



Article

# Effect of *Hippophae Rhamnoides L.* Leaves Treatment on the Radical Scavenging Activity, Reducing Power, Total Phenol Content and Sensory Profile of Dry White Wines Vinified with and without the Use of Sulphur Dioxide

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**Abstract:** This study evaluated the influence of the addition of increasing quantities of *Hippophae rhamnoides L.* leaves (HRL) on the radical scavenging activity, main oenological parameters and organoleptic characteristics of three white wines made from moschofilero and one white wine made from riesling grapes respectively. Radical Scavenging activity, reducing power, total phenol content (TPC) and color intensity increased in a linear manner in relation to HRL treatments. Indicatively the addition of 0.8 g/L of HRL increased the radical scavenging activity as determined via the inhibition of the DPPH radical from 23.2 to 58.4% in comparison to the initial values. Equally the reducing power as determined by the FRAP assay increased from 34.5 to 82.3%, TPC increased from 12.3 to 26.8% and the color intensity increased from 39-50%. The main oenological attributes examined, remained unchanged after the HRL addition. The addition of up to 0.4 g/L of HRL did not have a major impact on the organoleptic characteristics of the wines tasted whereas concentrations higher than 0.8 g/L were not considered beneficial. Results denote that the addition of *Hippophae rhamnoides L.* leaves to white wines contributes positively to the overall antioxidant capacity and could be used as an antioxidant agent in wines vinified in the absence of sulphur dioxide.

**Keywords:** Wine; DPPH; FRAP; *Hippophae rhamnoides L.* leaves; sulphur dioxide

## 1. Introduction

Wine is considered as one of the most important dietary sources of antioxidants for the human body. The antioxidant capacity of wines is mainly attributed to flavonoid and non-flavonoid compounds [1-3] macerated and/or produced during the alcoholic fermentation even though in certain cases the contribution of exogenous added antioxidants such as SO<sub>2</sub> and ascorbic acid has been found higher than the naturally existing antioxidants [4,5]. The polyphenolic content in white wines is generally lower than red wines, consisting mainly of hydroxycinnamic acids which are significantly crucial in oxidation related issues such as browning and flavor deterioration [6]. The contribution of the polyphenolic content to the overall antioxidant capacity in wines with sulphur dioxide additions (further down also referred to as conventional) is often overestimated due to the presence of sulphur dioxide [7].

Sulphur dioxide or SO<sub>2</sub> is the most widely used additive in the wine industry. The addition of sulphur dioxide in wine, inhibits oxidation and the growth of spoilage microorganisms, augments



pigment extraction and reduces the polymerization of phenolics [8] making it an indispensable partner of the vinification process. Nevertheless the use of sulphur dioxide has been regulated due to allergic reactions observed on hypersensitive individuals [9,10]. Furthermore the excessive use of sulphur dioxide raises qualitative issues since it has been involved in the presence of off flavours [11, 12] and in haze formation [13]. Currently there is a tendency of reducing the levels or replacing sulphites salts in order to produce minimum interventions wines and this is becoming relatively feasible with the use of physical and/or chemical methods [14-17].

*Hippophaë rhamnoides* L. leaves (HRL) (Elaeagnaceae), also mentioned as sea buckthorn, is an Eurasian nitrogen-fixing actinomycetes plant species, bearing yellow or orange berries [18-20] which has been used for centuries in both Europe and Asia for food, therapeutic, and pharmaceutical purposes. Apart from the berries and seeds, HR leaf extracts have recently gained in interest for their antimicrobial and antioxidant properties [19, 21] and for various pharmacological activities such as anti-inflammation, immunomodulation, radioprotection and tissue regeneration [22-23]. Proestos et al. (2015) [24] reported an increase in the antioxidant capacity of wines after the addition of 0.3 g/l of HRL which was found higher than the corresponding addition of HR berries.

The aim of this work was to evaluate the influence of the addition of increasing quantities of HR leaves on the free radical scavenging activity, reducing power, total phenol content, main oenological parameters and organoleptic characteristics of different white dry wines. Finally our goal was to investigate whether this natural byproduct could be used as an additive to enhance the antioxidant properties of wines produced without the use of sulphur dioxide.

## 2. Materials and Methods

### 2.1 Chemicals and equipment

2,2-Diphenyl-1-Picrylhydrazyl (DPPH), methanol, sodium acetate, iron(III)chloride, Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) and ascorbic acid were purchased from Chem Lab. Gallic acid and hydrochloric acid were obtained from Sigma-Aldrich, 2,4,6-Tri(2-pyridyl)-1,3,5-triazine (TPTZ) from Alfa Aesar, Glacial acetic acid from Carlo Erba and Folin-Ciocalteu Reagent (wine det.) was purchased from Merck.

All reagents and solvents used in this study were HPLC or of analytical grade.

### 2.2 Collection and preparation of plant material

The HR leaves were collected in 2 consecutive years (Sep 2014 & Sep 2015) from the hr crop in ELGO – Dimitra (<http://www.elgo.gr>, former NAGREF, <http://www.nagref.gr/>) in the region of Lykovrisi, Attica, Greece where the plant grows experimentally under natural conditions. Plant material was characterized by Dr. P. Zamanidis researcher in ELGO – Dimitra, Greece, where specimens were kept. Fresh leaves were cleaned thoroughly with nanopure water, dried at 55 °C to 3% of relative humidity and grinded in to powder (oven model ED 115, Binder, Tuttlingen, Germany).

Absorbance measurements were conducted on a UV mini 1240 UV-Visible spectrophotometer (Shimadzu, Japan).

### 2.3 Vinification of wine samples

All grapes were sourced from Arcadia, Peloponese, Southern Greece and vinifications were conducted in the winery of the Boutari s.a (Mantineia, Greece) in the same region. Four different white dry wines were produced in two equally sized batches each, resulting from the vinification in duplicate of approximately 1500 kg of local grapes as follows: a white dry wine made from local c.v. moschofilero grapes with typical white vinification followed by 6 months of maturation in stainless steel tanks referred to as mf15; a dry wine made from local c.v. riesling grapes matured for a period of 6 months in 225L French oak barrels referred to as rs15 and 2 dry wines from 2 consecutive vintages (2014, 2015) of the same vineyard, vinified without sulphur dioxide addition from c.v. moschofilero

grapes referred to as ss14, ss15 respectively matured for 6 months in 225 L French oak barrels (Table 1).

Table 1: Characterization and vinification notes of wine samples.

Sample Code	Variety	Region	Vintage year	Vinification
SS 14	moschofilero	Arcadia	2014	No Sulphites added. 6 months maturation in french oak barrels
SS 15	moschofilero	Arcadia	2015	No Sulphites added. 6 months maturation in french oak barrels
MF 15	moschofilero	Arcadia	2015	Conventional. 6 months maturation in inox tanks
RS 15	riesling	Arcadia	2015	Conventional. 6 months maturation in french oak barrels

Grapes were manually harvested at optimum technological maturity for each variety (20-24 °Brix), placed in small plastic containers and transferred immediately to the winery for the vinification process.

Upon receipt, the grapes were immediately destemmed and crushed, and during transfer the grape juice was treated with sulphur dioxide (50mg/L) to avoid must oxidation except for musts intended for vinifications without added sulphites. The mass was gently pressed under a dry ice blanket (30mm pellets, 10 kg pellets /1000 kg grapes), 2 g/100L of pectolytic enzymes were added (Depectil Clarification FCE) and the must was left to settle for 24h at 10° C. After clarification and under CO<sub>2</sub> blanket, clear juice was transferred to the fermentation tanks where 20 g/100L of yeast nutrients (Actiferm 1, Martin Vialatte, France) were added and the fermentation was quickly induced via inoculation of previously activated (24h before) *Saccharomyces cerevisiae* (Vitilevure 58W3 YSEO, Martin Vialatte, France) starter culture (20 g/100L). Fermentation was carried out in temperature-controlled stainless steel tanks at 15 -17 °C, lasted about 20 days with a complete consumption of the reducing sugars except for mf 15 sample where sugar levels were adjusted to 6 g/L. One week after the end of alcoholic fermentation for mf15 and rs 15 and one week after the end of the spontaneous malolactic fermentation for vinifications without added sulphites, the wines were separated from their lees and racked to clean containers. After the maturation phase the wines were further settled using a complex plant protein-based fining allergen free agent (ProvGreen Pure Wine, Martin Vialatte, France) (10 g/100L), and granular sodium bentonite (50 and 80 g/hL for Riesling and Moschofilero wines respectively). After cold stabilization the wines were racked, filtered (0.65µm) and bottled using a gravity filler.

In order to avoid macro-oxygenation and oxidation, all must and wine transfers, during the whole winemaking process until bottling, were made by previous saturation of pipes and tanks with a mixture of carbon dioxide and nitrogen.

#### 2.4 *Hippophae rhamnoides* L. leaves Treatment

Chemical composition, antioxidant and antimicrobial properties of HR leaves have been previously reported. [19,23,25]. The treatment of wines with *Hippophae Rhamnoides* L. leaves is not listed in the authorized oenological practices and processes in Annex 1A, EC Reg. 606/2009 [26] hence the aforementioned treatments serve experimental purposes solely.

HR leaves from 2014 harvest were used in sample SS 14 in order to initially evaluate HR leaves influence, whereas all the rest wine samples (SS15;MF15;RS 15) were treated with HR leaves from 2015 harvest. Powdered HR leaves were added in increasing quantities (0.00-0.13–0.40-0.60-0.80-1.60 g/L), the wines were capped using a technical stopper and left to macerate for 7 days at 20° C. Subsequently the wine was filtered for the removal of HR leaves and stored at 4° C till the day of analysis. The treatments were replicated in triplicate in each batch. All determinations thereafter were conducted in triplicate.

## 2.5 Main Oenological Attributes

The wine samples were characterised with regard to some common oenological characteristics related to wine quality with the aid of an NIR spectrometer (FOSS, NIR Systems, Inc., EL Leende, Netherlands). Free and total SO<sub>2</sub> were determined according to OIV methods of Analysis [27]. Results are depicted in Table 2.

Table 2: Main oenological parameters of wine samples untreated (0 g/L HRL) and treated with 1.6 g/L HRL. .

Sample Code	HRLT <sup>a</sup>	Free SO <sub>2</sub> <sup>b</sup>	Total SO <sub>2</sub> <sup>b</sup>	TA <sup>c</sup>	VA <sup>d</sup>	pH	Density	% Vol	Extract <sup>e</sup>	Sugar <sup>e</sup>	A420 <sup>f</sup>
SS 14	0	0	9	5.4	0.67	3.39	0.990	11.62	19.88	2.2	0.081
	1.6	0	10	5.4	0.68	3.38	0.990	11.63	19.92	2.2	0.156
SS 15	0	0	10	5.4	0.44	3.34	0.991	11.22	20.59	1.3	0.137
	1.6	0	10	5.4	0.43	3.35	0.991	11.21	20.62	1.2	0.215
MF 15	0	40	141	5.6	0.26	3.25	0.992	11.17	24.45	6.1	0.048
	1.6	37	142	5.6	0.24	3.25	0.992	11.16	24.51	6.0	0.110
RS 15	0	28	128	5.7	0.37	3.19	0.991	11.98	22.91	2.7	0.056
	1.6	26	127	5.8	0.38	3.20	0.991	12.03	22.87	2.6	0.107

Values are means of triplicate determination of each 1 of 3 bottles treated in each hrl concentration (n=18). <sup>a</sup>: HRLT: *Hippophae* leaves treatment expressed in g/L; <sup>b</sup>: Values are expressed as mg/L; <sup>c</sup>: Total Acidity (TA) expressed as g/L tartaric acid; <sup>d</sup>: Volatile Acidity (VA) expressed as g/L acetic acid; <sup>e</sup>: Values are expressed as g/L; <sup>f</sup>: Values are expressed as absorbance units at 420 nm

## 2.6 Sensory Evaluation

Sensory evaluation was conducted according to Castillo-Sánchez et al. [28] with minor modifications in the same month as the analysis. Wine samples were characterized with a three-digit code number which varied from tasting to tasting and were tasted blindly. Consecutively the samples were rated on a 0-10 scale by 8 wine professionals based on their organoleptic properties in an open tasting room using ISO standard tasting glasses. The radar plots 1- 4 contain ratings for 5 distinct descriptors of aroma (limpidity, intensity, fruit, vegetative and oak), and 4 distinct descriptors of taste (body and mouthfeel, balance, fruit, and aftertaste).

## 2.7 DPPH Assay

The determination of free radical scavenging activity by means of the Inhibition (I) of the DPPH radical is based on the quenching of the DPPH radical by antioxidants present in the wine samples. The antioxidant capacity of wines by the DPPH free radical scavenging method was determined following Porgali & Büyüktuncel, (2012) [29] with minor modifications. The procedure consists of adding 33 µL of wine, diluted in methanol, to 967 µL of a DPPH radical methanolic solution (6×10<sup>-5</sup> mol/L), and after 30 min in the dark, measuring the percentage of absorbance decrease at 515 nm. The percentage inhibition of initial concentration of DPPH· radical was calculated as:

$$\% \text{ Inhibition} = [(A_{\text{DPPH}} - A_{\text{wine}}) / A_{\text{DPPH}}] \times 100 \quad [30], \quad (1)$$

Quantification of the antiradical activity (A<sub>AR</sub>) was made by calibration curves obtained from methanolic solutions of Trolox, and the results were expressed in mM Trolox Equivalents (TE).

## 2.8. FRAP Assay

The reducing power of the samples was determined according to the method described by Benzie & Strain (1996), [31], with minor modifications. In this assay Fe(III) oxidizes wine antioxidants and the amount of Fe(II) produced is measured spectroscopically [32]. The Fe(II) selective ligand, 2,4,6- tripyridyl-s-triazine (TPTZ) is added which produces a colored Fe(II) complex that can be quantified spectroscopically at 593 nm and raises the reduction potential of Fe(III)/Fe(II) couple [32]. Procedure consists of mixing 30µL of diluted wine (1:5 for white wines) and 90µL water with 900µL

of freshly prepared, preincubated to 37 °C FRAP reagent, obtained by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solubilized in 40 mM HCl, and 20 mM FeCl<sub>3</sub> in a 10:1:1 ratio. Absorbance of the mixture was measured at 593 nm after 30 min and was deducted from the initial absorbance (M<sub>1</sub>) of FRAP Reagent. A calibration plot was generated based on known concentrations of Trolox; the results were expressed as mM Trolox Equivalents.

### 2.9 Analysis of Total Phenol Content (TPC)

The total phenol content was determined using the Folin-Ciocalteu reagent, as described by Singleton & Rossi (1965)[33]. The F-C reagent comprises of a phosphotungstate-molybdate complex, which on reduction by phenolic compounds in a one or two electron process produces a blue product with an absorbance maximum at 765 nm. A calibration plot was generated based on known concentrations of Gallic Acid. Results were expressed as mg/L Gallic Acid equivalents (GAE).

### 2.10 Statistical Analysis

Data are expressed as means ± SD of three parallel measurements. Correlation analysis between HRL Treatments and antioxidant properties of wine samples was performed by means of Spearman rho coefficient. One way ANOVA using Tukey HSD test was conducted for each assay to determine differences among treated wine samples.  $p < 0.05$  was regarded as statistically significant. All statistical analyses were performed using SPSS Statistical Package (version 25).

## 3. Results

In the current work, we determined the antioxidant capacity, reducing power, total phenol content and organoleptic effect on dry white wines treated with increasing quantities (0-1.6 g/L) of *Hippophae rhamnoides* L leaves. *H. rhamnoides* leaves are considered a good source of bioactive compounds, thus have attracted significant public and scientific interest because of their health promoting effects as antioxidants and antimicrobials [19].

Table 2. depicts the main oenological attributes of wine samples prior and after HRL treatments. Results are shown only for zero and highest HRL dose treatment as no statistically significant differences were observed in the parameters examined with the exception of colour intensity (Absorbance at 420nm), which is depicted in table 3. Colour intensity increased in a linear manner with the increasing HRL treatments. Values of alcohol content, total and volatile acidity, pH and sugar are in accordance with previously published data for Greek white wines [24,34].

Free radical scavenging activity ( $A_{AR}$ ), the ferric reducing power (FRP) and the total phenolic content (TPC), prior and after HR leaves treatments (in all the aforementioned concentrations) are presented in Table 3.

### 3.1. Radical scavenging activity as determined by the inhibition of the DPPH radical assay

The free radical scavenging activity prior and after HRL treatments was evaluated by the DPPH assay [35]. The DPPH assay relies on the neutralization of a methanol-soluble stable radical by direct reduction via electron transfer or by radical quenching via H atom transfer even though the latter could be considered as marginal reaction since it occurs very slowly in methanol [36].

It was observed that the radical scavenging activity of the treated wines increased linearly with the relevant increase in the HR leaves amount. The equations of radical scavenging activity via the DPPH Assay ( $A_{AR}$ ) for the wines treated and HR leaves amount (x) are presented in Figure 1. Regression analysis indicates that the  $A_{AR}$  correlated well with the amount of HR leaves added.

Sulphur dioxide plays a significant role in the antioxidant capacity of white wines [4,5,15]. The conventional white vinifications, even though conducted with different vinification protocols on their maturation phase, exhibited similar values of initial antioxidant capacity ( $A_{AR_{mf15}}$ :  $0.61 \pm 0.01$  mM TE;  $A_{AR_{RS15}}$ :  $0.62 \text{ mM} \pm 0.02$  TE) considerably higher than the sulphite free vinifications ( $A_{AR_{ss15}}$ :  $0.43 \pm 0.01$  mM TE;  $A_{AR_{ss14}}$ :  $0.53 \pm 0.01$  mM TE). Differences in the AC among sulphite free samples can be attributed to various reasons such vintage, vinification protocol or oxygen uptake. Treatment with



0.8 g/L HRL resulted in an increase in AAR by 28.7 to 58.4 % for sulphite-free vinifications ( $A_{AR\ SS14+0.8g/L\ HRL} = 0.69 \pm 0.01\text{mmTE}$ ;  $A_{AR\ SS15+0.8g/L\ HRL} = 0.68 \pm 0.01\text{mmTE}$ ) and 23.4 to 23.3% for conventional vinifications ( $A_{AR\ MF15+0.8g/L\ HRL} = 0.75 \pm 0.01\text{mmTE}$ ;  $A_{AR\ RS15+0.8g/L\ HRL} = 0.77 \pm 0.01\text{mmTE}$ ). Treatment with 1.6 g/L resulted in an increase ranging from 42.3% to 92.2 %.

As it is obvious treatment with 0.8 g/L of HR leaves to the white wines tested increased the radical scavenging activity of the wines without added sulphites to the levels of conventional wines demonstrating that HR leaves can have a positive effect on the AAR of white wines.

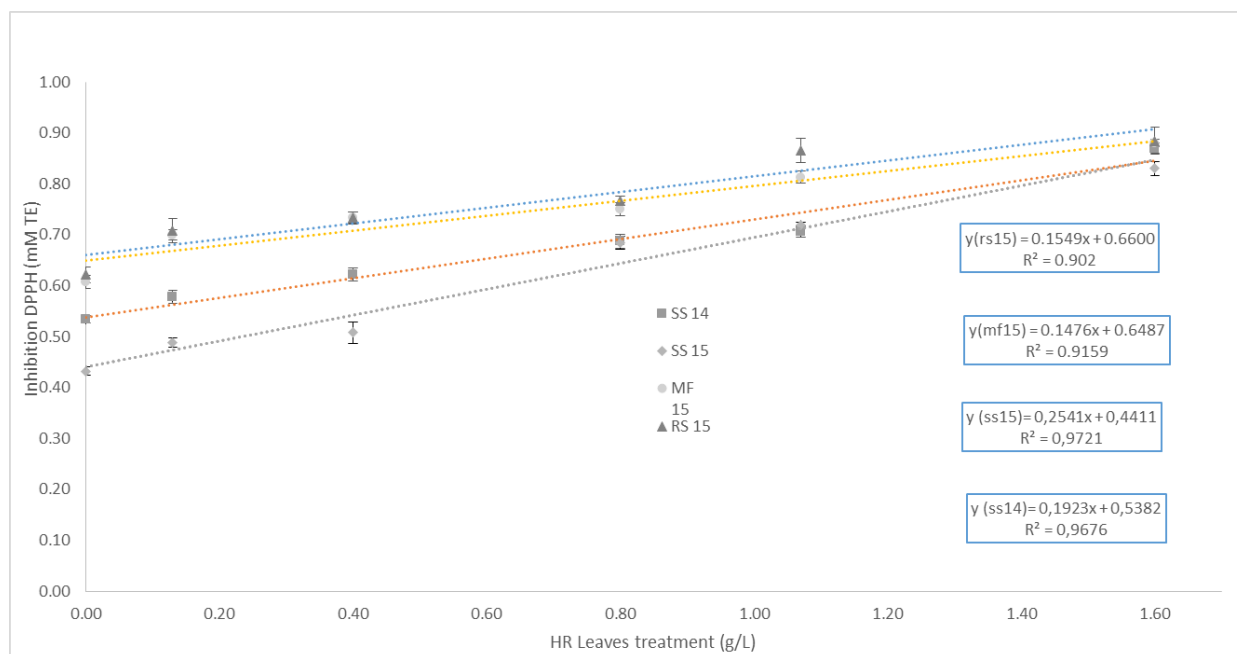


Figure 1: Radical Scavenging Activity via the inhibition of the DPPH radical vs increasing HRL treatments in each wine.

Table 3: Antiradical Activity (AAR), Ferric reducing antioxidant power (FRAP), Total phenol Content (TPC) and color intensity (A420) of wine samples prior and after the addition of increasing quantities of *Hippophaë Rhamnoides L.* leaves.

Source of variation <sup>a</sup>	HRL <sup>b</sup>	AAR <sup>c</sup>	FRAP <sup>d</sup>	TPC <sup>e</sup>	A420 <sup>f</sup>
SS14	0.00	0.53 <sup>a</sup> ± 0.01	1.44 <sup>a</sup> ± 0.09	233 <sup>a</sup> ± 7	0.081 <sup>a</sup> ± 0.001
	0.13	0.58 <sup>b</sup> ± 0.01	1.69 <sup>b</sup> ± 0.03	250 <sup>b</sup> ± 7	0.097 <sup>b</sup> ± 0.003
	0.40	0.62 <sup>b</sup> ± 0.01	1.95 <sup>c</sup> ± 0.04	269 <sup>b</sup> ± 7	0.112 <sup>c</sup> ± 0.003
	0.80	0.69 <sup>c</sup> ± 0.01	2.38 <sup>d</sup> ± 0.04	274 <sup>c</sup> ± 6	0.119 <sup>c</sup> ± 0.003
	1.07	0.71 <sup>c</sup> ± 0.01	2.63 <sup>e</sup> ± 0.03	283 <sup>c</sup> ± 6	0.135 <sup>d</sup> ± 0.003
	1.60	0.87 <sup>d</sup> ± 0.01	2.77 <sup>f</sup> ± 0.03	308 <sup>d</sup> ± 8	0.156 <sup>e</sup> ± 0.003
SS15	0.00	0.43 <sup>a</sup> ± 0.01	1.02 <sup>a</sup> ± 0.03	202 <sup>a</sup> ± 4	0.137 <sup>a</sup> ± 0.002
	0.13	0.49 <sup>b</sup> ± 0.01	1.09 <sup>a</sup> ± 0.02	210 <sup>a</sup> ± 9	0.147 <sup>b</sup> ± 0.003
	0.40	0.51 <sup>b</sup> ± 0.02	1.45 <sup>b</sup> ± 0.03	215 <sup>a</sup> ± 7	0.166 <sup>c</sup> ± 0.002
	0.80	0.68 <sup>c</sup> ± 0.01	1.70 <sup>c</sup> ± 0.02	256 <sup>b</sup> ± 9	0.191 <sup>d</sup> ± 0.003
	1.07	0.72 <sup>d</sup> ± 0.00	1.92 <sup>d</sup> ± 0.02	269 <sup>b</sup> ± 5	0.201 <sup>e</sup> ± 0.002
	1.60	0.83 <sup>e</sup> ± 0.01	2.13 <sup>e</sup> ± 0.07	300 <sup>c</sup> ± 11	0.215 <sup>f</sup> ± 0.003
MF 15	0.00	0.61 <sup>a</sup> ± 0.01	1.67 <sup>a</sup> ± 0.04	292 <sup>a</sup> ± 3	0.048 <sup>a</sup> ± 0.001
	0.13	0.70 <sup>b</sup> ± 0.01	1.83 <sup>a</sup> ± 0.12	303 <sup>a</sup> ± 3	0.060 <sup>b</sup> ± 0.002
	0.40	0.73 <sup>c</sup> ± 0.01	2.11 <sup>b</sup> ± 0.04	305 <sup>a</sup> ± 4	0.066 <sup>b,c</sup> ± 0.001
	0.80	0.75 <sup>d</sup> ± 0.01	2.24 <sup>b</sup> ± 0.06	328 <sup>b</sup> ± 5	0.072 <sup>c,d</sup> ± 0.003
	1.07	0.81 <sup>e</sup> ± 0.01	2.67 <sup>c</sup> ± 0.07	357 <sup>c</sup> ± 4	0.079 <sup>d</sup> ± 0.003
	1.60	0.88 <sup>f</sup> ± 0.01	2.85 <sup>d</sup> ± 0.07	385 <sup>d</sup> ± 4	0.110 <sup>e</sup> ± 0.002

RS 15	0.00	0.62 <sup>a</sup>	± 0.02	1.60 <sup>a</sup>	± 0.12	297 <sup>a</sup>	± 7	0.056 <sup>a</sup>	± 0.002
	0.13	0.71 <sup>b</sup>	± 0.02	1.91 <sup>b</sup>	± 0.05	320 <sup>a,b</sup>	± 11	0.063 <sup>b,c</sup>	± 0.002
	0.40	0.73 <sup>b</sup>	± 0.01	2.02 <sup>b</sup>	± 0.05	325 <sup>b</sup>	± 9	0.068 <sup>c</sup>	± 0.003
	0.80	0.77 <sup>c</sup>	± 0.01	2.44 <sup>c</sup>	± 0.07	350 <sup>c</sup>	± 7	0.080 <sup>d</sup>	± 0.002
	1.07	0.87 <sup>d</sup>	± 0.02	2.63 <sup>c</sup>	± 0.08	364 <sup>c</sup>	± 4	0.089 <sup>e</sup>	± 0.002
	1.60	0.88 <sup>d</sup>	± 0.03	2.94 <sup>d</sup>	± 0.05	396 <sup>d</sup>	± 8	0.107 <sup>f</sup>	± 0.002

All data are means of three independent measurements  $\pm$  std of 3 bottles treated in each HRL concentration of two equally sized batches of wines. Different superscript capital letters in the same column and for each wine(+ treatments) are significantly different ( $p < 0.05$ ) by Tukey HSD Test.

<sup>a</sup>: Source of variation different wine samples (1<sup>st</sup> column) vs HRL treatments (2<sup>nd</sup> column) ;<sup>b</sup>: Values are expressed as g/L; <sup>c</sup>: Values are expressed as mM trolox equivalents  $\pm$  std (n=3); <sup>d</sup>: Values are expressed as mM trolox equivalents  $\pm$  std (n=3); <sup>e</sup>: Values are expressed as mg/L gallic acid equivalents  $\pm$  std (n=3); <sup>f</sup>: Values are expressed as absorbance units at 420 nm  $\pm$  std (n=3)

Published data on the radical scavenging activity of 26 conventional commercial greek white wines with the DPPH assay ([37]) ranged from 0.47 to 0.60 mM TE (mean value: 0.52 mM TE) which were similar to the present study (range 0.43-0.62 mM TE ; mean value 0.55 mM TE) but with minor differences on the assay protocol. On the same study Psarra et al. (2002) [37] report values for conventional wines from moschofilero variety ranging from 0.53 to 0.58 mM TE. Tourtoglou, Nenadis & Paraskevopoulou (2014)[38] report higher values for wines from the malagouzia variety ranging from 0.70 to 0.81 mM TE (mean value 0.85 mM TE) whereas Stratil, Kubáň, & Fojtová, (2008)[39] report values for white wines ranging from 0.61 to 1.68 mM TE.

Proestos et al. (2015)[24] reported that a 0.3g/l HRL treatment resulted approximately in 3.5fold increase in AC via the DPPH assay (from 0.42 to 1.82 mM TE) which is significantly higher than the increase rate in the present study for all white wine samples and for the HR leaves from the 2 consecutive harvests as presented in table 3. This discrepancy may be attributed to several factors namely the chemical composition of the leaves which could be affected by the growing conditions, maturity level, time of harvest [40] as well as drying duration and temperature [21]. It was not possible to further compare the results for the DPPH, FRAP assays and TP content on the wines after HRL treatments since relevant bibliographic references were not found available.

### 3.2. Reducing capacity as determined by the FRAP assay

The FRAP assay is a simple and accurate assay which is based on the reducing capacity of a compound rather than its antiradical activity [5].

FRAP values prior HRL treatments ranged from  $FRAP_{SS15} = 1.02 \pm 0.03$  mM TE to  $FRAP_{MF15} = 1.67 \pm 0.04$  mM TE. Treatment with 0.8 g/L increased FRAP values from 34.5 to 66.9% whereas 1.6g/L almost doubled the initial FRAP values. The increase in ferric reducing power is depicted graphically in Figure 2. Correlation coefficients ( $R^2$ ) are in the range of 0.961 to 0.973 denoting a good correlation between FRAP values of wine samples and HRL additions. As expected sulphite free vinifications demonstrate considerably lower FRAP values but HR leaves treatment ranging from 0.13 to 0.8 g/L increased FRAP values to the level of sulphited samples.

Values of the wine samples prior to the HRL treatments are in the range of previously published literature. Stratil et al. (2008)[39] report FRAP values ranging from 0.86 to 2.14 mM TE for 8 commercial white wines from Czech republic whereas Proestos et al. (2015)[24] report a FRAP value equal to 1.79 mM TE for greek white wine from savatiano c.v. grapes.

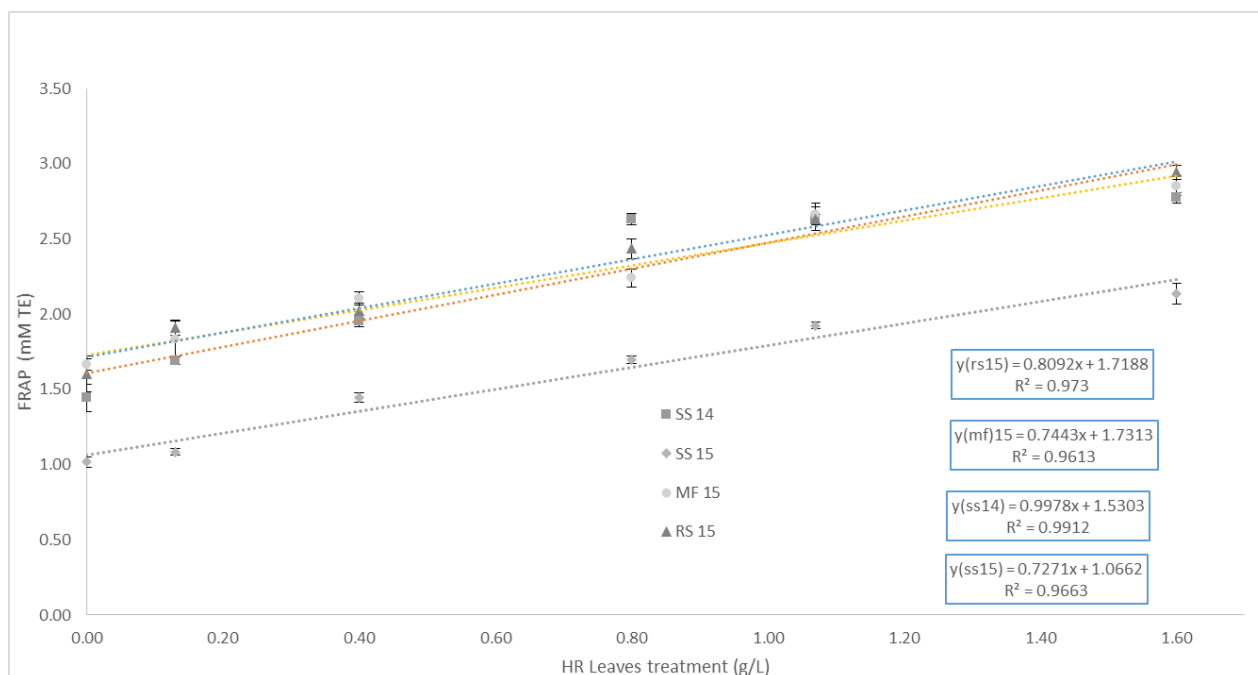


Figure 2: Ferric Reducing Antioxidant Power vs increasing HRL treatments in each wine.

### 3.3. Total phenol content

The contribution of phenolic compounds to the antioxidant activity of wines is considered crucial even though in some cases overestimated [7]. The concentration of different phenolic compounds vary significantly within wines from the same variety according to various factors such as vintage year, soil composition and climatic conditions. Moreover, significant changes may occur in the composition and content of phenolic compounds as a result of the vinification process and bottle storage [2,34].

The total phenol content of the wine samples prior HRL treatments ranged from  $202 \pm 4$  to  $297 \pm 7$  mg GAE/L (sulphite free vinifications mean TPC value 217 mg GAE/L; conventional vinifications mean TPC value 295 mg GAE/L; overall mean TPC value 256 mg GAE/L). As expected from previous assays, HRL treatments provoked a linear positive response to the values of TPC content. The increase in TPC content is demonstrated graphically in figure 3. Correlation coefficients ( $R^2$ ) were in the range of 0.970 to 0.982 denoting a good correlation between TPC content of wine samples and HRL additions. Treatments with 0.8 g HRL/L resulted in a 12.2 to 26.8% increase in TPC values in comparison to the initial values ( $TPC_{0.8 \text{ gHRL/L}}$  range  $283 \pm 6$  to  $350 \pm 7$ ; mean value 304 mg GAE/L) whereas treatment with 1.6 g HRL/L resulted in a mean 37.8% increase in TPC values.

TPC values prior HRL additions were in the range of previously published data. Stasko et al. (2008)[41] report values for Slovakian and Austrian white wines ranging from 210 to 390 mg GAE/L while Roussis et al. (2008)[42] report values ranging from 213 to 277 mg GAE/L.



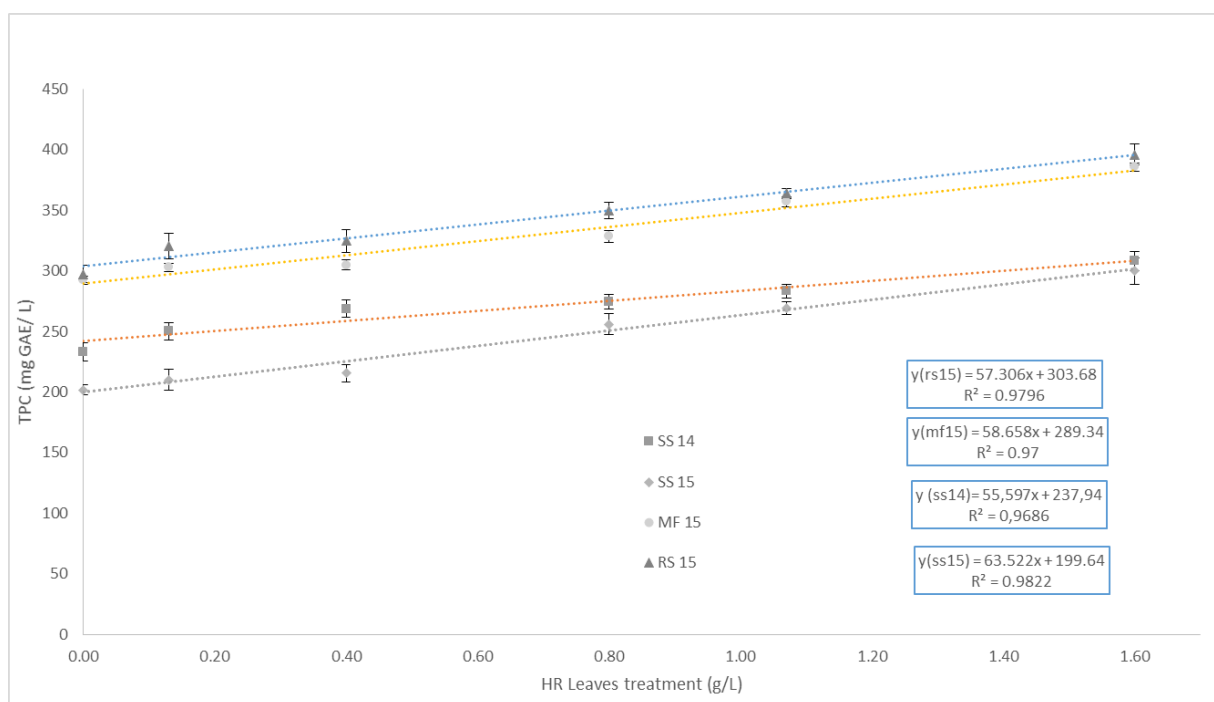


Figure 3: Total Phenol Content of wine samples vs increasing HRL treatments in each wine.

### 3.4. Sensory evaluation

Sensory characteristics of wines examined prior and after HRL treatments are presented in Figures A1 to A4 in appendix B. The specific wines were selected as different vinification protocols were conducted that resulted in white wines with different flavour profiles (mf15: light & fruity; rs15: oak aged white riesling; ss14: oak aged white moschofilero, no added sulphites, vintage 14; ss15: oak aged white, no added sulphites from moschofilero, vintage 2015). The purpose was to determine the organoleptic effect of HRL treatments to the wines with different vinification profiles.

Results denote that the treatment with 0,4 g/L HR leaves was evident from all tasters and for all samples but did not seem to have a major impact on the descriptors examined and was not statistically significant ( $p > 0.05$ ). Treatments with 0.8 g/L of HRL and higher resulted in a decrease in the rating of all sensory characteristics denoting that HRL provoked among others a decrease in limpidity, typicity and fruity aromas of the wines tested.

### 3.5. Correlation analysis

Correlation analysis was used to determine the relationships amongst the different antioxidant parameters measured for all wine samples via Spearman correlation coefficient Rho (Table 4). The total phenolic content of wine samples exhibited a very good correlation ( $p < 0.01$ ) with antioxidant properties. This findings are in accordance with previously published literature [43].

Table 4: Correlation between antioxidant assays and Total phenol content via Spearman Correlation Coefficient RHO (Correlation is significant at the 0.01 level (2-tailed)).

	DPPH	FRAP	F-C
DPPH	1		
FRAP	0.897	1	
F-C	0.871	0.797	1

## 4. Discussion

Experimental data above suggest that the treatment with *Hippophaë rhamnoides* L. leaves in dry white wines vinified with and without added sulphites contributed positively and in a linear manner

to the overall antioxidant capacity, total phenolic content and ferric reducing antioxidant power of the samples tested. Indicatively treatment with 0.8g/L HRL lead to an increase of the antioxidant capacity determined via the DPPH assay comparing to the initial values, ranging from 23.2 to 58.4% . Accordingly the increase in FRAP values ranged from 34.5 to 82.3% ,the increase in TPC ranged from 12,3 to 26,8% while color intensity values were also increased ranging from 39 to 50%. Treatment with 1.6 g/L HRL increased  $A_{AR}$  by a mean value of  $0.32 \pm 0.06$  mM TE , FRAP value by  $1.24 \pm 0.09$  mM TE and TPC by a mean value of  $91 \pm 11$  mg GAE/L. It was difficult to compare our experimental data on the antioxidant properties and TPC content of white wines without added sulphites as relevant studies are scarce in the literature. Findings above suggest that the use of HRL in all aforementioned concentrations in red wines would have had a limited effect comparing to their natural antioxidant properties and total phenolic content.

The increase in total phenolic content of wines was expected as aqueous and hydroalcoholic extracts *Hippophaë rhamnoides* L. leaves have been found to contain significant quantities of bioactive phenolic compounds [25] such as quercetin-3-O-galactoside, quercetin-3-O-glucoside, kaempferol and isorhamnetin [23]. Moreover HRL extracts have been found to exhibit potent antioxidant activity determined by the DPPH and FRAP assays [19-23].

The main oenological attributes examined, except from colour intensity (absorbance at 420nm) remained unchanged. The increase in color intensity by the HRL treatments was not considered as a positive contribution as seen by the values for colour in figures 1-4 by all tasters. Treatments up to 0.4 g/L HRL did not have a significant sensory impact on the samples tasted but treatments higher than 0.8 g/L of HRL resulted in a decrease in the rating of all sensory characteristics of the wines examined.

Full replacement of sulphur dioxide by a single additive has not been accomplished. Main efforts rely on the addition of compounds such as phenolic compounds, DMDC and bacteriocins but also on physical treatments such the application of Pulsed Electric fields, Ultrasounds, UV radiation and high pressure [15]. Our findings suggest that the use of *Hippophae Rhamnoides* L. leaves on white wines is a promising step towards this direction and this would be particularly helpful in the case of wines without added sulphites vinified with minimum interventions. Nevertheless we suggest that more research needs to be conducted in order to verify the lifespan of these wines over a specific period of time but also to test other variables such as different HRL species, treatments with larger concentrations in red wines or treatments in different stages of the vinification process.

**Author contributions:** P.T. and C.P. conceived the study; P.T. and A.T. contributed to the study design; M.L., P.T. and C.P. coordinated the study; A.T. conducted the vinifications and wine treatments; A.T. and K.P. performed the analytical determinations and analyzed the data; A.T. wrote the paper. All authors edited the paper.

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**Conflicts of Interest:** The authors declare no conflict of interest.

## Appendix A

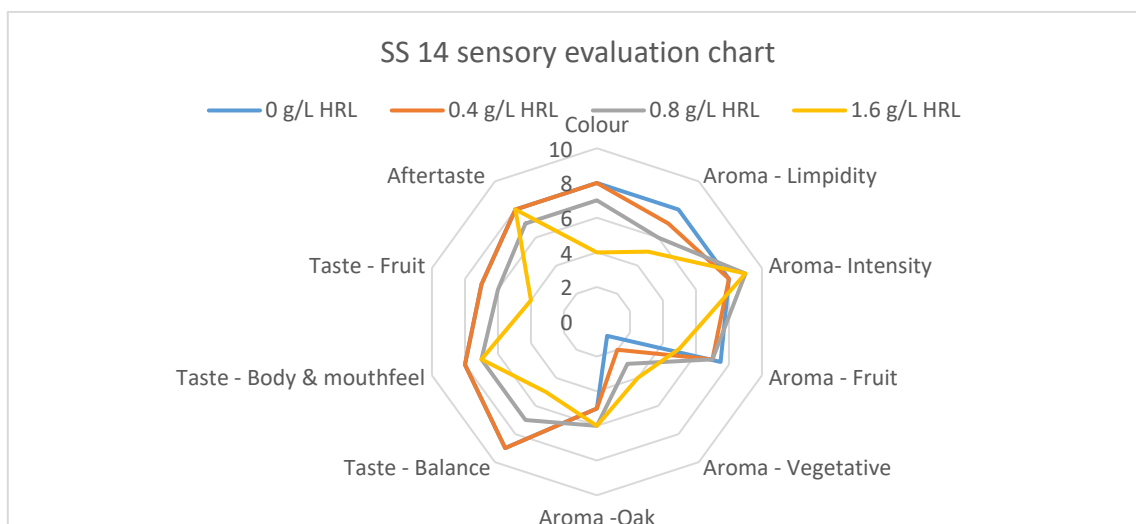


Figure A1: Sensory evaluation of ss 14 sample (moschofilero variety, vinified without added sulphites) treated with increasing quantities of HRL.

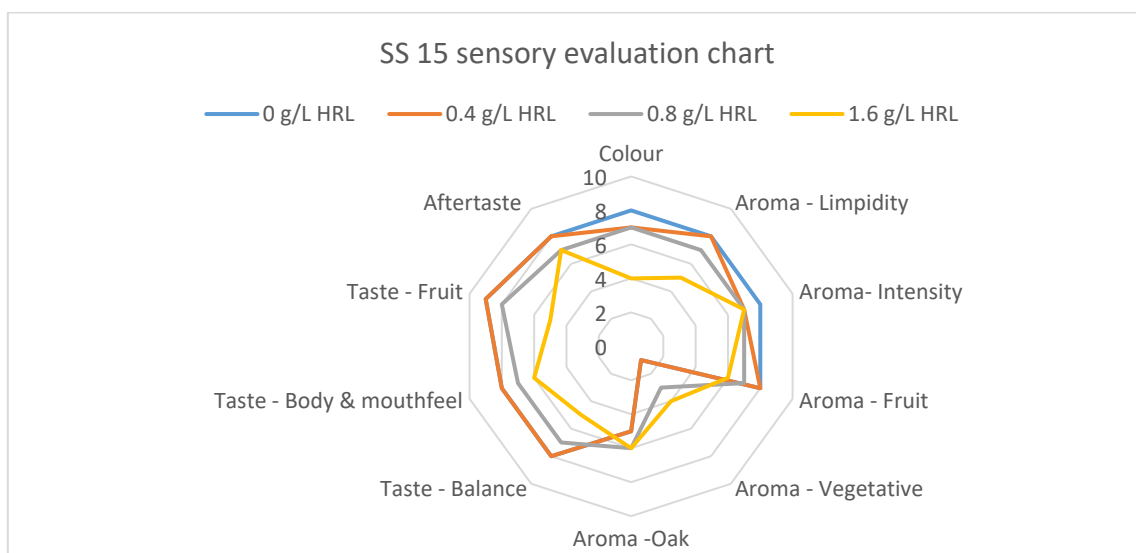


Figure A2: Sensory evaluation of ss 15 sample (moschofilero variety, vinified without added sulphites) treated with increasing quantities of HRL.

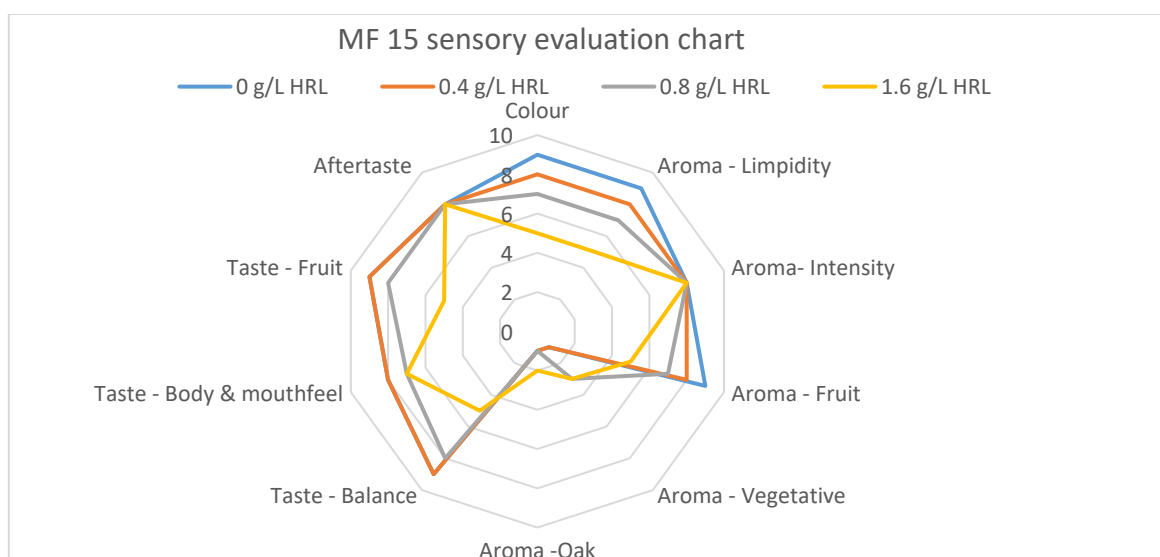


Figure A3: Sensory evaluation of mf 15 sample (moschofilero variety, conventional vinification) treated with increasing quantities of HRL.

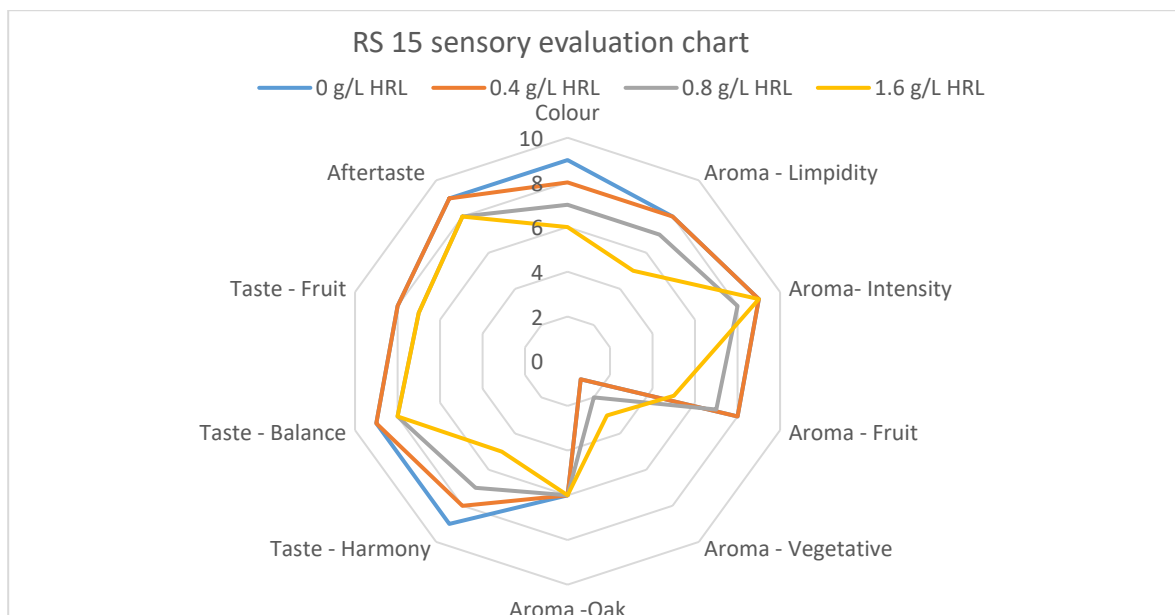


Figure A4: Sensory evaluation of mf 15 sample (riesling variety, conventional vinification) treated with increasing quantities of HRL.

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