The Study of Phenolic Compounds as Natural Antioxidants in Wine

M. López-Vélez, F. Martínez-Martínez, and C. Del Valle-Ribes

Department of Physical Chemistry, Faculty of Pharmacy, University of Granada, 18071 Granada, Spain

ABSTRACT: Plant phenolics present in fruit and vegetables, and that are particularly rich in red wine, have received considerable attention because of their potential antioxidant activity. Human consumption of antioxidants has many alleged health benefits, including protection against cardiovascular diseases, and, most recently, cancer. Red wines contain a variety of polyphenolic antioxidants. Five samples of commercial red wines from Spain and four phenolic compounds of red wine: gallic acid, trans-resveratrol, quercetin and rutin, have been studied. The total phenolics content and the total antioxidant activity (TAA) of wines was determined. The total phenolic content, determined according to the Folin-Ciocalteu method, varied from 1800 to 2300 mg/L, expressed as gallic acid equivalents (GAE). The antioxidative effects of wine phenolics were determined using a system based on the inhibition by antioxidants of the absorbance of the radical cation. The relationship between antioxidant activity of phenolic compounds, as hydrogen donating free radical scavengers, and their chemical structures was studied. Furthermore, the total antioxidant activity of the wines investigated was well correlated with phenol content. Thus, the results confirm that red wine polyphenols are, in vitro, significant antioxidants.

KEY WORDS: polyphenols, red wine, antioxidant activity.

I. INTRODUCTION

It is known that polyphenols are a large family of natural compounds that, from a chemical point of view, are characterized by the presence of one or more benzene-type rings. Polyphenols are directly related to some characteristics of foods, such as taste, palatability, and nutritional value, and have particular importance for the characteristics and quality of red wines. Recent studies in vitro and in vivo show that some polyphenols exhibit antioxidant and free radical-scavenging properties. Human consumption of antioxidants has many alleged health benefits, including protection against cardiovascular diseases, and, most recently, cancer. Wine, particularly red wine, is a rich source of antioxidant polyphenolic compounds. Recent research has suggested that dietary polyphenols, which possess antioxidant activity, may play a role in human health, particularly in diseases believed to involve, in part, oxidation, such as coronary heart disease, inflammation, and mutagenesis leading to carcinogenesis.

Grapes, wines, and grape byproducts contain large amounts of phenolic compounds, mostly flavonoids, at high concentrations of 1000 to 1800 mg/L, although their presence and structure are affected by a number of factors, including grape variety, sun exposure, vinification techniques, and aging. A large part of the phenolics in grapes, wines, and byproducts may act as antioxidants. The antioxidant capacity of phenolic compounds is essentially due to the ease with which a hydrogen atom from an aromatic hydroxyl group can be donated to a free radical and the ability of the phenolic moiety to support an unpaired electron due to delocalization around the \( \pi \)-electron system.

Red wine contains wood- and yeast-derived phenolics in addition to large amounts of phenolic components that originate from grapes, particularly the skins, which are removed during the vinification of wine. Although structurally diverse, phenolics are classified into two groups: the flavonoids and the nonflavonoids. The nonflavonoid compounds family includes the
hydroxycinnamates, hydroxybenzoates (e.g., gallic acid), and stilbenes (e.g., resveratrol). The flavonoids encompass flavan-3-ols (catechins), anthocyanins, and flavonols (e.g., quercetin and rutin). A significant proportion of the phenolic content of wine originates from the tannins, which are subdivided into condensed and hydrolyzable tannins. However, the phenolic profile of wine is not the same as that of fresh grapes because significant changes in phenolic composition occur during the wine-making process, both very early at the grape-crushing stage and during wine fermentation and aging.\footnote{13}

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. There are two basic categories of antioxidants, namely, synthetic and natural. Natural antioxidants present in foods and other biological materials have attracted considerable interest because of their presumed safety and potential nutritional and therapeutical effects. Because extensive and expensive testing of food additives are required to meet safety standards, synthetic antioxidants generally have been eliminated from many food applications.\footnote{14} The increasing interest in the search for natural replacements for synthetic antioxidants has led to the antioxidant evaluation of a number of plant sources. The most recent research on antioxidant action focuses on phenolic compounds such as flavonoids.\footnote{15}

The oxidative stress arising from an imbalance in the human antioxidant status (reactive oxygen species vs. defense and repair mechanisms) contributes to the pathology of oxidative diseases, such as cardiovascular diseases,\footnote{3} cancer,\footnote{16} inflammation,\footnote{17} and brain dysfunction.\footnote{18} Besides endogenous defenses, the consumption of dietary antioxidants plays an important role in protecting against these pathological events. In recent years, much attention has been devoted to ascorbic acid, tocopherol, tocotrienols, and \( \beta \)-carotene.\footnote{19} Recently, flavonoids and related phenolics have attracted increasing attention for their antioxidant properties, which may help to explain the protective effect of vegetable-rich diets on coronary heart disease (CHD).\footnote{20,21} Indeed, flavonoids and related phenolic acids are present in fruit, vegetables, and some beverages, being an integral part of the human diet. Among beverages, red wine has been reported to be more protective against CHD than other alcoholic beverages, thus confirming a possible role of red wine polyphenols in reducing thrombotic and atherogenic processes. In fact, it is well known that phenolic components of red wine may inhibit platelet aggregation and prevent the oxidation of the human low-density lipoproteins (LDL).\footnote{2,3,9,14} Moreover, recent clinical studies have shown that moderate consumption of red wine increases the total antioxidant capacity of human serum.\footnote{22,23} Therefore, the great interest in these phenolic constituents of red wine, including gallic acid, trans-resveratrol, quercetin, and rutin (Figure 1), has been stimulated by the potential beneficial effects on health. The purpose of this article was to study in vitro the antioxidant effects of these phenolic compounds present in red wine, using the Rice-Evans method,\footnote{24} which is based on the inhibition by antioxidants of the cation radical absorption. Total phenols were analyzed according to the Folin-Ciocalteu method, using gallic acid as the standard, and the resulting values were correlated with the total antioxidant activity.

\section*{II. MATERIALS AND METHODS}

\subsection*{A. Materials}

\subsubsection*{1. Apparatus}

Absorption spectra were recorded on a Perkin-Elmer (Beaconsfield, UK) lambda 16 UV-Vis spectrophotometer. Liquid chromatography was carried out using a Merck-Hitachi (Darmstadt, Germany) equipped with a diode-array detector (L-4500 Merck), biocompatible intelligent pump (Merck L-6200), eluent mixing chamber, a manual injector with 20-\( \mu \)l loop and chromatographic data processing software (model D-6500, Hitachi). The operating conditions were at room temperature.

\subsubsection*{2. Chemicals and Standards}

Standard substances of trans-resveratrol, quercetin, and rutin were purchased from Sigma.
Chemical Co. (St. Louis, MO, USA). Gallic acid was obtained from Merck (Darmstadt, Germany). Metmyoglobin (equine) was purchased from Sigma, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diaminonium salt (ABTS) from Aldrich Chem. Co (Milwaukee, WI, USA), and hydrogen peroxide from Panreac Quimica, SA (Barcelona, Spain). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Aldrich. The buffer used was isotonic 5 mM phosphate-buffered saline, pH 7.4 (PBS). Solvents used for chromatography were acetic acid and methanol of HPLC ultra-gradient grade, supplied by Merck, and deionized water. Ethanol and methanol of Uvasol grade from Merck were used for preparing standard solutions. Membrane filters of 0.45-μm pore size from Millipore were used for the filtration of the mobile phase and the samples.

3. Wine Samples

A group of commercially available red wines from different Spanish regions were analyzed. Samples were opened, protected against sunlight, and stored at 4°C. Analyses were carried out within a few days. The samples were filtered through a 0.45-μm membrane Millipore chromatographic filter.
B. Methods

1. Colorimetric Determination of Polyphenols Content

The concentration of wine phenolics was estimated by analyzing for total phenol by the Folin-Ciocalteu procedure and expressing results in micrograms per milliliter or molar equivalents of gallic acid. A calibration curve was prepared using concentrations of gallic acid ranging from 0 to 500 mg/L.

2. Chromatographic Conditions

The phenol contents of the red wine samples were determined by HPLC analysis.26

3. Measurement of Total Antioxidant Activity (TAA)

The TAA was evaluated according to the spectrophotometric method of Rice-Evans and Miller.24 The analytical strategy design was the inhibition assay with a fixed time point: ABTS, myoglobin, and a sample were mixed, and the reaction initiated with the addition of hydrogen peroxide. After a fixed time the absorbance of the solution was read. The blank absorbance minus the test absorbance, divided by the blank absorbance (expressed as a percentage), gives the percentage inhibition of the reaction.

III. RESULTS AND DISCUSSION

A. Total Polyphenols Content

Red wines contain large amounts of phenolic compounds. The concentration of phenolics, estimated by analyzing total phenols in wines, is presented in Table 3. The results show that the red wines contain high concentrations of phenolics. Similar results were obtained when wines were distilled to remove alcohol by vacuum and diluted to their original concentration with distilled water at room temperature.

B. Spectroscopic Identification and Maximum Wavelength of Standard Phenols

Phenols absorb in the ultraviolet (UV) region. Two absorption bands are characteristic of flavonoids. Band II, with a maximum in the 240 to 285 nm range, is believed to arise from the A-ring. Band I, with a maximum in the 300 to 550 nm range, presumably arises from the B-ring.28,29 Spectra of 175 flavonoids, their molecular extinction coefficients, and their UV spectral data in several solvents were published in a 1970 study.28 UV spectra of flavonols (quercetin and its glucoside rutin) exhibit two major absorption bands in the ultraviolet/visible region, Band I in the 320 to 385 nm range, and Band II in the 250 to 285 nm.28,30 Gallic acid and trans-resveratrol present a maximum of absorbance at 280 and 306 nm, respectively. The \( \lambda_{max} \) and \( \varepsilon \) values in methanol and ethanol at various wavelengths of the phenolic compounds studied in this work are presented in Table 1. Figure 2 shows UV-Vis spectra of each phenolic compound.

C. Analysis of Phenolic Compounds Using Photodiode Array Detection

The detection of polyphenols in food analysis is usually by UV-is with diode array detection (DAD). Typical wavelengths for analysis and quantification of flavonols and flavonol glycosides are 270 nm, 365 nm, and 370 nm.31 In this work, the flavonols quercetin and rutin were detected at 360 nm, the phenolic acid (gallic acid) at 280 nm, and the stilbene (trans-resveratrol) at 306 nm.32

236
TABLE 1
Absorbance Data for Standard Phenolic Compounds

<table>
<thead>
<tr>
<th>Phenolic compound</th>
<th>( \lambda_{\text{max}} ) (nm)</th>
<th>( \varepsilon_{\text{methanol}} ) (M(^{-1}) x cm(^{-1}))</th>
<th>( \varepsilon_{\text{ethanol}} ) (M(^{-1}) x cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>272</td>
<td>10 210</td>
<td>9 859</td>
</tr>
<tr>
<td>Trans-Resveratrol</td>
<td>306</td>
<td>29 352</td>
<td>31 041</td>
</tr>
<tr>
<td>Quercetin</td>
<td>373</td>
<td>24 373</td>
<td>26 097</td>
</tr>
<tr>
<td></td>
<td>256</td>
<td>23 797</td>
<td>24 670</td>
</tr>
<tr>
<td>Rutin</td>
<td>360</td>
<td>17 699</td>
<td>19 096</td>
</tr>
<tr>
<td></td>
<td>259</td>
<td>21 153</td>
<td>22 654</td>
</tr>
</tbody>
</table>

\( ^a \) From López et al.,\textsuperscript{26}

Once the chromatographic conditions for the separation had been studied, the procedure was applied to the determination of phenol components in five Spanish red wines from different geographical origins. The identification of the different compounds was achieved by a comparison of both retention time and the absorption spectra obtained for each eluted peak with those obtained for the standards. Table 3 shows the concentrations of the phenolic components in red wine samples. These results have already been published.\textsuperscript{26}

D. Measurement of Total Antioxidant Activity (TAA) of Polyphenols Against Radicals Generated in the Aqueous Phase and Structure-Activity Relationships

The ABTS radical cation assay, for total antioxidant activity in the aqueous phase, measures the collective abilities of the antioxidants in the medium concerned to scavenge the free radical in relation to that of Trolox, a water-soluble vitamin E analogue. Thus, the antioxidant activity can be defined in terms of the concentration of Trolox solution with an equivalent antioxidant potential to a standard concentration of the compound under investigation.\textsuperscript{19,24}

The constituents selected for this survey [trihydroxystilbene (trans-resveratrol), flavonoids (quercetin and rutin) and phenolic acids (gallic acid)] represent the most potent, potentially bioavailable, wine polyphenols in terms of their antiatherogenic, anti-cancer, and antiinflammatory activities,\textsuperscript{37,38,39,40} especially resveratrol and quercetin.

1. Total Antioxidant Activity (TAA) of Individual Phenolics in Pure Solution

The TAA of each of the polyphenols included in this study was measured at four to six concentrations prepared by diluting the pure standard in the appropriate solvent. A range of five dilutions (0 to 0.5 mmol/L) were used to measure TAA for each polyphenol, and the line relating TAA to concentration was derived from least-squares analysis of the data. From the midpoint of each line, the TAA/mmol of compound was calculated. The data of TAA (mmol/L), presented in Table 2, show the TAA of individual polyphenol constituents at a fixed concentration of 1.0 mmol/L derived from calibration curves of the type exhibited in Figure 3.

The ability of the polyphenolic compounds to act as antioxidants depends on the redox properties of their phenolic hydroxyl groups and the
FIGURE 2. UV-Vis spectra of phenolic compounds in methanol. In the case of nonflavonoid components: gallic acid (A) and trans-resveratrol (B) the spectra are shown in the range from 200 to 375 nm. The flavonoid components quercetin (C) and rutin (D) spectra are shown in the range from 200 to 475 nm.
TABLE 2
Hierarchy of Total Antioxidant Activity (TAA) of Polyphenols (TAA of Individual Phenolic Constituents at a Fixed Concentration of 1.0 mmol/L Derived from Calibration Curves of the Type Exhibited in Figure 3)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Free OH-Substituents</th>
<th>Glycosylated Position</th>
<th>TAA (mmol/L)</th>
<th>n</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>3,4,5</td>
<td>3</td>
<td>4.3 ± 0.05</td>
<td>3</td>
<td>hydroxybenzoleo</td>
</tr>
<tr>
<td>Trans-Resveratrol</td>
<td>3,5,4'</td>
<td>3</td>
<td>4.0 ± 0.07</td>
<td>3</td>
<td>stilbene</td>
</tr>
<tr>
<td>Quercetin</td>
<td>3,5,7,3',4'</td>
<td>3-rut</td>
<td>4.7 ± 0.08</td>
<td>3</td>
<td>flavonol</td>
</tr>
<tr>
<td>Rutin</td>
<td>5,7,3',4'</td>
<td>3-rut</td>
<td>3.8 ± 0.06</td>
<td>3</td>
<td>flavonol</td>
</tr>
</tbody>
</table>

TABLE 3
Details of Red Wine Samples Analyzed and Polyphenolic Content

<table>
<thead>
<tr>
<th></th>
<th>Wine 1</th>
<th>Wine 2</th>
<th>Wine 3</th>
<th>Wine 4</th>
<th>Wine 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>Valdepeñas</td>
<td>Ribera Duero</td>
<td>Valdepeñas</td>
<td>La Mancha</td>
<td>Rioja</td>
</tr>
<tr>
<td>Phenolics&lt;sup&gt;a&lt;/sup&gt; (mg/L)</td>
<td>1857</td>
<td>2315</td>
<td>1848</td>
<td>2050</td>
<td>1950</td>
</tr>
<tr>
<td>Antioxidant activity&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.6</td>
<td>14.3</td>
<td>11.1</td>
<td>13.2</td>
<td>12.5</td>
</tr>
<tr>
<td>Gallic acid&lt;sup&gt;c&lt;/sup&gt; (µg/ml)</td>
<td>53.3</td>
<td>46.2</td>
<td>48.1</td>
<td>38.8</td>
<td>27.7</td>
</tr>
<tr>
<td>Trans-Resveratrol&lt;sup&gt;c&lt;/sup&gt; (µg/ml)</td>
<td>nd</td>
<td>1.34</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Quercetin&lt;sup&gt;c&lt;/sup&gt; (µg/ml)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>4.66</td>
</tr>
<tr>
<td>Rutin&lt;sup&gt;c&lt;/sup&gt; (µg/ml)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total phenol content of red wines determined by the Folin-Ciocalteu method
<sup>b</sup> Antioxidant activity was determined by ABTS<sup>-</sup> method
<sup>c</sup> From López et al.,<sup>26</sup>
nd, not detected

239

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
potential for electron delocalization across the chemical structure. Table 2 compares the structures and the antioxidant activities of quercetin, gallic acid, rutin, and trans-resveratrol. The 3,4,5-trihydroxybenzoic acid (gallic acid) has an antioxidant capacity of 4.3 mM, corresponding to the three available hydroxyl groups. The esterification of the carboxylate group of gallic acid decreases the effectiveness and the substitution of the 3- and 5-hydroxyl with methoxy groups results in a decrease in antioxidant activity in comparison with the trihydroxy derivative.\[41\] The relationship between the structure of flavonoids and their antioxidant potential has been intensively studied by Rice-Evans and co-workers.\[41\] These authors reported the chemistry of flavonoids to be predictive for their free radical scavenging activity. Three criteria were found to enhance radical scavenging effectiveness: (1) an ortho-dihydroxy structure in the B ring; (2) a 2,3-double bond in conjugation with a 4-oxo function in the C ring, and (3) hydroxy groups in positions 3 and 5 in the A ring. These are some of the most important structural features defined by Bors\[42\] for the antioxidant activity of polyphenolic compounds. Our results concerning the antioxidant capacity of phenolic compounds studied agree with these findings. Quercetin, with the 2,3-double bond in conjugation to the 4-oxo group, exhibited the highest antioxidant activity value (Table 2).

The unsaturation in the C ring (Figure 1) and the electron delocalization across the molecule allow the stabilization of the arylxyl radical. The glycosylation of flavonoids reduces their activity compared with that of the corresponding aglycones.\[43\] Blocking the 3-hydroxyl group in the C
ring of quercetin as a glycoside (while retaining the 3',4'-dihydroxy structure in the B ring) as in rutin, or quercetin rutinoside, decreases the antioxidant activity value. The comparison of quercetin with rutin demonstrates the influence on antioxidant activity of the 3-OH in combination with the adjacent double bond in the C ring.

2. Total Antioxidant Activity of Spanish Red Wines

The total antioxidant activity (TAA) of five Spanish red wines from various origins was also investigated. TAA was in the range 11 to 15 mmol/L.

The polyphenolic content defines the antioxidant activity of the wines, and these are the major antioxidant constituents. The high antioxidant activity of the red wine sample could be attributed to synergistic effects in a mixture of natural phenolic compounds. Quercetin has a higher antioxidant activity and has been credited with being a major contributor to the antioxidant potential of red wines. However, our results for sample 2 of red wine show a synergistic effect involving gallic acid and trans-resveratrol. This sample showed greater TAA than the sample containing quercetin (sample 5), despite of the fact that, individually, quercetin shows the greatest antioxidant activity of the four phenolic compounds studied.

3. Relationship between Total Antioxidant Activity and Total Phenolic Content

The present study reveals a strong correlation between antioxidant activity and total phenolics quantified by the Folin-Ciocalteu assay (Figure 4). The total antioxidant activities of the wines studied are well correlated with total phenols, suggesting that the antioxidant activity may be obtained directly from gallic acid equivalents, according to this relationship:

\[
TAA = 0.0064 \text{GAE} - 0.2508 \quad (R^2 : 0.9268)
\]

These results confirm that red wine polyphenols are significant antioxidants in vitro and may explain the beneficial effects of a moderate daily intake of red wines.

The TAA varied from 11.1 to 14.3 mM Trolox equivalents for five red wines analyzed and the content of total phenols, as determined by Folin-Ciocalteu method, varied from 1 848 to 2315 mg/L GAE. These values are in agreement with those reported by Rice-Evans and Simonetti.

**FIGURE 4.** Relationship between total antioxidant activity (TAA) and phenolic content of red wines. Antioxidant activity was determined by ABTS\(^+\) method. Total phenolic content was determined by the Folin-Ciocalteu colorimetric assay [results expressed as mM gallic acid equivalents (GAE)].
A number of epidemiological studies have focused on the nonalcoholic components of wine as a “protective factor” in regard to human health, and several of these have suggested that the mortality rate from coronary heart disease can be decreased by moderate consumption of alcohol, particularly red wines. Antioxidant polyphenolic compounds, relatively abundant in red wines, have been considered to be mainly responsible for such epidemiological observations. The results of this current study suggest that phenolic components, characteristic of red wine, could be an important contributor to protective action because they have been shown to possess antioxidant activity and do not exclude the possibility of a synergistic action among the different classes of polyphenols. Red wine, consumed regularly at a moderate level of 150 to 300 ml/day, supplies natural antioxidants in their crude state, which may provide additional protection against in vivo oxidation of cellular components. However, sufficient data regarding their bioavailability and metabolic fate are not yet available. Consequently, further in vivo studies are essential in order to provide more evidence for the antioxidant role of the phenolic components of red wine.

REFERENCES


25. Reglamento (CEE) Nª 2676/90 de la Comisión de 17 de Septiembre de 1990 por el que se determinan los métodos de análisis comunitario aplicables en el sector del vino (Diario Oficial de las Comunidades Europeas).


