

A Novel Fluorimetric Method to Evaluate Red Wine Antioxidant Activity

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Received: 07 March 2018, Accepted: 20 June 2018, Published online: 17 July 2018

Abstract

In the light of the increasing use of red wine in the diet, due to its antioxidant properties, the availability of a simple and fast method to assay the antioxidant activity of this beverage is needed. Here we measured wine antioxidant capacity (WAC) via "crocin bleaching test", wine polyphenol content (WPC), via Folin Ciocalteu method, and fluorimetric response (WF) of 13 Italian red wines. A fairly good correlation between WAC and WF ($R = 0.82$) was found, thus validating the fluorimetric method as a simple, fast and cheap method to evaluate the antioxidant activity of red wines.

Keywords

red wine, antioxidant activity, fluorescence, polyphenols, crocin bleaching

1 Introduction

In discussing health attributes of wine the antioxidant capacity emerges as a major element [1, 2]. Flavonoids make up the bulk of the phenol content of wine, and there is wide evidence for prophylactic and therapeutic effects of these micro nutrients in a variety of human diseases [3, 4], although the absorption and metabolism require further investigation [5–8]. There is however evidence for the transfer of antioxidant capacity from wine to plasma [9–13]. On the other hand, wine antioxidants could play a protective role, independently from bioavailability, in reducing the content of peroxides of foods, and thus decreasing the amount assumed. Indeed it has been observed, using a chemiluminescent procedure, that lipid peroxides are detectable in plasma following a fat meal, and that the concentration is lower if the meal is assumed with red wine [14, 15]. Moreover, epidemiological studies have shown that moderate intake of red wine reduces incident of a variety of disease including coronary heart diseases [16]. In a pilot study it was shown that moderate red wine consumption, as a supplement to the standard treatment, reduces oxidative stress in patients recovering from an acute coronary syndrome [17]. It is assumed that beneficial effects of red wine are due to the antioxidant properties of its polyphenolic compounds which act in vivo against the reactive oxygen species (ROS) [18] which are responsible of the oxidative stress, one of the initial steps

of many chronic diseases including cardiovascular diseases, atherosclerosis, diabetes, cancer etc. [19, 20].

Thus, in evaluating the quality of wine, in addition to flavour, colour and fragrance, the antioxidant activity should also be considered [21–24], as also shown by the increasing number of papers dealing with this topic.

Indeed plenty of analytical methods were developed to assess the antioxidant activity of beverage and food. In particular, in addition to thiobarbituric acid [25] and thiocyanate [26] methods, a variety of methods are available including some based on free radical production as 4-amino-N,N-dimethyl aniline dihydrochloride (DMPD) [27], diphenylpicryl-hydrazyl (DPPH) [28], 2,2'-azino-bis(3-ethyl) benzotiazoline-6-sulphonic acid (ABTS) [29], other on crocin bleaching [30, 31], electron spin resonance [32] and on measurement of Oxygen Radical Absorbance Capacity (ORAC) [33]. Commonly, when dealing with peroxidation-driving peroxy radicals the antioxidant activity is monitored as the "chain breaking antioxidant capacity" (for ref see [34]). Differently, a competition kinetic procedure can be used by comparing the antioxidant activity of the sample under study with the antioxidant activity of either tocopherol or its water soluble analogue Trolox C [35, 36].

As known, fluorimetric measurements have been successfully applied to food and, more recently, the development

of front-face fluorescence takes advantage of fluorimetric properties of wine and other beverages [37, 38]. However, this technique is more time-consuming and not so easy to perform in the absence of qualified personnel, thus making these analyses difficult to apply routinely, especially for little wine makers. On the contrary, an easy, time and money saving measurement of wine antioxidant capacity could be extremely interesting for this kind of producers.

In this paper we measured the fluorescence of 13 Italian red wines; the fairly good correlation between red wine fluorescence values at 310 nm (280 nm excitation) and their antioxidant activity, measured by crocin bleaching test as Trolox C equivalents suggests a simple, fast and cheap novel fluorimetric method to evaluate the red wine antioxidant activity.

2 Materials and Methods

2.1 Wine samples and origin

13 red wines from different geographical areas (Regions) of Italy were purchased from local store: Grignolino (Piemonte), Trabucco (Molise), Cirò (Calabria), Frappato (Sicilia), Bardolino (Veneto), Tintilia (Molise), Primula (Trentino), Rosso di Montalcino (Toscana), Il Moro (Toscana), Montepulciano (Toscana), Cannonau (Sardegna), Chianti (Toscana), Merlot (Veneto).

2.2 Materials

2,2'-azobis (2-amidinopropane) dihydrochloride (ABAP), Folin-Ciocalteu reagent 2.0 N, Trolox C, catechin and gallic acid were from Sigma-Aldrich; all solvents (HPLC grade) and common reagents were from Merck. Saffron was bought from the local market.

Crocin was isolated from saffron by methanol extraction as in [39]. The concentration of crocin was determined photometrically ($\epsilon_{440} = 1.33 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) by using UV-VIS with a Spectrophotometer (Varian Cary 50).

2.3 The competition kinetics test

Thermal decomposition of ABAP generates carbon centered radicals, which react with molecular oxygen yielding peroxy radicals, in a reaction controlled by diffusion [30, 31]. These radicals bleach the carotenoid crocin, and the rate is followed as a specific decrease of absorbance at 440 nm [31, 39]. In the presence of another radical scavenger, competing with crocin for the reaction with peroxy radicals, the bleaching rate slows down, consistently with the rate constant of the reaction between the scavenger and peroxy radicals (see Scheme 1).

Unless otherwise stated, the reaction mixture contained in 2.0 mL of a 0.1 M phosphate buffer pH 7.0 (buffer A), 10 μM crocin (from a 0.6 mM solution in methanol) and increasing amounts of wine or the solution containing the antioxidant to be analysed. The reaction started by adding 12.5 mM ABAP (withdrawn from a fresh 0.5 M solution in buffer A kept at 0 °C) to the complete reaction mixture equilibrated at 40 °C.

The bleaching rate of crocin became linear approximately one or two min after the addition of the diazocompound and was usually recorded for 10 min. Only the last 5 min were considered for calculations.

Blanks without crocin were routinely run to rule out spectral interferences between the sample under analysis and crocin.

A mathematical transformation of the competition kinetics equation [31] yields the equation:

$$V_0/V = (k_c [C] + k_a [A]) / k_c [C] = 1 + k_a/k_c \times [A]/[C] \quad (1)$$

where:

V_0 = basal bleaching rate of crocin with peroxy radicals;

V = bleaching rate of crocin with peroxy radicals in the presence of the antioxidant;

k_c = rate constant of the reaction between peroxy radical and crocin;

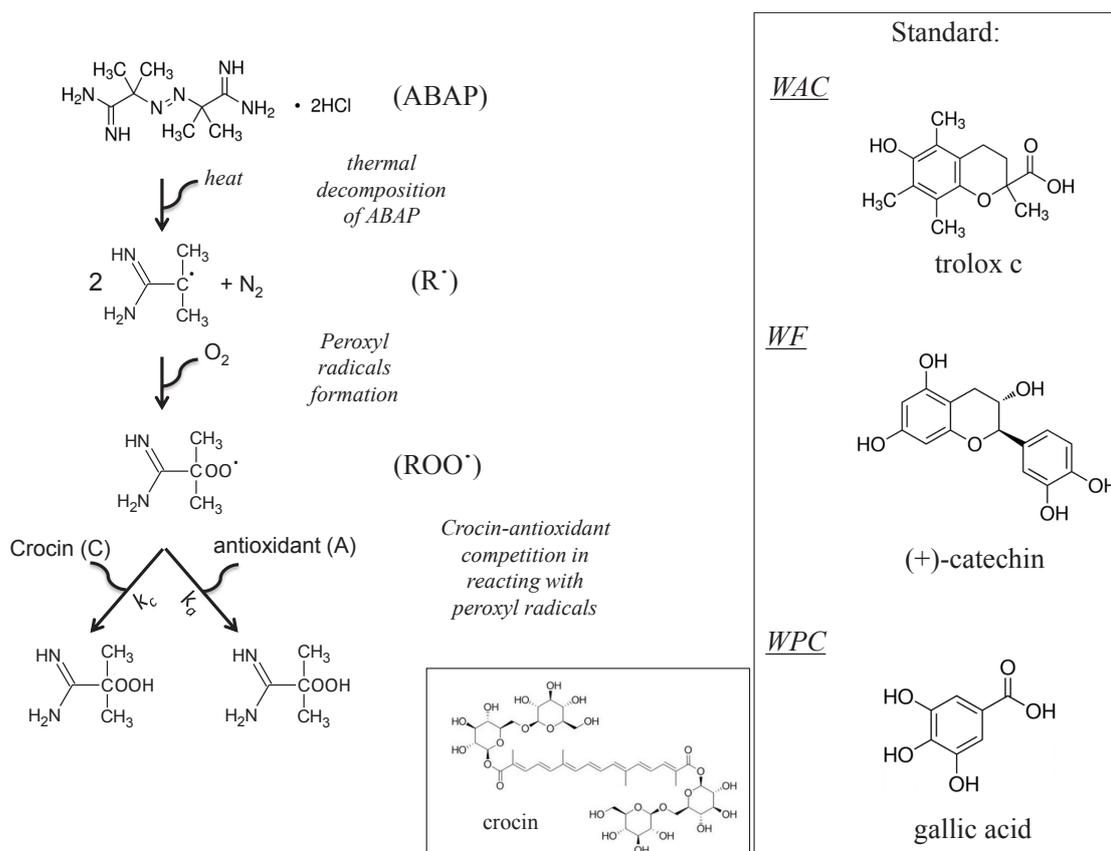
k_a = rate constant of the reaction between peroxy radical and antioxidant;

$[C]$ = concentration of crocin;

$[A]$ = concentration of antioxidant.

The value of k_a/k_c , corresponding to the slope of the linear regression of the plot of $[A]/[C]$ vs. V_0/V , indicates the relative capacity of the compound under analysis to interact with peroxy radicals.

The described kinetic procedure, set up for the analysis of the antioxidant capacity of single molecules, may be extended to complex mixtures where neither the concentration of antioxidants present nor their actual number are known. Wine and other food samples are of this kind. In fact, the overall antioxidant capacity of a complex mixture, corresponds to the sum of each antioxidant concentration multiplied by its rate constant for the interaction with a peroxy radical [31]. Moreover, those compounds possibly present in the mixture, which upon interaction with the peroxy radical produce new radicals and therefore are reactive with crocin, will affect the crocin bleaching rate, decreasing the antioxidant capability of the mixture, as measured by the kinetic test. Thus, from the



Scheme 1 Summary of reactions and chemical structures of compounds used in this work.

kinetics of crocin bleaching in the presence of a mixture of compounds, a value $k_a \times [A]$ can be calculated, where $[A]$ is the molar concentration of the theoretical antioxidant and k_a is the rate constant for its interaction with peroxy radicals. The $k_a \times [A]$ value accounts for the summed effect of all antioxidant and pro-oxidant agents present in the mixture which interact with free radicals.

The ratio between k_a/k_c of the mixture of antioxidants and k_a/k_c of Trolox C, expressed as "Trolox C equivalents", accounted for the antioxidant capacity of the analyzed sample. According to the competition kinetics, by plotting the bleaching rates obtained using the solution under analysis, a ratio k_a/k_c is obtained. By dividing this value by the k_a/k_c of Trolox C, a millimolar concentration of Trolox C accounting for the unknown solution antioxidant capacity is calculated [30, 36].

2.4 Wine sample preparation

To measure the antioxidant capacity by kinetic competition test, red wines aliquots were taken from closed bottles with a long stainless steel needle and diluted 1:10 with phosphate buffer pH 7.0.

2.5 Spectroscopic analyses

Spectrophotometric and fluorimetric analyses were performed with a Jasco V-570 spectrophotometer and FP-750 spectrofluorimeter, respectively.

Wine aliquots (5–40 μL range) were taken as in 2.4 and added with distilled water up to 2 mL in quartz cuvettes. Absorption spectra (from 250 to 600 nm) and fluorescence emission spectra (from 290 to 500 nm, λ_{ex} 280 nm) were recorded, in triplicate. All analyses were carried out immediately after wine sampling (less than 3 minutes) in order to limit oxidation reactions. For fluorimetric analysis reported in Table 1 and Figs. 4 and 6, 10 μL of wine sample were used in order to limit self-absorption; moreover, to rule out any instrumental variability intrinsic to the technique, a standard of catechin was used and wine fluorescence values were also expressed as catechin equivalents. Linearity between absorbance (280 nm) and fluorescence (280 / 310 nm) values vs. wine concentration was also checked (not shown).

Correlations among fluorescence values, antioxidant capacity and polyphenol content of wines were assessed through Pearson's correlation coefficients setting

significance threshold at the $P < 0.05$ level and $P < 0.01$ levels. All statistical tests were performed using the software package SPSS (15.0 version).

2.6 Polyphenol content detection

Folin-Ciocalteu reagent is frequently used to evaluate phenols and is utterly described by official analytical methods [40]. The reduction of phenolic substances by the Folin-Ciocalteu reagent involves reaction with a mixture of phosphotungstic acid and phosphomolybdic acid and subsequent spectrophotometric comparison at 765 nm. Gallic acid has been used as a standard thus the results were expressed as gallic acid equivalents. The method lacks specificity, as different phenolic substances can have different molar absorptivities. In addition, it also reacts with many other substances, such as sugars [41].

3 Results and Discussion

Both spectroscopic and fluorimetric measurement of solutions are simple, fast and relatively cheap needing only the experimental equipment. In the light of this and since wine contains a variety of chromophore, we compared the optical properties of diluted solutions of 13 Italian red wines. As expected, due to the presence of polyphenols, all these wines show absorbance maximum peak at 280 nm (Fig. 1). However, all the investigated wines differed one from another with respect to their absorption spectra, thus showing that spectroscopic properties could be used to characterize each wine. In order to gain further insight into this point, the fluorescence properties of each wine were investigated. In a first set of experiments, fluorescence spectra were obtained by using 280 nm as excitation wavelength. The investigated wines were found to differ one from another with respect to both the numbers (and wavelength) of peaks and fluorescence intensity (Fig. 2). As reported in introduction, this aspect was well-developed in front-face fluorescent measurements [37, 38]. On the other hand, besides the variability in fluorescence profile, all samples showed a peak at 310 nm. Thus, we tried to ascertain whether this spectroscopic property could somehow mirror the red wine antioxidant activity. To achieve this, comparisons were made among wine fluorescence (λ_{ex} 280 nm, λ_{em} 310 nm) (WF), wine total polyphenol content (WPC) and wine antioxidant capacity (WAC) measured by "crocin bleaching test" as in [30].

WPC and WAC were considered since both of them are used to measure the wine antioxidant activity having the latter the advantage to monitor the effective antioxidant

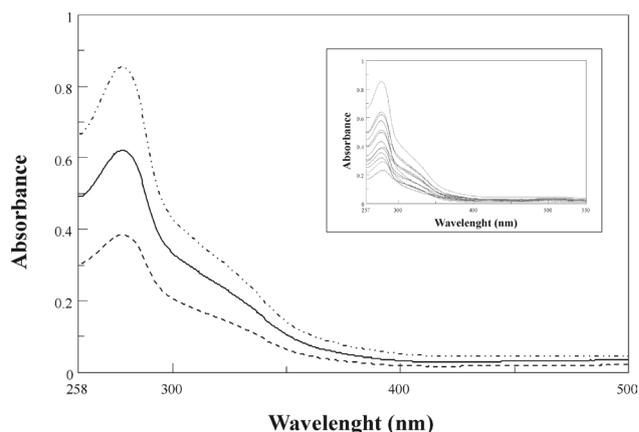


Fig. 1 Absorption spectra of different Italian red wines. Aliquots (40 μl) of each sample were added to water (final volume 2 ml) and the absorbance was measured in 250–550 nm range (see text for details). For more clarity, figure reports the absorption spectra of only three wines: Cirò (.....), Cannonau (—) and Frappato (---), while the absorption spectra of all wines are reported in the inset.

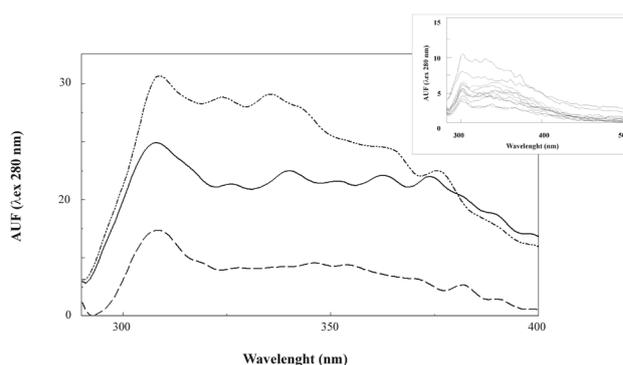


Fig. 2 Fluorescence spectra of different Italian red wines. Aliquots (40 μl) of each sample were added to water (final volume 2 ml) and emission fluorescence (λ_{ex} 280 nm) was measured in 290–500 nm range (see text for details). For more clarity, figure reports the spectra of three wines showing high (Cirò,), medium (Cannonau, —) and low (Frappato, ---) fluorescence values. In the inset, spectra of all wines (obtained diluting 15 μl of each wine in 2 ml final volume) were reported. AUF: arbitrary units of fluorescence.

activity rather than the content of the polyphenol and perhaps unknown compounds.

WAC was calculated from the slope of the fitting of the kinetic equation (for detail see [31]):

$$V_0/V = 1 + k_{\text{PseudoTrolox}}/k_c \times [\text{PseudoTrolox}]/[C] \quad (2)$$

where $k_{\text{PseudoTrolox}}$ is the rate constant for the interaction of Pseudo Trolox with peroxy radicals. Since: $k_a \times [A] = k_{\text{PseudoTrolox}} \times [\text{Pseudo Trolox}] = k_{\text{Trolox}} \times [\text{Trolox}]$ and since k_{Trolox} is known from independent measurements (0.76 ± 0.03), the ratio $[\text{PseudoTrolox}]/[\text{Trolox}]$ can be calculated. This ratio indicates the relative capacity of the

sample vs. Trolox to produce the same antioxidant effect. If a 1 mM solution of Trolox is used as a reference compound, a mM concentration of Pseudo-Trolox is obtained.

In Fig. 3 the plots $[A]/[C]$ vs. V_0/V of Trolox C and of two different wines are shown.

WPC was determined as reported in methods.

A typical experiment in which WF, WPC and WAC were determined is shown in Table 1. In this case, all the measurements have been carried out within 24 hours. Being fluorescence emission highly dependent on instrumental features (sensitivity, intensity of incident light, lamp age etc.), we measured a calibration curve with a standard of catechin and reported WF as catechin equivalents. Similar experiments carried out after weeks / months were in a fairly good agreement with data reported in Table 1. Except for "Bardolino" and "Frappato" (which also showed lowest fluorescence values), we measured an average high WAC in all wine samples, in fairly good agreement with what reported in other papers [42–45] as also occurs for WPC, although we measured values a little bit lower than ones reported in other papers for some Italian red wines [45, 46]. Unfortunately, due to technical reasons dependent on the experimental equipment, it was not possible to carry out a statistical analysis over 1 year. Nonetheless, the differences conformed to the same model.

In order to establish the correlation among the investigated parameters, Table 1 data for each wine were plotted as WF vs. WAC (Fig. 4), WPC vs. WAC (Fig. 5) and WF vs. WPC (Fig. 6).

Interestingly, a significant ($p < 0.01$) good correlation ($R = 0.82$) between WF and WAC was found (Fig. 4),

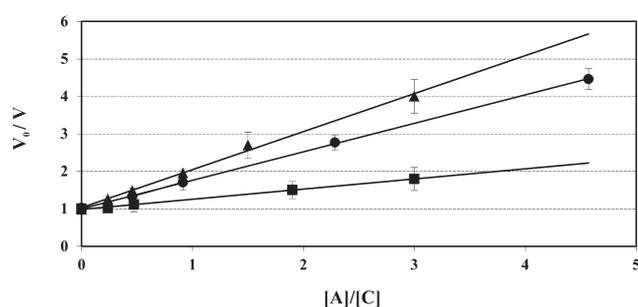


Fig. 3 Analysis of antioxidant capacity of wines by competition kinetics. An example of plot of competition kinetics equation is reported. V_0/V is the ratio between the crocin bleaching rate in the absence and in the presence of an antioxidant. $[A]/[C]$ is the ratio between the antioxidant and crocin concentration. ● Trolox ($y = 0.76x + 1.00$; $R = 0.99$); ▲ Cirò ($y = 1.01x + 1.03$; $R = 0.99$); ■ Bardolino ($y = 0.27x + 0.99$; $R = 0.99$). The data are expressed as means \pm SE obtained in three different measurements.

Table 1 Antioxidant activity (WAC), fluorescence (WF) and polyphenols content (WPC) of red wines. Analyses were carried out as described in Methods and in [25]. The value of k_a/k_c , corresponds to the slope of the linear regression of the plot of $[A]/[C]$ vs. V_0/V , where V_0 and V are the rates of crocin bleaching in the presence and in the absence of other antioxidants respectively, $[A]$ is the concentration of the wine antioxidants as PseudoTrolox and $[C]$ is the concentration of crocin, accounts for the antioxidant capacity. Trolox C equivalents were calculated by dividing the k_a/k_c of wines by the k_a/k_c of Trolox C. WF (10 μ l sample) was measured as described in Fig. 2, and reported as catechin equivalents.

Wine name	WAC Trolox eq. (mM)	WF Catechin eq. (mg/l)	WPC Gallic ac. eq. (mg/l)
Grignolino	9.24	220.4	1190
Trabucco	9.14	219.3	1150
Cirò	13.58	315.8	1550
Frappato	4.31	164.5	1100
Bardolino	3.12	115.1	150
Tintilia	10.23	246.7	1550
Primula	8.37	185.3	1200
Rosso di Montalcino	9.07	197.4	1400
Il Moro	9.79	170.0	1600
Montepulciano	9.72	183.1	1500
Cannonau	9.40	251.1	1220
Chianti	8.67	187.5	700
Merlot	10.19	191.9	1280

which results better than that obtained between WPC vs. WAC ($R = 0.76$, $p < 0.01$, Fig. 5).

This shows that, under the conditions reported above, measurements of red wine fluorescence mirror their antioxidant activity. The ratio WF / WPC was even lower

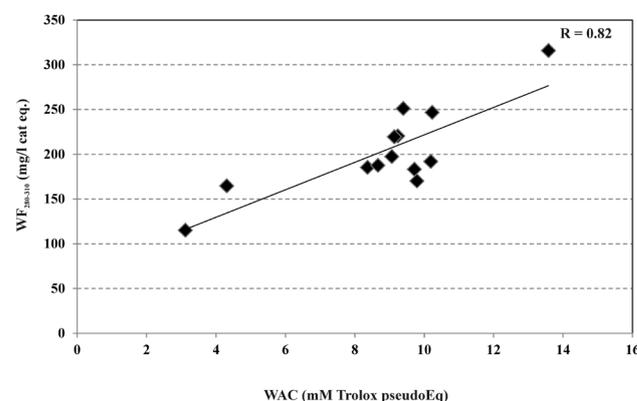


Fig. 4 Regression curve between antioxidant capacity (WAC) and wine fluorescence (WF). The same 13 wines were analysed in both ways, and the correlation between emission at 310 nm (see text for details) reported as catechin equivalents (mg/l) and equivalents of Trolox (mM) was calculated ($y = 15.33x + 68.27$; $R = 0.82$; $p < 0.01$).

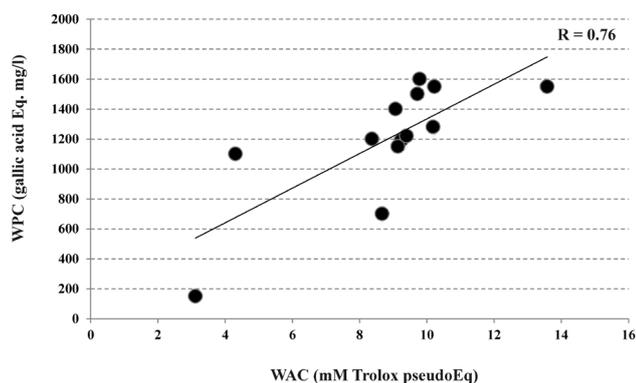


Fig. 5 Regression curve between wine polyphenol content (WPC) and antioxidant capacity (WAC). The antioxidant activity was measured by the kinetic competition test (see text for details). The total phenol content was determined by Folin-Ciocalteu reagent and the correlation was calculated ($y = 115.6x + 178.15$; $R = 0.76$; $p < 0.01$).

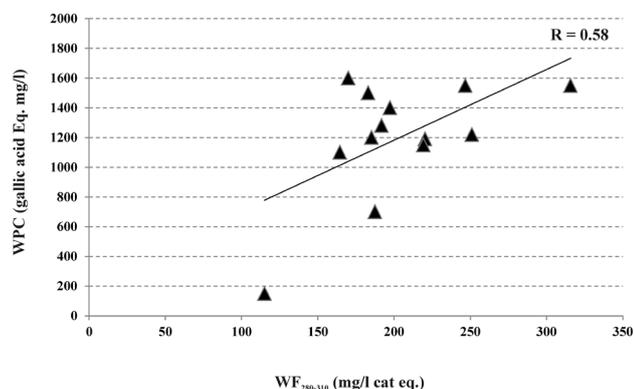


Fig. 6 Regression curve between polyphenol content (WPC) and fluorescence (WF) of wine. The same 13 wines were analyzed in both ways, and the correlation between emission at 310 nm (see text for details) and total phenol content was determined ($y = 4.75x + 230.62$; $R = 0.58$; $p < 0.05$).

($R = 0.58$, $p < 0.05$, Fig. 6) demonstrating that the fluorimetric approach on wine differs from WPC since the substances responsible for the antioxidant capacity probably are not only flavonoids in the checked wines.

In this case only red wines were investigated, but it could be that similar analysis can be applied to other

types of samples showing the same features. Flavonoids containing foods are good candidates for such an investigation.

Thus, here we show that the evaluation of antioxidant activity of red wines, and perhaps of other beverages, can be performed simply by measuring the fluorescence emission of diluted samples. The method strategy involves: i) investigation of photometric / fluorimetric of the sample; ii) identification of the absorbance / fluorimetric response and iii) comparison of these data with those obtained by traditional methods. Moreover, a standardization of this procedure can be made routinely by measuring simultaneously a catechin standard calibration curve, in order to rule out any instrumental variation. This novel method cannot substitute the more informative, but more complex, spectroscopic one as front-face fluorescence in the analyses of wine, especially in their characterization [38]. However, the sound correlation obtained here between spectroscopic data and those traditionally obtained clearly shows that this fluorimetric approach can be an extremely simple, fast and relatively cheap way for wine antioxidant determination. This simplicity makes the method as a routinely tool, not restricted to big wine companies, probably sound to investigate also the effects of technological treatment during wine preparation and aging.

4 Conclusion

Results reported here clearly show that fluorescence of diluted untreated samples of red wines used in this study mirrors their antioxidant capacity, thus suggesting this easy and (relative) cheap analysis as a promising tool for the wine industry.

Acknowledgement

This work was partially financed by an internal fund of Department of Medicine and Health Sciences "V. Tiberio", University of Molise.

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