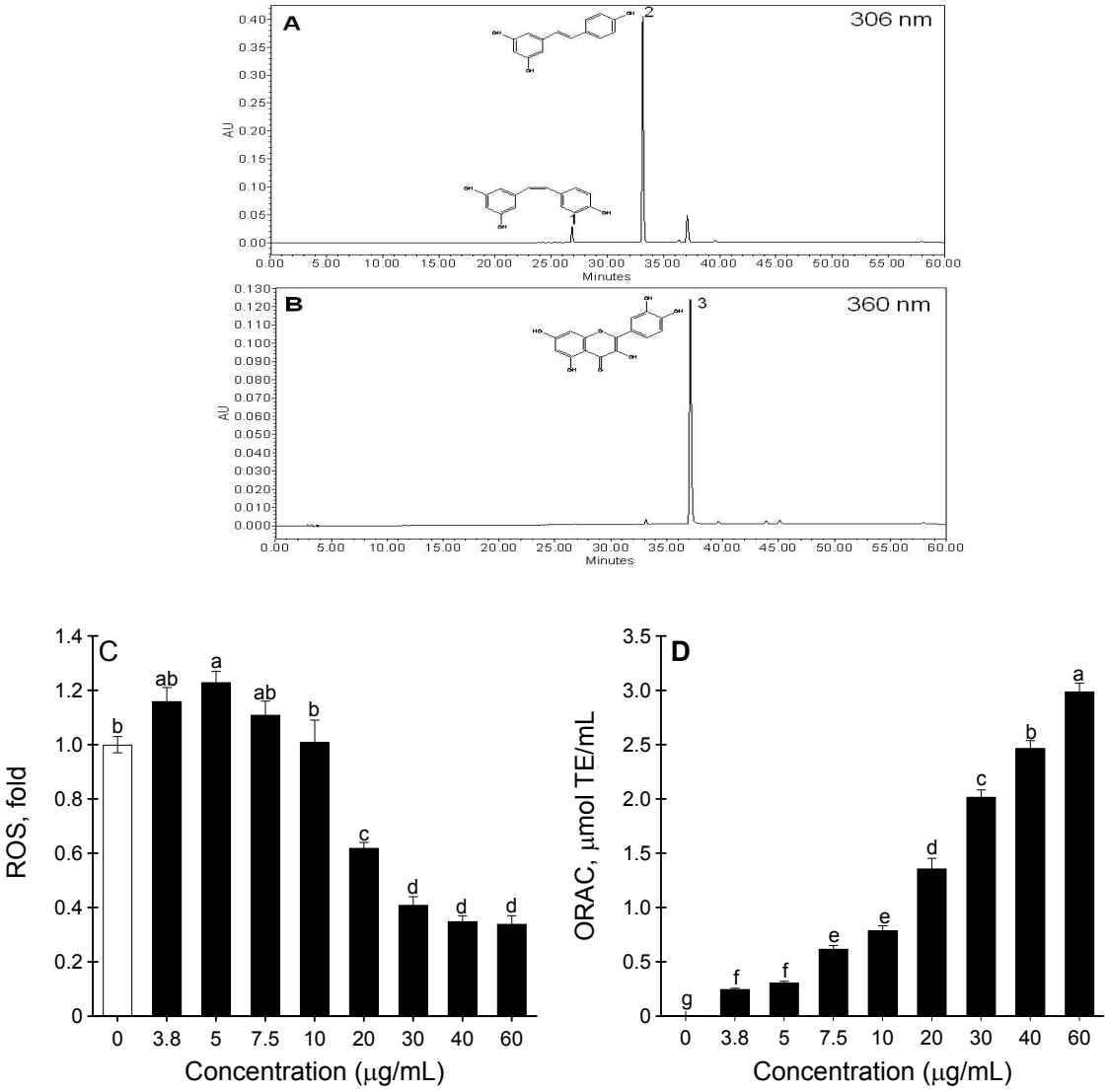


Figure 19. Chromatographic profile of resveratrol and quercetin in combination. ***A,*** stilbenes; ***B*****,** flavonols. Peak assignments: 1, *cis*-resveratrol; 2, *trans-resveratrol*; 3, quercetin. ***C,*** hydrogen peroxide-induced generation of reactive oxygen species (ROS) in HT-29 cells treated with RQ. ***D,*** ORAC-value of HT-29 cell-supernatant with resveratrol/quercetin after 24 h. Bars represent means ± SEM, n = 3. Bars with different letters are significantly different (LSD test, $p < 0.05$).

Generation of Intracellular Reactive Oxygen Species and ORAC

Intracellular generation of reactive oxygen species (ROS) was investigated after HT-29 cells were challenged with hydrogen peroxide. RQ slightly induced the generation of ROS at low concentrations (0 -10 μg/mL), whereas at concentrations higher than 20 μg/mL generation of ROS was significantly reduced (Figure 19C) by up to 66% compared to solvent-treated control cells. The antioxidant capacity values (ORAC) (Figure 19D) were significantly increased by all concentrations of RQ in a dose-dependent manner.

Cell Death and Cell Cycle Kinetics

HT-29 cell viability (Figure 20A) was significantly decreased by RQ in a dose- and time-dependent manner. The IC_{50} values were 24, 21, and 14 μg/mL, for 48, 72, and 96 h, respectively. RQ also inhibited cell proliferation, and after treatment for 72 h (Figure 20B) cell counts were decreased in a dose-dependent manner. The net numbers of cells remaining were the combined results of cancer cell proliferation as well as the cytotoxicity of RQ. Cell numbers were significantly decreased at all concentrations (3.75 - 60 μg/mL) and at 60 μg/mL RQ; cell proliferation was reduced by up to 73%.

The effects of RQ on cell cycle progression were determined by fluorescence-activated cell sorting (FACS) analysis (Figure 20C). In this study, there were minor but significant changes in the percentage of cells in different phases of the cell cycle using 2.5, 5, or 10 μg/mL of extract. There was a significant G_0/G_1 to S phase block compared to control cells when cells were treated with 20 μg/mL of the mixture (Figure 20C).

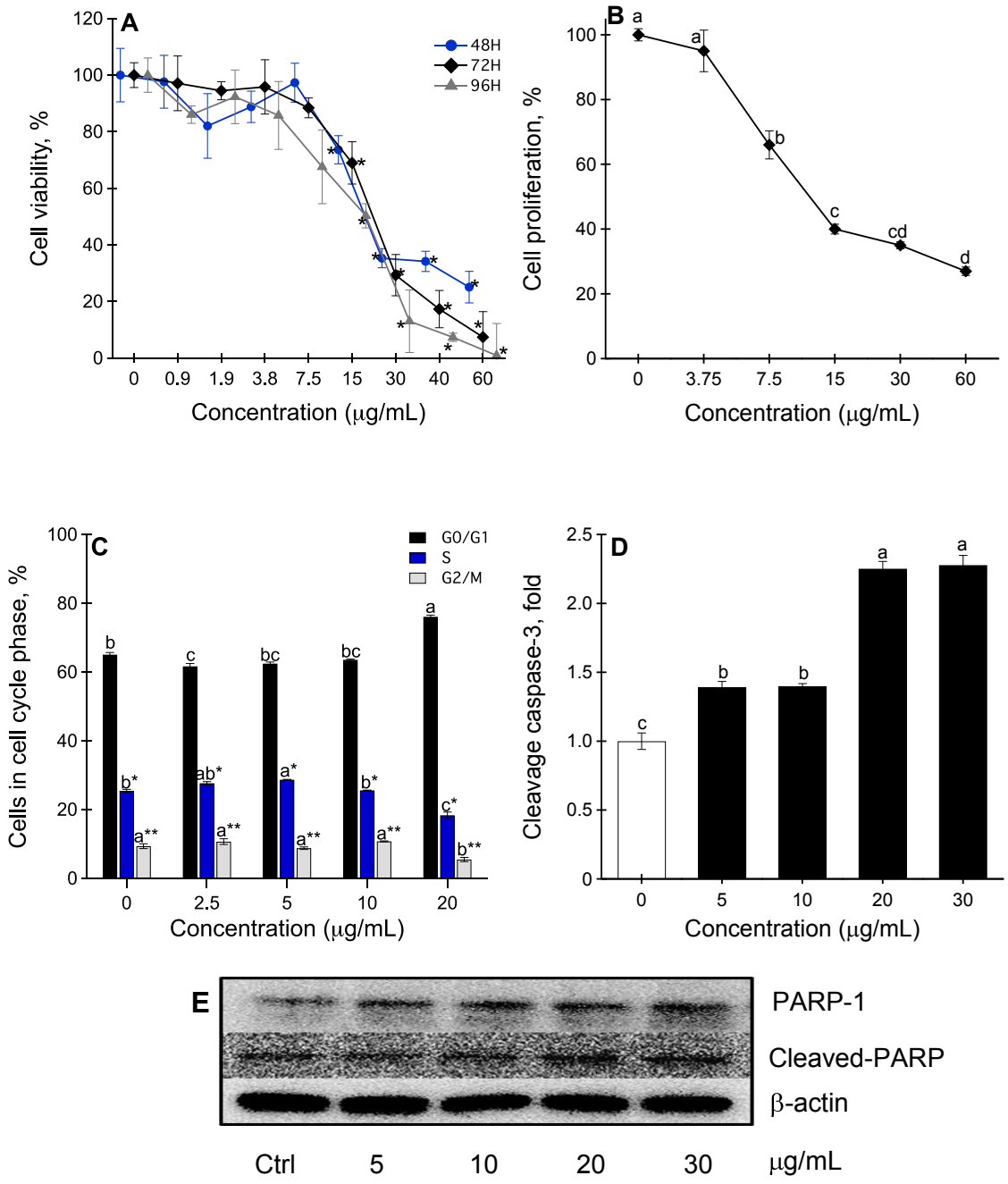


Figure 20. ***A,*** cell viability of HT-29 cells treated with RQ after 48, 72, and 96 h of incubation. ***B,*** cell proliferation of HT-29 cells after 72 h incubation. ***C,*** cell cycle kinetics in HT-29 cells treated with resveratrol/quercetin mixture for 24 h. ***D,*** protein levels of cleaved caspase-3. ***E,*** protein expression of Poly(ADP-ribose) polymerase-1 (PARP-1) and cleaved PARP (Asp214). Relative protein expression was normalized to β-actin. Bars represent means ± SEM, n = 3. Bars with different letters are significantly different (LSD test, $p < 0.05$).

Cleaved capase-3, the activated form, is involved in the execution of apoptosis and was induced up to 1.5-fold even at low concentrations of RQ (5 and 10 μg/mL) (Figure 20D). At higher concentrations (20 and 30 μg/mL), the induction was increased up to 2.3-fold. Poly(ADP-ribose) polymerase 1 (PARP-1) is a substrate for caspase-3 cleavage and produces cleaved PARP-1 (*208*). There was an increase in PARP cleavage when cells were treated with RQ (Figure 20E).

Modulation of Sp1 Transcription Factors and Dependent Genes

Previous studies with several botanical anticancer agents and their synthetic derivatives show that these compounds down-regulate Sp transcription factors and Sp-regulated genes (*209-214*). Results show that RQ (5 – 30 μg/mL) decreased Sp1, Sp3 and Sp4 mRNA levels and that there was also a parallel decrease in Sp1, Sp3 and Sp4 proteins (Figures 21A-E). In addition, RQ also decreased expression of survivin protein and mRNA; these results are consistent with RQ-mediated suppression of Sp transcription factors since survivin is an Sp-regulated gene.

Previous studies demonstrate that the inhibition of ZBTB10 expression by miR-27a is, at least in part, responsible for the increased expression of Sp-transcription factors in cancer cells and tumors (*129, 210, 215*). In this study, RQ significantly decreased miR-27a (Figure 22A) and up-regulated ZBTB10 mRNA (Figure 22B); this was accompanied by decreased Sp-proteins, and the Sp-regulated gene survivin (Figure 21E).

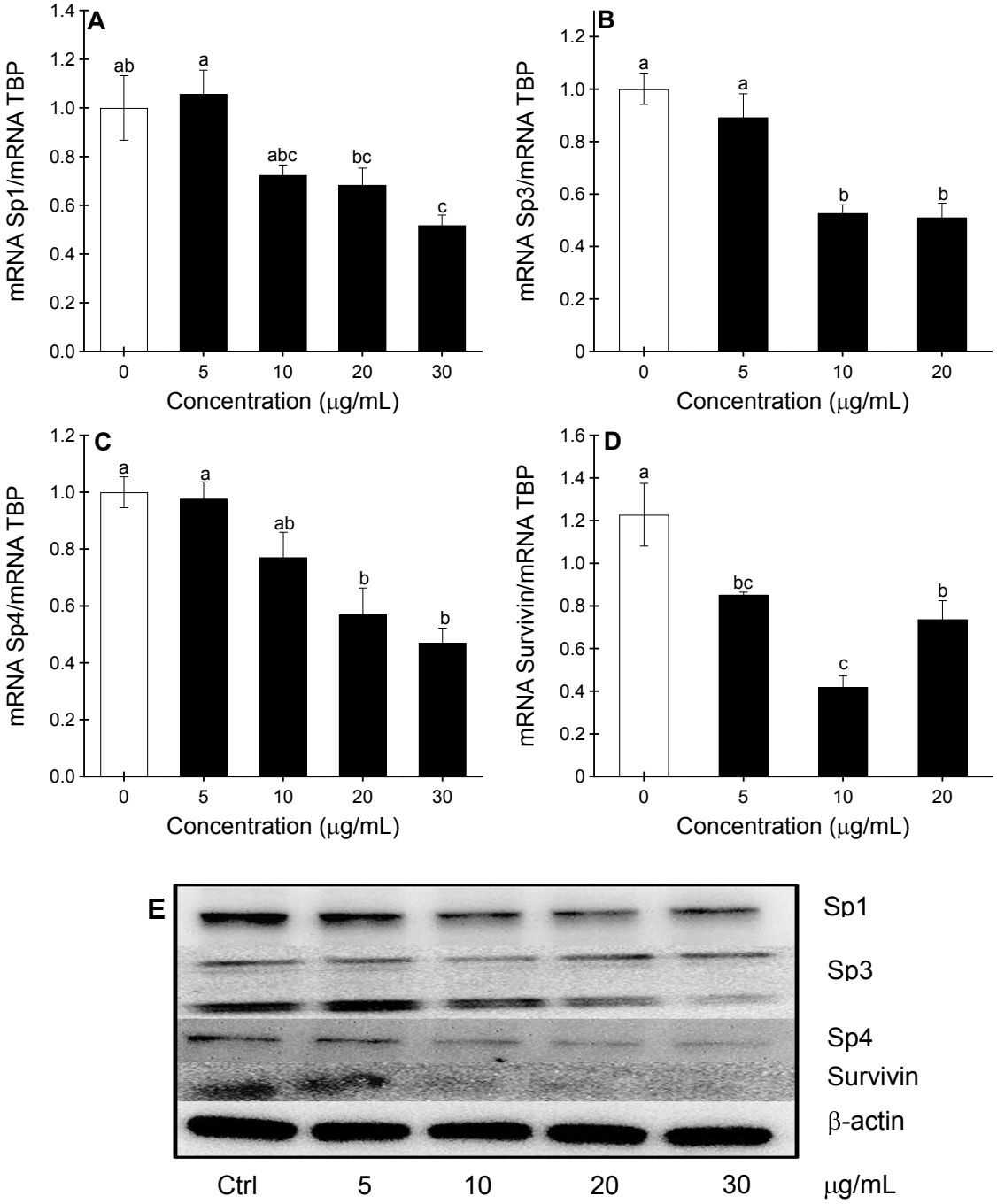


Figure 21. Expression of Sp1, Sp3, Sp4, and survivin in HT-29 cells after 24 h of incubation with resveratrol/quercetin. ***A*****,** mRNA expression of Sp1. ***B*****,** mRNA expression of Sp3. ***C*****,** mRNA expression of Sp4. ***D*****,** mRNA expression of survivin. ***E*****,** protein expression of Sp-proteins and survivin. Protein expression was normalized to β-actin. Bars represent means ± SEM, n = 3. Bars with different letters are significantly different (LSD test, $p < 0.05$).

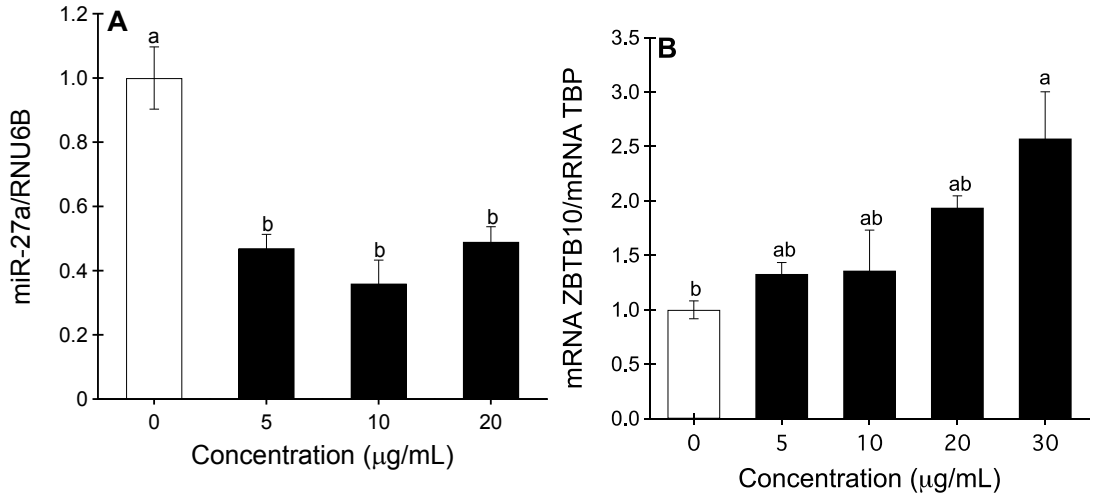


Figure 22. Expression of miR-27a and ZBTB10 in HT-29 cells 24 h after incubation with resveratrol/quercetin. ***A*****,** expression of miR-27a. ***B*****,** mRNA expression of ZTB10. Bars represent means ± SEM, n = 3. Bars with different letters are significantly different (LSD test, $p < 0.05$).

These results are consistent with previous studies regarding the effects of other botanicals and their derivatives on the miR-27a –ZBTB10-Sp1-axis in multiple cancer cell lines (*129, 209-214*). In this study, the over-expression of miR-27a mimic in HT-29 cells increased miR-27a, decreased ZBTB10 and increased Sp1 mRNA levels; this was consistent with the inactivation of endogenous ZBTB10 expressed in these cells (Figure 23A-D). Moreover, the miR-27a mimic also partially reversed RQ-induced down-regulation of miR-27a, induction of ZBTB10 and down-regulation of Sp1.

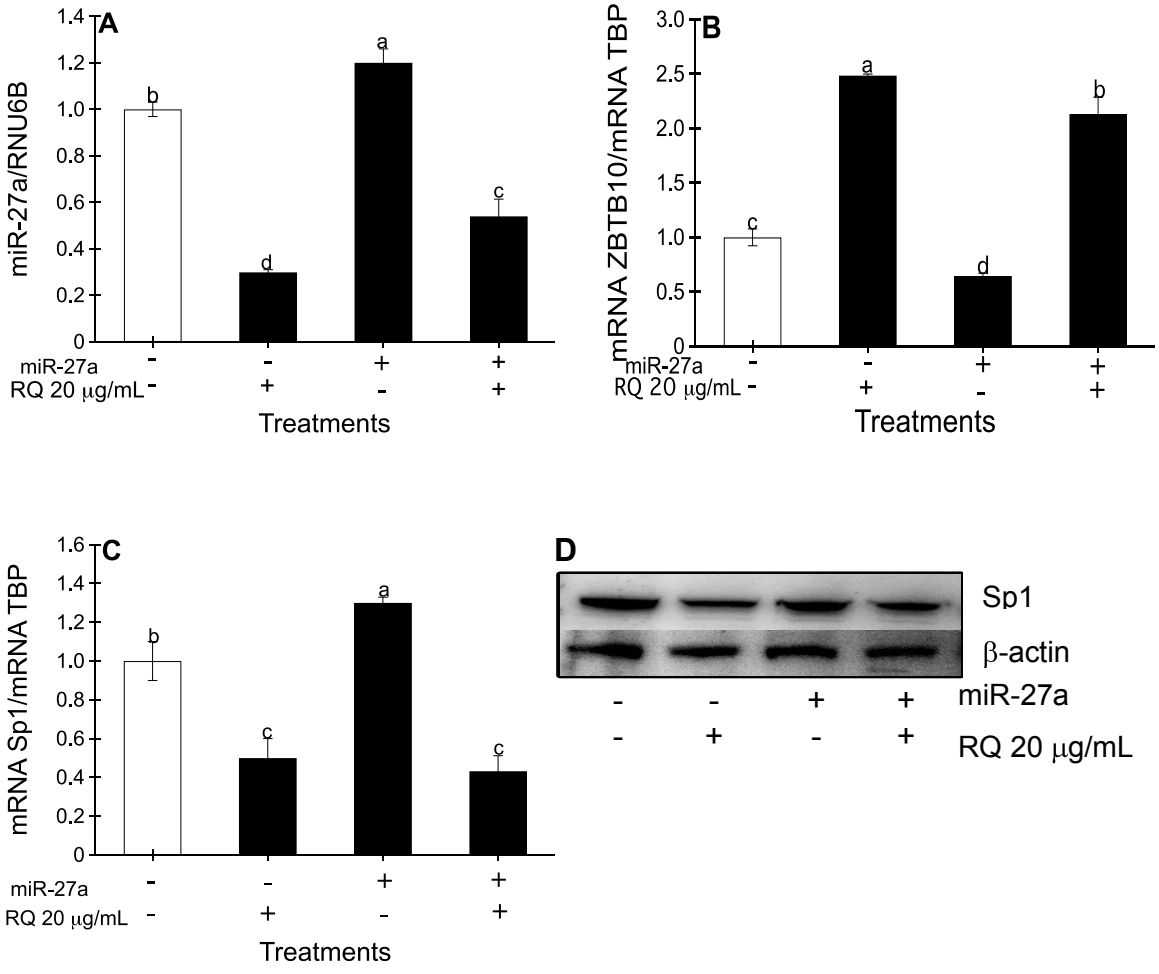


Figure 23. ***A,*** expression of miR-27a after transfection with miR-27a mimic in HT-29 cells. ***B,*** mRNA expression of ZBTB10. ***C,*** mRNA expression of Sp1. ***D,*** protein expression of Sp1 protein after transfection with miR-27a mimic. Protein expression was normalized to β-actin. Bars represent means ± SEM, n = 3. Bars with different letters are significantly different (LSD test, $p < 0.05$).

DISCUSSION

Generation of Intracellular Reactive Oxygen Species and ORAC

The generation of ROS has been associated with oxidative cellular damage that may be involved in the development of various pathological conditions (*216, 217*). The role of ROS in carcinogenesis is complex. Cancer cells tend to have higher constitutive levels of ROS than normal cells due in part to mutations in nuclear and mitochondrial genes responsible for the electron transport chain, and also due to increased metabolism and mitochondrial activity (*218*). Elevated levels of ROS may enhance cell proliferation and other events relevant to cancer progression (*218*). ROS can also cause DNA damage and oxidation of fatty acids in cellular membrane structures, thereby facilitating mutagenesis and cancer development (*217, 218*). However, many anticancer drugs are mitochondrotoxic and induce ROS, which in turn can lead to cancer cell death. Polyphenolics, such as resveratrol and quercetin, have the ability to scavenge free radicals (*206, 217*) and also induce activation of antioxidant and detoxifying enzymes, thus protecting cells against oxidative damage from carcinogenic compounds (*217, 219*). In this study, RQ induced a concentration-dependent increase in intracellular antioxidant capacity (Figure 19D). The intracellular concentration of ROS increased at a low RQ concentration (5 μg/mL) but decreased at high concentrations (20 - 60 μg/mL) (Figure 19C). One possible explanation for this bi-phasic effect may be the mitochondrotoxic properties of polyphenolics, which also induce ROS (*94, 220*). Studies with polyphenolics in cancer cells demonstrate their protective effects against oxidative damage in some conditions (*206, 221*). In this study, RQ induced ROS at lower

concentrations, potentially due to the additional formation of radicals where RQ concentrations were too low to have a protective effect. At higher concentrations, RQ inhibited the generation of ROS, potentially through scavenging ROS.

Cell Death and Cell Cycle Kinetics

The antiproliferative effects of resveratrol and quercetin were previously demonstrated in MOLT-4 leukemia cells. The combination of resveratrol and quercetin exhibited synergistic effects (*96*). Cell viability and cell proliferation were significantly decreased after the treatment with RQ (Figures 20A and 20B).

It was previously demonstrated that cell cycle arrest was induced by polyphenolics, including resveratrol and quercetin, in several cancer cell lines within different phases (*76, 89, 95, 96*). Tan and others, studied the effect of quercetin on HepG2 cells and found that after treatment with quercetin for 48h, cells were arrested in the G_0/G_1 phase (*222*). In MOLT-4 leukemia cells, polyphenolic-mediated cell cycle arrest was influenced by duration of treatment and type of polyphenolic (*96*). In this study, RQ (20 μg/mL) decreased the percentage of cells in the S-phase and increased the percentage of cells in the G_0/G_1 phase, which is consistent with the inhibition of the progression from G_0/G_1 to S-phase (Figure 20C).

Caspase-3 is a major executive enzyme in apoptosis and a commonly used indicator for the induction of apoptosis (*76, 223*). PARP-1, an abundant chromatin-associated protein (*224*), plays an important role in maintaining genome integrity (*224, 225*) and is cleaved during apoptosis by caspase-3. Cleaved PARP-1 is inactivated so that DNA damage cannot be repaired (*225*). Previous studies have demonstrated the

effects of resveratrol and quercetin, as well as some other polyphenolics on caspase-3 and PARP-1 activity (*96, 109, 223*). In general, findings from this study are in concordance with previous reports.

Modulation of Sp1 Transcription Factors and Dependent Genes

Many botanical compounds have previously been demonstrated to down-regulate Sp transcription factors and Sp-regulated genes (*209-214*). For example, curcumin decreased Sp1, Sp3 and Sp4 levels in bladder (*213*) and pancreatic (*214*) cancer cells. The terpenoid betulinic acid also decreased expression of these transcription factors in prostate cancer cells, and synthetic analogs of the triterpenoids, oleanolic and glycyrrhetinic acid, also exhibited comparable effects (*209-214*). The importance of Sp1 down-regulation in terms of the anticancer activity of these compounds is that Sp-regulated genes play an important role in cancer cell and tumor growth (cyclin D1, c-MET, EGFR), survival (NF-kB-p65, survivin, and Bcl-2) and angiogenesis (VEGF and its receptors) (*209-214, 226-229*).

Sp1 over-expression in gastric and pancreatic cancer patients is a negative prognostic factor (*230, 231*), as there is evidence that Sp1 exhibits oncogenic properties and plays a role in cell transformation and maintenance of the cancer phenotype (*211, 214, 215, 226, 232, 233*). The pathways associated with induction of high levels of Sp1, Sp3 and Sp4 during transformation are not known, however, our studies indicate that at least one mechanism for elevated expression in cancer cells and tumors is due to inhibition of ZBTB10 expression by miR-27a (*129, 210, 215*). ZBTB10 is a translational repressor and binds GC-rich sites to decrease Sp-dependent transactivation (*234*). Many

miRNAs play important roles in carcinogenesis, with either oncogenic or tumor-suppressing activities (*235, 236*). The over-expression of oncogenic miRNAs has been demonstrated in several cancer cell lines (*129, 237-239*). In a previous study, we showed that miR-27a expression was increased in six breast cancer cell lines (*129*) and also in colon cancer cells (*139*).

Results from this study show for the first time that apoptosis induced by RQ and inhibition of HT-29 cell growth was associated with decreased expression of Sp1, Sp3, Sp4 and Sp-regulated survivin. These latter responses were paralleled by perturbation of the miR-27a-ZBTB10 axis, resulting in the induction of ZBTB10 a potent Sp repressor gene (*234*). These effects of RQ on miR-27a-ZBTB10 were observed at concentrations of RQ that decrease ROS in HT-29 (Figure 19C) whereas previous studies with curcumin and the synthetic triterpenoid methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me) show that their effects on miR-27a-ZBTB10 were dependent on the generation of ROS (*214, 215*). Current studies in our laboratory focus on mechanisms of ROS-independent down-regulation of miR-27a by RQ and other anticancer agents since this pathway plays an important role in the anticancer activities of botanicals and their derivatives.

CONCLUSION

In conclusion, results indicated that a combination of resveratrol and quercetin had cytotoxic effects in colon cancer cells, resulting in apoptosis. Interactions of RQ and the miR-27a-ZBTB10-Sp1 axis were identified as one possible underlying mechanism.

Further studies are needed in order to assess the role of miR-27a and its clinical relevance in the anticancer effects exhibited by botanicals.

CHAPTER VI

POLYPEHNOLICS FROM BLACK SPANISH RED WINE (*VITIS AESTIVALIS)* HAVE ANTI-CANCER ACTIVITY IN COLON CANCER CELLS AND REPRESS ONCOGENIC MICRORNA-27a

SUMMARY

The anticancer activity of polyphenolics from a Lenoir red wine (*Vitis aestivalis*) was investigated in HT-29 colorectal adenocarcinoma cells. The extract significantly inhibited cell proliferation over a period of 36 h with an IC_{50} value of 258 μg GAE/mL at a concentration range of 18.7 - 300 μg gallic acid equivalents (GAE)/mL. The extract also decreased the generation of reactive oxygen species (ROS) and increased the antioxidant capacity as determined by the oxygen radical absorbance capacity assay (ORAC). The growth inhibitory effect of the wine phenolic extract was also accompanied by an induction of apoptosis (cleaved caspase-3 protein). Specificity protein (Sp) transcription factors Sp1, Sp3, Sp4 are over-expressed in colon cancer cell lines and regulate expression of genes required for proliferation and survival. The effects of the polyphenolic extract on the expression of Sp1, Sp3, and Sp4 were investigated. The expression of Sp1 was significantly decreased by the wine extract at promoter, mRNA, and protein levels; Sp3 and Sp4 transcription factors also were significantly decreased at mRNA and protein level. Furthermore, these responses were accompanied by decreased expression of the Sp-dependent survival gene survivin. The wine extract

also decreased the expression of microRNA-27a (miR-27a) and induced ZBTB10 mRNA. Results were confirmed by transfecting cells with the mimic for miR-27a, which could partially reverse the effects of the wine extract on miR-27a and Sp1 protein. Findings are consistent with previous studies in colon and other cancer cell lines where botanical-induced down-regulation of Sp1, Sp3 and Sp4 is associated with the modulation of miR-27a-ZBTB10 interactions, resulting in the induction of the Sp-repressor ZBTB10. These results link the anticancer activity of the wine extracts to the down-regulation of miR-27a.

INTRODUCTION

Consumption of fruits and vegetables has been associated with cancer prevention in several population studies and some reports have linked specific types of produce (e.g. cruciferous vegetables) to cancer prevention (*240-242*). Red wine has been found to have preventive effects in chronic diseases such as cancer (*75, 101, 243-246*). The chemopreventive properties associated with red wine consumption mainly have been attributed to the presence of polyphenolic compounds (*71, 247, 248*). The mechanisms of chemoprevention by phenolic compounds and other secondary plant compounds are unclear however; their antioxidant activities may be an important protective factor against several diseases, including cancer. The effects of phenolic fruit and vegetable extracts and their individual components as inhibitors of cancer cell and tumor growth have been extensively investigated. Polyphenolic extracts from blackberries, acai, grape seeds, etc., have been found to have anticancer effects in several cancer cell lines and in

animal models (*249-255*). Mechanisms of chemoprevention have been more intensively investigated with isolated polyphenolic compounds; several studies demonstrate the roles of polyphenolics in the induction of apoptosis and reduction of angiogenesis in multiple types of cancer (*89, 90, 111, 222, 256-258*). Moreover, polyphenolics from red wines including *Vitis vinifera* and *Vitis labrusca* varieties have been found to induce cell death, apoptosis, cell cycle arrest, and DNA-damage in various cancer cell lines, including colon cancer (*75, 76, 247, 248, 259-261*).

Research in our laboratories has focused on determining underlying mechanisms of action of various drugs, botanicals, and synthetic analogs as anticancer agents. The anticancer activity of the non-steroidal anti-inflammatory drugs (NSAIDs) tolfenamic acid, arsenic trioxide, the botanicals betulinic acid, curcumin, and the synthetic triterpenoid analogs methyl 2-cyano-3,12-dioxoooleana-1,9-dien-28-oate (CDDO-Me) and methyl-2-cyano-3,11-dioxo-18β-oleana-1,2-dien-30-oate (CDODA-Me) is due, in part, to down-regulation of Sp1, Sp3, and Sp4 which are over-expressed in cancer cells and tumors. The effects of these agents on Sp-proteins are accompanied by down-regulation of Sp-regulated genes involved in all proliferation (EGFR), survival (bcl-2, and survivin) and angiogenesis (VEGF1, VEGF-R) (*129, 139, 262*). In this study we show that a red wine extract also decreases Sp1, Sp3, and Sp4 expression in HT-29 cells and this is correlated with down-regulation of miR-27a and induction of the Sp1-repressor ZBTB10. This identifies, for the first time a novel mechanism of action for red wine extract in colon cancer cells.

MATERIALS AND METHODS

Extraction of Red Wine Polyphenols

A red wine, Port Barrel Reserve made of Black Spanish grapes, was provided by Messina Hof (Bryan, TX). Wine polyphenolics were extracted using a C_{18} cartridge (Waters, Inc., Milford, MA). The wine (5X diluted with acidified water) was applied to the C_{18} cartridge under vacuum and polyphenolics were eluted with acidified methanol after washing the cartridge with acidified water. The methanol was evaporated in a rotavapor (Büchi Labortechnik AG, Flawil, Switzerland) at 40 ^{o}C. The extract was stored at -80 ^{o}C and dissolved in dimethyl sulfoxide (DMSO) prior to use.

Determination of Total Soluble Phenolics

Total soluble phenolics were determined by Folin-Ciocalteu assay (*152*) using a microplate reader FLUOstar (BMG Labtech Inc., Durham, NC) with absorbance read at 726 nm, quantified by linear regression using a gallic acid standard, and expressed as gallic acid equivalents (GAE).

Determination of Monomeric and Polymeric Anthocyanins

Total anthocyanin contents were determined spectrophotometrically by pH differential method (*153*) using a Helios Gamma spectrophotometer (Thermo Electron, Waltham, MA) at 520 nm and quantified using μg/mL equivalents of malvidin-3-*O*-glucoside with a molar extinction coefficient of 28,000. The percentage of polymeric anthocyanins was determined based on color retention in the presence of sodium sulfite (*263*).

Determination of Oxygen Radical Absorbance Capacity

The antioxidant capacity was determined by means of oxygen radical absorbance capacity assay (ORAC) (*154*), using fluorescein as the fluorescent probe with a FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission, BMG Labtech Inc., Durham, NC). Results were reported in μmol of Trolox equivalents/mL.

HPLC-PDA-ESI-MS^n Analysis of Wine Polyphenols

Wine polyphenols were isolated into two fractions using C_{18} solid phase extraction cartridges (Waters, Inc., Milford, MA). Wine extract was diluted with acidified water and applied to the C_{18} cartridge under vacuum; the non-anthocyanin fraction was eluted with ethyl acetate, then the anthocyanin fraction was eluted with acidified methanol, and finally the C_{18} cartridge was washed with acidified water. Solvents were evaporated in a rotavapor (Büchi Labortechnik AG, Flawil, Switzerland) at 40 ^oC, and re-dissolved in methanol. The extracts were stored at -80 ^oC prior to HPLC-MS analyses.

Mass spectrometric analyses were performed on a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source (ThermoFisher, San Jose, CA). The non-anthocyanin fraction was analyzed using a Sunfire C_{18} column (Waters Inc., Milford, MA) (250 x 4.6 mm, 5 μm) at 25 ^oC. The chromatographic conditions consisted on mobile phase A 0.1% formic acid (v/v) in water, and mobile phase B 0.1% formic acid (v/v) in methanol, and ran at 0.40 mL/min. The non-anthocyanin fraction was separated with a gradient elution program in which phase A changed as follows: 0 min 100%, A; 1 min 95%, A; 15 min 70%, A; 40 min

35%, A; 50 min 5%, A; 55 min 5%, A; 56 min 100%, A. Ionization was conducted in the negative ion mode under the following conditions: sheath gas (N_2), 40 units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 4.5 kV; capillary temperature, 300 °C; capillary voltage, 7 V; tube lens offset, 55 V. The detection wavelengths were set at 280 nm and 360 nm, and the identifications were carried out based on fragmentation patterns and wavelengths.

Anthocyanin separation was carried out in a Symmetry C_{18}column (Waters Inc., Milford, MA) (250 x 4.6 mm, 5 μm) at 25 ^{o}C. The chromatographic conditions: mobile phase A water/formic acid/methanol (85:10:5), mobile phase B 0.1% formic acid (v/v) in methanol. A gradient program with 0.25 mL/min was used as follows 0 min 95%, A; 10 min 80%, A; 30 min 50%, A; 35 min 25%, A; 55 min 25%, A; 56 min, 95%, A; 59 min 95%, A. Ionization was conducted in positive mode as follow: sheath gas (N_2), 40 units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 4.5 kV; capillary temperature, 300 °C; capillary voltage, 3.3 V; tube lens offset, -60 V. The detection wavelength was set at 520 nm and the identification was carried out based on fragmentation pattern and wavelengths.

Cell Culture

Human adenocarcinoma cells HT-29 (American Type Culture Collection (ATCC, Manassas, VA) were cultured in McCoy's medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (100,000 U/L penicillin and 100 mg/L streptomycin). Cells were maintained in a humidified atmosphere of 95% air and 5%

CO_2 at 37 °C. For experiments, cells were seeded in DMEM/F-12 medium with 2.5% stripped serum and 1% antibiotic (100,000 U/L penicillin and 100 mg/L streptomycin).

Cell Proliferation

HT-29 cells ($2x10^4$ cells/well) were grown in a 24-well plate for 24 h, after which the growth medium was replaced with the experimental medium, containing different extract concentrations (from 0 to 300 μg GAE/mL). After 24 h, cell proliferation was determined using a cell counter (Beckman Coulter, Fullerton, CA). Cell counts were expressed as a percentage of control cells. The concentration at which cell proliferation was inhibited by 50% (IC_{50}) was calculated by linear regression analyses.

Generation of Reactive Oxygen Species

The generation of intracellular reactive oxygen species (ROS) was determined using the 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay (*206*). In brief, HT-29 cells ($1x10^4$ cells/well) were pre-treated with different concentrations of wine extract (0 - 300 μg GAE/mL) in a 96-well plate for 24 h. Cells were washed with phosphate buffer solution (PBS) and incubated with 200 μM hydrogen peroxide for 2 h at 37 °C. Hydrogen peroxide was washed away with PBS, and 10 μM DCFH-DA was added and incubated for 15 min at 37 °C. The fluorescence intensity was measured after 15 min at 37 °C using a FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emissions, BMG Labtech Inc., Durham, NC).

Cleaved Caspase-3 Activation

Cells were grown ($6x10^5$ cells/well) for 24h, after which the growth medium was replaced with medium containing different extract concentrations (0 - 300 μg GAE/mL). Caspase-3 activation was determined using an ELISA kit (Cell Signaling Technology Inc. Danvers, MA) according to the manufacturer's protocol on a microplate reader FLUOstar (BMG Labtech Inc., Durham, NC) at 450 nm.

Real-Time PCR Analysis of miRNA and mRNA

Colon adenocarcinoma cells HT-29 were grown ($2x10^5$ cells/well) in a 6-well plate for 24 h, after which the growth medium was replaced with medium containing different extract concentrations (0 - 300 μg GAE/mL). Total RNA for miRNA and mRNA was isolated using the mirVana™ miRNA Isolation Kit (Applied Biosystems, Foster City, CA) following the manufacturer's recommended protocol. Nucleic acid was evaluated for quality and quantity using the NanoDrop®*ND-1000 Spectrophotometer* (NanoDrop Technologies, Wilmington, DE).

The TaqMan® MicroRNA Assay for miR-27a and RNU6B (used as control) (Applied Biosystems, Foster City, CA) was used to reverse transcribe mature miRNA following the manufacturer's protocol in a MasterCycler (Eppendorf, Westbury, NY). Real-time PCR for miRNA was carried out with TaqMan® assay, which contained the forward and reverse primers as well as the TaqMan® probe and TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Foster City, CA).

SuperScript™ III First-Strand (Invitrogen, Carlsbad, CA)was used to reverse transcribe mRNA. TATA binding protein (TPB) was used as mRNA control. For real

time PCR, proprietary primers for Sp3 and Sp4 (Qiagen, Valencia, CA) were used. The following primers were purchased from Integrated DNA Technologies (Coralville, IA) and used for amplification as follows: TBP (sense 5'-TGC ACA GGA GCC AAG AGT GAA-3'; antisense 5'-CAC ATC ACA GCT CCC CAC CA-3'), caspase-3 (sense 5'-CTG GAC TGT GGC ATT GAG ACA-3'; antisense 5'-CGG CCT CCA CTG GTA TTT TAT G-3'), ZBTB10 (sense 5'-GCT GGA TAG TAG TTA TGT TGC-3'; antisense 5'-CTG AGT GGT TTG ATG GAC AGA G-3'), Sp1 (sense 5'-TCA CCA ATG CCA ATA GCT ACT CA-3'; antisense 5'-GAG TTG GTC CCT GAT GAT CCA-3'), survivin (sense 5'-CCA TGC AAA GGA AAC CAA CAA T-3'; antisense 5'-ATG GCA CGG CGC ACT T-3'), Myt-1 (sense 5'-TCC TTC CAA GAG TAG CTC CAA TCC-3'; antisense 5'-GCC GGT AGC TCC CAT ATG G-3'). RT-PCR for mRNA was performed using the SYBR GreenER qPCR SuperMix (Invitrogen, Carlsbad, CA) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). After completion of real time PCR, relative quantification of gene expression was evaluated by utilizing the comparative critical threshold (C_T) method as previously performed (*129*).

Cell Cycle Kinetics

Cells were grown ($2x10^5$ cells/well) for 24 h incubation and cell cycle was synchronized by FBS deprivation. Following FBS deprivation, cells were treated with extract (0 -75 μg GAE/mL) in medium supplemented with 2.5% FBS for 24 h. Cells were fixed in 90% ethanol as previously described (*264*). DNA was stained with propidium iodide containing RNAse (0.2 mg/mL) and analysis was carried out at 488 nm excitation and 620 nm emission wavelengths, on a FACScan flow cytometer

(Becton-Dickinson Immonunocytometry Systems, San Jose, CA). The percentage of cells in each cell cycle phase was analyzed using the ModFit LT version 3.2 for Macintosh by Verity Software House (Topsham, Maine).

Western Blotting

Cells were grown ($1x10^5$ cells/well) for 24 h and treated with wine extracts (0 - 300 μg GAE/mL) for 24 h. Cells were harvested and cell lysates were obtained using a high-salt buffer [1.5 mmol/L $MgCl_2$, 500 mmol/L NaCl, 1 mmol/L EGTA, 50 mmol/L HEPES, 10% glycerol, 1% Triton X-100; adjusted to pH 7.5] supplemented with protease inhibitors (Sigma-Aldrich, St. Louis, MO) (*129*). Samples were incubated at 100^{o}C for 5 min in 1X Laemmli buffer (0.1% bromophenol blue, 175 mM β-mercaptoethanol, 50 mM Tris-HCl, 2% SDS). Proteins were separated on a 10% SDS-PAGE at 120 V and transferred to PVDF membrane (Bio-Rad, Hercules, CA) as previously performed (*129*). Membranes were blocked and incubated with primary antibodies overnight 4 ^{o}C. After washing steps, membranes were incubated with secondary antibodies. Membranes were washed, incubated with chemiluminescence substrate (PerkinElmer Inc., Waltham, MA), and proteins were visualized with a Kodak Molecular Imaging System (Carestream Health, Rochester, NY).

Transfection with miR-27a Mimic

Transfection with 50 and 100 nM miR-27a mimic (Dharmacon, Lafayette, CO) was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 6 h as previously performed (*129*) for western blotting and RT-PCR, respectively. After transfection, cells were incubated with 300 μg GAE/mL extract for 24 h.

Promoter Transfection and Luciferase Assays

Cells were grown ($1x10^5$ cells/well) for 24 h and 0.5 μg of the reporter gene plasmid construct was transfected using Lipofectamine 2000 for 6 h as previously described (*129*). Cells were incubated with 300 μg GAE/mL extract for 18 h, then lysed with 200 μL of 1X reporter lysis buffer (Promega, Madison, WI) and used for the luciferase assays. A Lumicount microwell plate reader (Packard Instrument, Downers Grove, IL) was used to quantify luciferase activities, which were normalized to protein concentrations.

Statistical Analyses

Data from in vitro experiments were analyzed by one-way-analysis of variance (ANOVA) with the JMP-software version 8.0 (SAS Institute Inc., Cary, NC). Differences were deemed significant at $p \leq 0.05$ using a Tukey-Cramer HSD comparison for all pairs. The analysis of linear (pairwise) correlations was performed where correlations with a *p*-value less than 0.05 were deemed significant.

RESULTS

Chemical Composition

Chemical properties of wine extract have been determined using the ORAC, total anthocyanins and monomeric/polymeric anthocyanin, and total soluble phenolics (Table 15). The ORAC value of the concentrated wine extract was 1067 μmol TE/mL, while the red wine itself had an ORAC value of 60 μmol TE/mL. The concentrations of monomeric anthocyanins and polymeric anthocyanins in the concentrated extract were

4334 μg/mL and 2627 μg/mL, respectively. The wine itself had concentrations of monomeric anthocyanins and polymeric anthocyanins of 272 and 277 μg/mL, respectively. The concentration of total soluble phenolics was 28733 μg GAE/mL. The wine itself had 1692 μg GAE/mL, whereas a regular red wine on average contains 1600 μg GAE/mL (*76, 197, 198*).

Table 15. Chemical characterization of red wine extract and red wine.

Analyses	Red Wine Extract	Red Wine
Total soluble phenolics (μg GAE/mL)[1]	28733.27 ± 205	1692.07 ± 26.92
Monomeric anthocyanins (μg/mL)[2]	4334.44 ± 98.62	272.42 ± 1.84
Polymeric anthocyanins (μg/mL)[3]	2627.39± 64.04	277.88 ± 2.24
ORAC (μmol TE/mL)[4]	1067 ± 25	60.74 ± 2.18

[1]Quantified as equivalents of gallic acid. [2]Quantified as equivalents of malvivdin-3-*O*-glucoside. [3]Polymeric anthocyanins determined based on color retention in the presence of sodium sulfite as equivalents of malvivdin-3-*O*-glucoside. [4]Reported in μmol Trolox equivalents per milliliter of sample.

The chemical profile of the red wine extract is shown in Figures 24A-C, while the details of individual peaks are presented in Tables 16 and 17. The identification of wine polyphenols was performed by spectrophotometric characteristics and mass spectra when available. The polyphenolic compounds were detected at 280, 360 and 520 nm, consistent with the presence of four main groups of polyphenolic compounds such as phenolic acids, flavan-3-ols, flavonols and anthocyanins. The phenolic acids identified were hydroxybenzoic acids, such as gallic acid and syringic acid; whereas hydroxycinnamic acids included *p*-coumaric acid glycosides, caffeic acid and esters of

tartaric acid such as caftaric acid and *p*-coumaroyl tartaric acid, among others. In addition, the wine extract contained (+)-catechin and (-)-epicatechin, as well as procyanidin dimers and trimers. The main flavonols were myricetin and quercetin, along with its respective glucoside and glucoronide forms. With regard to the methanolic fraction rich in anthocyanins, malvidin derivatives such as 3,5-*O*-diglucoside, 3-*O*-glucoside, 3-*O*-(6-*p*-coumaroyl)-5-*O*-glucoside, and 3-*O*-(6-*p*-coumaroyl)-glucoside were the most abundant.

Generation of Intracellular Reactive Oxygen Species

The hydrogen peroxide induced generation of ROS was reduced by wine extract by 70% at a concentration of 300 μg GAE/mL (Figure 24D). The ORAC values of cell culture medium in each well (Figure 24E) showed significant differences among treatments (0 – 300 μg GAE/mL). While the ORAC value of cell culture medium was increasing with increasing extract concentration compared to the control, the generation of ROS was higher at 37.5 μg GAE/mL compared to the control.

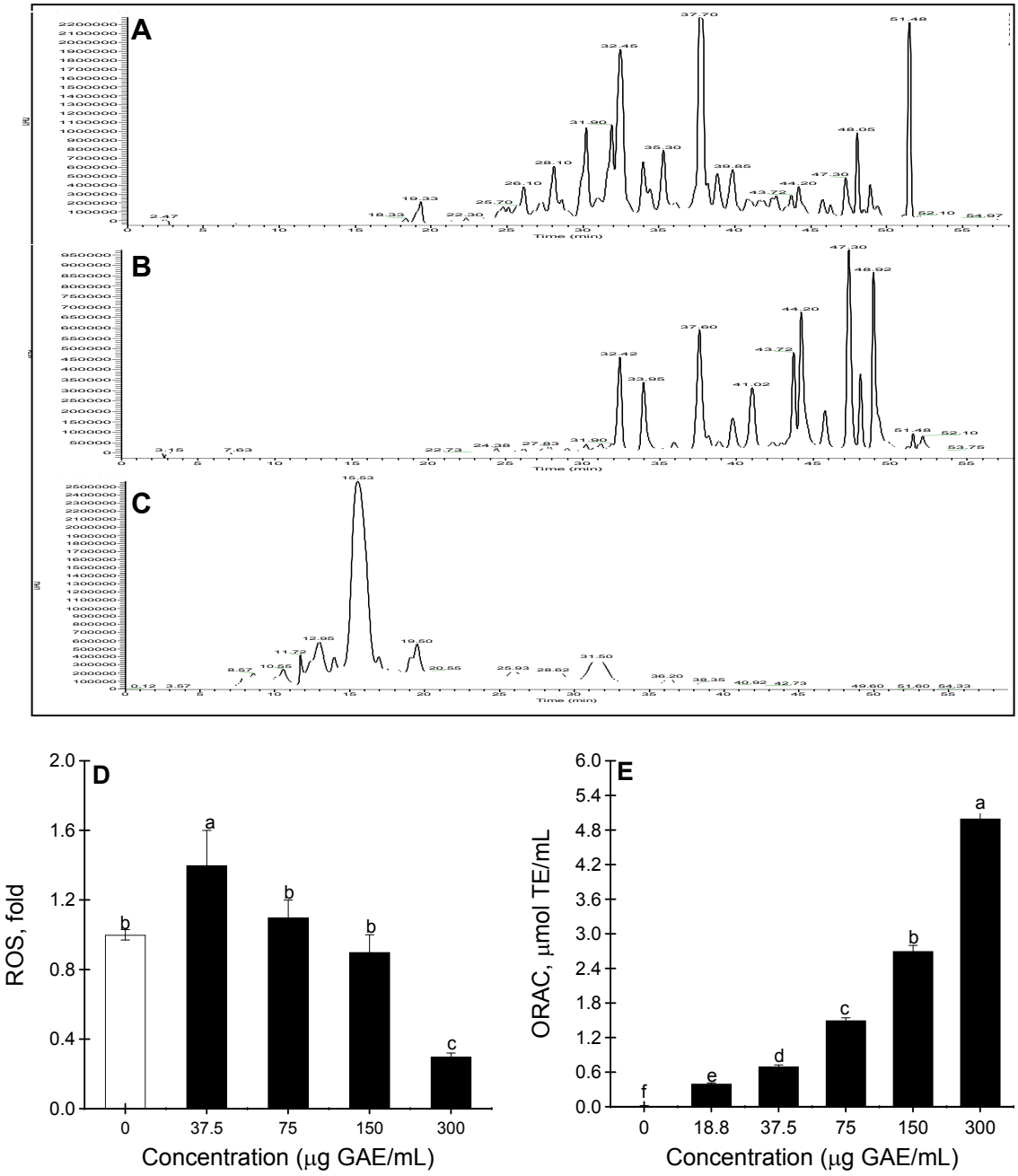


Figure 24. Representative chromatographic profile of polyphenolic compounds in Black Spanish red wine extract. ***A,*** phenolic acids, flavan-3-ols, and procyanidins at 280 nm; ***B,*** flavonols at 360 nm; and ***C,*** anthocyanins at 520 nm. ***D,*** hydrogen peroxide-induced generation of reactive oxygen species in HT-29 cells treated with wine extract. ***E,*** ORAC values from wine extract in which HT-29 cells were exposed. Each bar represents the means of three repetitions with ± SEM. Bars with different letters are significantly different (LSD test, $p < 0.05$).

Table 16. HPLC-MS-ESI-(-)-MS^n analyses of phenolics present in the ethyl acetate wine extract fraction.

T_R (min)	Compound	λ_{max}	MS $[M - H]^-$ (*m/z*)[1]	MS^2 (*m/z*)
17.9	Gallic acid	n.d.	169.2	125.3
21.5	Procyanidin dimer	281	576.9	559.0, 425.0, 407.1, 289.0
21.7	Procyanidin trimer	281	864.8, **577.0**	559.0, 425.0, 407.1, 289.0
22.3	Procyanidin dimer	280	576.9	
23.1	Procyanidin trimer	279	865.0, 577.2	
25.9	(+)-Catechin	279	289.0	
25.9	Procyanidin dimer	280	576.8	
30.1	*p*-Coumaroyl hexose	311	324.9	265.0, 243.9, 163.0
30.4	(-)-Epicatechin	280	289.0	
30.8	*p*-Coumaroyl hexose	311	324.9	265.0, 234.9, 163.1
32.4	Caffeic acid	320, 294	179.1	135.3
34.0	Caffeoyl tartaric acid	329	310.8	179.1, 149.2
35.3	Syringic acid	278	197.2	169.1
37.7	Myricetin-3-*O*-hexose	n.d.	479.1	316.1
38.8	*p*-Coumaroyl tartaric acid	314	294.9	163.0
39.7	Ferouyl tartaric acid	323, 294	324.9	193.0
41.0	Larcitrin-3-*O*-hexose	356, 307, 265sh, 256	493.1	331.1
41.1	Quercetin-3-*O*-glucoside	354, 265sh, 256	463.2	301.1
44.3	Myricetin	370, 306sh, 254	317.1	179.1
46.1	Methoxycinnamic acid hexoside	331, 294sh	338.9	292.9, 176.9, 161.2
47.3	Quercetin-3-*O*-glucoronide	357, 300sh, 256	477.0	301.1
48.9	Quercetin	371, 255	301.1	179.1, 151.1

[1]Ions in bold represent the most abundant ion on which further mass spectrometry analyses were conducted.

Table 17. HPLC-MS-ESI-(+)-MS^n analyses of anthocyanins present in the methanol fraction.

T_R (min)	Compound	λ_{max}	MS $[M + H]^+$ (*m/z*)	MS^2 (*m/z*)
10.6	Delphinidin-3-*O*-glucoside-5-*O*-glucoside	525, 278	627.1	464.8, 303.2
12.6	Cyanidin-3-*O*-glucoside-5-*O*-glucoside	522, 279	611.0	448.8, 287.2
13.0	Petunidin-3-*O*-glucoside-5-*O*-glucoside	524, 277	641.0	478.8, 317.1
13.9	Delphinidin-3-*O*-glucoside	525, 278	465.1	303.2
15.4	Peonidin-3-*O*-glucoside-5-*O*-glucoside	518, 278	625.0	462.8, 301.1
15.6	Malvidin-3-*O*-glucoside-5-*O*-glucoside	524, 277	655.2	492.8, 331.1
16.2	Cyanidin-3-*O*-glucoside	n.d.	449.3	287.1
17.0	Petunidin-3-*O*-glucoside	528, 279	479.0	422.9, 317.1
19.0	Peonidin-3-*O*-glucoside	523, 280	463.0	422.9, 301.1
19.5	Malvidin-3-*O*-glucoside	528, 279	493.0	331.0
20.8	Malvidin-3-*O*-(6-*O*-acetyl)-glucoside-5-*O*-glucoside	528, 282	697.0	492.7, 331.0
26.0	Delphinidin-3-O-(6-*p*-coumaroyl)-glucoside	531, 308, 285	773.0	610.9, 464.5, 303.2
28.7	Petunidin-3-*O*-(6-*p*-coumaroyl)-glucoside	527, 315, 282	787.1	624.2, 478.9, 317.1
31.5	Malvidin-3-*O*-(6-*p*-coumaroyl)-5-*O*-glucoside	533, 315, 283	801.1	638.9, 331.1
36.2	Malvidin-3-*O*-(6-*p*-coumaroyl)-glucoside	523, 282	639.1	331.1
38.3	Peonidin 3-*O*-(6-*p*-coumaroyl)-glucoside	506, 280	609.0	447.1, 301.1

Cell Death and Cell Cycle Kinetics

Cell proliferation was evaluated after 36 h of incubation where an IC_{50} value of 258 μg GAE/mL was determined (Figure 25A). Cell proliferation was dose dependent within the concentration-range of 18.75 - 300 μg GAE/mL. At the highest concentration (300 μg GAE/mL), cell proliferation was reduced by up to 70% when compared to the control. The wine extract induced apoptosis as evidenced by increased caspase-3 cleavage (Figure 25C) which was significantly increased by 1.7-fold only at the highest concentration of extract.

Growth inhibition was accompanied by alterations in cell cycle progression, as determined by fluorescence-activated cell sorting (FACS) analysis (Figure 25B). The highest extract concentration (75 μg GAE/mL) did not significantly affect the percentage of cells in G_o/G_1, non-significantly decreased the percentage of cells in the S phase and slightly but significantly, increased the percentage of cells in the G_2/M phase by 6% at the highest concentration (Figure 25B). Myt-1 is a known target gene of miR-27a and is involved in cell cycle regulation by inhibiting G_2/M through enhanced phosphorylation and inactivation of cdc2 leading to cell cycle arrest in the G_2/M phase (*129*). In this study, the mRNA of Myt-1 was significantly increased in a dose-dependent manner.

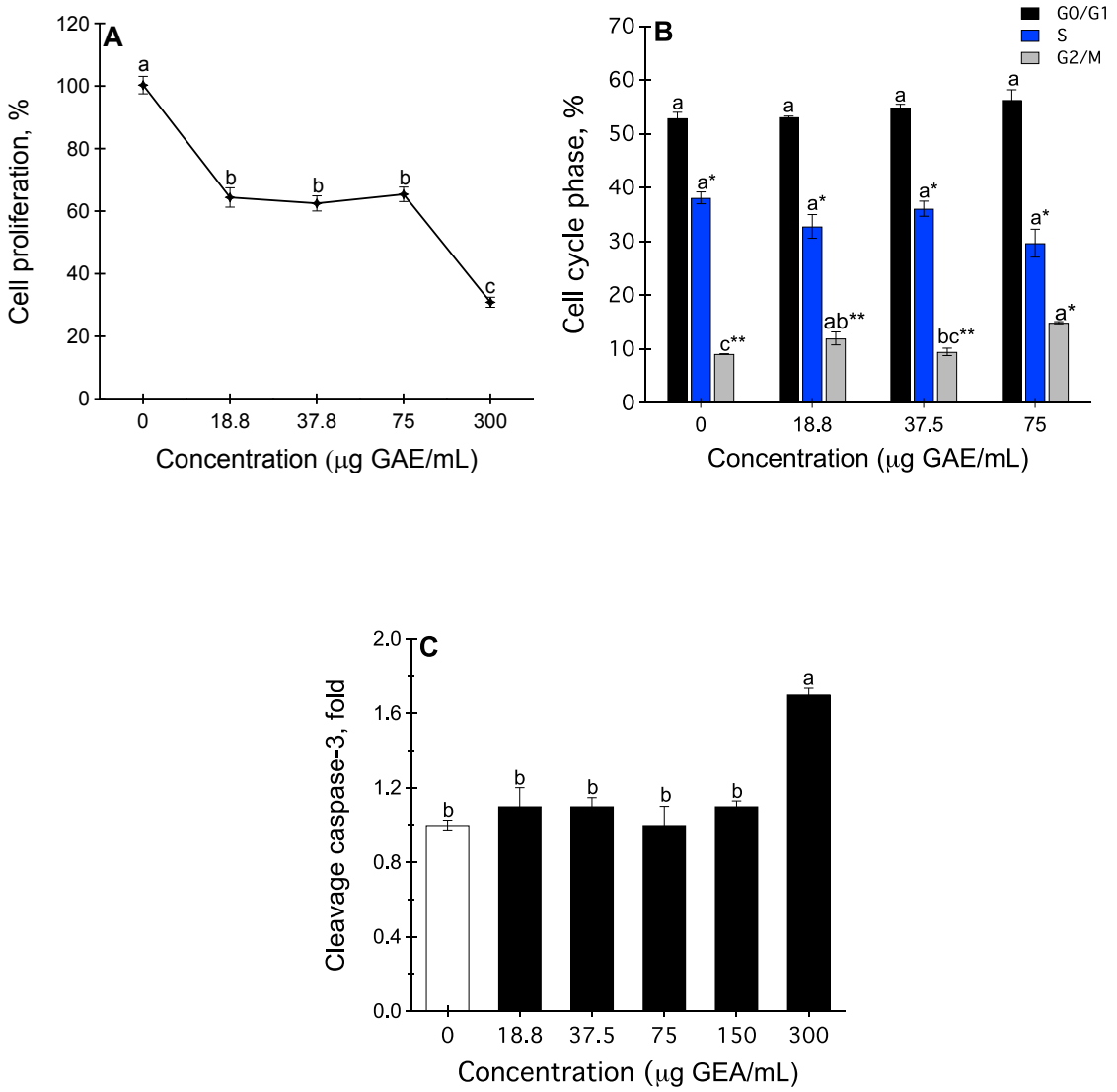


Figure 25. ***A,*** cell proliferation after 36 h incubation with wine extract. ***B,*** modulation of cell cycle in HT-29 cells treated with wine extract for 24 h. ***C,*** protein levels of cleaved caspase-3. Bars represent the mean of three repetitions with ± SEM. Bars with different letters are significantly different (LSD test, $p < 0.05$).

Modulation of Sp1 Transcription Factors and Dependent Genes

Sp1, Sp3 and Sp4 transcription factors are over-expressed in colon and other cancer cell lines (*265*) and the anticancer activity of botanicals such as curcumin and betulinic acid is, in part, due to the down-regulation of Sp-transcription factors and Sp-regulated gene products (*210, 211, 266*). The expression of Sp1 mRNA was significantly decreased at lower and higher concentrations (Figure 26A) by the extract. This also significantly decreased the luciferase activity in HT-29 cells, which were transfected with the promoter construct pSp1-For4 containing the GC-rich promoter region for Sp1 at 18.8 μg/mL and higher concentrations (Figure 26B). Protein concentrations of Sp1 seemed to be decreased only within the upper concentration range starting at 75 μg GAE/mL (Figure 26C), overall demonstrating that this transcription factor is targeted by the wine extract in colon cancer cells. Compounds that repress Sp1 in cancer cells also coordinately down-regulate Sp3 and Sp4; this is due in part to cross regulation of these genes by Sp-transcription factors (*265*). Results demonstrate that Sp3 and Sp4 transcription factors also were significantly decreased at mRNA (by up to 40 and 63% respectively) and at protein level (Figures 27A and 27C) by the wine extract, but only within a higher concentration range of 150-300 μg GAE/mL. This was accompanied by a significant decrease in survivin mRNA and protein level (Figures 27B and 27C) by the wine extract.

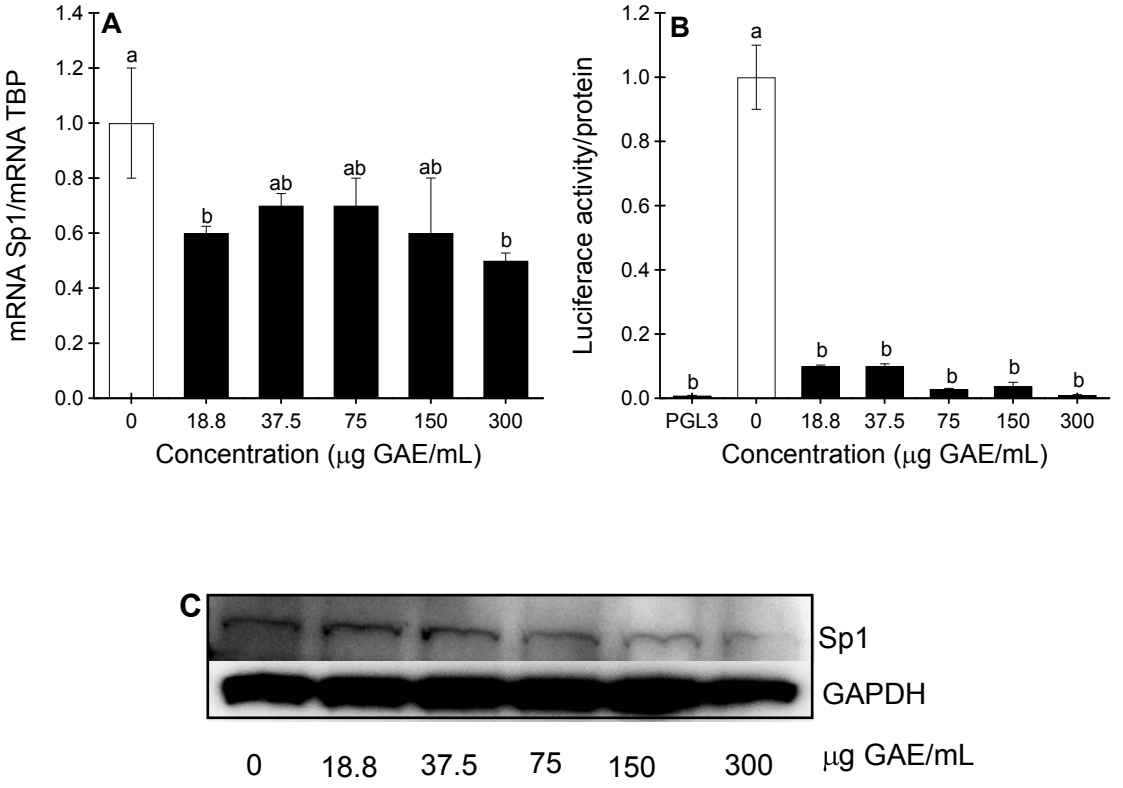


Figure 26. Expression of Sp1 protein in HT-29 cells 24 h after the incubation with wine extract. ***A***, expression of mRNA Sp1 determined by RT-PCR. ***B***, Sp1 promoter activity. ***C,*** western blot of Sp1 protein. Protein expression was normalized to GAPDH. Bars represent the mean of three repetitions with ± SEM. Bars with different letters are significantly different (LSD test, $p < 0.05$).

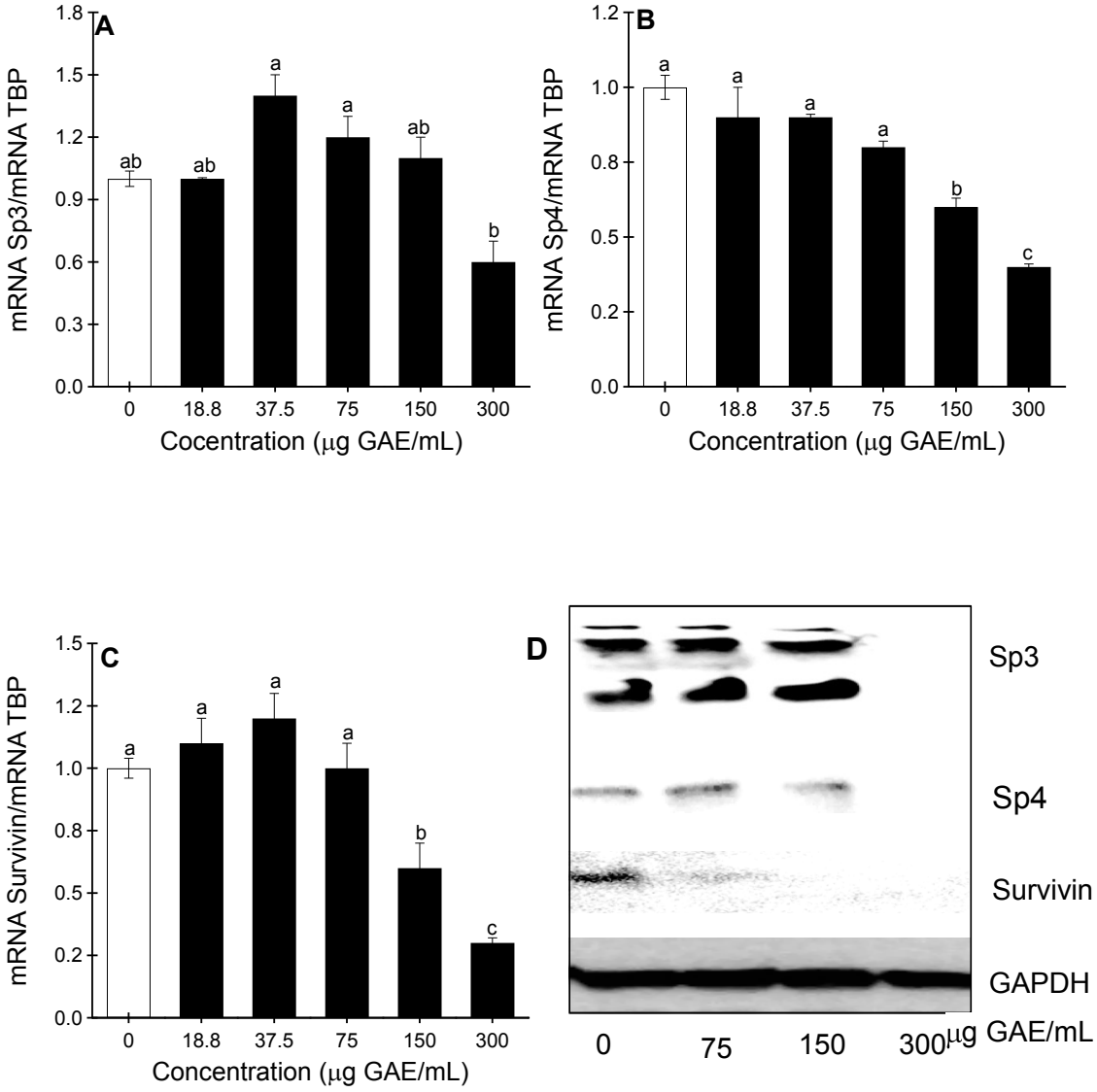


Figure 27. Expression of oncogenic transcription factors Sp3, Sp4, and anti-apoptotic protein survivin in HT-29 cells 24 h after incubation with wine extract. ***A,*** expression of mRNA Sp-3, ***B,*** Sp4 and ***C,*** survivin. ***D,*** protein expression of Sp3, Sp4 and survivin protein. Bars represent the means of three repetitions with ± SEM. Bars with different letters are significantly different (LSD test, $p < 0.05$). Protein expression was normalized to GAPDH.

Modulation of miR-27a and ZBTB10

Previous studies demonstrate that the down-regulation of Sp1, Sp3 and Sp4 in colon and breast cancer cells was due to decreased expression of miR-27a and induction of ZBTB10, a miR-27a-regulated putative suppressor of Sp1 (*210, 267*). Treatment of cells with wine extract significantly decreased miR-27a by up to 42% (Figure 28A) and induced the mRNA expression of ZBTB10, which was up-regulated (Figure 28B) dose-dependently by up to 1.5-fold. Myt-1 is another known target gene of miR-27a and is involved in cell cycle regulation by inhibiting G_2/M through enhanced phosphorylation and inactivation of cdc2 leading to cell cycle arrest in the G_2/M phase (*129*). In this study, the mRNA of Myt-1 (Figure 28C) was significantly increased in a dose-dependent manner, which correlates to the increase in the G_2/M phase.

In order to confirm the involvement of miR-27a in the regulation of ZBTB10, Sp1 and Sp1-dependent genes, cells were transiently transfected with the mimic of miR-27a (*267*). The transfection resulted in a significant increase of miR-27a (Figure 29A). Moreover, the mimic of miR-27a reversed the effects of the wine extract on Sp1 protein (Figure 29B). These results support a significant role of miR-27a in wine extract-mediated down-regulation of Sp1, Sp3, and Sp4 transcription factors in HT-29 colon cancer cells.

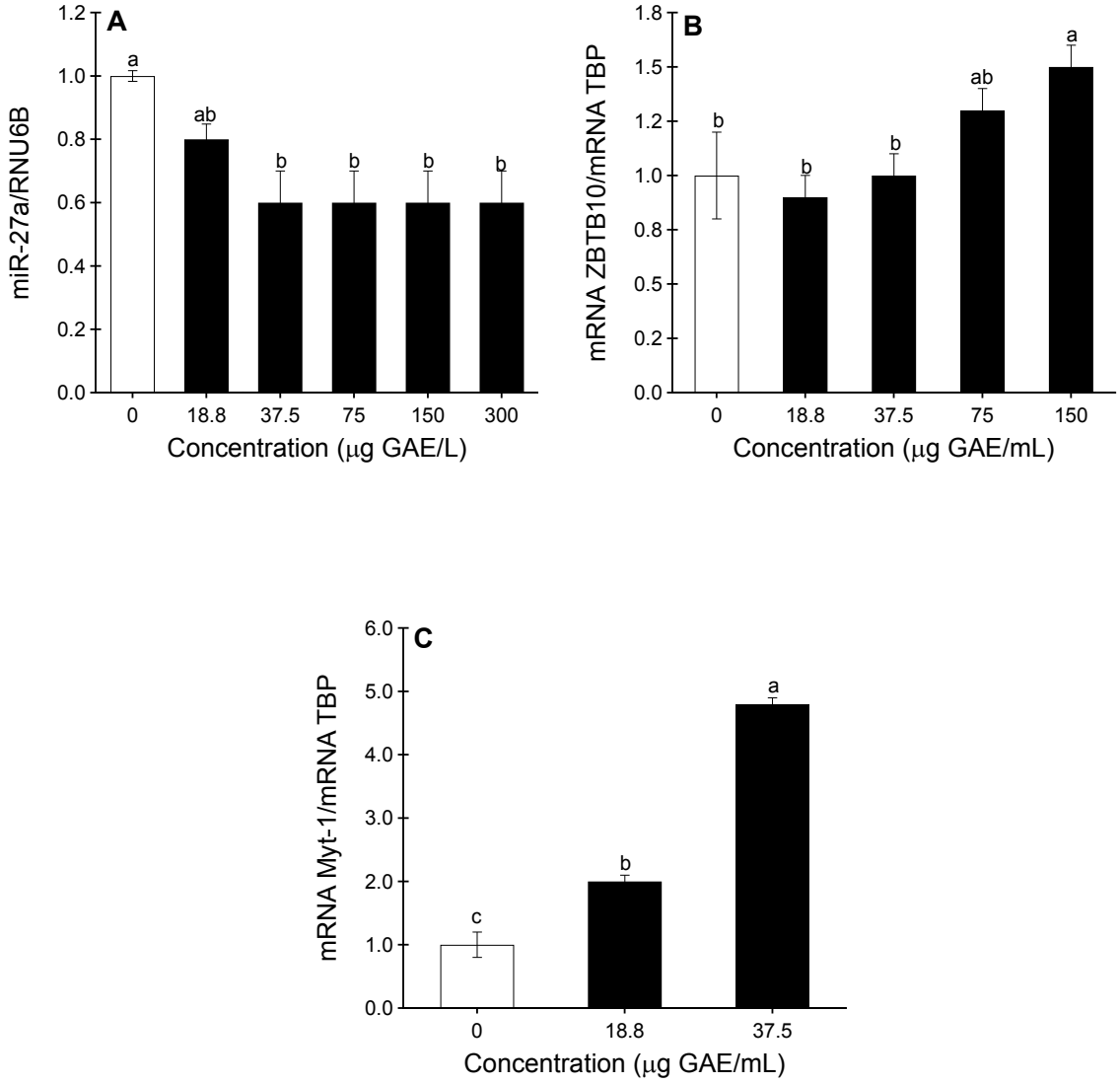


Figure 28. Expression of miR-27a, ZBTB10 and Myt-1 in HT-29 cells after 24 h of incubation with wine extract. ***A,*** expression of miR-27a. ***B*****,** ZBTB10 mRNA. ***C,*** Myt-1 mRNA. Bars represent the means of three repetitions with ± SEM. Bars with different letters are significantly different (LSD test, $p < 0.05$).

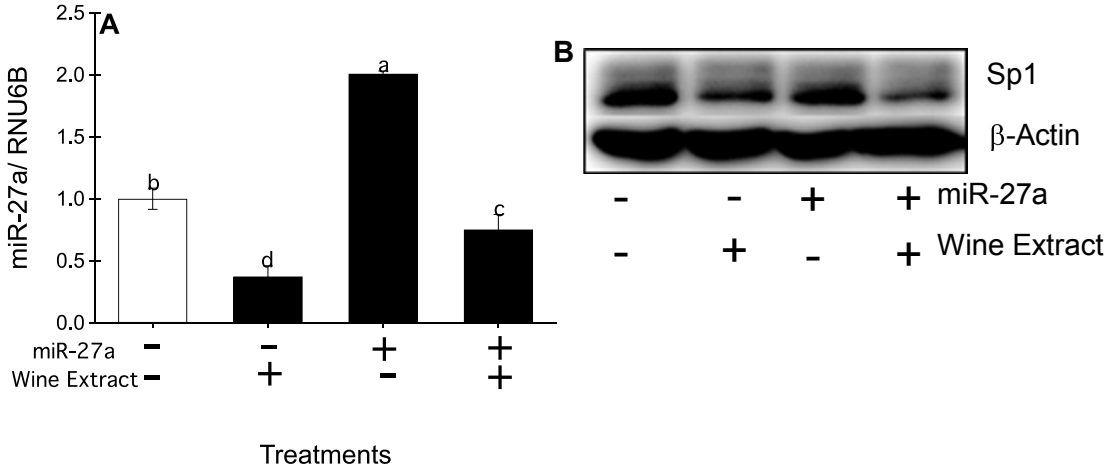


Figure 29. Effect of miR-27a mimic with and without wine extract on the expression Sp1-protein. ***A*****,** expression of miR-27a. ***B,*** protein expression of Sp1-protein. Bars represent the means of three repetitions with ± SEM. Bars with different letters are significantly different (LSD test, $p < 0.05$). Protein expression was normalized to β-actin.

DISCUSSION

Chemical Composition

The anticancer effects of extracts from various wines have been reported in numerous publications, however most reports focus on *Vitis vinifera* varieties (*76, 88*). The wine extracted for this study was made from Black Spanish grapes (Lenoir, *Vitis aestivalis*). Several studies have reported wine chemistry ORAC values within the range of 11 to 60 μmol TE/mL across different wine varieties. Differences in polyphenolic profile and content due to varietal differences, vinification, and maturation of wine contribute to this wide range (*76, 268*). The concentration of monomeric anthocyanins of the extract used in this study was in accordance with previously reported data for red wines, at 200 to 450 μg/mL (*76*); whereas the percentage of polymeric anthocyanins in

red wines may vary from 5 to 22 % (*269, 270*). The content of total soluble phenolics was shown to be within the same concentration range for red wines from different varieties with an average of 1600 μg GAE/mL (*76, 197*).

In this study, structural information and compound confirmation were obtained by means of mass spectrometric analyses. The chromatographic profile of a Black Spanish (*Vitis aestivalis*) wine showed the presence of similar phenolic acids and flavan-3-ols, which have been previously reported in grapes and wines (*76, 183*). Although gallic acid is one of the hydroxybenzoic acids with the highest concentration in wines, this was not the case for the wine extract made of Black Spanish grapes where it was found in relative minimum amounts in the wine extract and was identified with a parent ion at *m/z* 169.2 ($[M - H]^-$). Gallic acid may be lost during the extraction process, be present in naturally low concentrations in the fruit, or be affected by growing conditions (*23*). Furthermore, flavan-3-ols compounds such as (+)-catechin and (-)-epicatechin were also identified with similar parent ion at *m/z* 289.0 ($[M - H]^-$), and were confirmed by retention times (*183*). The wine extract contained relatively small amounts of procyanidin dimers and trimers, which were confirmed by UV spectroscopic properties (catechin UV spectra at 280 nm) and the characteristic signals at *m/z* 577.0 ($[M - H]^-$) and 865.0 ($[M-H]^-$) respectively (*183*).

Flavonols such as quercetin, myricetin, and kaempferol are usually found in glycoside and glucoronide forms in grapes, whereas in wines they may also be found in aglycon form due to the cleavage of the glycosidic bond during yeast fermentation and aging (*19, 22*). In the wine extract, the glycosidic, glucoronic, and aglycon forms were

identified for quercetin at *m/z* 463.2 ($[M - H]^-$), 477.0 ($[M - H]^-$), and 301.1 ($[M - H]^-$), respectively. For myricetin, just the glycosidic and aglycon forms were identified at *m/z* 479.1 ($[M - H]^-$) and *m/z* 317.1 ($[M - H]^-$), respectively.

Grapes from non *V. vinifera* varieties and hybrids contain not only 3-*O*-glucoside moieties, but also an extra sugar attached at the 5 position in the A ring of the anthocyanidin structure (*174*). The anthocyanin fraction of the Black Spanish wine showed these characteristic anthocyanins and were identified as delphinidin, cyanidin, petunidin, peonidin and malvidin 3,5-dilgucosides with parent ions at *m/z* 627.1 ($[M + H]^+$), *m/z* 611.0 ($[M + H]^+$), *m/z* 641.0 ($[M + H]^+$), *m/z* 625.0 ($[M + H]^+$),and *m/z* 655.2 ($[M + H]^+$), respectively. In addition, malvidin, with its different moieties, is the anthocyanin with more abundance in red wines (*23*). Overall, the chemical profile of the extract prepared from Black Spanish grapes was comparable to wines used in previous studies.

Generation of Intracellular Reactive Oxygen Species

In pathogenic situations, the generation of intracellular ROS has been associated with oxidative damage leading to degenerative and chronic diseases including cancer (*218, 271*). In this study, the polyphenolic extract reduced the generation of intracellular ROS (Figure 24D), most likely due to their antioxidant capacity (*272*). Evidence suggests that malignant cells have higher basal levels of ROS compared to normal cells, potentially as part of signaling relevant to tumor progression (*218*). The generation of ROS was increased at the lowest extract concentration, but otherwise decreased in a concentration dependent manner. Potentially, at the lowest concentration, the low

amount of antioxidants absorbed into the cells may have been oxidized themselves, instead of reducing the generation of ROS (*273*). Previous studies investigating the effects of botanical extracts on the generation of ROS in some cells have demonstrated protective effects against oxidative damage (*206*), but on the other hand polyphenolic extracts may also induce ROS generation and trigger apoptosis (*94, 220*).

Cell Death and Cell Cycle Kinetics

Red wine polyphenols from different wine varieties have shown to be effective in the reduction of cell proliferation in several types of cancer (*76, 88, 261*). The extract prepared from Black Spanish wine displays a similar concentration range compared to studies with *Vitis vinifera* varieties (*78, 88*). Several studies determined the pro-apoptotic effects of polyphenolics from red wine extracts (*88*). Caspases, a family of cystein proteases, are present in cells as an inactive form (*122*) and are activated by cleavage, which also can be induced by polyphenolics (*217*). Activated caspase-3 is one of the key elements in the execution of apoptosis (*76, 88*). In this study, an increase in activated caspase-3 indicated the induction of apoptosis by the wine extract. In previous studies, polyphenolic extracts from wine induced apoptosis in comparable concentration ranges (*88*). A combination of polyphenols present in red wines (quercetin and resveratrol) has been shown to exert to exert a synergistic effect on the activation of caspase-3 in MOLT-4 leukemia cell line (*96*).

Previous studies with wine extracts demonstrated arrest in different cell cycle phases. In a study with muscadine wine extract and leukemia, MOLT-4 cell line induced a significant increase in the percentage of cells in the S phase, while Cabernet Sauvignon

wine extract caused an increase in the percentage of cells in the G_2/M phase (*76*). In this study, a small increase in the G_2/M phase was observed (Figure 25B). In a previous mechanistic study with breast cancer cells, the reduction of miR-27a was accompanied by a significant increase in the G_2/M phase, and also by increased expression of Myt-1 and Wee-1 which are both target genes of miR-27a. Both are known regulators of cdc2 and cdc2/cyclin B-mediated arrest in the G_2/M progression, a critical step before cells can undergo mitosis. Myt-1 and Wee-1 both are known target genes of miR-27a (*129*). In this study, mRNA of Myt-1 (Figure 28C) was significantly increased, as expected, due to a decreased expression of miR-27a. However, the slight increase in the percentage of cells in the G_2/M phase indicates potential involvement by the wine extract in other factors affecting cell cycle regulation.

Modulation of Sp1 Transcription Factors and Dependent Genes

Specificity protein 1 (Sp1) was the first transcription factor identified and is a member of the Sp/Krüppel-like factor (KLF) family of transcription factors, which have been found to be over-expressed in tumor cells. Sp1, Sp3, and Sp4 transcription factors are considered oncogenic and their presence enhances the expression of angiogenic and anti-apoptotic genes, promoting cell growth and metastasis (*129, 265*). Moreover, a down-regulation of Sp proteins in cancer cells has widely been associated with cytotoxicity in cancer cells (*129, 265*). In previous studies, botanicals and their derivatives decreased the expression of Sp1, Sp3, and Sp4 transcription factors in several cancer cell lines at protein, mRNA and promoter levels (*210, 211, 266*). In this study, the expression of Sp1 transcription factor was decreased by the wine extract at protein and

promoter levels (Figures 26A-C). In previous studies, botanicals that repress Sp1 in cancer cells also coordinately down-regulated Sp3, Sp4, and Sp-dependent genes such as survivin, VEGF and VEGF-receptor (*265*) as was confirmed in this study where Sp3 and Sp4 and survivin were down-regulated at mRNA and protein levels (Figure 27D).

Modulation of miR-27a and ZBTB10

ZBTB10 is a putative inhibitor of Sp1 and its mRNA was identified as a target gene of miR-27a (*129*). MicroRNAs (miRNA) are small, non-coding RNA molecules that play an important role in regulating gene expression. MiRNAs are associated with cancer function either by tumor suppression or oncogenic activity (*129*). miR-27a belongs to a group of miRNAs which have been found to have oncogenic activity in several cancer cell lines (*129, 237*). In a previous study, an increase in miR-27a was observed in at least six breast cancer cell lines (*129*). Moreover, in a study with gastric adenocarcinoma cell line MGC803, miR-27a was the most highly over-expressed miRNA, compared to miR-9-1, miR-23a and miR-191, indicating oncogenic activity in gastric cancer (*237*). In a previous study with breast cancer cell lines, the expression of ZBTB10 was down-regulated by increased miR-27a (*129*). Only a few studies have investigated the effects of botanical compounds and their derivatives on the regulation of miR-27a and ZBTB10. Methyl 2-cyano-3,11-dioxo-18beta-olean-1,12-dien-30-oate (CDODA-Me), a synthetic derivative of glycyrrhetinic acid a triterpenoid phytochemical found in licorice extracts, induced apoptosis in RKO and SW480 colon cancer cells involving the miR-27a-ZBTB10-Sp1 axis (*139*). When pancreatic cancer cells were treated with a similar compound, 2-cyano-3,12-dioxoleana-1,9-dien-28-oic acid

(CDDO), and its methyl ester (CDDO-Me) the same mechanism seemed to be involved in the induction of cell death (*215*). In this study, the wine extract decreased miR-27a (Figure 28A), up-regulated ZBTB10 mRNA (Figure 28B), and decreased Sp1 at mRNA, protein and promoter levels (Figure 26A-C). This mechanism was previously observed in a study with the breast cancer cell line MDA-MB-231; data showed a reduction in ZBTB10 mRNA expression and an increase in Sp1 protein and Sp-dependent genes when cells were transfected with mimic of miR-27a. It was notable that a decrease in cell growth, increase in antioxidant capacity, Sp1 mRNA and promoter-activity were decreased across the lower and higher concentration-range, while Sp1 protein and Sp1-regulated mRNA and proteins seemed to predominantly respond to the wine extract within the higher concentration-range. This indicates that cell death may have been induced by an alternative mechanism within the lower concentration-range, while at higher concentrations it seems to be associated with the down-regulation of Sp1 protein and Sp1-protein-dependent genes regulated by the miR27a-ZBTB10-Sp1 axis. When breast cancer cells were transfected with the miR-27a antisense construct, the effects were the opposite: increased ZTB10 mRNA, reduction of Sp1 levels, as well as a reduction of Sp1-dependent genes (*129*). In this study, the decrease of the Sp1 transcription factor, at least in part, seems to have been induced by a decrease in miR-27a, as demonstrated in the transfection assay with the mimic of miR-27a (Figures 29A and 29B) where the miR-27a mimic significantly reversed the effects of the wine extract on miR27a and Sp1 protein. This may indicate that other factors are involved in the increase of ZBTB10 and decrease of Sp1 transcription factor. Mechanistically however,

results from this study seem to be in accordance with previous studies (*129, 139, 262*).

CONCLUSION

Results indicate that an extract prepared from red Black Spanish grapes reduced cancer cell proliferation, intracellular generation of ROS, and induced apoptosis through the induction of caspase-3. As a possible underlying mechanism for the anti-cancer activity, interactions of the wine extract with the miR-27a-ZBTB10-Sp1 axis were identified. Overall, results indicate that the miR-27a-ZBTB10-Sp1 axis plays a role in the anticancer effects of wine extract; however, additional mechanisms seem to be involved as indicated by some non-significant results from the transfection experiment with miR-27a mimic resulting in only a slight increase in the G_2/M phase. Further studies are required in order to identify miR-27a as a target for complex botanical extracts with anticancer effects.

CHAPTER VII

GENERAL CONCLUSIONS AND RECOMMENDATIONS

Texas grape and wine industries have experienced significant growth in recent years and are interested in developing and applying new technologies to improve the overall quality of grapes and wines. In addition, research has shown that moderate drinking of red wine, combined with a healthy diet may have potential health benefits against chronic-degenerative diseases.

Comparing chemical and phytochemical profiles of the American hybrid grapes Black Spanish (*Vitis aestivalis*) and Blanc Du Bois (*Vitis aestivalis hybrid*) the *Vitis vinifera* varieties, Cabernet Sauvignon and Merlot, the polyphenolic content was influenced more by grape skin color rather than grape variety. However, anthocyanin content and ORAC values varied by grape variety. No significant qualitative differences were observed between the four different grapes' chromatographic profiles for non-anthocyanins polyphenolics. Anthocyanin differences may possibly be explained by differences in genetic background and expression of pathways for synthesis and metabolism for secondary metabolites between *V. vinifera* and *V. aestivalis*. The diglucoside forms of anthocyanins were present in *V. aestivalis* but not in *V. vinifera* varieties.

Anthocyanin stability results indicated that oxygen is a key element to control during wine storage in containers and micro-oxygenation treatments. At early stages,

anthocyanin stability showed similar rates of degradation for all treatments (control, wine with oak pieces, oak barrel and micro-oxygenation). Based on these results, it was evident that the vessel size played an important role in anthocyanin stability, and uncontrolled oxygen exposure accelerated anthocyanin degradation in the small container when compared to the micro-oxygenation tank. Some small polyphenolic compounds may be extracted from the oak barrel; wood pieces and inner staves have been shown to promote copigmentation reactions and may help anthocyanin color stability. In general, micro-oxygenation treatments seemed to be an effective and controlled way to incorporate oxygen into the wine.

The anti-cancer effects of a combination of wine compounds (resveratrol/quercetin) and a polyphenolic extract from Black Spanish wine were investigated in colon cancer cells HT-29. Wine polyphenolics showed anti-cancer effects in colon cancer cells, had cytotoxic effects in colon cancer cells, and induced apoptosis. Additionally, as possible underlying mechanism for the anti-cancer activity, interactions of the wine polyphenolics with the miR-27a-ZBTB10-Sp1 axis were identified; however, additional mechanisms seem to be involved.

Further studies may address the profiles of volatile compounds in American hybrid grapes in order to identify characteristic volatiles that differ from *Vitis vinifera* varieties. In the area of micro-oxygenation more studies are needed which combine sensory data with instrumental techniques such as GC-MS to monitor volatiles during micro-oxygenation treatments. Health benefits of hybrid grapes should be compared to *Vitis vinifera* in human clinical trials.

In summary, this work will provide valuable information on grape varieties, wine-production, and potential health benefits with great relevance to the grape and wine industry in Texas and beyond.

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