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Climate change vs Wine industry in the Emilia-Romagna: Assessment of the climate change, influence on wine industry and mitigation techniques

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Summary

The present PhD thesis is organized in three sections as follows.

The first part of the PhD thesis was focused on the assessment of the climate change in the Emilia-Romagna, whereas considered research periods were during the both, past (1961–2015 for the entire Emilia-Romagna; 1953–2013 for the 'Romagna Sangiovese' appellation area) and future decades (2018–2027, 2011–2040 and 2071–2100 for the entire Emilia-Romagna). Two types of the spatially interpolated meteorological data for past periods (high resolution and low resolution), spatially interpolated climate data with corrected bias from Regional Climate Models for future periods, diverse statistical methods (trend analysis with Mann-Kendall test, trend homogeneity analysis with Pettitt test etc.) and appropriate bioclimatic indices developed particularly for the climatic classification of viticulture region were used to identify climatic suitability to cultivate grapes in the Emilia-Romagna. Additionally, a real case study was performed with data from seven Romagna's wineries in order to identify the potential impact of the climate change on the Sangiovese berry sugar content and grape yield.

The second part of the PhD thesis was focused on the development of mitigation techniques that may be used to face the impact of climate change in the future decades. In particular, late winter pruning was applied to *cv. Sangiovese* grapes aiming to reduce concentration of total soluble solids in berries. Additionally, dealcholization and acidification of Chardonnay wines were achieved by addition of must from unripe Chardonnay grapes and utilization of non-*Saccharomyces* yeast strains. Obtained results in the present PhD thesis may help viticulturists and winemakers to further develop wine industry by choosing climatologically appropriate grape varieties or researchers to further develop mitigation techniques which will allow sustainable grape production in the Emilia-Romagna.

The third part was related to the development of an analytical method to evaluate wine parameters affected by climate change and mitigation strategies, same as to analytical profiling of potential additives to face climate change.

Riassunto

La presente tesi di dottorato è organizzata in tre sezioni come di seguito indicato.

La prima parte della tesi di dottorato si è focalizzata sulla valutazione del cambiamento climatico nell'Emilia-Romagna, dove i periodi di studio considerati sono stati entrambi in passato (1961–2015 per l'intera Emilia-Romagna; 1953–2013 per la 'Romagna Sangiovese') e futuri decenni (2018–2027, 2011–2040 e 2071–2100 per tutta l'Emilia-Romagna). Due tipi di dati meteorologici interpolati spazialmente per periodi passati (alta risoluzione e bassa risoluzione), dati climatici interpolati spazialmente con bias corretto dai modelli climatici regionali per periodi futuri, metodi statistici diversi (analisi di tendenza con test Mann-Kendall, analisi di omogeneità di tendenza con test di Pettitt ecc.) E indici bioclimatici adeguati sviluppati in particolare per la classificazione climatica della regione viticola sono stati usati per identificare l'idoneità climatica per coltivare l'uva nell'Emilia-Romagna. Inoltre, è stato condotto uno studio di casi concreti con dati provenienti da sette cantine Romagnole per individuare l'impatto potenziale del cambiamento climatico sul contenuto di zucchero di bacche di Sangiovese e la resa dell'uva.

La seconda parte della tesi di dottorato è stata focalizzata sullo sviluppo di tecniche di mitigazione che possono essere utilizzate per affrontare l'impatto del cambiamento climatico nei prossimi decenni. In particolare, la potatura tardiva invernale è stata applicata a *cv. Sangiovese* per ridurre la concentrazione di solidi solubili totali nelle bacche. Inoltre, la degradazione e l'acidificazione dei vini Chardonnay sono stati ottenuti mediante l'aggiunta di mosti provenienti da uve Chardonnay non abbiate e l'utilizzazione di ceppi non-*Saccharomyces*. I risultati ottenuti nell'attuale tesi di dottorato possono aiutare i viticoltori e gli enologi a sviluppare ulteriormente l'industria del vino scegliendo le varietà di uve climatologicamente appropriate oi ricercatori per sviluppare ulteriormente tecniche di mitigazione che consentiranno la produzione sostenibile dell'uva in Emilia-Romagna.

La terza parte era legata allo sviluppo di un metodo analitico per valutare i parametri del vino influenzati di cambiamento climatico e di strategie mitigazione, anche per la profilazione analitica di potenziali additivi per affrontare il cambiamento climatico.

List of publications and submitted articles produced during the PhD program

1. **Teslić, N.,** Vujadinović, M., Ruml, M., Antolini, G., Vuković, A., Parpinello, Giuseppina P., Ricci, A., Versari, A., 2017. Climatic shifts in high quality wine production areas, Emilia Romagna, Italy, 1961–2015. *Climate Research* 73, 195–206. **Appendix A**

2. Versari, A., Ricci, A., **Teslić, N.,** Parpinello G.P., 2017. Climate change trends, grape production, and potential alcohol concentration in Italian wines. In Proceedings of the SIAVEN Symposium. Chile. **Appendix B**

3. **Teslić, N.,** Zinzani, G., Parpinello, G.P., Versari, A. 2018. Climate change trends, grape production, and potential alcohol concentration in wine from the "Romagna Sangiovese" appellation area (Italy). *Theoretical and Applied Climatology* 131, 793–803. **Appendix C**

4. **Teslić, N.,** Versari, A. 2016. Effect of late winter pruning on Sangiovese grape berry composition from organic management, in: Ventura, F., Pieri, L. (Eds.), Proceedings of the 19th conferences of Italian associtation of agrometeologists: New adversities and new services for agroecosystems. University of Bologna, Bologna, Italy, pp. 131–134. **Appendix E**

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11. Pavlić, B., Bera O., **Teslić, N.,** Vidović, S., Parpinello, G.P., Zeković, Z. Chemical profile and antioxidant activity of sage herbal dust extracts obtained by supercritical fluid extraction. *Industrial Crops and Products* (under review).

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Y1–must vinified with inoculation of *Saccharomyces cerevisiae/Saccharomyces paradoxus*; *Y2*–must vinified with sequential inoculation of *Candida zemplinina* and hybrid *Saccharomyces cerevisiae/Saccharomyces paradoxus*; *Y3*–must vinified with inoculation of *Saccharomyces cerevisiae*; H1–wine made with technologically mature (ratio total acidity/sugar content) Chardonnay grapes; H2–wine made with Chardonnay grapes obtained during '*delayed harvest*'; H3–wine made with blend of Chardonnay grapes obtained during 'early *green* harvest' and Chardonnay grapes obtained during '*delayed harvest*'; AAcid–acetic acid; VolAcid–volatile acidity; CafAcid–cafftaric acid; *p*-Cou–*p*-coumaric acid; TotPoly–total polyphenols; TotAci–total acidity; TAcid–tartaric acid; MAcid–malic acid; CAcid–citric acid; SAcid–succinic acid; Pref–panelist preference; pH–pH value; Alc–alcohol content. 130

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The list of abbreviations

BI	Bioclimatic index
CE	Catechin equivalent
CI	Cool night index
Cz	*Candida zemplinina*
DI	Dryness index
DOC	Controlled Denomination of Origin
DOCG	Controlled and Guaranteed Denomination of Origin
DOP	Protected Denomination of Origin
DSI	Dryness spell index
DTR	Diurnal temperature range
ER	Emilia-Romagna
GDD	Winkler index/Growing degree day
GMO	Genetic modification organism
HI	Huglin index
LA/FM	Leaf area to fruit mass ratio
MKT	Mann-Kendall test
ND 25–30°C	Number of days with max temperature in the range 25–30°C
ND > 30C°	Number of days with max temperature >30°C
OAV	Odor activity value
OIV	International organization of vine and wine
PCA	Principal component analysis
PLS	Partial least square
PreSc	Preference scores
PT	Pettitt test
RCM	Regional Climate Models
RCP	Representative concentration pathway
RMSE	Root mean square error
Sc	*Saccharomyces cerevisiae*
Sp	*Saccharomyces paradoxus*
T_{max}	Growing season maximum temperature
T_{mean}	Growing season mean temperature
T_{min}	Growing season minimum temperature
T_{prec}	Total precipitation
WVS	Waveguide Vector Spectrometer

CHAPTER 1

Introduction and Project aim

1 Introduction and Project aim

1.1 Introduction

'Climate change' is a change in the weather patterns that can be detected (e.g. using statistical test) as deviation in the mean and/or the variability of its features, which is persistent for the certain period (decades or longer). These deviations refer to any changes in the weather patterns, whether they occurred due to natural factors (e.g. volcano activities, forest fires, El Niño) or anthropogenic factors (e.g. exhaust gases from cars and factories) **(IPCC, 2007)**. Apart from constantly present changes in weather patterns due to natural factors (e.g. five major ice ages), since the middle of the 20th century exist also considerable influence of anthropogenic factors *(Fig 1.1)*. Influence of climate change may be manifested though many direct or indirect consequences (e.g. increase of Global temperature, increased risk of droughts, accelerated ice cape melting), which further alter vast number of ecosystems on the Earth.
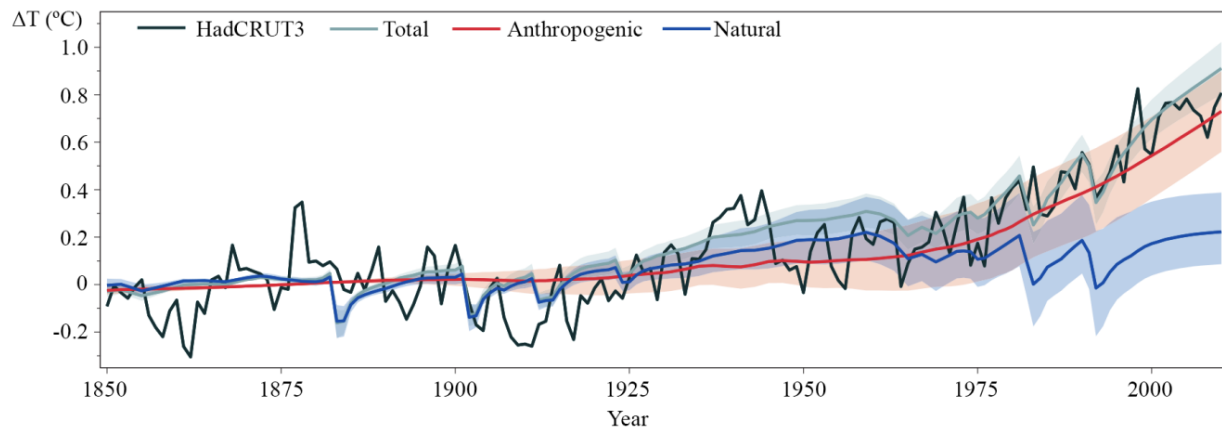
Figure 1.1 Contribution of natural (blue) and anthropogenic (red) factors to the observed (black) and simulated (gray) mean Global temperature increase (modified from Huber and Knutti, 2011).

Vitis vinifera is highly sensitive to climate conditions **(Fraga et al., 2012; Gladstones, 2011; Holland and Smith, 2014)** such as air temperature and precipitation, therefore climate change can modify grape and wine composition to large extent. Sensitivity to climate characteristic is reflected by narrow areas suitable for the high-quality wines production, often determined by growing season isotherms that vary from too cold (<12°C) to too hot (>22°C) **(Jones, 2006)** *(Fig. 1.2)*.
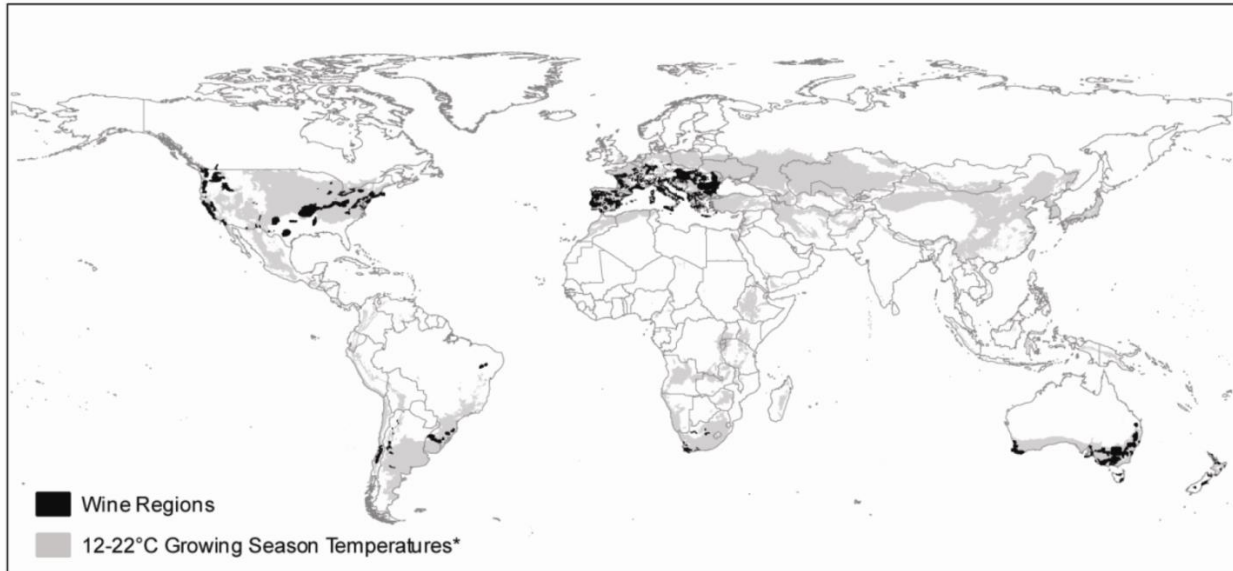
Figure 1.2 Global wine regions and 12–22°C growing season temperature zones (April–October in the Northern Hemisphere and October–April in the Southern Hemisphere) (adopted with permission from Jones, 2012).

Hypothesis that climate conditions, in particular temperature **(Jones, 2012)**, have strong influence on viticulture is also supported by historical evidence of vine-producing existence in the north coastal zones of the Baltics and southern England from 900 to 1300, due to the higher temperatures in that period **(Gladstones, 1992)**, and also production fade from the same regions making them inadequate due to the dramatic decrease of temperature, starting from the 14th until the 19th century **(Jones et al., 2005)** *(Fig 1.3)*.
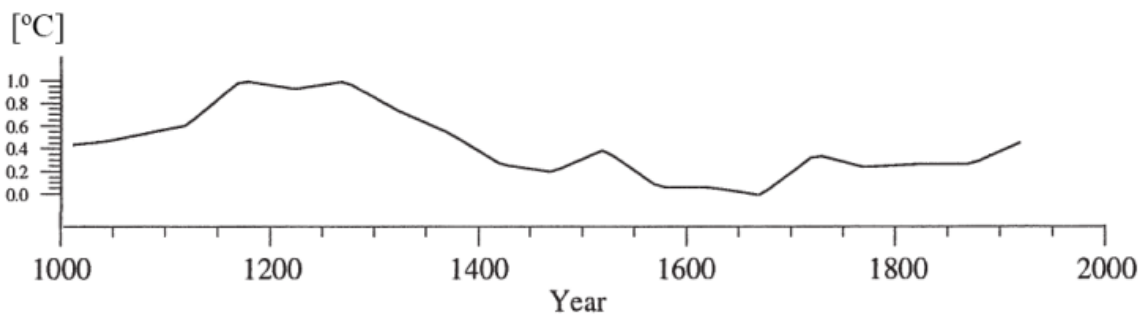
Figure 1.3 Standardized temperature variation in central England during last thousand years (modified from Crowley and Lowery, 2000).

The influence of the climate change on wine sector is depending on vast number of direct and indirect variables such are air temperature **(Neethling et al., 2012)**, precipitations (see 4.2.2.2), atmosphere level of CO_2 **(Kizildeniz et al., 2015)**, ultraviolet (UV-B) radiation **(Schultz, 2000)**, planted grape varieties **(Tomasi et al., 2011)**, application of adaptation techniques and husbandry practices **(Hunter et al., 2016; Palliotti et al., 2014; Varela et al., 2015)**, topography and soil characteristics **(Fraga et al., 2014a)** etc. Combination of all mentioned factors is in greater or lesser percent unique for each grape producing region, which is evident in many published works related to this topic **(Bonnefoy et al., 2013; Fraga et**

al., 2014b Hall and Jones, 2010; Lorenzo et al., 2013; Resco et al., 2016; Vršić et al., 2014), thus there is a need to examine also currently unstudied areas such as the traditional wine region Emilia-Romagna (Italy) due to its great importance at a national and international level. Assessment of climate change may be conducted whether for the past or the future, whereas both parts are required to fully understand climate change trends and gather information which later serve as a tool to develop adaptation strategies.

The final outcome and consequences of climate change influence on wine industry could rather be positive or negative. Negative consequences on wine industry are manifested as crop load reduction (**Ramos and Martínez-Casasnovas, 2010**), production of unbalanced wines with excessive alcohol (**Jones et al., 2005**), utilization of additional investment expenses in mitigation technologies, reduction of anthocyanins (**Mori et al., 2007**), lower must acidity (**Godden et al., 2015**) etc. On the contrary, in some high quality wine regions, such as Chianti (Italy), Bordeaux and Burgundy (France), Barossa and Margaret River (Australia), warming resulted in increasing trends of wine vintage ratings over the second half of the 20th century (**Jones et al., 2005**). Furthermore, warming in future decades may translocate zones with optimal growing season mean temperature (12–22°C) polewards, towards the coast and higher elevations (**Jones, 2012**) and transform non-traditional wine producing zones to suitable for grape cultivation (**Bardin-Camparotto et al. 2014**).

The wine and grape industry is widely spread over the world with approximately 7534 Kha of planted vineyard surfaces worldwide, with 274 MhL of wine and must production per year (harvest 2015). Even though, wine consumption was reduced worldwide after economic crisis in 2008, total volume of exported wine, same as the total value of exports is steadily growing from 2000's on a globe scale, suggesting that sustainable winemaking industry is an important factor for the economic stability in counties which are the largest wine exporters (France, Italy, Spain) (**OIV, 2016**). Nowadays, a sustainable wine industry in environment of accelerated climate change becomes a great challenge, thus it is necessary to develop appropriate adaptation techniques to mitigate upcoming events. In literature, there are already a various adaptation techniques that can be divided into four principal groups: (i) viticulture techniques, (ii) pre-fermentation techniques, (iii) biotechnological techniques and (iv) post-fermentation techniques. However, due to high diversity of climatic conditions over entire wine industry and everlasting trend to decrease cost of production and increase quality of final products there is a need to further develop new mitigation techniques and to examine synergistic effect of existing techniques.

1.2 Project aim

The aim of this PhD thesis titled: '*Climate change vs Wine industry in the Emilia-Romagna: Assessment of the climate change, influence on wine industry and mitigation techniques*' is to examine climate change trends in the Emilia-Romagna (ER) during both, past and future decades, with appropriate meteorological data base and suitable statistic tools. To identify the link, if any, between climate trends and grape quality/quality parameters and to develop new adaptation techniques to moderate the influence of climate change on wine industry.

To achieve this aim, several experiments were designed as followed:

I. Climatic shifts in the ER's high-quality wine production areas – covers examination of climate changes by calculating bioclimatic indices (BIs) for currently well-established high-quality wine production areas of the ER during the period 1961–2015; examination of climate changes in currently grape non-cultivated areas of the ER to identify, from climatological aspect, a new suitable area for grape production in the ER.

II. Projections of climatic shifts in the ER wine production areas – covers examination of climate projections in the periods 2018–2027, 2011–2040 and 2071–2100 under two possible scenarios (Representative concentration pathway (RCP) 4.5 and RCP 8.5) by calculating BIs for currently well-established high-quality wine production areas of the ER during.

III. Influence of climate change on grape yield and sugar content of Sangiovese grapes from the studied part of Romagna – covers assessment of climate change trends over 61 years (from 1953 to 2013) in the studied area by calculating BIs; relation between BIs and grape sugar content from seven wineries during the period 2001–2012; relation between BIs and grape yield during the period 1982–2012.

IV. Development of new adaptation techniques to climate change.

    a. Effect of late winter pruning on Sangiovese grape berry composition from organic management – covers examination of late winter pruning as potential technique to moderate effect of increasing total soluble solids must concentration in organic Sangiovese grapes caused by warmer and/or drier climatic conditions.

    b. Combination of viticulture and biotechnological techniques as a method to reduce alcohol content and pH – covers assessment of possibilities to use viticulture and biotechnological techniques as a combined method to mitigate negative impact of hot and dry vintages (e.g. excessive ethanol concentration and high pH) on Chardonnay wines.

V. Development of analytical method to evaluate wine parameters affected by climate change and analytical profiling of additives to face climate change.

    a. Development of method using Waveguide Vector Spectrometer to examine alcohol and glycerol content of red wines.

    b. Analytical profiling of commercial tannins by ICP-MS and spectrophotometric methods to identify potential additives in winemaking that could be used during hot vintages.

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CHAPTER 2

Climate change in the Emilia-Romagna's DOP appellation areas

(1961–2015)

2   Climate change in the Emilia-Romagna's high-quality wine DOP appellation areas (1961–2015)

Teslić, N., Vujadinović, M., Ruml, M., Antolini, G., Vuković, A., Parpinello, Giuseppina P., Ricci, A., Versari, A., 2017. Climatic shifts in high quality wine production areas, Emilia Romagna, Italy, 1961–2015. *Climate Research* 73, 195–206

Versari, A., Ricci, A., **Teslić, N.,** Parpinello G.P., 2017. Climate change trends, grape production, and potential alcohol concentration in Italian wines. In Proceedings of the SIAVEN Symposium. Chile

2.1   Introduction

As mentioned before grape production is strongly affected by climate variables (**Fraga et al. 2012a**), thus climate change may modify grape and wine composition to a great extent. However, due vast number of relevant climatic factors (e.g. temperature) and non-climatic factors (e.g. topography) the magnitude of climate change may diverse among wine regions (**Jones et al., 2005**). This was confirmed by Jones et al. (2005) that reported a significant growing season temperature trends for the majority of Europe and North-America wine regions during the last 50 years of the 20th century, with an average increase of 1.26°C. However, authors also reported the lack of statistically significant temperature trends for the majority of Southern Hemisphere wine regions. Thus, despite the importance of the global climate change trend, from the viticulturist/winemaker point of view it is also important to understand and examine regional climate change trends in order appropriately adapt to potential upcoming climate changes that could have impact on grape and wine composition. Therefore, climate change examination on regional level is particularly important for currently unstudied areas, such as the traditional Italian wine region ER due to its great importance at a national and international level.

Since the magnitude of climate modifications depends on mutual interaction of climatic and non-climatic variables, examinations of simple temperature and precipitation values are insufficient to explain climate change on regional level. Thus, certain BIs developed for effective monitoring of climate change in wine regions have to be used. Whereas computation of commonly used BIs allows easier comparison of climate characteristics and climate change shifts between wine regions. These BIs may be divided into three groups: (i) BIs derived from a single climatic variable (e.g. minimum temperatures during September – Cool night index (**Tonietto, 1999**)); (ii) BIs derived from two or more climatic variables (e.g. maximum and mean temperatures from April to September – Huglin index (**Huglin, 1978**)); (iii) BIs derived from climatic and non-climatic variables (e.g. monthly precipitation and evaporation of bare soil – Dryness index (**Tonietto and Carbonneau, 2004**)). In the last two decades, BI were computed by spatially interpolated meteorological data sets (**Fraga et al., 2012b; Hall and Jones, 2010**) or data sets directly from the meteorological stations (**Duchêne and Schneider, 2005; Tomasi et al., 2011**). Meteorological data sets from meteorological stations are surely a valuable tool for the regional climate change examination. However, spatially interpolated data sets may allow more precise estimates of climate variables at locations distant from the measuring meteorological stations. Furthermore, spatially interpolated data sets have often temporally complete series which allows easier implementation

(**Haylock et al., 2008**). The suitability of the spatially interpolated data sets for the regional climate change studies is strongly related to spatial resolution. This is of the paramount importance due to often complex topography of the grape cultivation regions, where data sets with relatively low spatial resolution provided by global climate models (up to 250 km) (**Jones et al., 2005; Webb et al., 2007**) or regional climate models (up to 25 km) (**Andrade et al., 2014; Lorenzo et al., 2013**) may be inadequate to present vineyard climate characteristics. Therefore, high-resolution, spatially interpolated climatic data (up to 5 km) (**Fraga et al., 2014; Lorenzo et al., 2016**) may be a valuable tool for the regional climate change examination of the grape growing areas.

Italy is one of the top world's wine producer with 49.5 10^6 hL of produced wine during the vintage 2015 and 682 000 ha of the total vineyard area (**OIV, 2016**). Total value of all exported wine reached 5.35 10^9 € during the 2015 (**OIV, 2016**), whereas approximately 50% of the total value of all exported Italian wine during 2015 was obtained by trading high-quality wine with Protected Denomination of Origin (DOP) (**www.italianwinecentral.com**). Therefore, high-quality wine industry affects the economic, social and cultural aspects of Italy to a great extent. Thus, the aim of this experiment was to ascertain the appearance, if any, of climatic change that could affect the winemaking industry in DOP appellation zones in the ER.

2.2   Materials and Methods

2.2.1   Study region

The traditional viticulture region ER is located in the northern Italy and stretches from ~ 43° 80' to 45° 10' N latitude and ~ 9° 20' to 12° 75' E longitude. Rich pedological and climatic diversity caused by the impact of the Adriatic Sea to the east and the mountains to the south, create a unique 'terroir' suitable for the cultivation of several grape varieties, both international and autochthonous. The ER counts about 55 000 ha of vineyards, representing 8.1% of the total Italian vineyard surface, with the main grape varieties such as Trebbiano Romagnolo white grape that covers 30.4%, Lambrusco red grape 17.7%, Sangiovese red grape 15.5%, Ancellota red grape that covers 7.9% of the total ER vineyard surfaces (**Pollini et al., 2013**). The total ER wine production is estimated on 7.91 10^6 hL during vintage 2014, placing the ER as the 2nd winemaking region with 18% of the total Italian wine production by volume. A considerable volume of the total ER's wine production (15.9%, vintage 2014) is high-quality DOP (Protected Denomination of Origin) wines, which are divided into subgroups, DOCG (Controlled and Guaranteed Denomination of Origin) and DOC (Controlled Denomination of Origin) wines. The production of the DOP wines is widespread over the entire ER region except for the mountain zones and certain northeastern and northwestern zones (*Fig. 2.1*).
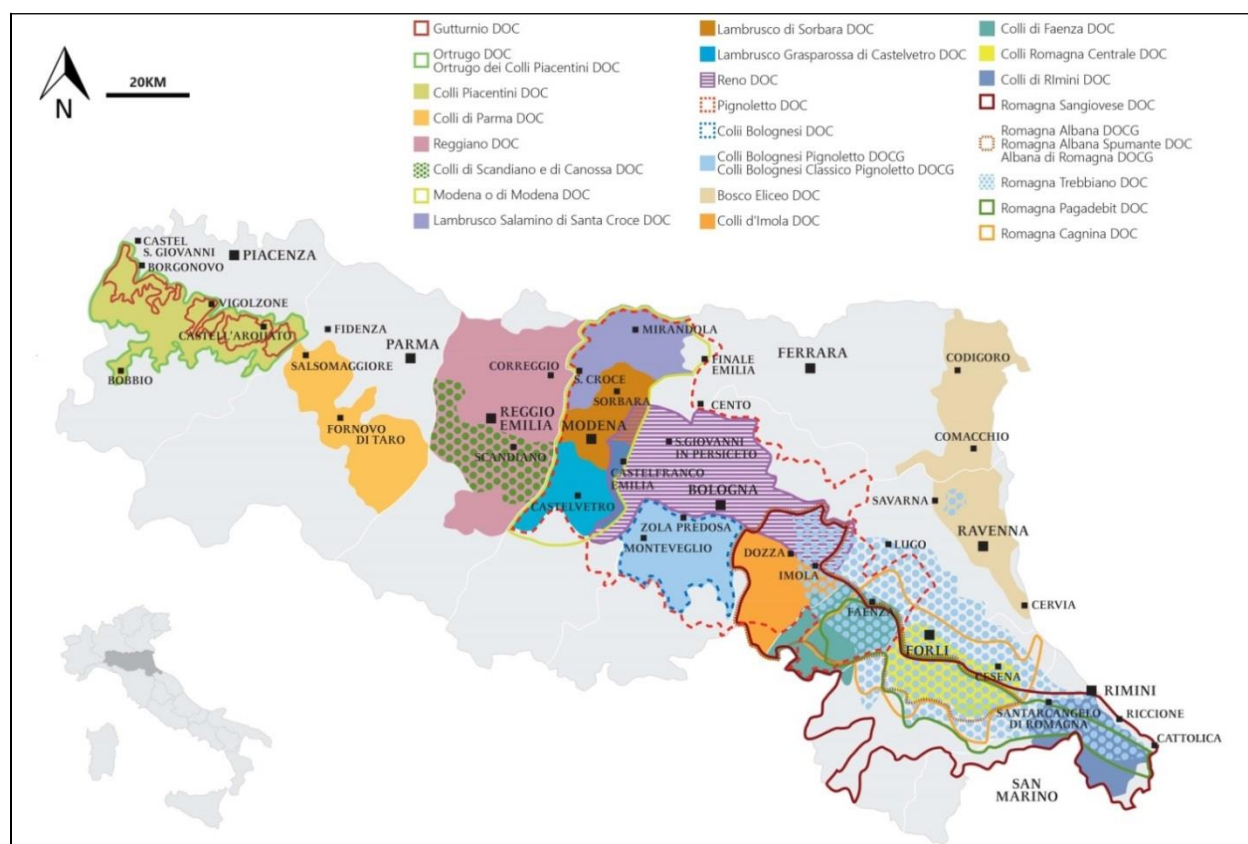
Figure 2.1 Location DOC (Controlled Denomination of Origin) and DOCG (Controlled and Guaranteed Denomination of Origin) grape production areas in the Emilia-Romagna (modified from www.enotecaemiliaromagna.it).

2.2.2   Meteorological data and bioclimatic indices

The experiment was conducted using a high-resolution gridded climate data provided by the Regional Agency for Prevention, Environment and Energy of the Emilia-Romagna **(www.arpae.it)**. Gridded meteorological data for the period 1961–2015 were obtained from precipitation (254 locations) and temperature (60 locations) time series, preliminarily checked for quality, temporal homogeneity and synchronicity. The daily climate data were interpolated on a 5 x 5 km grid, by the algorithms as described in details elsewhere **(Antolini et al., 2016)**. Algorithms consider topography (lapse rate examination, including thermal inversions; topographic barriers; topographic relative position), land use (urban fraction), and a day-by-day error minimizing procedure for the examination of the interpolation parameters. Specific BIs were computed for the DOP appellation viticulture zones over the two periods: 1961–1990, as a standard climatological period, and 1986–2015, as the latest 30-year time-series. The BIs used for this experiment were calculated as presented in ***Table 2.1.***

Table 2.1 Mathematic definitions and classes of used BIs.

Bioclimatic index	Mathematical definition	Classes
Temperature related indices		
Growing season mean temperature (T_{mean})[1]	$$Tmean = \frac{1}{N} \sum_{1.4.}^{31.10.} Tn$$ Tn – Mean air temperature (°C) N – Number of days	Too cool: < 12 Cool: 12–15 Intermediate: 15–17 Warm: 17–19 Hot: 19–21 Very Hot: 21–22 Too Hot: > 22
Number of days with max temperature in the range 25–30°C (ND 25–30°C)[2]	$$ND\ 25 - 30°C = \sum_{1.4.}^{31.10.} ND\ 25 - 30°C$$ ND 25–30°C – Number of days with max temperature in the range 25–30°C	Number of days with max temperature in the range 25–30°C (ND 25–30°C)[2]
Number of days with max temperature > 30°C (ND > 30°C)[2]	$$ND > 30°C = \sum_{1.4.}^{31.10.} ND > 30°C$$ ND > 30°C – Number of days with max temperature > 30°C	Number of days with max temperature > 30°C (ND > 30°C)[2]
Cool night index (CI)[3]	$$CI = \frac{1}{N} \sum_{1.9}^{30.9.} Tm$$ Tm – Min air temperature (°C) N – Number of days	Warm nights: > 18 Temperate nights: 14–18 Cool nights: 12–14 Very cool nights: < 12
Huglin index (HI)[4]	$$HI = \sum_{4.1.}^{30.9.} \frac{(Tx - 10°C) + (Tn - 10°C)}{2} * k$$ Tx – Max air temperature (°C) Tn – Mean air temperature (°C) k – Length of the day correction coefficient	Very warm: > 3000 Warm: 2400−3000 Temperate warm: 2100−2400 Temperate: 1800−2100 Cool: 1500−1800 Very cool: < 1500

Bioclimatic index	Mathematical definition	Classes
Growing degree day (GDD)[5,6]	$$GDD = \sum_{4.1.}^{31.10.} \frac{Tx + Tm}{2} - 10\,°C$$ Tm – Min air temperature (°C) Tx – Max air temperature (°C)	Too hot: > 2700 Region V: $2222-2700$ Region IV: $1944-2222$ Region III: $1667-1944$ Region II: $1389-1667$ Region I: $850-1389$ Too cool: < 850
Precipitation related indices		
Total precipitation (T$_{prec}$)	$$Tprec = \sum_{1.4.}^{31.10.} P$$ P – Precipitation (mm)	-
Dry spell index (DSI)[7]	$$DSI = \sum_{1.4.}^{30.9.} ND < 1\,mm$$ ND < 1 mm – Number of days with precipitation < 1mm	-
Temperature, precipitation and non-climatic variables related indices		
Dryness Index (DI)[4]	$$DI = Wo + \sum_{1.4.}^{30.9.} [Pm - (Et + Es)]$$ $$Et = aPET$$ $$Es = \frac{PET(1-a)Nef\,prec}{N}$$ W_0 – initial soil moisture (200 mm) P_m – monthly precipitation E_T – Water loss through transpiration E_S – Bare soil evaporation PET – Potential evaporation	Humid : > 150 Moderately dry: $50÷150$ Sub-humid: $-100÷50$ Very dry: < -100 a –Plant radiation absorption coef (a= 0.1,0.3,0.5 in April, May, June–September, respectively) N_{efprec} – Monthly effective soil evaporation N – Number of days in month

[1](Fraga et al., 2014), [2](Ramos et al., 2008), [3](Tonietto, 1999), [4](Tonietto and Carbonneau, 2004), [5](Hall and Jones, 2010), [6] (Winkler et al., 1974), [7](Dubuisson and Moisselin, 2006).

2.3   Results and Discussion

Narrow areas suitable for the production of high-quality wines are often determined by growing season isotherms that ranges from too cool until too hot (12°C<T_{mean}>22°C) (**Fraga et al., 2014**). Average T_{mean} in the ER's high-quality wine production areas during the periods 1961–1990 and 1986–2015 was 17.64 and 18.72°C, respectively *(Table 2.2)*. However, even if average T_{mean} in the Emilia-Romagna's DOP zones was characterized as 'warm' during the second period (1986–2015), in certain DOP zones of the ER, T_{mean} was characterized as 'hot' during the same period (**Teslić et al., 2017**).

Table 2.2 Average bioclimatic indices values during the two periods (1961–1990; 1986–2015) in the Emilia-Romagna's wine high-quality Protected Denomination of Origin appellation zones.

Period	T_{mean} [°C]	CI [°C]	ND25–30°C [days]	ND>30°C [days]	HI [units]	GDD [units]	T_{prec} [mm]	DSI [days]	DI [mm]
1961–1990	17.64	13.19	61.42	24.77	2048.35	1663.67	472.56	165.00	99.48
1986–2015	18.72	13.31	60.04	45.60	2299.90	1888.84	469.04	165.18	71.52

The increase of T_{mean} suggests different impact on regional viticulture suitability and production of high-quality wines. In particular, lesser appearance of vintages with 'warm' T_{mean} particularly after 2000's *(Fig. 2.2)*, which are optimal for Sangiovese, one of the main red cultivars in the ER (**Pollini et al., 2013**), may induce viticulturists to cultivate later maturing grapevine varieties in order to adapt to upcoming warming conditions that are expected for the entire northern Italy (**Ruml et al., 2012**). Grape varieties that may be potentially suitable for cultivation during the future decades in DOP areas of ER region include Grenache, Carignane, Zinfandel and Nebbiolo *(Fig. 2.3)*.
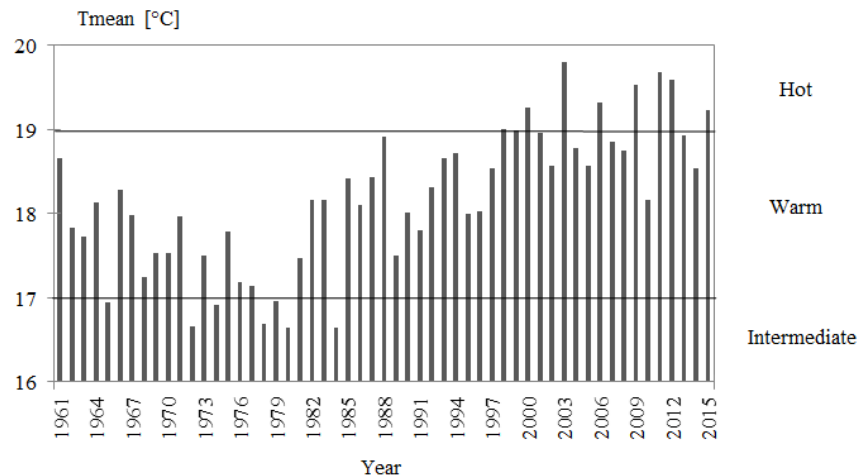
Figure 2.2 Average mean growing season temperature in the Emilia-Romagna's wine high-quality Protected Denomination of Origin appellation zones from 1961 until 2015.

Furthermore, as a direct consequence of increasing temperatures, an average number of days exceeding 30°C increased in the ER's DOP zones in the second period (45.60 days; 1986–2015) comparing to the first period (24.77 days; 1961–1990), *(Table 2.2)*. Inversely, an average number of days with maximum

temperatures was approximately constant in the ER's DOP zones *(Table 2.2)*. These changes may affect vine photosynthesis and growth process, since days with maximum temperature in the range of 25–30°C is optimal for the vine photosynthesis **(Carbonneau et al., 1992)**. On the other hand, a certain number of days exceeding 30°C may induce vine heat stress, premature véraison, berry abscission, reduced flavor development and enzyme activation **(Mullins et al., 1992)**. During the 21st century, daily maximum temperature in the vegetative period may even exceed 45°C, reaching upper-temperature limit for the photosynthesis process **(Greer and Weedon, 2012)**, and having a negative impact on the grape berry composition and crop load.
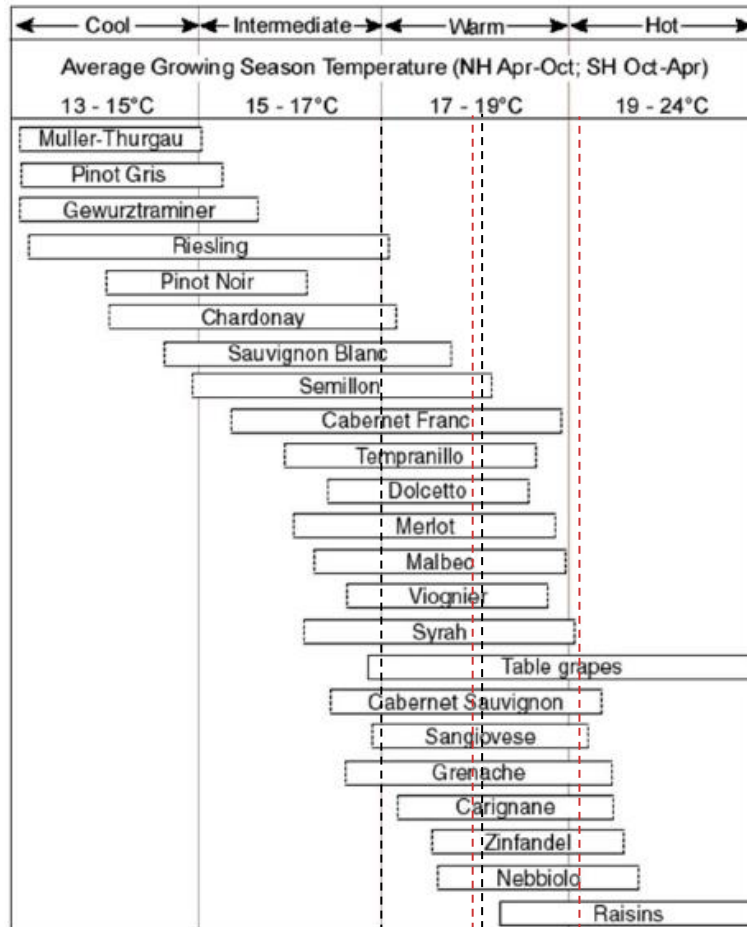
Figure 2.3 Optimal mean growing season temperatures (T_{mean}) for the cultivation of certain grape varieties. The range of the T_{mean} for two periods (1961–1990, black; 1986–2015, red) presents standard deviation of T_{mean} during respective periods (modified from Jones, 2006).

In the ER's DOP zones, CI which is related to the grape's synthesis of anthocyanins was approximately constant in the both periods *(Table 2.2)*, and nights were characterized as 'cool' during most of the vintages in the last 55 years *(Fig. 2.4)*. Several studies **(Kliewer, 1977; Tonietto and Carbonneau, 1998)** reported a positive effect of the night temperatures in an approximate range of 10–15°C on anthocyanin accumulation during the berry maturing period. Hence, obtained results in presented experiment suggested optimal night conditions for cultivation of red grape varieties which are used for red and rosé wine production that represents 55% of the total ER wine production **(Pollini et al., 2013)**.
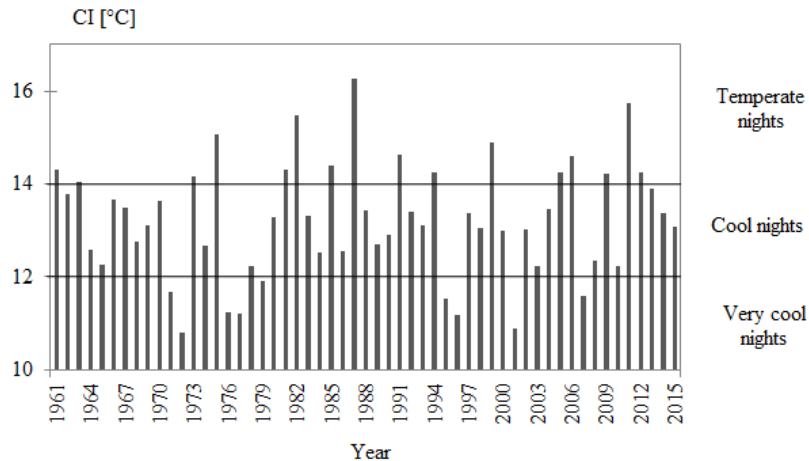
Figure 2.4 Average Cool night index in the Emilia-Romagna's wine high-quality Protected Denomination of Origin appellation zones from 1961 until 2015.

Average thermal accumulation in the ER's DOP areas presented as HI, was 2048.35 and 2299.90 units during the period 1961–1990 and 1986–2015, respectively *(Table 2.2)*. During the first period (1961–1990) vintages in the ER's DOP were mainly characterized as 'temperate/warm temperate' according to Huglin classification *(Fig. 2.5)*. However, due to warming, during the second period (1986–2015) same areas were characterized as 'warm temperate/warm' *(Fig. 2.5)*. This increase of thermal accumulation will most likely continue in the upcoming decades. It is predicted that the entire northern Italy, including the ER DOP zones, could be characterized as 'warm' (according to Huglin classification) wine region in the upcoming decades (2041–2070; A1B scenario) **(Fraga et al., 2013)**. In general, the magnitude of these changes will strongly depend on a level of anthropogenic carbon emissions into the atmosphere during the upcoming decades. Higher temperatures and consequently higher thermal accumulation may have a negative impact on grape/wine quality (see 4.1.1–4.1.6).
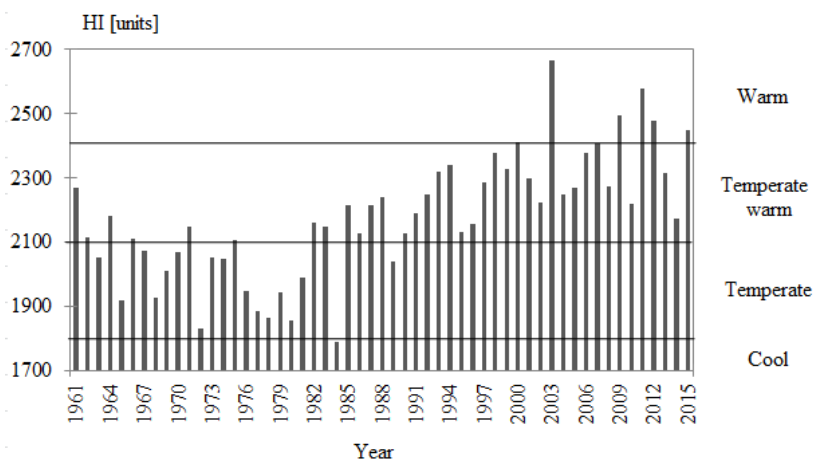
Figure 2.5 Average Huglin index in the Emilia-Romagna's wine high-quality Protected Denomination of Origin appellation zones from 1961 until 2015.

In the ER's DOP zones, average thermal accumulation in the presented as GDD was 1663.67 and 1888.84 units during the period 1961–1990 and 1986–2015, respectively *(Table 2.2)*. To produce high-quality wines about 1400–2000 GDD units are often required, depending on grape variety and environmental factors **(Gladstones, 1992)**. Thus, obtained results are suggesting that ER's DOP zones had optimal thermal accumulation for the production of high-quality wines during most of the vintages from 1961 until 2015. However, due to temperatures increase, after 2000's occurrence of vintages with thermal accumulation higher than 2000 GDD units is tending to be more frequent *(Fig. 2.6)*. Furthermore, a recent study reported that certain currently established DOP zones in the ER had more than 2000 GDD units during the period from 1986 until 2015 **(Teslić et al., 2017)**, suggesting that part of currently established DOP zones may become 'too hot' for the production of high-quality wines. This is especially related to white grape varieties that often demand lower temperatures for optimal cultivation conditions *(Fig. 2.3)*.
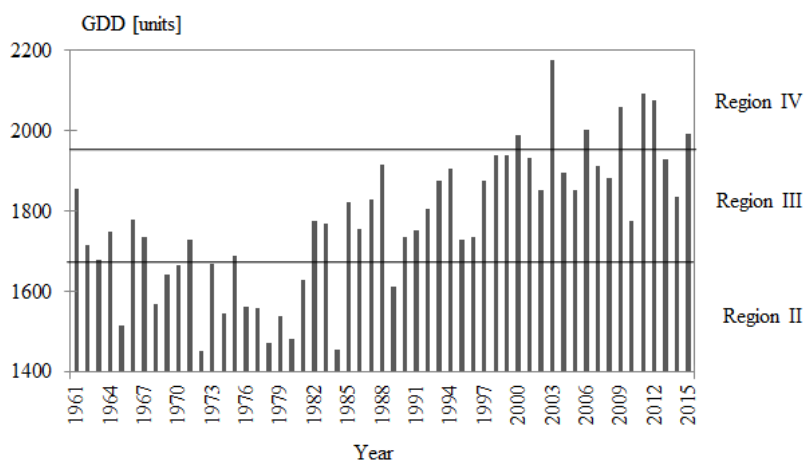
Figure 2.6 Average Growing degree day index in the Emilia-Romagna's wine high-quality Protected Denomination of Origin appellation zones from 1961 until 2015.

Average total precipitation in the ER's DOP areas was approximately constant and it was 472.56 and 469.04 mm during the period 1961–1990 and 1986–2015, respectively *(Table 2.2)*. A recent study also reported similar values of T_{prec} in the ER's DOP zones, whereas certain changes in precipitation patterns were observed during the period 1986–2015 compared to the period 1961–1990, however, mostly with a lack of statistical differences **(Teslić et al., 2017)**. DSI values on a regional level were also approximately constant and were 165 and 165.18 days during the period 1961–1990 and 1986–2015, respectively *(Table 2.2)*. However, in certain DOP zones ('Romagna Sangiovese' DOC) DSI had an increasing trend (9.33 days) which may result in a higher sugar content in Sangiovese berries (see 4.2.2.2). Drier conditions (evaluated with DI) were detected in the ER's DOP zones during the period 1986–2015 (71.52 mm; *Table 2.2*) comparing to the period 1961–1990 (99.48; *Table 2.2*) suggesting that besides precipitation, temperature as well, had an important role in soil water availability in the ER's DOP zones. The negative effect of the increasing temperatures on soil water availability may be due to higher evaporation from soil under warmer conditions **(Alcamo et al., 2007)**. The vintages in the ER's DOP areas were mainly characterized as moderately dry during the first period (1961–1990) *(Fig. 2.7)*. However, due to most likely higher soil evaporation, certain vintages in the ER's DOP areas, particularly after 2000's were characterized as sub-humid *(Fig. 2.7)*. The appearance of sub-humid vintages in the ER

DOP zones will most likely occur during the future decade as it was supported by a recent study (**Fraga et al., 2013**). Authors reported that during the period 2041–2070 under the A1B scenario certain areas in the ER may be characterized as 'sub-humid'. Detected changes may lead viticulturists to install irrigation systems, non-traditionally used for grape cultivation in the ER, to mitigate consequences caused by warmer and drier conditions (see 4.1.1–4.1.6).
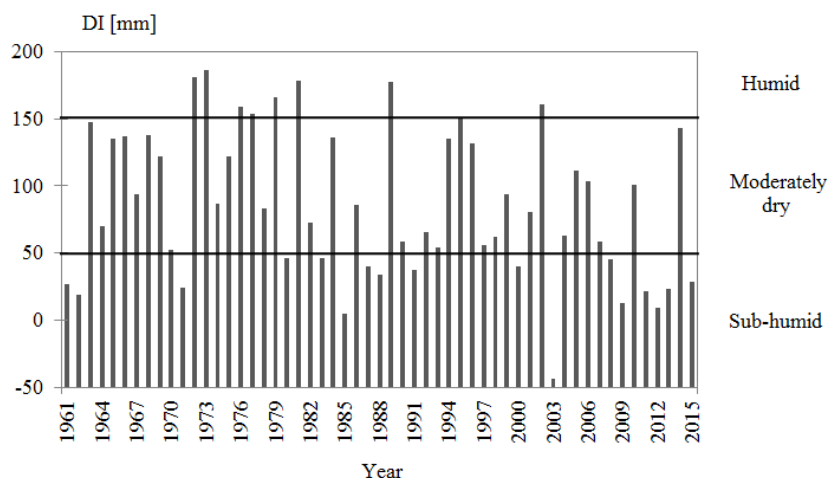
Figure 2.7 Average Dryness index in the Emilia-Romagna's wine high-quality Protected Denomination of Origin appellation zones from 1961 until 2015.

2.4   Conclusions

The findings of the present experiment highlighted the changes in climate related to the viticulture suitability of the ER's DOP zones during two periods, 1961–1990 and 1986–2015. Detected changes in the BI may affect suitability to produce high-quality wine in the ER's DOP areas, which could become 'too hot' for the production of these wines. The negative impact of rising temperatures on wine production could be mitigated by planting later ripening grape varieties comparing to those currently present in the ER DOP zones. Also, the experiment results suggested that warmer and drier conditions in the last 3 decades (1986–2015) decreased soil water availability necessary for plants development in the ER DOP zones, which implies the need of updated strategy for future implementation of irrigation systems in vineyards.

2.5　References

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Climatic shifts in high quality wine production areas, Emilia Romagna, Italy, 1961–2015

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ABSTRACT: In the presented work, daily observations of minimum and maximum temperatures and precipitation—spatially interpolated in a high-resolution grid (5 × 5 km)—were used to detect climate shifts in the viticultural appellation areas of the Emilia-Romagna (ER) region, in the periods 1961–1990 and 1986–2015. The growing season (April to October) minimum, mean, and maximum temperatures were significantly increased in the second period compared to the first over the majority of the ER. Precipitation did not differ significantly, with the exception of certain small northeastern areas of the ER. The detected changes affected the ER viticultural environment in several ways: (1) an increase in the number of days with maximum temperature exceeding 30°C, which can induce plant stress; (2) changes in starting and ending dates of the climatologically defined growing season, dates of the first fall frost and the last spring frost, and length of the frost-free period; (3) shifts of most vineyard areas from 'Region 2/Region 3' to 'Region 3/Region 4' (according to the Winkler Index); (4) shifts of the majority of the grape-producing zones from 'temperate/warm temperate' to 'warm temperate/warm' (according to the Huglin Index); (5) decreased availability of soil water, which is necessary for grapevine development.

KEY WORDS: Climate change · Emilia-Romagna · Vitiviniculture sustainability · Bioclimatic indices

1. INTRODUCTION

Italy is one of the top wine producers in the world (49.5×10^6 hL of wine produced during the harvest in 2015; cf. France, 47.6×10^6 hL, and Spain, 36.6×10^6 hL) and has 682 000 ha of total viticultural area, along with €5.35×10^9 total export value of all wines (Organisation Internationale de la Vigne e du Vin, OIV, 2016). Roughly 50% of the total Italian wine export value for 2015 was from high-quality wines with Protected Denomination of Origin (DOP) (http://

italianwinecentral.com). Thus, the winemaking industry and the production of high-quality wines influence the economic, social, and cultural aspects of Italy to a great extent.

Vitis vinifera (Vv) is highly sensitive to climate characteristics (Gladstones 2011, Fraga et al. 2012a, Holland & Smit 2014), such as temperature and precipitation, and therefore the weather can be considered as a major driver of grape production (Hannah et al. 2013). The strong influence of climate conditions on viticulture, in particular temperature (Jones

2012), is supported by historical evidence of vine production in the northern coastal zones of the Baltics and southern England from 900 to 1300, due to the higher temperatures in that period (Gladstones 1992), and its decline from the 14th century due to the dramatic decrease in temperature (Jones et al. 2005).

Due to the industrial revolution and anthropogenic carbon emissions, the atmospheric CO_2 concentration has increased from 280 to 400 ppm, with an estimated trend peaking at approximately 700 ppm by the end of the 21st century (Collins et al. 2013) and with corresponding temperature increase between 2.5 and 4.5°C in western and northern Europe (Schultz & Lebon 2005). Projected temperature trends, with side effects such as longer drought periods due to the higher soil evaporation (Alcamo et al. 2007), could have many negative consequences on the wine industry, including yield reduction (Ramos & Martínez-Casasnovas 2010), production of unbalanced wines with excessive alcohol and low pH (Jones et al. 2005), and the utilization of additional investment expenses in mitigation technologies. However, the impact of climate change is not necessarily detrimental to the wine industry. In some European high-quality wine regions, such as Chianti (Italy), Bordeaux, and Burgundy (France), warming has resulted in increasing trends for wine vintage ratings over the second half of the 20th century (Jones et al. 2005). Additionally, future warming could spatially shift zones with optimal growing season temperature (12 to 22°C) (Jones 2006) polewards, towards the coast and higher elevations (Jones 2012), and transform non-traditional wine areas into regions suitable for grape cultivation (Bardin-Camparotto et al. 2014).

Species such as Vv are affected by climatic and non-climatic factors (O'Donnell & Ignizio 2012). Therefore, temperature or precipitation measurements alone might be insufficient for the characterization and examination of viticultural suitability. For example, soil water availability presented only as seasonal or annual precipitation does not include non-climatic factors related to water loss through transpiration and evaporation from bare soil. On the other hand, the Dryness Index (DI), which was developed specially for viticultural use (Tonietto & Carbonneau 2004), incorporates particular climatic and non-climatic factors relevant for soil water availability. Hence, certain bioclimatic indices (BI) designed for effective monitoring of climate shifts in viticultural regions should be used.

In the last 2 decades, the potential impact of climate change on viticulture suitability has been stud-

ied in many different wine regions using either spatially interpolated meteorological data (Hall & Jones 2010, Fraga et al. 2012b) or data taken directly from meteorological stations (Neethling et al. 2012, Hannah et al. 2013, Neumann & Matzarakis 2014). Climate data from weather stations are clearly a valuable tool for regional climate change assessment. However, spatially interpolated data sets could allow better estimates of weather variables at locations distant from the measuring stations. Additionally, gridded data sets are often easier to implement due to temporally complete series (Haylock et al. 2008). The suitability of gridded data for regional climate change studies strongly depends on spatial resolution. This is particularly important for vitiviniculture studies, due to the often complex topography of the grape-growing regions, where data sets with spatial resolution provided by global climate models (up to 250 km) (Jones et al. 2005, Webb et al. 2007) or regional climate models (~25 km) (Lorenzo et al. 2013, Andrade et al. 2014) might be insufficient to present vineyard climate. Therefore, high-resolution, spatially interpolated climatic data (1 to 5 km) may be a valuable tool for the regional climate change assessment of grape-growing areas (Fraga et al. 2014, Lorenzo et al. 2016).

Based on previously published studies which addressed climatic changes in several wine regions (Hall & Jones 2010, Bonnefoy et al. 2013, Lorenzo et al. 2013, Fraga et al. 2014, Vrši et al. 2014, Resco et al. 2016), it is evident that each region has a unique combination of factors relevant to viticulture suitability. Thus there is a need to focus on the currently unstudied areas, such as the traditional Italian wine region Emilia-Romagna (ER), which has great importance at a national and international level. Therefore, the aim of this study was to ascertain the occurrence, if any, of climatic change that may affect the suitability of the high-quality wine production in the ER region.

2. MATERIALS AND METHODS

2.1. Study region

The traditional viticulture region ER is located in northern Italy, approximately between 43° 80' and 45° 10' N, and 9° 20' and 12° 75' E. Great pedological diversity and climatic variability, caused by the influence of the Adriatic Sea to the east and mountains to the south, create a unique 'terroir' suitable for the production of numerous grape varieties, both inter-
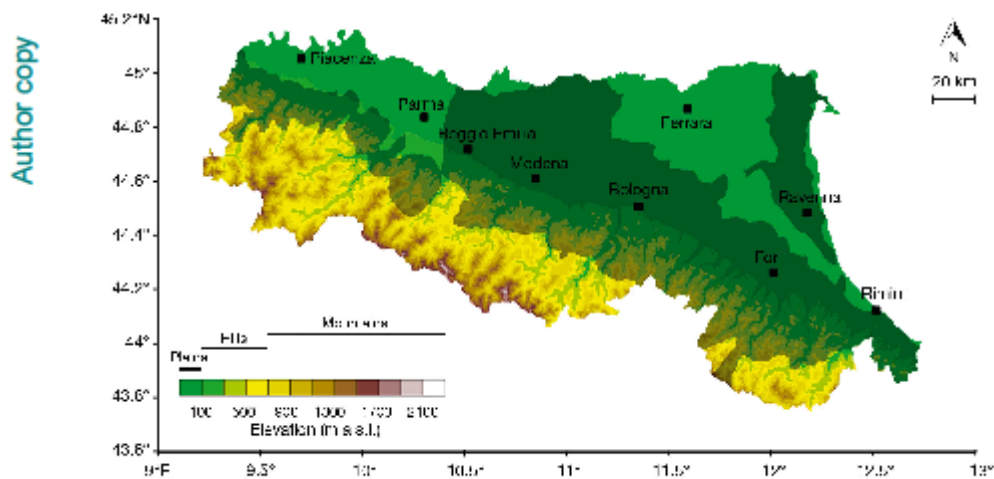
Fig. 1. Protected Denomination of Origin (DOP, shaded areas) grape production and topography in the Emilia-Romagna region

national and autochthonous. The total ER wine production is estimated at 7.91×10^6 hL (harvest 2014), placing the ER as the second largest wine-producing region in Italy, providing 18% of the total Italian wine production by volume. A considerable volume of the total wine production of the ER (15.9%, harvest 2014) consists of high-quality DOP wines. The production of DOP wines is widespread over the entire ER with the exception of the mountainous areas and certain northeastern and northwestern areas (Fig. 1).

2.2. Climate data

The study was conducted using high-resolution gridded meteorological data provided by the Regional Agency for Prevention, Environment and Energy of Emilia-Romagna (www.arpae.it). Gridded climate data for the period 1961–2015 were produced from precipitation (254 locations) and temperature (60 locations) time series, preliminarily checked for quality, temporal homogeneity, and synchronicity. The daily data were interpolated on a 5 × 5 km grid using the algorithms described by Antolini et al. (2016), which take into account topography (lapse rate estimation, including thermal inversions; topographic barriers; topographic relative position), land use (urban fraction), and a day-by-day error-minimizing procedure for the estimation of the interpolation parameters. An average elevation is available for each of the 1024 cells.

2.3. Bioclimatic indices

Specific BI were calculated for the viticultural appellation areas, as well as for the non-traditional grape cultivation zones, over the 2 periods: 1961–1990, as a standard climatological period, and 1986–2015, as the latest 30 yr time-series. The most common climate parameters and BI were considered (Table 1, Table S1 in the Supplement at www.int-res.com/articles/suppl/c073p195_supp.pdf).

Growing Degree Day (GDD) or the Winkler Thermal Index presents thermal accumulation during the growing season where only temperature accumulation above 10°C (base temperature) is taken into account (Winkler et al. 1974). Due to temperature increase and consequently prolongation of the growing season length, the standard growing season period relevant for GDD calculation (1 April to 31 October, northern hemisphere) may no longer be accurate. Therefore, in order to verify the suitability of standard GDD to display thermal accumulation in the ER, GDD was calculated using 2 methods: (1) calculating 'standard' thermal accumulation from 1 April to 31 October; (2) calculating thermal accumulation from the start of the growing season (GS_{st}, Table 1) until the end (GS_{ed}, Table 1).

The Heliothermal Index or Huglin Index (HI) presents thermal accumulation during the growing season, calculated using daily mean and maximum temperatures, providing more weight to maximum temperature compared to GDD. HI calculation also

Table 1. Description of growing season and frost indices used in the study

Variable	Description	Reference
Growing season indices		
T_{mean}	Growing season mean temperature (1 April–31 October)	(Jones 2006, Fraga et al. 2014)
T_{min}	Growing season minimum temperature (1 April–31 October)	(Ramos et al. 2008)
T_{max}	Growing season maximum temperature (1 April–31 October)	(Ramos et al. 2008)
ND 25–30°C	Number of days with a maximum temperature in the range of 25–30°C (1 April–31 October)	(Carbonneau et al. 1992)
ND > 30°C	Number of days with a maximum temperature >30°C (1 April–31 October)	(Neethling et al. 2012)
WI	Growing Degree Day or Winkler Thermal Index; see Section 2.3.	(Winkler et al. 1974)
HI	Heliothermal Index or Huglin Index (1 April–30 September); see Section 2.3.	(Huglin 1978)
GS_l	Growing season length (GS_{st}–GS_{ed}); the number of days between the start and end of the growing season	(Ruml et al. 2012)
GS_{ed}	Climatologically defined end of the growing season; day followed by a series of 5 sequential days with an average temperature below 10°C, which are not followed by a sequence of 5 days with an average temperature above 10°C	(Ruml et al. 2012)
GS_{st}	Climatologically defined start of the growing season; day preceded by the first occurrence of 5 consecutive days with an average temperature above 10°C, which are not followed by a sequence of 5 days with an average temperature below 10°C	(Jones & Davis 2000)
CNI	Cool Night Index (1–30 September); average of minimum temperatures during the ripening period	(Tonietto 1999)
DTR	Diurnal temperature range (1 August–30 September); thermal amplitude during the ripening months	(Ramos et al. 2008)
P_{tot}	Growing season total rainfall (1 April–31 October)	(Ruml et al. 2012)
WD	Number of wet days (1 April–31 October); the number of days with precipitation >5 mm	(Ruml et al. 2012)
DI	Dryness Index (1 April–30 September); see Section 2.3	(Tonietto & Carbonneau 2004)
Frost indices		
FFP	Length of the frost-free period (LF–FF); the number of days between the last spring frost and the first fall frost	(Ruml et al. 2012)
FF	Date of the first fall frost; the first fall day with temperature ≤0°C	(Ruml et al. 2012)
LF	Date of the last spring frost; the last spring day with temperature ≤0°C	(Ruml et al. 2012)

takes into consideration a correction factor related to increasing daylight length towards higher latitudes (Huglin 1978).

The DI was developed specially for vineyard use (Tonietto & Carbonneau 2004). The index characterizes soil water reserves at the end of the growing season, taking into account initial soil moisture (W_0 = 200 mm), monthly precipitation (P_m), water loss through transpiration (E_T) and evaporation (E_S) from bare soil. Transpiration losses are defined by potential evaporation (PET) and the plant radiation absorption coefficient (a), while bare soil evaporation is additionally driven by the number of days with effective soil evaporation.

2.4. Statistics

Statistical differences in the mean of a dependent variable (i.e. BIs) between the first period (1960–1991) and the studied period (1986–2015) were investigated by independent t-test with a confidence level of 95%. The test was performed for each of the 1024 grid cells.

3. RESULTS AND DISCUSSION

3.1. Growing season indices

Narrow areas suitable for the production of high-quality wines are often determined by growing season isotherms that vary from too cold (<12°C) to too hot (>22°C) (Jones 2006). In the ER region, the mean growing season temperature significantly increased, with a magnitude of up to 1.5°C in certain areas, causing classification shifts (according to Jones) in the majority of vineyard areas. In particular, in the period 1961–1990, grapes were mostly cultivated in the 'warm' areas of the ER (17 to 19°C), which shifted to 'hot' (19 to 21°C) in the later period 1986–2015 (Fig. 2a,b,c). This increase in the mean growing
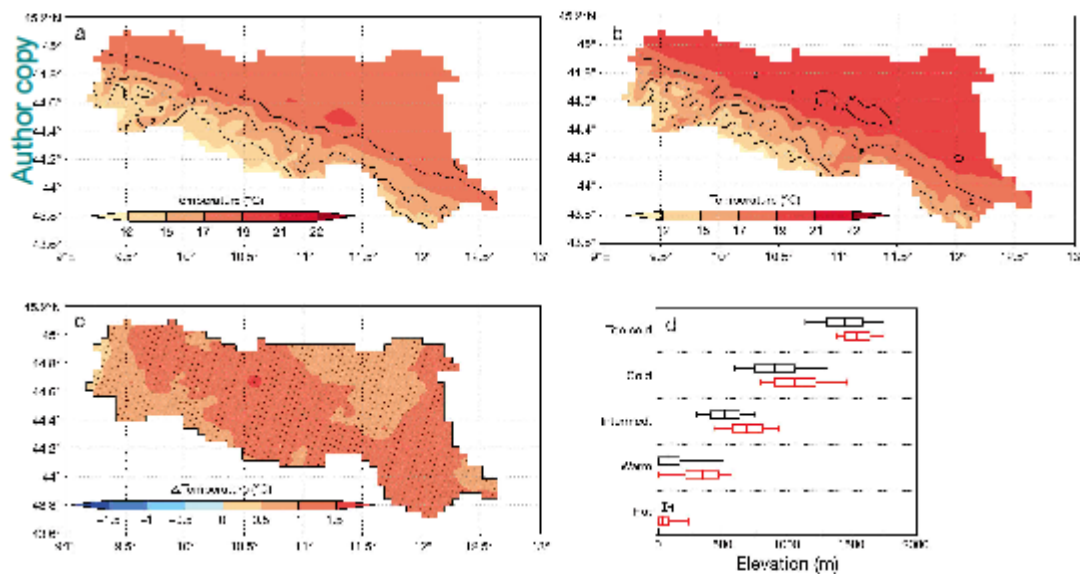
Fig. 2. Average growing season mean temperature in Emilia-Romagna for the periods (a) 1961–1990 and (b) 1986–2015. (c) Differences between the 2 periods in average growing season mean temperature, where areas with statistically significant ($p < 0.05$) differences are shown with hatched lines and a black border. (d) Elevation mean (central line), maximum and minimum values (whiskers), and standard deviation (box) of areas within the same classification group (see Table S1 in the Supplement); black: 1961–1990; red: 1986–2015

season temperature is likely to have an impact on regional viticulture suitability and the production of high-quality wines. For example, the reduction in areas with 'warm' temperature, which is optimal for Sangiovese, one of the main red cultivars in the ER, may induce viticulturists to cultivate later ripening grapevine varieties in order to adapt to the upcoming warming conditions which are predicted for the whole of northern Italy (Ruml et al. 2012). Apart from notable area reduction, zones with 'warm' mean growing season temperature shifted towards higher elevations, with mean heights of 67 and 335 m in the first (1961–1990) and second periods (1986–2015), respectively (Fig. 2d). In contrast, smaller elevation shifts were detected for zones with 'cold' and 'intermediate' mean growing season temperatures. Zones characterized by 'hot' mean growing season temperature had approximately constant mean height (Fig. 2d; see Table S1 in the Supplement for classification definitions).

Similarly to the mean temperature, the minimum and maximum temperatures mostly increased in the ER during the second studied period compared to the first, which is supported by other studies (Antolini et al. 2016) (see Figs. S1a,b & S2a,b in the Supplement). Unlike the maximum and mean temperatures, however, the increase in minimum temperature was not significant over the entire ER, and certain small areas even showed a decrease (Fig. 2c; see Figs. S1c & S2c in the Supplement). According to Tomozeiu et al. (2007), the maximum and minimum temperatures will continue to rise in future decades, with the minimum temperature increasing by 2°C during winter, summer and fall, and the maximum temperature increasing by 3 to 5°C during spring and summer by the end of the 21st century under the A2 scenario. The temperature rises caused spatial shifts of zones characterized by particular temperatures towards the south of the ER region, which is predominantly hilly and mountainous. Additionally, as a direct consequence of rising maximum temperatures, the number of days with a maximum temperature in the range of 25 to 30°C diminished (results not shown), while the number of days exceeding 30°C significantly increased over the ER grape-growing zones and most of the mountainous areas (Fig. 3) in the second period (1986–2015) compared to the first (1961–1990). These changes may affect plant photosynthesis and
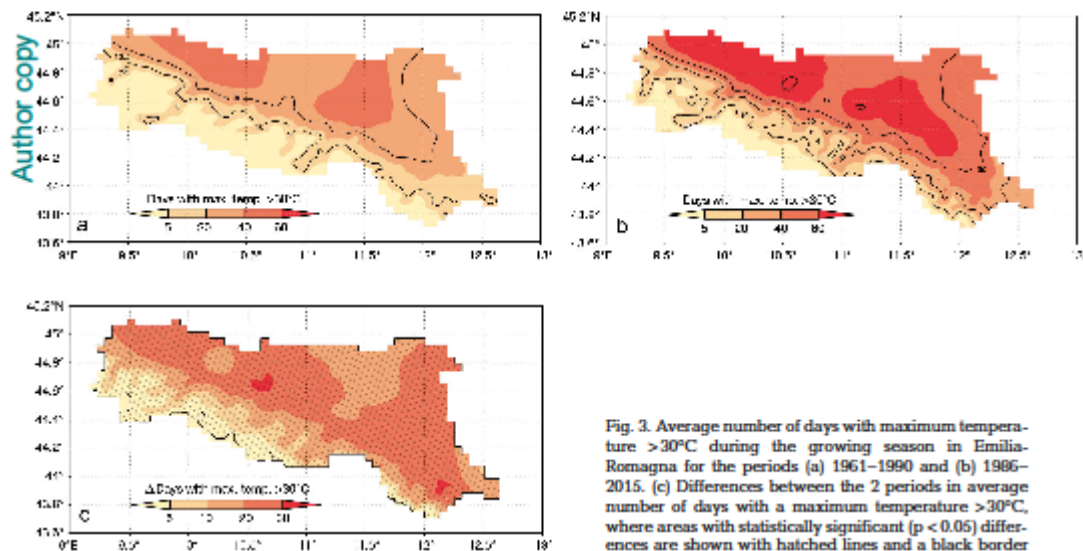
Fig. 3. Average number of days with maximum temperature >30°C during the growing season in Emilia-Romagna for the periods (a) 1961–1990 and (b) 1986–2015. (c) Differences between the 2 periods in average number of days with a maximum temperature >30°C, where areas with statistically significant (p < 0.05) differences are shown with hatched lines and a black border

growth, since days with maximum temperatures in the range of 25 to 30°C are optimal for grapevine photosynthesis (Carbonneau et al. 1992). On the other hand, several days exceeding 30°C can provoke plant heat stress, premature véraison, berry abscission, reduced flavor development, and enzyme activation (Mullins et al. 1992). During the 21st century, daily maximum temperature in the vegetative period could even exceed 45°C, reaching the upper temperature limit for photosynthesis (Greer & Weedon 2012), and having a detrimental impact on berry composition and yield.

The GDD values calculated using 2 methods (see Section 2 for details) were not significantly different for most of the ER area (see Fig. S3a,b in the Supplement). Hence, the 'standard' GDD calculation method (1 April to 31 October) was suitable for displaying thermal accumulation in the growing season. However, due to the higher temperatures, the climatologically defined growing season might start before 1 April and end after 31 October. Therefore, the 'standard' GDD calculation method might not be suitable in the future and may need additional adjustments (as presented in Method 2; see Section 2.3) in order to provide more accurate predictions of seasonal thermal accumulation in the coming decades. Using the standard method of calculation, a significant increase in the GDD value was detected for the entire region. The magnitude of the increase varied from

<100 GDD units (°D) in some mountainous areas to >350 GDD °D units in some central zones (Fig. 4c).

For the production of the high-quality wines, 1400 to 2000 GDD °D units are typically required, depending on vine variety and environmental factors (Gladstones 1992). The total area with optimal growing season thermal accumulation (1400 to 2000 GDD °D) for production of the high-quality wines was approximately constant in the ER during the 2 studied periods, despite the spatial shifts of these areas in the second period (1986–2015). In the center of the ER, some current grape cultivation areas became 'too hot' (>2000 GDD °D) while some areas in the south of the ER became climatologically optimal for the production of high-quality wines (Fig. 4a,b). According to the Winkler et al. (1974) classification (see Table S1 in the Supplement), the majority of the current grape cultivation areas in the ER were characterized as 'Region 2/Region 3' and 'Region 3/Region 4' during 1961–1990 and 1986–2015, respectively, with spatial shifts oriented towards zones with higher elevation and with the occurrence of 'heat islands' in central zones (Fig. 4a,b). Areas characterized as 'Region 3' had the strongest shift towards higher elevations, with mean heights of 41 and 271 m, in 1961–1990 and 1986–2015, respectively. Zones characterized as 'Regions 1 and 2' had a mean elevation difference in the range of 150 to 200 m between the 2 periods, while the mean elevation shift of areas classified as 'Region 4' was <10 m (Fig. 4d).
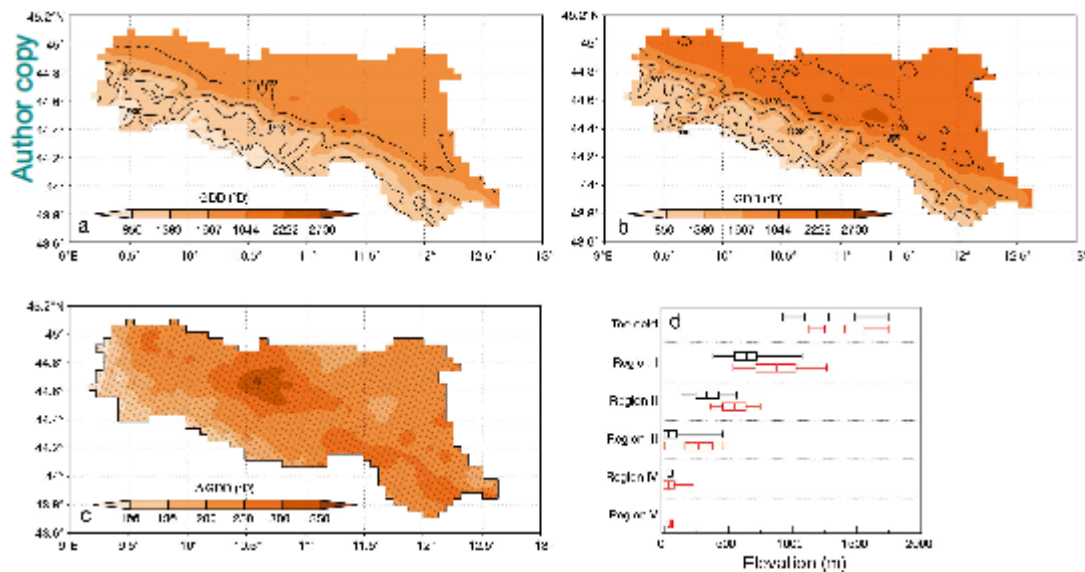
Fig. 4. Average thermal accumulation during the growing season in Emilia-Romagna presented as Growing Degree Day (GDD) for the periods (a) 1961–1990 and (b) 1986–2015. (c) Differences between the 2 periods in average GDD, where areas with statistically significant (p < 0.05) differences are shown with hatched lines and a black border. (d) Elevation mean (central line), maximum and minimum values (whiskers), and standard deviation (box) of areas within the same classification group determined by Winkler (see Table S1 in the Supplement); black: 1961–1990; red: 1986–2015

Displacement of the areas determined by the Huglin (1978) classification (see Table S1 in the Supplement) was oriented towards higher elevations, with the highest thermal accumulation detected in the central and northern grape cultivation zones in the second period (1986–2015) (see Fig. S4a,b in the Supplement). The majority of the high-quality grape-producing areas was classified as 'temperate/warm temperate' in the first period (1961–1990). However, these areas shifted to 'warm temperate/warm' in the second period (1986–2015), due to a significant increase in thermal accumulation (see Fig. S4a,b,c in the Supplement). This increase in thermal accumulation will likely continue in the future. It is predicted that the whole of northern Italy, including the ER, could be characterized as a 'warm' (according to the Huglin Index) grape-growing region in future decades (2041–2070; A1B scenario) (Fraga et al. 2013). In general, the magnitude of these changes will depend strongly on future anthropogenic carbon emissions into the atmosphere. Higher temperatures and consequently higher thermal accumulation could lead to an earlier appearance of the phenolog-

ical stages (e.g. bud-breaking, flowering, véraison) (Kartschall et al. 2015, Fraga et al. 2016, Ruml et al. 2016), in certain cases, a shorter time between 2 phenological stages (Fraga et al. 2016), and an earlier harvest (Jones & Davis 2000, Bock et al. 2011). This accelerated pace of phenological events and earlier harvest in warmer conditions may have negative impacts on berry composition and wine quality, e.g. changes in the sugar/acid ratio due to faster sugar accumulation and acid degradation (Urhausen et al. 2011), and reduction of aromatic compounds (Duchêne & Schneider 2005) and anthocyanins (Mori et al. 2007). However, the negative effect of higher temperatures will strongly depend on vineyard management and adaptation of grape varieties to the warmer conditions (van Leeuwen et al. 2013, Palliotti et al. 2014). However, it is possible that warming could also positively influence wine quality (Jones et al. 2005).

The climatologically defined start, end, and length of the growing season have a limited ability to predict the exact occurrence of phenological events for certain grape varieties in the ER. This is due to the

wide diversity of grape varieties planted in the ER and the important influence of the 'cultivar' factor on these predictions (de Cortázar-Atauri et al. 2009). However, the presented method could provide a valuable insight into the general changes in the starting and ending dates of the growing season for studies on a regional scale. Warming during the past decades has induced an extension of the growing season length in the ER, where areas with minimal indispensable vegetative season length, roughly 170 to 190 d (Mullins et al. 1992), shifted towards the south of the region to the non-traditional viticultural zones, thereby, making them climatologically suitable for the growing of early ripening grape varieties (see Fig. S5a,b in the Supplement). However, changes were not homogeneous, or statistically significant over the entire region. The central ER areas, like the hilly and mountainous areas, profited significantly in growing season prolongation (>10 d). In the rest of the ER, season length increase was not significant (see Fig. S5c in the Supplement). Growing season length extension was affected more by an earlier start than by a later end of the climatologically defined growing season (see Fig. S5d,e in the Supplement).

Over the entire ER region, the Cool Night Index (CNI), related to the synthesis of anthocyanins in grapes, was approximately constant in both studied periods. It ranged from 10°C in mountainous and hilly areas to 15°C in zones close to urban areas, with the majority of vineyard areas being under the cool night regime (see Table S1 in the Supplement). Similar observations were reported in a recent study conducted for the period 1950–2009 (Santos et al. 2012), in which the authors detected CNI values in approximately the same range (10 to 15°C) over the entire ER area. Several studies (Kliewer 1977, Tonietto & Carbonneau 1998) have reported a favorable effect of night temperatures in an approximate range of 10 to 15°C on anthocyanin accumulation during the ripening months. Therefore, the results obtained in this study suggest optimal night conditions for red and rosé wine production, which represents 55% of the total ER wine production (Pollini et al. 2013). The diurnal temperature range (DTR) during the ripening months, an index mostly related to red wine quality (Ramos et al. 2008), increased in the second period compared to the first for most of the ER. However, the changes were not necessarily significant (see Fig. S6a,b,c in the Supplement). A significant increase in DTR was detected in most of the ER high-quality wine-producing zones, suggesting an effect on red wine composition, which would be positive only up to a certain limit (Ramos et al. 2008). An ex-

cessively large DTR, which may occur in future decades due to the likely additional warming in the ER (Tomozeiu et al. 2007, Fraga et al. 2013), may also have negative effects on wine quality.

Growing season precipitation in the period 1961–1990 varied from approximately 1000 mm in the mountainous areas to below 400 mm in areas close to the River Po Delta, while most of the vineyard areas had precipitation in the range of 400 to 550 mm. In the second period, an increase in precipitation was detected in northeastern, certain central, and southwestern ER areas, while a reduction was detected for the rest of the ER (Fig. 5a,b). However, a statistically significant change was identified only in small sections of the northeastern area (Fig. 5c).

The changes in precipitation detected in the present study were accompanied by a significant reduction in wet days (precipitation >5 mm) in the small sections of the southeastern area, while a significant increase was detected in sections of the small central area and the area close to the River Po Delta (see Fig. S7 in the Supplementf). Even though certain areas in the ER had more precipitation in the second studied period (1986–2015) (Fig. 5c), the entire ER and northern Italy could see a decrease in precipitation, with a magnitude of up to 100 mm in certain areas (Ruml et al. 2012), by the end of the 21st century, suggesting longer droughts and drier conditions.

Drier conditions (evaluated with the DI) were detected in almost the entire ER during the second period compared to the first (Fig. 6). This was the case even in certain areas with increased precipitation (e.g. some parts of the central area), indicating that, besides precipitation, temperature also has a noticeable effect on soil water availability in the ER (Figs. 5c & 6c; see Fig. S2c in the Supplement). The negative effect of the increasing temperatures on soil water availability could be due to higher soil evaporation under warmer conditions (Alcamo et al. 2007). As expected, significantly drier conditions were detected in the areas that had less precipitation and the highest warming in the second period compared to the first (Figs. 5c & 6c; see Fig. S2c in the Supplement). Even though the majority of the vineyard areas were classified as 'humid/moderately dry' in both periods (Fig. 6a,b; see Table S1 in the Supplement), the detected drier conditions suggest potential challenges for the production of high-quality wines in the ER during future decades. This is due to the possible occurrence of 'sub-humid' (see Table S1 in the Supplement) conditions in some areas traditionally cultivated with grapes. The possibility of drier conditions in future decades is supported by a recent
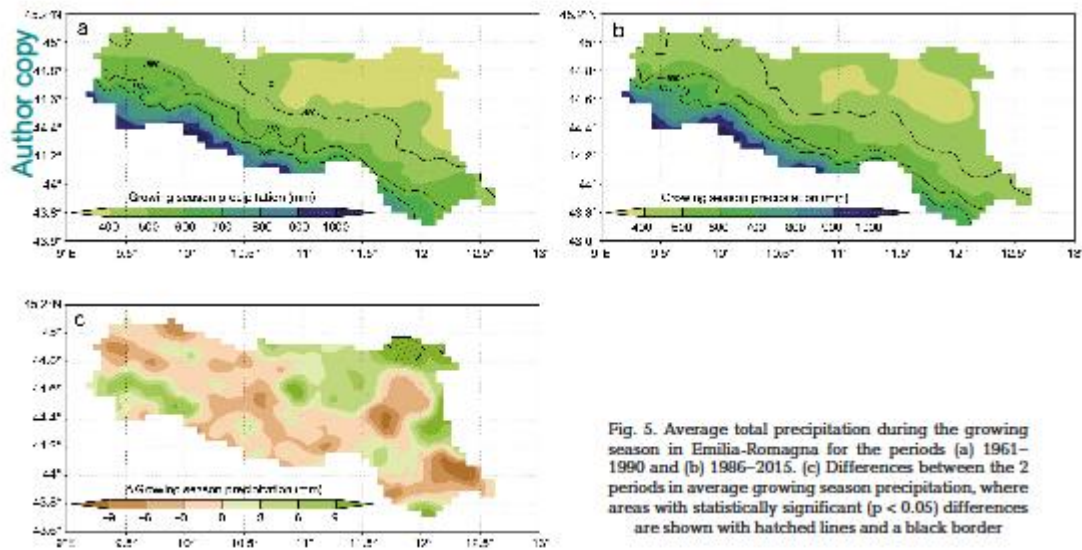
Fig. 5. Average total precipitation during the growing season in Emilia-Romagna for the periods (a) 1961–1990 and (b) 1986–2015. (c) Differences between the 2 periods in average growing season precipitation, where areas with statistically significant (p < 0.05) differences are shown with hatched lines and a black border
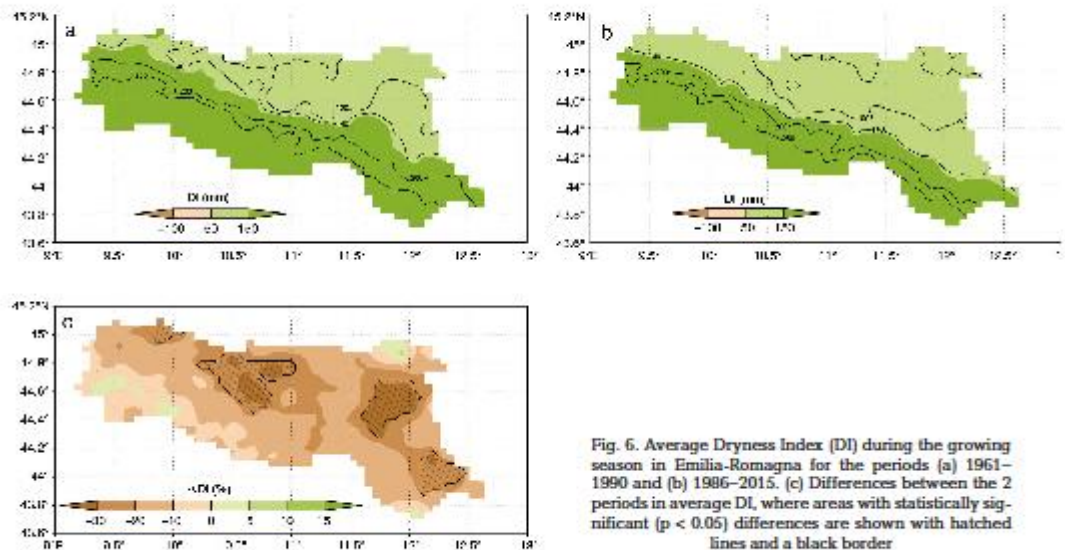
Fig. 6. Average Dryness Index (DI) during the growing season in Emilia-Romagna for the periods (a) 1961–1990 and (b) 1986–2015. (c) Differences between the 2 periods in average DI, where areas with statistically significant (p < 0.05) differences are shown with hatched lines and a black border

study (Fraga et al. 2013), in which certain areas in the ER were classified as 'sub-humid' during the period 2041–2070 under the A1B scenario. The detected changes could drive viticulturists to implement irrigation systems, which are not traditionally used for grape cultivation in the ER, in order to mitigate the consequences of warmer and drier conditions, such as crop load reduction (Ramos & Martínez-Casasnovas 2010) or higher sugar accumulation in berries (Poni et al. 2007).
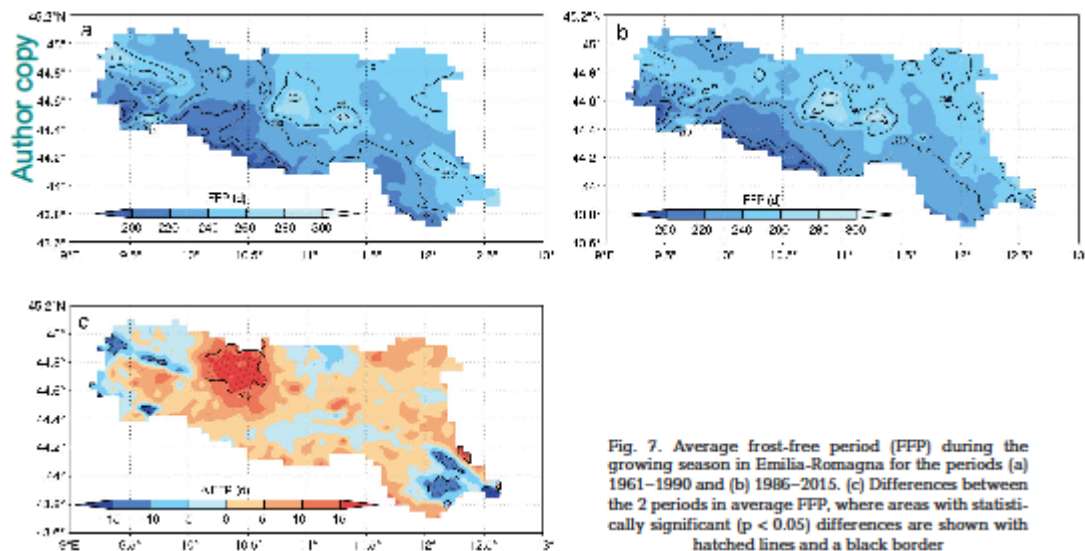
Fig. 7. Average frost-free period (FFP) during the growing season in Emilia-Romagna for the periods (a) 1961–1990 and (b) 1986–2015. (c) Differences between the 2 periods in average FFP, where areas with statistically significant (p < 0.05) differences are shown with hatched lines and a black border

3.2. Frost indices

The timing of frost occurrence, as well as the length of the frost-free period (FFP), may have a significant influence on viticulture. For example, spring frost occurrence after budburst could damage developing buds and decrease crop load, while the occurrence of fall frost before grape harvest could damage maturing canes and berries (Ruml et al. 2012). In our study, a significant reduction in the length of the FFP was detected in small sections of the southeastern and northwestern cultivated areas, while it significantly increased in small sections of the central and northern viticultural areas of the ER (Fig. 7a–c). The decrease in the length of the FFP in certain grape cultivation areas was most likely due to lower minimum temperatures in those areas during the second studied period (1986–2015) compared to the first (1961–1990) (Fig. 7c; see Fig. S1c in the Supplement). Furthermore, positive or negative variations in the FFP were caused by the different timing of both the first fall frost (FF) and the last spring frost (LF). Average FF occurrence, depending on the area, varied over the ER from late October to the beginning of December in the period 1961–1990 (data not shown). In the second studied period (1986–2015), significantly earlier FF occurrence was detected in small sections of the southeastern and southwestern culti-

vated areas, while delayed timing of FF was detected in small sections of the central and northern areas of the ER (see Fig. S8a in the Supplement). Average LF occurrence over the entire ER ranged from early March to late April in the period 1961–1990 (data not shown), with significantly earlier timing during the second period (1986–2015) in small areas spread over the ER and significantly later LF timing in small sections of the northwestern and northern areas (see Fig. S8b in the Supplement).

4. CONCLUSIONS

The findings of the present study highlight the changes in climate related to the viticultural suitability of the ER region during 2 periods, 1961–1990 and 1986–2015. Detected changes in the growing season temperatures and BI may affect suitability for the production of high-quality wine in the ER, which could become 'too hot' for this purpose. Negative impacts of increasing temperatures on wine production could be mitigated by planting later ripening grape varieties, compared to those currently present in the ER, or by establishing new vineyard areas at higher elevations. Also, the study results suggest that warmer and drier conditions in the last 30 yr (1986–2015) have decreased soil water availability,

which is important for grapevine development in certain areas of the ER. This implies the need for an updated strategy for the future implementation of irrigation systems in vineyards. Furthermore, the obtained results suggest that wine production expansion in the ER will depend greatly upon upcoming climatic changes. Thus, there is a need for further studies based on the assessment of BI for future decades, computed from climate model output data under different CO_2 emission scenarios.

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Appendix B – Climate change trends, grape production, and potential alcohol concentration in Italian wines

Climate change trends, grape production, and potential alcohol concentration in Italian wines

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Climate change and sustainability are two major elements of the ongoing international vitiviniculture worldwide scenarios. When dealing with sustainability in the wine sector, it is essential to bear in mind that it must be carried out on the basis of four fundamental pillars: environmental, social (safety and food hygiene), cultural and economic (resolution OIV CST 1/2004). Sustainable winegrowing is a comprehensive set of practices that are environmentally sound, socially equitable and economically viable. This applies to the whole production and processing chain, from grapes to grape juice and wines.

As a very simplification, the climate change is mainly due to CO_2 emission that is driven by the economic growth. The noticeable effects of climate change are dry and hot periods, high evaporation, unexpected heavy rainfall, and generally unfavorable distribution of precipitation. In particular, the release of CO_2 and other gases has increased the average temperature worldwide, and an increase of 1.0-5.0°C in the mean temperature is expected depending on the location. It is noteworthy that an increase of 1.0-2.5°C in average temperature can advance the grape phenological phases of about 6 to 22 days. However, as the grapevine developmental stages are regulated by temperature. the various parts of the grape berry do not necessarily mature at the same rhythm. From the practical point of view, the occurrence of warmer weather advances the technological maturity of grapes (i.e. grapes will become sweeter earlier) which cause a ripeness imbalance with the phenolic maturity (i.e. the seeds and skins ripen slower). Further practical implication related to climate change includes:

- Earlier phenology (plant growth events)
- Altered ripening profiles, wine styles:

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- Decrease in the total acidity content (of early-ripening varieties), nitrogen, primary aroma (atypical ageing), and anthocyanins (?)
- Decrease protection from oxidation (SO_2 less active), atypical ageing;
- Increase in potassium and sugar content of grapes, i.e. potential alcohol of wines;
- Increased water demand and timing of irrigation;
- Increase the irregularity of the yields;
- Altered/new disease/pest timing and severity (e.g. Botrytis, Peronospora)
- Changes in soil fertility and erosion (organic matter).

In this context, there several questions that deserve attention, including:
- What are the consequences of climate change on wine production and consumption?
- How will change the geography of wine production?
- To what extent can organic, biodynamic and natural wines be an alternative approach?

At local level, there is a need to consider the most appropriate short- and long-term adaptation strategy, the former includes:
- Use of unripe grapes harvested during cluster thinning;
- Techniques to increase wine acidity and decrease pH (e.g. new yeasts, resins);
- Organic wine (Reg EU No. 203/2012*) and biodynamic management with spontaneous fermentation;
- Partial dealcoholization of wines: 'sweet spot' approx. 2% v/v.

*The European Regulation on organic wines apply restriction on some physical techniques (e.g. Cation exchangers Partial dealcoholisation and Electrodialyses) and additives (e.g. malic acid).

Following the main results found in case studies of Emilia-Romagna region (Italy):
- Climate change in the viticulture appellation areas of the Emilia-Romagna region in the periods 1961–2015 showed (i) increased number of days with maximum temperature exceeding 30°C, which can induce plant stress; (ii) shift of the majority grape producing zones from the "temperate/warm temperate" to the "warm temperate/warm" (according to Huglin classification); (v) decreased soil water availability necessary for grapevine development (Teslić et al. 2017);
- Naturally occurring alcohol content in Sangiovese red wines showed a significant increasing trend with 0.07 % (v/v)/year and 0.83 % (v/v) over a 12-year period (2001–2012). Dry Spell Index (DSI) - the number of days with <1 mm of precipitation during the grape-growing season - showed high

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relationship with potential alcohol (adjusted R^2 =0.81) suggesting a large contribution of calculated climatic variables as a driver of increasing potential alcohol content in red wines from the Romagna Sangiovese appellation area (Teslić et al. 2016);

- The effects of biodynamic production practices on composition and sensory attributes of Sangiovese wines were examined for 2 years (2009 and 2010) in a vineyard that was converted from organic (ORG) to biodynamic (BDN) viticulture. During the first year (2009), the BDN wines were characterized by low alcohol strength, colour intensity, total polyphenols, monomeric anthocyanins and catechin. Conversely, the second year BDN wines differed from the organic wines in terms of total polyphenols and phenolic compounds, including polymeric pigments, co-pigmentation, tannins and iron-reactive polyphenols (Parpinello et al. 2015);

- Although the yeast population is mostly related to the grape management, i.e. organic or biodynamic, the wine composition is mainly affected by the winemaking process, and then by the grape management (Patrignani et al. 2017).

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3

CHAPTER 3

Predictions of climate change in the Emilia-Romagna's DOP appellation areas until the end of the 21st century

3   Predictions of climate change in the Emilia-Romagna's DOP appellation areas until the end of the 21st century

3.1   Introduction

Vitis vinifera is perennial plant allowing commercial exploitation during at least couple of decades **(Lereboullet et al., 2014)**, thus a long-term strategy needs to be applied to maximize commercial exploitation of planted vineyards. Therefore, climate change predictions are a valuable tool for development of long-term strategies (e.g. planting new cultivar). A recent study evaluated the importance of climate predictions for long-term decision making by examining 156 Australian vinegrowers/winemakers through the online survey **(Dunn et al., 2015)**. Results indicated that 42% of participants consider information related to climate predictions 'very much' useful to determine their long-term strategies while 35% of participants consider this information 'somewhat' useful. Furthermore, 48% of participants indicated that climate predictions in the 6–10 year's timeframe would help to develop long-term strategies while 73% of participants indicated that future climate information in timeframe 6–20 years may be useful to decide grape variety. Apart from timeframe, spatial scale and climate data resolution are playing important role in decision making. Dunn et al. reported that ~85% of participants consider that climate prediction information at 'somewhat local' (e.g. averaged over specific locality) are useful in long-term decision making.

It is well known that global warming is caused by human activities *(Fig. 1.1)*, related to the emission of greenhouse gases, aerosols and their precursors into the atmosphere. Thus, climate change prediction will strongly depend on the concentration of these compounds (whether already present or emitted afterwards) in the atmosphere during future decades and centuries. In that regard, in the Fifth Assessment Report (AR5), Intergovernmental Panel of Climate Change (IPPC) introduced 4 new climate scenarios called Representative Concentration Pathways (RCP), RCP 2.6, RCP 4.5, RCP 6 and RCP 8.5 **(Stocker et al., 2013)**. RCP scenarios are estimating the global warming according to trajectories of air greenhouses gases concentration (e.g. CO_2, CH_4, N_2O etc.), aerosols and their precursors, which concentration will depend on the socio-economic development of human society. Concentration of air CO_2 (not the only greenhouse gas, however the most relevant) during the pre-industrial era was ~280 ppm while a current concentration of air CO_2 is ~400 ppm. Whereas, according to RCP scenarios until the end of the 21st century air CO_2 concentration will reach ~420 ppm, ~540 ppm, ~670 ppm and ~940 ppm according to RCP 2.6, RCP 4.5, RCP 6 and RCP 8.5, respectively **(Stocker et al., 2013)**. Combined with other greenhouse gases, aerosols and their precursors, equivalent of air CO_2 concentration will be even higher until the end of the 21st century *(Fig. 3.1)*, which could cause increase of global temperature from 1–4°C, depending on RCP scenario *(Fig. 3.2)*.
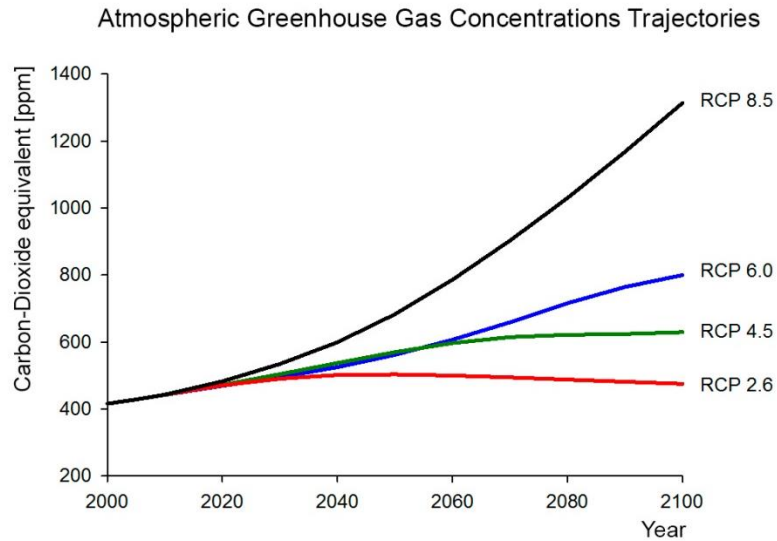
Figure 3.1 Air concentrations of greenhouse gases (e.g. CO_2, CH_4, N_2O etc.), aerosols and their precursors until the end of the 21st century according to RCP scenarios presented as the equivalent of air CO_2 concentration (modified from https://19january2017snapshot.epa.gov/climate-change-science/future-climate-change_.html).

For climatological studies often used RCP scenarios are 4.5 and 8.5 **(Fraga et al., 2015; Lee et al., 2015; Shope et al., 2016)**. RCP 4.5 is assuming that radiative forcing (difference between energy absorbed by the Earth and energy radiated back to space, in other words global warming) will be stabilized by the end of the 21st century, while RCP 8.5 represents the worst-case RCP scenario assuming that radiative forcing will continue to increase even after the 21st century *(Fig. 3.1; Fig. 3.2)*.
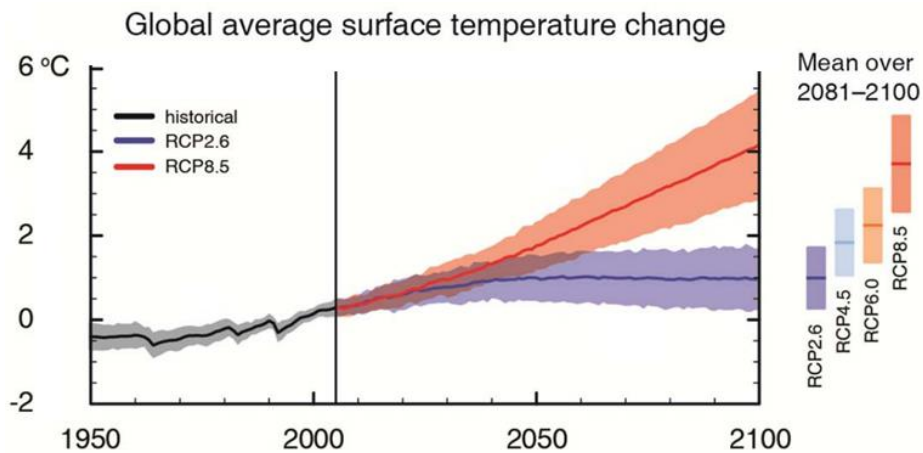
Figure 3.2 Average global surface temperature change until the 21st century according to RPCs scenarios (modified from Stocker et al., 2013).

Thus, evaluation of climate predictions at local spatial scale (11 x 11km) during the periods 2018–2027 and 2011–2040, under RCP 4.5 and RCP 8.5 scenarios, in the Emilia-Romagna DOP appellation areas may help local viticulturists and winemakers to develop long-term strategies. Additionally, the period 2071–2100 was studied as well.

3.2 Materials and Methods

3.2.1 Study region, model data and bioclimatic indices

Study region is the entire Emilia-Romagna with DOP areas (see 2.2.1). Mathematical definitions same as classifications of used BIs in the present study are presented in *Table 2.1*. Daily minimum temperatures, maximum temperatures and precipitation climate data were obtained from 'Coordinated Regional Climate Downscaling Experiment' (CORDEX) project **(http://cordex.org/)**. The core of CORDEX project presents an ensemble of Regional Climate Models (RCM) obtained with empirical statistical downscaling from Global Climate Models, which are made for two Representative Concentration Pathways scenarios (RCP 4.5 and RCP 8.5). Used climatological data for past period (1961–1990, as the standard climatological period) and future periods (2018–2027, 2011–2040 and 2071–2100) are in local spatial scale ~11 x 11km. Whereas, BIs for the past were calculated with historical data while BIs for the future were calculated from 9 RCM *(Table 3.1)*. The bias of RCM data used for BIs calculation was corrected and adjusted according to **http://cordex.org/data-access/bias-adjusted-rcm-data/**. Prior to calculation climate data were spatially interpolated to 5 x 5 km local scale to 1024 grid cells.

Table 3.1 List of all Global Climate Model/ Region Climate Model chains used in present study.

GCM	RCM
CNRM-CERFACS-CNRM-CM5	CCLM4-8-17
ICHEC-EC-EARTH	CCLM4-8-17
MOHC-HadGEM2-ES	CCLM4-8-17
MPI-M-MPI-ESM-LR	CCLM4-8-17
ICHEC-EC-EARTH	HIRHAM5
ICHEC-EC-EARTH	RACMO22E
MOHC-HadGEM2-ES	RACMO22E
MPI-M-MPI-ESM-LR	REMO2009
MPI-M-MPI-ESM-LR	REMO2009

3.2.2 Statistical analysis

Student t-test with 95% confidence level was applied to evaluate statistical differences in mean of a dependent variable (e.g. BI) for each grid cell between the standard period (1961–1990) and other studied periods (2018–2027, 2011–2040 and 2071–2100). Statistical test is run on one model (e.g. MPI-M-MPI-ESM-LR/CCLM4-8-17), one RCP scenario (e.g. RCP 4.5) and one studied period (e.g. 1961–1990 vs 2011–2040) each time. If ≥5 models (9 in total) are statistically significant according to Student t-test in the same grid cell for the same period (e.g. 1961–1990 vs 2011–2040), same BI (e.g. HI) under same scenario (e.g. RCP 4.5), those areas are marked as dotted on the figures. In other words, differences of ≥5 models are greater than 'natural' climatological variability or ensemble of models give statistically significant change.

3.3 Results and Discussion

Growing season mean temperature (T_{mean}), as it was discussed in Chapter 2 (see 2.3) during the period 1961–1990 was mainly characterized as 'warm' (for classification definition see *Table 2.1*) for most of the DOP zones in the ER *(Fig. 3.3)*. Comparing to the period 1961–1990, T_{mean} could significantly increase for the all other studied periods (2018–2027, 2011–2040 and 2071–2100) under both RCP scenarios (4.5 and 8.5) *(Fig. 3.4a; Fig. 3.4b; Fig. 3.4c; Fig. 3.5a; Fig. 3.5b; Fig. 3.5c)*. Due to temperature potential increase, most DOP zones in the ER may be characterized as 'hot' during periods 2018–2027 and 2011–2040 for both scenarios RCP 4.5 and RCP 8.5 *(Fig. 3.4a; Fig. 3.4b; Fig 3.5a; Fig 3.5b)*. A recent study reported that the majority of DOP zones in the ER were characterized as 'hot' during the period 1986–2015 **(Teslić et al., 2017)**. Thus, results from the present study are suggesting that T_{mean} may increase slightly until 2040 comparing to current conditions, under both RCP scenarios. According to RCP 4.5 scenario during the period 2071–2100 certain central and northeastern DOP areas of the ER may be characterized as 'very hot' while the rest of DOP areas may be characterized as 'hot', conditions which may be suitable for production of high-quality grapes/wines, however adjustments would be necessary (e.g. new grape varieties) *(Fig. 3.4c)*. On the other hand, according to RCP 8.5 scenario many DOP areas in the ER may be characterized as 'too hot', conditions in which production of high-quality grapes/wines with current technology and varieties would be questionable *(Fig. 3.5c)*. Obtained results are suggesting that the ER may be suitable for the production of high-grapes/wines at least until 2040, whereas further suitability will depend on factors related to wine industry development (vine adaptation on warmer conditions, technology development) and external factors (reduction of greenhouse emissions into the atmosphere).
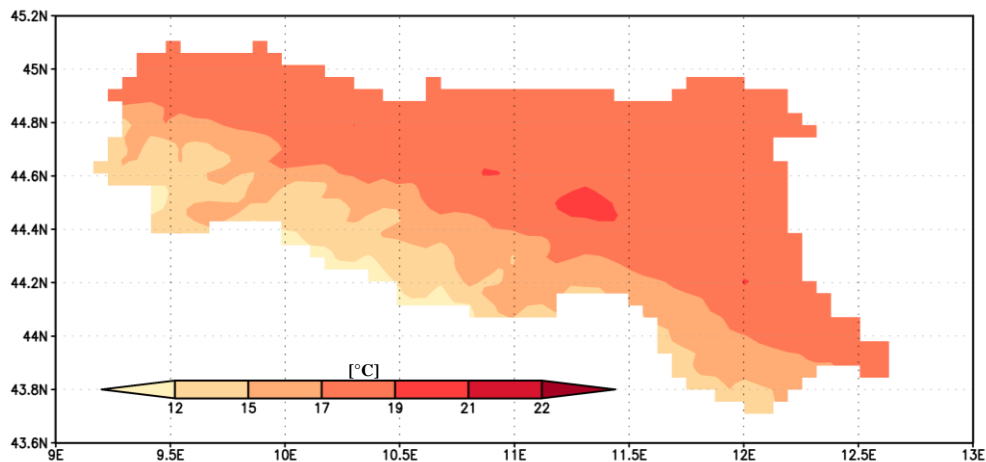
Figure 3.3 Average growing season mean temperature for the period 1961–1990 in the Emilia-Romagna, calculated with historical data previously described in Chapter 2 (see 2.2.2).
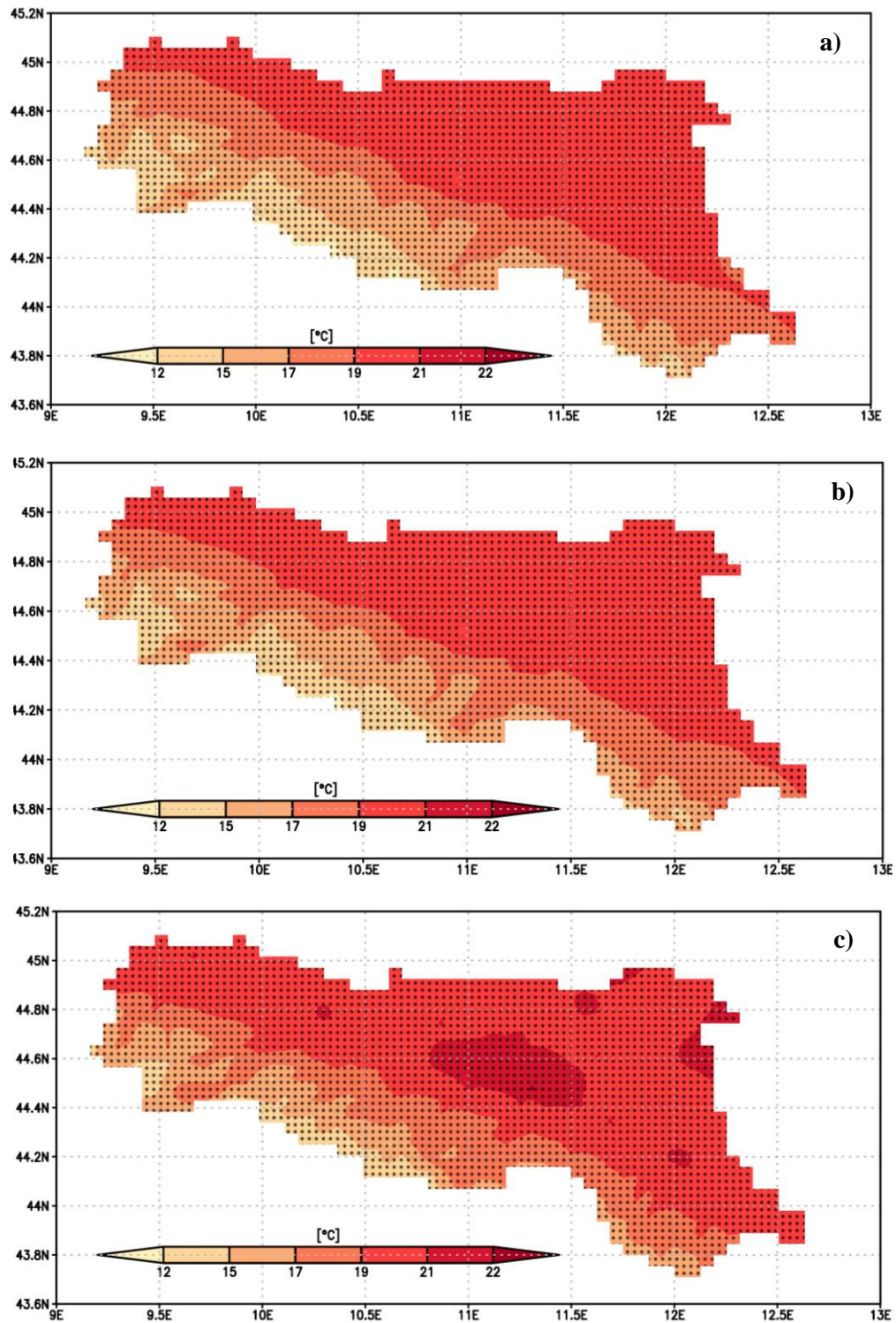
Figure 3.4 Growing season mean temperature in the Emilia-Romagna for the periods a) 2018–2027 b) 2011–2040 and c) 2071–2100, calculated as median of 9 models under RCP 4.5 scenario. Dotted areas are statistically significant (p < 0.05) according to t-test.
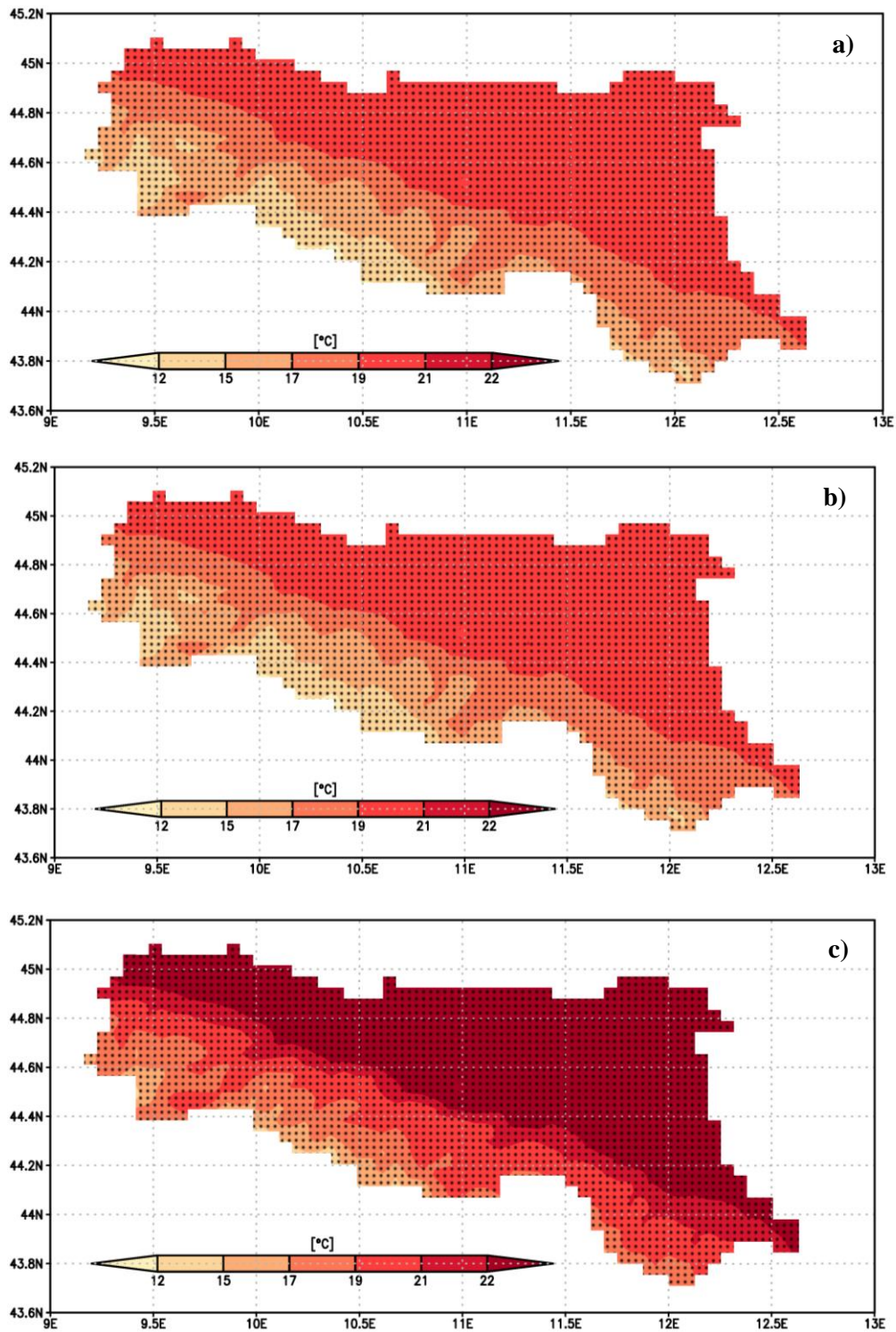
Figure 3.5 Growing season mean temperature in the Emilia-Romagna for the periods a) 2018–2027 b) 2011–2040 and c) 2071–2100 calculated as median of 9 models under RCP 8.5 scenario. Dotted areas are statistically significant (p < 0.05) according to t-test.

Comparing to the period 1961–1990 potential temperature increase could cause decrease number of days with maximum temperature in the range 25–30 °C (ND 25–30°C) during the studied periods 2018–2027 (data not shown) and 2011–2040, under both RCP scenarios (RCP 4.5 [data not shown] and RCP 8.5) in the entire ER *(Fig. 3.6a; Fig. 3.7a)*.
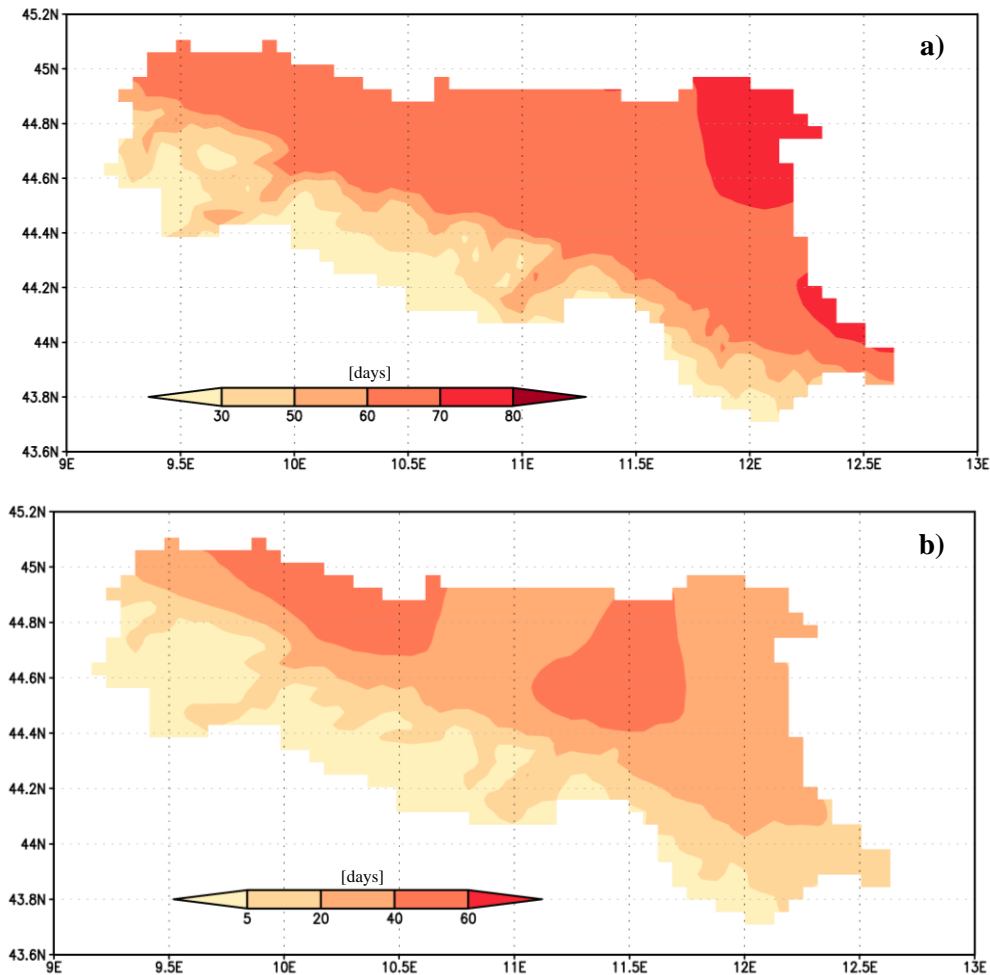
Figure 3.6 Average number of days with a) maximum temperature in the range 25–30°C b) maximum temperature >30°C, in the Emilia-Romagna during the period 1961–1990, calculated with historical data previously described in Chapter 2 (see 2.2.2).

Obtained results are also suggesting that differences in ND 25–30°C could be minor between 1961–1990 vs 2018–2027 and 1961–1990 vs 2011–2040 under same RCP scenario (e.g. RCP 4.5), similar as differences between same period (e.g. 1961–1990 vs 2011–2040) under RCP 4.5 and RCP 8.5 scenarios (data not shown). Potential decrease of ND 25–30°C could have a negative impact on grape quality as it was previously described in Chapter 2 (see 2.3). Interestingly, ND 25–30°C in the ER during the period 1961–1990 vs 2071–2100 under both RCP scenarios increased comparing to e.g. period 1961–1990 vs 2011–2040 under RCP 8.5 scenario *(Fig. 3.7a; Fig. 3.7b; Fig. 3.7c)*. In some mountain areas of the ER ND 25–30°C during e.g. 2071–2100 RCP 8.5 scenario, could be higher even that during 1961–1990 *(Fig. 3.7b)*.
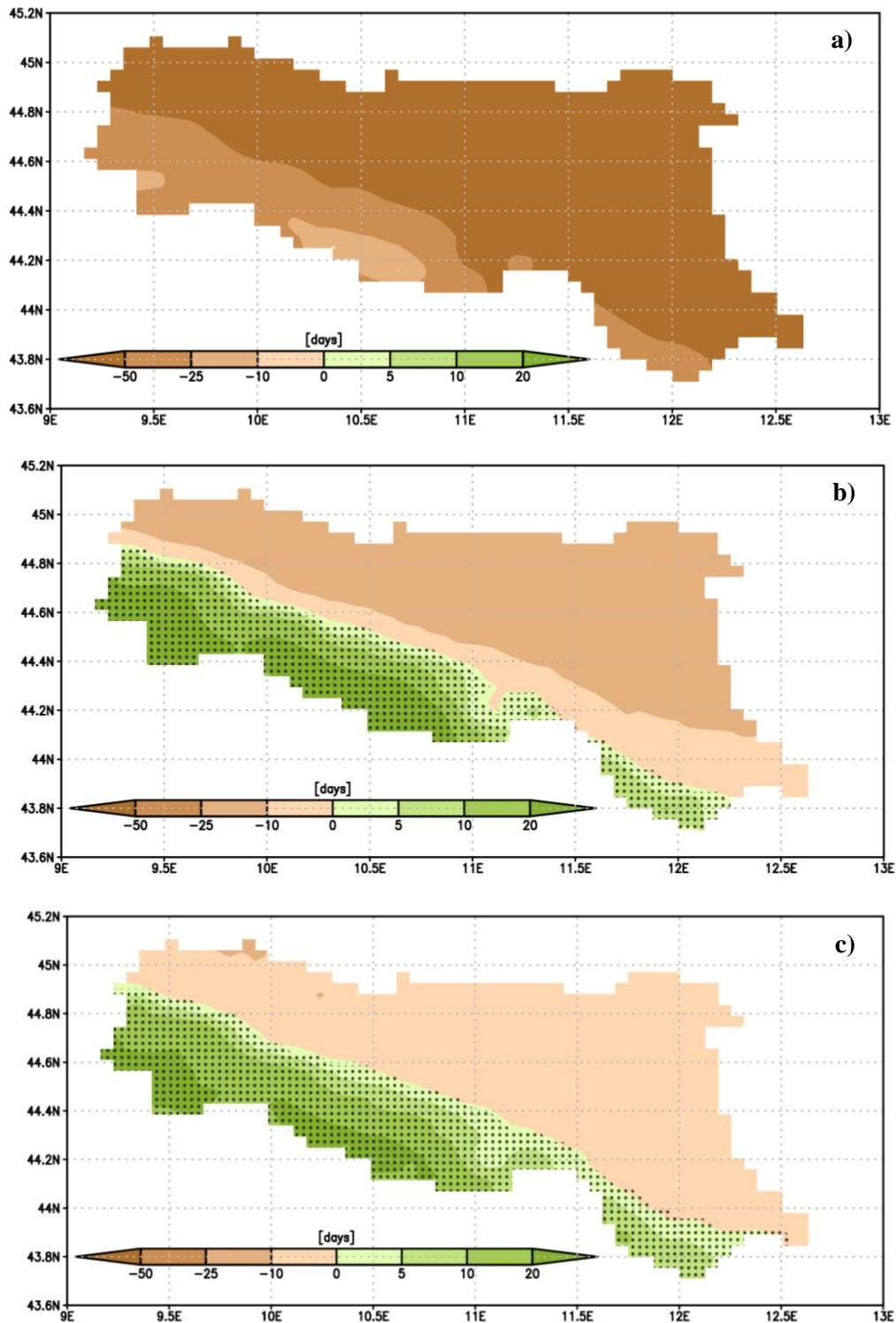
Figure 3.7 Difference in number of days during growing season with maximum temperature in the range 25–30°C between periods a) 1961–1990 vs 2011–2040 under RCP 8.5 scenario b) 1961–1990 vs 2071–2100 under RCP 8.5 scenario c) 1961–1990 vs 2071–2100 under RCP 4.5 scenario, in the Emilia-Romagna calculated as median of 9 models. Dotted areas are statistically significant (p < 0.05) according to t-test.
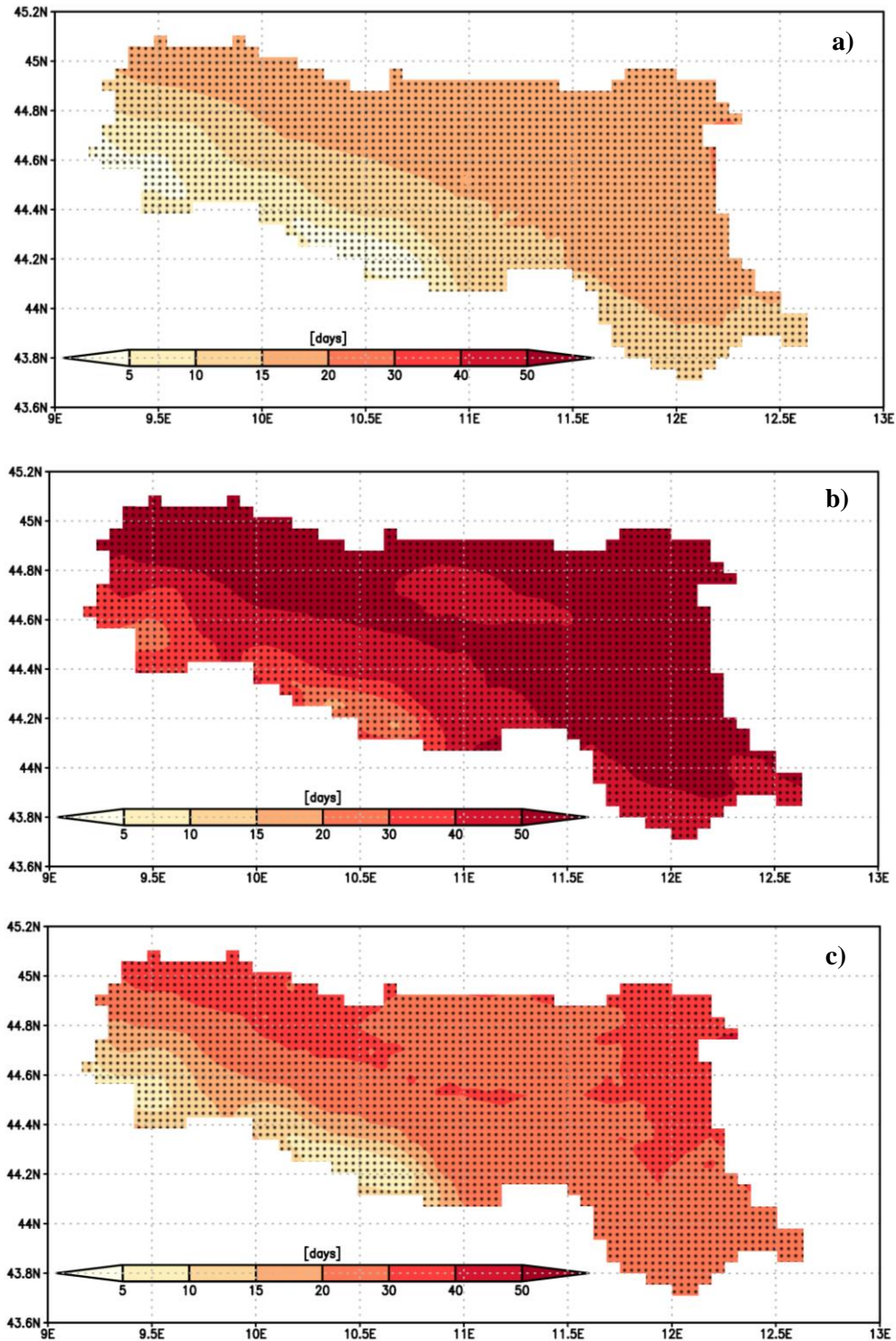
Figure 3.8 Difference in number of days during growing season with maximum temperature above 30°C between periods a) 1961–1990 vs 2011–2040 under RCP 8.5 scenario b) 1961–1990 vs 2071–2100 under RCP 8.5 scenario c) 1961–1990 vs 2071–2100 under RCP 4.5 scenario, in the Emilia-Romagna calculated as median of 9 models. Dotted areas are statistically significant (p < 0.05) according to t-test

In comparison with the period 1961–1990, number of days with maximum temperature above 30°C during growing season (ND > 30°C) could significantly increase over entire ER region in all studied periods (2018–2027 [data not shown], 2011–2041 and 2071–2100) under both RCP scenarios (*Fig. 3.6b; Fig. 3.8a; Fig. 3.8b; Fig 3.8c)*, which could negatively influence the grape quality (see 2.3). While in the closer future ND > 30°C in the ER according to RCP scenarios doesn't differ to a large extent (e.g. differences between 1961–1990 vs 2011–2040 under RCP 4.5 and 1961–1990 vs 2011–2040 under RCP 8.5), until the end of the 21st century ND > 30°C will be notably higher during the period 2071–2100 under RCP 8.5 scenario comparing to the period 2071–2100 under RCP 4.5 scenario *(Fig 3.8b; Fig 3.8c)*, suggesting that development of wine industry will also depend on external factors (reduction of greenhouse emissions into atmosphere).

Night temperatures over many DOP zones in the ER during the period 1961–1990 *(Fig 3.9)* same as during the period 1986–2015 *(Table 2.2)* were characterized as 'cool nights' (for classification definition see *Table 2.1*).
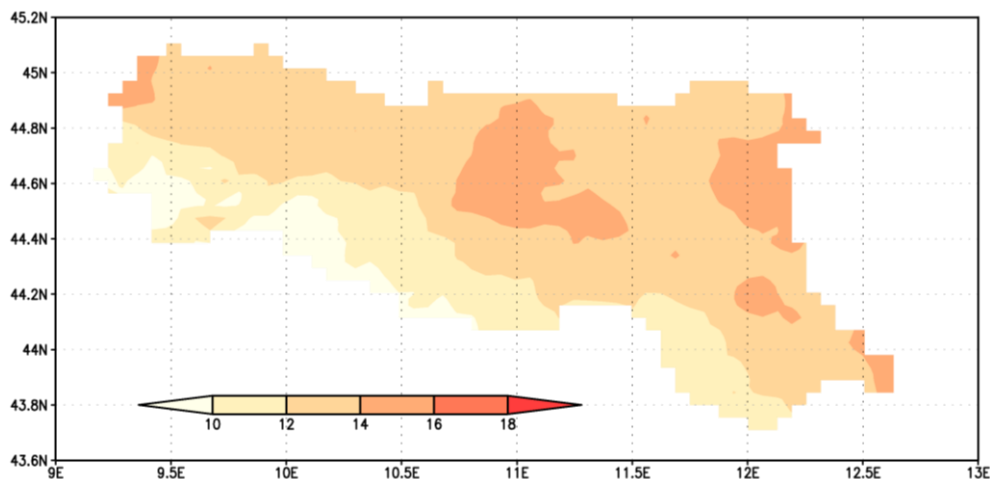
Figure 3.9 Average Cool Night Index for the period 1961–1990 in the Emilia-Romagna, calculated with historical data previously described in Chapter 2 (see 2.2.2).

However due to potential temperature increase during the following decades (2018–2027 [data not shown] and 2011–2041) according to both RCP scenarios (RCP 4.5 [data not shown] and RCP 8.5) night temperatures could be characterized 'temperate nights' over many DOP zones in the ER *(Fig. 3.10a)*. Until the end of the 21st century night temperatures could be even characterized even as 'warm nights' in certain DOP zones which will depend whether RCP 8.5 or RCP 4.5 scenario occur in the future *(Fig. 3.10b; Fig. 3.10c)*. Therefore, results are suggesting that in certain DOP zones night temperatures might exceed by far optimal conditions for anthocyanins synthesis which is in the range 10–15°**(Kliewer, 1977; Tonietto and Carbonneau, 1998)**. Thus, production of red grape varieties in those areas would be questionable.
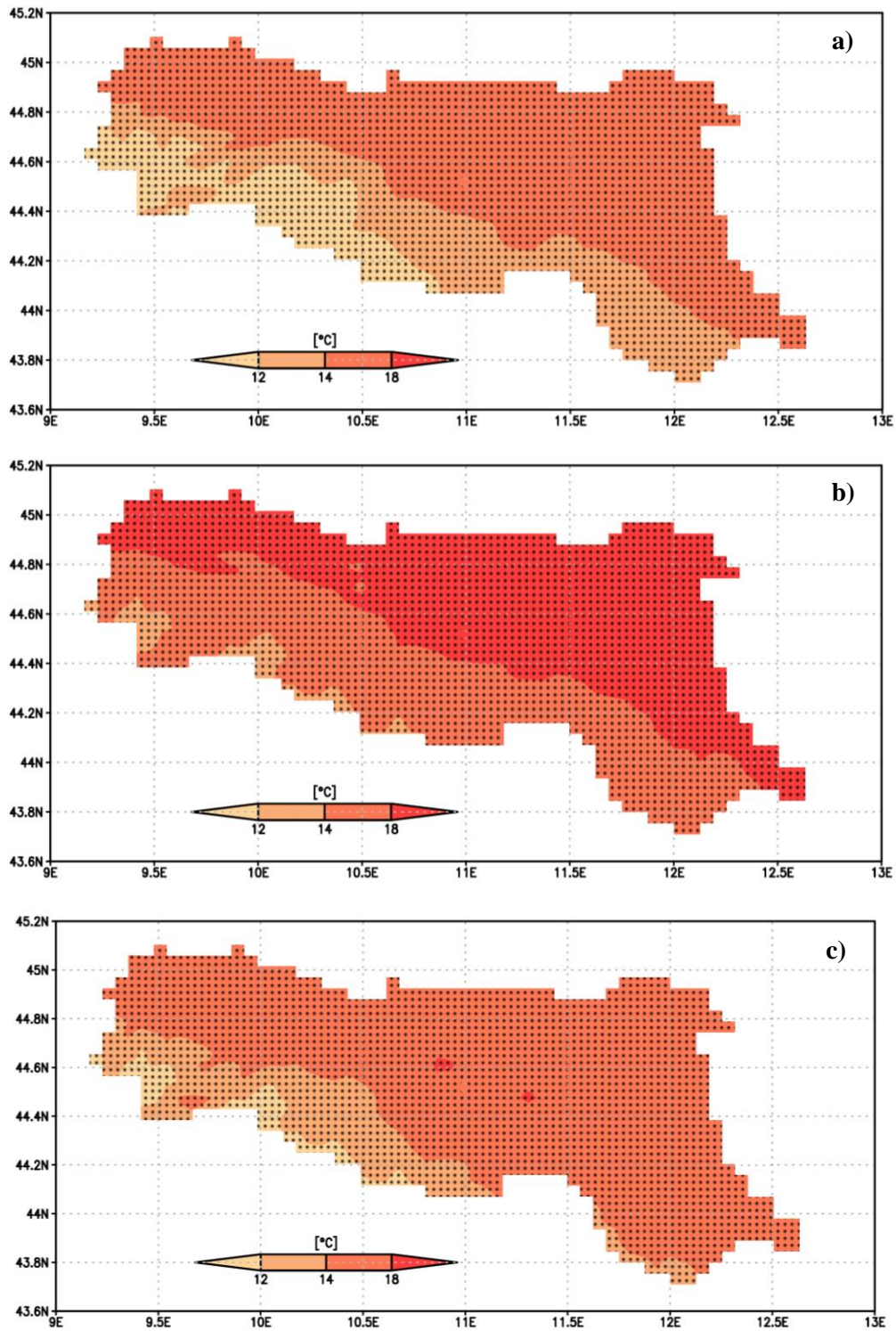
Figure 3.10 Cool Night Index in the Emilia-Romagna for the periods a) 2011–2040 under RCP 8.5 scenario b) 2071–2100 under RCP 8.5 scenario and c) 2071–2100 under RCP 4.5 scenario, calculated as median of 9 models. Dotted areas are statistically significant (p < 0.05) according to t-test.

According to Huglin classification (for classification definition see *Table 2.1*) most DOP zones in the ER were characterized as 'temperate/warm temperate' during the period 1961–1990 *(Fig. 3.11)*. However, due to a temperature increase during the period 1986–2015 majority of DOP zones were characterized as 'temperate warm/warm' **(Teslić et al., 2017)**.

Obtained results from present study are suggesting that according to both RCP (RCP 4.5 [data not shown] and RCP 8.5) scenarios until the 2040 (periods 2018–2027 [data not shown] and 2011–2040) the majority of DOP zones may be still characterized as 'temperate warm/warm' *(Fig 3.12a)*. Thus, strictly according to Huglin classification the ER would be suitable for cultivation of high-quality grapes until 2040. However, according to both RCP scenarios until the end of the 21st century many DOP zones will be characterized as 'warm' *(Fig. 3.12b; Fig. 3.12c)*. Therefore, according to Huglin classification, implementation of certain adjustments (planting new grape varieties) would be necessary to produce high-quality grapes in the DOP zones of the ER, if even possible, especially in case of RCP 8.5 scenario.
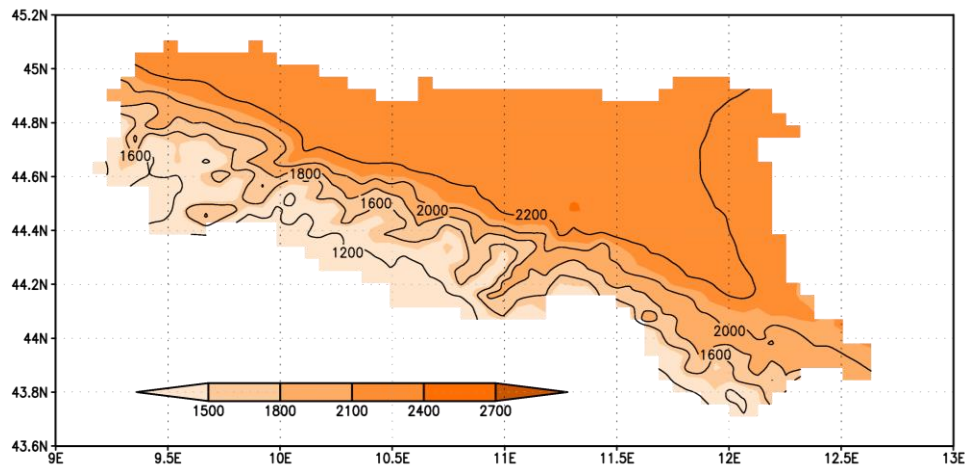
Figure 3.11 Average Huglin Index for the period 1961–1990 in the Emilia-Romagna, calculated with historical data previously described in Chapter 2 (see 2.2.2).
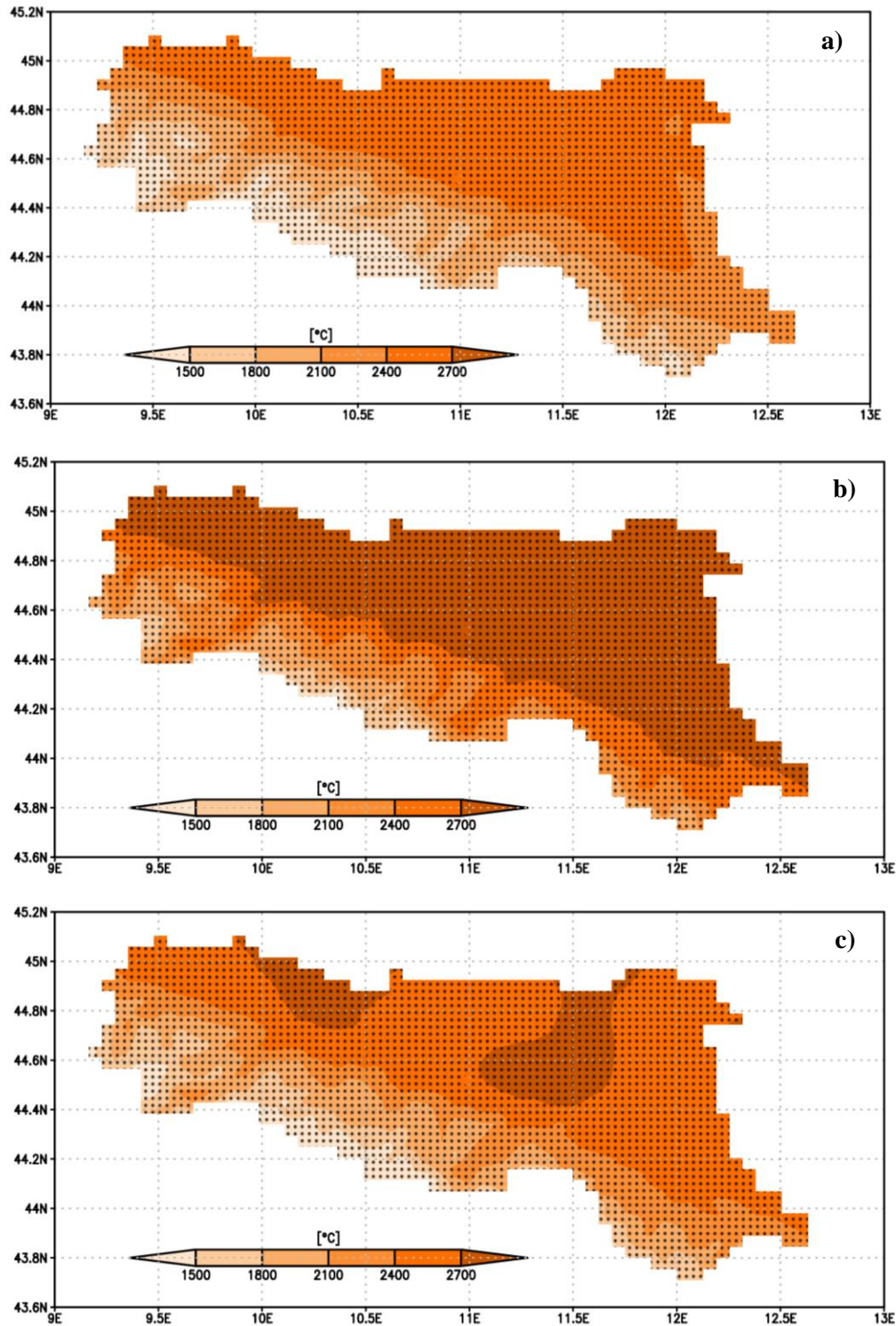
Figure 3.12 Huglin Index in the Emilia-Romagna for the periods a) 2011–2040 under RCP 8.5 scenario b) 2071–2100 under RCP 8.5 scenario and c) 2071–2100 under RCP 4.5 scenario, calculated as median of 9 models. Dotted areas are statistically significant (p < 0.05) according to t-test.

Due to temperature rising growing season thermal accumulation expressed as Growing Degree Day (GDD), increased significantly during the studied periods 2018–2027 (data not shown) and 2011–2040 comparing to the period 1961–1990 *(Fig. 3.13; Fig. 3.14a)*. Increase of GDD occurred according to both RCP scenarios (RCP 4.5 [data not shown] and RCP 8.5). Similarly to HI, warming resulted in classifications shifts of DOP zones, whereas during the period 1961–1990 the majority of DOP zones in the ER were characterized as 'Region 2/Region 3' according to Winker classification (for classification definition see *Table 2.1*) while during the studied periods 2018–2027 and 2011–2040 (under both RCP scenarios) those zones were characterized as 'Region 3/Region 4'. Furthermore, a recent study reported that DOP zones in the ER were characterized as 'Region 3/Region 4' during the 1986–2015 **(Teslić et al., 2017)**, suggesting that only slight warming could occur until 2040 comparing to nowadays conditions.
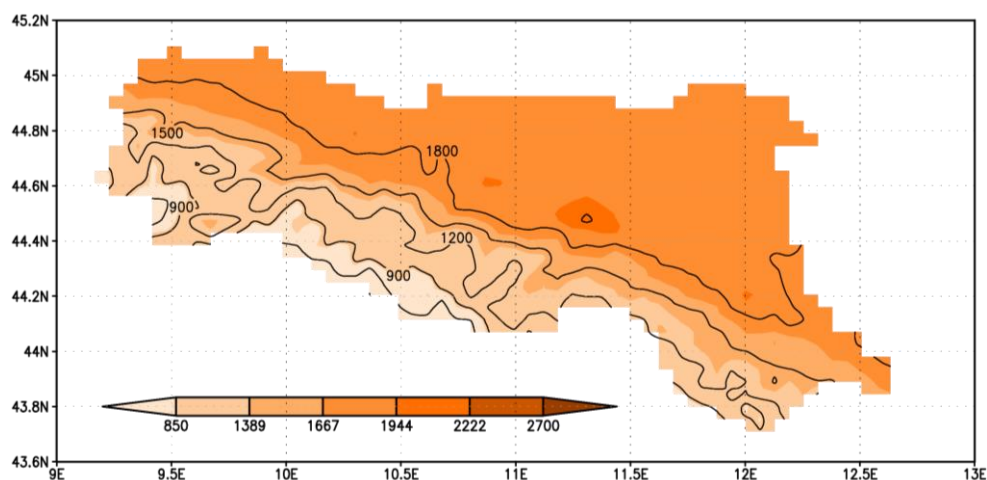
Figure 3.13 Average Growing Degree Day Index for the period 1961–1990 in the Emilia-Romagna, calculated with historical data previously described in Chapter 2 (see 2.2.2).
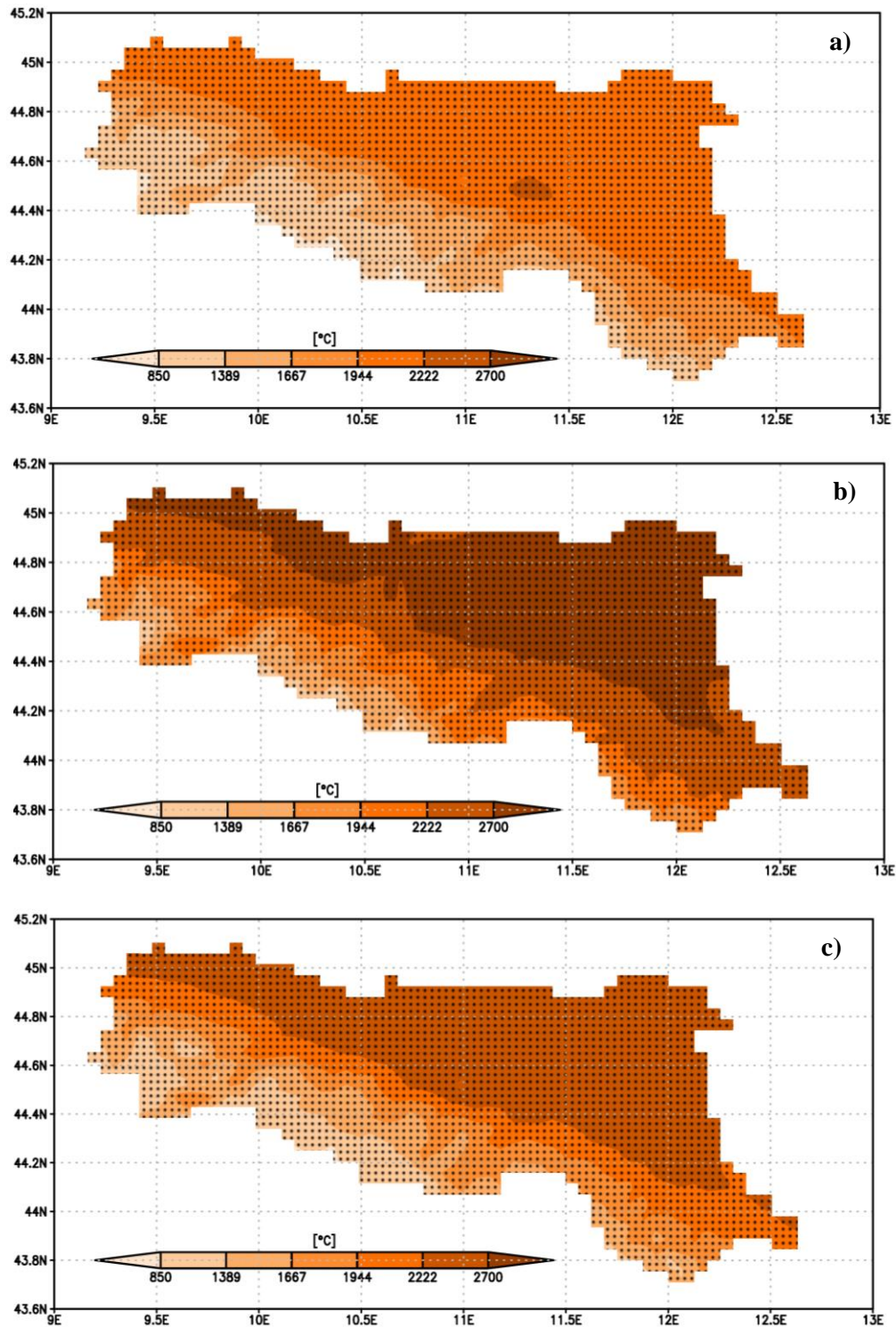
Figure 3.14 Growing Degree Day Index in the Emilia-Romagna for the periods a) 2011–2040 under RCP 8.5 scenario b) 2071–2100 under RCP 8.5 scenario and c) 2071–2100 under RCP 4.5 scenario, calculated as median of 9 models. Dotted areas are statistically significant (p < 0.05) according to t-test.

However, further warming towards the end of the 21st century (2071–2100) will cause that many DOP zones in the ER is going to be classified as 'Region 4/Region 5' if RCP 4.5 scenario occur or even 'Region 5/Too hot' if RCP 8.5 scenario occur *(Fig. 3.14b; Fig. 3.14c)*. Thus, high-quality grape production will be questionable, especially in the case of RCP 8.5 scenario.

During the period 1961–1990 in the ER, growing season precipitation varied from approximately 400 to 550 mm in most of the vineyard area *(Fig. 3.15)*.
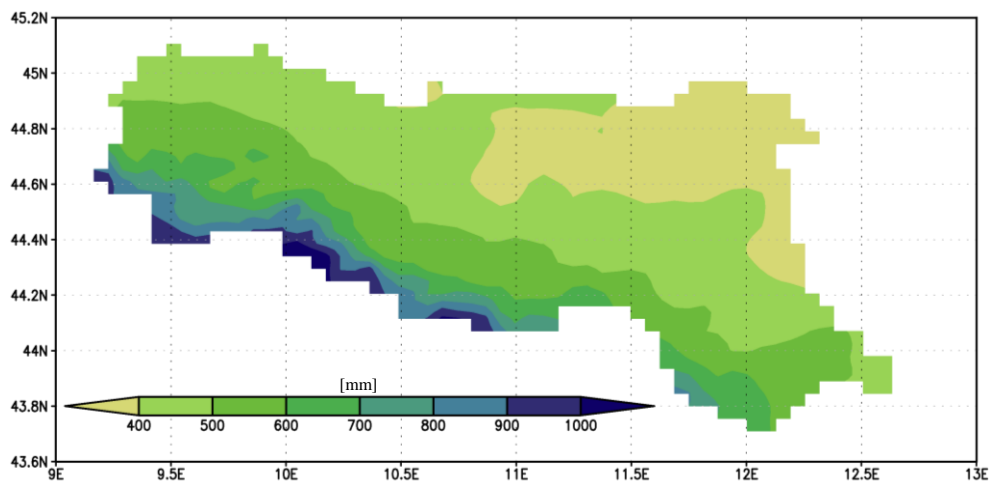
Figure 3.15 Total precipitation for the period 1961–1990 in the Emilia-Romagna, calculated with historical data previously described in Chapter 2 (see 2.2.2).

According to RCP 4.5 scenario total precipitation could decrease in the majority of DOP vineyard zones during the all studied periods (2018–2027, 2011–2040 and 2071–2100) comparing to the period 1961–1990 *(Fig. 3.16a; Fig. 3.16b; Fig. 3.16c)*. While, in certain central and northwestern vineyard areas, same as areas of Po River Delta could increase during the 2018–2027 and 2011–2040 *(Fig. 3.16a; Fig. 3.16b)*. Interestingly, according to RCP 8.5 scenario noticeably larger surface of DOP zones could have more growing season total precipitation during the periods 2018–2027 and 2011–2040 comparing to the same periods according to RCP 4.5 scenario *(Fig. 3.16a; Fig. 3.16b; Fig. 3.17a; Fig. 3.17b)*. On the other hand, during the period 2071–2100 growing season total precipitation could be noticeably lower in the most of DOP zones according to RCP 8.5 scenario comparing to the same period under RCP 4.5 *(Fig. 3.16c; Fig. 3.17c)*.
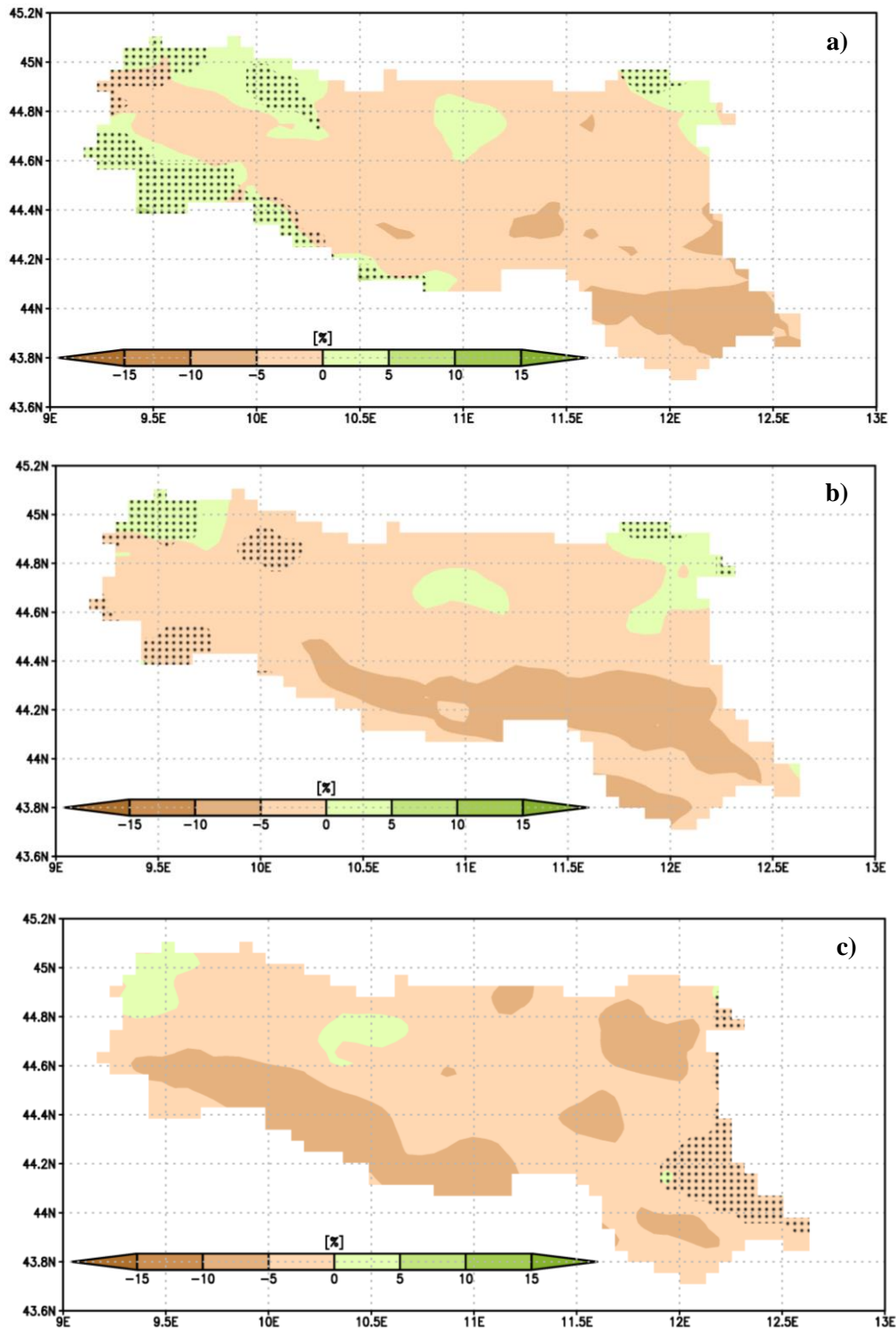
Figure 3.16 Relative difference in total precipitation during growing season between periods a) 1961–1990 vs 2018–2027 b) 1961–1990 vs 2011–2040 c) 1961–1990 vs 2071–2100, under RCP 4.5 scenario in the Emilia-Romagna calculated as median of 9 models. Dotted areas are statistically significant (p < 0.05) according to t-test.
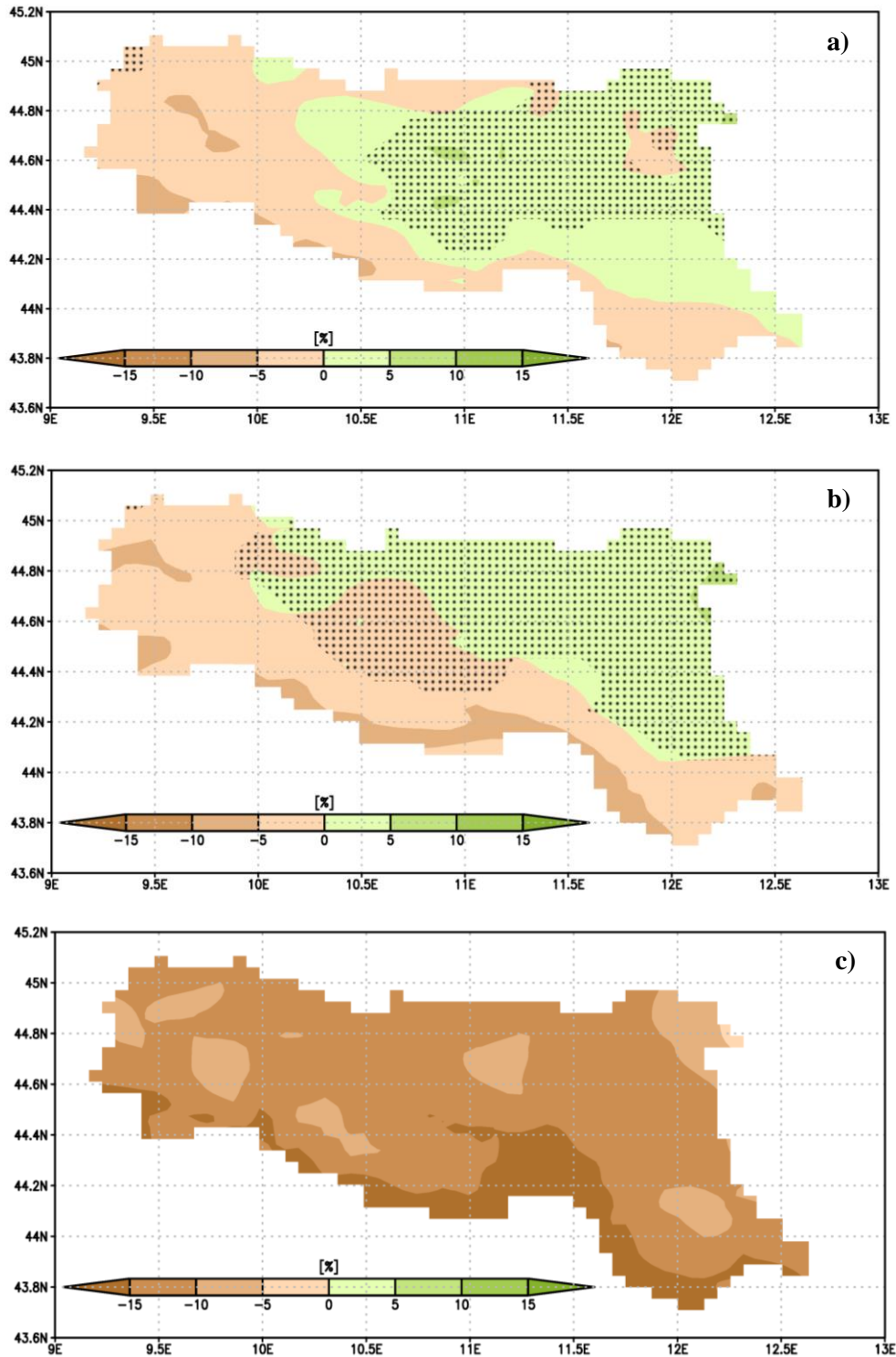
Figure 3.17 Relative difference in total precipitation during growing season between periods a) 1961–1990 vs 2018–2027 b) 1961–1990 vs 2011–2040 c) 1961–1990 vs 2071–2100, under RCP 8.5 scenario in the Emilia-Romagna calculated as median of 9 models. Dotted areas are statistically significant (p < 0.05) according to t-test.

Dry Spell Index (DSI) which was related to increase of grape sugar concentration in Sangiovese wines from 'Romagna Sangiovese' appellation area (see 4.2.2.2) could significantly increase during the all studied periods (2018–2027 [data not shown], 2011–2040 [data not shown] and 2071–2100) under both RCP scenarios *(Fig. 3.19a; Fig. 3.19b)* comparing to the period 1961–1990. The potential increase of DSI could be up to 5 days in most DOP zones during 2018–2027 and 2011–2040 under both RCP scenarios (data not shown). In certain DOP zones DSI could increase up to 10 days during the period 2071–2100 under RCP 4.5 scenario, or even up to 15 days during the same period under RCP 8.5 scenario *(Fig. 3.19a; Fig. 3.19b)*. Thus, obtained results are suggesting that grape sugar concentration might be even higher in the future decades. Normally, grape sugar concentration positively is related to increase DSI up to a certain limit, whereas too long drought periods could have a negative impact on grape quality (if not irrigated).
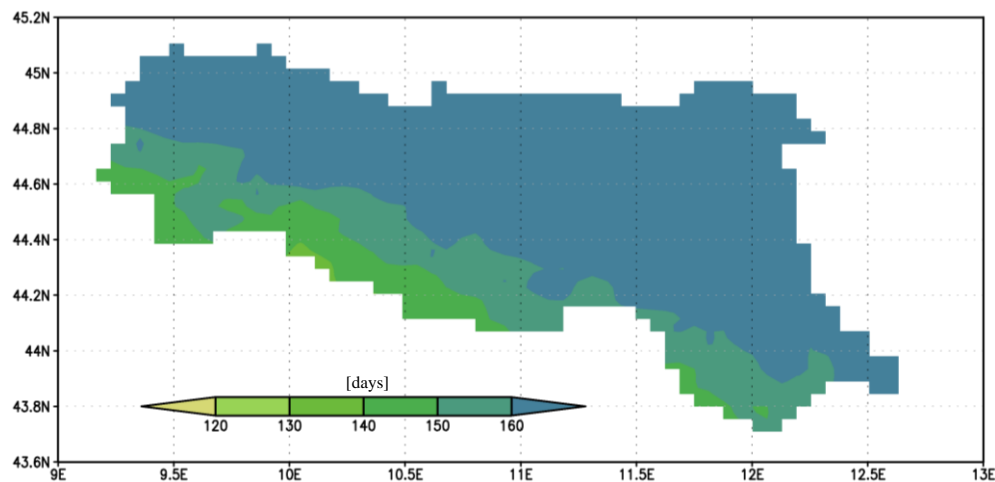
Figure 3.18 Dry Spell Index for the period 1961–1990 in the Emilia-Romagna, calculated with historical data previously described in Chapter 2 (see 2.2.2).
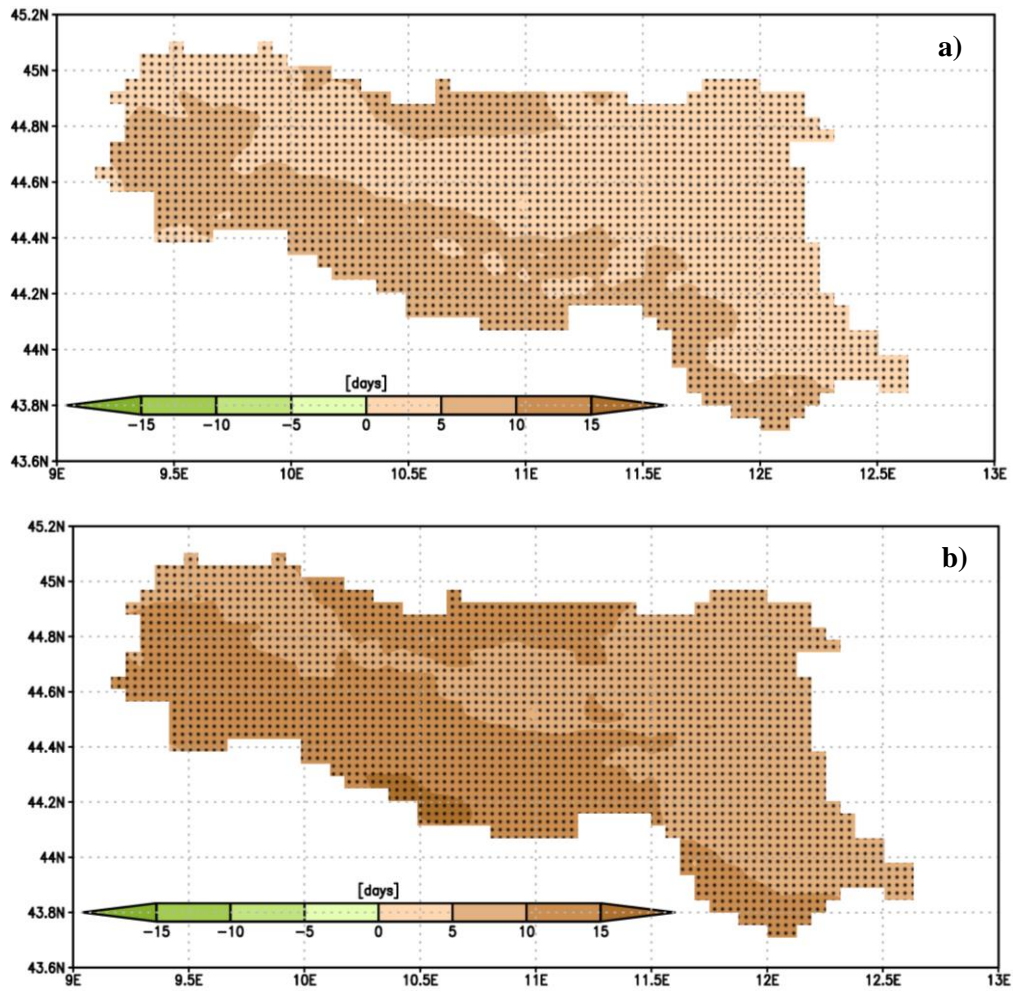
Figure 3.19 Difference in Dry Spell Index during growing season between periods a) 1961–1990 vs 2071– 2100 under RCP 4.5 scenario b) 1961–1990 vs 2071–2100 under RCP 8.5 scenario, in the Emilia-Romagna calculated as median of 9 models. Dotted areas are statistically significant (p < 0.05) according to t-test.

Water availability to vines during the growing season, expressed as Dryness Index (DI) could decrease in the ER during the periods 2018–2027 (data not shown) and 2011–2040 according to both RCP scenarios (RCP 4.5 [data not shown] and RCP 8.5) comparing to the period 1961–1991 *(Fig. 3.21; Fig. 3.22a)*.

Similar results were reported in a recent study whereas DI decreased during the period 1986–2015 comparing to the period 1961–1991 **(Teslić et al., 2017)**. However, even if potential decrease is possible, until 2040 most DOP zones in the ER should be characterized as 'humid/moderately dry' (for classification definition see **Table 2.1**) under both RCP scenarios (RCP 4.5 - data not shown) *(Fig. 3.21a)*.
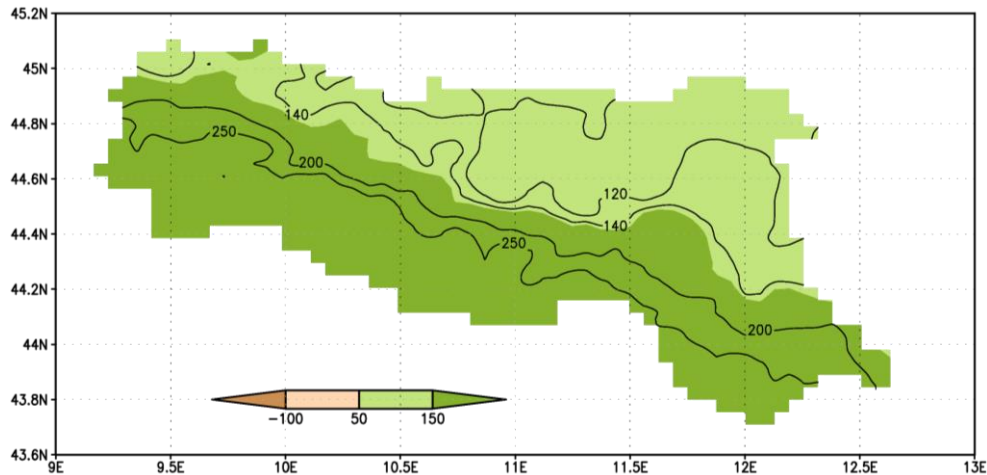
Figure 3.20 Dryness Index for the period 1961–1990 in the Emilia-Romagna, calculated with historical data previously described in Chapter 2 (see 2.2.2).

In this view, the ER should be suitable for production of high-quality grapes/wines until 2040. On the other hand, towards the end of the 21st century, due to precipitation decrease and temperature increase during the period 2071–2100 *(Fig. 3.4c; Fig. 3.5c; Fig. 3.16c; Fig. 3.17c)*, certain DOP zones in the ER could be characterized as 'sub-humid' according to RCP 4.5 scenario *(Fig. 3.21c)*. While during the same period (2071–2100) and according to RCP 8.5 scenario most DOP zones in the ER could be characterized as 'sub-humid' *(Fig. 3.21b)*. Obtained results are suggesting that those zones could potentially need an implementation of irrigation systems.
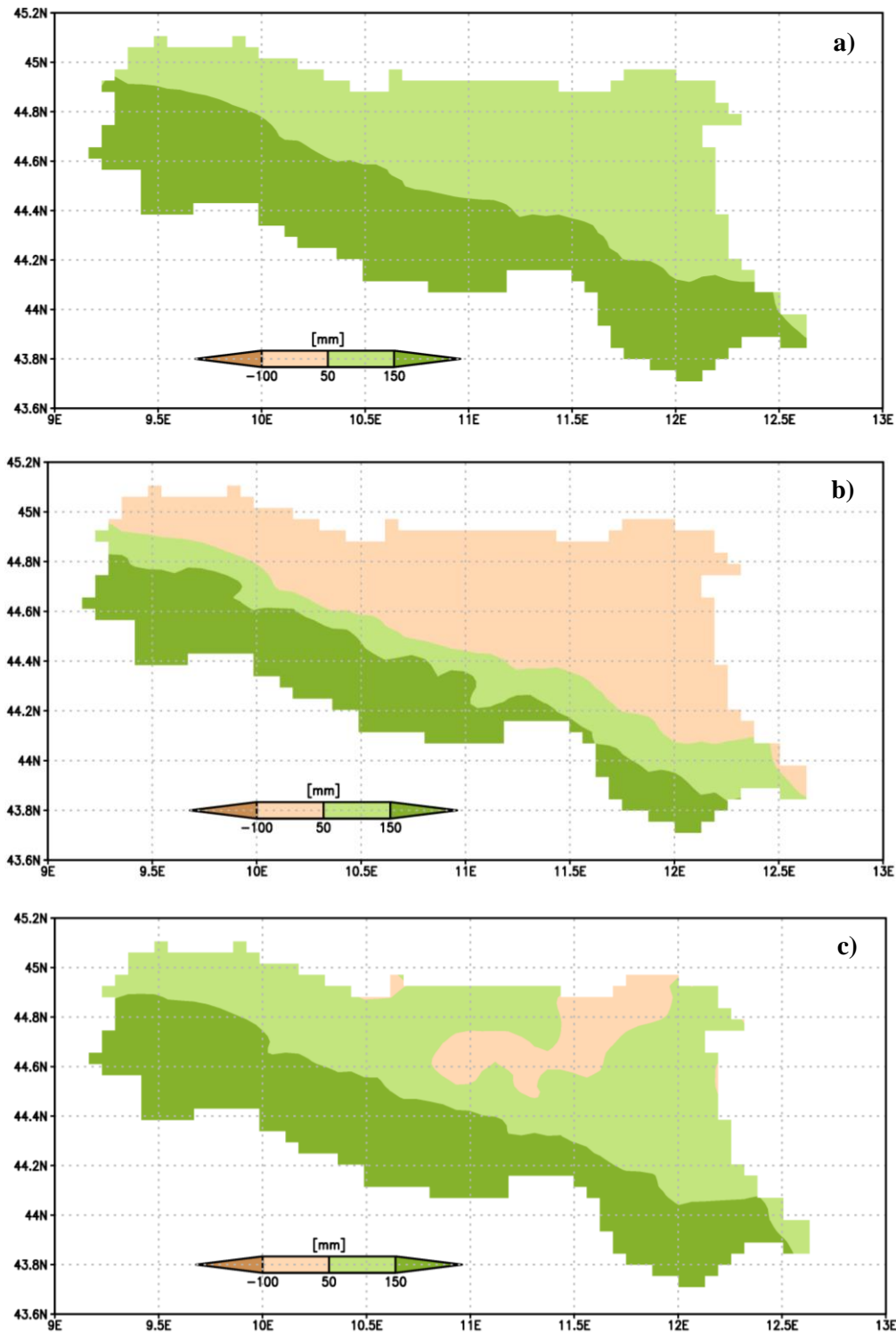
Figure 3.21 Difference in Dryness Index during growing season between periods a) 1961–1990 vs 2011–2040 under RCP 8.5 scenario b) 1961–1990 vs 2071– 2100 under RCP 8.5 scenario c) 1961–1990 vs 2071–2100 under RCP 4.5 scenario, in the Emilia-Romagna calculated as median of 9 models. Dotted areas are statistically significant (p < 0.05) according to t-test.

3.4   Conclusions

Results obtained in the present study are suggesting that weather conditions until 2040 in the DOP zones of the ER could be suitable for production of high-quality grapes. Whereas, comparing to nowadays weather conditions could be slightly hotter and dried under both RCP scenarios (RCP 4.5 and RCP 8.5). However, towards the end of the 21st century, certain DOP zones in the ER could become too hot and noticeably drier, whereas production of high-quality grapes with current technology and grape varieties could be questionable, particularly under RCP 8.5 scenario.

3.5   References

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CHAPTER 4

Influence of climate change on grape quality and quantity

4   Influence of climate change on grape quality and quantity

4.1   Introduction

Wine industry (from production aspect) could roughly be divided into grape production and wine production, whereas the influence of climate change is more directly related to grape production. On the other hand, even though wine production can be directly influenced by climate change (e.g. higher energy utilization for cooling systems), it is mostly indirectly affected through utilization of grapes. Thus, to fully understand the influence of climate change on wine industry it is required to find links between plant development and climatic factors.

4.1.1   Grape phenology

During the vegetative and reproductive cycles, *Vitis vinifera* undergoes diverse physiological and morphological changes. In the vine reproductive cycles, which are separated by dormancy period, there are four principal phenological stages: bud burst, flowering, véraison and maturity. The length and timing of each individual stage are determined by climatic and non-climatic factors. The most important climatic factor is air temperature (**Malheiro et al., 2013**) and certain temperature derived factors, such as thermal accumulation (**Urhausen et al., 2011**). While other climatic factors as precipitation and sunshine duration have often lesser influence on phenological stages (**Tomasi et al., 2011; Urhausen et al., 2013**). From the non-climatic factors, grape variety has a key role in vine development and length of phenological stages (**de Cortázar-Atauri et al., 2009; Jones and Davis, 2000; Tomasi et al., 2011**).

Bud break represents the beginning of growth cycle (**de Cortázar-Atauri et al., 2009**), which depending on vineyard location and variety occurs from February to April in the Northern Hemisphere (**Malheiro et al., 2013; Urhausen et al., 2011**) or from August to October in the Southern Hemisphere (**Hall et al., 2016**). Growth cycle or bud burst period starts with bud swelling (0 BBCH scale; for BBCH scale see Appendix D) and continues with gradual bud development, advances with full bud burst (green shoot tips are clearly visible on 50 % of buds, 8 BBCH scale) and last until the forming of the first leaves (11 BBCH scale). In literature, for easier comparison between different wine regions, it is often assumed and adopted that period of a few successive days with daily temperature of 10°C presents a minimum threshold required for bud burst initiation (**Winkler et al., 1974**), however minimum threshold is strongly determined by variety (**de Cortázar-Atauri et al., 2009**). Anyhow, air temperature or temperature derived factors (e.g. thermal accumulation) have a key role in bud burst initiation, thus warming and climate change could influence the beginning of growth cycle to a great extent. In fact, Urhausen et al. (2011) investigated phenology of *cv. Riesling* in Luxembourg during the period 1951–2005, and reported earlier bud burst occurrence under warmer conditions by two weeks. Another study investigated

phenology of several autochthonous grape varieties in Portuguese wine region during the 20 years period, and detected bud break precocity under higher temperatures by a week. However, earlier bud break was reported for only one grape variety, which suggested the importance of grape variety on the final outcome **(Malheiro et al., 2013)**. In certain cases, a significant warming period doesn't necessary involves earlier bud burst dates which was confirmed in Italian wine region Veneto. Whereas significant growing season temperature increases (2.3°C) were reported during the long-term period (1964–2009), however without earlier significant bud burst occurrence for all 18 varieties **(Tomasi et al., 2011)**. Further anticipation of bud burst timing under warmer conditions is also expected in the future decades (2041–2070), whereas depending on location, bud burst could occur up to 30 days earlier **(Fraga et al., 2016)**.

The period between bud burst and flowering (09–52 BBCH scale) are followed with accelerated expansion of leaf area. Apart from an earlier occurrence of phenological stages, even period between two events (e.g. bud burst to flowering) could be shorter as it was reported in Italian wine region **(Tomasi et al., 2011)**. Flowering period is the second phenological stage of vine reproductive cycle that starts with first clearly visible inflorescences (53 BBCH scale), advances with full flowering stage (50% of fallen flowerhoods; 65 BBCH scale) and finishes few days prior to fruit set (69 BBCH scale). In the Northern Hemisphere, full blooming occurs from the middle of May to the end of June **(Jones and Davis, 2000; Ramos et al., 2015; Tomasi et al., 2011)**, while in the Southern Hemisphere from the middle of October to the middle of December **(Fraga, 2014)**. Similarly to bud burst, air temperature has a paramount impact on triggering of full flowering. In particular, in Germany during long-term period (1968–2010), maximum air temperature increase (1°C) resulted in an advance of full flowering dates for approximately 6 days **(Bock et al., 2011)**. Advancing of flowering timing in the magnitude of 23 days was also reported in France during the second half of the 20th century **(Duchêne and Schneider, 2005)**, indicating a further earlier occurrence of blooming in the future decades, which was suggested by other study as well **(Fraga et al., 2016)**.

Weeks after full blooming (65 BBCH scale) and prior to full véraison (83 BBCH scale) are characterized by falling of remaining flowerhoods, fruit setting and gradual development of grape berries which is followed by synthesis and accumulation of sugars, acids, phenolic compound, aroma precursors etc. The length of the period between flowering and véraison is strongly determined by climatic factors. In particular warming from 1970 to 1997 in the Bordeaux (France) resulted in decrease duration of the period for 4 days **(Jones and Davis, 2000)**. Apart from temperature a grape variety is also essential for the length of this period. These conclusions were reported in a recent study, whereas in the Dois Portos (Portugal), higher temperatures caused a significant reduction in the length of the period between from blooming and véraison for two autochthon grape varieties which was not the case for other two grape varieties **(Malheiro et al., 2013)**. Véraison is the third phonological stage of vine growing cycle which starts with the first occurring of colored berries (81 BBCH scale), continues with full véraison (50% of colored berries; 83 BBCH scale) and finishes when all berries have characteristic variety color (85 BBCH scale). This phenological phase occurs from the end of July to the beginning of September in the Northern Hemisphere **(Jones and Davis, 2000; Tomasi et al., 2011; Urhausen et al., 2011)**, and from the middle of December to the middle of February in the Southern Hemisphere **(Fraga, 2014)**. As for all phenological stages, temperature and heat accumulation influence the occurrence of full véraison to large extent. The impact of temperature was reported by Tomasi et al. (2011), whereas an earlier occurrence of véraison was detected in Italy (13 days). These trends of earlier occurrence of véraison are also expected

to continue in the future decades up to 30 days in Spain and Italy **(Fraga et al., 2016)**, indicating the necessity to develop adaptation to these changes.

The period between full véraison (83 BBCH) and full maturity or harvest timing (89 BBCH) is followed by further accumulation of some grape berry compounds (e.g. sugars) and degradation of certain compounds (e.g. malic acid, aromatic precursors, see 4.1.2–4.1.5). Duration of this period depends on air temperature **(Malheiro et al., 2013)**, which increase in not necessarily sufficient for the period shortening **(Bock et al., 2011)**. The last stage of the berry growth cycles finishes with full maturity (89 BBCH) when grapes have reached technological maturity (optimal ratio between sugars and acids) and/or phenological maturity (developed secondary metabolites, e.g. phenolic compounds, aroma precursors). The harvest of fully mature grapes occurs from the middle of September to the beginning of November in the Northern Hemisphere **(Bock et al., 2011; Jones and Davis, 2000; Malheiro et al., 2013; Tomasi et al., 2011)** and from the middle of February to the middle of April in the Southern Hemisphere **(Fraga, 2014)**. Similarly, to other phenological stages, air temperature is the major climate factor which determines harvest date. This was clearly concluded in many studies which reported that increase of temperatures caused earlier occurrence of grape maturity in Italy **(Tomasi et al., 2011)**, Portugal **(Malheiro et al., 2013)**, France **(Jones and Davis, 2000)**, Slovenia **(Vršič et al., 2014)**, Germany **(Bock et al., 2011)**, Australia **(Petrie and Sadras 2008)**, Slovakia **(Jones et al., 2005)**, Spain **(Ramos et al., 2008)** etc. However, some studies reported lack to advanced maturity even if significant temperature increase was detected, indicating the importance of other factors as well (e.g. grape variety) **(Duchêne and Schneider, 2005; Jones et al., 2005; Malheiro et al., 2013)**. Due to most likely warming in the future decades earlier occurrence of harvest dates is expected to be continued, in the magnitude up to from 30 to 40 days in some parts of Spain, Italy, France, Greece **(Fraga et al., 2016)** etc.

4.1.2   Grape sugars

Vitis vinifera during the process of photosynthesis produces carbohydrates (sugars) which are natural reservoirs of energy required for plant development. Sugars are synthesized in leaves as sucrose, translocated via phloem to fruits **(Swanson and El - Shishiny, 1958)** and cleaved into hexoses by enzymatic activity of invertase for further utilization or storage in vacuoles **(Davies et al., 2012)**. Sugar concentration in berries, apart from obvious influence on sweetness of grapes/wines and alcohol level of wines, through genes expression and regulation can also influence secondary metabolites assimilation **(Davies et al., 2012)**. The influence of climate change on higher sugar content in grape berry and elevated ethanol content in wines could be cause by several factors such as atmospheric CO_2 concentration, higher temperatures and moderate water stress. In particular, temperature increase and elevated air CO_2 concentration (up to 30°C and 800 ppm CO_2, respectively) **(Greer and Weedon, 2012; Long et al., 2004)**, may enhance photosynthetic process and hasten pace of phenological events **(Duchêne and Schneider, 2005; Jones, 2012)**. This accelerated pace of phenological events causes faster sugar accumulation since their synthesis is preferential comparing to the synthesis path of secondary metabolites, such as anthocyanins **(Martínez-Lüscher et al., 2016)**. Furthermore, hasten pace of phenological stages is causing grapes to arrive earlier at technological maturity (optimum ratio between grape sugar content and acidity), while aroma and phenolic compounds remain undeveloped. On the other hand, if grape growers leave bunches to hang on vines and wait for aroma and phenolic compounds to develop, acidity values may reach level below optimum due to the respiration and malic acid degradation,

while sugar content reaches a higher than optimum level (**Jones, 2012**), which will finally cause production of unbalanced wines. The influence of air temperature on sugar content in berries was reported in the Upper Moselle wine region (Luxembourg), whereas in the period from 1965 to 2005 *cv. Riesling* must density increased for 0.3±0.2°Oe. Furthermore, in the same wine region similar observations were detected for another six grape varieties (e.g. *cv. Traminer, cv. Pinot Blanc* etc.) (**Urhausen et al., 2011**). Similar findings were also reported in the Lower Franconia (Germany), whereas during the 61 years period (1950–2010) grape sugar content increased for 2.4°Oe per decade (**Bock et al., 2011**). In another study, authors speculated that increasing warmth Alsace (France) could also cause production of grapes with elevated sugar content (**Duchêne et al., 2010**), confirming that temperature plays an important role on berry sugar content at the harvest. However, sugar content increase with increasing temperature trends during the relatively long period (few decades) is not a 'thumb rule'. Jones and Davis (2000) reported lack of sugar content increasing trends for *cv. Cabernet Sauvignon* and *cv. Merlot* in the Bordeaux (France), even if increasing temperatures were detected during the 28-year period (1970–1997). Apart from temperatures, moderate water stress may also accelerate sugar accumulation in berry as a result of inhibiting lateral shoot growth allowing transportation of carbohydrates to berries or as a direct effect of grapevine hormones (abscisic acid) activation during maturity process (**Coombe, 1989**). In this view, Poni et al. (2007) conducted partial root-zone drying on potted Sangiovese grapevine, simulating dry and wet conditions, whereas at harvest, vines submitted to dry conditions showed higher total soluble solids respect to the vines cultivated under wet conditions.

4.1.3   Grape acids

Grape berry contain a high number organic acids (**Kliewer, 1966**), whereas tartaric and malic acids are by far the predominant acids (over 90% of total berry acids) while other acids (e.g. citric, succinic, ascorbic etc.) are present in small concentrations (**Ford, 2012**). L-tartaric acid is stereoisomer naturally found in grape berries which synthesis occurs at the earliest stages of berry development and last until 40–50 days after blooming. Once synthetized, L-tartaric acid is accumulated in the berry vacuoles which concentration is generally constant (**Ford, 2012**). However, Ford (2012) suggested possible decrease of tartaric acid concentration in berries when exposed to high day temperatures (40°C) and night temperatures (30°C) during several days. Same as for tartaric acid, L-malic acid is naturally occurring stereoisomer in grape berries. The synthesis of malic acid in berries occurs in initial stages of berry development, primarily via β-carboxylation of phospho-enol-pyruvate to oxaloacetic acid, which is catalyzed by cytoplasmic enzyme phospho-enol-pyruvate carboxylase. Afterwards, oxaloacetic acid is used for malate synthesis which is catalyzed by malate dehydrogenase (**Ford, 2012**). Malic acid formed in early stages of berry development and peaking prior to véraison (**Ryona et al., 2008**). Malic acid is party degraded as berry ripening advances due to respiration and malic acid degradation (**Lakso and Kliewer, 1975**). The degradation of malic acid is strongly regulated by temperature, whereas at temperatures higher than 35°C have negative effect on malic acid concentration in berries (and wine total acidity afterwards) due inactivation of synthetic enzymes (**Lakso and Kliewer, 1975**). The impact of increasing temperatures on must acidity in general was reported in the Luxembourg, whereas from 1965 to 2005 a negative trend of must acidity was detected for *cv. Riesling* (~0.1 g/L) (**Urhausen et al., 2011**). Similar finding were reported in the France, whereas decreasing trends of wine acidity under warmer conditions were detected in the period 1970–1997 for *cv. Merlot* and *cv. Cabernet Sauvignon* (**Jones and Davis, 2000**). Recent study reported that apart from elevated temperatures, a combination of higher

temperatures with elevated air CO_2 concentration also have a negative effect on must acidity (**Martínez-Lüscher et al., 2016**). Lower water availability during dry vintages may also decrease wine acidity as it was reported in recent study (**Vršič et al. 2014**).

4.1.4   Grape aromatic compounds and aroma precursors

Wine headspace is very rich in aromas and contain from one to several hundred of aromatic compounds. Part of these compounds is released from grape aroma precursors via chemical and biochemical reactions during fermentation and wine ageing. The grape aroma precursors are primarily present in their non-volatile form while volatile form occurs rarely (**Darriet et al., 2012**). The synthesis and accumulation of grape aromatic precursors takes place in grape berry and for certain compounds (e.g. 3-Isobutyl-2-methoxypyrazine; IBMP) starts with fruit set, peaks prior to véraison and degrade as ripening advance (**Ryona et al., 2008**). Climate factors play an important role in final concentration of aromatic precursors in grape berries. In particular, IBMP present in *Cabernet Franc, Cabernet Sauvignon, Sauvignon blanc, Semillon* etc., contributing to 'bell pepper' sensation degrade at higher amount during vintages with higher temperatures (**Allen and Lacey, 1993**). Recent study reported that concentration of rotundone related to 'black pepper' sensation which is present in *cv. Syrah*, is negatively correlated with higher grape bunch zone temperatures (**Zhang et al., 2015**). Excessive berry temperatures are also related to lower concentration of terpenols (e.g. linanol, nerol, geraniol) in *cv.s Moscatel de Alejandria* and *Moscatel rosada* (**Belancic et al., 1997**). Vršić et al. (2014) reported that lower water availability during dry years may also cause reduction of aromatic compounds, confirming significance of the diverse climatic factors on final concentration of grape aroma precursors.

4.1.5   Grape phenolic compounds

The grape berries contain vast number of different phenolic compound which are important determinant of wine quality (**Castellarin et al., 2012**). The biosynthesis of all phenol compounds starts with production of phenylalanine amino acid via shikimate pathway, which links the synthesis of secondary metabolites (e.g. aromatic amino acids, phenols) with carbohydrate metabolism (**Castellarin et al., 2012**). Afterwards, formed phenylalanine is utilized for synthesis of phenolic compounds via phenylpropanoid, flavonoid and stilbenes pathways (**Sparvoli et al., 1994**). As for all components in grape berry, concentration of phenolic compounds if affected by climatic factors thus climate change may play an important role. In particular, water deficit resulted in increase of stilbene accumulation in *cv. Cabernet Sauvignon* berries which was absent in *cv. Chardonnay* berries, indicating also importance of varietal factor on final concentration of phenolic compounds (**Deluc et al., 2011**). Other studies reported negative impact excessive temperatures (>35°C) on anthocyanins concentration in *cv. Cabernet Sauvignon* (**Mori et al., 2007**), *cv. Pinot noir* (**Mori et al., 2007**), or *cv. Aki Queen* grapes (**Yamane et al., 2006**), due to inhibition of anthocyanin synthesis and degradation of existing anthocyanins (**Castellarin et al., 2012**). Furthermore, concentration of skin proanthocyanidins in *cv. Merlot* is also strongly related to heat summations, whereas both excessively lower and higher temperature decrease skin proanthocyanidins content at harvest (**Cohen et al., 2008**). UV-B radiation is another important factor for phenolic concentration which is not consequence of the climate change, but indirect cause of higher temperatures

and longer droughts. Higher UV-B radiation may promote stilbenes **(Versari et al. 2001)**, flavonol and anthocyanins synthesis **(Martínez-Lüscher et al. 2016)**.

4.1.6   Grape yield

Grape yield is also highly correlated to climatic factors thus climate change has a strong impact on crop load as well. Apart from climate factors alone, non-climatic factors such as soil fertility, air CO_2 etc. play an important role in determination of crop yield at harvest. For example, warming in Spanish region Rı´as Baixas had a positive correlation with grape production during the period 1987–2005 **(Lorenzo et al., 2013)**. Reversely, in another Spanish wine region warming resulted decrease of the grape yield during the period 1952–2006 **(Ramos et al., 2008)**. Ramos et al. (2008) also reported that precipitation reduction from blooming to véraison caused grape yield reduction. This may be explained with rapid cell division and reduced berry size that occurs with water stress **(Peacock, 2005)**. Another study reported that water deficit during the dry years resulted in in grape yield reduction up to 53% **(Ramos and Martínez-Casasnovas, 2010)**. This negative effect of water stress and excessive heating on the grape yield is however partly compensated with an increase of air CO_2 concentration as it was reported in several studies **(Bindi et al., 1996; Kizildeniz et al., 2015; Moutinho-Pereira et al., 2009; Schultz, 2000)**. As it is clearly evident, the future production of grapes and crop load will depend on combination of climatic (e.g. temperature) and non-climatic (e.g. nitrogen availability) factors, thus some areas such as France and Germany may have higher grape yield in the future decades while certain areas as Spain may have lower grape yield **(Fraga et al., 2016)**.

4.2   Climate change trends, grape sugar content and grape yield of Sangiovese grapes from the Romagna area

In this view, the present experiment aims to establish a relationship, if any, between total soluble solids in Sangiovese grapes and climate change trends evaluated with proper bioclimatic indices in the part of Romagna area *(Fig. 4.1)*. Moreover, the experiment evaluated the trend of grape production and its correlation with climate variables for the same area.

4.2.1   Materials and Methods

4.2.1.1   *Study region, grape sugar content and grape production data*

The Emilia-Romagna (ER) is located in the north of Italy and accounts for about 55,000 ha of vine cultivated surface which represent 8.1% of the total Italian vine cultivated surface and is the 2nd wine-producing region with 18% of the total Italian wine production by volume (harvest 2014). The Sangiovese (main red grape variety cultivated in Italy) wine production in the studied area represents approximately

70% of the entire ER region. The studied area is mostly located between 100 m and 300 m above sea level and stretches from 44°00' to 44°50' N latitude and from 11°50' to 12°50' E longitude *(Fig. 4.1)*.
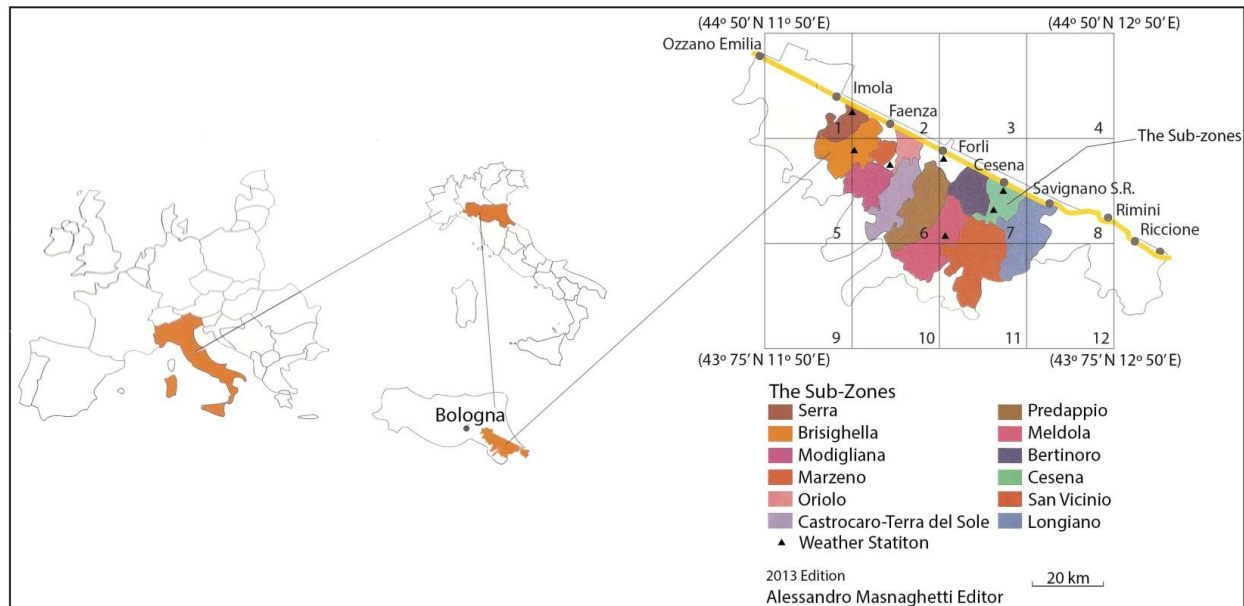
Figure 4.1 Location of the studied part of Romagna area (adopted with permission from Teslić et al., 2018).

Sugar content in Sangiovese berries was obtained from seven commercial wineries located in the studied area for the period from 2001 to 2012. Berry sugar content was measured directly in the field, day before harvest, using a portable digital refractometer. Measuring grape sugar content instead of the alcohol concentration in wines is more suitable for experiments consisted with presented study. This is due to avoidance of possible overestimates or underestimates of results caused by enrichment practices (e.g. grape sugar additions during fermentation).

Similarly, to the sugar content, the annual grape yield, produced on consistent vineyard surface, was obtained from the same seven wineries from 1982 to 2012. The dataset was averaged for every year between all seven wineries for grape sugar content and quantity of produced grapes.

4.2.1.2   *Meteorological data and bioclimatic indices*

Used bioclimatic indices (BI) were computed with values of daily maximum, mean, minimum temperatures and precipitation from the ENSEMBLES (E-OBS 0.25 deg. Regular grid, version 11.0; **www.ecad.eu**). Interpolated and gridded datasets were used for the period 1953–2013 from six grid cells (1, 2, 5, 6, 7 and 8; *Fig 4.1*) which covers the majority (~97%) of the total vineyards in studied area *(Fig. 4.1)*. Three grid cells in the bottom row (10, 11 and 12) were omitted from the calculations due to the small percentage (~3%) of vineyards in that area. Additional explanations related to the E-OBS dataset are described by Haylock et al. (2008). For validation purposes, all BI computed with E-OBS dataset were also computed with consistent data from seven weather stations **(www.arpa.emr.it)** for a short-term

period (2005–2013; *Fig 4.1*). A good correlation (r ≥0.9) was observed between BI values calculated from two datasets (E-OBS and weather stations), thus E-OBS dataset was suitable for the experiment. The bioclimatic indices used for this experiment were calculated as presented in *Table 2.1* and *Table 4.1.*

Table 4.1 Mathematic definitions and classes of used BIs.

Bioclimatic index	Mathematical definition	Classes
Temperature related indices		
Growing season max and min temperature (T_{max}; T_{min})	$$Tmax = \frac{1}{N}\sum_{1.4.}^{31.10.} Tx \; ; Tmin = \frac{1}{N}\sum_{1.4.}^{31.10.} Tm$$ Tx – Max air temperature (°C) Tm – Min air temperature (°C) N – Number of days	-
Diurnal temperature range (DTR)[1]	$$DTR = \frac{1}{N}\sum_{1.8.}^{30.9.} Tm - Tn$$ Tx – Max air temperature (°C) Tm – Min air temperature (°C)	-

[1](**Ramos et al., 2008**).

4.2.1.3   Statistical analysis

Basic descriptive statistics (e.g. mean value and standard deviation) for BIs, grape sugar content and grape yield data were calculated. Trend analysis was performed by Mann-Kendall test (MKT) (**Kendall and Stuart, 1967; Mann, 1945**), which is often used non-parametric test for detecting existing trends in meteorological, agrometeorological and hydrological datasets (**Bardin-Camparotto et al., 2014; Ramos et al., 2008**). To avoid over-fitting by insertion of auto-correlated data (**Von Storch and Navarra, 1995**), MKT was computed with Hamed and Ramachandra Rao (1998) modification.

The relationship between BIs, grape sugar content and grape yield was assessed using a multiple linear regression method. To avoid co-linearity, the BI were removed by using backward removal approach until remaining indices did not satisfy criteria of tolerance value >0.2 and VIF value <4 (**Neethling et al., 2012**). The determination coefficient 'adjusted R^2' was used as an estimator of the ability of calculated BI to explain the model (**Draper and Smith, 1981**). Data homogeneity and occurrence of breaking points in the datasets were assessed using non-parametric Pettitt test (PT) (**Pettitt, 1979**).

4.2.2 Results and Discussion

4.2.2.1 Bioclimatic indices

Growing season maximum, mean and minimum temperatures had significantly increasing trends over the studied period with an increase of 0.04, 0.03 and 0.02°C/year, respectively. The total increasing trends were estimated as 2.20, 1.65 and 1.40°C from 1953 to 2013 for maximum, mean and minimum temperatures, respectively *(Table 4.2)*.

Table 4.2 Pettitt test (PT) and Mann-Kendall test (MKT) applied to meteorological data (1953–2013) in the studied area. NS: no significant trend; *: 90% significant trend.

Index	PT p-level	PT Cutting point	MKT p-level	MKT Trend year^{-1}	MKT Total trend
T_{max} [°C]	<0.0001	1984	<0.0001	0.04	2.20
T_{mean} [°C]	<0.0001	1989	<0.0001	0.03	1.65
T_{min} [°C]	<0.0001	1984	<0.0001	0.02	1.40
DTR [°C]	0.049	1984	0.023	0.01	0.79
CI [°C]	0.648	NS	0.605	NS	NS
ND 25−30°C [days]	0.266	NS	0.096*	-0.11	-6.71
ND > 30°C [days]	<0.0001	1984	<0.0001	0.53	32.33
HI [units]	<0.0001	1989	<0.0001	5.88	358.62
GDD [units]	<0.0001	1984	<0.0001	6.1	371.98
T_{prec} [mm]	0.098*	1996	0.043	-1.94	-118.16
DSI [days]	0.055*	1996	0.026	0.15	9.33

As the growing season mean temperature (T_{mean}) value suitable for the growth of Sangiovese grapes ranges from 16.9 to 19.2°C **(Jones, 2006)**, the T_{mean} value of 18.49°C (for the period 1953–2013; *Fig. 4.2a Table 4.3*) found in this study showed that the examined area had optimum temperature conditions for the Sangiovese grapes cultivation. Results are suggesting that increasing of T_{mean} is more driven by growing season maximum than minimum temperature which was also reported for other viticulture regions in Europe *(Table 4.2)* **(Neethling et al., 2012; Malheiro et al., 2013; Ramos et al., 2008; Vršić et al., 2014)**. The possible ongoing increasing trend of growing season temperatures, if permanent, could become a long-term risk factor for a grape production as it was reported by some authors **(Hannah et al., 2013)**. However, its effects on the grape production will depend to a great extent on adaptation by viticulturists, including vineyard management and the use of grape varieties more resistant to warmer conditions **(van Leeuwen et al., 2013)**.

Table 4.3 Descriptive statistics applied to meteorological data (1953–2013) in the studied area.

Index	Mean	Std. dev.
T_{max} [°C]	23.65	1.01
T_{mean} [°C]	18.49	0.78
T_{min} [°C]	12.09	0.69
DTR [°C]	11.35	0.81
CI [°C]	13.66	1.21
ND25–30°C [days]	63.45	9.32
ND > 30°C [days]	30.57	14.69
HI [units]	2258.37	178.32
GDD [units]	1794.89	171.97
T_{prec} [mm]	463.88	121.39
DSI [days]	161.48	8.97
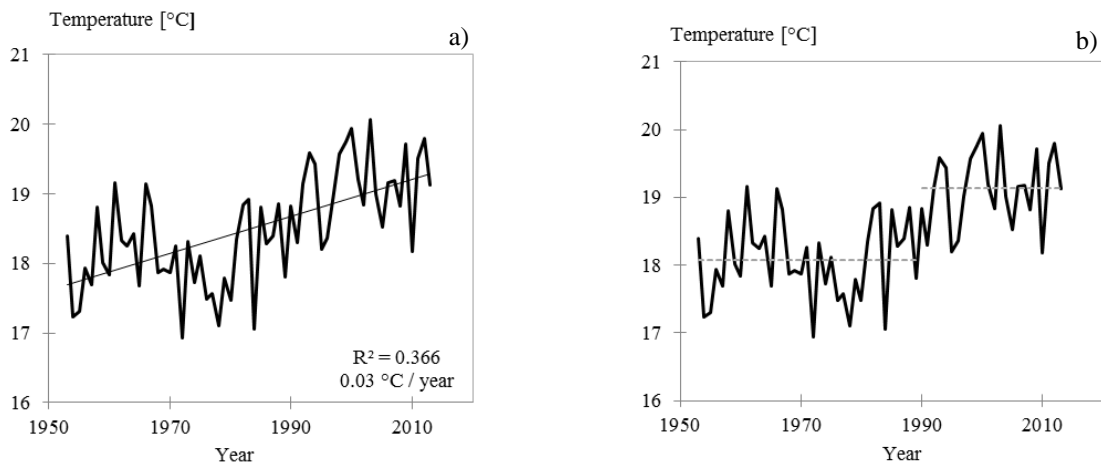
Figure 4.2 a) Linear trend of growing season mean temperature; b) Pettitt homogeneity test for a growing season mean temperature; in the studied area during the period from 1953 to 2013.

Cutting point of growing season mean temperature time series detected in 1989 can be explained by abrupt anomalies which started at the beginning of the 1970's, reaching maximum anomalies during the 1980's in the large-scale circulation patterns for the North Atlantic/European sector *(Table 4.2; Fig. 4.2b)* **(Mariani et al., 2012 Warmer et al., 2000)**.

Diurnal temperature range (DTR) showed a significant positive trend with an increase of 0.01°C/year and a total trend of 0.79°C from 1953 to 2013 *(Table 4.2)*. The DTR trend was mostly related to the increase of growing season maximum temperature over the grape ripening period (August–September). Thermal amplitude between the maximum and minimum temperatures may have a positive effect on berry composition **(Ramos et al., 2008)**. However, an excess in diurnal temperature range may have a negative effect on grape quality due to the plant stress with higher temperatures **(Ramos et al., 2008)**. The homogeneity test cutting point occurred in 1984 *(Table 4.2)*.

The Cool night index (CI) showed a lack of significant trend and homogeneity test cutting point due to the minor increase in minimum temperatures particularly during the grape ripening months *(Table 4.2)*. Night temperatures are correlated with the synthesis of secondary metabolites (e.g. anthocyanins) in red

grape varieties, whereas night temperatures in an approximate range of 10–15°C have a positive effect on anthocyanins accumulation **(Kliewer, 1977)**. Thus, the mean value of CI (13.66°C) was in the optimum range for the anthocyanins accumulation which is an important factor for production of Sangiovese grapes *(Table 4.3)*.

Number of days with maximum temperature in range from 25 to 30 °C (ND 25–30°C) showed a slightly significant negative trend, while number of days with maximum temperature > 30°C (ND > 30°C) had a significant positive trend with a total increasing trend of 32.33 days exceeding >30°C *(Table 4.2)*. Cutting points occurred in 1984 for ND > 30°C, whereas the ND 25–30°C was not significant for the homogeneity test *(Table 4.2)*. The increase of ND > 30°C may be beneficial during ripening **(Jones and Davis, 2000)**. However, too many days with temperature >30°C may stress the plant photosynthesis **(Mullins et al., 1992)**, since days with maximum temperatures ranging between 25–30°C are optimal conditions for the photosynthesis processes.

Two thermal indices often used to examine the suitability of selected area for grape production, Huglin index (HI) and Growing degree day (GDD), showed a significant increasing trend with 5.88 and 6.1 units per year and a total trend of 358.62 and 371.98 units from 1953 to 2013, respectively *(Table 4.2)*. According to the Huglin classification, due to the increasing temperatures in the Romagna area, HI trend shifted studied area from the temperate/warm temperate to the warm temperate/warm viticulture region *(Fig. 4.3a)*. Also, according to the Winkler classification, GDD regression trend shifted the Romagna area from the region II/III to the region III/IV *(Fig. 4.3b)*. The homogeneity test cutting points occurred in 1989 and 1984 for HI and GDD, respectively *(Table 4.2)*.
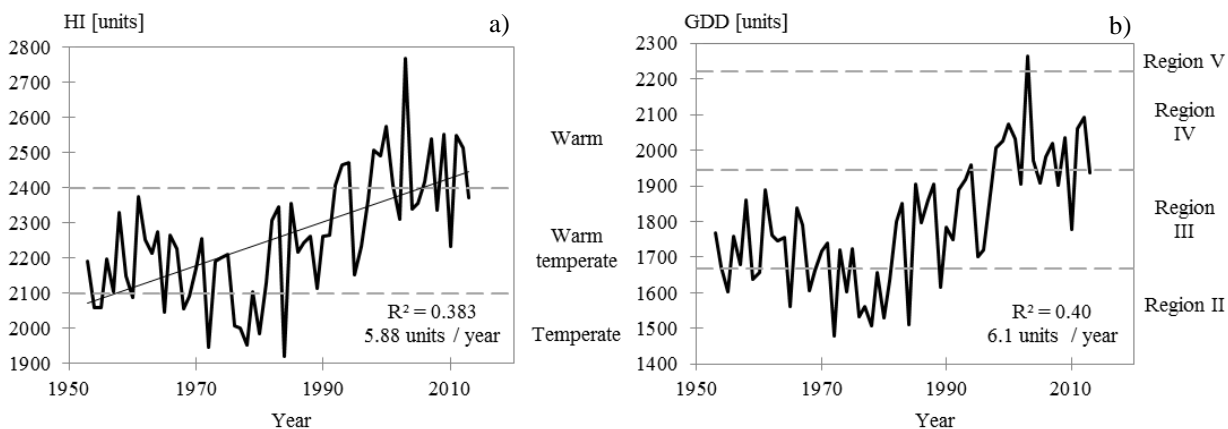
Figure 4.3 Linear trends of a) Huglin index; b) Growing degree day in the studied area during the period from 1953 to 2013.

Several studies reported a lack significant precipitation trends in certain European grape growing regions **(Jones et al., 2005; Neethling et al., 2012; Ramos et al., 2008)**. However, a significant negative trend was detected for precipitation in presented experiment, with a 1.94 mm/year and a 118.16 mm total trend decrease and with high annual variations over growing season period *(Table 4.2; Fig. 4.4a)*. These results are aligned with other studies, focused on Italy **(Brunetti et al., 2000)** and on ER **(Antolini et al., 2016)**. Furthermore, the positive Dry spell index (DSI) trend with 0.15 days/year and 9.33 days in total indicated on possible longer drought periods in the future decades *(Table 4.2; Fig. 4.4b)*. The homogeneity test

cutting point occurred in 1996 for total precipitation and DSI due to the mentioned abrupt anomalies in the large-scale circulation patterns *(Table 4.1)*.
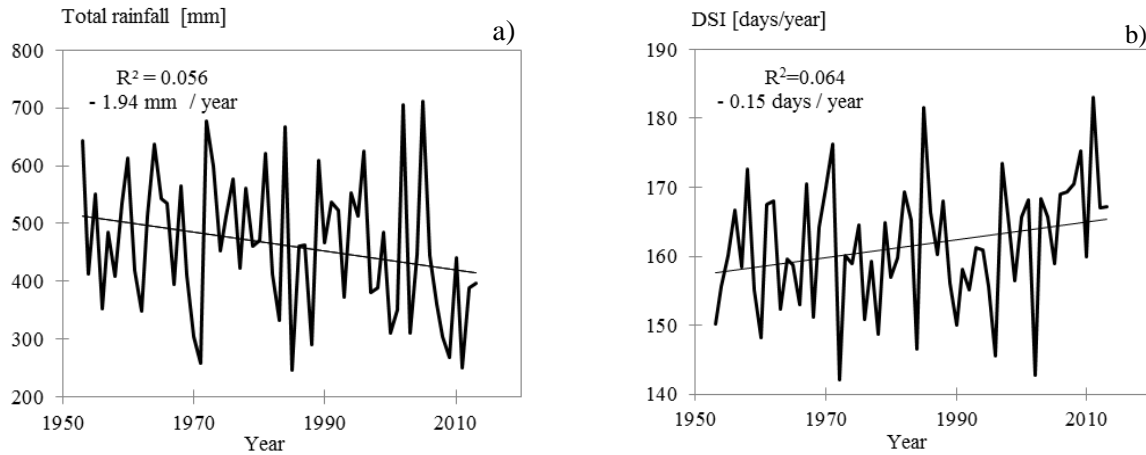
Figure 4.4 Linear trends of a) Total precipitation; b) Dry spell index in the studied area during the period from 1953 to 2013.

4.2.2.2   *Grape sugar content*

Sugar content in Sangiovese grape showed a significantly increasing trend with 0.12°Brix/year and 1.38°Brix during a 12 years period (2001–2012) *(Table 4.4; Table 4.5; Fig. 4.5a)*. High value of adjusted R^2 (0.81) obtained with multiple linear regression suggests a high contribution of computed bioclimatic indices on increasing berry sugar content in Sangiovese grapes from the studied part of Romagna area *(Table 4.6)*.

Table 4.4 Pettitt test (PT) and Mann-Kendall test (MKT) applied to grape sugar content (2001–2012) and grape yield data (1982–2012) in the studied area.

Parameter	PT p-level	PT Cutting point	MKT p-level	MKT Trend year^{-1}	MKT Total trend
Sugar content [°Brix]	0.017	2006	0.04	0.12	1.38
Grape yield [t]	0.003	1997	<0.01	33.49	1038.07

Table 4.5 Descriptive statistics applied to grape yield (1982–2012) and grape sugar content data (2001–2012) in the studied area.

Parameter	Mean	Std. dev.
Sugar content [°Brix]	17.17	0.83
Grape yield [t]	2216.47	486.59
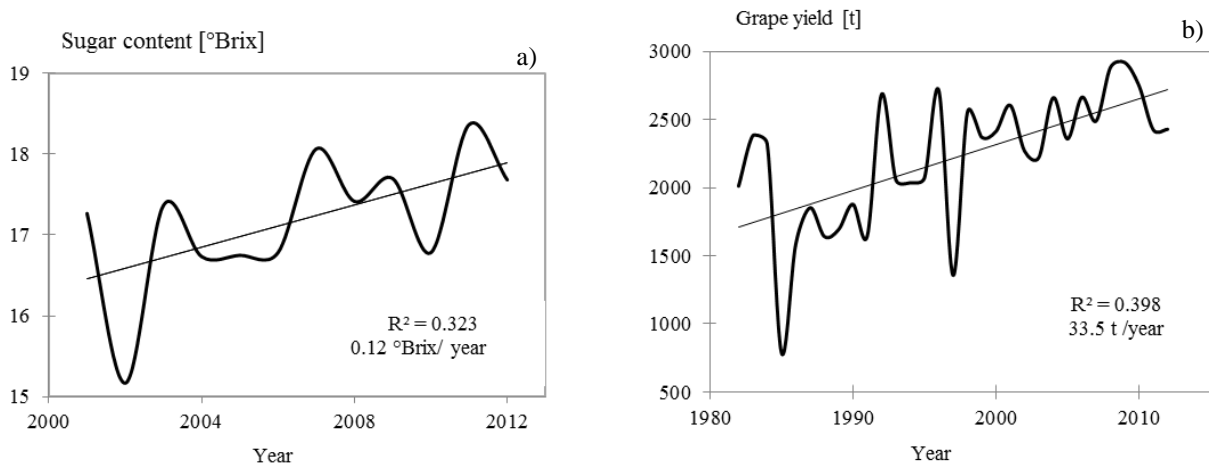
Figure 4.5 Linear trends of a) grape sugar content (2001–2012); b) grape yield data (1982–2012) in the studied area.

The positive standardized regression coefficient of DSI suggested that decrease of DSI had a positive effect on grape sugar content, which is possible only up to a certain limit *(Table 4.1; Table 4.6)*. Compared to DSI, HI regression coefficient suggests that increasing thermal accumulation had a lower impact on sugar content in Sangiovese grapes *(Table 4.1; Table 4.6)*. This, positive relation between HI and grape sugar content is possible up to the certain point due to photosynthesis process limitations. For additional information related to moderate water stress and temperature correlations with grape sugar content see 4.1.2.

Table 4.6 Standardized coefficients, adjusted R2 and p-level of multiple linear regression modeling applied to sugar content and bioclimatic indices (2001–2012); grape yield and bioclimatic indices (1982–2012) in the studied area.

Sugar content	Standardized coefficients			Adjusted R^2	p-level
	HI	DSI			
	0.14	0.84		0.81	0.0002
Grape yield	Standardized coefficients			Adjusted R^2	p-level
	T_{max}	DSI	ND 25–30°C		
	0.55	-0.42	-0.19	0.21	0.025

PT cutting point of grape sugar content occurred in 2006 *(Table 4.4)*. Hypothesis of drier conditions (DSI) coupled with increased temperature accumulation (HI) in the period after cutting point (2007–2012), comparing to the period before cutting point (2001–2006, with an exception of hot and dry 2003), may serve as an explanation for this outcome *(Fig. 4.6)*.
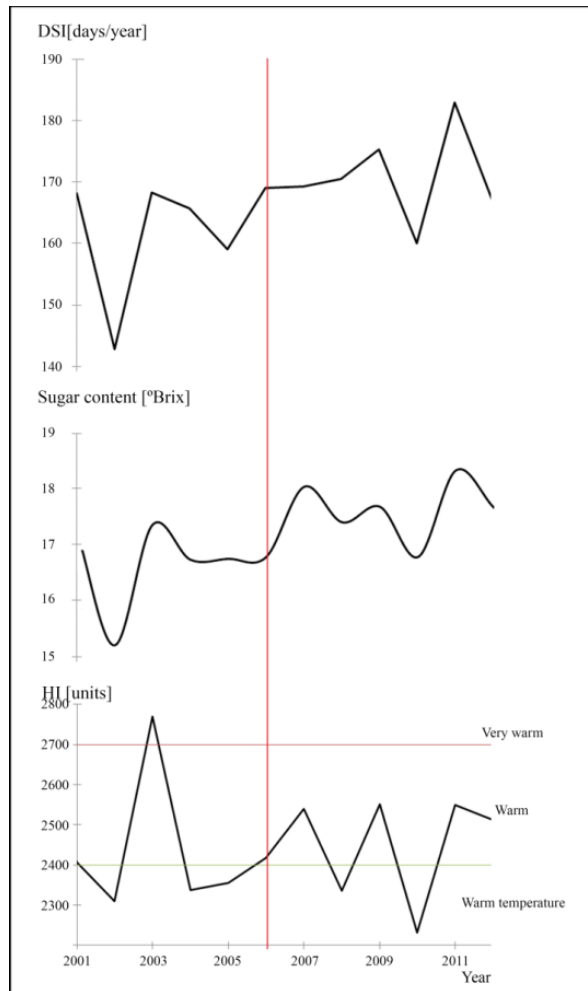
Figure 4.6 Growing season trends of Huglin index (HI); sugar content in Sangiovese grapes (Sugar content); Dry spell index (DSI) in the studied part of Romagna area from 2001 to 2012; red line – Sugar content breaking point.

4.2.2.3   Grape yield

Sangiovese grape yield showed a significant increasing trend of 33.49 tons/year and 1038.07 total tons from 1982 to 2012 *(Table 4.4; Table 4.5; Fig. 4.5b)*. In contrast to grape sugar content, a low value of adjusted R^2 (0.21) obtained with multiple linear regression indicate a low influence of computed bioclimatic indices on increase of Sangiovese grape production *(Table 4.6)*. Thus, suggesting that variables uncovered by this experiment, such as husbandry improvement (e.g. drainage, pesticides, canopy management, fertilizers) and soil characteristics, might had key a role on grape production increase in the studied part of Romagna area during the last 30 years. PT breaking point of increasing grape yield detected in 1997 and decreasing precipitation variables (T_{prec} and DSI) breaking points in 1996, are suggesting that higher grape yield occurred with lower water availability which is not aligned with other studies (**Ramos and Martínez-Casasnovas, 2010**). Therefore, minimal impact of calculated bioclimatic indices on Sangiovese grape yield, obtained with multiple linear regression is supported by PT.

The negative standardized regression coefficient of ND 25–30°C, suggested that lower ND 25–30°C had a negative impact on grape yield *(Table 4.1; Table 4.6)*. This may be due to decrease in the number of days with optimum temperature range for photosynthesis process (25–30°C) and increase in the number of days with temperatures that lead to initial plant stress (>30°C). T_{max} standardized coefficient obtained with multiple linear modeling, suggested a positive influence of increasing maximum temperature on grape yield in the studied part of Romagna during the last 30 years *(Table 4.1; Table 4.6)*. The negative DSI standardized coefficient suggested that increase in number of days without rain (<1mm) may decrease soil water availability causing drought stress to plants, which has a negative impact on grape yield **(Ramos and Martínez-Casasnovas, 2010)** *(Table 4.1; Table 4.6)*. For additional information related to moderate water stress and temperature correlations with grape yield see 4.1.6.

4.2.3   Conclusions

The studied part of Romagna area has been affected by weather anomalies in large-scale circulation patterns during the 1980's (Westerlies regimes). During the studied period (1953–2013), growing season mean temperature (18.49°C) and night temperatures during the ripening months of (13.66°C) were in the optimum range for Sangiovese production. The increase of T_{mean} was rather due to a rise in T_{max} than augmentation of T_{min}. The precipitation and DSI had a negative trend over the growing season with high annual variations, suggesting drier conditions. Multiple linear analysis coupled with PT, elucidated low impact of computed bioclimatic variables on increase of Sangiovese grape yield. Also displayed that variables which were not considered by this study, such as husbandry practices and soil characteristics, might had a significant role in grape yield determination. Using the same approach, the increase of berry sugar content in Sangiovese grapes during 2002–2012, was largely explained (81%) by computed bioclimatic indices, whereas DSI showed a higher correlation with increasing sugar content in berries respect to the HI. Furthermore, the experiment was done in collaboration with grape grower partners of the Caviro Coop (Faenza, Ravenna, Italy), thus the obtained results a valuable case study on the topic.

4.3   References

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Appendix C – Climate change trends, grape production, and potential alcohol concentration in wine from the 'Romagna Sangiovese' appellation area (Italy)

ORIGINAL PAPER

Climate change trends, grape production, and potential alcohol concentration in wine from the "Romagna Sangiovese" appellation area (Italy)

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Abstract The trend of climate change and its effect on grape production and wine composition was evaluated using a real case study of seven wineries located in the "Romagna Sangiovese" appellation area (northern Italy), one of the most important wine producing region of Italy. This preliminary study focused on three key aspects: (i) Assessment of climate change trends by calculating bioclimatic indices over the last 61 years (from 1953 to 2013) in the Romagna Sangiovese area: significant increasing trends were found for the maximum, mean, and minimum daily temperatures, while a decreasing trend was found for precipitation during the growing season period (April–October). Mean growing season temperature was 18.49 °C, considered as warm days in the Romagna Sangiovese area and optimal for vegetative growth of Sangiovese, while nights during the ripening months were cold (13.66 °C). The rise of temperature shifted studied area from the temperate/warm temperate to the warm temperate-/warm grape-growing region (according to the Huglin classification). (ii) Relation between the potential alcohol content from seven wineries and the climate change from 2001 to 2012: dry spell index (DSI) and Huglin index (HI) suggested a large contribution to increasing level of potential alcohol in Sangiovese wines, whereas DSI showed higher correlation with potential alcohol respect to the HI. (iii) Relation between grape production and the climate change from 1982 to 2012: a significant increasing trend was found with little effect of the climate change trends estimated with used bioclimatic indices. Practical implication at viticultural and oenological levels is discussed.

1 Introduction

Grape production is strongly affected by climate conditions (Jackson and Lombard 1993; Schultz 2000; van Leeuwen et al. 2004; Keller 2010; Fraga et al. 2014a); therefore, climate change can modify grape and wine composition to a great extent. Vine sensitivity to weather properties (Jones et al. 2005b; Gladstones 2011; Holland and Smit 2014), narrow spatial surfaces suitable for producing high-quality grapes as wine industry raw material, and possibility of perennial plant exploitation (Battaglini et al. 2009; Lereboullet et al. 2014) are indicative of the need for a climate change assessment associated with winemaking. Despite the importance of the global climate change trend, from the vine grower/winemaker perspective, it is more essential to understand regional atmospheric conditions (Jones et al. 2005b; Orlandini et al. 2009) and local microclimatic environment as well. Generally, increasing average global temperature over the last few decades is more than evident, as is the increasing temperature trend, although is not homogenous in every vine-growing region (Pielke et al. 2002; Jones et al. 2005b; van Leeuwen et al. 2013). For example, Jones et al. (2005b) confirmed a significant growing season temperature trend for the majority of northern hemisphere wine-producing regions between 1950 and 1999, with an average increase of 1.26 °C. However, there was also an insignificant trend in the majority of southern

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hemisphere wine regions, which emphasizes the necessity to focus study on smaller study areas.

Since climate modifications are vastly complex, examinations of simple temperature and precipitation values are insufficient to explain climate change trends. Therefore, several bioclimatic indices (e.g., Huglin index (HI) (Huglin 1978), Cool night index (CI) (Tonietto 1999), Winkler (WI) or growing degree day (GDD) index (Winkler et al. 1974), number of days with maximum temperatures higher than 30 °C (ND > 30 °C) (Ramos et al. 2008), number of days with precipitations <1 mm (Dry spell index, DSI) (Moisselin and Dubuisson 2006), etc.) are commonly used in viticulture to provide a better insight into climate change trends. However, the selected bioclimatic indices were mainly based only on air temperature, as it has the strongest influence on overall growth, productivity, and berry ripening of the grapevine (Jones 2012).

Jones et al. (2010) showed that climate change is responsible for over 50 % of alcohol trends. Moderate water stress may positively affect berry sugar accumulation during grape-growing season (Coombe 1989), while increasing temperature advances phenological stages and speeds up sugar accumulation in grape berries (Duchêne and Schneider 2005; Barbeau 2007; Jones 2012; Bonnefoy et al. 2013). Both water stress and increasing temperature later lead to the production of wines with higher alcohol content and other microbiological, technological, sensorial, and financial implications (Mira de Orduña 2010). In particular, increase of grape sugar content at harvest may cause slow/stuck alcoholic fermentations during hot years (Coulter et al. 2008) as well as alter sensory features due to the ethanol's tendency to increase bitterness perception (Fischer and Noble 1994; Vidal et al. 2004; Sokolowsky and Fischer 2012), suppress the perception of sourness (Williams 1972), and reduce astringency perception (Williams 1972; Vidal et al. 2004). Excess of alcohol in wine is also not desirable due to harmful effects on the health of consumers and civil restrictions (Catarino and Mendes 2011). Moreover, in the USA, winemakers need to pay additional taxes if the wine contains more than 14.5 % v/v of alcohol, whereas in EU, the alcohol limit for table wine is 15.0 % v/v. Recently, consumers showed a preference for wines with lower alcohol content (between 9 and 13 % v/v) (Massot et al. 2008).

Italy is one of the top wine producers in the world and its export represents the main income for the entire agro-food sector. Although the importance of climate change is well recognized by scientists worldwide, there is a need to improve its awareness among private companies as well.

In this view, the present study aims to establish a relationship between grape sugar content, presented as potential alcohol content in Sangiovese wines, and climate change trends based on selected bioclimatic indices of the specific area of interest (Fig. 1). Moreover, the study evaluated the trend of grape production for the same area and its correlation with

climate variables. It has to be noted that the examination of climatic trends and their influence on grape production and wine potential alcohol level in the "Romagna Sangiovese appellation area" was based on meteorological factors alone (e.g., temperature and precipitations). The effect of other possibly relevant factors, such as soil characteristic, effects of elevated atmospheric carbon dioxide concentration, influence of market decisions on alcohol level in wines, husbandry practices, etc., was not considered.

The study was done in collaboration with grape growers and local winery partners of the Caviro Coop (Faenza, RA, Italy), thus rendering the obtained results a valuable case study on the topic.

2 Materials and methods

2.1 Study region, potential alcohol concentration, and grape production data

The Emilia-Romagna (ER) is located in the north of Italy and accounts for about 55,000 ha of vineyards which represent 8.1% of the total Italian vineyard surface and is the second wine-producing region with 18 % of the total Italian wine production by volume. The ER include nine provinces, two of which are located within the "Romagna Sangiovese" appellation area, namely Ravenna and Forlì-Cesena, that account for 16,000 and 7000 ha of vineyard, respectively, thus representing 42 % of the total ER grape cultivated area for all varieties. The Sangiovese (main red grape variety cultivated in Italy) wine production, in the two provinces of Ravenna (1700 ha) and Forlì-Cesena (3300 ha) represents ca. 72 % of the entire Emilia-Romagna region. The studied area covers more than 97 % of the Romagna Sangiovese appellation area which is mostly located between 100 and 300 m above sea level and covering an approximate area from 44° 00′ to 44° 50′ N latitude and from 11° 50′ to 12° 50′ E longitude (Fig. 1).

Potential alcohol content of Sangiovese wines was calculated (Eq. 1) from sugar content in grapes that were harvested from seven commercial wineries in the studied area for the period from 2001 to 2012. Grape sugar content was measured directly in the field, day before harvest, using a portable digital refractometer. This approach is commonly used in enology to estimate the "natural" alcohol content of wine without any contribution due to the enrichment practices, if any (i.e., grape sugar additions during fermentation).

Equation 1 Bx, sugar content in grapes expressed as Brix degrees.

$$\text{Potential alcohol} \left[\%v/v \right] = Bx \times 0.6$$

Similarly to the potential alcohol content, the amount of grapes produced per year, produced on consistent vineyard
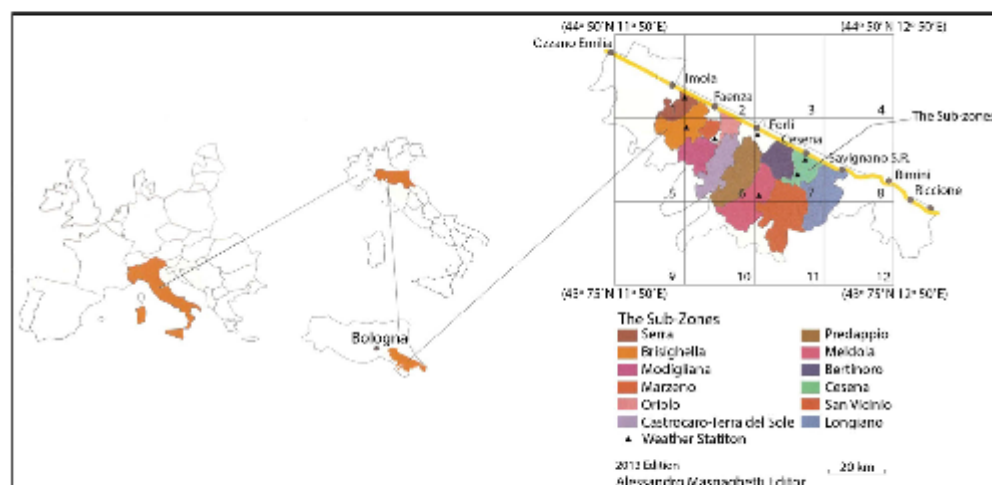
Fig. 1 Location of the Romagna Sangiovese appellation area (Italy)

surface, was obtained from the same wineries from 1982 to 2012. The dataset was averaged for every year between wineries for naturally occurring alcohol content and quantity of produced grapes.

2.2 Meteorological data and bioclimatic indices

Bioclimatic indices (BI) were computed with daily high-resolution observations of maximum, mean, and minimum temperatures and precipitation from the ENSEMBLES gridded observation dataset (E-OBS 0.25° Regular grid, version 11.0). Interpolated/gridded datasets were used for the period 1953–2013 from six grid cells which covered more than 97 % of the total vineyards in the studied area (Fig. 1; Fig. S1). Three grid cells in the bottom row (10, 11, and 12) were excluded due to the small percentage of vineyards in that area. Datasets produced by the ENSEMBLES project are used in several recent publications (Santos et al. 2012; Andrade et al. 2014; Fraga et al. 2014a, b; Männik et al. 2015; Konca-Kedzierska 2015), and further details about E-OBS dataset are described by Haylock et al. (2008).

For validation purposes, all bioclimatic indices used in the study were computed also with consistent data from seven weather stations (www.arpa.emr.it) for a short-term period (2005–2013). Locations of the weather stations are listed in Table S1.

The following bioclimatic indices were calculated (Table S2):

1. Growing season maximum (T_{max}), mean (T_{mean}), and minimum temperature (T_{min}) (April–October for the northern hemisphere).
2. Number of days with a maximum temperature in the range of 25–30 °C (ND 25–30 °C) over the growing season period.
3. Number of days with a maximum temperature >30 °C (ND > 30 °C) over the growing season period.
4. Winkler thermal index (WI) or Growing degree day (GDD) index was calculated during the grape-growing season by using daily minimum and maximum temperatures. Only days with a thermal base value above 10 °C were taken into account due to the minimal grapevine physiological activity threshold (Winkler et al. 1974). However, the GDD index does not take into account adjustment of increasing daylight duration with higher latitudes.
5. Heliothermal index or Huglin index (HI) is a thermal index that takes into account daily mean and maximum temperatures during the period April–September. HI gives more weights to maximum daily temperatures with respect to WI and displays improved fitting of potential sugar content of the grape; a correction factor ($k = 1.04$) was applied to the area of study to account the increasing length of the daylight towards higher latitudes (Huglin 1978; Tonietto and Carbonneau 2004).
6. Cool night index (CI) is an average value of minimum temperatures during September. CI is related to the grape's synthesis of anthocyanins (Tonietto and Carbonneau 2004), compounds responsible for the red

color of the wine that needs improvement in Sangiovese wines.

7. Diurnal temperature range (DTR) was calculated as a mean variation between daily minimum and maximum temperatures in the period from August to September (ripening months) (Ramos et al. 2008).

8. Total precipitation (T_{prec}) over the grape-growing season (April–October).

9. Dry spell index (DSI) presents the number of days with <1 mm of precipitation during the grape-growing season (Moisselin and Dubuisson 2006).

10. Selianinov index (SI) or Hydrothermal coefficient (HTC) was calculated from daily mean temperature and daily precipitation (Fregoni 2005; Selianinov 1928). Only days above 10 °C were considered. SI was used to examine hydric regimes and water supply of the vines in the studied area.

2.3 Statistical analysis

Basic descriptive statistics (i.e., mean value and standard deviation) for BI, potential alcohol level, and grape production data were calculated. Trend analysis based on the Mann-Kendal (MK) test (Mann 1945; Kendall and Stuart 1967), the most commonly used non-parametric test for detecting existing trends in meteorological, agrometeorological, and hydrological time series data (Ramos et al. 2008; Bardin-Camparotto et al. 2014), was computed with modification (Hamed and Ramachra Rao 1998) to avoid overfitting due to the auto-correlated data (Von Storch and Navarra 1995).

Relationship among BI, potential alcohol content, and grape production was examined using a multiple linear regression approach. To avoid mutual co-linearity, the number of bioclimatic indices was reduced using backward removal method until remaining indices did not satisfy criteria of tolerance value >0.2 and VIF value <4 (Neethling et al. 2012). The determination coefficient "adjusted R^2" was used as indicator of the ability of variables to explain the model (Draper and Smith 1981).

Homogeneity of data and breaking points were computed using non-parametric Pettitt test (PT) (Pettitt 1979). All statistical tests were performed at the 95 % confidence level, unless otherwise specified.

3 Results and discussions

3.1 Bioclimatic indices

BI calculated with both E-OBS and weather stations data showed a good linear correlation (>0.9); thus, the E-OBS data were suitable to use for selected area. Slightly hotter and drier

conditions of BI calculated with data from weather stations can be explained by predominant locations at lower elevations of weather stations than in E-OBS, especially in grid cell 5, which is mainly mountain area (data not shown).

Growing season T_{max}, T_{mean}, and T_{min} disclosed significant trends over the selected period with increase of 0.04, 0.03 and 0.02 °C/year, respectively, with total trends estimated as 2.20, 1.65, and 1.40 °C from 1953 to 2013 (Table 1; Fig. 2a).

As the T_{mean} value suitable for production of high-quality wines ranges from 12 to 22 °C (Jones 2006), the T_{mean} value of 18.49 °C (for the period 1953–2013) found in this study showed that the studied area was characterized by warm mean growing season temperature and had optimum temperature conditions for the growth of Sangiovese (16.9–19.2 °C (Jones 2006)). The result of increasing T_{mean} is more driven by T_{max} than T_{min} and similar results are found in other grape-growing regions in Europe (Ramos et al. 2008; Neethling et al. 2012; Malheiro et al. 2013; Vršič et al. 2014). The ongoing T_{mean} increasing trend, if persistent, is commonly considered a long-term risk factor by some authors (Hannah et al. 2013). However, its effects will depend to great extent on adaptation by growers, including vineyard management and the use of grape varieties adapted to warmer conditions (van Leeuwen et al. 2013).

Breaking point of T_{mean} detected in 1989 (Fig. 2b) can be explained by abrupt anomalies starting from the beginning of the 1970s reaching maximum anomalies during the 1980s in the large-scale circulation patterns (Westerlies regimes) which characterize the North Atlantic/European sector (Werner et al. 2000; Mariani et al. 2012).

ND 25–30 °C showed a slightly significant negative trend, while ND > 30 °C had a significant positive trend (Table 1) with a total increasing trend of 32.33 days exceeding >30 °C. Critical breaking points occurred in 1984 for ND > 30 °C, whereas the ND 25–30 °C was not significant for the Pettitt test (data not shown). Days with daily maximum temperatures ranging between 25 and 30 °C are critical for plant growth due to the optimum photosynthesis processes. Although several days with maximum temperature reaching over 30 °C may be beneficial during ripening (Jones and Davis 2000), too many days with temperature >30 °C may stress the plant photosynthesis (Mullins et al. 1992), while temperature >35 °C represent upper photosynthesis limits causing total inhibition of the process (Gladstones 1992; Jackson 2008).

Similarly, the two thermal indices often used to examine the suitability of selected area for grape production, HI, and GDD, showed a significant increasing trend with 5.88 and 6.1 units per year and a total trend of 358.62 and 371.98 units during the studied period, respectively (Table 1). According to the Huglin classification, approximately in the 1980s, due to the increasing temperatures in the ER, HI trend shifted studied area from the temperate/warm temperate to the warm temperate-/warm grape-growing region (Fig. 3a). In the same period,

Table 1 Descriptive statistics, MK test, and PT applied to bioclimatic data for the period 1953-2013

Index	Mean	Std. dev.	MK p level	MK trend year^{-1}	MK total trend	PT p-level	PT breaking point
T_{max}	23.65	1.01	<0.0001	0.04	2.20	<0.0001	1984
T_{mean}	18.49	0.78	<0.0001	0.03	1.65	<0.0001	1989
T_{min}	12.09	0.69	<0.0001	0.02	1.40	<0.0001	1984
ND 25–30 °C	63.45	9.32	0.096*	−0.11	−6.71	0.266	NS
ND >30 °C	30.57	14.69	<0.0001	0.53	32.33	<0.0001	1984
GDD	1794.89	171.97	<0.0001	6.1	371.98	<0.0001	1984
HI	2258.37	178.32	<0.0001	5.88	358.62	<0.0001	1989
CI	13.66	1.21	0.605	NS	NS	0.648	NS
DTR	11.35	0.81	0.023	0.01	0.79	0.049	1984
T_{prec}	463.88	121.39	0.043	−1.94	−118.16	0.098*	1996
DSI	161.48	8.97	0.026	0.15	9.33	0.055*	1996
SI	2.02	0.66	0.027	−0.01	−0.73	0.07*	1981

NS no significant trend

*90 % significant trend

according to the Winkler classification, GDD regression trend shifted from regions II/III to III/IV (Fig. 3b). The Pettitt test breaking points occurred in 1989 and 1984 for HI and GDD, respectively (data not shown).

The CI showed a lack of significant trend (Table 1) and lack of critical breaking point (data not shown) due to the slow increase of minimum temperatures particularly during the grape-ripening months. Mean value of CI was 13.66 °C, therefore, the Romagna Sangiovese appellation area was mostly characterized by cold nights (data not shown). Night temperatures are correlated with secondary metabolites (e.g., anthocyanins) of red grape varieties, whereat higher night

Fig. 2 a Linear trends of T_{max}, T_{mean}, T_{min}; b Pettitt homogeneity test T_{mean}; c linear trend of T_{prec} in the Romagna Sangiovese appellation area during the growing period from 1953 to 2013
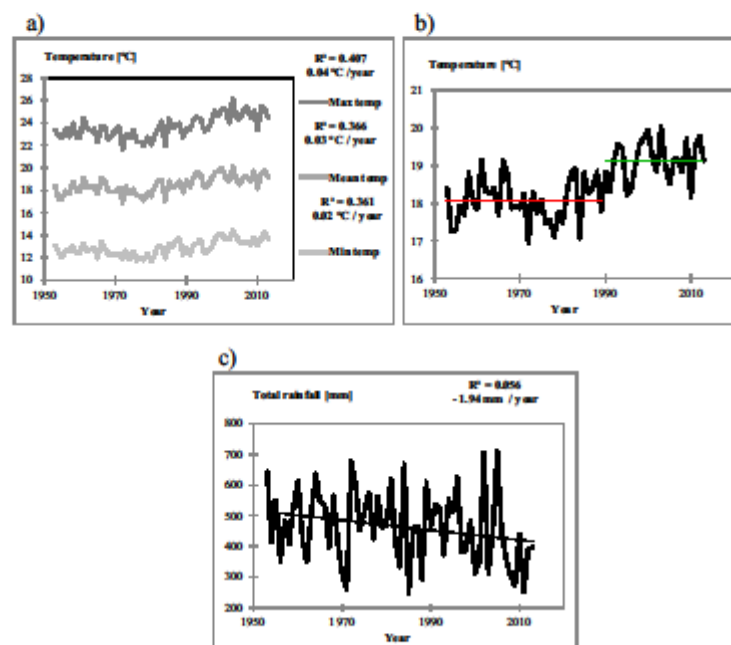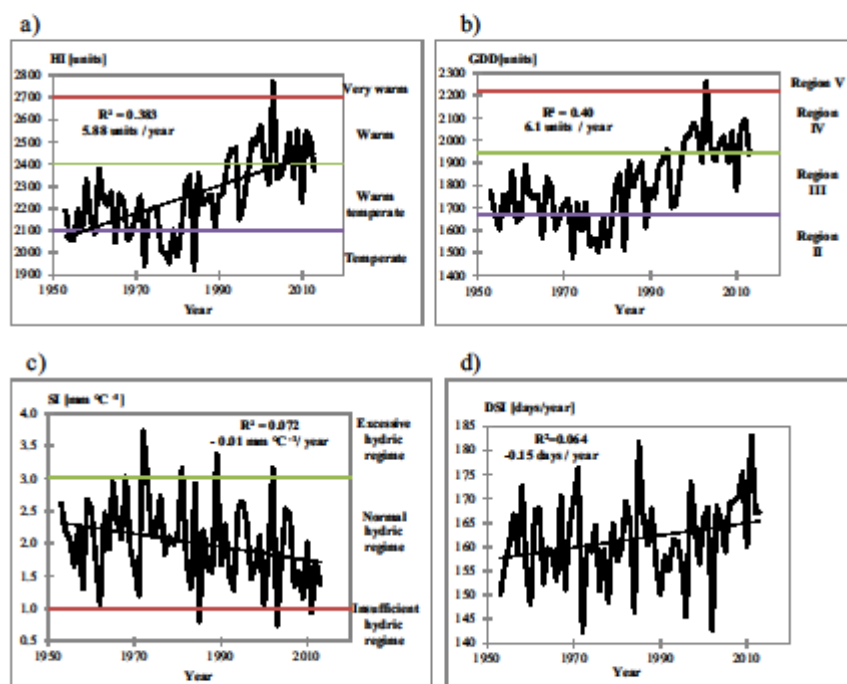
Fig. 3 Linear trends of a HI; b GDD; c SI; d DSI in the Romagna Sangiovese appellation area (Italy) during the period from 1953 to 2013

temperatures are causing higher loss of color and aroma (Jackson 2008).

DTR showed a significant positive trend with an increase of 0.01 °C/year and a total trend of 0.79 °C (Table 1) that is mostly related to the maximum temperature increase over the grape-ripening period (August–September). Although thermal amplitude between the maximum and minimum temperatures greatly affects berry composition, including its positive correlation with the synthesis of anthocyanins, an excess in diurnal temperature range negatively affects grape quality due to the plant stress with higher temperatures (Ramos et al. 2008). The Pettitt breaking point occurred in 1984 (data not shown).

Although many European vine-growing regions do not have significant precipitation trends (Ramos et al. 2008; Neethling et al. 2012) in this study, a significant negative trend

was detected for precipitation, with a 1.94 mm/year and a 118.16 mm total trend decrease with high annual variations over growing season period (Table 1; Fig. 2c). These findings are consistent with other authors, focused on Italy (Brunetti et al. 2000) and on Italian region Emila-Romagna (Antolini et al. 2016). Additionally, the positive DSI trend with 0.15 days/year and 9.33 days in total revealed possible longer drought periods over the growing season in the future (Table 1; Fig. 3d). The breaking point for both total precipitation and DSI occurred in 1996 due to the mentioned abrupt anomalies in the large-scale circulation patterns (data not shown).

The SI value showed a significant negative trend with high variation before the 2000s and minor amplitude in the past 10 years (2004–2013) (Fig. 3c). The total negative trend of

Table 2 Descriptive statistics, MK test, and PT test applied to potential alcohol concentration in wine (2001–2012) and grape production (1982–2012) in the Romagna Sangiovese appellation area (Italy)

	Mean	Std. dev.	MK p level	MK trend year^{-1}	MK total trend	PT p level	PT breaking point
Potential alcohol [% v/v]	10.30	0.5	0.04	0.07	0.83	0.017	2006
Grape production [t]	2216.47	486.59	<0.01	33.49	1038.07	0.003	1997

Fig. 4 Linear trends of a grape production (1982–2012) and b potential alcohol content in wines (2001–2012) in the Romagna Sangiovese appellation area (Italy)

3.2 Grape production

Grape production showed a significant increasing trend of 33.49 tons/year and 1038.07 total tons during 31 years (1982–2012), (Table 2, Fig. 4a). Low values of adjusted R^2 obtained from multiple linear regression approach (Table 3) elucidated low impact of computed climatic variables on increasing grape production, suggesting that variables uncovered by this study, such as husbandry improvement (e.g., drainage, pesticides, canopy management, fertilizers) and soil characteristics, might have predominant influence on grape yield in the Romagna Sangiovese appellation area during last 30 years. The influence of climate change may be underestimated as the upper level of CO_2 in the atmosphere increases crop load (Bindi et al. 1996; Schultz 2000; Moutinho-Pereira et al. 2009; Kizildeniz et al. 2015), whose effect may be relevant particularly after the 1970s due to the rapid increasing in the level of CO_2 (IPCC 2014). Cutting point of increasing grape production detected in 1997 and decreasing precipitation variables (T_{prec} and DSI) breaking point in 1996, implies higher yield with lower water availability which is opposite with other studies (Ramos and Martínez-Casasnovas 2010). Therefore, low influence of calculated

climate variables on grape production, obtained with multiple linear regression is supported by breaking point analysis.

The negative trend of the standardized ND 25–30 °C regression coefficient, which is related to the optimum temperature range for photosynthesis process, suggested negative impact on grape production; in other words, decrease of days with optimum temperature range may negatively affect crop load due to the reason that temperature increase leads to initial plant stress (several days exceeding >30 °C) and later to the total inhibition of the photosynthetic process (>35 °C).

T_{max} standardized coefficient obtained with multiple linear modeling, suggested a positive influence on crop load in the Romagna Sangiovese appellation area during the last decades. Similar results were found in the Rias Baixas wine region, Spain, whereas higher temperatures during the budburst and veraison significantly increased crop yield (Lorenzo et al. 2012). In the Bordeaux (France), higher temperatures shortened period from plant budburst to berry maturity into length favorable for higher yield (Jones and Davis 2000). Reversely, wine production decreased during warm seasons in the Penedès wine region, Spain (Ramos et al. 2008). Results from the mentioned studies indicate that positive/negative effect of the increasing temperature on the crop yield depends also on precipitation, atmospheric CO2 level, grape variety, and other factors. Even if detected in the Romagna Sangiovese appellation area, temperature increase may positively influence crop load up to a certain point, due to the detrimental influence of heating stress on grapevine photosynthetic process.

DSI standardized coefficient suggested that increasing number of days without rain (<1 mm) and longer drought

the SI was 0.73 mm °C^{-1}, while the annual decrease was 0.01 mm °C^{-1} (Table 1). The mean value of SI during the studied period was 2.02 mm °C^{-1}, a value actually considered as a normal hydric regime. The critical point for homogeneity test occurred in 1981 (data not shown).

Table 3 Standardized coefficients, standard errors, adjusted R^2, and p level of multiple linear regression modeling applied to grape production (tons) and bioclimatic indices found in the Romagna Sangiovese appellation area (Italy) from 1982 to 2012

	T_{max}	DSI	ND 25–30 °C	Adjusted R^2	p level
Standardized coefficients	0.55	-0.42	-0.19	0.21	0.025
Standard error	0.21	0.17	0.21		

Table 4 Standardized coefficients, standard errors, adjusted R^2, and p level of multiple linear regression modeling applied to naturally occurring alcohol content in Sangiovese red wines and bioclimatic indices in the Romagna Sangiovese appellation area (Italy) from 2001 to 2012

	HI	DSI	Adjusted R^2	p level
Standardized coefficients	0.14	0.84	0.81	0.0002
Standard error	0.15	0.15		

periods may reduce soil water availability causing drought stress to the grapevine, which negatively affects grape production (Ramos and Martínez-Casasnovas 2010).

3.3 Potential alcohol concentration

Naturally occurring alcohol content in Sangiovese red wines showed a significant increasing trend with 0.07 % (v/v)/year and 0.83 % (v/v) over a 12-year period (2001–2012) (Table 2, Fig. 4b).

In contrast to grape production, the high value of adjusted R^2 (0.81) suggests a large contribution of calculated climatic variables as a driver of increasing potential alcohol content in red wines from the Romagna Sangiovese appellation area (Table 4). The rest of the variables may be explained with consumer expectations in terms of full bodied, deeply colored, full-flavored red wines achieved with phenolic maturity, both skin and seed, which compels producers to prolong maturation of the grapes and increase the quantity of accumulated sugar in the grapes (García-Martín et al. 2010; Gil et al. 2013).

The increase of DSI coupled with decrease of SI and T_{psc} (not incorporated in the model due high co-linearity with DSI) suggested a positive impact on potential alcohol increase, possible only up to a certain limit. Moderate water stress may positively affect sugar accumulation in the berry as a result of inhibiting lateral shoot growth allowing transportation of carbohydrates to the fruit (Coombe 1989). Poni et al. (2007) conducted partial root-zone drying on potted Sangiovese grapevine, simulating dry (PRD) and wet conditions (WW), whereas at harvest, vines submitted to PRD showed higher total soluble solids respect to the vines treated with WW. Authors noted that use of potted vines approach may induce criticism due to the lack of real field conditions.

Compared to DSI, HI regression coefficient suggested lower impact on potential alcohol in wines. Detected higher temperatures and thermal accumulation may lead to earlier occurrence phenological stages (i.e., bud-breaking, flowering, veraison, full maturity/harvest) (Webb et al. 2007) and shorter time between two phenological phases (Jones et al. 2005a). Additionally, the combination of reduced precipitation with warming may provoke even faster passage through phenological stages of the vine (Webb et al. 2012). This accelerated pace of phenological events causes faster sugar accumulation
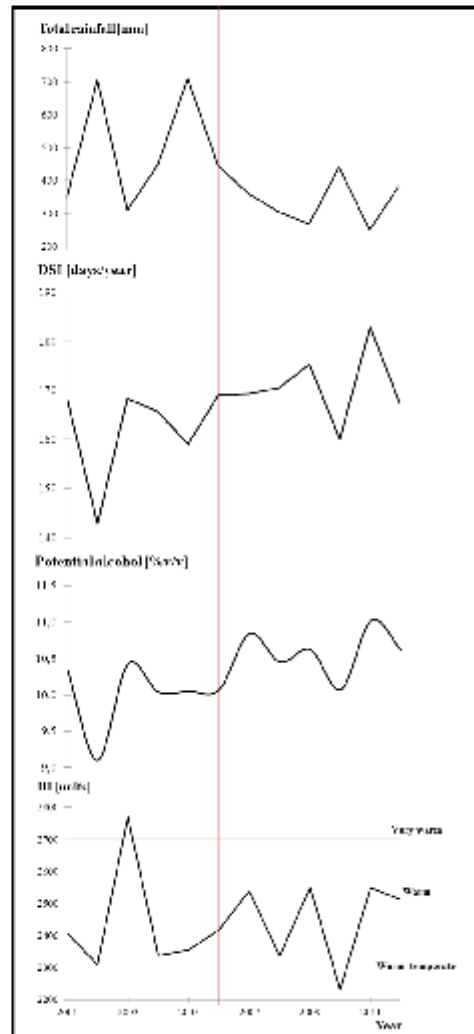
Fig. 5 Growing season trends of Huglin index (HI), potential alcohol level in Sangiovese wines (potential alcohol), dry spell index (DSI), and total precipitation (T_{psc}) in the Romagna Sangiovese appellation area (Italy) from 2001 to 2012; *red line* potential alcohol breaking point

and causing grapes to arrive earlier at technological maturity (optimum quantity of sugar content and acidity), while flavor compounds remain undeveloped. On the other hand, if vine growers wait for flavor compounds to develop, acidity values may reach a below optimum level due to the respiration, while sugar content reaches a higher than optimum level (Jones

2012). Therefore, producing wines with fully developed flavor and is often coupled with a high concentration of alcohol. However, positive relation is possible up to the certain point due to photosynthesic process limitations.

Breaking point of naturally occurring alcohol level in wines occurred in 2006 (Fig. 5). Hypothesis of reduced precipitation regime (DSI, T_{prec}) coupled with increased temperature accumulation (HI) in the period after breaking point (2007–2012), comparing to the period before breaking point (2001–2006, with an exception of hot and dry 2003), may serve as an explanation. These preliminary results require further and continuous monitoring to evaluate long-term effects of climate change on grape and wine parameters.

4 Conclusions

Overall, the Romagna Sangiovese appellation area has been affected with weather anomalies in large-scale circulation patterns during the 1980s (Westerlies regimes). During the period from 1953 to 2013, the mean growing season temperature was 18.49 °C, with night temperatures during the ripening months of 13.66 °C. The increasing T_{mean} trend was rather due to a rise in maximum daily temperatures than augmentation of minimum temperatures. The precipitation and SI had a negative trend over the growing season with high annual variations. Multiple linear analysis coupled with breaking point test, displayed low impact of calculated bioclimatic indices on increase of grape production in the Romagna Sangiovese appellation area, also indicated that variables uncovered by this study, such as husbandry practices and soil characteristics, might have a significant role in grape yield determination. Using the same approach, the increase of potential alcohol level in wines during 2002–2012, was largely explained (81 %) by conducted climatic variables, whereas precipitation decrease showed higher correlation with increasing potential alcohol content in wines respect to the rising temperatures. These preliminary results require further and continuous monitoring and to evaluate long-term effects of climate change on grape and wine parameters in the Romagna Sangiovese appellation area.

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CHAPTER 5

Techniques to adapt of wine industry to the climate change

5 Techniques to adapt of wine industry to the climate change

5.1 Introduction

It is well-established that in terms of quantity and quality grape production is strongly affected by climate conditions **(Jackson and Lombard, 1993; Mira de Orduña, 2010)**. Through vast number mutual interaction between indirect and direct climate variables (temperature, precipitation, UV-B radiation, air CO_2 concentration), grape growing areas could differ according to climate conditions on regional, local, vineyard or even row and plant level. The complexity of these interactions is augmented with accelerated climate change during the last decades, which are caused by human activities to a great extent *(Fig. 1.1)*. All together results in a wide range of differences between grape/wine producing areas in terms challenges and problems that wine industry need to confront.

Nowadays, challenges that wine industry needs to confront in changeable climatic conditions are often presented as crop load reduction, production of unbalanced wines with excessive alcohol and lower pH, reduction of anthocyanins in grape berries and wine, lower must acidity (see 4.1.1–4.1.6) etc. Among all mentioned challenges, redundant ethanol level in wines draws a significant attention to wine industry due to the fact that ethanol is the second most abundant wine component thus variations in ethanol content could cause diverse microbiological, technological, sensorial and financial implications **(Mira de Orduña, 2010)**. In particular, the increase of grape total soluble solids content at harvest may cause slow/stuck alcoholic fermentations during hot years **(Coulter et al., 2008)**, which could be even more expressed in the production of organic or biodynamic wines where producers rely on fermentation with spontaneous yeasts instead of commercial yeasts. Higher alcohol concentration could also alter wine sensory features due to the ethanol's tendency to increase bitterness perception **(Nurgel and Pickering, 2006; Sokolowsky and Fischer, 2012; Vidal et al., 2004; Villamor et al., 2013)**, suppress the perception of sourness **(William 1972)**, reduce astringency perception **(Vidal et al., 2004; William, 1972)**, add 'irritant' (heat) sensation **(Nurgel and Pickering, 2006; Villamor et al., 2013; William, 1972)**, elevate woody and spicy aroma/flavour **(Villamor et al., 2013)** suppress fruity, floral and caramel aroma/flavour **(Villamor et al., 2013)**. Excess of alcohol in wine is also not desirable due to the harmful effects on the health and behaviour of consumers **(Catarino and Mendes, 2011)**, which forced many countries to regulate civil restrictions related to legal limits of maximum ethanol content in human blood during driving, or minimum age limits required for purchase of alcohol-drinks **(Le Berre et al., 2007)**. Moreover, in the USA winemakers need to pay additional taxes if the wine contains more than 14.5% v/v of alcohol **(Massot et al., 2008)**. Recently, consumers showed a preference for wines with lower alcohol content (between 9% and 13% v/v) **(Massot et al., 2008)**.

Thus, certain adaptation techniques to reduce ethanol content in wines need to be applied to mitigate the influence of the climate change. By regulation of the International organization of vine and wine (OIV) ethanol content in wine generally should not be less than 8.5 % v/v. In certain specific cases when considering climatic conditions, soil or grape variety, specific qualitative factors or traditions related to producing process, ethanol content can be down to 7 % v/v or even less when allowed by the Code **(OIV, 2015)**. The example of specific cases is Italian sparkling white wine Moscato d'Asti, produced in the Piedmont with ethanol content reaching only 5.5% v/v. Reversely, maximum ethanol content is not

regulated by OIV, thus in specific cases of fortified wines such as Port, Marsala, Madeira and Sherry, ethanol content could reach ~20% v/v. Allowed dealcoholization was initially set by the European Commission at a limit of 2% v/v, irrespective of the initial alcohol level **(EC, 2009)**, whereas the limit was recently changed to 20% v/v of the initial effective alcohol content **(EC, 2013)**, thus allowing higher wine ethanol reduction for wines with more than 10% v/v alcohol. Adaptation techniques to remove excessive alcohol can be divided into four principal groups: (i) viticulture techniques, (ii) pre-fermentation techniques, (iii) biotechnological techniques and (iv) post-fermentation techniques.

5.1.1 Viticulture techniques

5.1.1.1 Late winter pruning

Winter pruning is primarily conducted to regulate grapevine yield, vigor and berry composition in the dormancy period (after leaf fall and before bud burst) **(Frioni et al., 2016)**. However, when winter pruning is applied after bud burst it may be used as a technique to delay the timing of harvest and to reduce the concentration of soluble solid in berries. This is due to nutrition competition between inflorescence primordial on the basal shoots (retained after winter pruning) and inflorescence primordial on the shoots in the upper part of cane (removed after winter pruning) **(Palliotti et al., 2014)**. In particular, application of late winter pruning before full flowering on *cv. Sangiovese* grapes during two seasons (2013–2014) caused significantly lower must sugar content (1.6°Brix), higher must titratable acidity concentration (1.8 g/L), higher must anthocyanins and phenolic concentration but also significantly lower grape yield **(Frioni et al., 2016)**. However, same authors reported that when treatment was applied after in the period close to full flowering (50% of flower caps fallen) no grape yield was obtained, thus application timing has a key role on the determination of final outcome **(Frioni et al., 2016)**.

5.1.1.2 Shoot trimming

The synthesis of sugars takes place in leaves which are afterwards transported via phloem to grape berries (see 4.1.2). Thus, regulating the leaf area and fruit mass ratio (LA/FM) with the application of shoot trimming may be used to reduce the concentration of sugars and/or slow-down sugar accumulation in berries and prevent earlier harvest. This was reported in a recent study that investigated the influence of shoot trimming after fruit set on *cv. Grenache* grape composition during three seasons (2010–2012), whereas sugar concentration was lower by ~3°Brix when compared to the control. However, shoot trimming also caused a reduction in anthocyanins by 10%, pH by 0.1 and bunch weight by ~10% **(Martinez De Toda et al., 2013)**. Other study investigated the impact of post-véraison shoot trimming on *cv. Sangiovese* grapes during two seasons (2011–2012) and reported a significant decrease of total soluble solids (1.2°Brix) while grape composition parameters (e.g. pH, titratable acidity, anthocyanins) and grape yield did not differ significantly **(Palliotti et al., 2013)**. Stoll et al. (2010) reported a delay of harvest timing by 20 days, sugar reduction in berries (~4°Brix) and berry weight reduction (~9%) by application of shoot trimming after fruit set on *cv. Riesling* grapes. Therefore, these findings are indicating that effect of shoot trimming on berry composition and grape yield depends on many different factors such as variety, vintage, climatic factors, timing and severity of application **(Palliotti et al., 2014)**.

5.1.1.3 Defoliation

Defoliation is another technique that regulates LA/FM, thus it may be used to reduce sugar content in grapes. In particular, Poni et al. (2013) studied the impact of leaf removal applied on *cv. Sangiovese* grapes and reported a significant reduction of must total soluble solids (1.3–2.4°Brix) and total acidity (~0.7–0.9 g/L) with a lack of differences in other yield and other grape composition parameters (e.g. anthocyanins). However, the final outcome may not be always positive e.g. significant decrease of sugar content in berries without significant differences in yield and other grape quality parameters. In fact, partial defoliation applied on *cv. Istrian Malvasia* grapes caused reduction of grape yield and the increase of sugar content in berries **(Bubola et al., 2009)**, which is controversial to previously cited study. Therefore, similarly to shoot trimming the effect of defoliation on grape yield and berry composition depends on several variables.

5.1.1.4 Late irrigation

Irrigation of plants in the period after véraison, especially when combined with shoot trimming could cause reduction of sugar accumulation. This is due to nutrients competition between grape berries and lateral shoots which are plant response on shoot trimming and applied irrigation **(Palliotti et al., 2014)**. In particular, with the application of later irrigation after véraison sugar content of *cv. Cabernet Sauvignon* grapes was reduced without differences in phenolic profile and wine quality **(Fernandez et al., 2013)**. Other author, reported only a minor reduction in sugar content of *cv. Cabernet Sauvignon* grapes when irrigation was doubled during the ripening period of a hot season **(McDonnell, 2011)**.

5.1.1.5 Growth regulation via hormones

Berry growth and accumulation of sugars may be regulated by different hormones which are stimulating or inhibiting these processes. Böttcher et al. (2011) investigate influence of 1–naphthaleneacetic acid (auxin) treatment when applied in pre-véraison period on *cv. Shiraz* grapes which resulted in slower berry development, increased berry size, improved synchronicity of total soluble solids accumulation and lack of differences in wine sensory. Other study reported that application of brassinazol and 1-methylcyclopropene inhibitors of epi-brassinolide and ethylene formation (growth hormones), respectively can slow-down growth process **(Symons, 2006)**. Sugar content may be regulated also by application of synthetic forchlorfenuron (cytokinin) on table 'Flame Seedless' grape with following effects of berry mass increase and berry color reduction **(Peppi and Fidelibus, 2008)**. Even if effective, the utilization of growth hormones is strictly regulated and often forbidden due to the uncertainty of results and partial understanding of physiological process regulation **(Palliotti et al., 2014)**, thus more studies need to be conducted to reveal the entire impact of these growth regulators on berry development before full-commercial exploitation.

5.1.1.6   Shading

Application of shading is a technique which could be used to mitigate plant heat stress, reduce berry sugar accumulation and slow-down berry development. For example, leaves shading was applied during two seasons (1987–1988) in *cv. Cabernet Sauvignon* vineyard resulting in a lower sugar content (~1°Brix), higher pH value and different wine aromas when compared to control treatments **(Morrison and Noble, 1990)**. Recent study, investigated the influence of several shading nets treatments applied in *cv. Shiraz* vineyards and concluded that overhead shading resulted in a lower berry sugar content due to lower water loss and a lower wine alcohol level. However, with phenolic compounds and wine color differences when compared to control **(Caravia et al., 2016)**. Application of shading nets doesn't necessarily involve a significant decrease of grape sugar content, Basile et al. (2015) reported a minor decrease (at the best 0.6°Brix; 90% shading) of total soluble solids content in *cv. Aglianico* grapes. Grape sugar decrease was followed with differences in grape yield, lack of differences in must pH value and must total acidity when compared to control treatment **(Basile et al., 2015)**. Thus, shading seems as a promising technique to reduce wine alcohol level in wines certain cases. However, further clarifications related to the appropriate timing of application, duration of shading and shading placement (whole plant, specific areas of a vine) are required to fully understand the influence on the final wine quality **(Palliotti et al., 2014)**.

5.1.1.7   Early harvest

Early harvest is a simple technique which can be used to reduce total soluble solids in grapes. It is may be achieved by blending of grapes and collected at different maturity stage (e.g. véraison and full maturity) and fermentation of obtained must mixtures. Kontoudakis et al. (2011a) reported that of mixing low-alcohol wines (~5% v/v ethanol content) obtained with grapes harvested at véraison and must of grapes harvested at full phenological maturity resulted in final wine alcohol level reduction of 0.9%, 1.7% and 3.0% v/v in Cabernet Sauvignon, Merlot and Bobal wines, respectively. This wine alcohol reduction was followed with the increase of wine total acidity and the slight increase of total anthocyanins in wines produced from mixture when compared to wines produced only with grape at full phonological maturity. However, through the sensory evaluation, judges were able to detect significant differences in Bobal wines between two mentioned trials (most likely due to excessive acidity). On the other hand, lack of differences was reported for Cabernet Sauvignon and Merlot wines **(Kontoudakis et al., 2011a)**. Similar results were obtained from the study, whereas mixing of *cv. Tempranillo* grapes harvested at véraison and full phenolic maturity caused the production of wines with lower alcohol level and 'good' acidity **(Martinez De Toda and Balda, 2011)**.

5.1.2   Pre-fermentation techniques

5.1.2.1   Nanofiltration

Nanofiltration is a technique based on physical separation of grape must on a high-sugar fraction (retentate) and low-sugar fraction (permeate) by utilization of semi-permeable membranes with pores size

from 1 to 10 nm. This technique is often used to remove excessive alcohol reduction from wines. However, nanofiltration can also be used for sugar removal from grape must which will afterwards result in the production of wines with a lower ethanol level. In that regard, García-Martín et al. (2010) investigated possibilities to use two-step nanofiltration for removal of excessive sugar content in one red and white grape variety, *cv. Tinta de Toro* and *cv. Verdejo*, respectively. The best balance between color, phenolic compounds, aroma compounds losses and sugar content removal was achieved by mixing the second permeate and untreated must (T+P2), resulting in lower ethanol level of produced wines by ~1.8–2.5% v/v **(García-Martín et al., 2010)**. A recent study investigated the possibilities to used one-step and two-step nanofiltration in order to remove excessive total soluble solids in red *cv. Granacha* and white *cv. Verdejo* grape variety **(Salgado et al., 2015)**. After grape must filtration, two-step nanofiltration of red wine showed the best results, obtaining wines with ~1.4% v/v lower ethanol level, minor differences in other wine quality parameters and lack of differences in sensory analysis results **(Salgado et al., 2015)**. Nanofiltration is offering a promising results and can be applied to reduce excessive ethanol level in wines, however in certain cases **(Salgado et al., 2015)**, grape must need to be pre-filtered in order to avoid rapid foul of membranes, thus entire process might become time-consuming and uneconomic **(Longo et al., 2017)**.

5.1.2.2 Ultrafiltration

Ultrafiltration is another membrane-based technique that utilizes semi-permeable membranes with bigger membrane pore size (10–100 nm) allowing separation of grape must on a high-sugar fraction (permeate) and low-sugar fraction (retentate). Cassano et al. (2008) investigated the possibilities to clarify must of white *cv. Verdeca* grape variety by utilization of cross-flow ultrafiltration. The must processing under different transmembrane pressure conditions resulted in total suluble solids reduction up to 1.8°Brix, but also decrease of total phenolics up to 30% and a slight increase of tartaric acid content (up to 0.12 g/L) **(Cassano et al., 2008)**. Similarly to nanofiltration, grape must need to be pre-filtered to allow effective filtering which is a down side of this technique **(Longo et al., 2017)**.

5.1.2.3 Dilution

Water addition and following dilution of grape sugar content and afterwards lower wine alcohol level is a simple technique. However, water addition dilutes also other wine compounds which is a down side of this technique. Water addition is not allowed in all countries (e.g. Italy, France) while certain countries as USA **(Bisson, 1999)** and Australia **(Varela et al., 2015)** permitted utilization of water. Harbertson et al. (2009) reported that water addition (~18% v/v) and partial removal of *cv. Merlot* grape must (~18% v/v) resulted in 4°Brix sugar content reduction compared to initial high sugar content must (28°Brix) and resulted in the production of wines with similar phenolic content and aroma attributes as control (only water addition ~18% v/v).

5.1.3   Biotechnological techniques

5.1.3.1   Genetic modification organism

During the last years, possibility of genetic modification organism (GMO) application is progressively wider. Genetic modifications can be also applied to *Saccharomyces cerevisiae (Sc)* yeasts cells, altering the genome and redirecting metabolic flux away from ethanol production towards the production of other compounds (e.g. glycerol, acetic acid) **(Kutyna et al., 2010)**. In particular, over-expression of certain genes (GPD2) caused a decrease of alcohol level in Chardonnay wine by ~0.75% v/v and the increase of glycerol **(de Barros Lopes et al., 2000)**. However, significant production of acetic acid was also detected which was confirmed by sensory evaluation **(de Barros Lopes et al., 2000)**. Genetic modifications approach may be also used to produce transgenic strains of *Aspergillus niger* and *Sc* which are able to produce glucose oxidase (not existing in *Sc*) **(Malherbe et al., 2003)**. Afterwards, transgenic strain may reduce ethanol level up to 2% v/v **(Malherbe et al., 2003)**. However, glucose oxidase converts glucose to gluconic acid and hydrogen peroxide which may have a negative impact on wine quality parameters **(Varela et al., 2015)**. As it is possible to conclude, genetic modifications and GMO are offering endless possibilities to regulate fermentation process. However, public restrictions to use GMO are down side of this technique.

5.1.3.2   Non-Saccharomyces cerevisiae

Saccharomyces cerevisiae is the most common specie used for the fermentation of grape must due to its relatively high resistance to ethanol, relatively low production of undesirable by-products (e.g. acetic acid), good fermenting capacity in high-sugar must conditions, ability to metabolize all sugars from grape must etc. However, without genetic modifications ethanol yield among strains of this specie seems to be approximately the same, even if certain variability may be found among wild isolated of *Sc* **(Ciani et al., 2016)**. Therefore, to reduce alcohol level in wines, many studies were conducted aiming to find appropriate alternative yeast specie which may be utilized for must fermentation. Reduction of ethanol content in wines is achieved by differences in production of by-products (e.g. glycerol, acetic acid) or biomass synthesis during fermentation of alternative species when compared to *Sc* **(Ciani et al., 2016)**. *Candida zemplinina (Cz)* (synonym *Starmerella bacillaris*) is one of the species that may be used to reduce alcohol level in wines. Apart from lower wine ethanol level up to 2% v/v when compared to *Sc* fermentation **(Englezos et al., 2016a)**, wines produced with whether only *Cz* or combined *Cz* and *Sc* may be characterized with higher glycerol production, higher acetic acid production, changes in aromatic profile e.g. lower isoamyl alcohol or 2-phenylethanol concentration **(Englezos et al., 2016a, 2016b; Giaramida et al., 2013; Romboli et al., 2015; Sadoudi et al., 2012)**. *Saccharomyces paradoxus (Sp)* may also be used to reduce ethanol level in wines up to 0.35% v/v when compared to *Sc* **(Orlic et al., 2007)**. Fermentation with *Sp* may also be followed by higher malic acid consumption, higher glycerol production, lower production of ethyl acetate and isoamyl acetate **(Orlic et al., 2007; Redzepovic et al., 2003)** etc. As an alternative to *Sc* for excessive alcohol removal, *Torulaspora delbrueckii (Td)* may serve as a solution. Several studies reported that utilization of *Td* as single specie or combined with *Sc* in fermentation, wine ethanol level may be reduced up to 0.4–0.5% v/v. These fermentations may be as well followed with lower malic acid content, higher volatile acidity and glycerol or different aromatic complex

in wines when compared to wines obtained with single *Sc* fermentations (**Loira et al., 2015; Puertas et al., 2017; Ramírez et al., 2016; Sadoudi et al., 2012**). The potential yeast species to remove excessive wine alcohol whether as single culture fermentation or coupled with *Sc* could be also *Candida stellata* (**Contreras et al., 2014b**), *Metschnikowia pulcherrima* (**Contreras et al., 2014a, 2014b; Sadoudi et al., 2012**), *Saccharomyces uvarum* (**Contreras et al., 2014a**), *Lachancea thermotolerans* (**Gobbi et al., 2013**) etc. Due to high diversity of potential alternatives to *Sc* this technique seems as a promising solution to address the production of wines with lower alcohol level and good sensory characteristics. Furthermore, this technique is inexpensive and simple.

5.1.4   Post-fermentation techniques

5.1.4.1   *Nanofiltration*

Nanofiltration as a post-fermentation technique works on a similar principle as for pre-fermentation technique (see 5.1.2.3), whereas by wine processing two fractions are obtained after separation, the high-ethanol fraction (permeate) and low-ethanol fraction (retentate). Similarly to all membrane separation process that have goal to reduce alcohol level in wines, the efficiency of nanofiltration depends on mixture of factors such are ethanol rejection coefficient, other wine compounds rejection coefficient (e.g. aroma compounds, acids, phenolic compounds) permeate flux, operating conditions (e.g. temperature, pressure, time) and membrane characteristics (e.g. material, pore size). In that regard, Catarino and Mendes (2011) conducted a study aiming to evaluate the efficiency of several nanofiltration membranes by regulating several of mentioned factors. Authors concluded that certain membranes may be used for the production of low-alcohol wines, especially if nanofiltration is combined with pervaporation (**Catarino and Mendes, 2011**). However, this additional equipment (e.g. pervaporation) may increase initial investment which is a down side of this combined approach. Other study reported that utilization of nanofiltration as a single technique may decrease ethanol content until 8% v/v which is followed by less than 15% w/v aroma compounds content decrease (**Labanda et al., 2009**).

5.1.4.2   *Reverse osmosis*

Reverse osmosis is a similar technique to nanofiltration and requires utilization of semi-permeable membranes with smaller pores size (0.1–1nm) when compared to nanofiltration. Thus reverse osmosis requires higher operating pressure and higher energy consumption when compared to nanofiltration which is one of the down sides of this technique (**Gonçalves et al., 2013**). Other down sides of reverse osmosis may be related lower permeate flux when compared to nanofiltation (**Catarino and Mendes, 2011**). However, several studies reported that utilization of reverse osmosis may be used for partial dealcoholization of wines (~2% v/v reduction) with hardly detectable sensorial differences (**Gil et al., 2013**) and with lack of differences in phenolic compounds content (**Bogianchini et al., 2011**) when compared to original wines.

5.1.4.3   Pervaporation

Pervaporation is another membrane technique, however comparing nanofiltration and reverse osmosis membrane, pervaporation needs utilization of hydrophobic membranes which are not allowing liquid (e.g. wine) passage through membrane pores. Instead, ethanol and other wine volatile compounds (e.g. aroma compounds) are partially evaporating on relatively low temperatures (~40°C) and migrating through the membrane as a vapor due to differences in a partial pressure created by the vacuum on the other side of membrane. Vapor rich in ethanol and with a certain amount of aromatic compounds is afterwards condensed **(Takács et al., 2007)**. Takács et al. (2007) evaluated possibilities to apply pervaporation as a technique to remove ethanol content from Tokaji Hárslevelű wines and concluded that working temperature plays a key role on the process efficiency, whereas 40°C was optimal to produce almost free alcohol product that matches organoleptic characteristics of a wines. Authors are also pointing the down side of this technique which is related to high initial economic investments (315k€) **(Takács et al., 2007)**. Other study, investigated the possibility to use pervaporation in combination with nanofiltation to remove excessive ethanol from a red wine, whereas high-quality low-alcohol wines were produced **(Catarino and Mendes, 2011)**. However, initial economic investments are most likely even higher when compared to single pervaporation technique.

5.1.4.4   Evaporative perstraction

Evaporative perstraction or also called osmotic distillation is a technique like pervaporation that use hydrophobic membranes, whereas separation of volatile compounds (e.g. ethanol, aromatic compounds) from the liquid (e.g. wine) is achieved by vapor pressure gradient between two sides of the membrane. Differences between two techniques are utilization of water that flows as stripping fluid in contra current on membrane side opposite to wine, and absorbs volatile permeate compounds. The up side of this technique is the fact that solubility of aroma compounds is higher in wine (feed fluid) when compared to pure water (stripping fluid), so the transfer of aromatic compounds in the water phase is limited **(Diban et al., 2008)**. Thus, evaporative perstraction may be used for production partially dealcoholized wines (2% v/v removal) with good sensory characteristics. In fact, Diban et al. (2008) reported that despite certain aroma compounds losses in Merlot wines during the partial alcohol removal (2% v/v), there was a lack of differences in wine sensory characteristics. Other studies also reported lack of difference in wine sensory characteristics as well **(Liguori et al., 2013; Lisanti et al., 2013)**, but also in volatile acidity, organic acids concentration, total phenolic content and color **(Liguori et al., 2013a)** in Aglianico wines once ethanol content was removed up to 2% v/v. However, Lisanti et al. (2013) also reported that differences in wine sensory characteristics were noticeable once ethanol content was reduced by 5% v/v, indicating that this technique might be suitable only for 'mild' ethanol removal from wines (up to 2% v/v). In fact, total dealcoholization (0.2% v/v remaining ethanol content) of Aglianico wines by evaporative perstraction caused reduction of aroma compounds by 98% **(Liguori et al., 2013b)**. Another study also reported a significant aroma compounds losses (44–70%) in red wine once ethanol content was reduced up to 38% **(Varavuth et al., 2009)**.

Spinning cone column is based on the production of low-alcohol wines in two steps. The first step presents dearomatization of wine in spinning cone column under vacuum and low temperatures (26°C). The products of the first step are the gas fraction (stripping agent and volatile compounds) and liquid fraction (dearomatized wine). The second step presents ethanol removal from the dearomatized wine in spinning cone column at equal pressure and slightly higher temperature (~30°C). Dealcoholized and dearomatized wine is afterwards mixed with aromatic fraction to obtain lower-alcohol level wines (**Belisario-Sánchez et al., 2012, 2009**). Lower alcohol level wines produced by a spinning cone column may have acceptable antioxidative ability, phenolic compound content (**Belisario-Sánchez et al., 2009**), and aromatic compounds content when compared to raw wines (**Belisario-Sánchez et al., 2012**). However, spinning cone column has a high demand of energy when compared to other techniques related to physical removal of ethanol (e.g. evaporative perstraction) which is down side of this technique (**Diban et al., 2013**).

5.1.4.6   Vacuum-distillation and supercritical CO_2 extraction

The combination of vacuum-distillation and supercritical extraction with CO_2 may also serve as technique to remove excessive alcohol from wine. The working principle is based on two-step processing. The first step presents vacuum distillation at a certain temperature range (24–28°C) and high vacuum (35–50mbar) that separates wine on a low-volatile fraction (wine base) and high-volatile fraction (alcohol and volatile aromas) due to differences in boiling temperatures. The second step presents supercritical CO_2 extraction at high pressure (80–100bar) and certain temperature range (25–35°C) that separate high-volatile fraction on liquid ethanol-water mixture and gas mixture (CO_2 and aromas) due to differences in extraction features. The gas mixture is afterwards adequately separated and aromas added into wine (**Seidlitz et al., 1992**). As for the majority of post-fermentation techniques down sides are certain sensorial differences that may occur due to partial removal of aromas and aimed ethanol removal (**Medina and Martinez, 1997**) and a high capital cost of the process (e.g. high-vacuum distillation) (**Schmidtke et al., 2012**).

5.2   Application of late winter pruning on *cv. Sangiovese* grapes from organic management and its impact on berry composition

Teslić, N., Versari, A. 2016. Effect of late winter pruning on Sangiovese grape berry composition from organic management, in: Ventura, F., Pieri, L. (Eds.), Proceedings of the 19th conferences of Italian association of agrometeologists: New adversities and new services for agroecosystems. University of Bologna, Bologna, Italy, pp. 131–134

Organic grape cultivation and winemaking need to be performed under stricter rules compared to a conventional approach. Therefore, application of many excessive alcohol removal techniques which are allowed in conventional winemaking (e.g. nanofiltration, spinning cone column) is prohibited (**EC, 2012**).

However, certain techniques are allowed in both approaches (e.g. late winter pruning). Thus, later winter pruning may have a paramount importance for organic farming since it may serve as a possible technique to reduce grape sugar content and wine alcohol level. The possibility of slowing down berry sugar accumulation by using late winter pruning was elaborated under viticulture techniques (see 5.1.2.1), whereas all cited studies were conducted on grapevines cultivated conventionally. To our best knowledge, there is a lack of information in literature related to late winter pruning application on *Vitis vinifera* from organic farming. Therefore, in presented experiment late winter pruning was applied on *cv. Sangiovese* from organic farming, aiming to reduce total soluble solids in berries at harvest period.

5.2.1   Materials and Methods

5.2.1.1   Vineyard management

The experiment was conducted during the vintage 2015, in a mature vineyard of *cv. Sangiovese* (clone FEDIT 30 ESAVE), trained to Cordon du Royat, grafted on Kober 5BB rootstock and with a 2.8 m x 1.0 m vine spacing (3,571 plants/ha). The vineyard is located in Tebano (44°17′7″ N, 11°52′59″E, Faenza, RA, Italy), in a medium hill slope (117 m a.s.l.), with south-east/north-west and downhill oriented rows. Since 2007, the vineyard was managed as organic in accordance with the European Council Regulations **(EC, 2007)**. Also starting from 2007, no irrigation and no fertilizers have been applied. The vineyard was protected from diseases and pests, using products for organic farming allowed by the European Council Regulations **(EC, 2002)**.

5.2.1.2   Design of experiment

The experiment was consisted of 3 trials that were performed in a block-randomized experimental design:
Trial 1 (T1– control) –winter pruning applied in December, BBCH=0 (for BBCH scale see Appendix D);
Trial 2 (T2) – winter pruning applied in March, BBCH=0;
Trial 3 (T3) – winter pruning applied in April, BBCH=12.
All trials were applied in 3 replications for 3 experimental plots, thus each trial included in total 9 vine samples (27 vine samples for all trials). The randomized blocks used for the experiment were in one row on the vineyard border and were spread along entire row. Selection of the plants within same block was made according to health condition and plant age, whereas plants more uniform according to these parameters were chosen for examination.

5.2.1.3   Vine development, berry composition and grape yield analysis

The vine development and occurrence phenological stages (e.g. bud burst) were monitored by one person during the growing season with a BBCH scale *(for BBCH scale see Appendix D)*. It was considered that bud burst occurred when green shoot tips were clearly visible on 50 % of buds (BBCH=8), flowering

when 50% of flowerhood were fallen (BBCH=65), véraison when 50% of bunches were colored (BBCH=83) and harvest when fruit reached maturity (BBCH=89).

Starting from véraison occurrence until the harvest, sampling was performed five times for each trial and in each experimental (9 samples in total). For each sample approximately 100 berries were randomly collected from the top, middle and bottom of the clusters to obtain berry composition parameters as followed: berry weight was measured with technical balance (Gibertini Elettronica S.r.l., Milan, Italy), sugar content was measured with electronic refractometer (Maselli Misure S.P.A., Parma, Italy), titratable acidity and pH were measured with automatic titrator (Crison Instrument SA, Barcelona, Spain). Additionally, at harvest, grape yield parameters, such as number of clusters per plant and yield per vine which were measured with digital dynamometer (Wunder SA-Bi S.r.l, Milan, Italy) and cluster weight that was measured with technical balance (Gibertini Elettronica S.r.l., Milan, Italy).

5.2.1.4   Statistical analysis

Parametric data were analyzed with one-way Anova to detect differences in berry composition or grape yield parameters and parameters with significant difference were afterwards evaluated with Least significant difference (LSD) post-hoc test to ascertain the difference between trials. All tests were conducted with a confidence level set at 90% and 95%.

5.2.1.5   Climatic characterization of the vintage 2015

For climatic characterization of the vintage 2015, meteorological data from a grid cell Tebano (11.7816E 44.2725N) during the period 1961–2015 was used to calculate T_{mean}, CI, GDD, DI and DSI (see 2.2.2 for details). Mean growing season temperature (T_{mean}) during the vintage 2015 was 19.6°C and characterized as 'hot' *(Table 5.1)* **(Fraga et al., 2014)**, which is noticeably higher compared to T_{mean} (17.70°C; *Table 5.1*) during the period 1961–2014 which was characterized as 'warm' **(Fraga et al., 2014)**. T_{mean} during the vintage 2015 was slightly higher compared to optimal T_{mean} for the cultivation of Sangiovese grapes (~16.9–19.4°C; *Fig. 2.3*). Cool night index (CI) during the vintage 2015 (12.79°C) was similar to CI during the period 1961–2014 (12.29°C; *Table 5.1*) and characterized as cool nights **(Tonietto, 1999)**, which is in optimal temperature range (~10–15°C) for anthocyanins synthesis as it was reported in several studies **(Kliewer, 1977; Tonietto and Carbonneau, 1998)**. Thermal accumulation during the vintage 2015 (1955.60 units; *Table 5.1*) presented as Growing degree day (GDD) was noticeably higher compared to same BI value during the period 1961–2014 (1675.27 units; *Table 5.1*). According to Gladstones (1992), thermal accumulation during the vintage 2015 was still in the range necessary for the production of high-quality wines (~1400–2000 units). However, due to ongoing warming it is expected that Tebano area becomes 'too hot' for the production of high-quality wines in future decades. Water availability presented as Dryness index (DI) was 37.94mm at the end of vintage 2015 *(Table 5.1)*, which is characterized as moderately dry **(Tonietto and Carbonneau, 2004)**. This moderately dry condition during the vintage 2015 comparing to sub-humid conditions (99.88 mm; *Table 5.1*) detected during the period 1961–2014 are suggesting that vintage 2015 required implementation of irrigation systems. During the vintage 2015 there were 163.59 days with less than 1mm of precipitation, which was slightly higher compared to the same BI value during the period 1961–2014 (160.00 days; *Table 5.1*). Obtained result is suggesting that sugar content in Sangiovese grape berries was higher during the vintage 2015 than

average sugar content in Sangiovese grape berries during the period 1961–2014, since a high correlation between DSI and sugar content in Sangiovese grape berries was detected previously (see 4.2.2.2). Due to mentioned, the vintage 2015 was appropriate for development of techniques which may be used to mitigate the influence of the climate change on Sangiovese grapes quality (e.g. late winter pruning), as it was noticeably hotter and drier compared to the period 1961–2014.

Table 5.1 Bioclimatic indices during the vintage 2015 and average bioclimatic indices values from the 1961 until the 2014.

Index	T_{mean} [°C]	CI [°C]	GDD [units]	DI [mm]	DSI [days]
1961–2014	17.70	12.29	1675.27	99.88	163.59
2015	19.6	12.79	1955.60	37.94	160.00

5.2.2   Results and Discussion

5.2.2.1   Vine development

Bud break (BBCH=8) appeared in the approximately same period for all trials *(Fig. 5.1)*. Starting from the bud burst until the middle of August, plant development of control grapevines (T1) was slightly faster when compared to vines submitted to T2. However, since the middle of August, T2 tended to develop slightly accelerated when compared to T1. In grapevines submitted to T3 a noticeable delay was detected in initial period of development when compared to vines submitted to T1 and T2. The maximum differences in plant development of T3 compared to T1 and T2 were reached between bud burst and flowering which were gradually compensated towards to véraison, ultimately leading, to a fastest development of plants submitted to T3 respect to the T1 and T2 that lasted until the harvest period *(Fig. 5.1; Fig. 5.2)*.
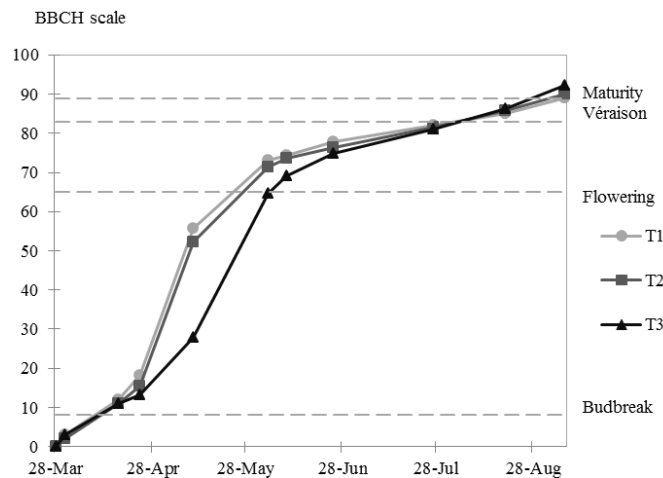
Figure 5.1 *cv. Sangiovese* vine development monitored over the vegetative period during the vintage 2015. T1 – winter pruning applied in December (BBCH=0); T2 – winter pruning applied in March (BBCH=0); T3 – winter pruning applied in April (BBCH=12).

Figure 5.2 cv. Sangiovese vine development progress on the 4th of May; left: T1 – winter pruning applied in December (BBCH=0); center: T2 – winter pruning applied in March (BBCH=0); right: T3 – winter pruning applied in April (BBCH=12).

Flowering (BBCH=65) occurred slightly earlier in T1 respect to T2, and noticeably earlier respect to T3. On the other hand, véraison (BBCH=83) occurred approximately at the same time for all trials while maturity appeared slightly earlier in plants submitted to T3 respect to T2 and T1.

<div align="center">

5.2.2.2   Grape yield

</div>

Grape yield parameters, such as the number of clusters, cluster weight and weight per berry were not significant within trials due to high variability among the same trial (***Table 5.2***). On the other hand, significant differences were detected in yield per plant (***Table 5.2***). The lowest crop load of T3 comparing to T1 and T2, same as the slowest grapevine development in early stages may be explained by different timing of winter pruning application, whereas for T1 and T2 pruning was applied before bud burst while for T3 after bud burst. Obtained results are aligned with a recent study where winter pruning was applied on *cv. Sangiovese* grapes causing a significant yield reduction when winter pruning was applied after bud burst **(Frioni et al., 2016)**.

Table 5.2 Grape yield and berry composition of *cv. Sangiovese*. T1 – winter pruning applied in December (BBCH=0); T2 – winter pruning applied in March (BBCH=0); T3 – winter pruning applied in April (BBCH=12). LSD: a – different from T3 with 95% significance; b – different from T1 with 90% significance; c – different from T1 with 95% significance.

Parameter	Trial 1	Trial 2	Trial 3	p-value
Yield [kg/plant]	2.24a	2.14a	1.25	0.068
Cluster weight [g]	158.76	138.90	114.16	NS
Number of clusters per plant	14	15	11	NS
Berry weight [g]	2.01	2.07	1.95	NS
Sugar content [°Brix]	23.0	24.13b	24.43c	0.052
Titratable acidity [g/L]	7.55	7.60	7.71	NS
pH	3.17	3.14	3.14	NS

At harvest, berry sugar content was 24.3, 24.13 and 23.0 °Brix in T1, T2 and T3, respectively *(Table. 5.2)*. Due to slightly faster grapevine development, berries from T1 had a slightly higher sugar content on

the 29th of July, compared with T2 and T3. However, differences within trials decreased until the 26th of August, when berry sugar content was same in all trials *(Fig. 5.3)*.
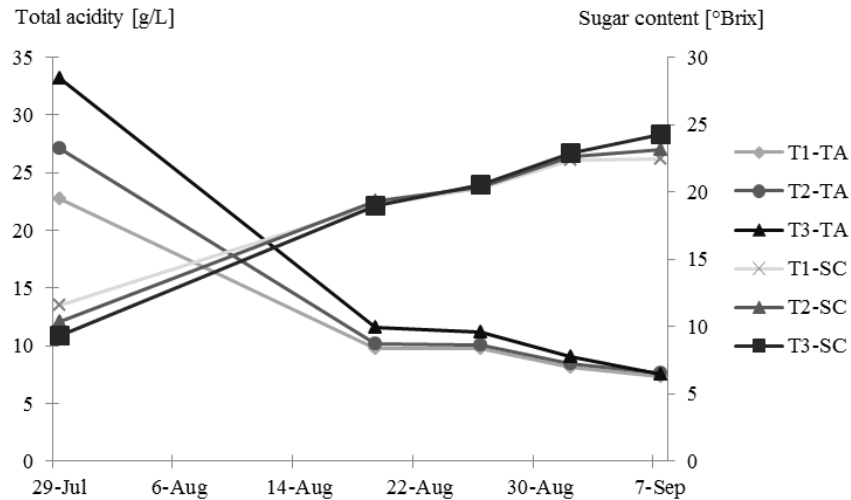
Figure 5.3 Chemical composition of *cv. Sangiovese* must during vintage 2015. SC – Sugar content; TA – Titratable acidity; T1 – winter pruning applied in December (BBCH=0); T2 – winter pruning applied in March (BBCH=0); T3 – winter pruning applied in April (BBCH=12).

Starting from the 26th of August, sugar accumulation in berries from T3 was faster respect to the T1 and T2, ultimately leading that at harvest T3 had higher sugar content respect to T1 and T2 *(Fig. 5.3; Table 5.2)*. The highest sugar content at harvest detected in berries of grapevines submitted to T3 may possibly be explained with different source-sink balance within trials. Grapevines with higher yield per plant (T1 and T2) have higher carbon demand in order to reach certain value of sugar content while plants with a lower yield per plant (T3) have lower carbon demand to reach the same value of sugar content (**Bobeica et al., 2015**).Thus, due to lower carbon competition within clusters, plants submitted to T3 had higher berry sugar content at harvest respect to T1 and T2. However, leaf to fruit area was not monitored, thus the last statement needs to be taken with caution. Although, the highest berry sugar content was detected in plants submitted to T3, which is opposite to the desired objective, further experiments need to be conducted in order better understand the possibilities of sugar content reduction in plants by application of late winter pruning. Apart from later winter pruning, experiments should also include monitoring of leaf to fruit area and if needed, application of cluster thinning and leaf removal to reduce the potential differences in source-sink balance among trials. Inversely to sugar content, at the end of July, total acidity was the highest in T3 comparing to T1 and T2. Differences in titratable acidity levels among trials were gradually smaller starting for the end of July, whereas at the beginning of September no differences were detected in total acidity levels among trials *(Fig 5.3)*. At harvest slightly higher total acidity levels were detected in berries from grapevines submitted to T3 compared with those of T2 and T1 *(Fig. 5.3)*.

However, even if detected differences were not significant *(Table 5.2)*. Berry juice pH value at harvest was similar in all trials 3.14, 3.14 and 3.17 in T3,T2 and T1, respectively. These findings are partly aligned with a recent study that reported a significant must sugar content decrease, significant must titratable acidity increase and lack of differences in must pH value when winter pruning was applied on *cv. Sangiovese* grapevines after inflorescence swelling (BBCH=55) respect to grapevines where pruning was applied before bud burst (BBCH=0) **(Frioni et al., 2016)**.

5.2.3   Conclusions

Late winter pruning had an influence on *cv. Sangiovese* grapevine development. In particular, grapevines submitted to T3 had a delay in early periods of plant development compared to T1 and T2. This delay in grapevines submitted to T3 was compensated until véraison, ultimately leading, to the fastest development of plants submitted to T3 respect to T1 and T2, until harvest period. The application of late pruning to grapevines significantly modified sugar content in plants submitted to T3 compared to control trial (T1). Also, the application of late pruning caused a significant reduction of yield per plant in T3 compared to T1 and T2. On the other hand, differences of pH, TA, berry weight, number of clusters and cluster weight were not significant. Although, the highest berry sugar content was detected in plants submitted to T3, which is opposite to the desired objective, further experiments need to be conducted in order better understand the possibilities of sugar content reduction in plants by application of late winter pruning. Apart from later winter pruning, experiments should also include monitoring of leaf to fruit area and if needed, application of cluster thinning and leaf removal to reduce the potential differences in source-sink balance among trials. Furthermore, trials need to be performed during at least two seasons to ascertain the conclusions.

5.3   Combination of 'early *green* harvest' and non-*Saccharomyces cerevisiae* yeasts as an approach reduce ethanol level in Chardonnay wines

Removal of excessive alcohol content from wine by utilization of single technique may have a negative impact on wine sensory characteristic due to aroma compounds removal **(Catarino and Mendes, 2011)**. Therefore, certain studies examined the possibilities to use two techniques (e.g. nanofiltration and pervaporation) as a combined method to remove excessive alcohol content from wines with a lesser impact on wine sensory characteristics compared to single technique method **(Catarino and Mendes, 2011)**. However, to install equipment for nanofiltration and pervaporation winemakers might require capital initial investments (315k€ only for pervaporation; **Takács et al., 2007**). Thus, presented experiment evaluated possibilities to use 'early *green* harvest' (viticulture technique) and non-*Saccharomyces cerevisiae* (biotechnological technique) as a combined method to remove excessive wine alcohol due to its simplicity and inexpensiveness.

5.3.1 Materials and Methods

5.3.1.1 Reagent and chemicals

Citric acid, lactic acid, L-malic acid, succinic acid, acetonitrile, (+)-catechin, (–)-epicatechin, caffeic acid, glycerol, sodium hydroxide, sodium carbonate, 2-ethyl butyric acid, dimethyl carbonate, Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) and gallic acid were purchased from Sigma-Aldrich (Steinheim, Germany). L-tartaric acid, sulfuric acid, *p*-coumaric acid, Folin-Ciocalteu's reagent, acetic acid, iodine and calcium hydroxide were purchased from Merck (Darmstadt, Germany). Glucose, yeast extract and peptone were purchased from Oxoid (Basingstoke, United Kindom). Ethanolic solution of phenolphthalein and potassium hydrogen sulfate were purchased from Carlo Erba (Milan, Italy). Ferulic acid was purchased from Extrasynthese (Genay, France). Glycerol enzymatic kit was purchased from Steroglass (Perugia, Italy). Methanol was purchased from VWR (Leuven, Belgium). Silicon antifoam was purchased from Ing. Castore Bullio (Milan, Italy).

5.3.1.2 Yeasts activation

The yeast strains utilized for the fermentation trials were: low-ethanol trial *(Y1)* – Exotics (hybrid *Saccharomyces paradoxus/Saccharomyces cerevisiae*) (Oenobrands, France), low-ethanol *alternative* trial *(Y2)* – *Candida zemplinina FT811* (yeast strain isolated at the University of Teramo) in sequential fermentation method with Exotics (*Y1*) and *control* trial *(Y3)* – Vin13 (*Saccharomyces cerevisiae*) (Oenobrands, France). For each fermentation (total n=18; **Fig. 5.4**), yeast strains were primarily inoculated in 10 ml of solution obtained with peptone (10 mg/mL), glucose (20 mg/mL) and yeast extract (10 mg/mL) and then incubated (24h at 25°C). Afterwards, the yeast strains were re-inoculated into 0.25 L of non-clarified and previously pasteurized must (must was pasteurized at 65°C for 30 min) and then incubated (24h at 25°C) to obtain sufficient quantity of active yeast cells for fermentations (at least 10^7 log CFU/mL). Once properly prepared, yeasts were added directly into grape must (for details see 5.3.1.3). In order to prevent the appearance of late spontaneous fermentation due to activity of wild *Saccharomyces cerevisiae* strains, trials inoculated with *Candida zemplinina* were inoculated in sequential fermentation method (at ethanol level ~7–8%) with 30 g/hL of yeast *Y1* (according to producer's instructions).
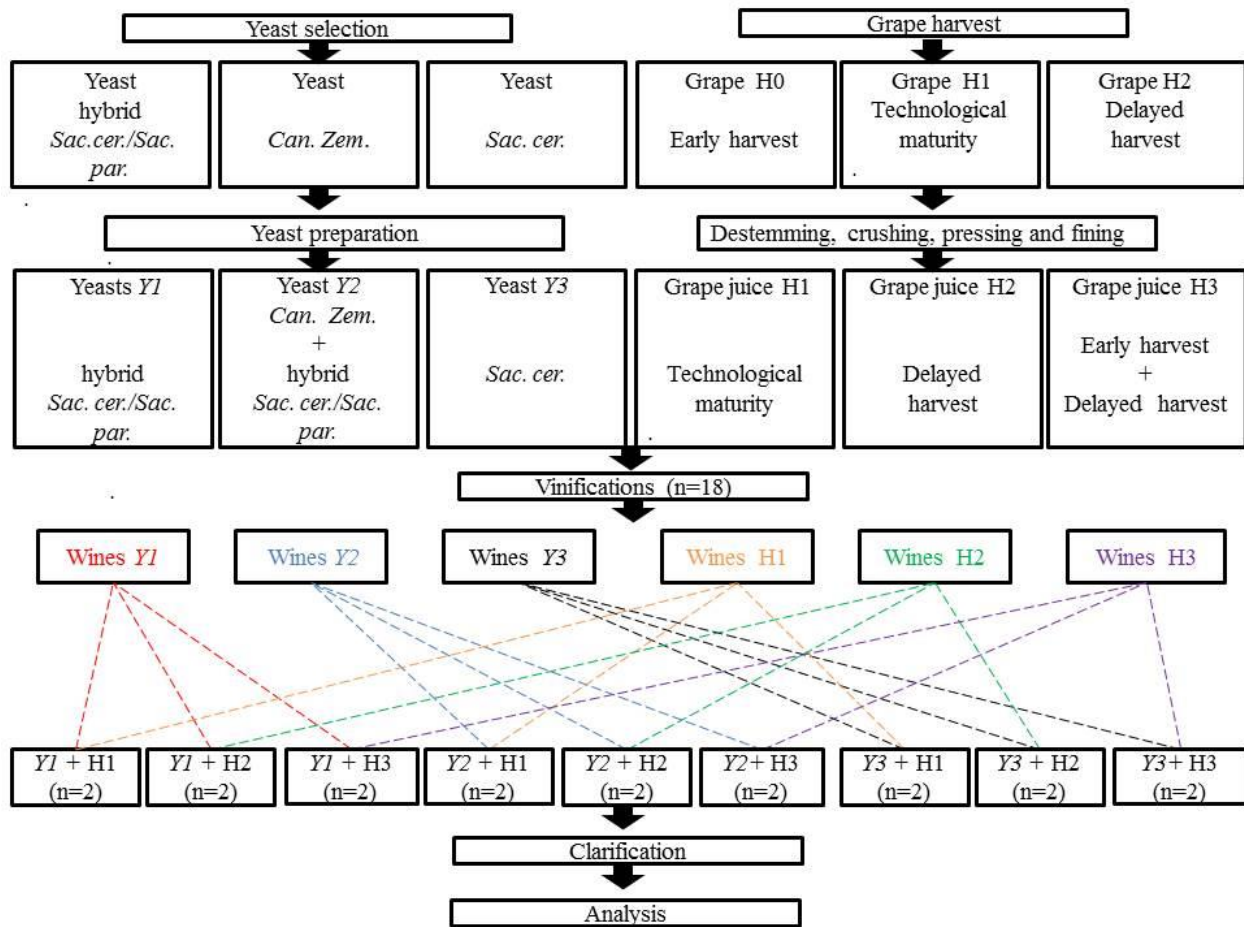
Figure 5.4 Experiment design and winemaking protocol (modified from Teslić et al., 2018).

5.3.1.3   Grape harvest and vinification procedure

The experiment was performed during vintage 2016 with *cv. Chardonnay* grapes. Grapevines were trained to free cordon with 2.5 m × 1.0 m spacing between plants (4000 plants/ha). The vineyard and experimental winery were located in Tebano (44°28'7" N; 11°77'5" E; Faenza, Italy). Harvest-0 (H0 - considered as 'early *green* harvest') was conducted at véraison, whereas 25 kg of manually thinned grapes was collected, which were afterwards manually destemmed and crushed. Obtained grape juice was treated with potassium metabisulfite (5 g/hL, AEB, Brescia, Italy) and stored into 10L plastic tank at low-temperature regime (-20°C) until fermentation.

Harvest-1 (H1) was conducted at grape technological maturity (*control*) - based on sugar concentration and total acidity – whereas ~100 kg of grapes was manually harvested and afterwards crushed and pressed using a semi-automatic press (22620M, Spedeil, Ofterdingen, Germany). Grape juice was racked into 100L stainless-steel tank, treated with potassium metabisulfite (8 g/hL), pectolytic enzymes (1 g/hL, Lafazym CL, Laffort, France), silica gel (30 g/hL, Baykisol 30, AEB, Brescia, Italy), gelatin (3 g/hL,

Gelsol, AEB, Brescia, Italy), bentonite (30 g/hL, Superbenton, Dal Cin, Italy), in respective order and stored at +4°C for clarification. After 48h of clarification, grape juice was transferred into six 20L stainless-steel tanks (in duplicate for each of 3 yeasts), treated with nutrients for yeasts (Nutristart, 30 g/hL, containing 0.39 mg/L of thiamine, Laffort, France), potassium metabisulfite (4 g/hL) and inoculated with yeast strains (for details see 5.3.1.2).

Four days after H1, ~200 kg of grape was manually harvested during harvest H2 ('*delayed maturity*') and treated as described for grapes at H1. The obtained must was split into two equal batches and placed into two 100L stainless-steel tanks. The first half of grape juice was processed similarly as grape juice at H1 (six 20L stainless-steel tanks, in duplicate for each of 3 yeasts). The second half was mixed with must H0 (~10% v/v, of added H0 grape juice) to match the sugar concentration of musts H2 *(Table 5.3)*. Afterwards, H3 grape juice was treated as must at H2 (six 20L stainless-steel tanks, in duplicate for each of 3 yeasts). Fermentation trials were run under controlled temperature regime (20°C *Y1* and *Y2*, 17°C *Y3*) whereas sugar consumption by yeasts was daily monitored using a Babo densimeter. At the end of fermentation processes (below 0.2 g/L of residual sugars), wines were treated with potassium metabisulfite (7 g/hL) and stored at +4°C for clarification. After five days of clarification, clear wine was transferred into glass containers and stored at 0°C for cold stabilization for 20 days. Stabilized wines were bottled into 1L glass bottles and sealed with crown caps, thus stored at +17°C until analysis.

Table 5.3 Chemical composition of Chardonnay grape juice obtained during vintage 2016.

Variables	pH	Total acidity (g/L)[1]	Sugar content (°Brix)
H0	2.61±0.06	26.29±0.05	9.6±0.1
H1	3.19±0.01	6.45±0.00	19.3±0.0
H2	3.30±0.02	6.28±0.04	20.5±0.1
H3	3.06±0.02	8.95±0.04	19.5±0.0

Table values present mean (±SD) of three analyses.
[1] – total acidity expressed as g/L of tartaric acid

5.3.1.4 Chemical analysis

Must sugar content, pH and total acidity

Grape sugar content was examined with a refractometer (PAL-1, Atago, Tokyo, Japan) while pH and total acidity of grape juice were analyzed with pH meter (pH 209, Hanna Instruments, Padova, Italy) according to the official European Commission methods **(EC, 1990)**.

Alcohol

Wine ethanol content was examined with hydrostatic balance and heat-stream distiller (Ing. Castore Bullio, Milan, Italy) according to the official European Commission methods **(EC, 1990)**.

Glycerol

Wine glycerol content was examined by UV-Vis spectrophotometer (Cary 60, Agilent Technologies, Santa Clara, USA) and enzymatic kit according to manufacturer's instructions (Steroglass, Milan, Italy).

Phenolics

Total polyphenol content was analyzed with colorimetric assay by UV-Vis spectrophotometer (Cary 60, Agilent Technologies, Santa Clara, USA) at 750 nm **(Singleton and Rossi, 1965)**. Calibration curve of gallic acid (0–0.665 mM; R^2=0.994) was used for quantification of phenolic compounds.

Individual phenolic compounds content was analyzed with high-performance liquid chromatography system (HPLC; Dionex IC-500, Milano, Italy), diode array detector (DAD) and Inertsustain C18 column (5µm, 4.6 × 250 mm; GL Science, Tokyo, Japan). Prior to manual injection into HPLC system that was conditioned at 30°C, wine samples were filtered with 0.2 µm cellulose acetate filter (GVC Filter Technology, Sanford, USA). Phenolic compounds separation was conducted at 0.5 ml/L flow rate with solvent A (distilled water:acetic acid = 95:5; % v/v) and solvent B (acetonitrile: distilled water = 80:20; % v/v) in following proportions: 15 min, 100% A; 30 min, 95% A; 50 min, 90 % A, 51 min, 89 % A; 70 min, 89% A; 82 min, 85% A; 90 min, 85% A; 95 min, 40% A; 109 min, 100% A. Identification and quantification of individual phenolic compounds was performed at 280 nm ((+)-catechin and (–)-epicatechin), 308 nm (coutaric as *p*-coumaric) and 324 nm (caftaric as caffeic, ferulic) with calibration curves of (+)-catechin (0–0.17 mM; R^2=0.999), (–)-epicatechin (0–0.17 mM; R^2=0.999), *p*-coumaric acid (0–0.30 mM; R^2=0.999), caffeic acid (0–0.28 mM; R^2=0.999) and ferulic acid (0–0.26 mM; R^2=0.999).

DPPH• radical scavenging

The sample antioxidative properties were analyzed via ability to scavenge DPPH• (2,2-diphenyl-1-picrylhydrazyl) free radicals by using a modified method which was originally described by Brand-Williams et al. (1995). In short, methanolic solution of the DPPH reagent (60 µM) was prepared and adjusted to absorbance of 0.70 (±0.02) nm by addition of methanol. DPPH reagent (2.9mL) and properly diluted samples (0.1mL) were blended in 1 cm plastic cuvettes, closed with parafilm and stored in dark at room temperature for 60 minutes. Quantification of wine antioxidative properties was performed at 517 nm with UV–Vis spectrophotometer (Cary 60, Agilent Technologies, Santa Clara, USA) and calibration curve of Trolox aqueous solutions (0–0.8 mM, R^2=0.999). Obtained results were reported as mg of Trolox equivalents per L of wine.

pH, total acidity, volatile acidity and organic acids

Total acidity, pH and volatile acidity were examined with heat-stream distiller (Ing. Castore Bullio, Milan, Italy) and pH meter (pH 209, Hanna Instruments, Padova, Italy) according to the official European Commission methods **(EC, 1990)**.

Content of individual organic acids in was analyzed by HPLC equipped with DAD and column Aminex HPX-87H (9 µm, 7.8 × 300 mm; Bio-Rad, Hercules, USA) according to a protocol previously described **(Castellari et al., 2000)**. Prior to manual injection into HPLC system that was conditioned at 45°C, the wine sample was filtered with 0.2 µm nylon filter (Gema Medical, Barcelona, Spain). Identification and

quantification of individual organic acids was performed at 210 nm with calibration curves of citric (0–31 mM; R^2=0.999), L-tartaric (0–66.5 mM; R^2=0.995), L-malic (0–110 mM; R^2=0.999), succinic (0–50 mM; R^2=0.999), lactic (0–115 mM; R^2=0.999) and acetic acid (0–105 mM; R^2=0.999).

Sulfur dioxide

Free and total wine sulfur dioxide content was analyzed by titration with N 0.02 I_2 in the presence of 1% starch solution as an indicator of titration ending point (**Ripper and Schmitt, 1896**).

Optical density

Optical density was analyzed by UV-Vis spectrophotometer (Cary 60, Agilent Technologies, Santa Clara, USA) at 420 nm according to the official European Commission methods (**EC, 1990**).

Volatile aromatic compounds

Prior to gas chromatography (GC) analysis, 0.3 ml of wine samples conditioned at room temperature were transferred into GC vials (Chromacol, Thermo Scientific) together with 0.185 ml of potassium hydrogen sulfate saturated aqueous solution and dimethyl carbonate for extraction. Additionally, 0.015 ml of 2-ethyl butyric acid aqueous solution (100µg/ml) was added as internal standard. Afterwards, the vials were centrifuged at 3800 rpm for 10 min (ALC4232, centrifuge) and analyzed by gas chromatography mass spectrometry (GC-MS). GC-MS analyses of wine samples were performed with a gas chromatograph (7820A, Agilent, Santa Clara, USA) equipped with a mass selective detector (5977E, Agilent, Santa Clara, USA). The autosampler was programmed for the injection of 1µl of extract at the sample depth of 10 mm. Splitless injection was selected with an inlet temperature of 250°C. Analytes were separated with a polar GC column Agilent DB-FFAP (0.25 mm × 30 m, i.d, 0.25 µm film thickness; Agilent, Santa Clara, USA) with the following thermal program: 50°C held for 5 min, ramp at 10°C/min until 250°C, held at 250°C for 5 min, with gas flow of 1ml/min. Detection was made with a quadrupole mass spectrometer operating under electron ionization at 70 eV with acquisition at 1 scan/s in the m/z 29 and 450 range. Mass spectra were acquired in full scan mode properly adjusting the electron multiplier voltage. Tentative identification was based on library mass spectra matching (NIST). Peak areas were integrated by extracting characteristic ions from total ion current.

5.3.1.5   Sensory analysis

Quantitative descriptive sensory analysis

As a preliminary step of sensory analysis, wines were assessed by winemakers and staff of the BSc program in Enology and Viticulture at the University of Bologna (3 females and 2 males, aged between 27 and 52), to ascertain the lack of differences between duplicate trials.
Afterwards, the nine wines were assessed, one randomly selected wine for each combination of yeasts (*Y1*, *Y2* or *Y3*) and harvest dates (H1, H2, H3). Firstly, wine sensory analysis was performed using a quantitative descriptive sensory analysis (**Stone et al., 1974**). Panelist evaluated samples in terms of

olfaction (alcoholic odor, fruity odor, flowery odor, herbal odor, odor complexity) and taste (alcoholic taste, acidity, sweetness, bitterness, structure, taste complexity, persistence) by marking 10 cm unstructured scale anchored with '0' (lack of presence) and '10' (extremely intensive). The panel accounted 25 panelists (12 females and 13 males, aged between 20 and 46) which were recruited among employees and students of the BSc program in Enology and Viticulture (University of Bologna, Campus of Food Science, Cesena, Italy), whereas all panelists were properly trained during the BSc program (in Enology and Viticulture) courses related to wine sensory evaluation. Wine tasting was performed in two sessions within the same day (4 samples in the 1st session and 5 samples in the 2nd session). Samples were numerically assigned with a three-digit number and randomly distributed to panelists in transparent and pear-shaped glasses containing 25 ml of wine **(ISO, 1977)**. For purpose of palate cleansing natural water was distributed to panelists (Levissima, Torino, Italy).

Preference test

Successively with quantitative descriptive analysis, panelists also evaluated samples in term of preference. Preference test was examined by utilization of a simple 10 cm unstructured linear hedonic scale anchored with '0' (as extremely disliked) and '10' (as extremely liked), since panelist have the possibility to express sensory perceptions more precise than with the nine-point hedonic scale **(Lawless and Heymann, 2010)**.

5.3.1.6   *Statistical Analysis*

The chemical composition data of grape juice and wines were statistically examined with one-factor analysis of variance (ANOVA) and parametric post-hoc Tukey tests with a confidence level set at 90% and 95%. Sensory data were statistically examined with non-parametric Kruskal-Wallis test with a confidence level set at 90% and 95%. Principal component analysis (PCA) was utilized to detect potential correlations between quantitative descriptive sensory analysis, preference test and wine chemical analysis variables.

5.3.1.7   *Climatic characterization of the vintage 2016*

Climatic characterization of the vintage 2016 was conducted with meteorological data from a grid cell Tebano (11.7816E 44.2725N) during the period 1961–2016 which were used to calculate T_{mean}, GDD and DI (see 2.2.2 for details). Results are suggesting that T_{mean} during the vintage 2016 was classified as 'warm' *(Table 5.4)* **(Fraga et al., 2014)**, which is noticeably higher compared to T_{mean} (17.72°C; *Table 5.4*) during the period 1961–2015 which was also classified as 'warm' **(Fraga et al., 2014)**. Furthermore, T_{mean} during the vintage 2016 was noticeably higher than optimal T_{mean} for Chardonnay cultivation (~14.0–17.1°C; *Fig. 2.3*). Thermal accumulation during the vintage 2016 was in upper limit of optimal thermal accumulation conditions to produce high-quality wines, which is often too hot for production of white wines (1400–2000; **Gladstones, 1992**). These higher temperatures and thermal accumulation caused moderately dry conditions during the vintage 2016 *(Table 5.4)*. Therefore, climate characteristic of the vintage 2016 in Tebano were suitable to evaluate the effect of the climate change on grape/wine quality since the temperature and soil water availability were far from optimal for cultivation of Chardonnay grapes.

Table 5.4 Bioclimatic indices during the vintage 2016 and average bioclimatic indices values from the 1961 until the 2015.

Index	T_{mean} [°C]	GDD [units]	DI [mm]
1961–2015	17.72	1680.37	98.76
2016	18.90	1906.80	89.57

5.3.2 Results and Discussion

5.3.2.1 *Influence of early green harvest and yeasts selection on wine quality parameters*

Alcohol

Selection of yeasts for vinification plays a key role in determining the final composition of wine, including ethanol content **(Contreras et al., 2014b)**, malic acid content **(Bovo et al., 2016)**, aroma profile **(Tofalo et al., 2016)**, glycerol and polyphenols contents **(Romboli et al., 2015)** and acetic acid content **(Rantsiou et al., 2012)**. In presented experiment, the average wine ethanol content of Chardonnay wine vinified by *Y1*, *Y2* and *Y3* yeasts was 11.83%, 11.87% and 12.04%, respectively *(Table 5.5)*; therefore, a drop of about 0.2% was achieved by using selected yeasts strains. This moderate ethanol decrease is consistent with a case study conducted with Chardonnay wines, whereas *Saccharomyces paradoxus* (*Sp*), compared to the *Saccharomyces cerevisiae (Sc)*, decreased the wine ethanol content of Chardonnay wines for ~0.35% **(Orlic et al., 2007)**. Other case study reported similar results, whereas alcohol level of Sangiovese wines was reduced up to 0.3% v/v by utilization of *Candida zemplinina (Cz)* **(Romboli et al., 2015)**. However, some case studies reported a higher ethanol removal by utilization of a *Cz* comparing to *Sc* for vinifications of red grape varieties **(Englezos et al., 2016a; Giaramida et al., 2013)**. Thus, it is postulated that yeasts can reduce wine ethanol level at the higher percent when fermenting high grape sugar content (often found in must of red grape varieties).

Since chemical composition of grape berries changes starting from the fruit set and last until the ripening (see 4.1.2–4.1.5), the date of grape harvest plays an important role in the final wine quality. As expected, all the 'mixed' samples – e.g. samples with part of their must replaced with the low-total soluble solids H0 grape juice – had significantly lower ethanol content (H3) compared to wines made with grape juice from H2. The average alcohol content for wines produced with grapes harvested at the H1, H2 and mixture of H0 and H2 (H3) was 11.62%, 12.50% and 11.60%, respectively *(Table 5.5)*. The results clearly indicated that the ethanol removal in Chardonnay wines was mainly related to grape harvest timing (~0.9%), respect to the yeast strains (~0.2%). Kontoudakis et al. (2011a) reported similar findings, whereas addition of low-ethanol wine (5%) in a high-sugar grape juice, resulted in ethanol content decrease of Cabernet Sauvignon wines (0.9%). Using the same method, the ethanol loss was enhanced of about 3.0% and 1.7% was accomplished in Bobal and Merlot wines respectively **(Kontoudakis et al, 2011)**.

Table 5.5 Chemical composition, optical density, SO_2 concentation and antioxidative capacity of Chardonnay wines produced during vintage 2016. Statistical analysis differences among trials based on one-way Anova with post-hoc test Tukey ($p < 0.05$; $p < 0.1$) are marked using different letters (see footnotes for explanation).

Variable	Alcohol (% v/v)	Glycerol (g/L)	Phenolics (mg/L)[3]	DPPH (mg/L)[4]	pH	Total acidity (g/L)[1]	Volatile acidity (g/L)[2]	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Optical density (420 nm)
H1	11.62±0.11b	5.1±0.7	125±6c	167.1±5.1	3.17±0.02b	7.07±0.26b	0.29±0.11	15±5	72±7a	0.08±0.01
H2	12.50±0.09a	5.5±0.7	132±3b	173.4±6.0	3.22±0.03a	6.88±0.27b	0.39±0.08	16±4	58±6b	0.09±0.01
H3	11.60±0.12b	5.2±0.9	138±2a	169.3±4.5	3.06±0.01c	8.80±0.14a	0.35±0.08	15±3	58±7b	0.08±0.01
Y1	11.83±0.48	6.2±0.2a	135±4	169.4±6.5	3.14±0.07	7.66±0.98	0.34±0.10d	13±1b	58±6	0.08±0.01
Y2	11.87±0.47	4.9±0.2b	131±7	170.5±5.3	3.17±0.08	7.55±0.97	0.43±0.05d	13±5b	66±9	0.09±0.01
Y3	12.04±0.45	4.7±0.4b	130±9	169.9±5.6	3.15±0.08	7.55±0.96	0.26±0.03e	18±2a	65±11	0.08±0.01

Table values present mean (±SD) of single analysis for six trials conducted with same grape picking date or yeast selection.

[1] – total acidity expressed as g/L of tartaric acid

[2] – volatile acidity expressed as g/L of acetic acid

[3] – concentration of total polyphenols expressed as mg/L of gallic acid

[4] – antioxidative capacity expressed as mg/L equivalents of Trolox

Average values assigned by different letter are statistically different from each other, by Tukey test at $p < 0.05$ (a, b and c), and at $p < 0.1$ (d and e).

The utilization of different harvest date and yeast strains selection effectively reduced the ethanol concentration (-1.21%) in Chardonnay wines from 12.68% (yeast *Y3* with H2) to 11.47% (yeast *Y1* with H3). According to other case studies, the utilization of suggested method in presented experiment can further reduce the wine ethanol content, especially in the case of red grape varieties **(Englezos et al., 2016a; Giaramida et al., 2013; Kontoudakis et al., 2011a)**.

Glycerol

Higher glycerol production often related to yeasts response to osmotic stress or low-temperatures stress **(Pérez-Torrado et al., 2016)**, may alter the wine sensory features due to sweetness perception **(Noble and Bursick, 1984)**, while glycerol contribution to the viscosity or wine 'structure', may be controversial due to relatively low glycerol concentration in wines **(Laguna et al., 2017)** (up to ~16 g/L; **Romboli et al., 2015**). In presented experiment, the different grape harvest dates did not significantly alter glycerol content that was 5.1, 5.5 and 5.2 g/L in H1, H2 and H3 wines, respectively *(Table 5.5)*. The highest glycerol content in H2 wines may be related to slightly higher osmotic stress comparing to H1 and H3 wines. Also, due to fact that H2 grape juice had a higher concentration of total soluble solids when compared to H1 and H3 grape juice that may be converted to more glycerol *(Table 5.3)*.

Comparing to grape picking date, yeast selection had more influence on wine glycerol concentration, whereas *Y1* (6.2 g/L) had significantly higher glycerol concentration when compared to *Y2* wines (4.9 g/L) and *Y3* wines (4.7 g/L) *(Table 5.5)*. Experiment results related to higher glycerol production in *Y1* wines compared to *Y3* wines are similar with other case study performed on Chardonnay wines **(Orlic et al., 2007)**.

Phenolics

Phenolics affect wine antioxidative features, color, taste etc., thus they have a significant impact on final wine quality **(Boulton et al., 1999)**. In presented experiment, phenolics content in Chardonnay wines was approximately from 120 to 140 mg/L (as gallic acid; *Table 5.5*), which value is close compared with other case studies related to phenolics quantity in Chardonnay wines **(Chamkha et al., 2003; Olejar et al., 2016; Ricci et al., 2017)**. The grape picking dates significantly affected the phenolics content of Chardonnay wines, whereas H1, H2 and H3 wines had 125, 132 and 138 mg/L of phenolic compounds (as gallic acid), respectively *(Table 5.5)*. The highest concentration of phenolics in wines H3 probably reflect the high content of phenolic compounds in juice obtained from grapes harvested at H0 that was added instead of H2 grape juice. At véraison, grapes (similarly to H0) have higher concentration of phenolic compounds compared to grapes after véraison (H1 and H2). This is explained by the increased synthesis of hydroxycinnamic acids and their derivatives that occurs before to véraison, followed by a decrease of hydroxycinnamic acids content after véraison due to 'dilution' by total soluble solids accumulation in grape berries **(Ong and Nagel, 1978)**. Hydroxycinnamic acids concentration was higher in H3 wines (22.5 mg/L) respect to H1 (20.7 mg/L) and H2 (20.9 mg/L) *(Table 5.6)*. Furthermore, concentration of caftaric acid which is the main hydroxycinnamic acid of white wines **(Adams, 2006)**, was the highest in H3 wines (18.2 mg/L) followed by H1 (17.1 mg/L) and H2 wines (16.8 mg/L). Comparing to caftaric acid other phenolic compounds were detected in low quantities *(Table 5.6)*. The values of caftaric acid found in this experiment are aligned with other studies related to Chardonnay wines (~8–44 mg/L) **(Cejudo-Bastante et al., 2011; Chamkha et al., 2003; Olejar et al., 2016)**.

Table 5.6 Phenolic compounds composition in Chardonnay wines produced during vintage 2016. Statistical analysis differences among trials based on one-way Anova with post-hoc test Tukey (p < 0.05; p < 0.1) are marked using different letters (see footnotes for explanation).

Var	Catechin (mg/L)	Epicatechin (mg/L)	Σ Flavan-3ols (mg/L)	Caftaric (mg/L)	Coutaric (mg/L)	Ferulic (mg/L)	Σ HCA (mg/L)	Σ Phenolics (mg/L)
H1	1.2±0.3	2.9±0.3d	4.1±0.3a	17.2±1.4b	1.2±0.1c	2.4±0.3b	20.7±1.3b	24.8±1.3b
H2	0.9±0.3	2.3±0.3e	3.1±0.4b	16.8±0.5b	1.4±0.1b	2.7±0.2a	20.9±0.4b	24.0±0.6b
H3	0.9±0.5	2.5±0.7e	3.4±0.5b	18.2±0.5a	1.7±0.1a	2.6±0.1a	22.5±0.5a	25.9±0.8a
Y1	1.0±0.3	2.6±0.6	3.6±0.7	17.2±0.6	1.4±0.2	2.4±0.3	21.1±1.1	24.6±0.6
Y2	1.1±0.5	2.3±0.5	3.4±0.6	17.9±1.0	1.4±0.2	2.6±0.3	22.0±0.7	25.3±1.3
Y3	0.9±0.4	2.8±0.3	3.7±0.5	17.1±1.3	1.4±0.2	2.6±0.2	21.1±1.5	24.8±1.5

Table values present mean (±SD) of single analysis for six trials conducted with same grape picking date or yeast selection.

Average values assigned by different letter are statistically different from each other, by Tukey test at p < 0.05 (a, b and c), and at p < 0.1 (d, e and f).

Compared to grape picking dates, the yeast strain had a minor effect on total polyphenols concentration and individual phenolic compounds content. Polyphenols content in Chardonnay wines fermented with yeast *Y1*, *Y2* and *Y3* was 135, 131 and 130 mg/L, respectively *(Table 5.5)*, which were partly identified by HPLC as low molecular weight phenolic compounds *(Table 5.6)*.

DPPH• radical scavenging

Antioxidative capacity of wines is important quality parameter since it reflects potential shelf-life and aging potential. Wine antioxidative capacity is mostly related to concentration of free SO_2, pH value, phenolic compounds composition and quantity. In presented experiment, wine antioxidative capacity was approximately 170 mg/L (as Trolox equivalent) for all wines and did not differ statistically according to grape harvest timing neither according to yeast selection *(Table 5.5)*. Obtained values are close to results reported in a recent study addressing antioxidative capacity of Chardonnay wines (**Olejar et al., 2016**).

pH, total acidity, volatile acidity and organic acids

Wine total acidity and pH values are among the most important wine quality parameters, due to their impact on wine color, organoleptic features, microorganism activity, content of active molecular sulfur dioxide etc. The average total acidity for H1, H2 and H3 wines was 7.07, 6.88 and 8.80 g/L (as tartaric acid), respectively, while pH values were 3.17, 3.22 and 3.06 for H1, H2 and H3 wines, respectively *(Table 5.5)*. These results are consistent with other case studies, whereas Chardonnay wines had total acidity in the range 4.2–10.1 g/L and pH value in the range 3.1–3.5 (**Cejudo-Bastante et al., 2011; Olejar et al., 2016; Orlic et al., 2007; Redzepovic et al., 2003; Ricci et al., 2017; Torrea et al., 2011**). As expected, the mix of H0 and H2 grape juices significantly increased the total acidity of Chardonnay wine (H3), thus presented method may serve, if necessary, as an alternative to chemical acidification of wines. These results are aligned with a case study (**Kontoudakis et al., 2011a**), whereas the mix of low-alcohol wine (produced with grapes collected at véraison) and grape juice (produced with ripen grapes) of Cabernet Sauvignon, Merlot and Bobal increased total acidity in the range of 0.8–2.2 g/L (as tartaric acid). Opposite to grape picking date, the selection of yeast strains had a minor impact on pH value and total acidity, whereas wines produced by *Y1*, *Y2* and *Y3* yeast strains had 3.14, 3.17 and 3.15 pH values and total acidity of 7.65, 7.55 and 7.55 g/L, respectively *(Table 5.5)*.

The increase of total acidity in samples H1 and H2 respect to the total acidity of corresponding grape juice prior to vinification *(Table 5.3; Table 5.5)* may be partially explained with the synthesis of succinic acid during the vinification process *(Table 5.7)*. On the other hand, the lower total acidity of H3 samples respect to corresponding grape juice *(Table 5.3; Table 5.5)* may be tentatively attributed to the consumption of malic acid by yeasts during vinification and the favored precipitation of potassium bitartrate during cold stabilization.

The presented combined method (different grape harvest dates with appropriate yeast selection) caused increase (2.55 g/L) of total acidity in Chardonnay wines from 6.37 g/L (yeast *Y2* with H2) to 8.92 g/L (yeast *Y1* with H3), clearly indicating that it may be used as an alternative to chemical acidification of wines.

Table 5.7 Organic acids composition of Chardonnay wines produced during vintage 2016. Statistical analysis differences among trials based on one-way Anova with post-hoc test Tukey ($p < 0.05$; $p < 0.1$) are marked using different letters (see footnotes for explanation).

Var	Lactic (g/L)	Succinic (g/L)	Acetic (g/L)	Citric (g/L)	Tartaric (g/L)	Malic (g/L)	Σ Acids (g/L)
H1	0.09±0.04	2.47±0.08a	0.12±0.15	0.19±0.01b	2.31±0.06e	3.58±0.15e	8.8±0.2b
H2	0.12±0.02	2.13±0.14b	0.20±0.18	0.20±0.02b	2.20±0.09f	3.25±0.28f	8.1±0.3c
H3	0.09±0.03	2.36±0.10a	0.19±0.14	0.24±0.01a	2.81±0.09d	4.71±0.29d	10.4±0.3a
Y1	0.12±0.01a	2.43±0.14	0.13±0.07b	0.21±0.03	2.45±0.30	3.69±0.65	9.0±1.0
Y2	0.07±0.03b	2.22±0.22	0.36±0.05a	0.20±0.03	2.38±0.28	3.72±0.71	8.9±1.1
Y3	0.11±0.03a	2.31±0.12	0.02±0.02c	0.22±0.02	2.49±0.31	4.13±0.72	9.3±1.1

Table values present mean (±SD) of single analysis for six trials conducted with same grape picking date or yeast selection.
Average values assigned by different letter are statistically different from each other, by Tukey test at $p < 0.05$ (a, b and c), and at $p < 0.1$ (d, e and f).

The grape picking date had a minor effect on volatile acidity and acetic acid concentration, which were 0.29, 0.39, 0.35 g/L, and 0.12, 0.20, 0.19 g/L (both as acetic acid) for H1, H2 and H3 wines, respectively *(Table 5.5; Table 5.7)*. Reversely, yeast strain selection had a significant effect on these parameters. In particular, wines vinified with yeast *Y3* had a significantly lower volatile acidity (0.26 g/L) respect to wines vinified by *Y1* (0.34 g/L) and *Y2* (0.43 g/L) yeasts *(Table 5.5)*. However, even if statistical differences were detected, wine content of volatile acids was below legal limits (1.2 g/L) for volatile acidity in wines set by OIV **(OIV, 2017)**. Detected higher wine volatile acidity content in samples vinified with *Y1* respect to *Y3* is partly consistent with other studies addressing similar topics (~0.2–0.4 g/L) **(Orlic et al., 2007; Redzepovic et al., 2003)**. The acetic acid content was lower in samples vinified with yeast *Y3* (0.02 g/L), respect to samples vinified with *Y1* and *Y2* yeasts: 0.13 and 0.36 g/L, respectively *(Table 5.7)*. The acetic acid content in samples vinified with *Y2* was lower in presented experiment (0.36 g/L) respect to other studies (~0.5–0.95 g/L) **(Giaramida et al., 2013; Romboli et al., 2015; Sadoudi et al., 2012)**.

Organic acids have a significant impact on wine stability and sensory features, especially in white wines **(Ribéreau-Gayon et al., 1982)**. Part of wine organic acids is present due to natural synthesis in grapes during grapevine reproduction cycle (tartaric, malic and citric acid), while other wine acids are present due to yeast and bacteria synthesis during vinification and ageing process (succinic, lactic and acetic acid). Content of wine organic acids obtained in the present experiment is reported in *Table 5.7*. Since

tartaric, malic citric acids are more related to berry ripening process, as expected their content was significantly affected by grape picking date *(Table 5.7)*. Apart from H3 wines, results related to content of tartaric, malic and citric acid are consistent with other case studies addressing Chardonnay wines, whereas tartaric acid was reported in the range 2.6–4.6 g/L **(Cejudo-Bastante et al., 2011; Wang et al., 2013)**, malic acid was reported in the range 1.5–3.3 g/L **(Cejudo-Bastante et al., 2011; Pan et al., 2011; Redzepovic et al., 2003; Torrea et al., 2011)** while citric acid was reported low as 0.1 g/L **(Wang et al., 2013)**. The highest content of these acids was detected in H3 wines since H0 grape juice had the highest total acidity *(Table 5.3)*.

Relatively low content of lactic acid detected in presented experiment (0.07–0.12 g/L; *Table 5.7*) respect to other studies addressing similar topic (0–0.7 g/L) **(Cejudo-Bastante et al., 2011; Redzepovic et al., 2003; Wang et al., 2013)** are suggesting that malolactic fermentation was partly completed.

Interestingly, succinic acid concentration in all samples (n.18 in total) varied from 1.94 (*Y2* yeast with H2) to 2.60 g/L (*Y1* yeast with H1), which is noticeably higher respect to concentrations of succinic acid often present in Chardonnay wines (~0.3–1.4 g/L) **(Redzepovic et al., 2003; Torrea et al., 2011; Wang et al., 2013)** or white wines in general (up to 1.7 g/L) **(Coulter et al., 2004; Patrignani et al., 2016)**. The high values of succinic acid could be explained with a relatively high content of vitamins (e.g. thiamin) added as Nutristart (see 5.3.1.3) that have a positive impact on succinic acid synthesis by yeasts **(Coulter et al., 2004)**. Furthermore, succinic acid is mostly synthesized during the first stages of vinification **(Arikawa et al., 1999; Thoukis et al., 1965)**, which synthesis may be increased in pasteurized **(Shimazu and Watanabe, 1981)** and non-clarified grape juice (see 5.3.1.2), due to high content of nutrients for yeasts. These results are indicating that regulation of yeasts nutrition may serve as an alternative approach to regulate wine total acidity and pH value during the 'hot' vintages which are often followed by low total acidity and high pH value.

Sulfur dioxide

Free SO_2 concentration is important parameter since it has antimicrobial and antioxidative properties **(Pezley, 2015)**. Quantity of free sulfur dioxide present in wine is mostly related to addition of potassium metabisulfite or similar compounds into must and/or wine during winemaking and wine storing. However, it could also be produced in small quantities (few mg/L) by yeasts during vinification **(Pezley, 2015)**. Thus, as expected, grape harvest dates did not influence free SO_2 that was 15, 16 and 15 in H1, H2 and H3 wines. On the other hand, significant differences in free SO_2 concentration were detected due to yeast selection, whereas *Y3* (18 mg/L) had higher free SO_2 content respect to *Y1* (13 mg/L) and *Y2* (13 mg/L) *(Table 5.5)*. Reversely, total SO_2 concentration did not differ significantly due to yeast selection and it was 58, 66 and 65 mg/L in wines vinified with *Y1*, *Y2* and *Y3*, respectively *(Table 5.5)*. Significant statistical differences in total SO_2 concentration were detected among trials with different grape harvest dates, whereas H1 (72 mg/L) had higher total SO_2 content comparing to H2 (58mg/L) and H3 (58 mg/L) wines *(Table 5.5)*. However, even if differences were significant, all trials had total SO_2 content far below legal limits set by OIV (200 mg/L for dry white wines) **(OIV, 2017)**.

Optical density

White wine optical density obtained at 420 nm may be used as control quality parameter since it reflects phenolic browning which is caused by oxidation. Apart from negative impact on white wine color, oxidation is followed also by negative impact on wine organoleptic characteristics **(Singleton and Cilliers, 1995)**. Optical density for Chardonnay wines obtained in the present study was similar for all wines (0.08–0.09) and did not differ statistically *(Table 5.5)*. Obtained results are aligned with other studies related to Chardonnay wines that reported optical density in approximate range from 0.07 until 0.095 **(Fu et al., 2009; Ricci et al., 2017)**. Furthermore, a relatively low optical density of wine samples is indicating that vinification processes were performed correctly, preventing significant phenolic browning.

Volatile aromatic compounds

Volatile aromatic profile and concentration of individual compounds determine the wine odor characteristic. Aromatic compounds in Chardonnay wines can be derived from grapes (e.g. linalool, β-damascenone etc.), alcohol and malolactic fermentation (e.g. ethyl hexanoate, diethyl succinate etc.) and from contact with Oak and ageing (e.g. vanillin, cis-oak lactone etc.) **(Gambetta et al., 2014)**. Thus, different harvest timing may influence the volatile aromatic profile. However, all detected aromatic compounds in the present experiment were mainly derived from alcohol and partial malolactic fermentation. Hence, differences among the trials according to grape harvest timing were mainly not significant *(Table 5.8)*.

On the other hand, significant differences were detected according to yeast selection *(Table 5.8)*. In particular, ethyl hexanoate, volatile aromatic compound important for Chardonnay characterization **(Gambetta et al., 2014)**, was significantly higher in *Y2* wines comparing to *Y1* wines *(Table 5.8)*. However, even if differences were detected, there was a lack of differences in fruity odor sensation *(Fig. 5.6)*. As expected, significant differences were detected also for acetic acid, whereas *Y2* has the highest concentration of acetic acid which is aligned with other analysis *(Table 5.5; Table 5.7; Table 5.8)*. Furthermore, isoamyl acetate was significantly higher in *Y3* comparing to *Y1* and *Y2* *(Table 5.8)*. which is aligned with literature, whereas *Sc* produces more isoamyl acetate comparing to *Cz* or *Sp* **(Orlic et al., 2007; Sadoudi et al., 2012)**. Significant differences were detected also for isoamyl alcohol, phenylethyl alcohol butanoic acid, isovaleric acid etc. *(Table 5.8)*.

Table 5.8 Volatile aromatic composition of Chardonnay wines produced during vintage 2016 expressed as mg/L. Statistical analysis differences among trials based on one-way Anova with post-hoc test Tukey ($p < 0.1$; $p < 0.05$) are marked using different letters (see footnotes for explanation).

Compounds	H1	H2	H3	Y1	Y2	Y3
Alcohols						
Isoamyl alcohol	82.6±21.0	88.6±20.7	91.8±20.7	108.7±2.9a	62.7±2.6c	86.7±9.5b
Phenylethyl alcohol	42.9±20.6	41.7±21.6	44.6±19.8	66.5±2.4a	20.5±2.7c	38.1±1.9b
Total alcohols	125.5±41.6	130.3±41.7	136.3±40.2	175.2±4.6a	83.2±4.0c	124.9±10.8b
Esters						
Isoamyl acetate	5.9±1.3	4.5±1.1	5.1±1.5	4.9±0.7b	3.8±0.7c	6.6±0.7a
Ethyl hexanoate	0.8±0.0	0.9±0.2	0.8±0.1	0.8±0.0b	0.9±0.2a	0.8±0.0b
Ethyl octanoate	1.3±0.0	1.5±0.2	1.3±0.2	1.3±0.1	1.5±0.2	1.3±0.2
Ethyl lactate	9.1±2.4b	12.5±2.5a	14.6±2.8a	14.5±2.9a	12.0±2.0a	9.2±2.7b
Mono-ethyl succinate	15.9±4.4	16.3±4.2	18.9±5.0	22.1±1.8a	13.8±1.8b	14.2±2.4b
Diethyl succinate	2.8±0.3	2.7±0.4	2.8±0.4	3.1±0.2a	2.4±0.2b	2.7±0.1b
Total esters	35.7±6.4	38.3±6.1	43.3±7.5	46.7±4.0a	34.4±1.7b	34.7±4.7b
Acids						
Acetic acid	51.4±48.5	72.5±54.6	55.8±51.0	45.3±16.2b	129.3±15.0a	17.3±1.8c
Butanoic acid	1.2±0.1	1.3±0.2	1.2±0.2	1.1±0.1b	1.4±0.1a	1.3±0.0a
Isovaleric acid	0.8±0.1	0.8±0.1	0.8±0.1	0.9±0.0a	0.7±0.1b	0.9±0.1a
Hexanoic acid	5.0±0.2	5.2±0.9	4.9±0.5	4.5±0.3b	5.6±0.8a	5.2±0.1a
Octanoic Acid	5.8±0.4	5.9±1.0	5.8±0.8	5.3±0.4f	5.9±0.9e	6.3±0.3d
Total acids	64.3±48.2	85.8±55.6	68.5±50.8	57.2±1.6b	142.9±1.6a	30.9±1.9c
Other						
γ-Butyrolactone	0.8±0.4	0.8±0.1	0.9±0.1	0.8±0.2	1.0±0.3l	0.7±0.1k

Table values present mean (±SD) of single analysis for six trials conducted with same grape picking date or yeast selection.

Average values assigned by different letter are statistically different from each other, by Tukey test at $p < 0.05$ (a, b and c), and at $p < 0.1$ (d, e and f).

However, only a few of detected compounds were present in concentrations higher than odor threshold limits *(Table 5.9)*. Volatile aromatic compounds that were present in concentration at least 50 times higher than threshold limit are characterized with fruity odor sensation, suggesting that wines were mostly perceived as fruity by the panelists *(Table 5.9)*. These findings are aligned with sensory analysis whereas fruity odor sensation had higher scores comparing to complexity, herbal, alcoholic and floral odor sensation *(Fig 5.6; Fig 5.7)*. Other compounds that were detected in the concentration higher than threshold limit (e.g. isovaleric acid, γ-butyrolactone, phenylethyl alcohol; *Table 5.9*) most likely contributed to the odor complexity sensation which was given higher scores by panelists comparing to alcoholic, herbal and floral odor sensation *(Fig 5.6; Fig 5.7)*.

Table 5.9 Volatile aromatic compounds odor activity values, description and odor threshold limits (µg/L). Odor activity values are ratio of certain compound concentration and odor threshold limit.

Compounds	Odor activity value (OAV)						Description	Odor threshold limits
	H1	H2	H3	Y1	Y2	Y3		
Alcohols								
Isoamyl alcohol	2.8	3.0	3.1	3.6	2.1	2.9	Applejack, spicy[1]	30000[1]
Phenylethyl alcohol	3.1	3.0	3.2	4.8	1.5	2.7	Sweet rose[1]	14000[1]
Total alcohols								
Esters								
Isoamyl acetate	196.1	150.7	168.6	164.1	125.5	218.7	Banana[2]	30[2]
Ethyl hexanoate	55.3	62.8	58.1	54.4	66.6	56.7	Fruity[1]	14[1]
Ethyl octanoate	258.4	291.9	254.3	253.4	291.4	266.0	Pineapple, pear, floral[1]	5[1]
Ethyl lactate	0.1	0.1	0.1	0.1	0.1	0.1	Fruity, buttery[3]	150000[3]
Mono-ethyl succinate	0.0	0.0	0.0	0.0	0.0	0.0	Caramel, coffee[4]	1000000[4]
Diethly succinate	0.0	0.0	0.0	0.0	0.0	0.0	Fermented, floral[1]	200000[1]
Total esters								
Acids								
Acetic acid	0.3	0.4	0.3	0.2	0.6	0.1	Acid, fatty, vinegar[2,5]	200000[2,5]
Butanoic acid	7.1	7.8	7.2	6.5	8.0	7.7	Cheese[6]	173[7]
Isovaleric acid	24.8	25.2	24.6	26.8	21.8	25.5	Cheese[6]	33.4[7]
Hexanoic acid	1.7	1.7	1.6	1.5	1.9	1.7	Rancid, cheese, copra oil[1,2]	3000[1,2]
Octanoic Acid	11.5	11.9	11.6	10.5	11.9	12.6	Rancid, cheese, fatty acid[1]	500[1]
Total acids								
Other								
γ-Butyro lactone	23.5	23.2	24.9	23.9	29.0	19.3	Caramel, sweet, toast[4]	35[4]

[1]**(Song et al., 2015)**, [2]**(Jiang et al., 2013)**, [3] **(Peinado et al., 2004)**, [4]**(Sánchez-Palomo et al., 2012)**, [5]**(Rapp and Mandery, 1986)**, [6]**(Aznar et al., 2001)**, [7]**(Ferreira et al., 2000)**.

The PCA plot allowed separation of Chardonnay wines according to volatile aromatic profile, whereas the 1st two PCs explained 91% of the variability *(Fig. 5.5a; Fig. 5.5b)*. As it was already explained, concentration of detected volatile aromatic compounds was more related to the yeast selection than to grape harvest dates, hence separation will be considered only according to yeast strain selection *(Table 5.9)*. Wines obtained with *Y1* were separated according to phenylethyl alcohol, isoamyl alcohol, diethyl succinate and mono-ethyl succinate, whereas only higher alcohols were detected in concentrations higher than odor activity value (OAV) *(Fig. 5.5a; Fig 5.5b; Table 5.9)*. Must fermentation with *Y2* resulted in the production of wines that were separated according to γ-butyro lactone, acetic acid, ethyl octanoate and ethyl hexanoate, whereas volatile aromatic compounds apart from acetic acid contributed to the odor characterization of *Y2* wines *(Fig. 5.5a; Fig 5.5b; Table 9)*. Vinification with *Y3* resulted in the production of wines that were separated according to isoamyl acetate which was present in concentration ~220 times higher than OAV, suggesting that isoamyl acetate contributed in fruity odor sensation of *Y3* wines *(Fig. 5.5a; Fig 5.5b; Table 9)*.
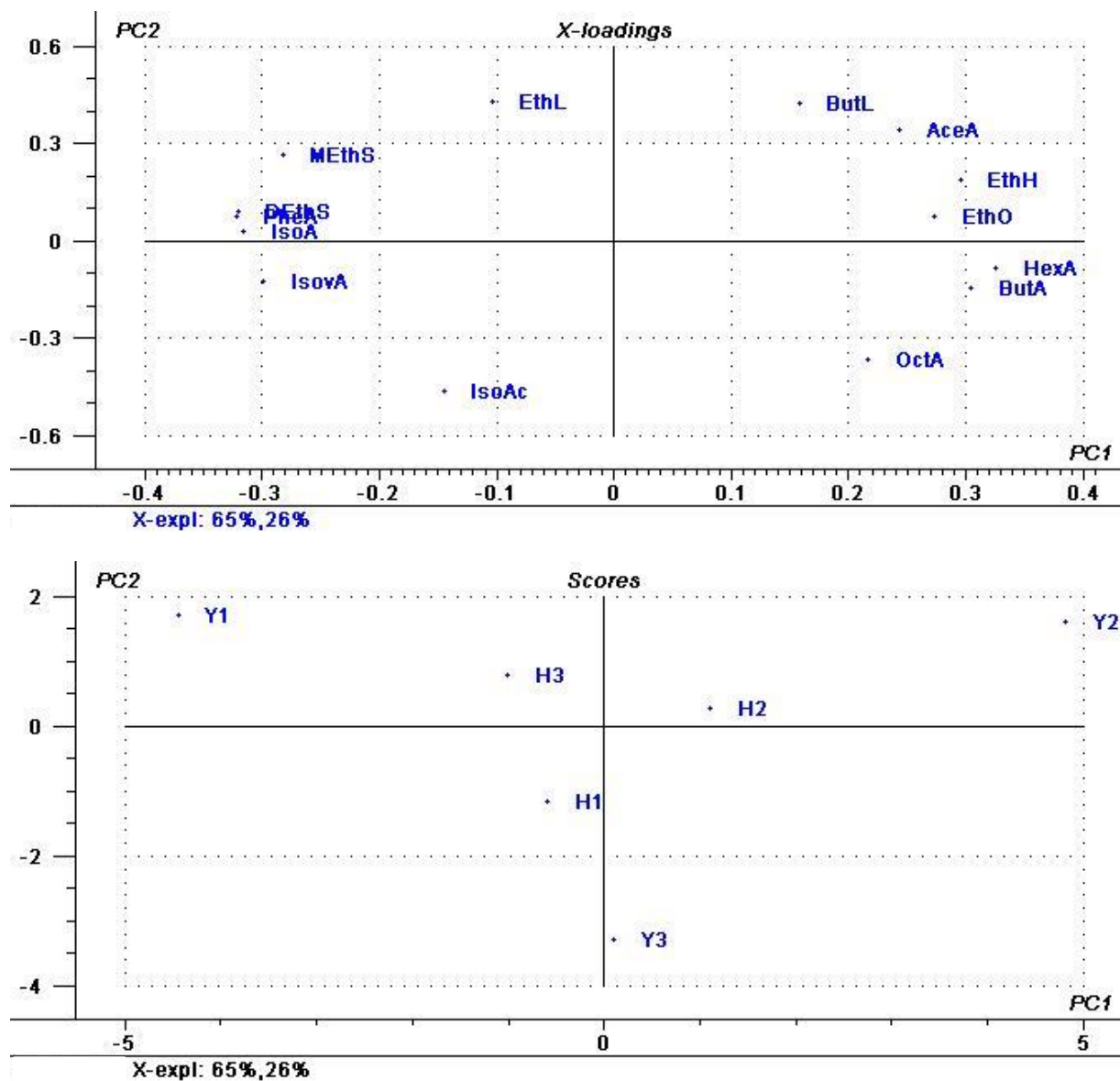
Figure 5.5 Principal component analysis a) scores plot of Chardonnay wines according to volatile aromatic compounds; b) correlation loadings plot of Chardonnay wines with volatile aromatic compounds profile. *Y1*–must vinified with inoculation of *Saccharomyces cerevisiae/Saccharomyces paradoxus*; *Y2*–must vinified with sequential inoculation of *Candida zemplinina* and hybrid *Saccharomyces cerevisiae/Saccharomyces paradoxus*; *Y3*–must vinified with inoculation of *Saccharomyces cerevisiae;* H1–wine made with technologically mature (ratio total acidity/sugar content) Chardonnay grapes; H2–wine made with Chardonnay grapes obtained during '*delayed harvest*'; H3–wine made with blend of Chardonnay grapes obtained during 'early *green* harvest' and Chardonnay grapes obtained during '*delayed harvest*'; AceA–acetic acid; ButA–butanoic acid; HexA–hexanoic acid; EthO–ethyl octanoate; PheA–phenylethyl alcohol; IsoA–isoamyl alcohol

Quantitative descriptive sensory analysis

Wine sensory characteristics is important wine quality parameter that depends on wine physical properties (e.g. density, color) and chemical composition (e.g. volatile esters content, organic acids content), and which determines final wine price to a great extent **(Gambetta et al., 2014)**. As concluded earlier, different grape picking timing altered chemical composition of Chardonnay wines *(Table 5.5; Table 5.6; Table 5.7)*, that caused certain significant differences in wine sensory characteristics *(Fig 5.6)*. These significant differences were more related to taste variables, while olfactory variables of Chardonnay wines did not differ significantly *(Fig 5.6)*. In particular, differences were detected in acidity, sweetness and bitterness. The highest acidity perception was detected in wines obtained with part of unripen grapes (H3; Acidity perception score=6.00) while other two trials H1 (4.67) and H2 (4.80) had approximately same acidity perception. This is aligned with total acidity and pH values whereas H3 wines had the significantly highest total acidity and significantly lowest pH value *(Table 5.3)*. Obtained results aren't surprising since addition of unripen grapes increases acidity as it was reported in several studies addressing similar topics **(Kontoudakis et al., 2011a, 2011b)**. Opposite to acidity perception, sweetness perception was lower in H3 wines (sweetness perception score=2.32) comparing to H1 (2.93) and H2 (2.92) that had approximately equal sweetness perception. Even if detected, differences in sweetness perception were not related to sugar quantity since all wine had residual sugar concentration below 0.2 g/L, these differences were most likely related to organic acids quantity. This phenomenon may be explained with mutual interaction of sweetness and acidity perception whereas addition of sugar suppress acidity and vice versa **(Green et al., 2011)**. Thus, in conditions of constant sugar concentration, increasing acids concentration may cause lower sweetness perception. Significant statistical differences were also detected for bitterness, whereas H3 wine had the highest bitterness perception (2.84), followed by H2 (2.50) and H1 wines (2.08) *(Fig. 5.6)*. The highest bitterness perception of H3 wine is again related to addition of unripen grapes which is aligned with literature **(Kontoudakis et al., 2011b)**. Thus, to avoid production of bitter and acidic wines, quantity of added unripen grapes need to be regulated with caution.
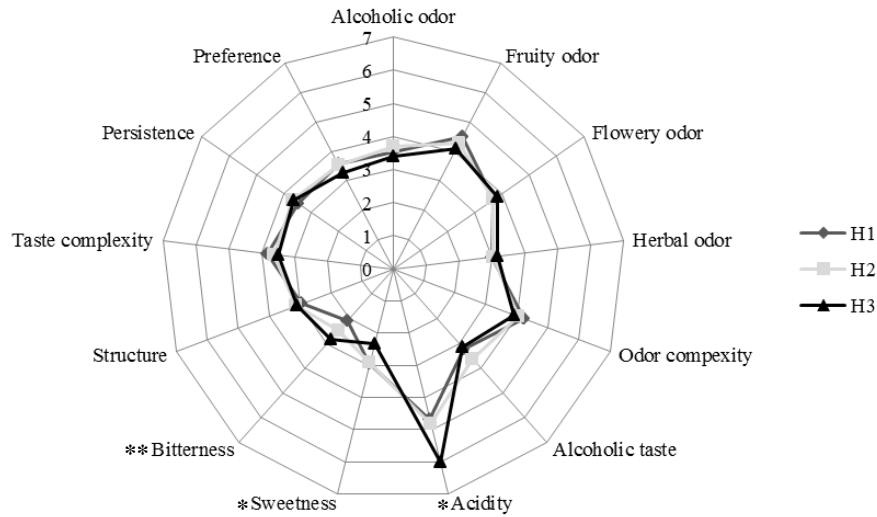
Figure 5.6 Sensory analysis scores of Chardonnay wines produced during vintage 2016 according to grape harvest timing. Values are the mean of 25 replicates of all samples (n=75). Statistical analysis was performed with Kruskal -Wallis test (* – $p < 0.05$; ** – $p < 0.1$).

On the other hand, sensory characteristics can be influenced by yeast selection as well. Those differences may be related to wine taste characteristics **(Gobbi et al., 2013; Tofalo et al., 2016)**, or to wine olfactory characteristics **(Varela et al., 2017; Wang et al., 2017)**. However, even if certain differences were detected (e.g. flowery odor) they were not statistical significant *(Fig 5.7)*.
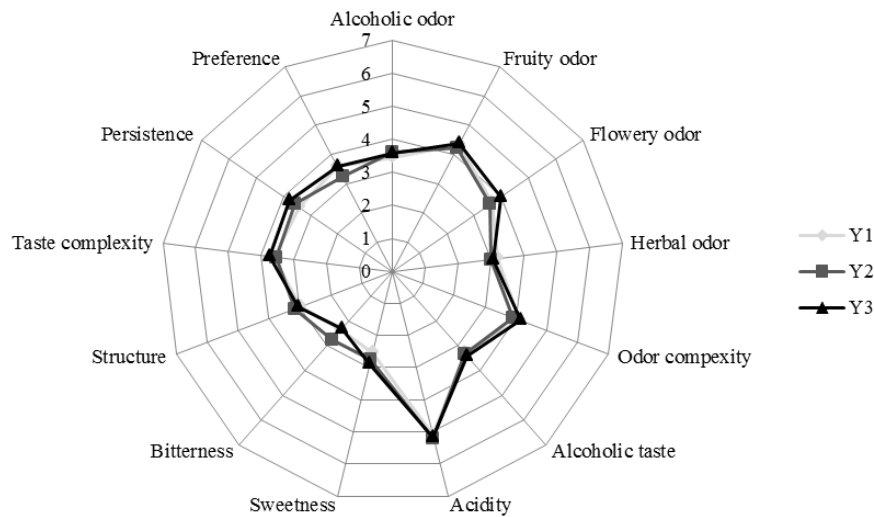
Figure 5.7 Sensory analysis scores of Chardonnay wines produced during vintage 2016 according to yeast strain selection. Values are the mean of 25 replicates of all samples (n=75). Statistical analysis was performed with Kruskal -Wallis test (* – $p < 0.05$; ** – $p < 0.1$).

According to non-parametric Kruskal-Wallis test wines did not differ statistically in regard to the average preference scores (*PreSc) (Table 5.10)*. The lowest *PreSc* were assigned to the H3 wines (3.27) and *Y2* wines (3.23), while other wines had approximately equal *PreSc* (3.55–3.59).

Table 5.10 Preference scores of Chardonnay wines. Statistical analysis of 75 replicates based on Kruskal - Wallis test ($p < 0.05$; $p < 0.1$).

Variable	Preference scores
H1	3.57±1.68
H2	3.55±1.72
H3	3.27±1.65
Y1	3.59±1.77
Y2	3.23±1.67
Y3	3.57±1.59

Values are the mean (±SD) of twenty-five analyses for three trials conducted with same grape picking date or yeast selection.

The PCA plot allowed disclosure of correlations between *PreSc* and the chemical composition of Chardonnay wines *(Fig 5.8a)*. The 1st two PCs explained 84% of the variability, whereas PC1 (57%) separated trials according to grape picking date, while PC2 (27%) separated trials according to yeast selection *(Fig 5.8b)*. Along PC1, *PreSc* was negatively correlated with total concentration of polyphenols, caftaric and coutaric acid same as with total acidity, tartaric, citric and malic acid *(Fig 5.8a)*. Thus, results are indicating that wines H3 may be perceived as acidic and bitter when compared to H1 and H2 *(Fig. 5.8a; Fig 5.8b)*. These results were confirmed by quantitative descriptive sensory analysis, whereas H3 wines had significantly higher acidity and bitterness sensation comparing to H1 and H2 wines *(Fig. 5.6)*.

Inversely, ethanol concentration and pH were positively related with *PreSc* along the PC1 *(Fig 5.8a)*. Since pH value is reversely proportional on a log-scale to total acidity which is negatively related to the *PreSc*, following outcome was expected.
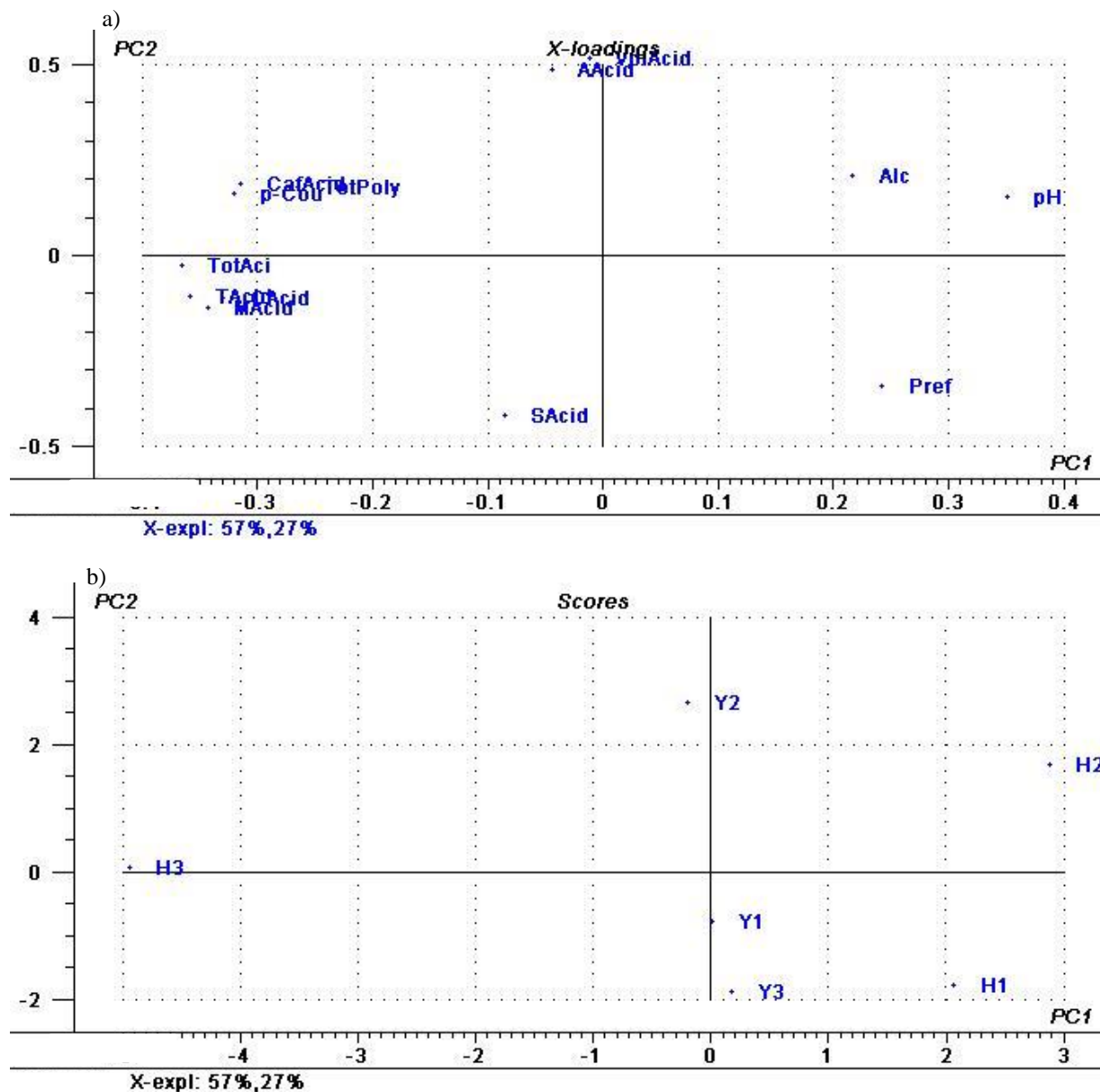
Figure 5.8 Principal component analysis a) scores plot of Chardonnay wines according to significant variables of chemical composition (without volatile aromatic compounds; b) correlation loadings plot of Chardonnay wines chemical composition (without volatile aromatic compounds) and panelist preference. *Y1*–must vinified with inoculation of *Saccharomyces cerevisiae/Saccharomyces paradoxus*; *Y2*–must vinified with sequential inoculation of *Candida zemplinina* and hybrid *Saccharomyces cerevisiae/Saccharomyces paradoxus*; *Y3*–must vinified with inoculation of *Saccharomyces cerevisiae;* H1–wine made with technologically mature (ratio total acidity/sugar content) Chardonnay grapes; H2–wine made with Chardonnay grapes obtained during '*delayed harvest*'; H3–wine made with blend of Chardonnay grapes obtained during 'early *green* harvest' and Chardonnay grapes obtained during '*delayed harvest*'; AAcid–acetic acid; VolAcid–volatile acidity; CafAcid–cafftaric acid; p-Cou–p-coumaric acid; TotPoly–total polyphenols; TotAci–total acidity; TAcid–tartaric acid; MAcid–malic acid; CAcid–citric acid; SAcid–succinic acid; Pref–panelist preference; pH–pH value; Alc–alcohol content.

Although the positive correlation between ethanol concentration and *PreSc* was detected, results suggested that low alcohol wines H1 and H3 were slightly preferred in that regard when compared to H2 wines *(Fig 5.8b)*.

On the other hand, along PC2 wines were separated according to yeast strain selection, with *PreSc* negatively related to acetic acid and volatile acidity, whereas succinic acid seemed to have a positive effect on *PreSc* *(Fig 5.8a)*. However, the results indicated that these variables and yeast strain selection had a minor impact on *PreSc* when compared to grape picking date and related variables separated along PC1.

The PCA allowed also disclosure of correlations between *PreSc* and results from quantitative descriptive sensory analysis of Chardonnay wines, whereas the 1st two PCs explained 88% of the variability *(Fig. 5.9a; Fig. 5.9b)*. Along PC1 (63%) *PreSc* was negatively correlated with bitterness and acidity, and positively correlated with taste and odor complexity, fruity odor sensation and sweetness *(Fig 5.9a)*. The negative correlation of *PreSc* with bitterness and acidity was expected since H3 wines which were characterized as the most acidic and bitter and had the highest total acidity and total polyphenol content *(Table 5.5; Fig 5.6)*. The positive correlation of sweetness perception with *PreSc* was not related to sugar quantity since all wines had residual sugar concentration below 0.2 g/L, these differences were most likely (inversely) related to organic acids quantity, as it was already explained. The importance of odor and taste complexity on *PreSc* of Chardonnay wines that were detected in the present experiment is aligned with a recent study related to quality ratings of Chardonnay wines **(Gambetta et al., 2017)**. Authors pointed out that richness on palate was assigned by the expert panel as a property of the highest quality Chardonnay wines. However, authors also reported that all young Chardonnay wines (similar to wines in the present experiment) corresponded to fruitier and fresher sensation which was not considered as a property of the highest quality Chardonnay wines. These results are suggesting that positive correlation of *PreSc* with fruity odor sensation is related only to young Chardonnay wines, whereas fruitier young Chardonnay wines are more preferred compared to less fruity young Chardonnay wines. The most important variables along the PC2 (25%) were alcoholic taste and odor and herbal odor which were all distant from *PreSc* *(Fig 5.9a)*. Obtained results are suggesting that lower alcohol content of H1 wines is more preferred compared to H2 wines *(Fig 5.9a)*, which was also confirmed by PCA loading plot of chemical composition and *PreSc* *(Fig 5.9a)*. The negative correlation of herbal odor perception with *PreSc* is might be related to high acidity and bitterness, therefore high total acidity and polyphenol content, whereas such wines (H3 wines in the presented experiment) might give a general impression of wines made from unripe grapes with green grapes odor nuances or harshness on the palate. In fact, a recent study reported that Chardonnay wines with the highest acidity and polyphenol content were significantly different among other Chardonnay wines according to green odor and harshness on the palate **(Olejar et al., 2016)**.
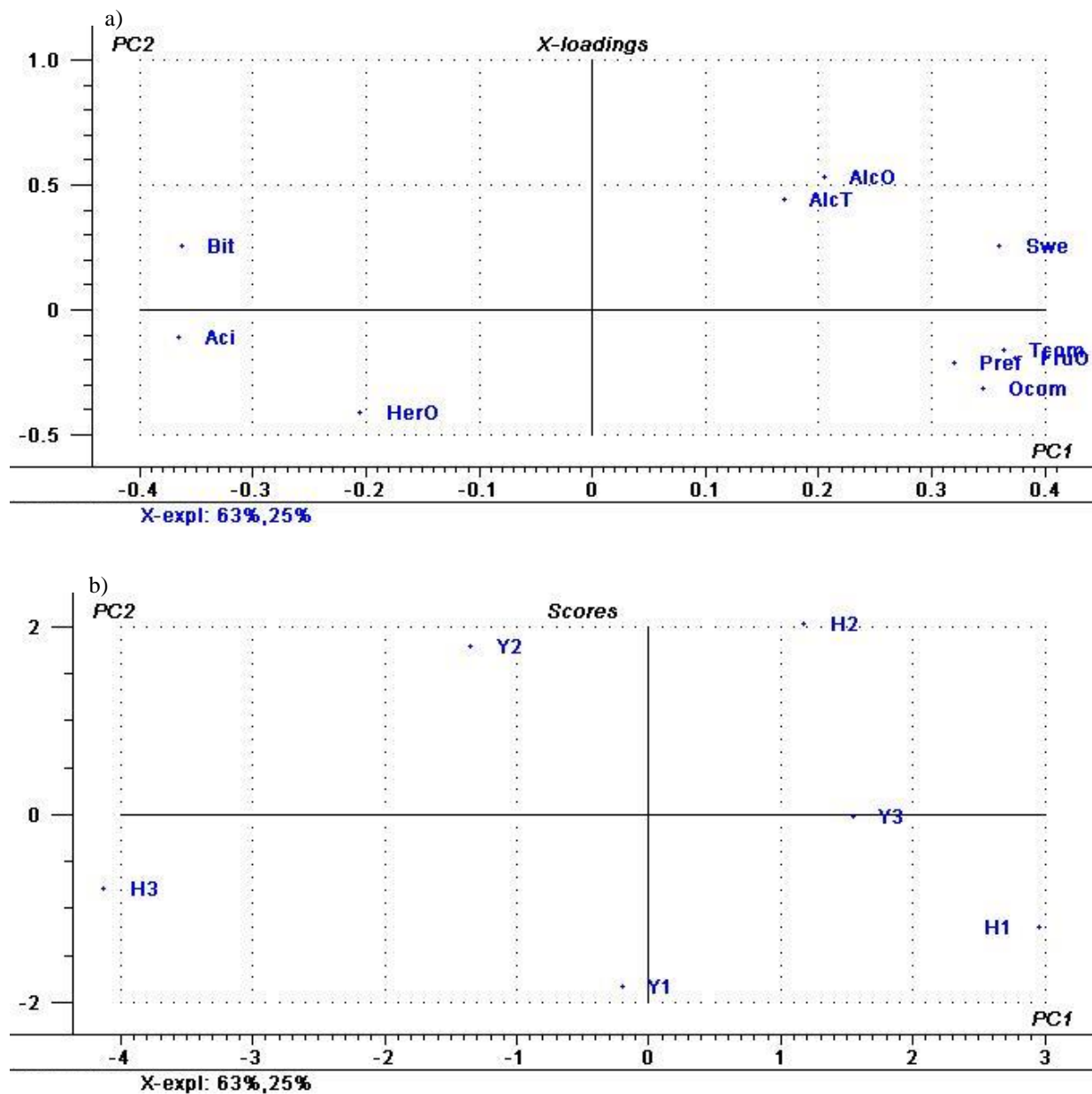
Figure 5.9 Principal component analysis a) scores plot of Chardonnay wines according to significant variables of quantitative descriptive sensory analysis; b) correlation loadings plot of Chardonnay wines quantitative descriptive sensory analysis and panelist preference. *Y1*–must vinified with inoculation of *Saccharomyces cerevisiae/Saccharomyces paradoxus*; *Y2*–must vinified with sequential inoculation of *Candida zemplinina* and hybrid *Saccharomyces cerevisiae/Saccharomyces paradoxus*; *Y3*–must vinified with inoculation of *Saccharomyces cerevisiae;* H1–wine made with technologically mature (ratio total acidity/sugar content) Chardonnay grapes; H2–wine made with Chardonnay grapes obtained during '*delayed harvest*'; H3–wine made with blend of Chardonnay grapes obtained during 'early *green* harvest' and Chardonnay grapes obtained during '*delayed harvest*'; Aci–acidity; HerO–herbal odor; AlcT–alcoholic taste; AlcO–alcoholic odor; Swe–sweetness; Tcom–taste complexity; Ocom–odor complexity; FruO–fruity odor; Pref–panelist preference.

5.3.3   Conclusions

Preliminary results obtained in this experiment suggested that combined approach of 'early *green* harvest' and lower ethanol yield yeasts can reduce wine ethanol content (~1.2% v/v) and increase wine total acidity (~2.5 g/L as tartaric acid) in Chardonnay wines. Grape picking date had a greater effect on dealcoholization and acidification of Chardonnay wines when compared to yeast selection. However, wines produced with combined method were less preferred for consumption due to acidic and bitter perception of these wines. Hence, grape juice obtained from unripe grapes can require further chemical deacidification and fining to eliminate redundant acidity and bitterness. The grape juice acidity obtained from unripe grapes may also be reduced by vinification of these grape juices, due to higher yeast consummation of malic acid under these conditions **(Bovo et al., 2016)**. Therefore, the presented experiment has pointed out advantages and drawbacks of a combined strategy to mitigate the impact of most likely upcoming hotter and drier vintages in the future decades.

5.4   References

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Appendix D – Phenological growth stages and BBCH-identification keys of grapevine

Grapevine Lorenz et al., 1994

Phenological growth stages and BBCH-identification keys of grapevine
(Vitis vinifera L. ssp. vinifera)

Code	Description
Principal growth stage 0: Sprouting/Bud development	
00	Dormancy: winter buds pointed to rounded, light or dark brown according to cultivar; bud scales more or less closed according to cultivar
01	Beginning of bud swelling: buds begin to expand inside the bud scales
03	End of bud swelling: buds swollen, but not green
05	"Wool stage": brown wool clearly visible
07	Beginning of bud burst: green shoot tips just visible
08	Bud burst: green shoot tips clearly visible
Principal growth stage 1: Leaf development	
11	First leaf unfolded and spread away from shoot
12	2nd leaves unfolded
13	3rd leaves unfolded
1 .	Stages continuous till . . .
19	9 or more leaves unfolded
Principal growth stage 5: Inflorescence emerge	
53	Inflorescences clearly visible
55	Inflorescences swelling, flowers closely pressed together
57	Inflorescences fully developed; flowers separating
Principal growth stage 6: Flowering	
60	First flowerhoods detached from the receptacle
61	Beginning of flowering: 10% of flowerhoods fallen
62	20% of flowerhoods fallen
63	Early flowering: 30% of flowerhoods fallen
64	40% of flowerhoods fallen
65	Full flowering: 50% of flowerhoods fallen
66	60% of flowerhoods fallen
67	70% of flowerhoods fallen
68	80% of flowerhoods fallen
69	End of flowering

Grapevine Lorenz et al., 1994

Phenological growth stages and BBCH-identification keys of grapevine

Code	Description
Principal growth stage 7: Development of fruits	
71	Fruit set: young fruits begin to swell, remains of flowers lost
73	Berries groat-sized, bunches begin to hang
75	Berries pea-sized, bunches hang
77	Berries beginning to touch
79	Majority of berries touching
Principal growth stage 8: Ripening of berries	
81	Beginning of ripening: berries begin to develop variety-specific colour
83	Berries developing colour
85	Softening of berries
89	Berries ripe for harvest
Principal growth stage 9: Senescence	
91	After harvest; end of wood maturation
92	Beginning of leaf discolouration
93	Beginning of leaf-fall
95	50% of leaves fallen
97	End of leaf-fall
99	Harvested product

Grapevine

00   01   05   09   11   13   15   55

57   61   63   65   68

71   73   75   77

81   89   91   93   97

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Appendix E – Effect of late winter pruning on Sangiovese grape berry composition from organic management

EFFETTO DELLA POTATURA TARDIVA SULLA COMPOSIZIONE DI UVE BIOLOGICHE SANGIOVESE
EFFECT OF LATE WINTER PRUNING ON SANGIOVESE GRAPE BERRY COMPOSITION FROM ORGANIC MANAGEMENT

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Abstract
Mitigation strategies, such as late winter pruning, can be imposed in order to slow down shoot growth and sugar accumulation in the berry. During 2015 vintage, three different timing of winter pruning were applied to organic cv. Sangiovese vines: control T1 (December), T2 (March) and T3 (April). At harvest, the concentration of total soluble solids were significantly higher in vines submitted to T3 (24.43 Bx°) and T2 (24.13) respect to T1 (23.0) while yield in plants treated in T1 (2.24 kg/plant) and T2 (2,14) was significantly higher compared to T3 (1.25). Berry weight was lower without significant difference in vines submitted to T3 (1.95 g/berry) respect to T (2.01) and T2 (2.07). Berry juice titratable acidity was lower without significant difference in T1 (7.55 g tartaric acid/l) compared with T2 (7.60) and T3 (7.71), while pH was similar within treatments, 3.17, 3.14 and 3.14 in T1, T2 and T3, respectively. Number of bunches per plant was 14, 15, 11 in T1, T2, T3, respectively. Although, T3 displayed significantly higher berry TSS compared with control T1 and T2 a slower shoot growth was observed during the early phenological stages and significantly lower yield compared to T2 and T1 was detected. Therefore, further studies of late winter pruning application on grapevine need to be conducted with included monitoring of leaf to fruit area and techniques which control sink-source balance, such as cluster thinning, in order better understand potential of TSS reduction in vines.

Keywords
Berry composition, climate change, mitigation strategies, organic Sangiovese grape, sustainability.
Parole chiave
Composizione uve Sangiovese, cambiamento climatico, strategie di mitigazione, biologico, sostenibilità.

Introduction
Grape and wine production is affected by climate change, (Fraga et al. 2014), which is responsible for over 50 % of higher alcohol content in wines during past years (Jones 2010). In particular, increasing temperature during vegetative season boosts phenological phases and sugar accumulation in the berry, which may lead to the production of wines with high alcohol levels (Duchêne and Schneider 2005; Jones 2012). Excessive alcohol in wines may cause various microbiological, technological, sensorial and financial implications to the wine industry (Mira de Orduña 2010). In particular, during warm seasons high grape sugar concentration at harvest may cause slow/stuck alcoholic fermentations (Coulter et al. 2008), as well alter sensory features due to ethanol's tendency to suppress the perception of sourness (William 1972), reduce astringency perception (Vidal et al. 2004) and increase bitterness perception (Sokolowsky and Fischer 2012). Moreover, in the USA winemakers need to pay additional taxes if wine contains more than 14.5% v/v of alcohol, whereas in EU the alcohol limit for table wine is 15.0% v/v. Recently consumers showed preference for wines with lower alcohol content (between 9 and 13% v/v) (Massot et al. 2008). Therefore, excessive wine alcohol removal or sugar accumulation reduction techniques needed to be applied in order to mitigate mentioned implications.

Mitigation techniques can be divided in three groups: (I) vineyard management – shoot trimming (Filippetti et al. 2011; Rombolà et al., 2011; Rombolà et al., 2014; Rombolà et al., 2015; Bondada et al., 2016), late irrigation (Fernandez et al 2013) etc, (II) biotechnological approach – yeast with lower ethanol production (Giaramida et al. 2013) and (III) winery management – nanofiltration, reserve osmosis (Massot et al. 2008), spinning cone column (Belisario-Sanchez et al. 2009) etc.
Possibility of slowing down berry sugar accumulation through canopy management practices, is of paramount importance in organic farming due to prohibition of techniques, such as dealcoholization, to correct wine alcohol (Reg. EC 203/2012).
Therefore, this study was focused on late winter pruning, as one the vineyard management techniques to mitigate total soluble solids (TSS) in grapes, applied to the organically cultivated Sangiovese grapes which is main red cultivar cultivated in Italy (planted in approximately 10% of overall vineyards) .

Materials and Methods
The experiment was performed in 2015, in a mature vineyard of cv. Sangiovese (clone FEDIT 30 ESAVE), *Vitis vinifera* L., grafted on Kober 5BB rootstock and trained to Cordon du Royat. The vineyard is located in Tebano (Faenza, RA), Italy (44°17'7" N, 11°52'59"E, 117

m a.s.l.), in a medium hill slope, with South-East/North-West and downhill oriented rows. Vines were spaced 2.8 m x 1.0 m (3,571 plants/ha). Starting in 2007, the vineyard was managed as organic in accordance with Reg. EC 834/2007 (EC., 2007). Since 2007, no irrigation water has been supplied and no fertilizers have been provided. The vineyard was treated to control diseases and pests, using organic products allowed by the EC Regulation (EC, 2002). Yearly, at the end of the vegetative season, herbaceous species were sowed in alternate planting rows, such as fava bean (*Vicia faba*), barley (*Hordeum vulgare*) and in the row strip, *Trifolium subterraneumon*. Soil was managed by moving the vegetation in late spring and successively maintaining the biomass on the soil surface.

The experiment included 3 treatments, in a block-randomized experimental design:

(i) Treatment 1 (T1-control) – pruning performed in December, BBCH=0;

(ii) Treatment 2 (T2) - pruning performed in March, BBCH=0;

(iii) Treatment 3 - pruning performed in April, BBCH=12.

Each treatment was applied to 3 replications, for a total of 3 experimental plots, each including 9 sample plants (total 27 sample plants).

Grapevine development was followed during whole vegetative season with BBCH scale (Lorenz et al, 1994) The occurrence of bud break (BBCH=7; green shoot tips clearly visible), flowering (BBCH=65; 50 % of flowerhood fallen), veraison (BBCH=83; 50% of colored bunches) and technological maturity (BBCH=89; optimal ratio between total soluble solids and titratable acidity) were monitored.

Samples of 100 berries, randomly chosen from the top, middle and bottom of the bunches were collected, in each experimental plot, five times from veraison to harvest and following parameters were evaluated: berry weight (technical balance Gibertini Elettronica S.r.l., Milan, Italy). total soluble solids (TSS, Electronic Refractometer Maselli Misure S.P.A., Parma, Italy), titratable acidity (TA) and pH (Crison Compact Titrator, Crison Instrument SA, Barcelona, Spain).

At harvest, productive parameters, such as number of clusters per plant, productivity per vine (Wunder Digital Dynamometer, Wunder SA-Bi S.r.l, Milan, Italy), and bunch weight.

Parametric data were submitted to one way Anova in order to detect differences within parameters. For the further exploration of differences, parameters with significant difference detected with Anova (TSS and yield per plant), were submitted to the Least significant difference (LSD) post hoc test. Tests were performed with significanse level of 95 and 90 %.

Result and Discussion
Plant development
Bud burst (BBCH=7) occurred in the same period for all treatments. From bud burst until the middle of August, plant development of control plants and vines submitted to

T2 were slightly different, whereas since middle of August, T2 had tencency to develop faster. In plants submitted to T3 a delay in early periods of plant development was observed compared to T1 and T2. This delay in plants submitted to T3 was compensated at veraison, ultimately leading, to a fastest development, respect to the T1 and T2, until harvest period *(Fig. 1; Fig.2)*.
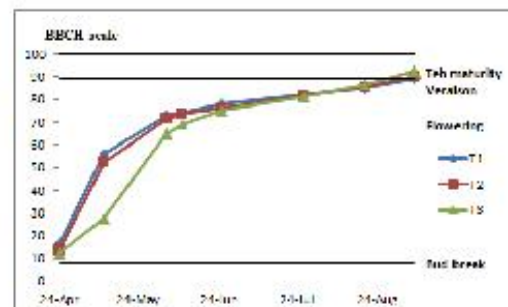
Fig 1 - Lorenz et al. extended BBCH scale of grapevine development submitted to T1, T2 and T3 from plant dormancy period (BBCH=0) until technological maturity (BBCH=89). T1 - winter pruning performed in December; T2 - winter pruning performed in March; T3 - winter pruning performed in April.

Fig. 2 - Shoot growth progress on the April 24th; up, T1 - winter pruning performed in December; center, T2 - winter pruning performed in March; down, T3 - winter pruning performed in April.

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Plants submitted to T3 had tendency to develop slower compared to T1 and T2, maximum differences were reached between bud burst and flowering. Flowering (BBCH=65) occurred slightly earlier in T1 compared with T2, while in T3, flowering was significantly delayed respect to T1. Veraison (BBCH=83) occurred slightly earlier in T1 compared with other treatments. Technological maturity was observed slighly earlier in vines subbmited to T3 respect to T2 and T1.

Technological paremeters

At harvest, TSS was 24.3, 24.13 and 23.0 Bx° in T1, T2 and T3 respectively (Tab.1).

Tab. 1 - Grape composition and productive parameters of organically cultivated cv. Sangiovese, submitted to T1, T2 and T3 treatments during vintage 2015; one way Anova: a - 90 % significance, NS - not significant; LSD: b – different from T1 with 90 % significance, c – different from T1 with 95 % significance, d – different from T3 with 95 % significance.

	Treatment 1	Treatment 2	Treatment 3	p-value
Total soluble solids [Bx°]	23.0	24.13b	24.43c	0.052a
Titratable acidity [g tar acid/l]	7.55	7.60	7.71	NS
pH	3.17	3.14	3.14	NS
Yield [kg/plant]	2.24d	2.14d	1.25	0.068a
Berry weight [g]	2.01	2.07	1.95	NS
Bunch weight [g]	158.76	138.9	114.16	NS
Number of bunches per plant	14	15	11	NS
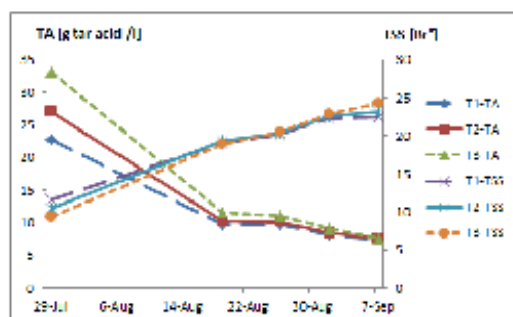
Fig. 3 - Seasonal trends of total soluble solids (TSS, Brix) and titratable acidity (TA, g/L tartaric acid), recorded in 2015 on cv. Sangiovese vines submitted T1, T2 and T3. Data are means of n=3 values. T1 - winter pruning performed in December; T2 - winter pruning performed in March; T3 - winter pruning performed in April.

Due to slighy faster plant development, berries from T1 plants had higher TSS at 29th of July, compared with T2

and T3. However, differences within treatments decreased until 26th of August, when TSS was same in all treatments. (Fig. 3).

Berries from plants submitted to T3 displayed faster total soluble solids accumulation respect to the T1. At harvest, T3 had significanly higher TSS respect to T1, while no differences were observed between T2 and T3. Highest TSS concentration in berries of vines submitted to T3 at harvest may possibly be explained with different source-sink balance within treatments caused by different yield per plant between treatments. Plants with higher yield per plant (T1 and T2) have higher carbon demand in order to reach certain value of TSS while plants with lower yield per plant (T3) have lower carbon competiton within bunches resulting faster sugar accumulation (Bobeica et al, 2015). Since leaf to fruit area was not monitored, last statement need to be taken with caution. Although, highest quantity of soluble solids vines submitted to T3 was achieved, which is contrary to the desired objective, further studies need to include, next to later winter pruning, monitoring of leaf to fruit area and techniques which control sink-source balance, such as cluster thinning, in order better understand potential of TSS reduction in plants subbmited to late winter pruning. Opposite to TSS, at the end of July, TA was highest in T3 comparing to T1 and T2 to T1. Differences in titratable acidity levels among treatments decreased until the beginning of September. At harvest slighly higher TA values were detected in berries from plants submitted to T3 compared with those of T2 and T1 vines, however, detected differences, were not significant. Similarly, berry juice pH at harvest was similar in all treatments 3.14, 3.14 and 3.17 in T3,T2 and T1, respectively. In other studies (Palliotti et al. 2014) significant TSS, pH, titratable acidity and berry weight reduction was detected when winter pruning was applied on vines before bud burst (BBCH=0) respect to vines where pruning was applied after inflorescence swelling (BBCH=55).

Productive parameters

Parameters, such as number of bunches, weight per berry, cluster weight were not significant within treatments while significant differences were detected in yield per plant (Tab. 1). Lowest plant yield within T3 comparing to T1 and T2, same as plant development in early stages, may be explained by different timing of pruning imposition, before bud burst (T1 and T2), after bud burst (T3), which is in agreement with literature (Palliotti et al. 2014). Palliotti et al. (2014) applied pruning on cv. Sangiovese grapes and detected significant yield reduction when winter pruning was applied after bud burst event.

Conclusion

Late pruning influenced plant development. In particular, plants submitted to T3 had a delay in early periods of plant development compared to T1 and T2. This delay in vines submitted to T3 was compensated at veraison, ultimately

Appendix F – Utilization of 'early *green* harvest' and non-*Saccharomyces cerevisiae* yeasts as a combined approach to face climate change in winemaking

ORIGINAL PAPER

Utilization of 'early *green* harvest' and non-*Saccharomyces cerevisiae* yeasts as a combined approach to face climate change in winemaking

Nemanja Teslić[1] · Francesca Patrignani[1,2] · Michele Ghidotti[3] · Giuseppina Paola Parpinello[1] · Arianna Ricci[1] · Rosanna Tofalo[4] · Rosalba Lanciotti[1,2] · Andrea Versari[1]

Abstract
Present study aimed to ascertain whether the combination of two factors, i.e., time of harvest and type of yeast, can significantly moderate the effect of climate change on Chardonnay wine composition. In this view, three Chardonnay musts obtained from grapes at different harvest date [technological maturity 'as control'; delayed harvest; a mixture of 'early (*green*) harvest' with delayed harvest 'as alternative approach'] and three selected yeast strains [*Saccharomyces cerevisiae* 'as control'; hybrid *Saccharomyces cerevisiae/Saccharomyces paradoxus*; scalar *alternative approach* with *Starmerella bacillaris* and hybrid *Saccharomyces cerevisiae/Saccharomyces paradoxus*] were used to design and compare six different trials, replicated at pilot level (*n.* total fermentations: 18). Wines were evaluated in terms of sensory and chemical parameters (alcohol, acidity, organic acids, phenolic compounds and glycerol) and results tested by statistical analysis. Although the wine alcohol content decreased at the best by – 1.2% v/v, whereas the total acidity increased up to – 2.5 g/L, the results from sensory evaluation highlighted that the proposed 'alternative approach' may cause excessive acidity and bitterness perception, therefore, further deacidification and fining treatments may be needed. The present approach to reduce the alcohol content of wine and increase its total acidity is simple, inexpensive and applicable in all wineries.

Keywords Chardonnay · Climate change · Harvest date · Low alcohol wine · Yeasts

Introduction

It is well known that climate change is partly responsible for the elevated sugar concentration and lower acidity in grape must [1–4]. Since there is a growing trend that fully matured and fully flavored wines are more valued and demanded by consumers, wine styles and market trends may also be responsible for the elevated sugar concentration and lower acidity [5, 6]. Higher temperatures often coupled with elevated air CO_2 concentration are main climate driver of higher sugar accumulation in grape berries due to hastened pace of phenological events [7, 8]. Under these climate condition, synthesis path of carbohydrates is preferential comparing to the synthesis path of secondary metabolites, such as anthocyanins [9]. In addition, moderate water stress may accelerate sugar accumulation in berry as a result of inhibiting lateral shoot growth allowing transportation of carbohydrates to berries or as a direct effect of grapevine hormone (abscisic acid) activation during maturity process [10]. On the other hand, elevated temperatures and air CO_2 concentration have a negative influence on total acidity and

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malic acid content in grape must due to lower efficiency of malate synthesis and higher malic acid degradation [9, 11]. In practical view, during warm and dry vintages, grapes will reach technological maturity earlier (optimal sugar/acidity ratio), while flavor compounds could remain undeveloped. However, if viticulturists wait for full flavor development, sugar/acidity ratio could be undesirable [8], and lead to the production of 'flabby' wines (elevated alcohol content and low total acidity) [12]. Elevated temperatures may also alter the aromatic profile, i.e., due to excessive berry temperatures synthesis of terpenols (i.e., linanol, nerol, geraniol) in *cv. Moscatel de Alejandria* is tending to be lower [13]; degradation of aromatic precursor 3-Isobutyl-2-methoxypyrazine present in *cv. s Semillon, Sauvignon blanc, Cabernet Sauvignon*, etc. is higher during warmer vintages [14]. Reduction of precipitation may affect aromatic profile as well, i.e., content of primary aromas typical for *cv. Muscat blanc* was usually lower during the dry years over the last two decades in the northeastern Slovenia [15].

With the assumption that climate conditions similar to 2007 in Europe will most likely occur frequently—i.e., extremely high spring temperatures, advanced phenology [16], high must sugar content and low must total acidity [17]—winemakers need more practical tools to handle with potential change in alcohol level and total acidity of their wines.

Many studies were already conducted on this field testing different techniques and methodologies which may be divided into four groups: viticulture techniques, pre-fermentation techniques, biotechnological techniques and post-fermentation techniques. Viticulture techniques are related to vineyard management which could significantly change development of vines during the growing season, thus alter the berry composition at harvest, i.e., well-timed application of winter pruning may decrease berry total soluble solids content by 1.6°Brix, increase must total acidity by 1.8 g/L, increase must anthocyanins and phenolic concentration and significantly lower grape yield [18]. Post-véraison shoot trimming may also decrease sugar content in berries for 1.2°Brix [19]. Early harvest may be used as well to decrease alcohol content and increase acidity in wines. A study reported that of mixing low-alcohol wines (– 5% v/v alcohol content) obtained with grapes harvested at véraison and grapes harvested at full phenological maturity resulted in final wine ethanol level reduction of 0.9, 1.7 and 3.0% v/v and wine total acidity increase of 0.8, 0.8 and 2.2 g/L in Cabernet Sauvignon, Merlot and Bobal wines, respectively [20]. Pre-fermentation techniques may include certain physical treatments such is nanofiltration, whereas utilization of semi-permeable membranes allows removal of excessive sugar. Two-step nanofiltration was tested on red grape must, obtaining afterwards wines with – 1.4% v/v lower alcohol content with minor differences on other wine quality parameters and lack of differences in sensory analysis results [21]. Dilution by water addition may be another pre-fermentation technique, whereas water addition (– 18% v/v) and partial removal grape must (– 18% v/v) resulted in 4°Brix sugar content reduction compared to initial high sugar content must (28°Brix) and resulted in the production of wines with similar phenolic content and aroma attributes as control (only water addition – 18% v/v) [22]. However, water addition is not allowed in all countries (i.e., Italy and France). Biotechnological techniques may include utilization of genetically modified microorganisms (GMO), whereas altering the genome is possible to redirect metabolic flux away from ethanol synthesis path towards the production of other metabolites (i.e., glycerol, acetic acid) [23]. Similarly, to water addition utilization of GMO is restricted in certain countries. The most popular and widely accepted biotechnological technique is utilization of various non-*Saccharomyces cerevisiae* species as inoculation cultures instead of traditional *Saccharomyces cerevisiae* (*Sc*) yeasts. Researchers already investigated many different species as a single inoculation culture or in scalar inoculation of two species (one is usually *Sc*) which resulted in alteration of alcohol content, aromatic profile, volatile acidity, total acidity, glycerol content etc.; i.e., sequential inoculation of *Metschnikowia pulcherrima* with *Sc* resulted in ethanol reduction by 1.6% v/v, increase of higher alcohols and decrease of volatile acids in Shiraz wines [24]. A recent study investigated sequential inoculation of *Lachancea thermotolerans* and *Sc* which comparing to inoculation with *Sc* resulted in lower ethanol yield (– 0.7% v/v), higher total acidity (– 5.4 g/L) and volatile acidity (0.18 g/L), higher glycerol content (2.2 g/L), etc. [25]. Post-fermentation techniques similarly to pre-fermentation techniques may utilize semi-permeable membranes to remove excessive alcohol content. There are various techniques, such as nanofiltration and reverse osmosis (hydrophilic membranes), pervaporation and evaporative perstraction (hydrophobic membranes); i.e., alcohol was partially removed (2% v/v) from Merlot wines by utilization of evaporative perstraction which was followed by loss of certain aroma compounds, however, with lack of differences in wine sensory characteristics [26].

However, the most of previous investigations on the field generally focus on a single approach only (viticulture management or microbiology or physical/chemical treatments), while at real industry level, the proper solution is a combination of different techniques most of the time. Furthermore, most often the fermentation trials are tested at laboratory level only and there is a need to scale up the trials to larger experimental volumes prior to final industrial scale. Therefore, the present study investigated a combined approach—i.e. viticulture and biotechnology—based on different timing

of grape harvest and the use of three selected yeast strains to reduce the alcohol content and increase the total acidity of Chardonnay wine.

Materials and methods

Chemicals and reagents

Citric acid, lactic acid, L-malic acid, succinic acid, acetonitrile, (+)-catechin, (−)-epicatechin, caffeic acid, glycerol, sodium hydroxide, sodium carbonate and gallic acid were purchased from Sigma-Aldrich (Steinheim, Germany). L-tartaric acid, sulphuric acid, p-coumaric acid, Folin–Ciocalteu's reagent, acetic acid and calcium hydroxide were purchased from Merck (Darmstadt, Germany). Glucose, yeast extract and peptone were purchased from Oxoid (Basingstoke, United Kindom). Ethanolic solution of phenolphthalein was purchased from Carlo Erba (Milan, Italy). Ferulic acid was purchased from Extrasynthese (Genay, France). Glycerol enzymatic kit was purchased from Steroglass (Perugia, Italy). Silicon antifoam was purchased from Ing. Castore Bullio (Milan, Italy).

Yeasts selection and preparation

The strains used for the fermentations were: Vin13 (V; *Saccharomyces cerevisiae 'as control'*) (Oenobrands, France), Exotics (E; hybrid *Saccharomyces cerevisiae/Saccharomyces paradoxus*) (Oenobrands, France) and *Starmerella bacillaris FT811* (strain isolated at the University of Teramo) in *scalar alternative approach* with Exotics (S). For each fermentation (in total n. 18; Fig. 1), yeasts were activated in 10 mL of liquid yeast extract peptone dextrose (10 g/L peptone, 10 g/L yeast extract, 20 g/L glucose) and incubated for 24 h at 25 °C. Then, the yeasts were re-inoculated into 250 ml of non-clarified and pasteurized must (pasteurization conditions; 65 °C for 30 min) and then incubated for 24 h at 25 °C to obtain at least 10^7 log CFU/mL. Activated yeasts were directly transferred into grape must (for details see following 'Grape-harvesting- and winemaking procedure'). The fermentations inoculated with *Starmerella bacillaris* were inoculated in scalar approach (at alcohol content – 7–8% v/v) with 30 g/hL of yeast E to prevent the occurrence of late spontaneous fermentation and the incidence of wild *Sc* strains.
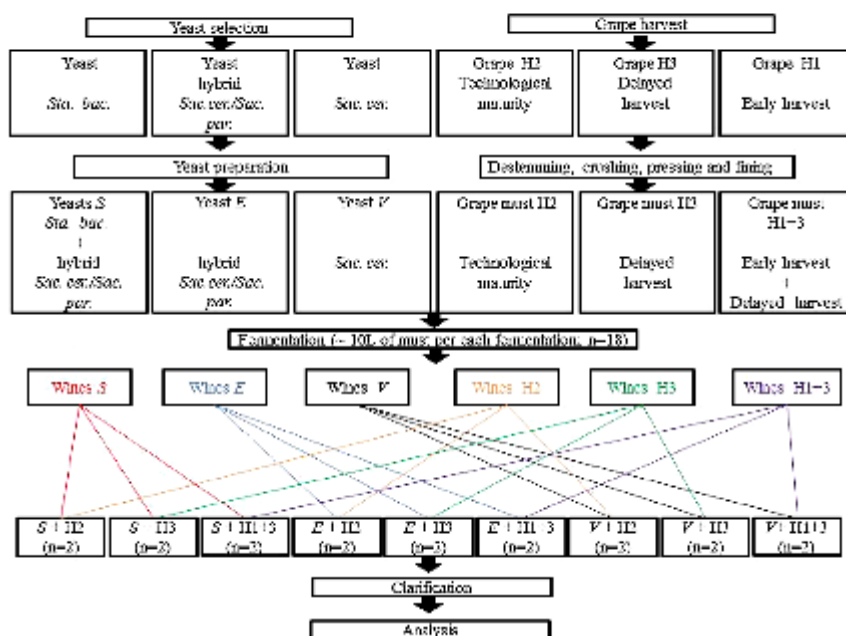
Fig. 1 Design of the experiment and winemaking procedure

Grape-harvesting- and winemaking procedure

The fermentations were performed on 2016, with grapes from *cv. Chardonnay* trained to free cordon with a 2.5 m × 1.0 m vines spacing (4000 plants/ha). The vineyard and winery were located in Tebano (44°28'7"N; 11°77'5"E; Faenza, Italy). Harvest-1 (H1) was considered as 'early *green* harvest' (at véraison), with manual harvest of 25 kg of thinned grapes, that were hand destemmed and crushed (Table 1; Fig. 1). The grape must was supplemented with $K_2S_2O_5$ (5 g/hL, AEB, Italy) and stored into PVC tank at − 20 °C until fermentation, as described in the following.

Harvest-2 (H2) was performed at technological maturity (*control*)—based on sugar content and total acidity—with grapes (− 100 kg) manually harvested, crushed and pressed using a semi-automatic press (22620M, Spedeil, Ofterdingen, Germany) (Table 1; Fig. 1). Grape must was transferred into stainless-steel tank, supplemented with $K_2S_2O_5$ (8 g/hL), pectolytic enzymes (1 g/hL, Lafazym CL, Laffort, France), silica gel (30 g/hL, Baykisol 30, AEB, Italy), gelatin (3 g/hL, Gelsol, AEB, Italy), bentonite (30 g/hL, Superbenton, Dal Cin, Italy) and stored at + 4 °C for fining. After 48 h, must was racked into six stainless-steel fermenters (duplicate fermentations for each of three yeasts; − 10 L of must was used for each fermentation), supplemented with complex nutrients (Nutristart 30 g/hL, containing 0.39 mg/L of thiamine, Laffort, France), $K_2S_2O_5$ (4 g/hL) and inoculated with yeast strains (see 'Yeast preparation' section).

4 days after H2, 200 kg of grapes were manually collected (harvest-3: H3) and processed as described for grapes H2 already (Table 1; Fig. 1). The H3 must, considered as *'delayed harvest'*, was split into two equal parts and part of which was treated similarly as must H2 (six stainless-steel fermenters, duplicate fermentations for each of three yeasts; − 10 L of must was used for each fermentation). The second half was mixed with must H1 (in a volume ratio H1:H3 = 1:10) to match the sugar content of musts H2 (Table 1). Later, must H1 + 3 was treated as must H2 (six stainless-steel fermenters, duplicate fermentations for each of three yeasts; − 10 L of must was used

for each fermentation). Fermentations of all trials (*S, E, V,* H2, H3, H1 + 3; Fig. 1) were run in stainless-steel tanks with controlled temperature (17 °C *V,* 20 °C *E* and *S*), and the ongoing of fermentation was monitored by daily measuring sugar consumption with Babo densimeter. Once fermentations were complete (below 0.2 g/L of residual sugars), wines were supplemented with $K_2S_2O_5$ (7 g/hL) and stored for 5 days at + 4 °C for clarification. Clear liquid was racked into glass containers and stored at 0 °C for cold stabilization during 20 days. Stabilized wines were finally bottled in glass and closed with crown cap, thus stored at + 17 °C until analysis.

Basic enological analysis and glycerol analysis

Must total acidity was analyzed with volumetric method, whereas sodium hydroxide solution (0.1 N) was added into the sample until pH reached value of 7 which was controlled by pH meter (pH 209, Hanna Instruments, Padova, Italy). Must pH was analyzed as well with pH meter (pH 209, Hanna Instruments, Padova, Italy), whereas total soluble solid content was analyzed with a digital refractometer (PAL-1, Atago, Tokyo, Japan), according to official European Commission methods [27]. Effective alcohol content of wines was analyzed with hydrostatic balance and heat-stream distiller (Ing. Castore Bullio, Milan, Italy), according to official European Commission methods [27], same as wine total acidity (method used for grape musts), pH and volatile acidity. Glycerol concentration in wines was analyzed by enzymatic kit and UV–Vis spectrophotometer (Cary 60, Agilent Technologies, Santa Clara, USA) according to producer's instruction (Steroglass, Milan, Italy).

Organic acid analysis

Organic acids in wines were analyzed by high-performance liquid chromatography (HPLC) (Dionex IC-500, Milano, Italy) using a system with diode array detection (DAD), column Aminex HPX-87H (9 µm, 7.8 × 300 mm; Bio-Rad, Hercules, USA) according to a method previously described [28]. Prior to direct injection into HPLC system, the sample was filtered with 0.2 µm nylon membrane (Gema Medical, Barcelona, Spain). Organic acids were identified and quantified at 210 nm with calibration curves of citric (0–31 mmol/L; $R^2 = 0.999$), L-tartaric (0–66.5 mmol/L; $R^2 = 0.995$), L-malic (0–110 mmol/L; $R^2 = 0.999$), succinic (0–50 mmol/L; $R^2 = 0.999$), lactic (0–115 mmol/L; $R^2 = 0.999$) and acetic acid (0–105 mmol/L; $R^2 = 0.999$). Example of chromatogram was added as supplementary material (Fig. S1).

Table 1 Chemical composition of Chardonnay grape must vintage 2016

Variables	Total soluble solids (g/L)	pH	Total acidity (g/L)*
H1	99.7 ± 1.0	2.61 ± 0.06	26.29 ± 0.05
H2	208.4 ± 0.0	3.19 ± 0.01	6.45 ± 0.00
H3	222.5 ± 1.0	3.30 ± 0.02	6.28 ± 0.04
H1 + 3	211.7 ± 0.0	3.06 ± 0.02	8.95 ± 0.04

Values are the mean (± SD) of three analysis conducted for must harvested at different date

*Total acidity expressed as g/L of tartaric acid

Phenolic compound analysis

Colorimetric assay of total polyphenol concentration was expressed at 1 cm path length according to the literature [29]. Quantification of phenolic compounds was performed at 750 nm with calibration curve of gallic acid (0–0.665 mmol/L; $R^2 = 0.994$).

Concentration of individual phenolic compounds was analyzed with HPLC-DAD and Inertsustain C18 column (5 μm, 4.6×250 mm; GL Science, Tokyo, Japan). Prior to direct injection into HPLC system conditioned at 30 °C, samples were filtered with 0.2 μm cellulose acetate membrane (GVC Filter Technology, Sanford, USA). Separation of phenolic compound was performed at 0.5 ml/L flow rate with solvent A (acetic acid:water= 5:95 mL/dL) and solvent B (acetonitrile:water= 80:20 mL/dL) in the following proportions: 15 min, 0 mL/dL B; 30 min, 5 mL/dL B; 50 min, 10 mL/dL B, 51 min, 11 mL/dL B; 70 min, 11 mL/dL B; 82 min, 15 mL/dL B; 90 min, 15 mL/dL B; 95 min, 60 mL/dL B; 109 min, 0 mL/dL B. Phenolic compound were identified and quantified at 280 nm [(+)-catechin and (−)-epicatechin], 308 nm (coutaric as *p*-coumaric) and 324 nm (caftaric as caffeic, ferulic) with calibration curves of (+)-catechin (0–0.17 mmol/L; $R^2 = 0.999$), (−)-epicatechin (0–0.17 mmol/L; $R^2 = 0.999$), *p*-coumaric acid (0–0.30 mmol/L; $R^2 = 0.999$), caffeic acid (0–0.28 mmol/L; $R^2 = 0.999$) and ferulic acid (0–0.26 mmol/L; $R^2 = 0.999$). Example of chromatogram was added as supplementary material (Fig. S2).

Sensory analysis

As a preliminary step, wines were tasted by enologists and trained staff of the BSc program in Enology and Viticulture at the University of Bologna (three females and two males, aged between 27 and 52), to ascertain the lack of differences between duplicate vinifications.

Thereafter, one wine was randomly selected for each combination of yeast (S, E or V) and harvest date (H2, H3, H1+3), and the nine selected wines were evaluated in terms of preference by 25 consumers (12 females and 13 males, aged between 20 and 46) recruited among employees and students of the BSc program in enology and viticulture (University of Bologna, Campus of Food Science, Cesena, Italy). Sample preference was evaluated using simple 100 mm unstructured linear hedonic scale anchored with '0' (as extremely disliked) and '10' (as extremely liked), since consumers have the possibility to express sensory perceptions more accurately than with the nine-point hedonic scale [30]. Sensory evaluation was performed in two sessions within the same day (four wines in the first session and five wines in the second session). Wines were coded with a three-digit number and randomly distributed to consumers in transparent and pear-shaped glasses containing 25 ml of wine [31]. Natural water was distributed to assessors for palate cleaning (Levissima, Torino, Italy).

Statistical analysis

The chemical composition of grape musts and wines was statistically evaluated with one-factor analysis of variance (ANOVA) and parametric post hoc Tukey's tests with a confidence level set at 90 and 95%. Sensory data were statistically evaluated with non-parametric Kruskal–Wallis test with a confidence level set at 90 and 95%. Principal component analysis (PCA) was used as unsupervised method to disclose the hidden structure of sensory evaluation of wines.

Results and discussions

Influence of harvest date and yeast strains on basic enological parameters and glycerol content

It is well known that yeast strains play a key role in determining the final composition of wine including alcohol, total acidity, malic acid, acetic acid, glycerol, polyphenols, aroma profile, etc. [32]. In our study, the average alcohol content of Chardonnay wine produced by S, E and V yeasts was 11.87% v/v, 11.83% v/v and 12.04% v/v, respectively (Table 2); therefore, alcohol content had a tendency to drop of about 0.2% v/v using selected yeasts. This moderate alcohol decrease is consistent with the literature, in particular *Saccharomyces paradoxus* (*Sp*), compared to the *Saccharomyces cerevisiae* (*Sc*), produced less alcohol in Chardonnay wines of about −0.35% v/v [33], while Sangiovese red must fermented with *Starmerella bacillaris* (*Sb*) yeast showed lower alcohol up to 0.3% v/v [34]. Although some studies reported further alcohol reduction using a *Sb* comparing to *Sc* for fermentations of red grape varieties [35], it is postulated that this occur at the higher extent when fermenting grapes with high sugar content (situation often found in red wines compared to white wines, when same yeast species are used). Apart from *Sb* and *Sp*, other non-*Saccharomyces* species may be used for alcohol reduction as well. A recent study reported alcohol reduction in Chardonnay wines of 1.0 and 1.1% v/v by utilization of *Saccharomycess uvarum* and *Metschnikowia pulcherrima* (*Mp*) both in sequential inoculation with *Sc* [36]. Other study reported that *Schizosaccharomyces malidevorans* in sequential inoculation with *Sc* reduced ethanol content by 0.6% v/v in growing medium [24]. Thus, these findings are suggesting that utilization of, i.e., *Mp* may decrease alcohol content in Chardonnay wines at higher amount than selected yeasts in our study.

Since the chemical composition of berries changes with time during ripening, the date of grape harvest plays an

Table 2 Chemical composition of Chardonnay wines produces during vintage 2016

Variables	Alcohol (% v/v)	Glycerol (g/L)	pH	Total acidity (g/L)*	Volatile acidity (g/L)**	Phenolics (mg/L)***
H 2	$11.62 \pm 0.11b$	5.1 ± 0.7	$3.17 \pm 0.02b$	$7.07 \pm 0.26b$	0.29 ± 0.11	$125 \pm 6c$
H 3	$12.50 \pm 0.09a$	5.5 ± 0.7	$3.22 \pm 0.03a$	$6.88 \pm 0.27b$	0.39 ± 0.08	$132 \pm 3b$
H 1+3	$11.60 \pm 0.12b$	5.2 ± 0.9	$3.06 \pm 0.01c$	$8.80 \pm 0.14a$	0.35 ± 0.08	$138 \pm 2a$
S	11.87 ± 0.47	$4.9 \pm 0.2b$	3.17 ± 0.08	7.55 ± 0.97	$0.43 \pm 0.05d$	131 ± 7
E	11.83 ± 0.48	$6.2 \pm 0.2a$	3.14 ± 0.07	7.66 ± 0.98	$0.34 \pm 0.10d$	135 ± 4
V	12.04 ± 0.45	$4.7 \pm 0.4b$	3.15 ± 0.08	7.55 ± 0.96	$0.26 \pm 0.03e$	130 ± 9

Statistical analysis based on one-way ANOVA with Tukey's post hoc test ($p < 0.05$; $p < 0.1$) using different letters for significantly different groups (see footnotes for explanation)

Values are the mean ($\pm$ SD) of single analysis for six fermentations conducted with same harvest date or selected yeasts

Average values assigned by different letter are statistically different from each other, by Tukey's test at $p < 0.05$ (a, b and c), and at $p < 0.1$ (d and e)

*Total acidity expressed as g/L of tartaric acid

**Volatile acidity expressed as g/L of acetic acid

***Concentration of total polyphenols expressed as mg/L of gallic acid

important role on the wine composition. In this study, the so-called 'delayed harvest' (must H3) occurred only 4 days after H2 to minimize the loss of the primary aroma of Chardonnay grapes. As expected, the 'blended' samples (H1 +3) had a significantly lower alcohol content compared to wines made with H3 must. The average ethanol concentration for wines produced with grapes harvested at the H2, H3 and blend H1 +3 was 11.62, 12.50 and 11.60% v/v, respectively (Table 2). The results clearly showed that the alcohol reduction in Chardonnay wines was mainly affected by harvest date (– 0.9% v/v), compared to the yeast strains (– 0.2% v/v). Other authors reported similar results, whereas the addition of low-alcohol wine (5% v/v) in a high-sugar must, afterwards caused reduction of alcohol content in Cabernet Sauvignon wines by 0.9% v/v and about 3.0 and 1.7% v/v in Bobal and Merlot wines, respectively [20].

The use of selected yeasts and different timing of grape harvest effectively decreased the alcohol content (– 1.21% v/v) in Chardonnay wines from 12.68% v/v (yeast V with H3) to 11.47% v/v (yeast E with H1 +3). According to the literature, the use of a combined approach can further decrease the alcohol content in wines, especially in the case of red grape varieties [20, 35, 37].

Production of glycerol, often related to yeast adaptation to osmotic or cold stress [38], can affect the sensory characteristics of wine due to sweetness perception [39], whereas its contribution to the viscosity or body, may be controversial due to relatively low concentration of glycerol in wines [40]. In this study, the date of harvest did not significantly affected the concentration of glycerol that was 5.1, 5.5 and 5.2 g/L in H2, H3 and H1 +3 wines, respectively (Table 2). The higher glycerol concentration in H3 wines could be related (1) to slightly higher osmotic stress comparing to H2 and H1 +3 wines and (2) due to the fact that H3 must had higher

concentration of sugars when compared to H2 and H1 +3 must, which could be converted to more glycerol (Table 1).

On the other hand, the selected yeasts had more influence on glycerol content, whereas V (4.7 g/L) and S wines (4.9 g/L) showed a significantly lower concentration when compared to E wines (6.2 g/L) (Table 2). Results related to higher glycerol production in E wines compared to V wines is consistent with the literature [33]. Furthermore, various Sp strains can produce from – 5.6 to 9.2 g/L of glycerol in Chardonnay wines [33], indicating the importance of yeast strain selection among same species.

It is well known that wine total acidity and pH values are among the most important wine parameters, due to their effect on sensory characteristics, color stability, malolactic fermentation, concentration of active molecular SO_2, etc. The average total acidity for wines produced with grapes harvested at the H2, H3 and H1 +3 was 7.07, 6.88 and 8.80 g/L (as tartaric acid), respectively, while pH values were 3.17, 3.22 and 3.06 for H2, H3 and H1 +3 wines, respectively (Table 2). Both parameters were aligned with the literature, in which Chardonnay wines had pH value in the range 3.1–3.5, and total acidity in the range 4.2–10.1 g/L [33, 41–44]. As expected, the blend of must H1 +3 significantly increased the total acidity of Chardonnay wine, thus representing an alternative 'natural' approach to chemical acidification to tailor wine acidity, if needed. These findings are consistent with a recent study [20], in which the blend of low-alcohol wine (obtained with grapes harvested at the beginning of véraison) and must (obtained with ripen grapes) of Cabernet Sauvignon, Merlot and Bobal increased total acidity in the range of 0.8–2.2 g/L (as tartaric acid). On the other hand, compared to harvest date, the selection of yeasts had a little effect on wine total acidity and pH, whereas wines produced

by *S*, *E* and *V* yeast had 7.55, 7.66 and 7.55 g/L of total acidity, and pH value of 3.17, 3.14 and 3.15, respectively (Table 2). However, a study reported that total acidity in Chardonnay wines was 7.3 and 6.8 g/L when must was inoculated with *Sc* and *Sp*, respectively [43]. Suggesting that *Sp* may also decrease wine total acidity due to higher degradation of malic acid during the fermentation with *Sp* comparing to *Sc*. Fermentation with *Schizosaccharomyces pombe* may also decrease total acidity (4.2 g/L) and increase pH (3.36) comparing to *Sc* (7.3 g/L; 3.27) [43]. On the other hand, sequential inoculation of *Lachancea thermotolerans* and *Sc* may increase wine total acidity (12.40 g/L) and reduce pH (3.2) comparing to *Sc* (7.0 g/L; 3.4) [25].

The increase of total acidity in wines H2 and H3 compared to the corresponding must before fermentation (Tables 1, 2) can be partially explained with the production of succinic acid during the fermentation process (Table 3). On the other hand, the lower total acidity of wine H1+3 compared with the respective musts (Tables 1, 2) can be tentatively ascribed to the consumption of malic acid and the favored precipitation of potassium bitartrate. The combined approach herein proposed, i.e., selected yeasts and different grape harvest date, increased (2.55 g/L) total acidity of Chardonnay wine from 6.37 g/L (yeast *S* with H3) to 8.92 g/L (yeast *E* with H1+3).

The harvest date had a little impact on volatile acidity that was 0.29, 0.39 and 0.35 g/L (as acetic acid) for H2, H3 and H1+3 wines, respectively (Table 2). On the other hand, selected yeasts had a significant impact on volatile acidity. In particular, wines fermented with yeast *V* had a significantly lower volatile acidity (0.26 g/L), comparing to wines produced by yeast *S* (0.43 g/L) and *E* (0.34 g/L) (Table 2). Even though statistical differences were detected, concentration of volatile acids in wines was far below 1.2 g/L, which is the legal limit of volatile acidity in wines set by International Organization of Vine and Wine [45]. The concentration of volatile acids in wines fermented with *E* was higher comparing to *V* which is partly aligned to other studies (−0.2–0.4 g/L) [33, 43].

Influence of harvest date and yeast strains on organic acid profile and content

Organic acids are present in wine as naturally occurring in grapes (tartaric, malic and citric acid) or as products of yeast and bacteria metabolism during the fermentation (succinic, lactic and acetic acid). The concentration of organic acids in wine is depending on grape variety, selected yeast, vinification, storage conditions, etc. Since tartaric, malic citric acids are more related to grape variety and grape maturity process their concentration was significantly influenced by harvest date (Table 3). Except H1+3 wines, concentrations of these acids are aligned with other studies related to Chardonnay wines, with tartaric acid was in the range from −2.6 to 4.6 g/L [41, 46], malic acid from −1.5 to 3.3 g/L [41, 43, 44, 47] and citric acid −0.1 g/L [46]. As expected, the highest concentrations of these acids were detected in H1+3 wines as H1 must had the highest total acidity (Table 1). The higher concentration of tartaric and malic acid in H2 wine compared to H3 wines may be due to formation of their respective potassium salts as the ripening process advances [48].

Similar to volatile acidity, the harvest date had a little impact on acetic acid content which was 0.12, 0.20 and 0.19 g/L (as acetic acid) for H2, H3 and H1+3 wines, respectively (Table 3). Reversely, the yeast selection had a significant impact on acetic acid concentration that was 0.36, 0.13 and 0.02 g/L in *S*, *E* and *V* wines, respectively (Table 3). Results are suggesting that concentration of acetic acid in wines fermented with *S* was lower in our study (0.36 g/L) when compared to other studies (−0.5–0.95 g/L) [34, 35, 49].

Table 3 Composition of organic acids of Chardonnay wines produced during vintage 2016

Variables	Citric (g/L)	Tartaric (g/L)	Malic (g/L)	Lactic (g/L)	Succinic (g/L)	Acetic (g/L)	Σ Acids (g/L)
H 2	0.19±0.01b	2.31±0.06e	3.58±0.15e	0.09±0.04	2.47±0.08a	0.12±0.15	8.76±0.19b
H 3	0.20±0.02b	2.20±0.09f	3.25±0.28f	0.12±0.02	2.13±0.14b	0.20±0.18	8.09±0.30c
H 1+3	0.24±0.01a	2.81±0.09d	4.71±0.29d	0.09±0.03	2.36±0.10a	0.19±0.14	10.40±0.29a
S	0.20±0.03	2.38±0.28	3.72±0.71	0.07±0.03b	2.22±0.22	0.36±0.05a	8.94±1.09
E	0.21±0.03	2.45±0.30	3.69±0.65	0.12±0.01a	2.43±0.14	0.13±0.07b	9.03±1.05
V	0.22±0.02	2.49±0.31	4.13±0.72	0.11±0.03a	2.31±0.12	0.02±0.02c	9.28±1.12

Statistical analysis based on one-way ANOVA with Tukey's post hoc test ($p < 0.05$; $p < 0.1$) using different letters for significantly different groups (see footnotes for explanation)

Values are the mean (±SD) of single analysis for six fermentations conducted with same harvest date or selected yeasts

Average values assigned by different letter are statistically different from each other, by Tukey's test at $p < 0.05$ (a, b and c), and at $p < 0.1$ (d, e and f)

Succinic acid content in all wines (*n.* 18 trials in total) ranged from 1.94 (*S* yeast with H3) to 2.60 g/L (*E* yeast with H2), which is appreciably higher when compared to concentrations of succinic acid usually found in Chardonnay wines (–0.3–1.4 g/L) [43, 44, 46], or white wines in general (up to 1.6 g/L) [50]. The reason for these high values of succinic acid could be due to the relatively high concentration of thiamine added as Nutristart (see 'Grape-harvesting- and winemaking procedure') [50]. Succinic acid is metabolized by yeasts via Tricarboxylic acid (TCA) cycle, whereas cofactors required for TCA cycle-related enzymatic reactions (i.e., entry of pyruvic acid and glutamate to TCA cycle) are derived from vitamins (i.e., thiamine, biotin, pantothetic acid). Thus, changes in concentration of these compounds could alter the quantity of succinic acids [50]. Furthermore, succinic acid is mainly produced during the first stages of fermentation [51, 52], which concentration could be higher in pasteurized [53] and non-clarified must (see 'Yeast preparation'), due to high content of yeast nutrients. These findings suggested that regulation of yeast nutrition represents a further tool to regulate wine total acidity and pH value during the 'hot' vintages by low total acidity and high pH value.

Influence of harvest date and yeast strains on phenolic compound profile and content

Phenolic compounds are important for wine quality as they affect color, flavor and taste [32]. In our study, polyphenol concentration in Chardonnay wines was – 120–140 mg/L (as gallic acid; Table 2), which value is close compared with other studies addressing phenolic compound content in Chardonnay wines [42, 54]. The harvest date significantly affected the polyphenols content of Chardonnay wines, with grapes harvested at H2, H3 and H1 + 3 with 125, 132 and 138 mg/L of phenolic compounds (as gallic acid), respectively (Table 2). The highest concentration of phenolic compounds in wines H1 + 3 most likely reflect the high content of polyphenols in must obtained from grapes harvested at H1 which was added instead of H3 must. Grapes harvested at véraison (similarly to H1) have higher concentration of polyphenols comparing to grapes harvested after véraison (H3 and H2). This finding is explained by the increased synthesis of hydroxycinnamic acids and their derivatives that occurs prior to véraison, followed by a drop of hydroxycinnamic acids content after véraison due to sugar accumulation and berry growth [55]. In fact, hydroxycinnamic acid content was the highest in wines made with grapes harvested at H1 + 3 (22.5 mg/L) compared to H2 (20.7 mg/L) and H3 (20.9 mg/L) (Table 4). As expected, concentration of caftaric acid, the main hydroxycinnamic acid of white wines [56], peaked in wine H1 + 3 (18.2 mg/L) followed by H2 (17.1 mg/L) and H3 (16.8 mg/L) (Table 4). The values of caftaric acid found in this study are consistent with other studies on Chardonnay wines (– 8–44 mg/L) [41, 42, 54]. (+)-Catechin was detected in the range 0.9–1.2 mg/L for all wines and did not differ statistically according to harvest date or yeast selection (Table 4). Obtained results in our study are aligned with other studies, whereas (+)-catechin concentration in Chardonnay wines was in the range – 0.7–33 mg/L [41, 42, 54]. On the other hand, (–)-epicatechin was significantly higher in H2 (2.9 mg/L) comparing to H3 (2.3 mg/L) and H1 + 3 (2.5 mg/L), while (–)-epicatechin did not differ according to yeast selection (Table 4). Other studies reported similar concentrations of (–)epicatechin in Chardonnay wines (0.5–56 mg/L) [41, 42, 54].

Compared to harvest date, the yeast strain had a little impact on total polyphenols content and concentration of individual phenolic compounds. Polyphenols content in Chardonnay wines fermented with yeast *S*, *E* and *V* was 131, 135 and 130 mg/L, respectively (Table 2), part of which was identified by HPLC as low molecular weight phenolic compounds (Table 4).

Table 4 Composition of phenolic compounds in Chardonnay wines produced during vintage 2016

Variables	Caftaric (mg/L)	Coutaric (mg/L)	Ferulic (mg/L)	ΣHCA (mg/L)	Catechin (mg/L)	Epicatechin (mg/L)	Σ Flavan-3ols (mg/L)	Σ Phenolics (mg/L)
H 2	17.2 ± 1.4b	1.2 ± 0.1c	2.4 ± 0.3b	20.7 ± 1.3b	1.2 ± 0.3	2.9 ± 0.3d	4.1 ± 0.3a	24.8 ± 1.3b
H 3	16.8 ± 0.5b	1.4 ± 0.1b	2.7 ± 0.2a	20.9 ± 0.4b	0.9 ± 0.3	2.3 ± 0.3e	3.1 ± 0.4b	24.0 ± 0.6b
H 1 + 3	18.2 ± 0.5a	1.7 ± 0.1a	2.6 ± 0.1a	22.5 ± 0.5*	0.9 ± 0.5	2.5 ± 0.7e	3.4 ± 0.5b	25.9 ± 0.8a
S	17.9 ± 1.0	1.4 ± 0.2	2.6 ± 0.3	22.0 ± 0.7	1.1 ± 0.5	2.3 ± 0.5	3.4 ± 0.6	25.3 ± 1.3
E	17.2 ± 0.6	1.4 ± 0.2	2.4 ± 0.3	21.1 ± 1.1	1.0 ± 0.3	2.6 ± 0.6	3.6 ± 0.7	24.6 ± 0.6
V	17.1 ± 1.3	1.4 ± 0.2	2.6 ± 0.2	21.1 ± 1.5	0.9 ± 0.4	2.8 ± 0.3	3.7 ± 0.5	24.8 ± 1.5

Statistical analysis based on one-way ANOVA with Tukey's post hoc test ($p < 0.05$; $p < 0.1$) using different letters for significantly different groups (see footnotes for explanation)

Values are the mean (± SD) of single analysis for six fermentations conducted with same harvest date or selected yeasts

Average values assigned by different letter are statistically different from each other, by Tukey's test at $p < 0.05$ (a, b and c), and at $p < 0.1$ (d and e)

Influence of harvest date and yeast strains on consumer preference

Variable	Preference scores
H 2	3.57 ± 1.68
H 3	3.55 ± 1.72
H 1+3	3.27 ± 1.65
S	3.23 ± 1.67
E	3.59 ± 1.77
V	3.57 ± 1.59

Values are the mean ($\pm$SD) of 25 analysis (25 consumers) for three randomly chosen fermentation conducted with same harvest date or selected yeast (see text for variables code)

Statistical analysis based on Kruskal–Wallis test ($p < 0.05$; $p < 0.1$)

The average preference scores (*Pref*) did not differ statistically according to non-parametric Kruskal–Wallis test (Table 5). The lowest values were assigned to the H1+3 wines (3.27) and *S* wines (3.23), while other wines had approximately equal scores (3.55–3.59). The PCA plot allowed to disclosure of correlations between *Pref* and the chemical composition of Chardonnay wines. The first two PCs explained 84% of the variability, whereas PC_1 (57%) separated trials according to harvest date, while PC_2 (27%) separated trials according to yeast selection (Fig. 2a, b). Along PC_1, *Pref* was negatively associated with total acidity, tartaric, citric and malic acid, same as with total concentration of polyphenols, caftaric and coutaric acid (Fig. 2a), hence suggesting that wines H1+3 may be perceived as acidic and bitter when compared to H2 and H3 (Fig. 2a, b). It is well known that organic acids have different dissociation constant and sensory properties, whereas the chemistry of bitter compounds is more complex and needs to be further investigated. Reversely, alcohol content and pH were positively correlated with *Pref* along the PC_1 (Fig. 2a). Since pH value is inversely proportional on a log-scale to total acidity

Fig. 2 Principal component analysis a correlation-loading plot of chemical and sensory data; b scores plot of Chardonnay wines according to significant variables. *Alc* alcohol content, *pH* pH, *Pref* consumer preference, *VA* volatile acidity, *Ace* acetic acid, *Suc* succinic acid, *TP* total polyphenols, *Caf* caftaric acid, *Cou* p-coumaric acid, *TA* total acidity, *Tar* tartaric acid, *Mal* malic acid, *Cit* citric acid, *H2* wine made with Chardonnay grapes at technological maturity as *"control"* (ratio total acidity/sugar concentration), *H3* wine made with delayed harvest Chardonnay grapes, *H1+3* wine made with mixture of Chardonnay grapes from 'early green harvest' and delayed harvest of Chardonnay, *S* must fermented with scalar inoculation of *Starmerella bacillaris* and hybrid *Saccharomyces cerevisiae/Saccharomyces paradoxus*, *E* must fermented with hybrid *Saccharomyces cerevisiae/Saccharomyces paradoxus*, *V* must fermented with *Saccharomyces cerevisiae*

which is negatively correlated to the *Pref*, this outcome was expected.

On the other hand, along PC₂ wines were separated according to yeast selection, with *Pref* negatively correlated to acetic acid and volatile acidity, whereas succinic acid seemed to have a positive impact on consumer preference (Fig. 2a). However, the results showed that these variables and yeast selection had a minor influence on *Pref* when compared to harvest date and related variables separated along PC₁.

Conclusions

Preliminary results obtained in this study suggested that combined approach of 'early *green* harvest' and selected yeasts with low ethanol yield can decrease alcohol content (– 1.2% v/v) and increase in total acidity (– 2.5 g/L as tartaric acid) in Chardonnay wines. Harvest date had a greater impact on final alcohol and acidity content of Chardonnay wines when compared to selected yeasts. However, wines obtained with the combined approach were questioned by consumers most likely due to their acidic and bitter perception. Therefore, must obtained from unripe grapes can require further chemical deacidification and fining to eliminate excessive acidity and bitterness. Thus, the present study has highlighted some advantages and drawbacks of a combined strategy to counteract the impact of warm dry vintages.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Human and animal right statement This article does not contain any studies with human or animal subjects.

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CHAPTER 6

Development of analytical method to examine wine parameters affected by climate change and mitigation techniques; Identification of potential additives to face the climate change

6 Development of analytical method to examine wine parameters affected by climate change and mitigation techniques; Identification of potential additives to face the climate change

6.1 Introduction

The increase of alcohol in wines may be partly related to the climate change (see 4.2.2.2). The excessive alcohol in wines could be removed by various techniques (see chapter 5). To properly determine desired level of dealcoholization, prior to application of post-fermentation techniques (e.g. nanofiltration) it is necessary to accurately measure the alcohol level in wines. Furthermore, to properly follow dealcoholization process and terminate the process at desired alcohol level it is necessary to have appropriate analytical equipment/methods. Thus, in modern winemaking apart from techniques to reduce excessive alcohol in wines, winemakers need rapid and accurate equipment/methods to measure alcohol level in wines as well. Therefore, the part of presented experiments was related to the assessment of red wine alcohol level with Waveguide Vector Spectrometer. Additionally, the same equipment was utilized to measure glycerol content in red wines which may be significantly altered during ethanol reduction by biotechnological techniques (see 5.3.2.1).

Temperature increase and elevated air CO_2 concentration may enhance the photosynthetic process and accelerate pace of phenological events **(Duchêne and Schneider, 2005; Jones, 2012)**. This, accelerated pace of phenological events may induce earlier technological maturity of grapes (optimum ratio between grape sugar content and acidity) followed with undeveloped aroma and phenolic compounds. In order to reach full phenolic maturity viticulturist often leave grape bunches to hang on plants, which is may be followed with acidity values below optimum level and sugar content above optimum level **(Jones, 2012)**, which will finally cause the production of unbalanced wines. However, in a case of different timing of technological and phenolic maturity some viticulturists may choose to harvest grapes at technological maturity instead at full phenolic maturity. Wines obtained with grapes that reached only technological maturity may have 'poor' sensory characteristics (e.g. lack of color) or low antioxidant activity if anthocyanins are scarce. Thus, winemakers may choose to improve wine quality by addition of certain commercial tannins. The utilization of commercial tannins is widely accepted in winemaking due to their antioxidant, antimicrobial and flavouring features. However, certain information (e.g. toxic metals content) are not always clearly demonstrated to winemakers. Therefore, the second part of the trial is related to analytical characterization determination of food-grade commercial tannins with different botanical origin.

6.2 Rapid assessment of red wine compositional parameters by means of a new Waveguide Vector Spectrometer

6.2.1 Materials and Methods

6.2.1.1 The Waveguide Vector Spectrometer and signal acquisition

The Waveguide Vector Spectrometer (WVS) works in the 1.6–2.7 GHz frequency range, and it is made of a rectangular aluminium waveguide with a closable opening for the positioning of the 24 ml glass container *(Fig. 6.1)*. The WVS is connected to a PC by a USB port, and it is equipped with a control unit with A/D and D/A converters. Spectral acquisitions of both gain and phase (resolution: Gain, 0.03 dB; Phase, 0.18°; frequency, 0.35 MHz; A/D conversion, 10 bit; acquisition time: 36 s, number of recorded points: 3130) were conducted in triplicate at a constant temperature of 25°C($\pm$1°C) by Java program. The glass container was filled with a sample volume of ~19.79 ml using a syringe. To eliminate the interference related to the WVS warming condition state and to ambient air variations (e.g. humidity), for each sample a background spectrum was subsequently subtracted from acquired signal.
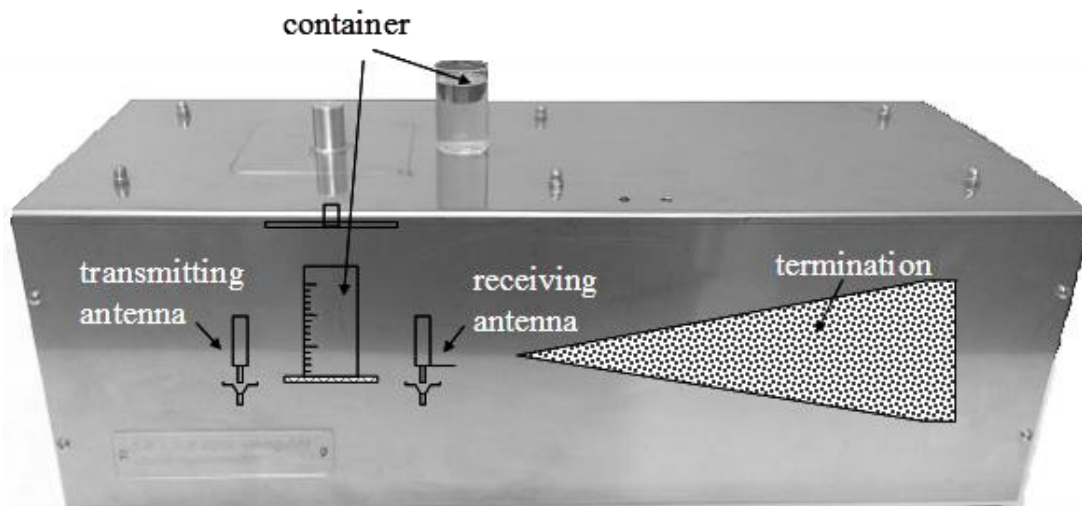
Figure 6.1 Schematic of the internal structure of the Waveguide Vector Spectrometer (adopted with permission from Teslić et al., 2017).

For the experiments, forty-two red wines were produced during the vintage 2015. Red wines alcohol (v/v %) and glycerol (g/L) contents were analyzed in triplicates according to OIV method (Resolution OIV/OENO 390/2010) with Fourier Transform Infrared Spectroscopy, FTIR (Winescan™ SO2, Hilleroed, Denmark), which results are presented in ***Table 6.1***.

Table 6.1 Red wine composition.

Wine parameter	Mean	SD	Min	Max
Alcohol (% v/v)	14.0	±0.6	12.8	15.5
Glycerol (g/L)	9.1	±0.8	8.0	11.0

Mean, average value of wine parameters (n=46); SD, standard deviation; Min, minimum value of wine parameters; Max, maximum value of wine parameters.

6.2.1.3   Statistical analysis, calibration and validation PLS

Partial least squares (PLS) regression analysis was explored to estimate the content of the selected wine qualitative parameters by the acquired Gain and Phase spectra. The accuracy of the model was examined in terms of R^2 and RMSE (Root Mean Square Error) for both calibration and full cross validation. Test set validation was also performed by a randomly selected 25% of the data set.

6.2.2   Results and Discussion

The PLS regression parameters (R^2 and RMSE) obtained for both Gain and Phase spectra are presented in ***Table 6.2*** for the frequency range 1.6–2.1 GHz, since this frequency range provided an improved PLS prediction models compared to the entire spectrum (1.6–2.7 GHz). Overall, in the test set validation, Gain spectra usually showed improved prediction capability for selected parameters compared to Phase once, most likely due to lesser effect by matrix complexity on Gain spectra compared to Phase spectra. Test set validation for red wine ethanol content showed the highest R^2 values of 0.961 (RMSE=0.11% v/v) and 0.955 (RMSE=0.13% v/v) for Gain and Phase spectra, respectively ***(Table 6.2)***. Test set validation for red wine glycerol content disclosed an R^2 values of 0.834 (0.31 g/L) and for Gain, while for Phase a R^2 values of 0.809 (0.33 g/L) ***(Table 6.2)***.

Table 6.2 Partial least square regression of red wine spectra for the prediction of alcohol and glycerol content from Gain and Phase spectra in the frequency range 1.6–2.1 GHz.

	Wine parameter	PCs	Calibration		Full cross validation		Test set validation	
			R^2	RMSE	R^2	RMSE	R^2	RMSE
Phase	Alcohol (% v/v)	5	0.965	0.11	0.957	0.12	0.955	0.13
	Glycerol (g/L)	14	0.995	0.05	0.809	0.33	0.809	0.33
Gain	Alcohol (% v/v)	5	0.972	0.10	0.968	0.11	0.961	0.11
	Glycerol (g/L)	14	0.988	0.09	0.843	0.31	0.834	0.31

PCs, number of principal components; R^2, coefficient of determination; RMSE, root mean square error.

Until today, rapid and extensively explored methods for wine composition estimation include vibrational spectroscopy based mainly on NIR and MIR spectral analysis which is supported by the multivariate approach of data evaluation **(Teixeira dos Santos et al., 2017)**. According to the vast number of conducted studies related to this topics, the assessment accuracy is shown to be influenced by the used technique, the composition of the wine samples and by the reference method **(Canal and Ozen, 2015; Friedel et al., 2013)**. In particular, in validation with NIR reflectance spectra, PLS regression models of different types of red, rosé and white wines had R^2 values of 0.978 (SEP=0.24% v/v) and 0.845 (SEP=0.72 g/L) for ethanol and glycerol content, respectively **(Urbano-Cuadrado et al., 2004)**. PLS regression models were also set up to predict ethanol concentration of red and white wines from ATR-MIR spectra with an R^2 value in validation of 0.99 (SEP=0.11% v/v) **(Cozzolino et al., 2011)** and glycerol content from FT-IR spectra obtained from a large number of wines with an r value of 0.96 (SEP=0.40 g/L) **(Nieuwoudt et al., 2004)**. The combination of visible and MIR spectra with orthogonal PLS regression technique produced R^2 values of 0.83 (RMSEP=0.47 g/L) for the prediction of glycerol content of red and white wines **(Sen et al., 2016)**. Hence, RMSE values up to 0.11% v/v for ethanol content and 0.31 g/L for glycerol content are acceptable for wine quality control analysis and the WVS system may be considered as an alternative to the NIR and MIR spectroscopic devices.

6.2.3   Conclusions

The global wine industry is always looking for new rapid analytical methods with high performance to monitor product quality with respect to regulation, as well as to improve the winemaking process. Thus, based on the study results tested WVS appeared to be able for a rapid estimation of the main wine compositional parameters (e.g. alcohol and glycerol content) in the process control.

6.3   Analytical characterization of commercial tannins

Ricci, A., Olejar, K.J., Parpinello, G.P., Mattioli, A.U., **Teslić, N.,** Kilmartin, P.A., Versari, A. 2016. Antioxidant activity of commercial food grade tannins exemplified in a wine model. *Food Additives & Contaminants: Part A* 33, 1761–1774

Ricci, A., Parpinello, G.P., Palma, A.S., **Teslić, N.,** Brilli, C., Pizzi, A., Versari, A. 2017. Analytical profiling of food-grade extracts from grape (*Vitis vinifera sp.*) seeds and skins, green tea (*Camellia sinensis*) leaves and Limousin oak (*Quercus robur*) heartwood using MALDI-TOF-MS, ICP-MS and spectrophotometric methods. *Journal of Food Composition and Analysis* 59, 95–104

6.3.1   Materials and Methods

In purpose of analytical characterization of food-grade commercial tannins from the different botanical origin, several analytical methods such as DPPH• radical scavenging, ICP-MS, total polyphenols and tannins assays were performed (**Ricci et al., 2017, 2016**).

6.3.2   Results and Discussion

Total polyphenols content varied in commercial tannins, and it was the lowest in samples obtained from leaves of *Vitis vinifera* (1.17 mM CE; expressed as catechin equivalent) and the highest in samples obtained from selected *Quercus* woods (2.77 mM CE; *Table 6.3*). Similarly to total polyphenols content, tannins concentration varied in samples, and it was the lowest in samples obtained from leaves of *Vitis vinifera* (0.71 mM CE) and the highest in samples obtained from Malbec red grape seeds (1.60 mM CE) *(Table 6.3)*. These differences in total polyphenols and tannins contents resulted in variation of tannins/polyphenols ratio, indicating that polymeric and monomeric fraction varied among samples *(Table 6.3)*. The highest percentage of tannins was detected for samples obtained from red fruits tree woods while the lowest tannins percentage was detected in samples obtained from grape seeds *(Table 6.3)*. These results are suggesting that sample obtained from grape seed was mostly composed of monomeric fraction while sample obtained from red fruits tree woods was mostly composed of polymeric fraction.

Table 6.3 Total polyphenols content, tannins content and DPPH radical scavenging potential of commercial tannins from different botanical origin.

Botanical Origin	Total polyphenols (mM CE)	Tannins (mM CE)	Tannins/Polyphenols ratio [%]	DPPH (%inhibition)
Leaves of *Vitis vinifera* red grapes[1]	1.17±0.00	0.71±0.03	60.68	22.4±0.2
Grape seeds[1,2]	2.64±0.09	0.82±0.01	31.06	73.9±0.3
Grape berry[1]	2.11±0.01	1.03±0.02	48.82	44.9±0.2
Grape skins and seeds[3,4]	2.75±0.00	1.16±0.04	42.18	72.9±0.3
White grape seeds[1,2]	1.94±0.01	1.05±0.04	54.12	55.6±0.3
Grape seeds[2,5]	2.33±0.00	1.08±0.04	46.35	68.6±0.3
Malbec red grape seeds[3]	2.33±0.02	1.60±0.01	68.67	63.0±0.4
Unfermented grape skins[1,3]	2.48±0.02	1.09±0.01	43.95	76.7±0.4
American Oak[3]	1.42±0.00	1.06±0.03	74.65	45.8±0.3
Limuosin Oak[3]	2.23±0.00	1.27±0.05	56.95	77.4±0.2
French Oak[3]	1.77±0.01	1.15±0.04	64.97	63.6±0.4
Selected *Quercus* woods[3,6]	2.77±0.01	1.35±0.02	48.74	76.6±0.5
Red fruits tree wood[7]	1.91±0.00	1.62±0.00	84.82	72.0±0.2

[1]Antioxidant; [2]Color stabilizer; [3]Fining agent; [4]Clarifying agent; [5]Cross-linker for anthocyanins; [6]White wine body supporter; [7]Red wine clarifying agent.

Different chemical composition (e.g. Tannins/Polyphenols ratio) affected the antioxidant capacity of commercial tannins *(Table 6.3)*. In particular, samples with higher tannins percentage (lower Tannins/Polyphenols ratio) or higher degree of polymerization had lower antioxidative capacity *(Table 6.3; Fig. 6.2)*. These results are indicating that monomeric fraction of total polyphenols content has a paramount role in the determination of antioxidative capacity of commercial tannins. Thus, commercial tannins aimed to be used as antioxidants should have a lower degree of phenols polymerization. In fact, commercial tannins obtained from grape seeds had most likely relatively low degree of polymerization which resulted in high antioxidative capacity *(Table 6.3)*. Furthermore, sample obtained from grape leaves had most likely high degree of polymerization indicating that botanical source or extraction methods might not be suitable to produce commercial tannins which will be used as antioxidants.
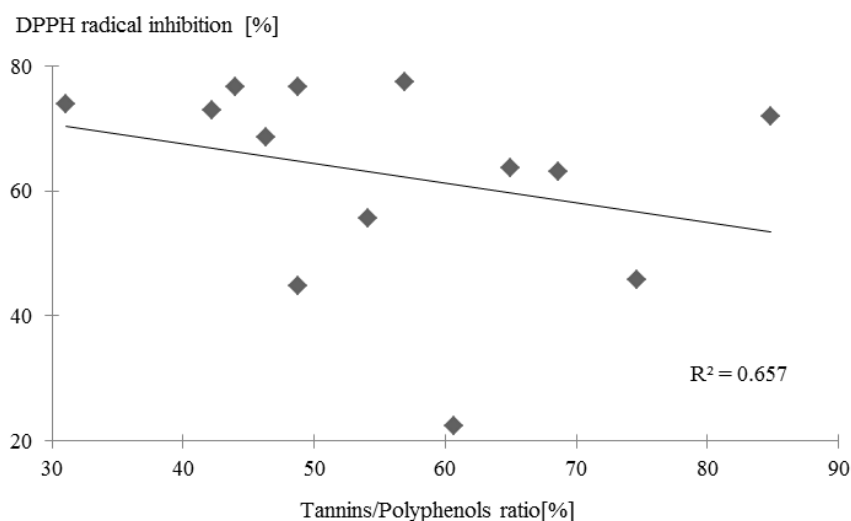
Figure 6.2 Correlation between antioxidative capacity and Tannins/Polyphenols ratio of studied commercial tannins.

Four commercial tannins were characterized by four macroelements (Mg, K, Ca and Mn) and twelve microelements (rest of the elements) which concentrations are listed in **Table 6.4**. Elemental composition is an important quality parameter of commercial tannins due to their diverse roles. In particular, Cu, Fe and Mn are oxidation catalysators of polyphenols which is afterwards responsible for alterations of wine sensory characteristic **(Waterhouse and Laurie, 2006)**. An element such as K influence the bitartrate stability/instability etc. Most of the detected elements in presented experiment were present in a concentration far below limits set by World Health Organization (WHO), while a concentration of Pb exceed these limits *(Table 6.4)*. However, in necessary to point out that concentration of Pb didn't exceeds limits set by International organization of vine and wine (0.15 ppm) **(OIV, 2017)**. Thus, commercial tannins could be used in winemaking in that regard.

Table 6.4 Elemental composition of commercial tannins from different botanical origin expressed as ppm.

Element	Green tea leaves	Limousin oak	Grape skin	Grape seed	WHO Guidelines[1]
^{7}Li	0.002	0.002	0.001	0.001	Nl
^{24}Mg[He]	0.500	0.200	0.090	0.080	Nl
^{27}Al	Nd	Nd	Nd	Nd	0.200
^{39}K[He]	0.800	0.600	1.200	1.400	Nl
^{44}Ca[He]	0.400	0.400	0.220	0.200	Nl
^{52}Cr[He]	0.008	0.008	0.011	0.009	0.050
^{55}Mn[He]	0.010	0.004	0.002	0.001	0.400
^{56}Fe	0.060	0.030	0.050	0.050	1.000–3.000
^{59}Co	0.0001	0.0001	0.0001	0.0001	Nl
^{60}Ni	Nd	Nd	Nd	Nd	0.070
^{63}Cu	0.040	0.020	0.020	0.020	2.000
^{66}Zn	0.990	0.250	Nd	Nd	3.000
^{75}As	0.002	0.002	0.002	0.002	0.010
^{88}Sr	0.020	0.020	0.004	0.003	Nl
^{137}Ba	0.020	0.020	0.010	0.010	0.700
^{207}Pb	0.033	0.033	0.033	0.031	0.010

[He] – assay conducted in under He flow; Nl – not listed; Nd – not detected; [1]**(World Health Organization, 2004)**.

6.3.3   Conclusions

Commercial tannins differed according to a chemical composition which resulted in differences of antioxidative capacity, whereas samples with a lower degree of polymerization had higher antioxidative capacity thus they are appropriate to be used as an antioxidative agents in winemaking in that regard. However, additional test related to the sensory properties of these tannins need to be evaluated. Elemental composition characterization of four commercial tannins revealed that all elements a part of Pb were present in concentrations lower that limits set by WHO. However, a concentration of Pb was lower than limits set by OIV, thus they could be used in winemaking in that regard.

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Appendix G – Rapid assessment of red wine compositional parameters by means of a new Waveguide Vector Spectrometer

Rapid assessment of red wine compositional parameters by means of a new Waveguide Vector Spectrometer

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ABSTRACT

A new method for rapid assessment of selected wine parameters based on a 1.6—2.7 GHz Waveguide Vector Spectrometer (WVS) is proposed for the first time. The Gain and Phase spectra of forty-two samples of Sangiovese red wines were acquired in the best frequency range (1.6—2.1 GHz) and modelled using Partial Least Square (PLS) regression to predict alcohol, dry extract and glycerol contents. The prediction of alcohol content showed test set validation R^2 values of 0.961 (RMSE = 0.11 ml/dL) and of 0.955 (RMSE = 0.13 ml/dL) for Gain and Phase spectra, respectively. Glycerol and dry extract were predicted at the best using Gain spectra with R^2 values of 0.834 (RMSE = 0.31 g/L) and 0.861 (RMSE = 0.51 g/L), respectively. A test was also conducted to assess the technique ability in the detection of alcohol and glycerol contents by preparing and using samples of model wines with known added content of these substances. R^2 values up to 0.992 (RMSE = 0.16 ml/dL) for alcohol and 0.999 (RMSE = 0.04 g/L) for glycerol contents were obtained using Gain spectra. The suggested technique has shown to be promising for the rapid assessment of the control process of these important attributes of wines.

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1. Introduction

Rapid and inexpensive techniques for the assessment of the quality parameters of foods are known to be highly attractive both for off-line and on-line process control. The analysis of the interaction of food materials with an electromagnetic wave has been shown to provide valuable information related to the product composition (Berardinelli et al., 2013; García, Torres, Prieto, & De Blas, 2001; Ragni, Al-Shami, Mikhaylenko, & Tang, 2007), water activity (Iaccheri et al., 2015), density (Kent, 1977) and structure (Ragni, Cevoli, Berardinelli, & Silaghi, 2012). Apart from the temperature and used frequency, the result of such interaction is affected by the ability of sample to initially accumulate the electromagnetic energy - this property is associated with the dielectric

constant - and then to dissipate it into heat, which originates the loss factor. Dielectric constant and loss factor are, respectively, the real and the imaginary component of the complex permittivity, which is mainly driven by the electric conduction, dipoles, electronic, ionic and Maxwell-Wagner mechanisms (Nelson, 1999).

It is well known that the dielectric properties of sample are affected by the moisture, salt, carbohydrates, fat, protein and alcohol content. During the last 50 years, extensive literature was devoted to the investigation of agri-food dielectric properties, and the moisture content of the cereal grain products was undoubtedly the main explored parameters (Jha et al., 2011; Venkatesh & Raghavan, 2004). This analytical approach was also applied to investigate different parameters in fruits and vegetables (Guo, Nelson, Trabelsi, & Kays, 2007; Nelson, 2003; Sipahioglu & Barringer, 2003), beans (Berbert, Queiroz, & Melo, 2002), bakery products (Kim, Morgan, Okos, & Stroshine, 1998), meat (Lyng, Zhang, & Brunton, 2005; Sipahioglu, Barringer, Taub, & Yang, 2003), fish (Liu & Sakai, 1999; Wang, Tang, Rasco, Kong, & Wang, 2008), shell eggs (Ragni et al., 2007, 2008), dairy products (Ragni et al., 2016; Shiinoki, Motouri, & Ito, 1998), and extra virgin olive

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oil (Berardinelli et al., 2013) as well. Previous works conducted with a system composed by an aluminium rectangular waveguide, a sweeper oscillator and a spectrum analyser showed that gain waveforms (from 2.0 to 2.2 GHz and from 2.9 to 3.0 GHz) contain information related also to soluble solids content (°Brix) and Magness–Taylor flesh firmness of kiwifruits (Ragni et al., 2012) or to the ripening of Parmigiano–Reggiano cheese (2–3 GHz) (Cevoli, Ragni, Gori, Berardinelli, & Caboni, 2012).

Red wine is the beverage obtained from the partial or complete alcoholic fermentation of fresh grapes, whether crushed or not, or of grape must. According to the International organization of vine and wine (OIV), its actual alcohol content should not be less than 8.5 mL/dL (definition 18/73; OIV 2017). In general, the most abundant components of red wine include water (85–90 mL/dL), ethanol (10–15 mL/dL), glycerol (5–12 g/L) and organic acids (3–5 g/L) while all other components are in traces (0.3%) (Geana et al., 2016; Giaramida et al., 2013; Godden, Wilkes, & Johnson, 2015; Laguna, Bartolomé, & Moreno-Arribas, 2017). For high-quality wines, quality control during production process and storage plays an important role. Certain official OIV (International organisation of vine and wine) methods for alcohol, glycerol and dry extract content determination require sample preparation (OIV-MA-AS312-01B; OIV 2016) and are set up for only one wine parameter (OIV-MA-AS312-05; OIV 2016). Rapid, inexpensive, simple to use, accurate and methods that are also suitable for determination of more than one compositional parameter are however requested.

Alcoholic content in beverages has been proven to affect both the dielectric constant and loss factor. Ethanol solutions (0.5 mL/dL, 10 mL/dL and 40 mL/dL) added with sugar have been analysed by dielectric properties in the 0.5–50 GHz range (Abidin, Omar, & Biak, 2014), which measurements addressed discrimination of alcoholic beverages with alcohol content ranging from 4.2 to 99.8 mL/dL (Abidin, Omar, Yogarajah, Biak, & Man, 2014). Moreover, assessment of dielectric constant and loss factor in combination with linear discriminant analysis have been investigated to discriminate between red grape juices and red wines samples at 200 MHz and at 3 GHz with a classification rate of 100% (García, Torres, De Blas, De Francisco, & Illanes, 2004) while Bohigas and Tejada (2010) developed a method to determine the alcohol content of alcoholic beverages; the method based on the Debye model and on measurements of the permittivity at 1 GHz and 7 GHz by using water solutions of ethanol (alcoholic levels up to 40 mL/dL).

It is noteworthy that previous studies were conducted by using an open-ended coaxial probe, which requires special care to avoid errors at very low and very high frequency, as well as at low values of dielectric constant and loss factor. Particularly for liquid and semi-solid foods, density variation, air gaps or bubbles between the end of the coaxial probe and the sample can compromise the results (Nelson and Datta, 2001). In this view, less expensive instrumental chains, such as systems using a waveguide probe, could be a valuable alternative depending on the nature of dielectric material, frequency of interest, and degree of accuracy required.

The waveguide is a method that can exploit the propagation of an electromagnetic wave within a closed cavity for the detection of the dielectric properties of the material (Belrhiti, Bri, Nakheli, & Mamouni, 2013). The values of dielectric constant and loss factor can be derived from the transmission line theory, which indicates that these properties can be calculated by measuring the phase and amplitude of a reflected microwave signal from a dielectric material placed against the end of a transmission line, such as a waveguide (von Hippel, 1954).

In this way, the waveguide setup can be a suitable equipment to obtain accurate measurements of permittivity, and could be attractive as a function of its user friendliness, simple sample preparation, convenient temperature control, and affordability of owning this equipment.

The Waveguide Vector Spectrometer (WVS) used in the present research (Italian patent No. 1421744, 2016, application n. MI2014A000039, 2014; International application: PCT/IB2015/050246; WO2015/107455A1, 2015, Alma Mater Studiorum, University of Bologna, Italy) operating in the low region of the microwave spectrum, was set up with the purpose to provide a comprehensive, rapid and simple tool for the assessment of the main compositional parameters of agri-food products. An introductory article, focusing the performances and novelty of the instrument, is reported in literature (Ragni et al., 2017).

This study aims to investigate the potentiality of the WVS for the rapid assessment of selected wine compositional parameters, e.g. alcohol, glycerol and dry extract contents. These wine parameters play an important role in the certification of appellation wine (e.g. DOP - Protected Denomination of Origin, IGP - Protected Geographical Indication).

2. Materials and methods

2.1. The Waveguide Vector Spectrometer and signal acquisition

The waveguide vector spectrometer (Fig. 1) operates in the 1.6–2.7 GHz frequency range, and it is composed of a rectangular aluminium waveguide with a closable opening for the placement of the 24 ml cylindrical glass sample container. The container is placed between a transmitting antenna (connected to a radio frequency signal generator) and a receiving antenna (a gain and phase comparator is connected to the antennas). The WVS is interfaced to a computer by a USB port, and it is equipped with a control unit with A/D and D/A converters. Spectral acquisitions of both gain and phase (resolution: Gain, 0.03 dB; Phase, 0.18°; frequency, 0.35 MHz; A/D conversion, 10 bit; acquisition time: 36 s, number of recorded points: 3130) were acquired in triplicate at a constant temperature of 25 °C (± 1 °C) by using a dedicated Java programme.

The glass container was filled with an approximate volume of 1.979×10^{-5} m^3 using a syringe. The constant sample volume was enabled by using equipment with fixed needle indicating the attainment of the level).

In order to eliminate the influences related to the WVS warming condition state and to ambient air variations (e.g. humidity), from each sample spectrum was subsequently subtracted the signal acquired with the empty container (blank spectrum).

2.2. Standard solutions

A preliminary study with a standard solution (called 'model wine') was designed to ascertain the effect of alcohol (e.g. ethanol) and glycerol on gain and phase spectra. Glycerol (99%) and ethanol (96%) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium hydroxide ($\geq$99%) and L (+)-tartaric acid ($\geq$99.5) were purchased from Merck (Darmstadt, Germany). For model wine preparation tartaric acid (16.65 mmol/L) was dissolved in pure distilled water (18 MΩcm). To obtain pH value often found in wines (3.6), solution pH was adjusted with 1n NaOH. Prepared solution was divided into two batches: (I) with fixed glycerol content (7 g/L) and ethanol concentration varying by 1 mL/dL from 10 mL/dL to 15 mL/dL; (II) with fixed ethanol content (12 mL/dL) and glycerol content varying by 1 g/L from 5 g/L to 10 g/L) (Table 1). Ranges of ethanol and glycerol concentration were selected according to common values found in red wines (Giaramida et al., 2013; Godden et al., 2015; Laguna et al., 2017). For each standard solution, 3 spectral acquisitions were conducted.
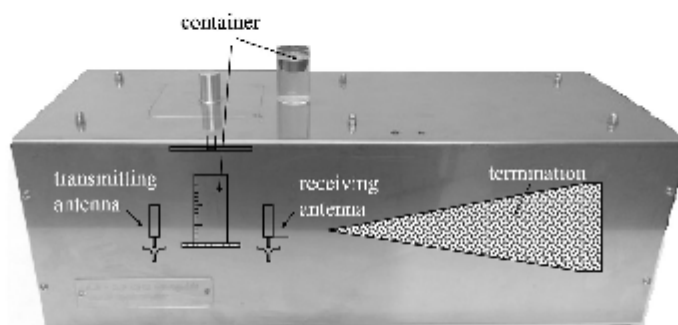
Fig. 1. Front view of the waveguide vector spectrometer and a schematic of the internal structure.

Table 1
Model wines composition.

Batch I		Batch II	
Sample	Alcohol (mL/dL)	Sample	Glycerol (g/L)
A	10	G	5
B	11	H	6
C	12	I	7
D	13	J	8
E	14	K	9
F	15	L	10

2.3. Wine samples

During the vintage 2015, approximately seven hundred kilograms of mature Sangiovese grapes was manually harvested and transported to the Unibo experimental winery (Tebano, RA, Italy). Grapes were divided (42 batches), manually destemmed and crushed. Obtained grape must (with grape skin) was transferred into stainless steel fermenters, treated with $K_2S_2O_5$ (100 mg/L, AEB, Italy), Nutristart (30 g/hL, Laffort, France) and inoculated with yeasts (Lalvin EC-1118; Lallemand, Switzerland; Exotic, Anchor, South Africa; Zymaflore RX60, Laffort, France; CHT07, Enobiotech, Italy). Fermentations were carried at the constant temperature of 22 °C (±1). Once fermentations were done (residual sugars less than 2 g/L), grape skin was separated from wine using a semi-automatic press (22620M, Spedeil, Ofterdingen, Germany). Obtained wines were bottled, sealed with a crown cap and stored at 15 °C until analysis. Alcohol mL/dL), glycerol (g/L) and dry extract (g/L) contents were analysed in triplicates according to OIV method (Resolution OIV/OENO 390/2010) with Fourier Transform Infrared Spectroscopy, FTIR (Winescan™ SO_2, Hilleroed, Denmark). Mean, standard deviation, minimum and maximum values of the parameters of analysed wines are summarised in Table 2. For each of

Table 2
Sangiovese red wine composition.

Wine parameter	Mean	SD	Min	Max
Alcohol (mL/dL)	14.0	±0.6	12.8	15.5
Glycerol (g/L)	9.1	±0.8	8.0	11.0
Dry extract (g/L)	28.3	±1.3	26.1	31.8

Mean, average value of wine parameters (n = 46); SD, standard deviation; Min, minimum value of wine parameters; Max, maximum value of wine parameters.

the analysed 42 wine samples, 3 spectral acquisitions were conducted.

2.4. Statistical analysis, calibration and validation

PLS regression analysis was explored to estimate the content of the selected wine qualitative parameters starting from the reference data and the acquired Gain and Phase spectra, either in the preliminary trials conducted on standard solutions (alcohol, glycerol) or on Sangiovese red wines (alcohol, glycerol and dry extract).

Independent variables were arranged in a 6 × 3 (samples) × 3128 (variables, Gain or Phase) matrix for the model wine solutions and in a 42 × 3 (samples) × 3128 (variables, Gain or Phase) matrix for wine samples and the Unscrambler X software was used (Camo Asa, Oslo, Norway).

The accuracy of the model was assessed in terms of R^2 and RMSE (Root Mean Square Error) for both calibration and full cross validation. Test set validation was also conducted by considering only wine samples matrices and by using a randomly selected 25% of the data set. The optimal number of PCs (PLS components) was selected by analysing the validation residual variance plot and calculated where the prediction error is minimized (Esbensen, 1994).

On the basis of the model accuracy (R^2 and RMSE) and on the exploration of both loadings and scores plots, an optimum spectrum frequency range was selected for wine sample models.

3. Results and discussion

3.1. Standard solutions

Gain and Phase waveforms for different concentrations of alcohol (Fig. 2; Fig. 3) and glycerol (Fig. 4; Fig. 5) are shown. For both Gain and Phase waveforms, a trend passing from low to high concentrations of both compounds, e.g. ethanol and glycerol, was observed. This trend can be appreciated in different regions of the explored frequency range depending on the compounds selected. In the described figures, focus windows are included as examples. PLS regressions conducted in these spectral ranges showed, respectively for Gain and Phase waveforms the following R^2 values: 0.993 (RMSE = 0.14 mL/dL) and 0.964 (RMSE = 0.33 mL/dL) in calibration and 0.992 (RMSE = 0.16 mL/dL) and 0.961 (RMSE = 0.36 mL/dL) in full cross validation for the alcohol content; 0.999 (RMSE = 0.03 g/L) and 0.914 (RMSE = 0.52 g/L) in calibration and 0.999 (RMSE = 0.04 g/L) and 0.886 (RMSE = 0.65 g/L) in full cross validation for the glycerol content (Table 3).
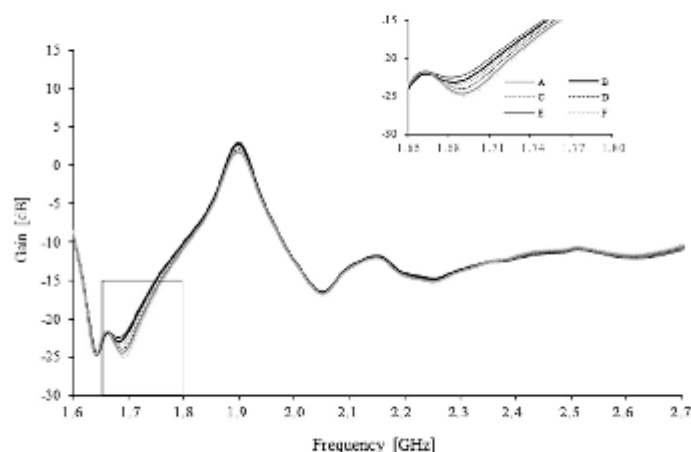
Fig. 2. Gain spectra for different concentrations of alcohol (A–F) in wine models (see Table 1).
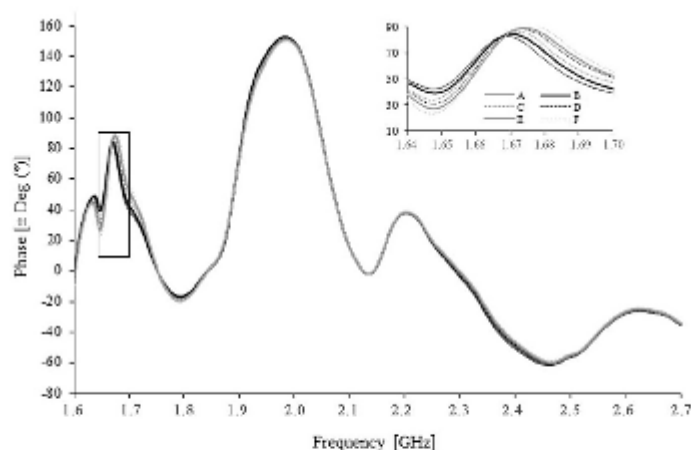
Fig. 3. Phase spectra for different concentrations of alcohol (A–F) in wine models (see Table 1).

3.2. Red wine samples

The PLS regression parameters (R^2 and RMSE) obtained for both Gain and Phase spectra are summarised in Table 4 for the frequency range 1.6–2.1 GHz. This range provided an improved prediction models compared to the entire spectrum (1.6–2.7 GHz).

The calibration process plays an important role in the final wine parameter measurement values. Once calibrated for wine parameter measurements (e.g. alcohol content in red wines) with one type of wines, WVS could be also used for wine parameter measurements in other types of wines (e.g. alcohol content in white wines) at a satisfactory level (Ragni et al., 2017). However, as it was reported by the authors (for alcohol content only), the prediction is a bit better when calibration is done for each wine type separately (e.g. red and white wines).

Overall, in the test set validation, Gain spectra usually showed better prediction capability compared to Phase once, most likely due to lesser effect by matrix complexity on Gain spectra compared to Phase spectra. Test set validation for alcohol showed the highest R^2 values of 0.961 (RMSE = 0.11 mL/dL) and 0.955 (RMSE = 0.13 mL/dL) for Gain and Phase spectra, respectively (Table 4). Test set validation for glycerol and dry extract disclosed an R^2 values of 0.834 (0.31 g/L) and 0.861 (0.51 g/L) for Gain, while for Phase a R^2 values of 0.809 (0.33 g/L) and 0.848 (0.48 g/L), respectively.

The low accuracy expected and found for glycerol and dry extract was due to their lower content compared to alcohol. Predicted and observed values (test set validation) are shown in Figs. 6, 7 and 8 respectively for alcohol (mL/dL), glycerol (g/L) and extract (g/L) contents.

Linear correlations between alcohol and glycerol contents and
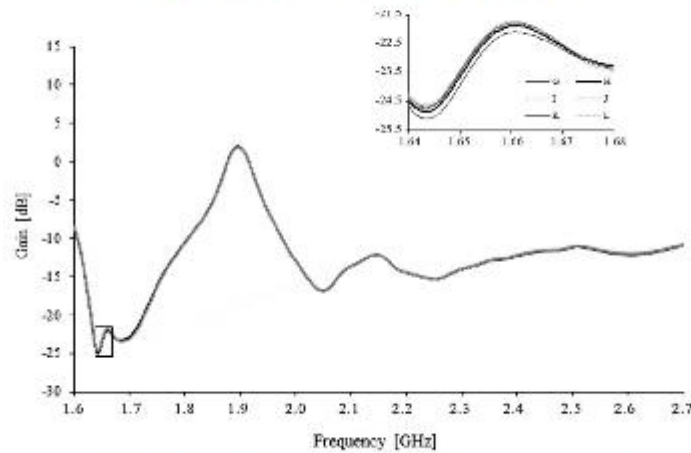
Fig. 4. Gain spectra for different concentrations of glycerol (G–L) in wine models (see Table 1).
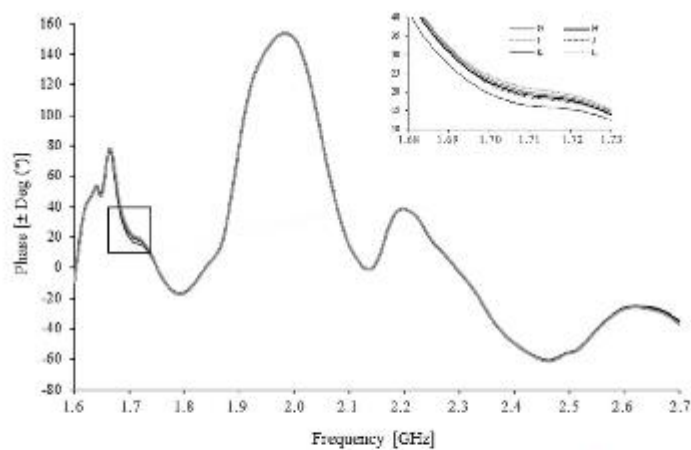
Fig. 5. Phase spectra for different concentrations of glycerol (G–L) in wine models (see Table 1).

Table 3
Calibration and validation of model wine spectra of selected parameters from Gain and Phase spectra.

Parameter	Model wine parameter	PCs	Calibration		Full cross validation	
			R^2	RMSE	R^2	RMSE
Gain	Alcohol (mL/dL)	1	0.993	0.14	0.992	0.16
	Glycerol (g/L)	4	0.999	0.03	0.999	0.04
Phase	Alcohol (mL/dL)	1	0.964	0.33	0.961	0.36
	Glycerol (g/L)	4	0.914	0.52	0.886	0.65

PCs, number of PLS components; R^2, coefficient of determination; RMSE, root mean square error.

between alcohol and dry extract contents are summarised in Fig. 9. Alcohol content showed a poor linear relationship with glycerol ($R^2 = 0.346$) and dry extract contents ($R^2 = 0.520$), therefore it seems that the spectra contain specific information for the prediction of the two latter parameters.

At date, rapid and extensively explored methods for wine compositional parameter estimation include vibrational spectroscopy based mainly on NIR and MIR spectral analysis supported by multivariate data analysis (Teixeira do Santos et al., 2017). According to the numerous works conducted on this topic, the estimation accuracy is shown to be affected by the kind of the used technique, the composition of the wine samples and the reference method (Canal & Ozen, 2015; Friedel, Patz, & Dietrich, 2013). In particular, in validation with NIR reflectance spectra, PLS estimations of different types of red, rosé and white wines were

Table 4
PLS regression of red wine spectra for the prediction of selected parameters from Gain and Phase spectra in the frequency range 1.6–2.1 GHz.

Parameter	Wine parameter	PCs	Calibration		Full cross validation		Test set validation	
			R^2	RMSE	R^2	RMSE	R^2	RMSE
Gain	Alcohol (mL/dL)	5	0.972	0.10	0.968	0.11	0.961	0.11
	Glycerol (g/L)	14	0.988	0.09	0.843	0.31	0.834	0.31
	Extract (g/L)	14	0.988	0.14	0.879	0.44	0.861	0.51
Phase	Alcohol (mL/dL)	5	0.965	0.11	0.957	0.12	0.955	0.13
	Glycerol (g/L)	14	0.995	0.05	0.809	0.33	0.809	0.33
	Extract (g/L)	14	0.993	0.10	0.851	0.45	0.848	0.48

PCs, number of PLS components; R^2, coefficient of determination; RMSE, root mean square error.
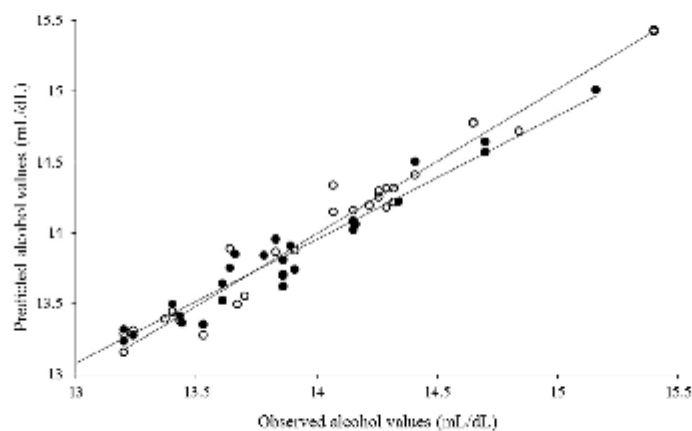
Fig. 6. Predicted versus observed values of alcohol content (mL/dL) in red wines using Gain (○) and Phase signal (●) (test set validation, see Table 4). Coefficients of determination were $R^2 = 0.961$ and $R^2 = 0.955$ for Gain and Phase signal, respectively.
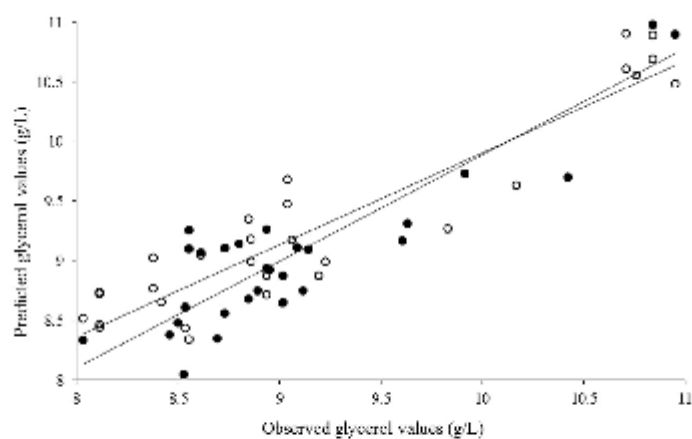
Fig. 7. Predicted versus observed values of glycerol content (g/L) in red wines using Gain (○) and Phase signal (●) (test set validation, see Table 4). Coefficients of determination were $R^2 = 0.834$ and $R^2 = 0.809$ for Gain and Phase signal, respectively.
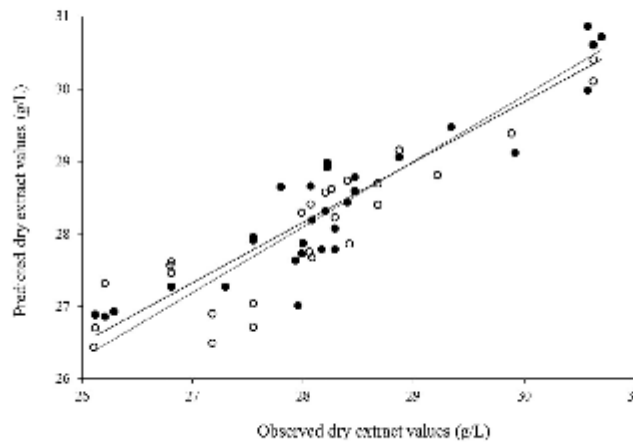
Fig. 8. Predicted versus observed values of dry extract content (g/L) in red wines using Gain (○) and Phase signal (●) (test set validation, see Table 4). Coefficients of determination were $R^2 = 0.861$ and $R^2 = 0.848$ for Gain and Phase signal, respectively.
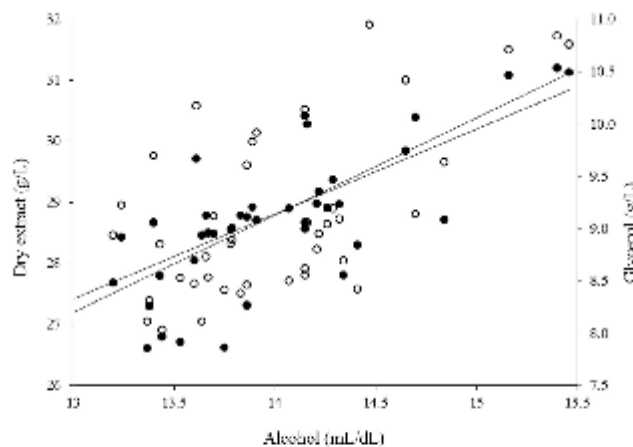
Fig. 9. Correlation between alcohol (mL/dL) and glycerol (g/L) content (○) and correlation between alcohol (mL/dL) and extract (g/L) content (●), for the analysed red wines. Coefficients of determination were $R^2 = 0.520$ and $R^2 = 0.346$ for alcohol/extract content and alcohol/glycerol content, respectively.

characterized by R^2 values of 0.978 (SEP = 0.24 mL/dL) and 0.845 (SEP = 0.72 g/L) respectively for alcohol and glycerol content (Urbano-Cuadrado, Luque de Castro, Pèrez-Juan, García-Olmo, & Gómez-Nieto, 2004). PLS regression models were also set up to estimate alcohol content of red and white wines from ATR-MIR spectra with an R^2 value in validation of 0.99 (SEP = 0.11 mL/dL) (Cozzolino, Cynkar, Shah, & Smith, 2011) and glycerol content from FT-IR spectra acquired on a large number of wines of various styles with an r value of 0.96 (SEP = 0.40 g/L) (Nieuwoudt, Prior, Pretorius, Manley, & Bauer, 2004). The combination of visible and MIR spectra with orthogonal PLS regression technique produced R^2 values of 0.83 (RMSEP = 0.47 g/L) for the estimation of glycerol content of red and white wines (Sen, Ozturk, Tokatli, & Ozen, 2016). About dry extract content, FT-MIR measurements conducted on 327 typical German wines showed, in validation, PLS estimations characterized by an R^2 of 0.999 (RMSEP = 0.99 g/L) (Patz, Blieke, Ristow, & Dietrich, 2004). Thus, RMSE values up to 0.11 mL/dL for alcohol, 0.31 g/L for glycerol and 0.51 g/L for dry extract are acceptable for wine quality control analysis. Furthermore, the integration in a single instrument of both the sensor (waveguide) and the measuring system — which extract in a vector fashion both gain and phase shifts occurring through the sample — represents the novelty of the proposed device in the dielectric panorama. The system could be also considered as an alternative to the NIR and MIR spectroscopic devices.

The global wine industry is looking for new analytical methods to monitor product quality with respect to regulation, as well as to improve the winemaking process. In this view, the proposed work

exploits an emerging analytical technique (WVS) that represents an important advance in the field with great forthcoming in the wine industry.

4. Conclusion

The potentiality of a full integrated system for the measurements of the Gain and Phase waveforms in the 1.6–2.7 GHz frequency range combined with PLS regression analysis was tested in terms of prediction of the main compositional parameters Sangiovese red wines: alcohol, glycerol and dry extract contents. Preliminary analyses conducted on standard solutions simulating common red wines ethanol and glycerol contents, evidenced changes in the acquired spectra according to the levels of the two parameters. PLS models obtained from acquisition with real wine samples showed R^2 values in test set validation up to 0.961 (RMSE $=$ 0.11 mL/dL), 0.834 (RMSE $=$ 0.31 g/L), and 0.861 (RMSE $=$ 0.51 g/L), for the prediction of the alcohol, glycerol and dry extract contents, respectively. For both standard solutions and real wine samples, highest accuracies were observed with Gain waveforms. Based on these results the tested waveguide vector spectrometer appeared to be able for a rapid prediction of the main wine compositional parameters in the process control.

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Appendix H – Antioxidant activity of commercial food grade tannins exemplified in a wine model

Antioxidant activity of commercial food grade tannins exemplified in a wine model

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ABSTRACT

Although commercial tannins are widely used in foods and beverages, an improved understanding of the structure and composition of vegetable tannins is needed to promote the exploitation of agri-food by-products and waste and their valorisation in more sustainable industrial applications. This study aims to characterise the phytochemical composition and antioxidant activity of 13 food grade tannins using multiple analytical approaches, including spectrophotometry and HPLC-ECD to determine the amount of targeted polyphenolic compounds. Moreover, the antioxidant activity of tannins was assessed in terms of radical scavenging activity (DPPH• assay), reducing power (FRAP assay), and redox properties (cyclic voltammetry, CV). A statistical univariate and multivariate correlation analysis was performed on 17 parameters including tannin content (range: 0.71–1.62 mM), gallic acid, (+)-catechin, syringic acid and (–)-epicatechin. The compositional profile of tannins was related to their chemical moiety, antioxidant activity and the botanical origin of the extracts. In particular, the CV signal at 500 mV was highly correlated with DPPH• value due to the catechol ring of flavonoids and trigalloyl moieties of gallic acid–based compounds. Practical examples of tannins application in winemaking are discussed.

Introduction

Commercial tannins are plant polyphenolic compounds extracted from different botanical components (e.g. gall-nuts, chestnut, oak, quebracho, grape seeds) and are composed of different chemical structures including gallic and ellagic acids oligomers (hydrolysable tannins) or proanthocyanidins polymers (i.e. condensed tannins) (see Supplemental Data). In the food and beverage industry they are considered as flavourings, whereas the International Organisation of Vine and Wine (OIV) authorises the addition of commercial tannins to facilitate the clarification of grape musts and wines. In particular, winemaking takes advantage of these compounds to enhance the colour stability, to improve the structure of light-bodied wines, and to provide additional protection from oxidation (Versari et al. 2013), thus possibly minimising the need for synthetic antioxidants (mainly sulphur dioxide). A high content of polyphenolic compounds is commonly associated with higher market value grades (Mercurio et al. 2010).

Based on the current EU wine regulation (No. 1308/2013), it is not mandatory to declare on the label the addition of tannins to wine. Similarly, the labelling of commercial tannins provides basic information only, without any specification of the effective 'active principle' (e.g. proanthocyanidins) in terms of content, purity, structure, activity, etc. That information would be helpful to winemakers to tailor the dosage and the style of their wines. This topic of investigation is consistent with the 2015–2019 OIV strategic plan (http://www.oiv.int/public/medias/3345/ps-2015-2019-en.pdf), which includes the need to (i) study and develop methodologies that tend to guarantee product authenticity; (ii) ensure consistency about traceability and labelling; and (iii) draw up specifications for products for oenological use.

Usually, commercial tannins are supplied with limited information on their botanical source and composition, therefore improved understanding is needed of the chemical composition and properties of commercial tannins (Magalhães et al. 2014) to optimise their use for specific chemical (Lambri et al. 2014) and sensory applications

(Villamor et al. 2013). In particular, the analytical characterisation of the composition of commercial tannins provides useful information on the structure-activity relationships of these phytochemicals (Rice-Evans et al. 1996). Several spectrophotometric methods are available to obtain a preliminary index of total polyphenols and tannins in a complex mixture (De Beer et al. 2004; Sarneckis et al. 2006) and total antioxidant capacity (Hagerman et al. 1998; Yokozawa et al. 1998). On the other hand, structural chemical information requires separation techniques, which are useful tools to discriminate the single phenolic compounds and the extent of polymerisation of larger structures. The most widespread method to determine the simple phenolic fraction is HPLC with diode-array (DAD) or electrochemical (ECD) detection, which allows quantification of the monomeric polyphenols, and provides additional information on the polymerised fraction (Gòmez-Alonso et al. 2007; Versari et al. 2008).

In addition to conventional spectrophotometric assays, a further evaluation of commercial tannins involves the characterisation of their redox properties as antioxidants. Among the electrochemical methods, a particular emphasis has been given to the use of cyclic voltammetry, being an attractive analytical option for the rapid screening of single compounds and complex matrices (Kilmartin et al. 2002). Antioxidant activity is commonly evaluated in terms of ability to scavenge the DPPH• free radicals (DPPH• assay), reducing power against Fe(III) salts (FRAP assay), and oxidisability seen via the redox process during cyclic voltammetry. The latter approach was found to be an alternative and effective method to assess the antioxidant power of phenolic compounds, in addition to its ability to discriminate between reversible and non-reversible redox processes (Kilmartin et al. 2001; Kilmartin & Hsu 2003).

In the current work, a selection of commercial food grade tannins was analysed using a combined analytical approach focusing on their phytochemical composition and antioxidant activity. The relationship among tannins and various selected parameters was evaluated to improve their application in winemaking and other industrial applications.

Materials and methods

Samples

Thirteen samples of dry commercial food grade tannins with general information from the producers (Table 1) (Enologica Vason, Verona, Italy; HTS enologia, Marsala, Italy; Laffort, Bordeaux Cedex, FR; AEB Group, Brescia, Italy) were representative of proanthocyanidins, commonly called condensed tannins (labelled 'Pr'), and hydrolysable tannins (labelled 'Hy'). Stock solutions of dry tannins were prepared at 1 g/L in model wine solution, equivalent to 3.45 mM (+)-catechin, which is a suitable concentration for analytical purposes. The model wine solution was made up of 12% ethanol (> 99%) in distilled water, with the addition of L-tartaric acid 0.033M and NaOH to reach pH 3.6 (Merck Darmstadt, Germany).

The samples, all of commercial food grade, were directly provided by producers who claim their authenticity with general information on the label. Indeed, the aim of the work was to ascertain the authenticity (and purity) of the selected tannins using multiple analytical approaches, which are based on previous findings.

Chemicals and reagents

Sodium carbonate anhydrous (≥99.5%) used for the Folin-Ciocalteu's assay (Merck), bovine serum albumin (BSA, fraction V, lyophilised powder), sodium dodecyl sulphate (SDS; lauryl sulphate, sodium salt, 95%), triethanolamine (TEA, 98%), $FeCl_3.6H_2O$ (98%) used for the Adams-Harbertson's assay, and $FeCl_3$ anhydrous and 2,4,6-tris-pyridyl triazine (TPTZ) for the FRAP assay, standards of (+)-catechin monohydrate (98%) and $FeSO_4.7H_2O$ (≥

Table 1. List of commercial oenological tannins (samples) and their main applications in winemaking.

Samples code	Description	Oenological applications
Pr1	Leaves of *Vitis vinifera* red grapes	Antioxidant
Pr2	Grape seeds	Antioxidant; colour stabilisation
Pr3	Grape berry	Antioxidant
Pr4	Grape skins and seeds	Fining and clarifying agent
Pr5	White grape seeds	Antioxidant; colour stabilisation
Pr6	Grape seeds	Cross-linker for anthocyanins; colour stabilisation
Pr7	Malbec red grape seeds	Fining agent
Pr8	Unfermented grape skins	Antioxidant; fining agent
Hy1	American Oak	Fining agent
Hy2	Limousin Oak	Fining agent
Hy3	French Oak	Fining agent
Hy4	Selected *Quercus* woods	Fining agent; provides body to white wines
Hy5	Red fruits tree wood	Clarifying agent for red wines

99%) were used for the calibration of Folin-Ciocalteu and FRAP assays (Sigma-Aldrich, Saint Louis, MO).

For HPLC analyses, eight phenolic standards – gallic acid, caffeic acid, ferulic acid, syringic acid, (+)-catechin, (-)-epicatechin, epicatechin gallate (ECG) and rutin – were used (Sigma-Aldrich). Methanol, acetonitrile, monosodium and disodium phosphate (Scharlau, Sentmenat, Spain), ortho-phosphoric acid (Ajax Finechem Pty Ltd, Sydney, NSW, AU) and 18-Ohm purified water (Barnsted Nanopure water system, Thermo Scientific, Waltham, MA) were also used.

Total polyphenols and tannins analysis: Folin-Ciocalteu and Adams-Harbertson assays

Total polyphenolic compounds (TPC) were quantified as mM (+)-catechin equivalent (CE) (Singleton & Rossi 1965). Tannins (mM CE) were determined using the protein precipitation-based tannin method developed for wine that uses bovine serum albumin (BSA), re-suspension of the precipitate and a subsequent colour development reaction using iron(III) chloride that has been shown to react with vicinal di-hydroxylated phenols making an indigo complex absorbing at 510 nm (Harbertson et al. 2002). Briefly, 500 µL of each stock tannin solution was added to the reaction mixture to assess the reactivity of each sample against the Bovine Serum Albumin (BSA) protein. The sample was centrifuged to pellet the tannin-protein complex. Then, the pellet was re-suspended in a cuvette to which a ferric (III) chloride reagent was added and incubated at room temperature for 10 minutes, and the absorbance was read at 510 nm (tannin final measurement) against TEA buffer as a blank (tannin background measurement) using a Shimadzu UV mini 1240 spectrophotometer (Kyoto, Japan). The guidelines for dilution set forward by Jensen et al. (2008) were used for the tannins' analysis by protein precipitation assay.

HPLC analysis of simple phenolics

Phenolic monomers were assessed using the method described in Olejar et al. (2015). Briefly, samples and standards were weighed on a Shimadzu UW2200H analytical balance (Kyoto, Japan) and diluted in model wine solution to a final concentration of 0.1 mM (+)-catechin equivalent (CE) (29 mg/L) for the tannin samples, whereas the standards covered the range between 0.1 and 100 mg/L. Solution of both samples and standards were then filtered through a 0.45 µm syringe filter prior to assay on an Agilent 1100 HPLC (Agilent Technologies Inc., Santa Clara, CA) equipped with column heater and ESA CouloChem III electrochemical

detector (Thermo Fisher Scientific, Waltham, MA). Separation was undertaken using a Supelco Ascentis RP-Amide column, 3.0 × 100 mm, 3 µm (Sigma-Aldrich, St. Louis, MO) at 42°C. The system operated in the gradient mode with mobile phase-A being 30 mM phosphate buffer at pH 2.6, and mobile phase-B being a 10:60:30 v/v/v ratio of methanol, acetonitrile, and 100 mM phosphate buffer. Compounds of interest were detected at 450 mV and 750 mV and the chromatograms were processed with Agilent OpenLAB CDS ChemStation Edition software (version C.01.06 (61)).

Ferric-reducing antioxidant power: FRAP assay

The ability of tannins to reduce Fe(III) ions in acidic conditions was assayed according to the original colorimetric method (Benzie & Strain 1999). Briefly, samples were diluted in model wine to reach a 0.2 mM CE concentration (58 mg/L for each tannin sample), then 100 µL solutions were added to 1900 µL of FRAP reagent; the resulting mixtures were stored at 37°C; FRAP values were obtained after 30 minutes using a Shimadzu UV mini 1240 spectrophotometer (Kyoto, Japan) and results were expressed as mM Fe^{2+}, in the range 0.02–0.31 mM, using a $FeSO_4 \cdot 7H_2O$ calibration curve.

Radical scavenging activity: DPPH• assay

The DPPH• assay was performed using the original procedure (Brand-Williams et al. 1995) and further developed (Villaño et al. 2007). Briefly, for the radical scavenging assay, 100 µL of tannin solutions 0.2 mM CE were added to 2.9 mL of 200 µM DPPH• solution in methanol. Solutions were incubated in the dark and at room temperature for the time required to reach the steady-state of reaction, then absorbance was measured at 517 nm in 10 mm plastic cuvettes against pure methanol using a Shimadzu UV mini 1240 spectrophotometer (Kyoto, Japan) and expressed as percentage of inhibition (Dudonné et al. 2009), using the following formula:

$$\%inhibition = [(A_b - A_s)/A_b] \times 100 \qquad (1)$$

where A_b = absorbance of the reagent blank, and A_s = absorbance of the sample. The absorbance values have been further used to estimate the total stoichiometry of the radical scavenging reaction for tannins, as calculated using the formula (Villaño et al. 2007):

$$n = [(A_b - A_s)/\varepsilon] \times c_0 l \qquad (2)$$

where A_b = absorbance of the reagent blank, A_s = absorbance of the sample at the steady state,

c_0 = the initial concentration of antioxidant (0.2 mM CE), l = optical path (1 cm) and ε = the molar extinction coefficient of DPPH• at the working conditions (ε = 11563 /M cm), as calculated by (Villaño et al. 2007).

Redox process: cyclic voltammetry experiments

Cyclic voltammetry of tannins in model wine solution (0.2 mM CE) was performed according to the method previously described (Kilmartin 2001). A Bioanalytical Systems (BAS) 100A electrochemical analyser was used to record the voltammograms. The BAS C2 electrochemical cell stand was used with a 3 mm glassy carbon disc electrode (BAS M-2012), a platinum counter electrode and an Ag/AgCl reference electrode (+207 mV versus SHE). Prior to each experimental run, the glassy carbon electrode was polished with 3 μm alumina powder (PK-4 polishing kit), then rinsed with ultrapure water. The voltammograms were recorded at a scan rate of 100 mV/s and a sensitivity of 1 μA. A blank run was recorded daily in the model wine solution, and was used to subtract the background current. The initial scan was taken from −200 to 500 mV, in order to record the first anodic peak. Further scans were taken from −200 to 1000 mV to detect subsequent oxidation peaks. Each experiment was run in triplicate and the average value of the current integrated under the anodic peaks (increase in anodic current with potential, μA•mV) were expressed in mM CE. A calibration curve of (+)-catechin monohydrate over the concentration range 0–1.0 mM was used for the measurement of the first anodic peak, following integration of the anodic current produced by the standard solutions. A second calculation was made based upon the integral of the total anodic current to 1000 mV.

Data processing and statistical analysis

Microsoft Excel was used for data entry, whereas derivatives of the cyclic voltammograms were calculated using OriginPro 8 (Origin Lab Corp., Northampton, MA), and statistical analyses, including Principal Component Analysis (PCA) were performed with Unscrambler X.1 (Camo ASA, Oslo, NO) and XLSTAT (Addinsoft 40, Paris, FR) software. All analyses were performed in triplicate and results were provided as average values.

Results and discussion

Characterisation of food grade tannins

HPLC–ECD analysis and spectrophotometric assays allowed characterisation in terms of polyphenol content, highlighting different composition of samples (Tables 2 and 3). Total polyphenolic compounds (TPC) content ranged from 1.17 to 2.77 mM CE, of which tannins varied between 0.71 and 1.62 mM CE, which corresponds to 34–80% of the potential TPC considered. The wide range of TPC is most likely related to several variables, including the botanical source, the effectiveness of extraction methods and the purity of commercial formulation (Versari et al. 2013).

The commercial tannins based on proanthocyanidins (Pr) showed an overall high content of total polyphenols, in particular the sample from unfermented grape skin (Pr8) with 2.48 mM of total polyphenols, 1.09 mM of which were tannins, and a significant content in flavan-3-ols and flavonol monomers (Table 2). Among the 'Pr' series (i.e. condensed tannins), the sample Pr4 – a blend of grape skins and seeds extracts – showed the highest TPC value (2.75 mM CE) with a significant tannin (1.16 mM CE) and quercetin

Table 2. Phytochemicals characterisation of oenological tannins: total polyphenols, tannins and monomers. *RU = Rutin Equivalent; n.d. = concentration was below the limit of quantification (mean±SD).

	Gallic acid (μM)	(+)-Catechin (μM)	Syringic acid (μM)	(−)-Epicatechin (μM)	Ferulic acid (μM)	ECG (μM)	Quercetin glycoside (μM RU*)
Pr1	8.6 ± 0.1	8.7 ± 0.1	n.d.	12.4 ± 0.4	5.0 ± 0.4	9.0 ± 1.1	n.d.
Pr2	8.3 ± 0.0	26.9 ± 0.2	17.2 ± 0.2	18.1 ± 0.3	8.7 ± 0.7	6.6 ± 0.5	n.d.
Pr3	8.3 ± 1.7	13.7 ± 0.1	14.4 ± 0.0	10.2 ± 0.2	n.d.	4.8 ± 0.0	9.7 ± 2.7
Pr4	24.8 ± 0.1	11.1 ± 0.5	13.5 ± 0.1	13.6 ± 0.1	8.1 ± 3.6	6.0 ± 0.9	17.7 ± 1.2
Pr5	9.3 ± 0.0	35.6 ± 1.0	18.0 ± 0.6	36.2 ± 1.1	5.4 ± 0.3	10.1 ± 2.2	n.d.
Pr6	10.1 ± 0.4	20.0 ± 0.5	14.3 ± 0.2	21.1 ± 0.5	5.1 ± 0.3	6.5 ± 0.9	n.d.
Pr7	4.9 ± 0.2	20.1 ± 1.0	23.5 ± 0.7	16.1 ± 0.8	10.5 ± 0.6	8.1 ± 0.3	24.2 ± 11.1
Pr8	4.2 ± 0.1	42.8 ± 1.7	26.1 ± 1.7	36.8 ± 1.0	11.9 ± 0.1	5.1 ± 0.0	11.9 ± 4.7
Hy1	16.6 ± 0.1	4.6 ± 0.4	12.2 ± 0.4	n.d.	6.3 ± 0.5	2.8 ± 0.0	n.d.
Hy2	21.2 ± 0.1	5.0 ± 0.2	12.2 ± 0.4	n.d.	7.0 ± 0.0	3.0 ± 0.2	n.d.
Hy3	15.3 ± 0.0	4.7 ± 0.1	12.7 ± 0.0	n.d.	9.3 ± 1.0	2.9 ± 0.0	n.d.
Hy4	13.4 ± 0.0	16.6 ± 0.2	15.3 ± 0.2	13.5 ± 0.7	6.7 ± 0.4	20.9 ± 1.3	n.d.
Hy5	14.7 ± 0.1	4.5 ± 0.0	n.d.	n.d.	5.0 ± 0.0	2.8 ± 0.0	n.d.

Table 3. Antioxidant activity of food grade tannins determined with multiple analytical approaches (mean ± SD).

Sample	TPC (mM CE)	Tannins (mM CE)	Radical scavenging (DPPH) (% inhibition)	Reducing power (FRAP) (mM $FeSO_4.7H_2O$)	CV_{500mV} (mM CE)	CV_{1000mV} (mM CE)
Pr1	1.17 ± 0.000	0.71 ± 0.029	22.4 ± 0.20	0.520 ± 0.007	0.148 ± 0.000	0.697 ± 0.007
Pr2	2.64 ± 0.09	0.82 ± 0.012	73.9 ± 0.30	0.638 ± 0.001	0.229 ± 0.002	0.802 ± 0.006
Pr3	2.11 ± 0.009	1.03 ± 0.015	44.9 ± 0.20	0.628 ± 0.002	0.176 ± 0.006	0.744 ± 0.004
Pr4	2.75 ± 0.004	1.16 ± 0.039	72.9 ± 0.30	0.624 ± 0.003	0.221 ± 0.004	0.773 ± 0.011
Pr5	1.94 ± 0.009	1.05 ± 0.037	55.6 ± 0.30	0.513 ± 0.004	0.185 ± 0.007	0.693 ± 0.006
Pr6	2.33 ± 0.004	1.08 ± 0.037	68.6 ± 0.30	0.662 ± 0.023	0.227 ± 0.009	0.748 ± 0.002
Pr7	2.33 ± 0.018	1.60 ± 0.010	63.0 ± 0.40	0.641 ± 0.007	0.200 ± 0.004	0.738 ± 0.013
Pr8	2.48 ± 0.022	1.09 ± 0.015	76.7 ± 0.40	0.628 ± 0.007	0.240 ± 0.008	0.747 ± 0.009
Hy1	1.42 ± 0.000	1.06 ± 0.029	45.8 ± 0.30	0.504 ± 0.004	0.175 ± 0.015	0.714 ± 0.022
Hy2	2.23 ± 0.004	1.27 ± 0.049	77.4 ± 0.20	0.642 ± 0.003	0.233 ± 0.004	0.737 ± 0.012
Hy3	1.77 ± 0.009	1.15 ± 0.037	63.6 ± 0.40	0.614 ± 0.004	0.201 ± 0.000	0.735 ± 0.006
Hy4	2.77 ± 0.013	1.35 ± 0.019	76.6 ± 0.51	0.650 ± 0.006	0.256 ± 0.003	0.734 ± 0.010
Hy5	1.91 ± 0.004	1.62 ± 0.002	72.0 ± 0.20	0.592 ± 0.005	0.239 ± 0.005	0.725 ± 0.006

glycoside content (17.7 µM RU). These results are consistent with data from berry skin extracts of *Vitis vinifera L.* white grape cultivars, whose total polyphenolic compounds averaged at 3.01 mM CE, including 0.190 mM CE of total flavonoid compounds (Katalinić et al. 2010). Extracts from white grape skin rich in phenolics and stilbenes are useful in winemaking to enhance the co-pigmentation phenomena, which increases the colour intensity of red wines (Boulton 2001). Gallic acid peaked on sample Pr4 (24.8 µM) followed by sample Hy2 (21.2 µM) and the effect of this molecule on wine is controversial as it may enhance the bitter and astringent sensory perceptions (Robichaud & Noble 1990). Tannin Pr5 from white grape seeds had the highest content in flavan-3-ol compounds, namely (+)-catechin (35.6 µM), (−)-epicatechin (36.2 µM), and ECG (10.1 µM), with a lack of quercetin glycosides as previously reported (Rodríguez Montealegre et al. 2006). The grape seed samples Pr5 and Pr7 resembled each other with the exception of increased level of (+)-catechin and (−)-epicatechin in Pr5, and quercetin glycoside in Pr7 (Table 2). Although the differences in flavonols can be partly due to grape varietals (Pinelo et al. 2006), and genus (Yilmaz & Toledo 2004), the presence of large amounts of flavonols in Pr7 suggests that this sample does not correspond to a pure grape seed extract.

Similarly, the sample Pr1 – an extract from *Vitis vinifera* leaves – despite of the low content of total polyphenols (1.17 mM CE) and tannins (0.71 mM CE) and a lack of flavonol glycosides and hydroxycinnamic acids is recommended by producers as an antioxidant. HPLC analysis revealed the presence in sample Pr1 of (−)-epicatechin and ECG (12.4 and 9.0 µM, respectively) followed by (+)-catechin (8.7 µM) and gallic acid (8.6 µM). The origin and/or the purity of this sample seems questionable as the leaves of *Vitis vinifera* were reported to be rich in

flavonol glycosides and hydroxycinnamic acids (Monagas et al. 2006). Alternatively, the hypothesis that the extraction method needs to be improved, e.g. in terms of solvent, temperature, time, solvent-to-solid ratio, was formulated.

About hydrolysable commercial tannins (Hy series): samples Hy1, Hy2 and Hy3 were ellagitannins from American and European – Limousin and French – oak, respectively, that are routinely used to produce barrels, chips and exogenous tannins to be used in winemaking. Limousin and French oak extracts (samples Hy2 and Hy3, respectively) showed a high content in TPC (2.23 and 1.77 mM CE) and tannins (1.27 and 1.15 mM CE, respectively), whereas American oak (Hy1) had the lowest content of both TPC and tannins (1.42 and 1.06 mM CE, respectively). Sample Hy4, a blend of selected *Quercus* woods, revealed an extended tannin fraction (1.35 mM CE) that accounts for almost 48.7% of the total polyphenolic compounds (2.77 mM CE). The significant amount of (−)-epicatechin monomer (13.5 µM) and the high content of tannins found in Hy4 suggested the presence of bark blended in this sample. Among 'Hy' series, the sample Hy5 showed the highest concentration in tannins (1.62 mM CE) that accounted for 84.8% of the total polyphenols content (1.91 mM CE).

Cyclic voltammetry

Cyclic voltammetry provided an insight into electrochemical properties of the tannins, which can be related to the redox properties of the plant phenolic compounds (Kilmartin et al. 2001; Kilmartin & Hsu 2003). Figure 1 shows cyclic voltammograms of samples with high hydrolyzable tannins (Hy series, Figure 1 (a)) and those related to condensed tannins (Pr series, Figure 1(b)). The electrochemical parameters derived from the voltammograms are listed in Table 4.
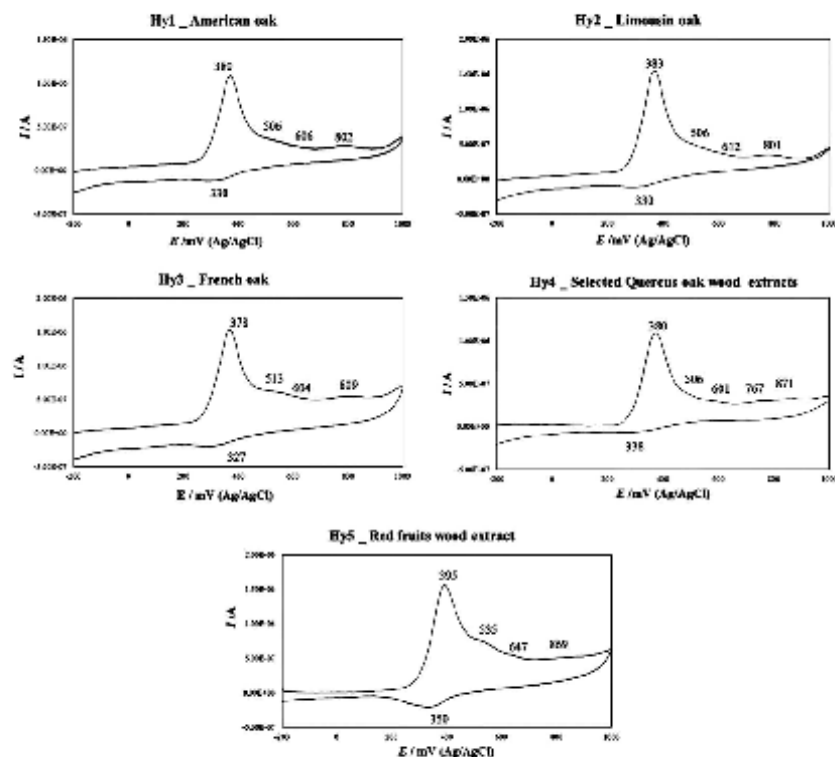
Figure 1. Cyclic voltammograms (background subtracted). Samples 'Hy' ((a) and samples 'Pr' (b) in the model wine solution, measured at 100 mV/s above the potential range: −200–1000 mV, at a 3 mm glassy carbon electrode.

Anodic oxidation peaks, which were largely irreversible and located around 380 mV on the voltammograms of the Hy series with hydrolyzable tannins, were attributed to gallate (and ellagic acid) groups attached to central sugar moieties. Small amounts of flavanol compounds contribute to the small return cathodic peak in each case (see Table 4), while the peak separation values ($E_{p,a} − E_{p,c}$) were also much larger than the 29 mV expected for a fully reversible system (Kilmartin et al. 2001). Although the further presence of an irreversible oxidation peak in the range 506–510 mV for the Hy extracts might reveal the presence of rutin (Wang et al. 2012; Ervin & Kariuki 2016), this attribution seems controversial when applied to the analysed tannins, given the lack of this peak in the grape extracts 'Pr' (see Table 4), which generally contain rutin (Sovak 2001; Iacopini et al. 2008; Castillo-Muñoz et al. 2010). The hypothesis is formulated that the peak characterising the 'Hy' series would be attributable to molecular features of hexahydroxydiphenic acid derivatives that

occur as glucose esters in ellagitannins and are not present in the grape extracts (Singleton 1992; Bors et al. 2001).

Samples Hy4 and Hy5 exhibited anodic peaks at slightly higher potentials (871 and 869 mV, respectively), similar to oxidation potentials occurring in the Pr series and previously attributed to secondary electrochemical oxidation of flavan-3-ol (Kilmartin et al. 2002). This attribution was consistent with the composition of sample Hy4, containing a significant amount of ECG (13.5 M) and (−)-epigallocatechin (20.9) monomers when compared with the other hydrolysable extracts. Conversely, its occurrence was controversial in sample Hy5, which showed an amount of flavan-3-ols comparable with the other Hy extracts. Sample Hy5 also showed a consistent reverse cathodic peak at 350 mV (see Figure 1(a), Table 4) which is related to the reduction of quinone to catechol and could support the hypothesis of the occurrence of a flavonoid-based compound, but the selectivity of the electrochemical
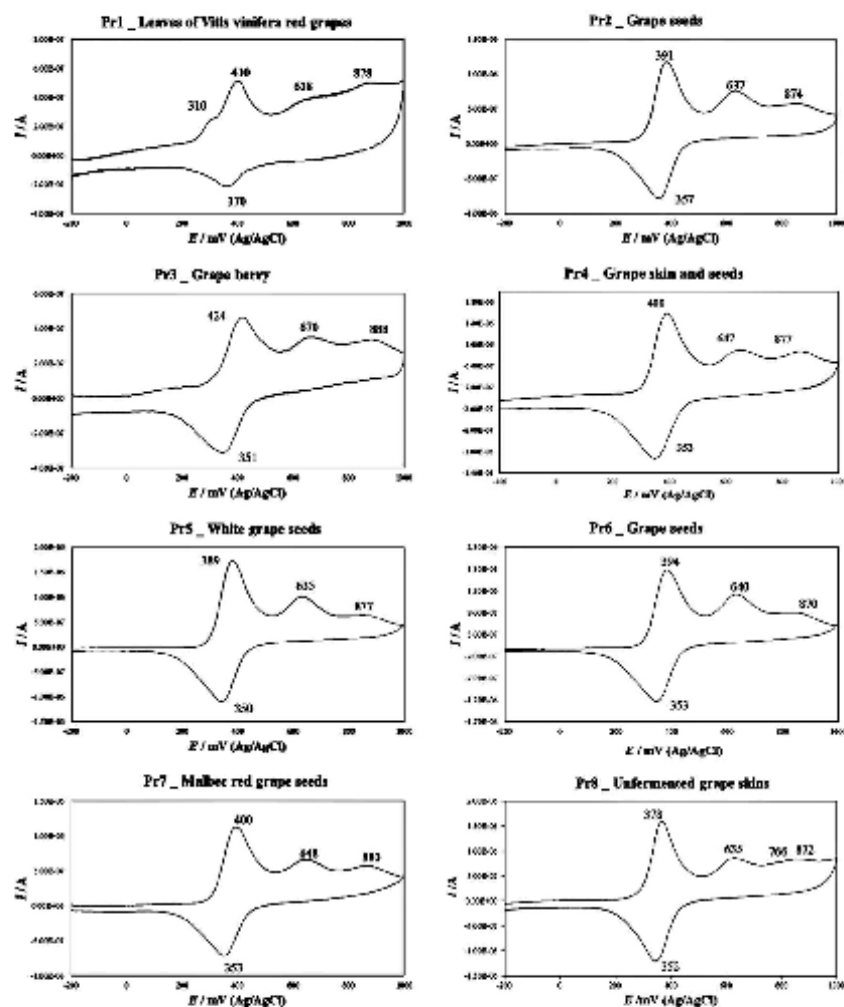
Figure 1. (continued)

method and the compositional information were not sufficient to unambiguously attribute this peak; a more detailed analysis of the composition is required for the red fruit tree wood extract.

Even though the ECG compound, with a gallate group, was also detected in the condensed tannins 'Pr' series, this anodic peak likely overlapped the main anodic potential in the range 390–410 mV, which originated from the oxidation of catechol ring of (+)-catechin (and (−)-epicatechin) units for proanthocyanidins and of the triphenol of gallic acid present (Kilmartin et al. 2001, 2002). A prominent reverse cathodic peak, typical of catechin or epicatechin, was always observed (Figure 1(b)), consistent with the oxidation of ortho-diphenols to quinones being followed by the reverse process on the return scan (Janeiro & Oliveira Brett 2004). The presence of an earlier well-defined peak at 378 mV for sample Pr8 suggested a predominance of galloylated flavanols in the larger oligomeric phenolic structures of the

Table 4. Anodic and cathodic peaks in the order they occur in the respective voltammograms; approximate values are given for broad peaks. The redox couples from semi reversible processes are shown in bold italics.

Sample	$E_{p,a}$											$E_{p,c}$		$E_{p,a}$-$E_{p,c}$*	E_{mid}
Pr1	310	-	***410***	-	-	-	638	-	-	-	878	-	***370***	40	390.0
Pr2	-	-	***391***	-	-	-	637	-	-	-	874	-	***357***	34	374.0
Pr3	-	-	–	***424***	-	-	-	670	-	-	888	-	***351***	73	387.5
Pr4	-	-	***400***	-	-	-	647	-	-	-	877	-	***353***	47	376.5
Pr5	-	-	***390***	-	-	-	635	-	-	-	877	-	***350***	40	370.0
Pr6	-	-	***394***	-	-	-	640	-	-	-	870	-	***353***	41	373.5
Pr7	-	-	***400***	-	-	-	648	-	-	-	880	-	***353***	47	376.5
Pr8	-	***378***	–	-	-	-	635	-	766	-	872	-	***350***	28	364.0
Hy1	-	***380***	–	-	506	606	-	-	-	802	–	***330***	-	50	355.0
Hy2	-	***383***	–	-	506	612	-	-	-	801	–	***330***	-	53	356.5
Hy3	-	***378***	–	-	513	604	-	-	-	809	–	***327***	-	51	352.5
Hy4	-	***380***	–	-	506	601	-	-	767	-	871	***338***	-	42	359.0
Hy5	-	-	***395***	-	535	-	647	-	-	-	869	-	***350***	45	372.5

*Distance between anionic and cathodic peaks for redox couples.

unfermented grape skin extract. This hypothesis was confirmed in previous work by HPLC and MALDI-TOF analyses on crude grape skin extracts (Katalinić et al. 2010; Monagas et al. 2010), and suggested by the authors as a cause of the high antioxidant activity of the analysed unfermented grape skin extract. The oxidation peak at 310 mV – detected on sample Pr1 only – can be ascribed theoretically to quercetin (Kilmartin et al. 2001; Zielińska et al. 2008), which was not detected by HPLC in Pr1, and to ascorbic acid (Loewus et al. 1987) which oxidises at the same potential under similar analytical conditions (John 2005).

Further anodic peaks or shoulders around 650–670 mV could be due to malvidin-type anthocyanin units, or to stilbenes, which can produce peaks at potentials greater than those seen for the catechol-containing phenolic compounds (Corduneanu et al. 2006). All condensed tannin extracts showed an oxidation peak in the 870–888 mV range, attributable to second oxidation peaks of catechin–based compounds.

Antioxidant activity

Table 3 summarises results for the antioxidant assays, alongside the TPC and tannin content. Information about reducing power, redox properties and radical scavenging activity related to effective electron and hydrogen-transfer processes were combined to provide a more comprehensive overview of the antioxidant activity of the extracts (Table 4). In the Pr series, sample Pr8 showed the highest radical scavenging activity followed by Pr2 and Pr4 (DPPH• = 76.7%, 73.9% and 72.9%, respectively), which correlated well with the redox properties given by the peaks seen at potentials less than 500 mV (CV_{500mV} = 0.240, 0.229 and 0.221 mM CE, respectively). The reducing power

given by the FRAP assay was high (FRAP = 0.628, 0.638 and 0.624 mM Fe(II), for Pr8, Pr2 and Pr4, respectively), but the values were not significantly different when compared with the other Pr samples, showing similar reducing effectiveness for flavanol-based extracts derived from the grape source, regardless of the extent of the tannin fraction. Samples Pr6 and Pr7, procyanidins from grape seeds, were effective reducing agents in accordance with the FRAP assay results (0.662 and 0.641 mM Fe(II), respectively), with good redox properties according to the cyclic voltammetry response (CV_{500mV} = 0.227 and 0.200 mM CE, respectively; CV_{1000mV} = 0.748 and 0.738 mM CE, respectively). On the other hand, the low concentration in phenolic compounds in sample Pr1 leads to a low response according to all of the antioxidant mechanisms investigated (DPPH• = 22.4%; FRAP = 0.52 mM Fe(II); CV_{500mV} = 0.15 mM CE; CV_{1000mV} = 0.70 mM CE).

The 'Hy' series was characterised by a high antioxidant activity for samples Hy2 and Hy4, which were rich in phenolic compounds. Sample Hy2 was especially effective as radical scavenger (DPPH• = 77.3%) with the highest redox value of the series (CV_{500mV} = 0.256 mM CE), while the reducing power ($FRAP_{Hy2}$ = 0.642 mM Fe(II); $FRAP_{Hy4}$ = 0.650 mM Fe(II)) and redox activities over the 1000 mV potential range (CV_{Hy2} = 0.737 mM CE; CV_{Hy4} = 0.734 mM CE) were quite similar for the two compounds.

The Hy1 sample, American oak, exhibited the lowest antioxidant activity, in agreement with the lowest content in phytochemicals (DPPH• = 45.8%; FRAP = 0.504 mM Fe (II); CV_{500mV} = 0.175 mM CE; CV_{1000mV} = 0.714 mM CE).

The Pearson correlation analysis (Table 5) showed the best correlation to be between the DPPH• assay and the cyclic voltammetry using the current integrated

Table 5. Pearson correlation matrix of phenolics and tannins content and antioxidant activity determined with different analytical approaches.

	TPC (mM CE)	Tannins (mM CE)	Radical scavenging (DPPH)	Reducing power (FRAP)	CV$_{500mV}$ (mM CE)	CV$_{1000mV}$ (mM CE)
TPC	1					
Tannins	0.781	1				
Radical scavenging	0.811	0.808	1			
Reducing power	0.791	0.659	0.704	1		
CV_500mV	0.779	0.817	0.949	0.704	1	
CV_1000mV	0.739	0.393	0.591	0.714	0.525	1

under the anodic peak to 500 mV ($r = 0.949$). The anodic current to 500 mV accounted for the antioxidant effect of chemical moieties such as catechol and galloyl groups having faster reaction rates with the DPPH• radical, itself a weak oxidising agent (Kilmartin et al. 2001). The *ortho* -di (catechol) and -tri (pyrogallol) hydroxyl substitutions on flavonoids and benzoic acids increase the antioxidant activity of these compounds (Rice-Evans et al. 1996; Leopoldini et al. 2011). Furthermore, galloylation of the flavanol monomers increases the effectiveness of phenolic compounds as radical scavengers (Yokozawa et al. 1998), and this structure is likely to occur in oligomeric proanthocyanidins derived from grape sources, but to be less prominent in highly polymerised tannins (Kuhnert et al. 2015). The correlation between DPPH• value and the full anodic current calculated to 1000 mV curve ($r = 0.59$) dropped as the additional phenolic compounds with a high redox value are weaker reducing agents, which are not readily oxidised by the DPPH• radical. On the other hand, although the cyclic voltammetry response to 1000 mV provided higher correlation with total polyphenols ($r = 0.74$), the low correlation with tannins ($r = 0.39$) suggested that they underwent oxidation at low potential values (<500 mV, see Table 3). The correlation between DPPH• value and the TPC as calculated using the Folin-Ciocalteu method ($r = 0.811$) is consistent with results from Magalhães et al. (2014), where a correlation coefficient of 0.86 between the same methods was observed for a selection of commercial tannins. Moreover, the additional correlation found between DPPH• assay and tannin fraction calculated in this work ($r = 0.811$) indicates that most of the activity is attributable to the polymerised polyphenol compounds, regardless the botanical origin.

The FRAP assay was similarly correlated with both the anodic current up to 500 mV ($r = 0.70$) and to 1000 mV ($r = 0.71$), and most of the contribution was likely provided by the current in the 200–700 mV range. Fe^{3+}, the ionic species that is reduced during the ferric-reducing antioxidant assay, has a cathodic potential of 180 mV versus SHE (Firuzi et al. 2005); in our working conditions none of the phenolic compounds oxidise at such low potential value to couple with the Fe(III) present (or generated from coupled oxidation processes). However, the formation of coordination complexes between phenolics and iron makes the Fe(III) a stronger oxidant in the FRAP assay system (Danilewicz 2015), which makes the reaction possible. The satisfactory correlation between DPPH• and FRAP values ($r = 0.704$) underlines the important contribution provided to the electron transfer process involving the most active phenolic molecular features. Discrepancies between the two methods were due to lability of the DPPH reagent compared with the increased stability of the reagent Fe(III)/TPTZ (FRAP reagent) in the buffered model wine solution (Danilewicz 2015).

The discrepancy between the TPC values calculated with the Folin-Ciocalteu and the CV$_{1000\ mV}$ is most probably related to the nature and purity of the commercial products, which might contain significant amount of lignin degradation products, resinous substances, reducing sugars, and artificial food additives such as dextrin, starch, and other vegetal amides (Villavecchia & Eigenmann 1976). The total antioxidant stoichiometry of tannin samples was calculated as the number of radical molecules reduced by one molecule of antioxidant (Equation (2)), and correlation between total polyphenols and n_{tot} calculated was good ($r = 0.811$). Table 6 shows the radical scavenging stoichiometry calculated for tannins and compared with the literature (Villaño et al. 2007) for the monomeric constituents of tannins (gallic acid and flavan-3-ols derived structures). It is well known that the ability of monomers to scavenge radicals effectively increases with the number of –OH substituents, however the increase of hydroxyl groups following polymerisation does not provide an additive effect in terms of availability in the proton-transfer process. In fact, the occurrence of polymerisation slightly increased the stoichiometry of the radical scavenging reaction when compared with the reaction of phenolic monomers against the DPPH• radical. Two hypotheses were considered to mainly influence the availability of hydroxyl groups to participate in the radical scavenging reaction: (i) the steric arrangement following the polymerisation

Table 6. Stoichiometry of scavenging of DPPH• radical with enological tannins (n_{tot}) and data from the literature for monomeric and oligomeric phenolic compounds (n_{tot}*) (Villaño et al. 2007).

Sample code	Sample description	n_{tot}
Pr1	Vitis vinifera leaves	2.7
Pr2	Grape berries	9.0
Pr3	Grape	5.4
Pr4	Grape skin and seeds	8.8
Pr5	White grape seeds	6.7
Pr6	Grape seeds	8.3
Pr7	Malbec red grape seeds	7.6
Pr8	Unfermented grape skins	9.3
Hy1	American oak	5.5
Hy2	Limousin oak	9.4
Hy3	French oak	7.7
Hy4	Selected Quercus wood	9.3
Hy5	Trees wood	8.7
	Polyphenolic compound	**n_{tot}***
	Gallic acid	5.6
	(+)-Catechin	4.5
	(−)-Epicatechin	6.6
	(−)-Epigallocatechin	5.4
	(−)-Epicatechin gallate	7.3
	(−)-Epigallocatechin gallate	7.9
	Procyanidin B1	7.6
	Procyanidin B2	7.4
	Ascorbic acid	2.3
	Trolox	2.0
	Resveratrol	1.0

process, and (ii) the stability of intermediate products, such as dimers or quinones, which could further react with the radical increasing the stoichiometry. The compounds with high stoichiometry n-value are characterised by an extended polymerisation of flavonoid monomers and/or a high degree of galloylation, as gallic acid has three –OH groups and the n-value calculated for the reaction with DPPH• radical was 6 (Villaño et al. 2007).

From the practical point of view, the sample derived from Limousin oak and from selected *Quercus* woods showed a higher content of active compounds and a stronger total antioxidant capacity. When considering the condensed tannins series, the extract from unfermented grape skins showed the highest performances, despite a content of phenolic compounds that is lower than other 'Pr' samples, probably due to the high occurrence of flavonoid gallate structures that implement the antioxidant mechanisms. The stoichiometry of radical scavenging reactions was not additive with respect to the related monomers, suggesting a contribution of the spatial arrangement in the availability of active functional groups.

Principal component analysis (PCA)

Principal Component Analysis (PCA) was used to disclose the hidden structure of extracts from different botanical sources on the basis of HPLC,
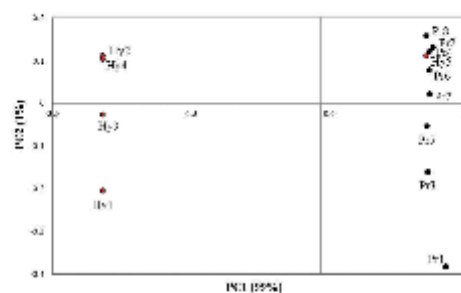
Figure 2. (colour online) Principal component analysis of investigated tannins: score plot for PCA first and second factors.

spectrophotometric and CV results. The first two principal components accounted for the total variance, in particular PC1 (explained variance: 99%) provided the most useful subset for explaining the data. The projection of the tannin samples over the first two principal components (Figure 2) clearly grouped the condensed tannins (series 'Pr', right-side of the quadrant) separating them from the hydrolysable extracts (series 'Hy', left-side of the quadrant); the separation was easily achieved showing how the two chemical subclasses exhibit distinctive chemical-physical and compositional characteristics. The ability to separate the commercial tannins by their composition in polyphenolic compounds and antioxidant mechanisms (recently highlighted by Magalhães et al. 2014) was further confirmed in this work by using the PCA.

The model provided an unexpected prediction for the exceptional sample Hy5, grouped between the 'Pr' compounds. Tannin Hy5, a hydrolysable extract, differed from the other samples to the high extent of a polymerised fraction (84.8% of the total polyphenol content); moreover, it was distinguished within the 'Hy' series for the presence of a reverse cathodic peak at 350 mV, as detected in cyclic voltammetry. This electrochemical feature suggested the presence of a flavonoid-based compound that was not detected in HPLC, which influenced the PCA classification of the sample.

The discrimination was mainly based on the presence of a reversible cathodic peak in the cyclic voltammogram of condensed tannins; this cathodic current peaked at 350 mV (see Table 3) and is generated following the oxidation of catechol to quinone, which is reversible at the time-scale and diffusion limit of the voltammetry experiment. Flavonoid compounds and the content in syringic acid were also responsible for the discrimination between flavonoid-based tannins and the 'Hy' series. A significant fraction of flavonoid features (resorcinol

group and the heterocycle of catechin-based compounds) oxidises in the range 500–1000 mV, and this is the reason for a high anodic current observed up to 1000 mV for sample Hy5 and the 'Pr' series.

The peculiar composition of tannins based on flavan-3-ols make them suitable for several technological applications. In more detail, proanthocyanidins are flavan-3-ol oligomers and polymers, containing resorcinol, oxygenated heterocycle, catechol ring, and occasionally catechol-gallated structures. Their structure-activity effect is also related to their interaction with the chemical environment, in particular the pH value and alcohol content affect the efficiency of the antioxidant mechanisms and the balance between antioxidant and pro-oxidant activities.

It is generally established that the catechol and pyrogallol rings act as electrophilic centres, and are mainly responsible for the antioxidant activity of these compounds; conversely, the resorcinol ring is less active as an antioxidant (lower electrophilic effect) but it is characterised by high nucleophilicity. Phenolic compounds can easily deprotonate to form a phenoxide, undergoing resonant stabilisation. In general, condensed tannins are suitable for wastewater treatments (e.g. winery effluents), since the deprotonation is promoted by electronic delocalisation within aromatic ring, thus increasing the electronic density to the unpaired oxygen atom. It follows that the more phenolic groups are available in a tannin structure, the more effective its coagulation capability (Özacar & Şengil 2003). On the basis of this assumption, we can predict the coagulation capability of these compounds using an 'effectiveness scale' for the tannin series investigated based on the cyclic voltammetry response of the anodic current up to 1000 mV, which is related to the number of active sites in the extract (–OH groups). The series was classified as follow: Pr2>Pr4>Pr6>Pr8>Pr3>Pr7>Hy2>Hy3>Hy4>Hy5>Hy1>Pr1>Pr5. The series begins with Pr2, Pr4 and Pr6 samples, all extracts from grape seeds, whose proanthocyanidins are partly galloylated (about 20% galloylated units), followed by Pr8, a grape skin proanthocyanidin that is characterised by (-)-epigallocatechin as a major component (Souquet et al. 1996; Sarni-Manchado et al. 1999). The proanthocyanidins Pr1 and Pr5, located at the end of the series, are characterised by a low content in total polyphenols and tannins when compared with the 'Pr' series, and the low electrochemical activity could be related to an occasional low extraction yield for these compounds.

A similar consideration makes grape tannins extracts suitable for the production of high-performances polymeric films. In a previous work, a plastic film based on ethyl cellulose and blended with a grape seed extract was

prepared, taking advantage of the ordered condensed structures of the natural extract based on C4–C8 linkages between monomers. Therefore, the tannin molecules allowed the –OH sites to be available for interaction with the active sites of the plastic, and to keep some active sites available for antioxidant activity (Olejar et al. 2014). According to the PCA classification (Figures 2 and 3), the Pr series is confirmed to be the main candidate for this application, with high TPC values and a significant electrochemical activity in the whole range up to 1000 mV. On the basis of these considerations, a tentative classification of the condensed tannins investigated in view of their application as plastic additives could be made on the basis of the total polyphenol/tannin ratio: Pr2 (3.22) > Pr4 (2.37) > Pr8 (2.28) > Pr6 (2.16) > Pr3 (2.05) > Pr5 (1.85) > Pr1 (1.65) > Pr7 (1.46). This classification was based on the premise that most of the monomeric fraction consists of flavanols and flavonols (Table 2, Figure 3) able to interact with the active sites of the plastic and to support the antioxidant activity of tannin molecules.

High oxidation current values were calculated up to 500 mV (378–383 mV) for the 'Hy' series, also showing a high correlation with the ability to scavenge the DPPH synthetic radical of these molecules; the scatter plot shows the classification of hydrolysable compounds as active radical scavengers (Figures 2 and 3). The scatterplot shows a correlation between the redox activity up to 500 mV, the radical scavenging capacity and the percentage of polymerised fraction in tannin extracts. In particular, samples Hy2 and Hy4, which are
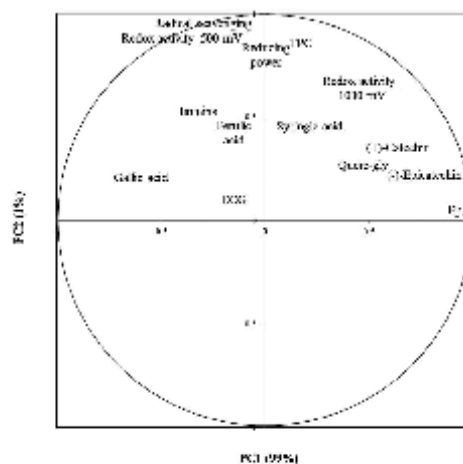
Figure 3. Principal component analysis of investigated tannins: loading plot for PCA first and second factors.

extracts from Limousin oak and a blend of selected European *Quercus* woods, respectively, were particularly rich in tannin fraction and strong radical scavengers. Furthermore, extracts located at the left-side of the plot (Figure 2) are generally characterised by a high content of gallic acid monomer, as expected for the hydrolysable series of tannins; the hydrolysis of glycosylated chains and the degradation of lignin of heartwood following extraction are the two main breakdown processes responsible for the high occurrence of gallic acid (Quinn & Singleton 1985). The effectiveness of the radical scavenging mechanisms could be related to the high resonant stabilisation for gallic acid compounds, 5.14 kcal/mol in water (Leopoldini et al. 2004), which make this monomer and its derivatives suitable as radical scavengers. Hydrolysable extracts, especially derived from European oak species, could be the basis for formulations of additives for long-term conservation of foodstuffs; nevertheless, the presence of these compounds influences the performances of hydrolysable tannins when used as food additives, due to the astringent perception supplied by gallic and ellagic acid-based compounds. A tailored introduction of these extracts in food technologies would require a detailed investigation of the relationship between chemical composition, dosage and sensory impact (Ricci et al. 2016).

Conclusions

The proposed approach was successfully applied for the elucidation of the structure-activity relationship of food grade tannins. It reflected their effectiveness as natural antioxidants due to their complex combination of reducing and redox activities, which also contributes to their ability to scavenge radicals. In particular, the tunability of the oxidising potentials enabled a discrimination between the high-reactive polyphenol fractions, which oxidise at lower potentials within 500 mV, and polyphenolic compounds, which are less reactive and participate in the antioxidant activity with low-rate secondary reactions.

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Disclosure statement

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Appendix I – Analytical profiling of food-grade extracts from grape (*Vitis vinifera sp.*) seeds and skins, green tea (*Camellia sinensis*) leaves and Limousin oak (*Quercus robur*) heartwood using MALDI-TOF-MS, ICP-MS and spectrophotometric methods

Original Research Article

Analytical profiling of food-grade extracts from grape (*Vitis vinifera* sp.) seeds and skins, green tea (*Camellia sinensis*) leaves and Limousin oak (*Quercus robur*) heartwood using MALDI-TOF-MS, ICP-MS and spectrophotometric methods

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ABSTRACT

Tannins are food additives widely used due to their antioxidant and antimicrobial activities, and flavouring properties. Nevertheless, the information provided by the manufacturers are often generic, and data on the presence of specific phenolic compounds and potentially toxic elements are needed. In this work a selection of food-grade plant extracts – also called 'commercial tannins' – from different botanical sources: *Vitis vinifera* sp., *Camellia Sinensis*, *Quercus robur*, were profiled using UV–vis spectrophotometry, matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS), and inductively coupled plasma-mass spectrometry (ICP-MS). The combined analytical approach was suitable for quality control of the polyphenolic fraction to highlight authenticity markers (e.g. galloylated flavonoids, glycosides, ellagitannins), and to ascertain the content of toxic elements (Cu, Sr, As, Co, Cr, Fe, Zn, Li, Ba and Pb) respect to their legal and/or recommended limits.

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1. Introduction

There is an increasing interest in the exploitation of phytochemicals from agri-food by-products and wastes. In this perspective, tannins – secondary phenolic metabolites produced by plants – have gained increasing interest for their exploitation in the food industry, due to their application in human and animal nutrition as natural antioxidants (Arogba, 2000; Chung et al., 1998) and feed flavourings (EFSA FEEDAP Panel, 2014).

Commercial tannins are natural polyphenolic compounds occurring in plant woods, fruits, seeds, and extracted from different plant tissues. Few selected species are employed in the food technologies, due to their high content in bioactive compounds which reflects their technological performances. Procyanidins are condensed molecular structures composed of flavan-3-ol monomeric units; the main natural sources are constituted by grape seed, skin and leaves, mimosa bark, Quebracho wood, and tea leaves. Hydrolysable tannins consists of glycosylated units of gallic and ellagic acid monomers and polymers; the oak, chestnut and gallnut woods provide primary sources of hydrolysable tannins for the food industry (Versari et al., 2013).

Tannins consist of a complex matrix including polymerized fraction and additional components such as phenolic monomers, oligomeric fractions, phenolic aldehydes, stilbenes, sugars and glycosylated compounds, among others (Versari et al., 2013). The properties of tannins are strongly dependent on the composition of specific classes of bioactive compounds occurring as a consequence of the extraction process, and on the lack of contaminants (Vivas et al., 2004a). In particular, the phenolic compounds can

remove free radicals and chelate transition metals and proteins, improving quality and stability of food and beverages (Haslam, 1998). However, tannins could induce astringent, antinutritional and toxic effects (Avallone et al., 1997; Makkar, 2003). Therefore their analytical characterization is a suitable tool for the valorisation of plant extracts (e.g. tea leaves) and for their exploitation as sources of food supplements for human nutrition and animal feed. In particular, the bioaccessibility and fermentability of tannins by colonic microflora is highly affected by their degree of polymerisation (Serrano et al., 2009).

The use of tannins in the food industry is regulated by Communitarian and International legislations (FDA- Code of Federal Regulations Title 21; Directive 2012/12/EU of the European Parliament and of the Council, amending Council Directive 2001/112/EC; Beer Judge Certification Program (BJCP) Style Guidelines 2015; Resolution OIV-OENO 554-2015, among others) but limited recommendations are provided for the authentication and typification of raw materials. The composition of the monomeric polyphenolic fraction and building blocks of oligomers are specific for each botanical class, and provide information on the effective origin of the extracts. The main markers of quality and authenticity of commercial tannins are related to the content of condensed and hydrolysate structures, also including (i) the degree of galloylation of polygalloylglucose chains and flavanol monomers, (ii) the glycosylation patterns derived from degradation of plant tissues, and (iii) their molecular arrangement following extraction (Amakura et al., 2009; Nonier et al., 2005)

One of the most beneficial groups of polyphenolic compounds obtained by natural extracts is constituted by proanthocyanidins, with procyanidins as the most prominent subclass; mixtures of procyanidolic oligomers naturally occur in fruits, with red fruits and grapefruit as the main sources, and tea leaves. The related extracts have technological and nutritional impact, being exploited both in the beverages industry and as food supplement. The grape extracts are commonly composed of flavonoid monomers and procyanidin oligomers and polymers extracted from *Vitis vinifera* sp. grape seeds and skin; among them, a large amount of flavan-3-ol monomers esterified with gallic acid units has been observed in the grape seed procyanidins (Krueger et al., 2000), resulting in an enhanced bioactivity and a stronger sensory impact when compared to the skin extract.

Due to the steric hindrance produced by esterification of building monomers, the degree of polymerisation of procyanidins could reach a maximum of 16 units; the occurrence of higher polymers may derive from oxidative condensation of oligomers following extraction (Prieur et al., 1994). The grape skin extract is characterised by a higher degree of polymerisation, related to the low degree of esterification of flavan-3-ol monomeric units. Both skin and seed extracts from grape are effective antioxidants and can be added to foods and beverages to retard deterioration.

Green tea (*Camellia sinensis*) leaves are recognized as a major source of galloflavonoid and flavonoid gallates, with a prevalence of (−)-epigallocatechin gallates (Graham, 1992; Yoshizawa et al., 1987); the peculiar composition makes the extract a suitable candidate as a protection from oxidative stress (Frazier et al., 2010; Perumalla and Hettiarachchy, 2011). Nevertheless, the ability of galloylated structure to interact with proteins is also responsible for the astringent perception induced by gallic acid-based compounds (Harbertson et al., 2012; Obreque-Slier et al., 2010); the use of both grape seed extract and green tea tannins would require a detailed investigation on the chemical composition of the phenolic fraction and on the sensory impact for their addition.

The water-soluble hydrolysable tannins contained in European oak (*Quercus* species) heartwood have been historically exploited for cooperage, and they are recognized as providing beneficial stabilising and flavouring effects. Oak wood is mainly used for the conservation of fine beverages: aging wines, spirits, and balsamic vinegars are traditionally stored in barrels, where the continuous contact with wood naturally extracts and releases ellagitannins in solution; ellagitannins contained in the oak extracts increase the antioxidant activity, due to the high content in gallic and ellagic acids-based compounds (Landete, 2011). Several scientific surveys have explored the differences between European and American *Quercus* species used for cooperage, to determine the most suitable formulation for food industry needs. Among them, the work of Chatonnet and Dubourdieu (1998) has highlighted the supremacy of oak obtained from Limousin forests in relation to its content in water-soluble precursors (Chatonnet and Dubourdieu, 1998). Limousin oak forests are located in the southwest area of France, with a prevalence of *Q. robur* trees; this species has a high content in ellagitannins, vanillin and phenolic aldehydes when compared to *Q. petraea* and *Q. alba* oak species (Chatonnet and Dubourdieu, 1998).

Since there is no regulation constraining manufacturers to provide a detailed composition of the extracts or the methods of extraction used, information on adulteration and possible contamination is not available to the consumer, and the purity and the healthiness of commercial tannins can be questionable. Monitoring the composition of food additives is a key step to guarantee consumer safety, through the identification of markers (both qualitative and quantitative) which inform on their effective content in bioactive compounds, their authenticity and non-toxicity (Li and Vederas, 2009; Pennington, 2002). Moreover, a correct balance in macroelements (Ca, K, Mg, Zn, among others) could enhance the nutritional value of this food additive (Sulaiman et al., 2011; Vin et al., 2014).

Due to the large variety of commercial botanical extracts used in the food industry, there is a need to explore both targeted and non-targeted approaches to assess the quality/authenticity of commercial tannin formulations. In this work, the compositional parameters of four tannins employed for human consumption and selected to represent commercially available extracts from: grape seed, grape skin, green tea leaves, and Limousin oak heartwood, were investigated, and quality and authenticity markers were identified. MALDI-TOF MS, ICP-MS and spectrophotometric assays were used for qualitative and quantitative analysis; in particular, the elemental and molecular composition, along with the polyphenolic content and antiradical activity were assessed for these commercial food additives, and briefly discussed.

2. Experimental

2.1. Chemicals

Solvents, L-tartaric acid (≈100%) pure ethanol (>99%) to prepare tannin solutions, and HNO_3 and H_2O_2 reagent grade used for ICP-MS analysis were supplied by Merck (Darmstadt, Germany). Bovine serum albumin (BSA), anhydrous $FeCl_3$ and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) for spectrophotometric determinations were purchased by Sigma Aldrich (St. Louis, MO). Phosphorus red reference standard (99,999%) used for MALDI-TOF analyses was purchased from Acros Organics (Fair Lawn, NJ).

2.2. Samples preparation

Four food-grade commercial tannins from different botanical sources: grape seed (SEP) and skin (SKP) proanthocyanidins, green tea procyanidins (GTP), and Limousin oak ellagitannin (LOE), were purchased from the Enologica Vason company (Enologica Vason S. p.A., Verona, Italy) as lyophilized powders and stored at room temperature; any purification treatment was carried out on commercial samples before analyses. According to Harbertson

et al. (2002) 1 g/L of tannin powders were dissolved in hydro-alcoholic solution (12% (v/v) ethanol in distilled water, with addition of L-0.033 M tartaric acid and 1 M NaOH to reach pH 3.6) to obtain a "stock solutions" of each sample; then, stock solutions were properly diluted in distilled water for spectrophotometric assays.

For MALDI-TOF experiments, powder samples were dissolved in acetone/water 50/50% (v/v) at a concentration of 5 mg/mL, and then the solutions were mixed (1:1, v/v) with a standard solution of 2,5-dihydroxybenzoic acid at 10 mg/mL dissolved in the same solvent. Then, 1.5 μL of the mixed solution were spotted on a 384-well MALDI-TOF plate, followed by evaporation of the solvent at ambient temperature before analysis.

For ICP-MS analysis the stock solutions (5 mL) were added with 3 mL of HNO_3 and 2 mL of H_2O_2 reagent grade, then heated to 130 °C (digester DigiBlock ED365; LabTech, Hopkinton, MA) for 3 h; subsequently the mixture was diluted with 25 mL of ultrapure H_2O before analysis.

2.3. Determination of total polyphenols, tannin fraction and in vitro antioxidant capacity

Total (iron reactive) polyphenols and tannins were quantified using the method of Harbertson et al. (Harbertson et al., 2002) which is based (i) on the ability of protein (i.e. bovine serum albumin, BSA) to precipitate tannins, and (ii) on the reactivity of ferric chloride with phenolic compounds that possess ortho-dihydroxyl groups, as previously described (Versari et al., 2007). This method is particularly suitable for commercial tannins analysis as it estimates the degree of purity and the amount of iron-reactive polyphenolics, which play a critical role in redox systems.

The in vitro antioxidant capacity of tannin samples was determined using the DPPH• radical scavenging method (Brand-Williams et al., 1995) that evaluate of the decay of radical absorbance at 517 nm; results were expressed as Trolox equivalents (mmol TE/L).

All spectrophotometric determinations were performed using a Shimadzu UV Mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan).

2.4. MALDI–TOF mass spectrometry

MALDI-TOF spectra were recorded using a Kratos compact MALDI Axima Performance TOF 2 instrument (Shimadzu Biotech, Manchester, UK), equipped with a nitrogen laser (337 nm), an ion gate for the selection of precursor ions, and a collision cell, according to Lagel et al. (2014). The windows for separation of precursor ions were approximately 4 Da. Argon has been used as the collision gas. All data were obtained in positive ion linear mode applying the accumulation of 441 scans per spectrum. Linear negative mode was used for the investigation of hydrolysable and

glycosylated molecular patterns which characterise the Limousin oak extract. The calibration of the linear modes was done using phosphorus red pigment as a reference over a mass range up to 2500 Da. NaCl was added in the sampling wells as the salt to enhance ion formation previous deposition of samples[S1] . The MALDI-TOF target was then analysed to give the resulting spectra, using a raster analysis over the target; Maldi-MS software was used for data treatment (Shimadzu Biotech, Manchester, UK).

2.5. ICP-MS analyses

Metals were analyzed using an Agilent ICP-MS equipped with a 7700 Q Apex + Spiro TMD (Agilent Technology, Santa Clara, CA); the standard nebulizer for sample introduction was replaced with a desolvation system. The APEX Q atomizes the sample into a cyclonic chamber heated to 140 °C and subsequently cooled, thus inducing the removal of most of the aqueous component of the sample. After condensation; this process enables concentration of the sample by about 10 times. The Spiro TMD (Teflon membrane desolvator) is composed of a spiral interfaced with a Teflon membrane heated at 100 °C that further removes the water vapor from the sample aerosol stream, enabling only the dry component to reach the plasma. The acid digestion step and the combined use of APEX Q and Spiro TMD technologies enable the simultaneous determination of macro, micro and trace elements, and it is routinely used as an internal laboratory protocol for elemental profiling; nevertheless, in this work only the first two categories showed significant concentrations and are accordingly discussed in the Results and Discussion section. The instrument is also equipped with a collision chamber sparged with He for the removal of interferences, such as oxides (below 0.1%); analyses were performed with and without He stream, to avoid overestimation due to occasional adducts formed.

Internal standards Ge and Tl were added through a tee into the Apex, before the introduction of the sample and used for quantitation.

2.6. Statistical analysis

Microsoft Excel was used for data entry, and statistical analysis was performed with Unscrambler X.1 (Camo ASA, Oslo, Norway). All analyses were performed in triplicate and the results expressed as mean ± standard deviation (SD). The statistically significant level was considered at $\alpha = 0.05$.

3. Results and discussion

3.1. Total (iron reactive) polyphenols, tannin fraction and in vitro antioxidant capacity

The total polyphenol content, tannin fraction and the in vitro antioxidant capacity of the four commercial extracts (Table 1) was

Table 1
Phytochemical composition and antioxidant capacity of selected commercial tannins. Results from replicates (n=3) are reported as mean values ± standard deviation. Legend: grape seed (SEP) and skin (SKP) proanthocyanidins, green tea procyanidins (GTP), Limousin oak ellagitannin (LOE).

	Tannin samples			
	SEP	SKP	GTP	LOE
Total polyphenols (mM CE[a])	1.62 ± 0.12	2.83 ± 0.01	2.11 ± 0.01	2.34 ± 0.03
Tannins (mM CE)	1.49 ± 0.02	1.23 ± 0.01	1.04 ± 0.02	1.42 ± 0.0
[c]Tannins/total polyphenols (%)	93.8	42.9	47.6	60.9
DPPH (mM TE[b])	0.26 ± 0.0	0.24 ± 0.01	0.42 ± 0.01	0.39 ± 0.01
[d]Non-phenolic compounds (%)	46.7	6.7	30.0	23.3

[a]Expressed as (+)-catechin equivalent. [b]Expressed as Trolox equivalent. [c]Calculated as % weight: tannins/total polyphenols. [d]Calculated as % weight: 1 g/L dry powder – estimated total polyphenols.

carried out to set-up a rapid screening approach to provide both qualitative and quantitative information useful for quality control of commercial products. Samples were ranked in terms of total polyphenols content as follows: SKP > LOE > GTP > SEP.

The SKP extract peaked in the total polyphenol content (2.83 mM CE), of which 42.9% is the polymeric tannin fraction (1.23 mM CE). The seed procyanidin (SEP) was lowest in total polyphenols (1.62 mM CE), most of which (93%) comprised the polymerised fraction (flavonoid oligomers and polymers). The SKP/SEP ratio of proanthocyanidins estimated in this work (value = 1.3) is almost doubled when compared to the value of 0.55 obtained by Vivas et al. (2004a, b) when studying similar commercial formulations. This result emphasised the high variability of bioactive compounds in commercial extracts, which is affected by several factors, including the extraction process (e.g., time, temperature, solvent, etc.) and the raw material (e.g., grape variety and maturity). Although the tannin fraction (% of the total polyphenol content) defines the effectiveness of the extraction process and influences the technological potential of the extracts for industrial applications (Kallithraka et al., 1995), the monomeric fraction contributes to the antioxidant properties, and constitutes an important parameter to be monitored. The green tea extract showed a high content in polyphenolic compounds (2.11 mM CE), part of which was tannins (47.6%), and the highest antioxidant capacity as radical scavenging (0.42 mM TE). The green tea leaves are rich in flavonoid-based monomers with a high degree of galloylation (Perumalla and Hettiarachchy, 2011), and the effectiveness of the galloylation patterns in the radical scavenging activity was also observed in the SEP sample (0.26 mM TE) compared to SKP (0.24 mM TE). The enhanced antioxidant capacity of Limousin oak extract (0.39 mM TE) compared to the grape extracts can be explained by the high content in total polyphenols

(2.34 mM CE), with a great content in hydrolysable tannins (60.9%) that provides an effective protection against oxidation.

In general, samples showed a variable content in non-phenolic compounds (SEP > GTP > LOE > SKP), which was attributable to the presence of degradation by-products following extraction or addition of stabilising additives during processing, i.e. arabic gum powder, proteinaceous material, cellulose (Romani et al., 2006).

3.2. Targeted analysis by MALDI-TOF mass spectrometry

3.2.1. Seed proanthocyanidin (SEP)

The MALDI-TOF profile of SEP sample highlighted the galloylation patterns that are a valuable marker of extracts from grape seeds and skin (Souquet et al., 1996), the latter with high polymerization index and less astringent sensation (Vidal et al., 2003). The SEP showed monomers from fragmentation patterns of flavonoid compounds (231 Da), whereas the 271 Da peak was attributable to fisetinidin (or catechin with loss in –OH), while the catechin was present in protonated form (291 Da). The occurrence of (–)-gallocatechin/(–)-epigallocatechin compounds was represented by the peak at 303 Da, together with typical fragments 258–220 Da [MS^2 (m/z)]. The 152 Da fragment was related to the presence of galloyl units released during fragmentation. The major flavonoid monomeric units were found to be (+)-catechin/(–)-epicatechin (mass increment: 289 Da). However the building block of the first series of polymeric structures was represented by a fisitinedin-fisetinidin dimer (540 Da), increasing its degree of polymerisation with catechins as repeating unit (Fig. 1): 827 Da (trimer); 1125 Da (tetramer); 1416 Da (pentamer); 1703 Da (hexamer); 1992 Da (heptamer); 2278 Da (octamer). Although the MALDI-TOF analysis was not able to discriminate between stereoisomers, due to the steric hindrance provided by flavanol
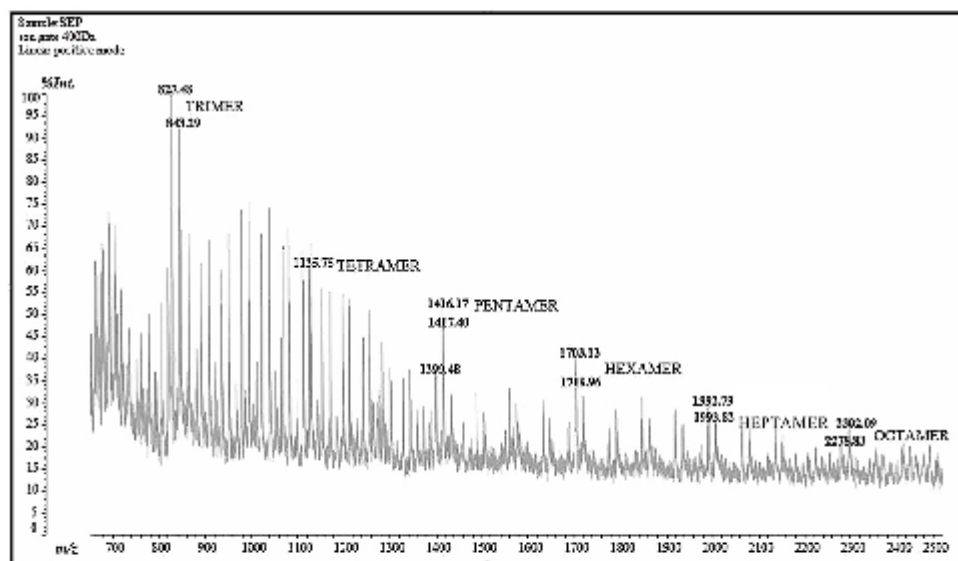
Fig. 1. The MALDI-TOF MS spectrum of sample SEP (linear positive mode, ion gate: 400 Da) recorded in the range 650–2500 Da: procyanidins series (repeat unit: catechins) for the grape seed extract.

Table 2
Calculated and experimental MALDI-TOF peaks related to the galloylated procyanidins series in the SEP sample.

DP*	no. of galloyl units (+152 Da)	Calculated (M+Na⁺)	Observed (M+Na⁺) Linear positive mode
Trimer	0	890	891
	1	1041	–
	2	1194	1194
	3	1344	1344
Tetramer	0	1178	1178
	1	1330	1328
	2	1482	1482
	3	1634	1633
Pentamer	0	1466	–
	1	1618	–
	2	1770	–
	3	1922	1922
	4	2074	2080
Hexamer	0	1754	–
	1	1906	–
	2	2058	–
	3	2210	–
	4	2363	–
Heptamer	0	2042	2037
	1	2194	2197
	2	2347	2348
	3	2495	2497
	4	2939	–
Octamer	0	2331	–
	1	2483	2480
	2	2635	–
	3	2787	–
	4	2939	–

*DP: degree of polymerisation. (–) Not present.

units, we hypothesized a (–)-epicatechin-like structure as a building block for grape seed procyanidins, with (+)-catechin as a possible terminal unit (Vivas et al., 2004b). The second series of polymeric structures was characterised by the presence of galloylated compounds with a variable number of galloyl units, as summarized in Table 2. Flavonoid compounds were also found

in the grape seed extract, as sodium adducts (309 Da, kaempferol; 324 Da, quercetin); on the basis of their molecular weight, the 463 peak was attributed to the quercetin-3-glycoside monomer, and the 609 Da peak was related to a quercetin-3-rutinoside (rutin) – like structure.

3.2.2. Skin proanthocyanidin (SKP)

The MALDI-TOF spectra of the grape skin proanthocyanidin (SKP) showed (+)-catechin/(–)-epicatechin (peak 283 Da) and gallocatechin/epigallocatechin (peak 303 Da with additional fragments 250 and 226 Da) as the main units, in agreement with the literature (Souquet et al., 1996). Noteworthy, the peaks at 166/169 Da (gallic acid), 436 Da ((–)-epicatechin-3-gallate) and 462–464 Da ((–)-epicatechin-3-gallate sodium adduct) were markers for the presence of galloylated units. Catechin gallates are generally recognized as the main constituents of grape seed extracts (Vivas et al., 2004b; Yang and Chien, 2000), with a variable degree of galloylation in grape skins, mainly featured as (–)-epicatechin-3-O-gallate structures (Souquet, Cheynier, Brossaud, & Moutounet, 1996). The 463 Da peak could be alternatively assigned to the occurrence of quercetin-3-glycoside (isoquercetin), although not associated with complimentary fragmentation patterns. The only confirmation for this attribution derived from the presence of the peak at 617 Da, as a possible isoquercetin gallate dimer. The 605 Da peak was referred to as quercetin-3-rutinoside (rutin) with loss in protons.

The composition of procyanidins for the SKP sample is listed in Table 3. It is mainly composed of three building blocks: catechin dimers (procyanidins B), catechin-gallocatechin dimers, fisetinidin dimers; among these three combinations, the main repeat unit consists of catechins (+288 Da, Fig. 2).

3.2.3. Green tea procyanidins (GTP)

The green tea leaves were characterized by prodelphinidin compounds and flavonol-glycosides (Table 4). According to the procyanidins composition, two series were detected in the MALDI-TOF spectra: the first series was characterised by procyanidin dimers, trimers and tetramers (mostly prodelphinidin), with various combinations of monomeric units. The 467 Da peak was attributed to catechin gallate or robinetinidin gallate, whereas the 628 Da was tentatively attributed to a catechin digallate protonated adduct that might represent a marker for authenticity of the

Table 3
Calculated and experimental MALDI-TOF peaks for procyanidins detected in the SKP sample.

Calculated (M, Da)	Observed (M, Da) Linear positive mode	Attribution
542	544	Fisetinidin dimer
572	572	Catechin-gallocatechin with loss of water
578	581	A-/B- type procyanidins
591	592	Catechin-gallocatechin dimer
604	600	B-type procyanidin, (+Na⁺)
633	634	Gallocatechin-gallocatechin dimer (+Na⁺)
715	713	Catechin gallate+ catechin dimer, with loss in –OH
823	828	Gallocatechin+ catechin+ flavanol fragment, trimer
847	847	Gallocatechin+ catechin+ flavanol fragment, trimer (+Na⁺)
1111	1114	Gallocatechin+ 2*catechin+ flavanol fragment, tetramer
1129	1131	2*gallocatechin+ catechin+ flavanol fragment, tetramer
Oligomers with increase in catechin unit (+288 Da)		
1419	1415	Pentamer
1707	1702	Hexamer
1995	1990	Heptamer
2300	2298	Octamer (+gallocatechin*)

*Octamer, gallocatechin as a possible terminal unit.
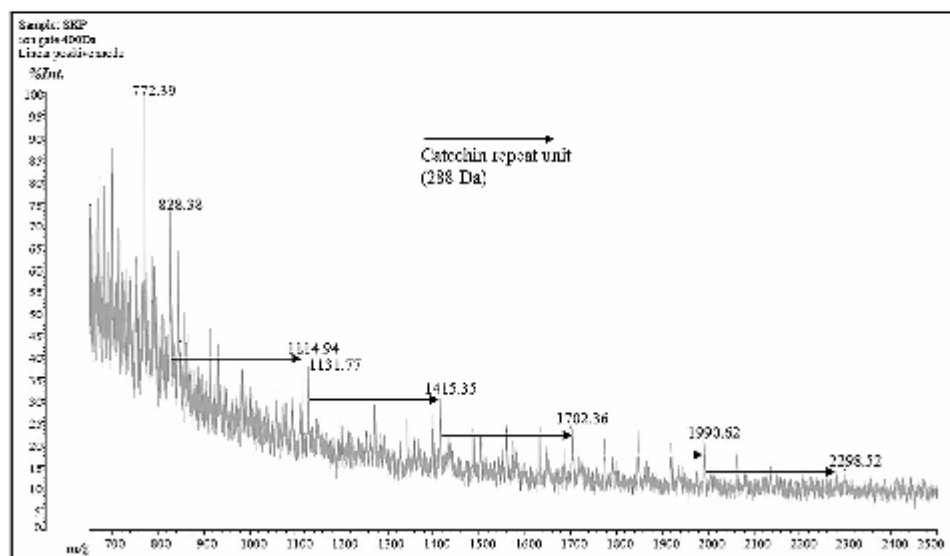
Fig. 2. The MALDI-TOF MS spectrum of sample SKP (linear positive mode, ion gate: 400 Da) recorded in the range 650–2500 Da: procyanidins series (repeat unit: catechins) for the grape skin extract.

extract. Despite the presence of the 574 Da peak – attributed to a procyanidins B-like structure – the oligomers series was mainly characterised by gallate compounds: 735 Da (catechin gallate-fisetinidin, dimer); 889 Da (catechin gallate-fisetinidin gallate, dimer); 903 Da (catechin gallate-catechin gallate, dimer); 1775 Da (3 catechin gallates units-fisetinidin gallate, tetramer). The 1978 Da and 2419 Da were assigned to the same series and were composed of catechin gallate unit linked to higher oligomers.

Remarkably, the mass region of ion peaks of the second series (951-1112-1274-1436-1602-1765-1927-2088-2247-2406), with occasional loss in protonated adducts, was observed with a peak-to-peak mass difference of 162 Da, consistent with the repeating unit of a hexose structure, possibly glucose (Fig. 3). This finding suggested the presence of glycosylated chains, either derived from degradation products of the foliar tissue or related to rearrangement of glucose release during extraction, since

flavonoids in green tea are predominantly present as glycosides rather than non-glycosylated forms (Wang and Helliwell, 2001). As an alternative hypothesis, the use of a sugar-based chain as a stabilising agent was formulated (although not labelled by suppliers).

3.2.4. Limousin oak ellagitannin (LOE)

The Limousin oak heartwood extract was rich in distinctive compounds, such as valoneic acid, polygalloylglucose structures, aldehyde derivatives and ellagitannins. The occurrence of a hydrolysable tannin is unambiguously confirmed by the presence of fragmentation patterns related to the polygalloylglucose structures: the 127 Da peak is related to cleavage mechanisms involving the glucose ring; the 152 Da peak is attributed to galloyl moieties (coupled to the 166 Da peak, due to the presence of gallic acid molecules). Moreover, ellagic acid (305 Da) and hexahydrox-ydiphenic acid (HHDP, 343 Da) monomers were detected in the extract, which are typically found in ellagitannin-based extracts. Several patterns were attributed to the fragmentation of poly-galloyl compounds and sugar chains, which are likely to derive from oak wood following extraction; the basic unit for this series was a glucose dimer (glucose + glucose fragment, 219 Da; sodium adduct: 248 Da). Table 5 provides a list of the fragmentation patterns produced by the Limousin oak tannin, including poly-galloyl glucoses: the gallic acid multiprotonated residues linked as esters, through carboxylic acid moiety, to sugar or gallic acid molecules (Fig. 4). Vescalagin/castalagin (934 Da) were detected as the main constituents of the extract, as confirmed by the 593, 623 and 609 Da peaks attributed to castalin/vescalin fragments (most likely castalin with loss in —OH moieties, and castalagin molecule with loss in an ellagic acid function and a water molecule, respectively). The occurrence of these compounds in the Q. robur extract is consistent with the literature (Nonier et al., 2005). The

Table 4
Calculated and experimental MALDI-TOF peaks for flavonol and flavonol glycosides detected in the GTP sample.

Calculated (M, Da)	Observed (M, Da) Linear positive mode	Attribution
309	309	Kaempferol (+Na⁺)
318	318	Myricetin
325	325	Quercetin (+Na⁺)
448	444	Kaempferol-3-glycoside (astragalin)
464	464	Quercetin-3-glycoside (isoquercetin)
510	510	Myricetin-3-glycoside (+Na⁺)
610	609	Quercetin-3-rutinoside (rutin)
617	617	Kaempferol-3-rutinoside (+Na⁺)
847	847	Quercetin-3-rutinoside (+Na⁺)
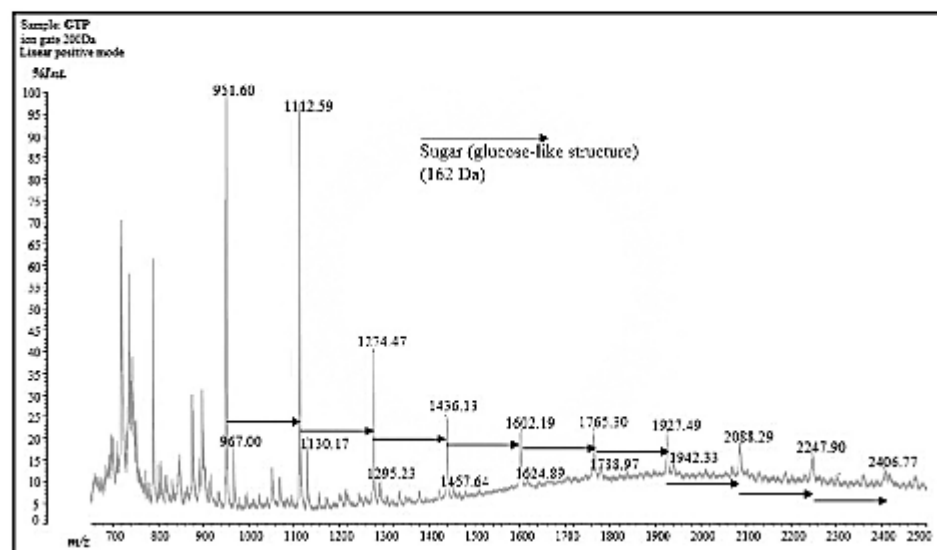
Fig. 3. The MALDI-TOF MS spectrum of sample GTP (linear positive mode, ion gate: 200 Da) recorded in the range 650–2500 Da: glycosylated chain series.

sodium adduct of roburin A/roburin D dimers (1873 Da peak) can be considered a marker for the *Quercus* wood extract.

3.3. Elemental profiling

Besides four macroelements (^{24}Mg, ^{39}K, ^{44}Ca and ^{55}Mn), twelve trace isotopes (^{7}Li, ^{27}Al, ^{52}Cr, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{88}Sr, ^{75}As, ^{138}Ba and ^{207}Pb) were investigated as possible contaminants derived from the botanical source or processing. Overall, the concentrations of macroelements ranged from 0.08 to 0.5 ppm for Mg, and from 0.2 to 0.4 ppm for Ca. The microelements ranged between 0.001–0.01 ppm for Mn, up to 0.99 ppm for Zn, and around 0.0001 ppm for Co (Table 6). Iron and copper were generally present in samples at very low levels (GTP sample: 0.06 ppm ^{56}Fe and 0.04 ppm ^{63}Cu, respectively) which is suitable for beverage application. Cu, Fe and Mn are important catalysts of oxidation of organic substrates (Danilewicz, 2007) and they form stable complexes with polyphenols, affecting the aroma, taste and color of many beverages, including wine (Waterhouse and Laurie, 2006) and beer (Bamforth, 2011).

As, Ni, Cu, and Ba, with respective security limits of 0.01, 0.07, 2.00, and 0.70 ppm recommended by WHO (2004), were far below the legal limits for all samples assayed. Although Cr, Li, Al and Sr are not included in the WHO guidelines, their concentrations lay within the ppb level in the four samples. In particular aluminum, which is likely to induce clouding and decreasing wine stability at increasing pH values (Mrak et al., 1937), was below the limit of quantitation (in the order of ppt) for all samples investigated. The content of Cr, which is crucial in foods due to its toxicity, ranged between 8.2–10.7 ppb, which is far below the ppm levels previously published for tannin extracts (Zmozinski et al., 2015; Mehra et al., 2013).

The Pb levels exceeded the limit set up by WHO for drinking waters (Table 6); the occurrence of the same Pb level in all samples suggested that a possible source of contamination occurred during processing rather than from the botanical sources themselves. Besides the WHO recommendations, several Community regulations have focused on exposure of humans to lead through food, and the SCF suggested a PTWI (provisional tolerable weekly intake) of 25 μg/kg bw (Reports of the Scientific Committee for Food, 32nd

Table 5
MALDI-TOF fragmentation patterns for ellagitannins and polygalloylglucoside compounds detected in the LOE sample.

Calculated (M, Da)	Observed (M, Da) Linear negative mode	Attribution
364	362	Gallic acid dimer + sugar fragment
390	394	Galloyl moiety + sugar + sugar fragment
424	424	Gallic acid dimer + sugar fragment
463	463	Ellagic acid hexoside
493	494	Valoneic acid dilactone (+Na$^+$)
507; 506	506	Digalloyl glucose (+Na$^+$); Valoneic acid
567	566	Gallic acid trimer + sugar fragment (+Na$^+$) loss in two −OH
634	633–634	Galloyl HHDO glucose
1085	1038	Isamer[S2] : 2 gallic acids and 4 hexosyl polysaccharide + glucose residue
1463	1463	Castalagin or pentagalloylglucose linked to a nonahydroxytriphenoic acid (+Na$^+$)
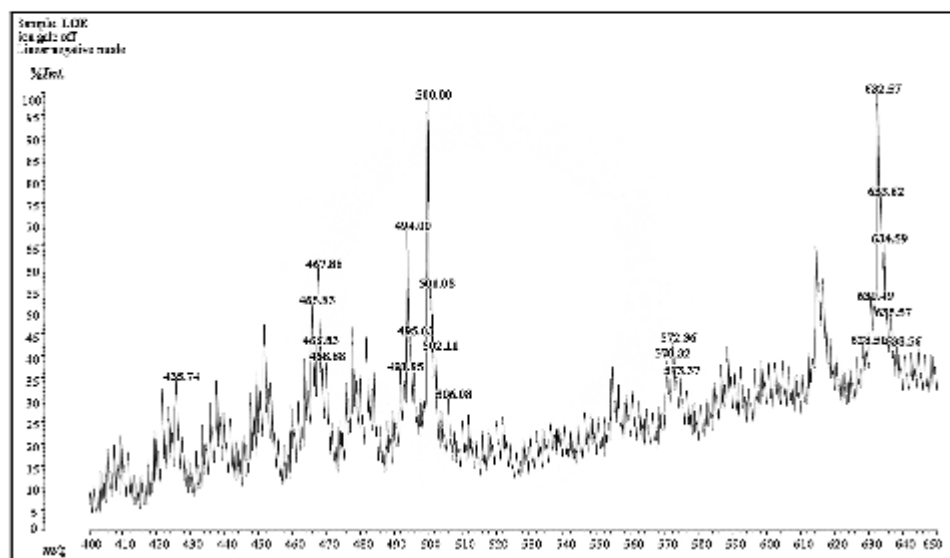
Fig. 4. The MALDI-TOF MS spectrum of sample LOE (linear negative mode, ion gate: off) recorded in the range 400–650 Da showed basic units of ellagitannins and glycosylation patterns (see Table 5).

Table 6
Elemental composition of selected commercial tannins used as food additives; results are provided as mean values (n=3).

Element	Tannin samples (ppm)				(ppm)	(%)	Guideline values (WHO, 2004)
	SEP	SKP	GTP	LOE	LOD	RSD*	
^{7}Li	0.001	0.001	0.002	0.002	8.53E−06	2.44	na
^{24}Mg [He]	0.080	0.090	0.500	0.200	1.34E−04	1.64	na
^{27}Al	nd	nd	nd	nd	1.01E−03	2.48	0.200
^{39}K [He]	1.400	1.200	0.800	0.600	2.31E−04	1.39	na
^{44}Ca [He]	0.200	0.220	0.400	0.400	7.81E−04	2.28	na
^{52}Cr [He]	0.009	0.011	0.008	0.008	9.09E−06	1.89	0.050
^{55}Mn [He]	0.001	0.002	0.010	0.004	5.81E−06	1.96	0.400
^{56}Fe	0.050	0.050	0.060	0.030	9.77E−05	2.28	1.000–3.000
^{59}Co	0.0001	0.0001	0.0001	0.0001	3.30E−07	2.03	na
^{60}Ni	nd	nd	nd	nd	7.95E−05	1.94	0.070
^{63}Cu	0.020	0.020	0.040	0.020	1.35E−05	2.06	2.000
^{66}Zn	nd	nd	0.990	0.250	5.51E−04	1.95	3.000
^{75}As	0.002	0.002	0.002	0.002	1.30E−03	2.52	0.010
^{88}Sr	0.003	0.004	0.020	0.020	2.22E−05	1.96	na
^{137}Ba	0.010	0.010	0.020	0.020	7.02E−04	2.17	0.700
^{207}Pb	0.031	0.033	0.033	0.033	5.74E−05	1.61	0.010

RSD*: Average values for each element. nd: not detected or below the quantitation limit; na: not available. [He]: analysis was performed under He stream conditions.

series, 19 June 1992); in more recent times, the Scientific Committee has stressed the hazard related to metal contaminant intake with a directive that encourage the minimisation of lead levels in food (Reports on tasks for scientific co-operation, task 3.2.11: Assessment of dietary exposure to arsenic, cadmium, lead and mercury of the population of the EU Member States, Directive 93/5/CEE, 2004).

It has to be noticed that the maximum concentration of Pb allowed in wine is 0.3 mg/L according to the International Organization of Vine and Wine (OIV); accordingly, the lead level in the extracts lies far below the safety threshold defined for enological applications (Ivanova-Petropulos et al., 2013). Regardless of the ambiguity in the definition of a common risk threshold for the Pb content, the hypothesis of occasional contaminations occurring along the supply chain would require a detailed investigation in a representative number of samples to be confirmed, and the implementation of purification processes.

4. Conclusions

In this work, the composition of commercially available food-grade tannins was explored to achieve two objectives: (i) identify

markers for the authentication of commercial products according to the declared botanical origin, and (ii) monitor the presence of potentially toxic contaminants. Objective (i) was achieved through the combined use of MALDI-TOF MS and UV–vis spectrophotometric methods which showed variable degree of purity and variable percentage of tannins; in particular, the highest content in polyphenols was reached by the SKP extract (93.3%), followed by LOE > GTP > SEP. The quantitative analysis of polyphenols accounted for the antiradical activity of the extracts, which reflects the nutritional properties of additives. According to the chemical composition of the polyphenolic fraction the main discriminant fingerprints between the plant extracts evaluated as authentication tools were found: the composition in flavonoid and their degree of galloylation for SEP, SKP and GTP samples, and the specific glycosylation patterns and castalagin/vescalagin derivatives occurring in the LOE sample. The GTP sample showed a high variety of molecular fragments attributable to flavonols, which have a high antioxidant power and may legitimize the use of this extract as a food supplement. Recognition patterns were detected for the GTP sample (628 Da protonated fragment) and for the LOE extract (sodium adduct of roburin dimer at 1873 Da); the two molecular fingerprints were suggested as authenticity markers for the tea leaves and *Quercus* wood extracts. In objective (ii), the ICP-MS elemental profile confirmed the levels of potentially toxic contaminants; Pb levels were found to exceed the level suggested by the WHO regulations for drinking water, despite higher concentrations are allowed in other food applications. The present work has highlighted the need for correct production practices along the supply chain, to minimize the occurrence of external contamination sources. To our knowledge, the elemental composition of a Limousin oak food-grade tannin was reported for the first time.

The present work constitute a preliminary study, and will be implemented increasing the number and variety of samples to build a database of commercial products currently available in the market, thus contributing to their proper exploitation in the food industry.

Acknowledgments

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7 Final conclusions

7   Final conclusions

In the Emilia-Romagna region, evidence of 'climate change' was detected during the recent past decades (1961–2015). In general, viticulturists faced a significant increase of daily mean temperatures during the growing seasons in the magnitude up to 1.5°C (depending on the DOP areas) in the last 30 years (1986–2015) comparing to the period 1961–1990. Furthermore, climate conditions in the ER were also drier during the last 30 years in certain DOP zones. Due to high sensitivity of vines to climate conditions, these changes affected grape production and grape quality at a certain level in the ER. In particular, sugar concentration in Sangiovese grapes from the Romagna area has increased up to 1.38 °Brix from 2001 until 2012. Higher berry sugar content in Sangiovese grapes occurred most likely also due to climatological factors (81% probability according to multiple linear regression), such are longer drought periods (DSI) and higher thermal accumulation (HI) during growing seasons. Normally, apart berry sugar concentration other grape quality parameters, such as organic acids concentration, aromatic compounds concentration, phenolic compounds concentration etc., may be also influenced by warmer and drier conditions in certain cases as well. Grape yield could also be affected by the climate change even if not concluded for the Sangiovese grapes from Romagna area (21% probability according to multiple linear regression). Further increase of temperatures and drier conditions comparing to nowadays conditions are expected according to 9 Regional Climate Models simulated based on two potential trajectories of air greenhouses gases concentration until the end of the 21st century (RCP 4.5 and RCP 8.5 scenarios). The magnitude of warming and droughts would depend on the socio-economic development of human society, with logical outcome that scenario with higher pollution and higher emission of greenhouse gases into atmosphere (RCP 8.5 scenario) will cause higher variations in climatological patterns comparing to scenario with reduced pollution and emission of greenhouse gases into atmosphere (RCP 4.5 scenario). In any case scenario (RCP 4.5 and RCP 8.5 scenarios), according to models simulations until 2040 the majority of DOP zones in the ER should be still suitable for production of high-quality grapes, at least for the later ripening varieties (e.g. currently produced Sangiovese) while production of high-quality white grape varieties would be questionable (e.g. currently produced Chardonnay). On the other hand, toward the end of the 21st century mean growing season temperature could rise even above 22°C in certain areas of the ER. This could be particularly possible under RCP 8.5 scenario where most of the ER DOP zones could be characterized as 'too hot' (mean growing season temperature above 22°C) suggesting that production of high-quality grapes would be highly questionable. Therefore, to enable production of high-quality grapes and wines (at least until 2040) certain techniques need to be applied to moderate the impact of upcoming warming and drier conditions. Logically, mitigation techniques may 'correct' grape and wine quality up to a certain limit point. In particular, a combined method of 'early *green* harvest' and *non-Saccharomyces* may be used to reduce excessive alcohol in wine (~1.20% v/v alcohol removed in Chardonnay wines during vintage 2016) and to increase wine total acidity (~2.5 g/L total acidity increased Chardonnay wines during vintage 2016). However, certain mitigation technologies (a combined method of 'early *green* harvest' and *non-Saccharomyces*) may also cause a negative 'side effects' (too bitter and too acidic wines), thus development of reliable mitigation techniques which are causing minor (or not at all) negative 'side effects' should be the direction of wine industry development. A future research should particularly direct towards the development of a combined method with two or more techniques involved since at real industry level, the proper solution is a combination of different techniques most of the time.

Appendix J – Utilization of sage by-products as raw material for antioxidants recovery -Ultrasound versus microwave-assisted extraction

Utilization of sage by-products as raw material for antioxidants recovery—Ultrasound versus microwave-assisted extraction

CrossMark

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ABSTRACT

In this work, sage (Salvia officinalis L.) by-products from filter tea factory, i.e. sage herbal dust, was valorized as raw material for extraction of phenolic antioxidants. Ultrasound-assisted (UAE) and microwave-assisted extraction (MAE) of polyphenols from sage herbal dust were separately optimized by simultaneous maximization of total phenols (TP) and total flavonoids (TF) yields. Box-Behnken experimental design and response surface methodology (RSM) was used for extraction optimization. In case of UAE, temperature (40, 60 and 80 °C), extraction time (40, 60 and 80 min) and ultrasonic power (24, 42 and 60 W/L) were independent variables, while optimized MAE parameters were ethanol concentration (40, 60 and 80%), extraction time (10, 20 and 30 min) and liquid to solid ratio (20, 30 and 40 mL/g). Antioxidant activity of sage extracts was determined by DPPH•, FRAP and superoxide anion radical neutralization assays, and good correlation between polyphenols content and antioxidant activity was observed. According to results, it could be concluded that novel extraction techniques (UAE and MAE) provided significant advantages for recovery of sage polyphenols comparing to traditional methods.

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1. Introduction

Sage (Salvia officinalis L.) is a valuable medicinal plant from Lamiaceae family, which has been recognized for many medicinal plants with designated radical scavenger activity (Babovic et al., 2010). Traditionally, it has been widely used as herbal tea, spice and food flavouring agent, while industrially it found application as fragrance agent in cosmetics, perfumery and pharmaceutical industry. Up to date, sage has been utilized in production of various pharmaceutical formulations, due to wide range of biological activities, namely: antimicrobial (Bozin et al., 2007), preservative (Hayouni et al., 2008), immunomodulatory (Capek and Hřibalová, 2004), antioxidant (Babovic et al., 2010) and anticancer (Sertel et al., 2011) properties. Different functional preparations have been prepared from sage such as essential oil, and both lipophilic and hydrophilic extracts. Content of volatile fraction in sage, isolated by, either, solvent extraction, hydrodistillation, supercritical fluid extraction or subcritical water extraction, varies from 0.7 to 5.2% (Glisic et al., 2010), while α-thujone, β-thujone and camphor are its most abundant terpenoid compounds (Aleksovski and Sovová, 2007). Moreover, sage represents a good source of polyphenols, particularly phenolic acids derivatives (rosmarinic, carnosic, caffeic, ferulic, cinnamic and chlorogenic acid) (Hossain et al., 2010; Dragovic-Uzelac et al., 2012) and certain flavonoids (Roby et al., 2013), which has been recognized as bioactive compounds with high antioxidant activity.

Nowadays, people are returning to the use of traditional herbal preparations, rather than synthetic drugs, for treatment of various medicinal conditions. Therefore, herbal tea is rapidly becoming more and more popular beverage worldwide. During the production of filter tea, plant material is subjected to various unit operations, such as drying, cutting, grinding, fractionation, etc., described in details by Vidović et al. (2013). During hammer mill grinding, certain amount of fine powder (approx. 20%) is being produced, which has been recognized as herbal dust and has been considered as by-product. This fraction could not be used for filter tea packing since its particle size (<0.315 mm) is lower that filter pore size, therefore, this fraction is usually being discarded

from the factory as by-product (Ramić et al., 2015). Although, processing of plant material during the production of filter tea causes loss of bioactive compounds (essential oil, polyphenols, ascorbic acid, etc.) (Vidović et al., 2013), this material still possesses certain amount of health benefit compounds and could be potentially utilized as raw material for extraction. Volatile compounds from essential oils rather easily evaporate from the herbal dust, however, significant amount of low-volatile compounds such as polyphenols is being retained in plant matrix. Moreover, solid–liquid extraction from herbal dust occurs rather quickly due to particularly low mass-transfer limitations, since particle size of plant material is rather small. Ultrasound and microwave-assisted extraction of polyphenols from tea factory by-products, i.e. herbal dust, has been recently performed from various plant materials such as horsetail (Milutinović et al., 2014), yarrow (Milutinović et al., 2015) and chokeberry (Ramić et al., 2015). The most commonly used filter tea in Balkan countries is being produced from aromatic medicinal plants (chamomile, mint, lemon balm, sage, etc.), or fruit (apple, rose hip, etc.), and amount of herbal dust generated from these plants is particularly high. Therefore, there is an initiative to valorize utilization of herbal dust as raw material for extraction of various bioactive compounds.

Classical extraction techniques commonly used for chemical standardization of botanicals and herbal preparations have been overcome by emerging green extraction technologies such as ultrasound-assisted (UAE), microwave-assisted (MAE) and pulsed-electric fields assisted extraction, as well as, high pressure techniques such as accelerated-solvent (ASE), subcritical water (SWE) and supercritical fluid extraction (SFE) (Heng et al., 2013). All these techniques have the same goals: 1) increase of yield of target compounds, 2) reduction of time, solvent and energy consumption, 3) minimization of environmental impact using green solvents. UAE and MAE have been particularly useful for extraction of polyphenols from various plant materials (Chan et al., 2011; Roselló-Soto et al., 2015). Recently, MAE has been utilized for the recovery of polyphenols from sage (Putnik et al., 2016). According to Tiwari (2015), the most important parameters influencing UAE are ultrasonic power and frequency, temperature, extraction time, matrix and solvent properties. Whilst, solvent polarity (dielectric constant), extraction time, irradiation power, temperature and contact surface area have been recognized as the main MAE parameters affecting the polyphenols extraction (Routray and Orsat, 2012).

In this work, the most important UAE and MAE parameters affecting the polyphenols extraction from sage herbal dust have been identified and these extraction techniques have been optimized separately. Response surface methodology (RSM) was used for optimization of UAE and MAE of polyphenols extraction from sage herbal dust, which was valorized for utilization as raw material for extraction of polyphenols. Influence of UAE (temperature, extraction time and ultrasonic power) and MAE (ethanol concentration, extraction time and solid to liquid ratio) parameters on sage polyphenols extraction was evaluated by RSM influence analysis. Moreover, antioxidant activity of obtained extracts was evaluated by DPPH•, FRAP and superoxide anion radical neutralization assays.

2. Materials and methods

2.1. Plant material

Sage (Salvia officinalis L.) originated from Montenegro was kindly donated by domestic filter tea factory, Fructus (Bačka Palanka, Serbia). Dry plant material was subjected to processing in the filter tea factory and production of herbal dust (by-product) has been described elsewhere (Ramić et al., 2015). Herbal dust fraction with <0.315 mm mean particle size was discarded as by-product, was used as raw material for present study.

2.2. Chemicals

Following reagents were purchased from Sigma-Aldrich Chem, Steinheim, Germany: Folin-Ciocalteu reagent, (±)-catechin, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3,5-di-tert-butyl-4-hydroxytoluene (BHT), phenazine methosulfate (PMS), 2,3,5-triphenyltetrazolium chloride (TPTZ, Tetrazolium Red) and β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetra-sodium salt hydrate (NADH). Nitroblue tetrazolium (NBT) was purchased from Alfa Aesar (Karlsruhe, Germany), while ferric chloride-6-hydrate and ascorbic acid were purchased from Centrohem (Stara Pazova, Serbia). All other reagents used in this study were of analytical reagent grade.

2.3. Conventional solid–liquid extraction (CE)

Conventional solid–liquid extraction was performed in order to determine experimental domain for the ethanol concentration used in optimization study and to compare yield of polyphenols obtained by conventional and novel extraction techniques. In each experimental run, 5.0 g of sample was extracted with different solvent (100 mL). Extractions were performed at room temperature for 24 h with 150 rpm shaking speed. Water and following mixtures of water and ethanol were used: 20%, 40% 60%, 80% and 96% ethanol. After extraction, obtained extracts were filtrated through filter paper. Extracts were collected into glass vials and stored at 4 °C prior analysis.

2.4. Novel extraction techniques

2.4.1. Ultrasound-assisted extraction (UAE)

In all UAE experimental runs, 5.0 g of sample was mixed with 100 mL of 60% ethanol in 250 mL glass flasks. Ultrasound-assisted extraction was performed in sonication water bath (EUP540A, Euinstruments, France) with frequency fixed at 40 kHz. Temperature (40, 60 and 80 °C), extraction time (40, 60 and 80 min) and ultra-sonic power (24, 42 and 60 W/L) were independent variables which were set by the control panel of the instrument. In order to prevent evaporation of the extraction solvent, condenser was added on the flask during extraction. Flasks were always positioned in the position of the ultrasonic bath in order to provide constant ultrasonic power. After extraction, extracts were filtered through filter paper, collected into glass vials, sealed and stored at 4 °C prior analysis.

2.4.2. Microwave-assisted extraction (MAE)

Mono-mode microwave-assisted extraction (MAE) was performed in experimental setup described in details by Zeković et al. (2016). In all MAE experimental runs, certain mass of sage herbal dust (depending on applied liquid to solid ratio) was mixed with 100 mL of extraction solvent (40, 60 and 80% ethanol) in 250 mL round glass flasks. Selection of experimental domain for the ethanol concentration was based on results of CE, and optimal ethanol concentration obtained in CE was chosen as the middle level of this variable for MAE. Extractions were performed at fixed microwave frequency and irradiation power (600 W), always positioned at the same distance from the magnetron. Ethanol concentration (40, 60 and 80%), extraction time (10, 20 and 30 min) and liquid to solid ratio (20, 30 and 40 mL/g) were independent variables. After the extraction, crude extracts were filtered through filter paper under vacuum, collected into glass vials, sealed and stored at 4 °C prior analysis.

2.5. Total phenols content (TP)

Total phenols content (TP) in all liquid extracts, obtained by both conventional and novel (UAE and MAE) extraction techniques was determined using Folin–Ciocalteu procedure (Singleton and Rossi, 1965). Gallic acid was used as standard polyphenol compound for standard diagram, and absorbances were measured at 750 nm (6300 Spectrophotometer, Jenway, UK). Content of total polyphenols was expressed as grams of gallic acid equivalents (GAE) per 100 g of sample dry weight (DW). All experiments were performed in triplicate, and results were expressed as mean values.

2.6. Total flavonoids content (TF)

Total flavonoids content was determined using aluminum chloride colorimetric assay (Harborne, 1984). Standard diagram was obtained using catechin as flavonoid compound and absorbances were measured at 510 nm. Results were expressed as grams of catechin equivalents (CE) per 100 g DW. All experiments were performed in triplicate, and results were expressed as mean values.

2.7. Antioxidant activity of sage extracts

2.7.1. Reduction of DPPH radical
The ability of the plant extracts to scavenge the DPPH radical was tested using a previously described, customized method for 96-well microplates (Soler-Rivas et al., 2000; Beara et al., 2009). Briefly, ten microliters of examined extract solutions, in series of different concentrations, were added to 100 μL of 90 μmol/L DPPH solution in methanol, and the mixture was diluted with 190 μL of methanol. In the control, the exact amount of extract was substituted with solvent, and in the blank probe, only methanol (290 μL) and extract (10 μL) were mixed. The absorbance was measured after 1 h at 515 nm. Well known synthetic antioxidants, PG (propyl gallate) and BHT (butylated hydroxytoluene) were used as a positive control. All samples and the control were done in triplicate and results were expressed as mean ± standard deviation. Results of the antioxidant potential were expressed as the concentration of plant extract which decreased the initial DPPH• concentration by 50% (IC_{50}; μg/mL).

2.7.2. FRAP assay
To evaluate the reducing power of extracts, the ferric ion reducing antioxidant power (FRAP) assay (Benzie and Strain, 1996), modified for 96-well microplates (Lesjak et al., 2011), was conducted. Extracts were prepared in series of different concentrations, whereas the ascorbic acid ranging from 1.25 to 140 μg/mL was used to create a standard curve. FRAP reagent was prepared by mixing 10 mmol/L 2,4,6-tripyridil-s-triazine (TPTZ) in 40 mmol/L HCl, 0.02 mol/L $FeCl_3$, and acetate buffer, pH 3.6, in ratio of 1:1:10, respectively. Following the addition of the extract or ascorbic acid (10 μL) to 290 μL of FRAP reagent (substituted with distilled water in the blank probe), the absorbance was measured at 593 nm after 6 min. All samples were made in triplicate and mean values of reducing power were expressed as milligrams of ascorbic acid equivalents (AAE) per gram of DW, calculated according to the standard calibration curve.

2.7.3. Superoxide anion scavenger capacity
The potential of the extracts to neutralize superoxide anion radical formed by reduction of NBT with NADH mediated by PMS, under aerobic conditions, was conducted according to the method of Nishikimi et al. (1972), adapted for 96-well microplates. The mixture of 50 μL NBT (144 μmol/L), 10 μL extract (concentrations ranging from 0.625 to 5.00 mg/mL for UAE extracts and from 0.15 to 10.0 mg/mL for MAE extracts), 20 μL NADH (0.68 mmol/L) and

20 μL of freshly prepared PMS (60 μmol/L) was diluted with 220 μL of phosphate buffer pH 8.3. In the control, the extract was substituted with the solvent, while the blank probe was prepared by mixing 310 μL of buffer and 10 μL of extract. The absorbance was measured at 560 nm after 5 min. All samples and the control were done in triplicate and the result was presented as the IC_{50} value (μg/mL).

2.8. Design of experiments

The RSM was applied to evaluate the effects of extraction parameters and optimize extraction conditions TP and TF as investigated responses. Box-Behnken experimental design with three numeric factors at three levels was used for both UAE and MAE. Design consisted of seventeen randomized runs with five replicates at the central point. Investigated UAE variables used in present experimental design were temperature (40, 60 and 80 °C), extraction time (40, 60 and 80 min) and ultrasonic power (24, 42 and 60 W/L). Whilst investigated independent MAE parameters were ethanol concentration (40, 60 and 80%), extraction time (10, 20 and 30 min) and liquid to solid ratio (20, 30 and 40 mL/g). Each of the coded variables was forced to range from −1 to 1, in order to normalize parameters so the units of the parameters are irrelevant (Baş and Boyacı, 2007). The natural and coded values of independent UAE and MAE variables used in BBD are presented in Tables 1 and 2, respectively. The response variables were fitted to the commonly used second-order polynomial model (Eq. (1)) (Ferreira et al., 2007; Leardi, 2009):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i<j=1}^{3} \beta_{ij} X_i X_j \tag{1}$$

where: Y represents the response variable, X_i and X_j are the independent variables affecting the response, and β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for mean, linear, quadratic and cross-product terms. Optimal extraction conditions were determined considering total phenols and total flavonoids content, while selection of optimal conditions were based on desirability function, D (Derringer, 1980). Design-Expert v.7 Trial (Stat-Ease, Minneapolis, Minnesota, USA) was used for multiple linear regression analysis. The results were statistically tested by analysis of variance (ANOVA), while model adequacy was evaluated by the coefficient of determination (R^2), coefficient of variance (CV) and p-values for the model and lack of fit. In order to verify obtained empirical models, validation was performed by extracts preparation at optimized UAE and MAE conditions. Confidence interval (95%) of predicted values was compared with experimentally observed TP and TF in optimized extracts.

2.9. HPLC analysis of phenolic acids

HPLC system (Dionex DX500, Milano, Italy) equipped with temperature control oven (30 °C), photodiode array detector (DAD), Chromeleon chromatography manager software v. 6.60 SP2 and an Aquapore ODS-300 RP-C18 column (250 × 4.6 mm; 7 μm particle size; Applied Biosystems, San Jose, CA, USA) was used for identification and quantification of phenolic acids according to Ivanova-Petropulos et al. (2015). The standards and samples were filtered using 0.20 μm cellulose acetate membrane (GVS Filter Technology, Indianapolis, USA) before manual injection into the HPLC system. Mobile phases were water/formic acid (98/2, v/v, solvent A) and acetonitrile/water/formic acid (80/18/2, v/v/v, solvent B) at flow rate of 0.5 mL/min. The proportions of solvent B were: 0–50 min, 9%; 65–70 min, 10%; 77 min, 30%; 80–97 min, 0%. Caffeic,

Table 1
Box-Behnken experimental design with natural and coded parameters of ultrasound-assisted extraction and experimentally observed and predicted values of total phenols content (TP) and total flavonoids content (TF).

Run[a]	Independent variables						Responses			
	Temperature [°C]		Extraction time [min]		Ultrasonic power [W/L]		TP [g GAE/100 g DW]		TF [g CE/100 g DW]	
	coded	natural	coded	natural	coded	natural	Experimental	Predicted	Experimental	Predicted
1	0	60	0	60	0	42	9.061	9.247	6.510	6.375
2	0	60	−1	40	1	60	8.713	8.729	6.560	6.458
3	1	80	−1	40	0	42	8.926	9.064	6.617	6.693
4	0	60	0	60	0	42	8.904	9.247	6.345	6.375
5	0	60	1	80	1	60	9.308	9.347	6.768	6.764
6	−1	40	1	80	0	42	7.230	7.093	5.464	5.387
7	1	80	0	60	1	60	9.195	9.041	6.739	6.765
8	−1	40	−1	40	0	42	6.893	6.778	5.112	5.135
9	0	60	0	60	0	42	9.386	9.247	6.488	6.375
10	−1	40	0	60	1	60	6.848	6.947	5.241	5.321
11	0	60	0	60	0	42	8.982	9.247	6.223	6.375
12	0	60	−1	40	−1	24	8.690	8.651	6.302	6.306
13	1	80	0	60	−1	24	9.319	9.221	6.875	6.795
14	0	60	1	80	−1	24	9.049	9.033	6.524	6.626
15	−1	40	0	60	−1	24	6.220	6.740	5.027	5.001
16	1	80	1	80	0	42	9.633	9.746	7.090	7.068
17	0	60	0	60	0	42	9.903	9.247	6.309	6.375

[a] randomized.

Table 2
Box-Behnken experimental design with natural and coded parameters of microwave-assisted extraction and experimentally observed and predicted values of total phenols content (TP) and total flavonoids content (TF).

Run[a]	Independent variables						Responses			
	Ethanol concentration [%]		Extraction time [min]		Liquid/solid ratio [mL/g]		TP [g GAE/100 g DW]		TF [g CE/100 g DW]	
	coded	natural	coded	natural	coded	natural	Experimental	Predicted	Experimental	Predicted
1	0	60	0	20	0	30	9.642	9.750	6.997	7.253
2	0	60	0	20	0	30	9.440	9.750	7.312	7.253
3	−1	40	1	30	0	30	9.946	9.740	7.366	7.264
4	0	60	0	20	0	30	9.845	9.750	7.377	7.253
5	0	60	0	20	0	30	10.283	9.750	7.334	7.253
6	−1	40	−1	10	0	30	8.597	8.934	7.366	7.235
7	0	60	1	30	−1	20	9.757	9.902	7.312	7.193
8	1	80	−1	10	0	30	7.012	7.217	5.857	5.959
9	1	80	0	20	1	40	8.217	8.156	6.886	6.665
10	1	80	0	20	−1	20	7.747	7.939	6.280	6.269
11	0	60	−1	10	−1	20	9.252	8.854	6.187	6.097
12	0	60	1	30	1	40	9.407	9.805	7.087	7.177
13	−1	40	0	20	1	40	10.126	9.934	7.803	7.815
14	−1	40	0	20	−1	20	9.667	9.728	6.667	6.889
15	0	60	−1	10	1	40	9.519	9.374	7.316	7.435
16	0	60	0	20	0	30	9.541	9.750	7.247	7.253
17	1	80	1	30	0	30	8.226	7.889	6.639	6.770

[a] randomized.

ferulic and rosmarinic acids were quantified at 256 nm, whereas p-coumaric acid was quantified at 308 nm.

3. Results and discussion

3.1. Model adequacy

Results of the experimentally obtained values of the total phenols (TP) and total flavonoids (TF) content obtained by ultrasound-assisted (UAE) and microwave-assisted (MAE) extraction, presented in Tables 1 and 2, respectively, have been fitted to quadratic polynomial model (Eq. (1)). Analysis of variance (ANOVA) was employed in order to check adequacy of the applied models and p-values of regression coefficients for each investigated response are summarized in Table 3. Coefficient of multiple determination (R^2) was used as first indicator of model fitness. According to particularly high R^2 for TP and TF obtained by UAE (0.958 and 0.984, respectively), it has been suggested that applied second-order polynomial model represents good approximation of experimen-

tal results. Similar situation was with TP and TF obtained by MAE, where observed R^2 were 0.910 and 0.932, respectively. Coefficient of variance (CV) was observed as another descriptive statistics parameter. In case of UAE, observed CV of applied models for TP and TF were 3.95 and 1.98%, while in case of MAE, these values were slightly higher (4.50 and 2.90%, respectively). According to reasonably low CV (<10%) for all responses, good reproducibility of the investigated systems was suggested, since CV describes dispersion of the data and small values indicates low variation in the mean value. Since, descriptive statistics often does not often provide sufficient data of the model adequacy, ANOVA, i.e. decision making statistics, should be applied. Therefore, model adequacy was determined by Fisher's test (F-test) for the model and lack of fit (Table 3). Good adequacy of the models indicated by descriptive statistics was confirmed by highly significant p-values (<0.01) for all applied models (Table 3). Proper model fitness has been also confirmed by insignificant lack of fit (p> 0.05) for all models. This means that dispersion of experimental results was model-independent measure of the pure error (Myers et al., 2009). Therefore, according to

Table 3
Analysis of the variance (ANOVA) of the fitted second-order polynomial models.

Ultrasound–assisted extraction		Microwave–assisted extraction	
Total phenols content			
Source	p-value	Source	p-value
Model	0.0005*	Model	0.0064*
X_1–Temperature	<0.0001*	X_1–Ethanol concentration	0.0005*
X_2–Extraction time	0.0760***	X_2–Extraction time	0.0393**
X_3–Ultrasonic power	0.4402	X_3–Liquid/solid ratio	0.4925
X_1X_2	0.6023	X_1X_2	0.8749
X_1X_3	0.3046	X_1X_3	0.9895
X_2X_3	0.7387	X_2X_3	0.4792
X_1^2	0.0004*	X_1^2	0.0025*
X_2^2	0.9257	X_2^2	0.1009
X_3^2	0.1220	X_3^2	0.5894
Lack of fit	0.8463	Lack of fit	0.2245
Total flavonoids content			
Model	<0.0001*	Model	0.0025*
X_1–Temperature	<0.0001*	X_1–Ethanol concentration	0.0005*
X_2–Extraction time	0.0089*	X_2–Extraction time	0.0224**
X_3–Ultrasonic power	0.1406	X_3–Liquid/solid ratio	0.0025*
X_1X_2	0.6371	X_1X_2	0.0959
X_1X_3	0.1983	X_1X_3	0.2332
X_2X_3	0.9554	X_2X_3	0.0126**
X_1^2	0.0002*	X_1^2	0.0361**
X_2^2	0.0647***	X_2^2	0.0966***
X_3^2	0.6167	X_3^2	0.4044
Lack of fit	0.4561	Lack of fit	0.1647

* highly significant ($p < 0.01$).
** significant ($0.01 < p < 0.05$).
*** moderately significant ($0.05 < p < 0.10$).

ANOVA and descriptive statistics, applied quadratic model (Eq. (1)) represented good approximation of experimental results for both UAE and MAE. Method of least square (MLS) was used for calculation of regression coefficients in Eq. (1), which provided descriptive model equations (Eqs. (2)–(5)) for TP and TF, obtained by UAE and MAE, respectively:

- Ultrasound-assisted extraction:

$$TP = 9.25 + 1.24X_1 + 0.25X_2 - 1.06X_1^2 \tag{2}$$

$$TF = 6.38 + 10.81X_1 + 0.16X_2 - 0.44X_1^2 + 0.13X_2^2 \tag{3}$$

- Microwave-assisted extraction:

$$TP = 9.75 - 0.89X_1 + 0.37X_2 - 0.92X_1^2 \tag{4}$$

$$TF = 7.25 - 0.44X_1 + 0.21X_2 + 0.33X_3 - 0.34X_2X_3 - 0.26X_1^2 + 0.19X_2^2 \tag{5}$$

where: X_1, X_2 and X_3 are temperature, extraction time and ultrasonic power in case of UAE, and ethanol concentration, extraction time and solid to liquid ratio in case of MAE. Predictive model equations (Eqs. (2)–(5)) represented reduced Eq. (1), since coefficients of variables with insignificant influence could be neglected.

3.2. Total phenols content (TP)

Results of TP in extracts obtained by conventional solid–liquid extraction (CE) are presented in Fig. 1. TP varied between 1.761 and 5.872 g GAE/100 g DW, depending on the proportion of water and ethanol in extraction solvent. The highest TP was obtained using 60% ethanol as extraction solvent, suggesting that this solvent would be suitable for UAE. On the other hand, TP observed in sage extracts obtained under different UAE conditions (temperature, extraction time and ultrasonic power) was between 6.220 and 9.903 g GAE/100 g DW. It has been known that *Salvia officinalis* L. represents a good source of polyphenols, however, particularly high TP obtained by both UAE and MAE showed that sage herbal dust
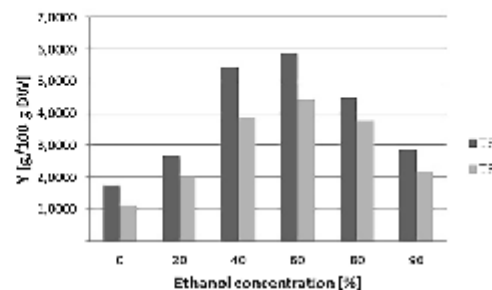
Fig. 1. Yield of polyphenols compounds (TP and TF) observed in extracts obtained by conventional solid–liquid extraction.

could be potentially utilized as raw material for polyphenols extraction. In case of UAE, the lowest TP was observed at temperature of 40 °C, extraction time of 60 min and ultrasonic power of 24 W/L, while the highest TP was obtained at central point (run 17; middle level of UAE parameters) at temperature of 80 °C, extraction time of 60 min and ultrasonic power of 24 W/L. All UAE extracts obtained at 40 °C had significantly lower TP (6.220–7.230 g GAE/100 g DW), comparing to extracts obtained at 60 and 80 °C (>8.5 g GAE/100 g DW) (Table 1). This indicated that temperature could be designated as the most influential UAE parameter. Roby et al. (2013) determined TP in sage extracts obtained by maceration using methanol, ethanol, diethyl ether and hexane as extraction solvents, and the highest TP was obtained using ethanol and methanol as solvents (5.80 and 5.95 mg GAE/g dry extract (DE)). Farhat et al. (2013) reported that geographical location significantly influences TP in methanolic *S. officinalis* extracts (approx. 10–16 g GAE/100 g DW), however, the same authors investigated only sage from different sites in Tunisia, while sample used in this work, originated from Montenegro. Therefore, when comparing polyphenolic content of one plant species, one must have in mind that different cultivars,

geographical origin and cultivation could have significant effect on TP, besides applied extraction solvent and technique. Hossain et al. (2010) applied pressurized-liquid extraction using methanol-water mixtures (32–88%) as extraction solvent in order to maximize TP from sage and reported that 60% methanol and increased temperature (129 °C) significantly increase TP (6.16 g GAE/100 g DW).

In case of MAE, TP varied between 7.747 and 10.283 g GAE/100 g DW, depending on applied extraction conditions (ethanol concentration, extraction time and solid to liquid ratio). It could be seen that both UAE and MAE provided significant increase of TP yield, comparing to CE. The highest TP obtained by MAE was observed in sample 5, which was obtained at central point (60% ethanol, 20 min of extraction time and 30 mL/g of liquid to solid ratio). Comparing maximal TP obtained by UAE and MAE, it could be seen that MAE provides slightly higher TP, however, the main difference in applied conditions could be higher liquid to solid ratio used in MAE (20–40 mL/g), comparing to UAE (20 mL/g). Higher liquid to solid ratio increases concentration gradient between solid and liquid phase, therefore, it is rather expected that TP would similarly increase, if higher liquid to solid ratio was applied in UAE. Dragovic-Uzelac et al. (2012) performed MAE of sage on similar extraction conditions and the highest TP (4.70 g rosmarinic acid equivalents (RAE)/100 g DW) was observed after 9 min of extraction, using 500 W of irradiation power and 30% ethanol as extraction solvent. On the other hand, Dent et al. (2013) investigated influence of ethanol concentration, extraction time and temperature, and reported that extraction with 30% ethanol at 60 °C for 30 min provided the highest TP (6.278 g RAE/100 g DW). Chemical profile of sage polyphenols have been investigated in detail and it has been reported that phenolic acid derivatives (rosmarinic, carnosic, caffeic, ferulic, cinnamic and chlorogenic acid) and certain flavonoids (luteolin and apigenin) were the most abundant compounds (Hossain et al., 2010; Dragovic-Uzelac et al., 2012; Dent et al., 2013; Roby et al., 2013).

Durling et al. (2007) reported that ethanol-water mixtures (55–75% ethanol) is the most suitable solvents for simultaneous extraction of the most important phytochemicals from sage (rosmarinic acid, carnosic acid and essential oil), therefore, 60% ethanol was used as extraction solvent in all UAE runs in present work. Effects of UAE and MAE parameters on TP has been presented on Fig. 2, while their significance was determined by RSM influence analysis expressed as p-value (Table 3). In case of UAE, linear and quadratic terms of temperature exhibited highly significant influence ($p < 0.01$), while linear term of extraction time exhibited moderately significant influence on TP (Table 3). Positive influence of liner term of temperature was the most noticeable effect, which is rather expected, since temperature directly affects mass transfer increasing diffusion, causing degradation of the plant matrix and improving physical solvent properties in terms of penetration and solubility power (Ramić et al., 2015). This was in accordance with previously reported works investigated UAE of polyphenols from different plant samples, but in similar experimental domain (Ramić et al., 2015; Tomšik et al., 2016). According to Eq. (2), quadratic term of temperature exhibited significant negative influence, meaning that TP will increase with increase of temperature and after certain point (70–75 °C), it will start to decrease with further temperature increase, probably due to degradation of polyphenols at increased temperature and extraction time. Moderately significant influence of extractions time causes slight, but constant increase of TP with increase of this variable in investigated experimental domain. This was in accordance with study of UAE kinetics of total extractive substances from sage, since rapid extraction of solutes occurs in the first ten minutes of extraction and further increase of extraction time (up to 80 min) causes slight increase in extraction yield (Veličković et al., 2006).

In case of MAE, linear and quadratic terms of ethanol concentration exhibited highly significant influence on TP, while linear influence of extraction time was significant (Table 3). Linear term of ethanol concentration exhibited negative influence on TP, according to Eq. (4). However, influence of quadratic term of ethanol concentration was also negative, meaning that TP will start to decrease ethanol concentration is too low (40%), suggesting that optimal ethanol concentration for TP extraction will be higher than 40%, but still near that value. Again, positive influence of linear extraction time was observed, as it was the case with UAE, however, it has been reported that TP would start to decrease if prolonged MAE was applied (Dragovic-Uzelac et al., 2012; Dent et al., 2013). Results obtained in this work were in accordance with previous claim since MAE sample obtained using 60% ethanol and 40 mL/g liquid to solid ratio at 10 min of extraction time (Run 15) was slightly higher than TP in sample obtained using same ethanol concentration and liquid to solid ratio for 30 min of extraction time (Run 12) (Table 2). Dent et al. (2013) reported that 30% ethanol provides significantly higher TP in sage extracts obtained by MAE, comparing to 50% ethanol, which was in accordance with results from present work (Table 2).

3.3. Total flavonoids content (TF)

Experimentally obtained values of TF observed in sage extracts obtained by UAE and MAE are presented in Tables 1 and 2, respectively, while results of TF obtained by CE were presented in Fig. 1. Application of water as extraction solvent in CE provided the lowest TF (1.112 g CE/100 g DW), while 60% ethanol provided the highest TF (4.452 g CE/100 g DW), as it was the case with TP. TF in sage extracts obtained by UAE was between 5.027 and 7.090 g CE/100 g DW. The same UAE conditions that provided the lowest TP (temperature of 40 °C, extraction time of 60 min and ultrasonic power of 24 W/L; run 15) also provided the lowest TF yield, suggesting good correlation between influence of UAE parameters and TP and TF, respectively. Again, relatively low TF (<5.464 g CE/100 g DW) was observed when UAE were performed at 40 °C, as it was the case with TP, meaning that temperature would be crucial UAE parameter affecting TF (Table 1). On the other hand, the highest TF was obtained at temperature of 80 °C, extraction time of 80 min and ultrasonic power of 42 W/L (Table 1). Gird et al. (2014) reported that TF in sage extracts obtained by heating in reflux with 70% ethanol were between 1.1323 and 2.0994 g rutin equivalents (RE)/100 g DW, and flavonoids content significantly varies with time of harvest. According to Veličković et al. (2006), TF in sage extracts obtained by UAE using methanol and 70% ethanol as extraction solvent were 59.6 and 63.2 mg rutin equivalents (RE)/g dry extract. According to results obtained in present work, UAE significantly increases TF yield which was in accordance with previous reports (Ramić et al., 2015; Tomšik et al., 2016), however, Veličković et al. (2006) found no significant difference in TF observed in sage extracts obtained by classical extraction and UAE.

TF observed in MAE extracts was between 5.857 g CE/100 g DW, obtained with 80% ethanol, 10 min of extraction time and 30 mL/g of liquid to solid ratio, and 7.803 g CE/100 g DW obtained with 40% ethanol, 20 min of extraction time and 40 mL/g of liquid to solid ratio (Table 2). Again, novel extraction techniques (UAE and MAE) provided significant advantages in terms of yield of polyphenols compounds, comparing to CE. Dragovic-Uzelac et al. (2012) reported that MAE significantly increases TF yield (1.61 g/100 g DW) comparing to classical extraction (0.54 g/100 g DW). TF obtained in present work (Table 2) was significantly higher comparing to TF determined in sage extracts obtained by MAE in previously reported works (Dragovic-Uzelac et al., 2012; Dent et al., 2013), due to difference in applied assays. Colorimetric assay with aluminum chloride applied in this work was rather nonselective,

Fig. 2. Response surface plots showing combined effects of a) ultrasound-assisted extraction and b) microwave-assisted extraction parameters on total phenols content (TP).

comparing to TF obtained as sum of individual flavonoids determined by HPLC (Dragovic-Uzelac et al., 2012; Dent et al., 2013).

According to ANOVA results from Table 3, it could be seen that linear terms of temperature and extraction time and quadratic term of temperature exhibited highly significant influence on TF, while influence of quadratic term of extraction time was moderately significant. Both linear terms of temperature and extraction time exhibited positive influence on TF (Fig. 3a), suggesting that prolonged UAE at elevated temperature is mandatory for complete flavonoids recovery, which is in accordance with previous reports (Ramić et al., 2015; Tomšik et al., 2016). However, the highest applied temperature (80 °C) would not be optimal for flavonoids extraction due to significant negative effect of its quadratic term (Table 3). Therefore, TF would increase with increase of temperature and eventually start to decrease due to possible thermal degradation, similar to TP. In case of MAE, all linear terms of inde-

pendent variables exhibited significant influence, which was highly significant (p < 0.01) in case of ethanol concentration and liquid to solid ratio, and moderately significant (p < 0.05) for extraction time. Extraction time and liquid to solid ration exhibited positive influence on TF, while ethanol concentration influence was negative which imply that sage flavonoids were better extracted if polar solvent mixtures, i.e. reduced ethanol concentration in extraction solvent, were applied. Negative effect of quadratic term of ethanol concentration on flavonoids extraction was previously reported by Silva et al. (2007). This suggested that TF reaches maximum with decrease of ethanol concentration then starts to decrease as ethanol concentration is approaching its lower level (40%). This is in accordance with results reported by Dent et al. (2013), where MAE of sage was investigated at different ethanol concentrations (30, 50 and 70%) and higher flavonoid yields are observed when 30 and 50% ethanol were applied as extraction solvent, suggesting that moder-
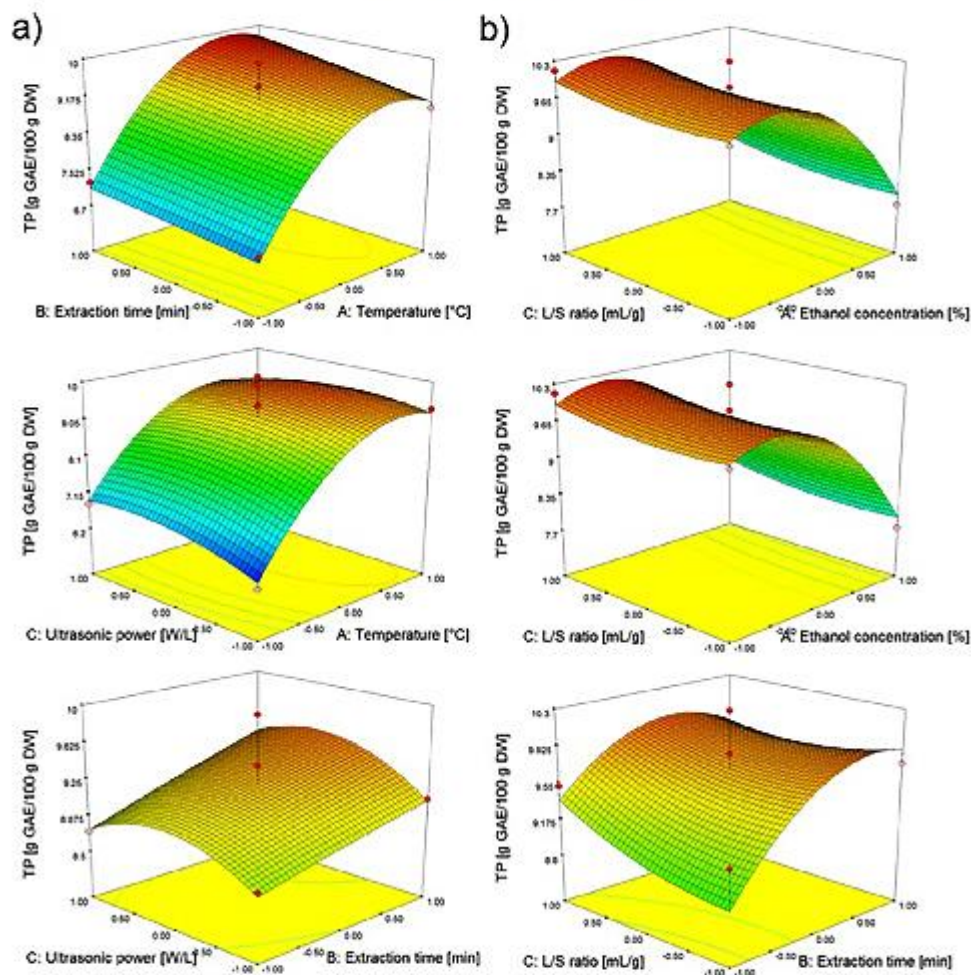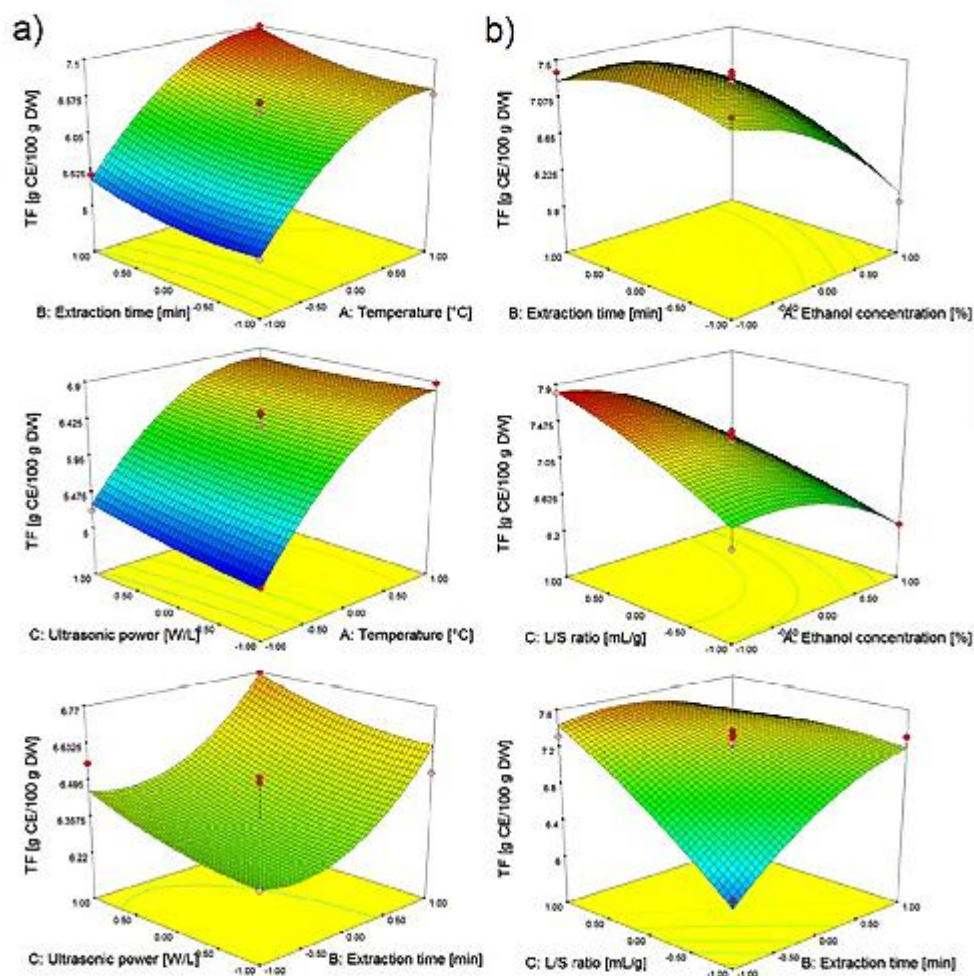
Fig. 3. Response surface plots showing combined effects of a) ultrasound–assisted extraction and b) microwave–assisted extraction parameters on total flavonoids content (TF).

ately polar solvent provides good recovery of sage flavonoids which were dominantly in the form of flavone (luteolin and apigenin) glycosides.

3.4. Antioxidant activity

An imbalance between the production of free radicals and antioxidants present in the organism can lead to a state known as oxidative stress (Wen et al., 2013). Oxidative stress is characterized by an increased production of free radicals, which include reactive oxygen species (ROS), reactive nitrogen species (NOS), carbon-centered and sulfur-centered radicals (Wu et al., 2013). ROS, such as superoxide anion ($O_2^{\cdot-}$), hydroxyl ($^\cdot OH$), peroxyl radical ($ROO\cdot$) and hydrogen peroxide (H_2O_2), can induce oxidative degradation of proteins, unsaturated fatty acids, carbohydrates and nucleic acids leading to many pathophysiological conditions

such as atherosclerosis, cardiovascular disease, arthritis, asthma, Parkinson's and Alzheimer's disease, autoimmune disorders and cancer (Sindhi et al., 2013). Antioxidants are molecules that can slow down or completely inhibit the harmful effects of free radicals. Due to numerous side effects of synthetic antioxidants, PG and BHT (Pop et al., 2013), there is an increasing interest for antioxidants from natural sources. Owing to their structure, plant's secondary biomolecules, phenols and flavonoids, exhibit strong antioxidant activity. These compounds are capable of neutralizing free radicals by transferring electrons or hydrogen atoms onto them, chelating and reducing transition metals, inhibiting oxidizing enzymes and, also, regenerating essential vitamins (Sharma, 2014). Having in mind the role of oxidative stress in the development of severe chronic diseases, it was worthwhile to investigate the antioxidant activity of sage extracts and dependency of this activity on phenolic and flavonoid content. Since different mechanisms of antioxidant

Table 4
Antioxidant activities of sage herbal dust extracts obtained by UAE and MAE.

Run	Ultrasound–assisted extraction			Microwave-assisted extraction		
	DPPH[a] [μg/mL]	FRAP [mg AAE/g DW]	$O_2^{\bullet-}$ [μg/mL]	DPPH[a] [μg/mL]	FRAP [mg AAE/g DW]	$O_2^{\bullet-}$ [μg/mL]
1	9.76 ± 0.3787	221.0 ± 9.441	41.76 ± 2.093	12.40 ± 0.525	92.57 ± 4.234	52.30 ± 0.537
2	16.51 ± 0.6126	184.9 ± 3.056	53.44 ± 2.977	11.88 ± 0.165	86.49 ± 4.007	43.64 ± 2.708
3	9.02 ± 0.4864	207.8 ± 8.581	42.07 ± 3.230	10.40 ± 0.213	90.59 ± 0.552	45.27 ± 2.732
4	11.57 ± 0.2348	190.4 ± 8.514	48.37 ± 3.020	12.85 ± 0.922	80.72 ± 3.501	65.32 ± 3.917
5	10.35 ± 0.3525	196.3 ± 19.25	42.44 ± 2.023	13.25 ± 0.474	74.98 ± 0.767	54.49 ± 2.149
6	21.44 ± 2.1247	167.1 ± 9.025	50.66 ± 1.292	11.18 ± 0.478	83.16 ± 1.899	55.65 ± 0.459
7	9.29 ± 0.8527	194.8 ± 11.69	37.99 ± 1.579	13.34 ± 0.444	81.48 ± 2.419	66.57 ± 4.779
8	13.89 ± 1.3693	148.6 ± 6.764	72.88 ± 3.197	16.38 ± 0.639	68.19 ± 4.349	59.97 ± 0.828
9	10.22 ± 0.5164	195.9 ± 7.796	66.49 ± 0.989	15.19 ± 0.355	68.41 ± 3.403	95.48 ± 1.329
10	12.30 ± 0.4074	176.3 ± 8.014	71.18 ± 4.151	17.24 ± 0.587	71.43 ± 2.538	85.04 ± 5.042
11	11.03 ± 0.723	183.7 ± 4.675	41.19 ± 0.393	14.12 ± 0.828	76.39 ± 1.640	53.89 ± 3.831
12	10.35 ± 0.7944	211.2 ± 11.44	71.66 ± 2.761	13.66 ± 0.566	75.84 ± 1.438	56.28 ± 3.206
13	11.49 ± 0.6677	199.9 ± 17.41	55.44 ± 5.510	11.40 ± 0.605	84.04 ± 1.938	59.46 ± 1.478
14	10.73 ± 0.3736	170.1 ± 8.674	34.34 ± 1.871	15.07 ± 0.503	85.39 ± 4.046	69.09 ± 2.588
15	15.41 ± 0.7871	124.1 ± 9.799	34.03 ± 2.136	13.67 ± 0.226	79.83 ± 1.562	48.73 ± 0.947
16	10.49 ± 0.1957	203.1 ± 11.89	38.93 ± 3.438	13.03 ± 0.836	85.19 ± 0.980	59.34 ± 2.566
17	11.89 ± 0.9599	190.6 ± 8.252	81.09 ± 1.351	15.20 ± 0.478	71.03 ± 4.171	65.35 ± 5.197
PG[a]	24.99 ± 0.300		17.00 ± 1.000			
BHT[b]	50.77 ± 4.000					

[a] propyl gallate.
[b] butylated hydroxytoluene.

Table 5
Correlation matrix with Pearson's correlation coefficients (r) for TP, TF, DPPH•, FRAP and $O_2^{\bullet-}$.

r[a]	TP	TF	DPPH•	FRAP	$O_2^{\bullet-}$	TP	TF	DPPH•	FRAP	$O_2^{\bullet-}$
TP	1					1				
TF	0.932*	1				0.754*	1			
DPPH•	−0.612*	−0.590*	1			−0.700*	−0.811*	1		
FRAP	0.785*	0.793*	−0.602*	1		0.689*	0.581*	−0.774*	1	
$O_2^{\bullet-}$	−0.078	−0.293	0.098	0.045	1	−0.476	−0.331	0.658*	−0.582*	1

[a] Pearson's correlation coefficient.
* $p < 0.05$

activity were involved, different methods should be used to elucidate the antioxidant potential of plant extracts or pure compounds responsible for the beneficial effect on human health (Sharma, 2014). Therefore, extracts, as well as the standard antioxidant BHT, were examined for their reducing power and scavenging capacity towards superoxide anion and DPPH radical.

Radical scavenging capacity of sage herbal dust extracts towards DPPH radicals, expressed as IC_{50} value, was between 9.02 and 21.44 μg/mL for sage extracts obtained by UAE and from 10.40 to 17.24 μg/mL for extracts obtained by MAE. The highest antioxidant activity of UAE extracts (9.02 μg/mL) was obtained at 80 °C, 40 min of extraction time and 42 W/L of ultrasonic power, while extract obtained with 40% ethanol, 30 min of extraction time and with 30 mL/g of liquid to solid ratio provided the highest antioxidant activity (10.40 μg/mL) among extracts obtained by MAE. Moreover, moderate correlation between IC_{50} and TP and TF, respectively ($p < 0.05$) (Table 5), suggesting that polyphenols were the most important compounds responsible for antioxidant activity. Moreover, all sage extracts obtained by both UAE and MAE exhibited higher activity than commonly used synthetic antioxidants, propyl gallate (24.99 μg/mL) and butylated hydroxytoluene (50.77 μg/mL). Jeshvaghani et al. (2015) reported IC_{50} value of *S. officinalis* extract obtained by classical extraction with methanol was 233.0 μg/mL, which was rather high IC_{50} value for sage. This indicated that application of modern extraction techniques, in this case UAE and MAE, could significantly improve antioxidant capacity of sage extracts, however, Veličković et al. (2006) found no significant difference between antioxidant activity of sage extracts obtained by classical extraction and UAE. On the other hand, Farhat et al. (2013) reported that antioxidant activity of methanolic sage

extracts varies from 3.37 to 10.08 μg/mL, depending of geographical location of plant collection.

Although, there was no significant difference between TP, TF and radical scavenging capacity of towards DPPH radicals between sage extracts obtained by UAE and MAE (Tables 1, 2 and 4), ferric reducing antioxidant power (FRAP) of sage extracts obtained by UAE (124.1–221.0 mg AAE/g DW) was approximately twice higher comparing to FRAP in extracts obtained by MAE (68.19–92.57 mg AAE/g DW). Possible explanation for this phenomenon could be oxidation of certain antioxidant compounds during severe MAE conditions which were rougher comparing to UAE conditions. The highest FRAP of sage extracts obtained by UAE (221.0 mg AAE/g DW) and MAE (92.57 mg AAE/g DW) were observed at central point (Run 1) of both applied techniques. Relatively high values of TP and TF were also observed at central points for both UAE and MAE, suggesting good correlation between content of polyphenol compounds and FRAP. According to Pearson's correlation coefficients (r) from Table 5, good correlation between FRAP and TP and TF, respectively, was confirmed ($p < 0.05$) and it was better in case of UAE. According to literature data, FRAP of sage extracts obtained by classical extraction were 167 μM Trolox/100 g DW (Wojdyło et al., 2007) and 178.65–197.33 mM Fe(II)/mg (Farhat et al., 2013), while FRAP of sage extracts obtained by pressurized liquid extraction using aqueous methanol as extraction solvent was between 5.84 and 20.34 g Trolox/100 g DW (Hossain et al., 2010).

Superoxide radical-scavenging activities of sage herbal dust extracts obtained by UAE and MAE, expressed as IC_{50} value, were presented in Table 4. In case of UAE, extract with the highest $O_2^{\bullet-}$ scavenging activity, i.e. the lowest IC_{50} (34.03 μg/mL), was obtained at temperature of 40 °C, extraction time of 40 min and ultrasonic power of 24 W/L, which were relatively mild UAE con-

Table 6
Estimated optimal UAE and MAE parameters for simultaneous maximization of total phenols and total flavonoids content.

Extraction technique	Optimized conditions	Predicted responses[a]	Observed responses[b]
Ultrasound-assisted extraction	Temperature: 75.4 °C Extraction time: 80 min Ultrasonic power: 42.54 W/L	TP = 9.876 ± 0.584 g GAE/100 g DW TF = 7.052 ± 0.209 g CE/100 g DW	TP = 9.881 ± 0.157 g GAE/100 g DW TF = 6.997 ± 0.341 g CE/100 g DW
Microwave-assisted extraction	Ethanol concentration: 46.2% Extraction time: 18.7 min L/S ratio: 40 mL/g	TP = 10.105 ± 0.685 g GAE/100 g DW TF = 7.802 ± 0.338 g CE/100 g DW	TP = 10.379 ± 0.193 g GAE/100 g DW TF = 7.544 ± 0.098 g CE/100 g DW

[a] predicted value ± 95% confidence interval.
[b] mean value ± standard deviation (n = 4).

ditions, comparing to optimal parameters for TP and TF extraction. This indicated weak correlation between $O_2^{\bullet-}$ scavenging activity and polyphenols content which was confirmed with low Pearson's correlation coefficients from Table 5. On the other hand, the highest $O_2^{\bullet-}$ scavenging activity of sage extracts was obtained on following MAE parameters: 60% ethanol, 20 min and 30 mL/g liquid to solid ratio. Even though, polyphenols yield at these conditions was rather good, week correlation between IC_{50} and polyphenols content (TP and TF) was observed ($p > 0.05$) (Table 5). Evidently, some other compounds may contribute to $O_2^{\bullet-}$ scavenging activity. Phenolic diterpenoids, mostly derivatives of carnosoic acid, were reported as the most significant antioxidants in sage (Babovic et al., 2010). Propyl gallate exhibited higher $O_2^{\bullet-}$ scavenging activity (17.00 μg/mL) than sage extracts obtained in this work (>34.03 μg/mL). Lu and Foo (2001) reported that caffeic and rosmarinic acid were the most important compounds from sage responsible for $O_2^{\bullet-}$ scavenging activity, which was approx. 15–20 times higher than Trolox activity.

3.5. Process optimization

Optimization of UAE and MAE processes in order to maximize polyphenols yield was the primary objective of this work. In order to simultaneously maximize investigated responses (TP and TF), desirability function was applied (Derringer, 1980). The optimal UAE parameters for maximized polyphenols yield and predicted values of responses were presented in Table 6. It could be observed that UAE conditions which would provide the highest TP and TF were temperature of 75.4 °C, extraction time of 80 min and ultrasonic power of 42.54 W/L. On the other hand, optimized MAE parameters were application of 46.2% aqueous ethanol as extraction solvent, extraction time of 18.7 min and liquid to solid ratio of 40 mL/g. Optimized UAE and MAE conditions were in accordance with analysis independent variables parameters determined by RSM and ANOVA (Table 3). It could be seen that MAE provides slightly higher yields of polyphenols, which could be explained that higher liquid to solid ratio applied in certain MAE. MAE also provided certain advantages in terms of time consumption, however, UAE should be more suitable for polyphenols extraction in terms of instrumentation, operational costs and potential scale up of the extraction process. In order to validate predictive mathematical models, validation was performed by separate extraction at optimal conditions for UAE and MAE. According to results from Table 6, it could be seen that experimentally obtained values were in accordance with predicted results, i.e. both TP and TF for UAE and MAE were within 95% confidence interval for predicted results. This suggested that UAE and MAE optimizations were adequately performed and that obtained model equations could be used for point prediction within investigated experimental domain.

HPLC analysis of sage extracts obtained by CE using 60% ethanol as extraction solvent and by UAE and MAE at optimal conditions was performed in order to determine content of main phenolic acids, particularly rosmarinic acid. According to results from Table 7, it could be seen that both UAE and MAE provided signifi-

Table 7
Yield of main phenolic acids observed in extracts obtained by CE (60% ethanol), and separately optimized UAE and MAE.

Compound	Extraction technique		
	CE	UAE	MAE
	Yield [mg/100 g DW]		
Caffeic acid	33.69	30.93	18.07
p-Coumaric acid	5.40	5.03	5.53
Ferulic acid	13.14	14.08	4.72
Rosmarinic acid	840.43	1623.21	1724.81

cantly higher (approximately twice higher) yield of rosmarinic acid, suggesting again that novel extraction techniques would provide significant advantages in terms of polyphenols extraction, comparing to traditionally applied techniques. Significant increase in yield was observed only in case of rosmarinic acid, while in case of caffeic, p-coumaric and ferulic acid, changes in yield were rather insignificant.

4. Conclusions

According to satisfactory statistical parameters (R^2 and CV) and analysis of variance (ANOVA) for the model and lack-of-fit testing, it could be concluded that second-order polynomial model provided adequate mathematical description of the UAE and MAE of sage polyphenols. Therefore, RSM could be successfully applied for simultaneous optimization for maximized TP and TF yields. Temperature (for UAE) and ethanol concentration (for MAE) have been designated as the most influential extraction parameters affecting polyphenols yield, while all other parameters influence was moderate. Therefore, optimized UAE conditions for maximized extraction of TP and TF were temperature of 75.4 °C, extraction time of 80 min and ultrasonic power of 42.54 W/L, while optimized MAE parameters for achieving the same goal were ethanol concentration of 46.2%, extraction time of 18.7 min and liquid to solid ratio of 40 mL/g. In vitro antioxidant activity of extracts was determined through ferric reducing antioxidant power and scavenging capacity towards superoxide anion and DPPH radical. Results indicated that obtained sage extracts possessed high antioxidant activity, which was particularly increased towards scavenging capacity of DPPH radicals and reducing power towards ferric ions, while $O_2^{\bullet-}$ scavenging activity of extracts was rather moderate. According to results presented in this work, it could be concluded that sage herbal dust could be considered as interesting raw material for polyphenols extraction. Therefore, it could be used for production extracts rich in antioxidants, instead of being discarded from filter tea factory as by-product.

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Appendix K – Sage processing from by-product to high quality powder: I. Bioactive potential

Sage processing from by-product to high quality powder: I. Bioactive potential

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ABSTRACT

Nowadays, valorization of food industry by-products and agricultural waste has been in the focus of investigation worldwide. Therefore, the aim of this study was utilization of sage (*Salvia officinalis* L.) herbal dust, by-product from filter tea factory, for production of high-quality dry extracts by spray drying technique. Liquid feed for spray drying was obtained using conventional (maceration (MAC) and hydrodistillation (PHD)) and novel (ultrasound-assisted (UAE), microwave-assisted (MAE) and subcritical water extraction (SWE)) extraction techniques, whereas sage by-product from filter tea factory was used as raw material. Dry powders were characterized in terms of polyphenol content (total phenols (TP) and total flavonoids (TF)), antioxidant activity and antimicrobial activity towards selected gram-positive bacteria strains. Particularly high TP and TF were observed in all dry extracts (90.20–290.28 mg GAE/g and 56.98–154.88 mg CE/g, respectively), while applied extraction technique had significant effect on polyphenol content. Antioxidant activity was determined by DPPH, FRAP and ABTS assays and extracts obtained by MAC, UAE and MAE were more potent comparing with SWE and PHD. In case of antimicrobial activity, all investigated samples exhibited certain antimicrobial effects towards selected Gram positive bacteria (MIC < 50 mg/mL). Good correlation between polyphenol content, antioxidant potential and antimicrobial activity was observed, whereas sample obtained by UAE had the best balance of biochemical properties.

1. Introduction

Medicinal plants from *Lamiaceae* family (rosemary, thyme, sage, peppermint, etc.) have been widely used in traditional medicine due to their specific health benefit actions. Sage (*Salvia officinalis* L.) has been recognized for its different pharmacological effects, such as antimicrobial (Bozin et al., 2007), anti-inflammatory, immune-modulatory (Capek and Hřibalová, 2004), antioxidant (Ivanovic et al., 2009) and anticancer (Kamatou et al., 2008) effects, while it has been also used in for the treatment of nervous conditions (Baricevic and Bartol, 2000). Beside its medicinal applications, it has gained commercial interest as flavouring agent in food industry (Gali-Muhtasib et al., 2000) and fragrance in perfumes. Various classes of secondary plant metabolites have been isolated from sage. Among them, terpenoids and polyphenols have exhibited certain bioactive potential. Main terpenoids in sage essential oil are α- and β-thujone, camphor and 1,8-cineole (Aleksovski and Sovová, 2007), while polyphenolic fraction consisted of three main subgroups: phenolic acids (rosmarinic, ferulic and caffeic acid) (Farhat et al., 2013), flavonoids (luteolin, apigenin and naringenin derivatives) (Farhat et al., 2013; Dent et al., 2013) and phenolic diterpenes (carnosic acid, carnosol, rosmanol, epirosmanol and isorosmanol (Babovic et al., 2010). Sage has been widely used in the form of filter tea. Therefore, a large quantity of by-product is being generated within filter tea factory. Plant processing and generation of sage by-product, i.e. herbal dust, in filter tea factory has been described in details by Pavlić et al. (2016). Moreover, it has been proposed that sage herbal dust could be used as raw material for extraction of polyphenolic bioactive compounds by novel extraction techniques (ultrasound-assisted extraction, microwave-assisted extraction and subcritical water extraction) (Pavlić et al., 2016). This way, relatively cheap raw material could be used for recovery of valuable health benefit compounds in the form of liquid extracts. However, liquid extracts are commonly more sensitive to effects of environmental factors and bioactives could easily degrade due to water, light and oxygen effects. Therefore, it is

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necessary to transform liquid extracts in their solid (powder) form, which is usually more stable (Munin and Edwards-Lévy, 2011), easier to handle, standardize, transport and store (Oliveira et al., 2006). This is in accordance with universal five recovery stages of high-added value components from food wastes and by-products (Galanakis, 2012), since dry extract could be directly incorporated in other food and pharmaceutical product, without further processing. Finally, it must be further investigated, is it more economically feasible to apply conventional or emerging processing technologies, which are able to answer to a specific challenges and opportunities (Galanakis, 2013).

Furthermore, instant solubility and consumers' acceptance are advantages of dry extracts, which are commonly produced from liquid feed using spray drying technology. The main reasons for wide application of spray drying for production of dry extracts from either fruit juices and medicinal plant extracts (Đorđević et al., 2014; Caliskan and Dirim, 2016) are: 1) short contact with drying medium and high evaporation rate, 2) easy handling and low operating cost, 3) wide range of carrier materials, 4) good reproducibility and 5) high production rate at industrial level. Although, relatively high temperatures are used in spray drying (> 100 °C), good recovery of thermo-sensitive compounds is provided due to fast evaporation and short contact of extract and heated air at elevated temperature. Fast evaporation is provided by the generation of small droplets which will significantly increase specific surface area, which is in contact with a hot flow of drying air (Cortés-Rojas et al., 2015). Therefore, spray drying technique has been successfully applied for drying of medicinal plant (Şahin-Nadeem et al., 2013), fruit (Daza et al., 2016), food industry by-products (de Souza et al., 2015) extracts. Obtained high-quality powders could be further incorporated in food products, as well as in solid dosage forms (tablets and capsules), which are the most commonly used forms of pharmaceutical products and dietary supplements (Leuenberger and Lanz, 2005).

Different polymer materials from proteins, polysaccharides and gums could be used as carrier material in spray drying process. Among them, maltodextrin has been usually applied due to its wide range of assets such as low cost, high solubility, low hygroscopicity, low viscosity and good bulking and film-forming properties (Goula and Adamopoulos, 2005; Şahin Nadeem et al., 2011; Caliskan and Dirim, 2016), while poor emulsifying properties has been underlined as its main disadvantage. Maltodextrins with different degree of dextrose equivalents are obtained by acid hydrolysis of corn starch. The main aspects of application of carriers during spray drying is increase of liquid feed glass transition temperature in order to avoid collapse and its adherence to the dryer's chamber (Marques et al., 2014).

Şahin-Nadeem et al. (2013) investigated influence of inlet air temperature and carrier concentration on the spray drying process of Salvia fruticosa Miller. According to them, addition of different carrier materials and their concentrations provided powders with unique physico-chemical properties, i.e. instant soluble powder which had improved solubility, colour, total phenols content and antioxidant activity. Furthermore, spray drying technique was successfully applied for production of high-quality powders from Achillea millefolium herbal dust obtained from filter tea factory (Vladić et al., 2016). Therefore, it was assumed that spray drying of sage herbal dust liquid extracts obtained by novel extraction techniques would also result with dry extracts with specific chemical properties and biological activities.

The main aim of this work was application of spray drying technique for production of dry extracts from sage by-product liquid extracts obtained by conventional (maceration and hydrodistillation) and novel (ultrasound-assisted extraction, microwave-assisted extraction and subcritical water extraction) extraction techniques. Furthermore, drying was performed with and without addition of carrier (maltodextrin), in order to compare its impact on dry powder properties. Chemical characterization of dry extracts was examined by determination of polyphenol content, while their bioactive potential was determined for antioxidant (DPPH, FRAP and ABTS) and anti-

microbial activity towards selected microbial strains.

2. Materials and methods

2.1. Plant material

Sage (Salvia officinalis L.) originated from Montenegro was kindly donated by local filter tea factory, Fructus DOO (Bačka Palanka, Serbia). Details of plant material processing within the filter tea factory were described elsewhere (Ramić et al., 2015). Sage herbal dust, obtained as by-product, was used as raw material in this research. Sample had mean particle size less than 0.315 mm.

2.2. Chemicals

Following reagents were purchased from Sigma-Aldrich Chem (Steinheim, Germany): Folin-Ciocalteu reagent, (±)-catechin, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ABTS (2,2'-azino-bis-(-3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), TPZT (2,4,6-tris (2-pyridil)-s triazine), iron (III)-chloride and Iron (II)-sulfathepta-hydrate and potassium persulfate. Trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid) was purchased from Sigma–Aldrich (Milano, Italy). Sodium acetate and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Formic acid was obtained from VWR International (Milan, Italy). Maltodextrin (DE19.7) was purchased from Brenntag (Mülheim, Germany). All other reagents used in this study were of analytical reagent grade.

2.3. Extraction

2.3.1. Conventional extraction techniques
2.3.1.1. Maceration. Conventional solid-liquid extraction, i.e. maceration, was performed using previously optimized ethanol concentration for polyphenol extraction (Zeković et al., 2016b). Plant material (200 g) and 60% ethanol (4 L) were mixed in glass flask. Extraction was performed at room temperature for 5 days. Flask was thoroughly shaken twice a day. After extraction, obtained extracts were immediately filtrated through filter paper under vacuum. Extract was collected into glass flask and stored at 4 °C until further experiments.

2.3.1.2. Hydrodistillation. Hydrodistillation was performed in order to separate essential oil from low-volatile compounds which were retained in postdistillation waste. The official procedure from Ph.Jug IV was applied for hydrodistillation. After that, postdistillation liquid (waste) was immediately filtrated through filter paper under vacuum, collected into glass flask and stored at 4 °C until further experiments.

2.3.2. Novel extraction techniques
2.3.2.1. Ultrasound-assisted extraction (UAE). Ultrasound-assisted extraction was performed in sonication water bath (EUP540A, Euinstruments, France) with fixed frequency at 40 kHz. Extraction was performed at previously optimized conditions: temperature of 75 °C, extraction time of 80 min and sonication power of 43 W/L (Zeković et al., 2016b). Therefore, 5 g of sage herbal dust was mixed with 100 mL of 60% ethanol in 250 mL glass flasks. Condenser was added on glass flask in order to prevent any possible evaporation of the extraction solvent. Flasks were always positioned in the same distance from the transducer. Extraction at same conditions was continuously repeated in order to collect enough liquid feed for spray drying (2 L). After extraction, extracts were immediately filtered through filter paper under vacuum, collected into glass flasks and stored at 4 °C until further experiments.

2.3.2.2. Microwave-assisted extraction (MAE). Mono-mode microwave-assisted extraction (MAE) was performed in experimental setup described elsewhere (Zeković et al., 2016a). Extraction was

performed at previously optimized MAE conditions: ethanol concentration of 46%, extraction time of 18.7 min and liquid/solid ratio of 1:20 (m/v) (Zeković et al., 2016b). Sample was mixed with extraction solvent in glass flask and placed in MAE apparatus and extractions were performed on fixed frequency and irradiation power (600 W). Flasks were always positioned on the same distance from the magnetron and no additional agitation was applied. In this case, extraction was also repeated at same conditions in order to collect enough liquid feed for spray drying (2 L). After extraction, extracts were immediately filtered through filter paper under vacuum, collected into glass flasks and stored at 4 °C until further experiments.

2.3.2.3. Subcritical water extraction (SWE).

SWE was performed at previously optimized extraction conditions: temperature of 201 °C, extraction time of 15.8 min and no addition of HCl in extraction solvent (Pavlić et al., 2016). Comparing with previously published optimization study, SWE in this work was performed in pilot plant batch-type high-pressure extractor (Parr Instrument Company, USA) with internal volume 15 L and maximum operating pressure of 20 MPa and temperature 350 °C, connected with temperature controller (4848BM, Parr Instrument Company, USA). For extraction, 1 kg of plant material and 10 L of water were mixed and extraction was performed at isobaric conditions (3 MPa) and nitrogen was used to pressurize extractor. Stirring (1000 rpm) was employed in order to increase mass and heat transfer, and prevent local overheat on the inner walls of extractor. After the extraction, extractor was cooled in iced bath to room temperature and crude extract was filtered through filter paper under vacuum, collected into glass flasks and stored at 4 °C until further experiments.

2.4. Spray drying

Liquid extracts obtained by conventional and novel extraction techniques were dried using spray drying process in pilot Anhydro spray dryer plant (APV Anhydro AS, Denmark). Process temperatures for spray drying were: inlet temperature of 120–125° C, outlet temperature 75–85° C. Liquid feed was transferred in drying chamber using laboratory peristaltic pump (FH100 Series, Thermo Scientific, USA) with 1.36 L/h flow rate. For each experimental run, 2 L of liquid feed was dried. Rotary disk with speed range from 20,000 to 21,000 rpm was used for atomization of liquid feed. After drying of droplets, dry extract was separated from heating medium in cyclone and collected in plastic vessel. All liquid extracts were spray dried with (20%) and without addition of carrier. Maltodextrin (DE19.7) was used as carrier material and its solution was added to liquid feed, and both were mixed continuously with a magnetic stirrer at temperature of approx. 40 °C prior drying. Obtained dry extracts were collected in glass bottles, sealed and kept protected from air and humidity.

2.5. Chemical analysis

2.5.1. Total phenols content (TP)

Total phenols content (TP) was determined in both liquid extracts and dry powders. Liquid extracts were directly used for analysis, while dry powders were reconstituted in their respective extraction solvent prior analysis. TP was determined using Folin-Ciocalteu procedure (Singleton and Rossi, 1965). Gallic acid was used as standard compound for preparation of calibration curve, and absorbance of the samples was measured at 750 nm (6300 Spectrophotometer, Jenway, UK). Content of phenolic compounds in liquid extracts was expressed as mg of gallic acid equivalents (GAE) per ml, while content in dry extracts was expressed as mg GAE per g of dry extract. All experiments were performed in triplicate, and results are expressed as mean values ± standard deviation (SD).

2.5.2. Total flavonoids content (TF)

Similarly to TP, total flavonoids content (TF) was also determined in liquid and dry extracts using aluminum chloride colorimetric assay (Harborne, 1984). Catechin was used for preparation of standard diagram and absorbance was measured at 510 nm. Results for liquid extracts were expressed as mg of catechin equivalents (CE) per ml, while TF in dry extracts were expressed as mg CE per g of dry extract. All experiments were performed in triplicate, and results were expressed as mean values.

2.6. Biological activity

2.6.1. In vitro antioxidant activity

2.6.1.1. DPPH assay. The sample ability to scavenge 2,2-diphenyl-1-picrylhydrazyl free radicals (DPPH) was measured using a modified method originally presented by Brand-Williams et al. (1995). Briefly, methanolic solution of the DPPH reagent (65 μM) was freshly prepared and adjusted with methanol to reach absorbance of 0.70 (± 0.02). DPPH reagent and properly diluted samples, reconstituted in their respective solvents, were mixed (2.9 mL + 0.1 mL) in the 10 mm plastic cuvettes and incubated at room temperature for 60 min. Free radical scavenging measurements were performed at 517 nm, in triplicates with UV–VIS spectrophotometer (Shimadzu, UVmini 1240, Milan, Italy). Calibration curve was obtained by measuring free radical scavenging of freshly prepared Trolox aqueous solutions (0–0.8 mM, r^2 = 0.99). Obtained results were reported as mg of Trolox equivalents per g of dry extract.

2.6.1.2. Ferric reducing antioxidant power (FRAP) assay. The sample ability to reduce Fe^{3+} was measured using slightly modified method firstly presented by Benzie and Strain (1996). The FRAP reagent was freshly prepared from 300 mM acetate buffer (pH = 3.6), 10 mM 2,4,6-tris(2-pyridil)-s triazine (TPZT), 40 mMHCl solution and 20 mM $FeCl_3$ aqueous solution. Solutions were mixed in ratio 10:1:1 (v/v/v). Properly diluted samples, reconstituted in their respective solvents, and FRAP reagent were mixed (0.1 mL + 1.9 mL) and stored to incubate in the dark at 37 °C for 10 min. Measurements were performed at 593 nm, in triplicates, with UV–VIS spectrophotometer (Shimadzu, UVmini 1240, Milan, Italy). Calibration was performed using freshly prepared Fe^{2+} aqueous solutions (0–0.23 mM, r^2 = 0.99). Results were finally reported as mg of Fe^{2+} equivalents per g of dry extract.

2.6.1.3. ABTS$^+$ free radical-scavenging assay. The ABTS free radical scavenging ability of samples was measured using a modified method originally described by Re et al. (1999). ABTS stock solution was freshly prepared from mixture (1:1, v/v) of 2.45 mM potassium persulfate aqueous solution and 7 mM ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) aqueous solution and left in the dark at room temperature for 16 h. A stock solution was diluted using 300 mM acetate buffer (pH = 3.6) to an absorbance of 0.70 (± 0.02). Properly diluted samples, reconstituted in their respective solvents, and ABTS reagent were mixed (0.1 mL + 2.9 mL) and stored in the dark at room temperature for 300 min. Measurements were performed at 734 nm, in duplicates with UV–VIS spectrophotometer (Shimadzu, UVmini 1240, Milan, Italy). Freshly prepared Trolox aqueous solutions (0–0.8 mM, r^2 = 0.97) were used to obtain the calibration curve. Results were finally reported as mg of Trolox equivalents per g of dry extract.

2.6.2. Antimicrobial activity

2.6.2.1. Bacterial and yeast strains. The bacterial strains used to evaluate the antimicrobial activity of different extracts from S. officinalis were: Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus ATCC 25923, Bacillus cereus ATCC 11778, Listeria ivanovii ATCC 19119, Enterococcus faecalis ATCC 19433,

Staphylococcus saprophyticus[w], *Bacillus cereus*[w], *Listeria monocytogenes*[w], *Enterococcus faecalis*[w] ([w]- wild strain). Wild strains were obtained from food and tap water and identified by Vitek°2 Compact System (BioMérieux, France).

The yeast strains used in this study were: *Saccharomyces cerevisiae* ATCC 9763 and *Candida albicans* ATCC 10231.

2.6.2.2. Antimicrobial screening. Two different methods were applied for the estimation of the antimicrobial activity: disk diffusion method and assessment of minimal inhibitory concentration (MIC) by the method of serial dilutions in microtiter plates.

2.6.2.3. Disk diffusion method. Bacterial strains were grown on Müller-Hinton agar (HiMedia, Mumbai, India) at 37 °C for 24 h and at 30 °C (*Bacillus cereus* ATCC 11778 and *Bacillus cereus*[w]) for 18 h. Yeast strains were grown on Sabouraud Maltose agar (HiMedia, Mumbai, India) at 25 °C (*Saccharomyces cerevisiae* ATCC 9763) or at 37 °C (*Candida albicans* ATCC 10231) for 48 h.

Cells were suspended in a sterile 0.9% NaCl solution. Suspensions were adjusted to a concentration of $1 \cdot 10^6$ cfu/mL (estimated by Densichek; BioMérieux, France). Afterwards, 2 mL of the prepared suspensions for inoculation were homogenized with 18 mL of melted (45 °C) media (the same as for suspension preparation) and poured into Petri dishes.

After the solidification, four sterile discs (6 mm in diameter) (HiMedia, Mumbai, India) were placed onto the previously inoculated agar plates. Applied disks were impregnated with 15 μL of the MAC, UAE, MAE, SWE or PHD extracts water solution (50 mg/mL). Sterile distilled water was used as negative control, while as a positive control the following antibiotics and antimycotics were used: solution of ACV supplement 1 mg/mL and 0.8 mg/mL (HiMedia, Mumbai, India) for *P. aeruginosa* ATCC 27853, actidion (3 mg/mL) (Acros Organic, New Jersey, USA) for *S. cerevisiae* ATCC 9763 and *C. albicans* ATCC 10231 and for all the other tested microorganisms CTC (cefotaxime 30 μg and clavulanic acid 10 μg per disk) (HiMedia, Mumbai, India) were used. After the incubation period, the diameter of the inhibition halo zone was measured for each disk using HiAntibiotic Zone Scale™ (HiMedia, Mumbai, India). Each experiment was performed in triplicate (n = 3).

2.6.2.4. Microdilution method. Minimal inhibitory concentration was assessed for gram-positive bacteria using the microdilution method in sterile flat-bottom 96-well microtiter plates. The preparation procedure of suspensions for inoculation is previously described *Disk diffusion method.* 1 mL of the prepared suspension (1×10^6 cfu/mL.) was homogenized with 9 mL of Müller-Hinton broth (HiMedia, Mumbai, India). In order to obtain final concentration in each well (n = 3) 100 μL of inoculated media were mixed with 100 μL of extract dilutions. In each test microtiter plate there were a positive control (inoculated media without extracts) and a negative control (100 μL of medium mixed with 100 μL of extracts). All test plates were incubated for 24 h at 37 °C or at 30 °C (*Bacillus* strains). Afterwards, a 100 μL aliquot was poured into Petri dishes and homogenized with Plate Count agar (HiMedia, Mumbai, India). Petri dishes were incubated under identical conditions as microtiter plates and the colonies were enumerated by viable count following the incubation period.

Minimal inhibitory concentration (MIC) is known as the lowest concentration of antimicrobial agent that, under defined *in vitro* conditions, prevents the appearance of visible growth of a microorganism within a defined period of time. MIC is usually calculated as $100 \times (N_c - N_t)/N_c$ (%), where N_c and N_t are numbers of cells of positive control and treatment, respectively.

2.7. Statistical analysis

All experiments were performed in triplicate for statistical purpose and results were presented as mean value ± standard deviation (SD),

Table 1
Characterization of liquid feed obtained by different extraction techniques.

Sample name	Extraction technique	Y [%]	Total solids [mg/ml.]	TP [mg GAE/ml]	TF [mg CE/ml]	TP/TF
MAC	S/L extraction (maceration)	25.82	12.91	3.95	2.71	1.46
UAE	Ultrasound-assisted extraction	30.44	15.22	4.92	2.86	1.72
MAE	Microwave-assisted extraction	32.18	16.09	5.29	3.37	1.57
SWE	Subcritical water extraction	35.62	35.62	6.90	4.57	1.51
PHD	Hydrodistillation (postdistillation waste)	31.38	15.69	3.38	2.22	1.52

while significant levels were defined at $p < 0.05$ using *t*-test. Statistical analysis was carried out using Statistica 10.0 (StatSoft Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. Characterization of liquid feed

Liquid feed used for spray drying process was first characterized in terms of total solids, total phenols (TP) and total flavonoids (TF) content (Table 1). Moreover, total extraction yield (Y) obtained by each extraction technique was also determined. According to data from Table 1, it could be observed that the highest Y (35.62%) was obtained using subcritical water extraction (SWE). This is rather expected results, since severe SWE conditions, pressurized solvent and increased temperature, disrupt the cell structure of the plant matrix and allow easier diffusion of the extractable compounds from solid to liquid phase (Mustafa and Turner, 2011). Furthermore, Y obtained by SWE in this work was lower that Y achieved at optimized SWE conditions at laboratory plant (Pavlić et al., 2016). In this work, SWE was also performed at optimized conditions, however, it was performed at pilot plant which significantly prolonged heating and cooling phase of the SWE. The lowest Y (25.82%) was achieved by maceration. This technique demanded the highest time consumption (5 days), however, extraction was performed at room temperature. All other techniques were performed at increased temperature, which caused increase in Y, together with other mechanisms specific for each applied technique. Total solids content in sage by-product liquid extracts was between 12.91 and 35.62 mg/mL. Evaluation of this parameter was essential since it can significantly affect physico-chemical properties of the dry powders obtained by spray drying (Couto et al., 2012). In case of polyphenol content, the highest TP and TF were observed in extract obtained by SWE, while the lowest contents were in postdistillation waste (Table 1). On the other hand, TP yield obtained by MAC, UAE, MAE, SWE and PHD was 7.90, 9.83, 10.57, 6.90 and 6.75 g GAE/100 g dry plant material, respectively. The highest TP and TF yields were achieved by MAE, since solid-liquid ratio was 1:10 (m/v) in case of SWE, while applied solid-liquid ratio in all other techniques was 1:20 (m/v). TP and TF obtained by UAE and MAE were in accordance with previous report (Zeković et al., 2016b), while pilot plant SWE provided lower TP and TF, comparing with results obtained at laboratory level (Pavlić et al., 2016). The TP/TF ratio is almost consistent among the several extraction methods (range: 1.46–1.72), therefore the selectivity versus these two aggregate groups (i.e. TP and TF) seems affected to little extent.

3.2. Polyphenol content in dry extracts

The main issue of management of plant extracts is a lack of

Table 2
Total phenols and total flavonoids content in dry extracts obtained by spray drying.

Sample	MD[a] [%]	TP [mg GAE/g]	TF [mg CE/g]
MAC	0	280.35 ± 5.24[c]	154.88 ± 1.33[a]
MAC-20	20	226.90 ± 4.00[d]	133.55 ± 1.69[b]
UAE	0	290.28 ± 2.31[c]	147.70 ± 2.24[a]
UAE-20	20	281.88 ± 1.57[d]	135.41 ± 1.32[d]
MAE	0	254.43 ± 2.51[e]	150.07 ± 1.69[a]
MAE-20	20	210.79 ± 2.80[f]	119.41 ± 1.80[b]
SWE	0	106.26 ± 2.68[g]	58.97 ± 1.02[e]
SWE-20	20	91.35 ± 1.72[g]	56.98 ± 1.00[f]
PHD	0	106.59 ± 1.52[b]	74.02 ± 0.98[g]
PHD-20	20	90.20 ± 1.58[i]	59.87 ± 0.44[h]

Values followed by the same letter did not share significant difference at 5% (t-test).
[a] Maltodextrin concentration [%].

standardization procedures in terms of bioactive compound(s) content. Since, polyphenolic compounds have been regarded with high bioactive potential, it is essential to determine their content in dry extracts which could be further used in pharmaceutical formulations, dietary supplements and for fortification of food products. The total phenols (TP) and total flavonoids (TF) contents observed in spray dried sage extracts with and without addition of carrier materials are presented in Table 2. The highest TP (290.28 mg GAE/g) was observed in extract prepared by UAE, while the lowest (90.20 mg GAE/g) was in extracts prepared by PHD with addition of maltodextrin. To our best knowledge, there are no previous reports about spray drying of *S. officinalis* extracts. However, rosemary has been regarded as plant very similar to sage in terms of polyphenol content and antioxidant activity. According to Couto et al. (2012), TP in spray dried rosemary extract prepared by maceration varied between 132 and 174 mg GAE/g. Since all extracts were dried under the same spray drying conditions, TP is directly connected to applied extraction technique used for preparation of liquid feed and to addition of carrier material. Şahin-Nadeem et al. (2013) reported that TP in spray dried *Salvia fruticosa* was between 132.8 and 297.2 mg/g, similarly to TP obtained in this work. As expected, TP in extracts prepared with addition of maltodextrin (20%) was lower comparing with their respective extracts spray dried without carrier. This is rather expected since concentration of sage extract is diluted with maltodextrin in spray dried powder. It could be observed that maceration, UAE and MAE provided dry extract with relatively high polyphenol content (Table 2). However, it is also important to consider total extraction yield (Table 1), which was the lowest in case of maceration. Although maceration can provide dry powders with high TP, global yield would be lower comparing with extracts obtained by UAE and MAE. This suggests on the advantage of application of novel extraction techniques, rather than conventional. Relatively low TP in extracts obtained by SWE and PHD could be explained by the applied extraction solvent. In these cases it was water which exhibited significantly lower selectivity towards polyphenol extraction comparing with aqueous ethanol applied in case of MAE, UAE and MAC. The total extraction yield obtained by SWE (35.62%) would still provide satisfying global yield of polyphenols in dry extracts, however, their content would be diluted with concomitant compounds extracted during solid-liquid extraction.

On the other hand, the highest TF (154.88 mg CE/g) was observed in extract prepared by maceration, while the lowest (56.98 mg CE/g) was in extract prepared by SWE with addition of maltodextrin. Again, TF observed in MAC, UAE and MAE powders was significantly higher comparing with SWE and PHD (Table 2). TF in spray dried rosemary extracts obtained by maceration with aqueous ethanol varied between 46 and 76.4 mg/g, which was significantly lower comparing with sage dry extract similarly prepared (154.88 mg CE/g) (Couto et al., 2012). MAC, UAE and MAE samples showed high content of TF, comparing with SWE and PHD, as it was the case with TP. This indicated that UAE and MAE would be the most suitable techniques which could be applied for both total polyphenols and flavonoids extraction due to high total
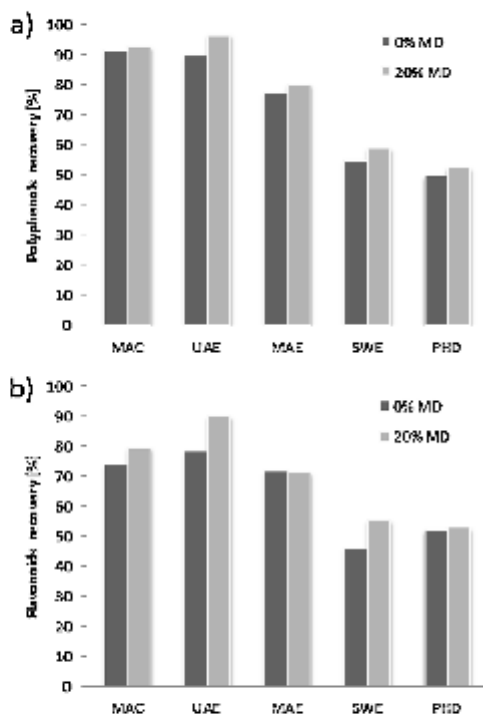

Fig. 1. Recovery of a) polyphenols and b) flavonoids achieved by spray drying with and without addition of carrier material.

extraction yield (Table 1) and high content of these compounds in dry powders (Table 2). Besides advantages in terms of product yield and quality, UAE and MAE would significantly reduce time consumption, since extraction time for these processes was 80 and 18.7 min, respectively.

In order to determine degradation percentage of polyphenols, recovery was calculated according to the following equation (Shofinita and Langrish, 2014):

$$\text{polyphenols recovery [\%]} = \frac{TP_{\text{spray dried powder}}}{TP_{\text{extract}}} 100 \tag{1}$$

Similarly, recovery was also calculated for flavonoids using values of TF observed in spray dried powder and in extract, respectively. Recovery of polyphenols and flavonoids achieved by spray drying of sage by-product extracts obtained by conventional and novel extraction techniques is presented on Fig. 1. Polyphenols recovery was the highest for UAE-20 sample (96.62%) and the lowest for PHD (49.55%). Similarly, the highest flavonoids recovery (90.08%) was observed in UAE-20, as it was the case with polyphenols, while recovery was the lowest for SWE (45.96%). According to Souza et al. (2008), degradation of polyphenolic compounds could occur during spray drying process due to phenomena of oxidative condensation and thermal degradation induced by elevated temperature. However, besides beneficial effects on powder stability, carrier could also reduce degradation of target compound(s) during spray drying process (Krishnaiah et al., 2014). It could be observed that in almost all cases, recovery of both polyphenols and flavonoids was higher when maltodextrin was added as carrier material (Fig. 1), suggesting of maltodextrin's ability to protect polyphenols from thermal degradation. Furthermore, maltodextrin exhib-

ited the highest positive effect on polyphenols recovery comparing with whey protein isolate and pea protein isolate (Moreno et al., 2016). Recovery was in correlation with applied extraction solvent, decreasing with the increase of water proportion in extraction solvent. The highest recovery was observed for MAC and UAE, than for MAE and the lowest was observed for SWE and PHD samples, while applied extraction solvents in each extraction technique was 60% ethanol (MAC and UAE), 46% ethanol (MAE) and water (SWE and PHD). This could be explained with the increase of the boiling point when water is used as solvent, meaning that evaporation in spray drying would occur at higher temperature which allows degradation of certain polyphenols and flavonoids.

3.3. Antioxidant activity

Polyphenols are a group of naturally occurring plant compounds synthesized as secondary metabolites during plant growth in response to stress and external aggressors such as pathogens and ultraviolet radiation. They may be divided into two major classes, phenolic acids and flavonoids. Phenolic acids include benzoic acid, cinnamic acid and their derivatives, while flavonoids include several sub-classes, flavonols, flavones, flavanones, flavanols, anthocyanins and isoflavones. Due to specific composition and ability to act as a free radical scavenger, consummation of food rich in polyphenols such as dark chocolate, black berry, black olives, green tea, red wine, blueberry, etc. (Pérez-Jiménez et al., 2010) may help in prevention of heart diseases, cancer and aging effect among many other beneficial for human health (Pandey and Rizvi, 2009). Apart of oxidative stability, in food polyphenols may contribute to the bitterness, astringency, colour, flavour and odour. Since sage has been recognized as plant with high antioxidant potential, it is essential to perform in vitro analysis of dry extracts antioxidant capacity.

Antioxidant activity of sage by-product spray dried extracts was comparatively determined by DPPH, FRAP and ABTS assays. Antioxidant activity determined by DPPH assay varied between 244.26 and 476.00 mg Trolox/g. Antioxidant activity was particularly high in samples prepared by UAE and MAE (Table 3). In case of ferric reducing antioxidant power, the highest was also achieved in UAE (52.56 mg Fe^{2+}/g), while the lowest was observed in PHD-20 (25.74 mg Fe^{2+}/g). Results of free radical-scavenging capacity towards $ABTS^+$ radical were between 377.52 and 811.68 mg Trolox/g. According to results from Table 3, it could be concluded that sage dry extracts possessed particularly high antioxidant capacity.

Similarly to polyphenol content, antioxidant activity decreased with the addition of maltodextrin due to dilution of dry extracts bioactive compounds with inert carrier. SWE and PHD extracts exhibited significantly lower antioxidant activity comparing with other techniques (Table 3), which is directly connected with polyphenol content in dry extracts (Table 2). Furthermore, essential oil of sage, which possess certain antioxidant activity (Bozin et al., 2007), was removed from the

PHD sample, since only postdistillation waste was used as feed for spray drying. This led to significantly lower antioxidant activity of PHD sample, comparing with other extraction techniques (Table 3). According to all antioxidant activity parameters, MAC, UAE and MAE expressed significantly higher antioxidant capacity comparing with SWE and PHD (Table 3). Even though, MAC sample exhibited similar antioxidant capacity as UAE and MAE samples, higher yield of dry extract per mass of plant material could be achieved when novel extraction techniques are being applied. This justified application of emerging extraction techniques (UAE and MAE) in initial step of solid-liquid extraction in order to achieve satisfying antioxidant properties of dry extracts.

Antioxidant activity of dry sage extracts was in accordance with polyphenol content. According to Fig. 2, good linear correlation between TP, TF and antioxidant activity parameters (DPPH, FRAP and ABTS) was observed. Similar correlation between TP and antioxidant activity of Salvia fruticosa and Rosmarinus officinalis spray dried extracts has been observed (Şahin-Nadeem et al., 2013; Couto et al., 2012). Linear regression of TP and antioxidant activity was higher comparing with TF, which was displayed as higher r^2 (Fig. 2). This suggested that polyphenols are main compounds responsible for antioxidant effects in these extracts, although additional plant metabolites could exhibit antioxidant effects as well. Furthermore, it is indicated that addition of MD would prevent or reduce loss of antioxidant activity with increase of TP and TF recovery (Fig. 1). Previously, antioxidant activity of spray dried extracts of Saturija montana and Achillea millefolium were determined only by DPPH assay in our previous research (Vidović et al., 2014; Vladić et al., 2016). In case of sage herbal dust, antioxidant activity was determined by three in vitro assays and good correlation was observed according to data from Fig. 2. According to particularly underlined antioxidant potential, it is suggested that sage herbal dust dry extracts could be used in plant pharmaceutical formulations, as well as food additives with preservative effect.

3.4. Antimicrobial activity

From ancient times, mankind has been used medicinal plants to treat common infectious diseases, due to their healing potential. Medicinal plants may be used in traditional and modern medicine as crude drugs, or as source of natural active compounds. The antimicrobial potential of sage essential oils and extracts has been proven in numerous papers (Bozin et al., 2007; Stanojević et al., 2010; Witkowska et al., 2013). Despite a good antibacterial efficiency, it is very difficult to compare the results among authors due to differences in origin of examined plant, its composition, employed techniques, tested microorganisms, growth medium, etc. For that reason, it is necessary to determine the antimicrobial activity of sage by-products spray dried extracts (MAC, UAE, MAE, SWE and PHD). Only spray dried samples without addition of maltodextrin were evaluated for antimicrobial activity.

The preliminary evaluation of antimicrobial activity was performed by disk diffusion method. According to the obtained results (Table 4), gram-negative bacteria and yeast strains were found to be resistant to all extracts. The resistance of yeasts may be referred to more complex cell structure (eukaryotic cell) in comparison with bacteria (prokaryotic cell). No efficiency of tested extracts against gram-negative could be a consequence of the cell envelope structure. Namely, gram-negative bacteria are surrounded by a thin peptidoglycan cell wall, which itself is surrounded by an outer membrane containing lipopolysaccharide, while gram-positive bacteria lack an outer membrane (Silhavy et al., 2010). The outer membrane is a barrier that limits the diffusion of active compounds trough external membrane (Pierozan et al., 2009). The gained results are in accordance with other studies, where similar effect of sage extracts and its essential oil towards investigated microorganisms has been reported (Pierozan et al., 2009).

Table 3

Antioxidant activity of spray dried sage by-product extracts determined by DPPH, FRAP and ABTS assays.

Sample	DPPH [mg Trolox/g]	FRAP [mg Fe^{2+}/g]	ABTS [mg Trolox/g]
MAC	418.11 ± 6.24[a]	45.14 ± 0.93[a]	670.55 ± 36.48[a]
MAC-20	379.47 ± 12.95[b]	39.27 ± 0.28[b]	600.79 ± 19.10[b]
UAE	442.58 ± 3.19[c]	52.56 ± 1.30[c]	669.26 ± 25.91[a]
UAE-20	369.16 ± 5.27[b]	37.42 ± 0.87[d]	538.89 ± 30.13[b,d]
MAE	476.00 ± 17.01[e]	42.56 ± 0.51[a]	811.69 ± 34.97[e,d]
MAE-20	372.24 ± 1.69[b]	40.19 ± 1.11[b,c]	595.91 ± 9.63[c]
SWE	298.63 ± 7.54[d]	31.09 ± 0.33[e]	431.88 ± 11.88[f]
SWE-20	244.26 ± 6.90[e]	28.30 ± 0.97[g]	377.52 ± 15.59[g]
PHD	275.99 ± 13.01[d]	28.45 ± 1.01[g]	496.81 ± 11.88[h]
PHD-20	247.60 ± 11.60[e]	25.74 ± 0.73[h]	429.77 ± 15.59[f]

Values followed by the same letter did not share significant difference at 5% (t-test).
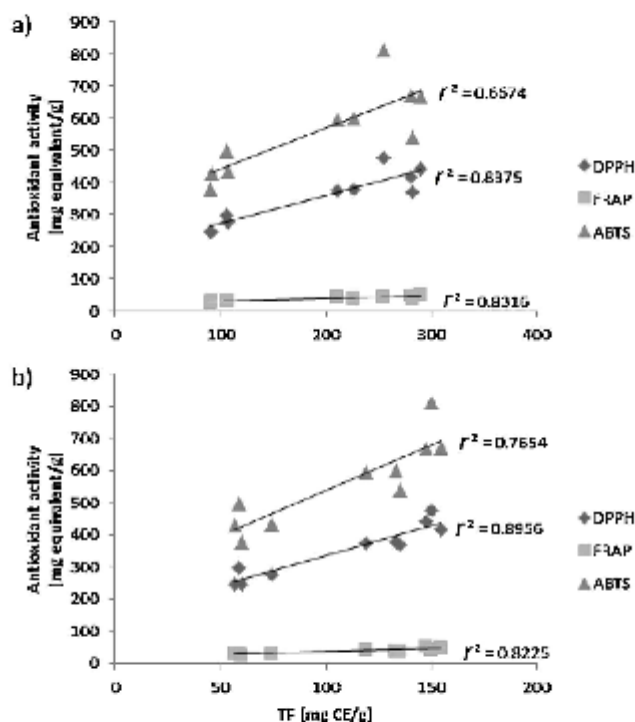
Fig. 2. Linear regression between antioxidant activity parameters (DPPH, FRAP and ABTS) and a) TP and b) TF.

Table 4
Antimicrobial activity of *Salvia officinalis* dry extracts (mean value diameter of the inhibition zone (mm) including disc (6 mm) ± standard deviation).

Group	Tested strains	Disc diffusion method (15 µL, extract concentration 50 mg/mL)					Positive control CTC
		Tested samples					
		MAC	UAE	MAE	SWE	PHD	
G(−) bacteria	*Escherichia coli* ATCC 25922	nd[a]	nd	nd	nd	nd	14.0 ± 0.0
	Pseudomonas aeruginosa ATCC 27853	nd	nd	nd	nd	nd	15.3 ± 1.7[c]
G(+) bacteria	*Staphylococcus aureus* ATCC 25923	12.33 ± 0.58[b]	14.33 ± 0.[b]	nd	10.67 ± 0.58[b]	12.00 ± 0.00[b]	12.00 ± 0.00
	Bacillus cereus ATCC 11778	20.00 ± 1.00	18.00 ± 2.65	20.33 ± 2.08	8.67 ± 0.58	11.00 ± 0.00	nd
	Listeria innocui ATCC 19119	18.67 ± 0.58	16.67 ± 0.58	19.67 ± 0.58	7.33 ± 1.15	13.33 ± 0.58	7.67 ± 0.58
	Enterococcus faecalis ATCC 19433	15.67 ± 2.31	16.33 ± 0.58	17.00 ± 0.00	nd	10.00 ± 0.00	9.00 ± 1.20
	Staphylococcus saprophyticus[w]	23.00 ± 1.00	21.33 ± 2.89	24.00 ± 0.00	7.33 ± 0.58	11.00 ± 0.00	11.00 ± 0.58
	Bacillus cereus[w]	8.00 ± 0.58	8.00 ± 0.00	8.00 ± 2.08	nd	nd	nd
	Listeria monocytogenes[w]	15.67 ± 0.58	15.67 ± 1.53	22.33 ± 0.58	8.00 ± 0.00	nd	10.20 ± 0.42
	Enterococcus faecalis[w]	12.00 ± 1.73	nd	13.33 ± 2.08	nd	11.00 ± 0.00	8.00 ± 0.00
Yeast	*Saccharomyces cerevisiae* ATCC 9763	nd	nd	nd	nd	nd	13.33 ± 0.58[d]
	Candida albicans ATCC 10231	nd	nd	nd	nd	nd	12.00 ± 0.00[d]

[a] Non detected inhibition zone.
[b] Clear zone around disc.
[c] ACV supplement.
[d] Actidion.

Table 5

Minimal inhibitory concentration (mg/mL) assayed by the microdilution method.

Group	Tested strains	Minimal inhibitory concentration (MIC) [mg/mL]				
		MAC	UAE	MAE	SWE	PHD
G(+) bacteria	*Staphylococcus aureus* ATCC 25923	25	12.5	12.5	50	25
	Bacillus cereus ATCC 11778	37.5	9.4	37.5	37.5	37.5
	Listeria ivanovii ATCC 19119	6.25	9.4	6.25	6.25	9.4
	Enterococcus faecalis ATCC 19433	25	50	50	25	25
	Staphylococcus saprophyticus[*]	6.25	9.4	9.4	18.8	9.4
	Bacillus cereus[*]	> 50	> 50	> 50	> 50	> 50
	Listeria monocytogenes[*]	3.12	6.25	12.5	6.25	12.5
	Enterococcus faecalis[*]	6.25	6.25	9.4	18.8	18.8

[*] wild strain.

On the other hand, the results presented in Table 4 shows that gram-positive bacteria show different sensitivity to tested extracts. In general, MAE, MAC and UAE show similar antibacterial activity against tested gram-positive bacteria, while SWE and PHD manifest slightly weaker (around 10 mm on average) or no antimicrobial activity. In general, the highest antimicrobial activity of tested extracts was determined against *Staphylococcus saprophyticus*[*]. The lowest antimicrobial activity was assessed against *Bacillus cereus*[*]. In addition, the minimal inhibitory concentration was determined for all tested gram-positive bacteria.

The antibacterial activity of spray dried sage extracts against selected strains of gram-positive bacteria determined by microdilution method is presented in Table 5. It could be observed that all investigated samples exhibited satisfying antimicrobial activity due to low MIC (< 50 mg/mL), which was higher only in case of *Bacillus cereus*[*]. According to MIC values from Table 5, it could be noted that *Staphylococcus aureus* ATCC 25923, *Listeria ivanovii* ATCC 19119, *Enterococcus faecalis* ATCC 19433, *Staphylococcus saprophyticus*[*], *Listeria monocytogenes*[*] and *Enterococcus faecalis*[*] were particularly sensitive to antimicrobial effects of MAC, UAE and MAE samples. However, their respective MIC values significantly increased for SWE and PHD samples (Table 5). Furthermore, investigated samples exhibited weak antimicrobial activity towards vegetative cells of *Bacillus cereus*[*], while MIC for vegetative cells of *Bacillus cereus* ATCC 11778 was lower, which suggested on higher resistance of wild strains. However, this was not the case with *Enterococcus faecalis*, since wild isolate was more sensitive to effects of investigated extracts. Pierozan et al. (2009) reported that MIC of sage essential oil dissolved in dimethyl sulphoxide (DMSO) was < 10 mg/mL for *Enterococcus faecalis* and *Staphylococcus aureus*, whereas water was used as solvent in all investigated samples for microdilution method thus completely neglecting antimicrobial effects of solvent. Furthermore, Witkowska et al. (2013) reported that MIC of sage extracts prepared with water, ethanol and hexane as extraction solvents were 10–40 mg/mL for *Staphylococcus aureus*, however, DMSO was also used as solvent for determination of antimicrobial activity. Antimicrobial activity was in accordance with polyphenol content (Table 2) and antioxidant activity (Table 3), since MAC, MAE and UAE samples were generally more potent comparing with SWE and PHD. This also indicated that extracted sage polyphenols were main compounds responsible for biological activity (antioxidant and antimicrobial effects), while their content in dry extracts was directly connected to applied extraction technique and process conditions.

Comparing the results obtained by disk diffusion method and microdilution method it can be concluded that the results are not uniform. Nevertheless, both methods indicate the sensibility of gram-positive bacteria to sage extracts; therefore the disk diffusion method may be applied for preliminary studies, while the microdilution method should be used for quantification of antimicrobial activity. According to data from Tables 4 and 5, it could be observed that UAE sample exhibited the highest antimicrobial activity. Due to certain antimicrobial effects, spray dried sage extracts could be used as food preservatives, particularly in solid food. Furthermore, they could be incorporated in pharmaceutical formulations with antimicrobial effects, fortified with particularly high antioxidant activity.

4. Conclusions

The results of the present study described the exploitation of the sage by-product from filter tea factory for production of dry extracts with effective biochemical properties. Initial step was the production of liquid feed for spray drying using conventional (maceration (MAC) and hydrodistillation (PHD)) and novel (ultrasound-assisted (UAE), microwave-assisted (MAE) and subcritical water extraction (SWE)) extraction techniques. Obtained liquid extracts were spray dried under the same conditions and dry extracts were characterized in terms of polyphenol content, antioxidant activity and antimicrobial activity. According to TP and TF content, it could be concluded that UAE and MAE could provide significant advantage comparing with other techniques, thus justifying application of emerging techniques over traditional. All investigated samples exhibited high antioxidant activity, which was particularly high in case of MAC, UAE and MAE, however, MAC sample had certain disadvantage in terms of total yield of recovered polyphenols. Investigated antimicrobial activity of dry extracts towards selected gram-positive bacteria, gram-negative bacteria and yeasts suggested that sage extracts exhibited antimicrobial effects only towards gram-positive bacterial. Furthermore, good correlation between polyphenols and antioxidant and antimicrobial activity parameters was observed suggesting that these were main responsible compounds for these effects. According to all investigated biochemical parameters, extract obtained by UAE had the best properties, therefore, it was proposed that this technique would be the most suitable for production of sage extracts. Obtained dry extracts could be used as food preservatives, or in pharmaceutical formulations and dietary supplements with the aim to exhibit antioxidant and antimicrobial activity.

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